Methodology for collection, processing, and storage of biological samples in a longitudinal Australian pregnancy cohort: The Newcastle 1000 Study

Tegan Grace, Joshua J Fisher, Nathan A Castles, Elizabeth A Jones, Sarah J Delforce, Alexandra E Peters, Gabrielle K Crombie, Emily C Hoedt, Kirby E Warren, Richard GS Kahl, Jonathan J Hirst, Kirsty G Pringle, Craig E Pennell

Submitted to: JMIR Research Protocols
on: June 24, 2024

Table of Contents

Methodology for collection, processing, and storage of biological samples in a longitudinal Australian pregnancy cohort: The Newcastle 1000 Study

Tegan Grace[1] BSc, PhD; Joshua J Fisher[1] BSc, PhD; Nathan A Castles[1] BSc, MA; Elizabeth A Jones[1] BMID, MAPH; Sarah J Delforce[1] BSc, PhD; Alexandra E Peters[2] BSc; Gabrielle K Crombie[3] BSc, PhD; Emily C Hoedt[2] BSc, PhD; Kirby E Warren[2] BSc, PhD; Richard GS Kahl[4] BSc; Jonathan J Hirst[2] BSc, PhD; Kirsty G Pringle[2] BSc, PhD; Craig E Pennell[1] MBBS, PhD

[1]School of Medicine and Public Health College and Health, Medicine and Wellbeing The University of Newcastle Callaghan AU
[2]School of Biomedical Sciences and Pharmacy College of Health, Medicine and Wellbeing The University of Newcastle Callaghan AU
[3]School of Life and Medical Science Faculty of Population Health Sciences University College London London GB
[4]School of Environmental and Life Sciences College of Engineering, Science and Environment The University of Newcastle Callaghan AU

Corresponding Author:
Tegan Grace BSc, PhD
School of Medicine and Public Health
College and Health, Medicine and Wellbeing
The University of Newcastle
University Drive
Callaghan
AU

Abstract

Background: Research in the developmental origins of health and disease (DOHaD) provides compelling evidence that adverse events during the first 1000 days of life from conception can impact life course health. Despite many decades of research, we are still lacking a complete understanding of the mechanisms underlying some of these associations. The Newcastle 1000 (NEW1000) Study is a comprehensive pregnancy cohort study, based in Newcastle, New South Wales, Australia which will provide detailed data about the first 1000 days of life to investigate the developmental origins of non-communicable disease.

Objective: NEW1000 is a prospective population-based cohort study which will recruit pregnant women and their partners between 11-14 weeks gestation, with assessments at 20-, 28-, and 36-weeks, birth, 6-weeks and 6-months. The study will provide a longitudinal multisystem approach to phenotyping, supported by robust clinical data and collection of biological samples.

Methods: This manuscript describes in detail the large variety of samples collected within the study, the method of collection, storage, and utility of the samples within the biobank. With a particular focus on incorporation of the samples into emerging and novel large scale “omics” platforms including genome, microbiome, epigenome, transcriptome, fragmentome, metabolome, proteome, exposome and cell free DNA and RNA. Specifically, this manuscript details the methods used to collect, process and store biological samples including maternal, paternal and fetal blood, microbiome (stool, skin, vaginal, oral), urine, saliva, hair, toenail, placenta, colostrum and breastmilk.

Results: N/A

Conclusions: The NEW1000 Study will generate a multigenerational, deeply phenotyped cohort with a comprehensive biobank of samples relevant to a large variety of analyses including multiple ‘-omics’ platforms. Clinical Trial: N/A

(JMIR Preprints 24/06/2024:63562)
DOI: https://doi.org/10.2196/preprints.63562

Preprint Settings

1) Would you like to publish your submitted manuscript as preprint?

✓ **Please make my preprint PDF available to anyone at any time (recommended).**
Please make my preprint PDF available only to logged-in users; I understand that my title and abstract will remain visible to all users.
Only make the preprint title and abstract visible.

No, I do not wish to publish my submitted manuscript as a preprint.

2) If accepted for publication in a JMIR journal, would you like the PDF to be visible to the public?

✔ **Yes, please make my accepted manuscript PDF available to anyone at any time (Recommended).**

Yes, but please make my accepted manuscript PDF available only to logged-in users; I understand that the title and abstract will remain v

Yes, but only make the title and abstract visible (see Important note, above). I understand that if I later pay to participate in <a href="http

Original Manuscript

Methodology for collection, processing, and storage of biological samples in a large Australian pregnancy cohort: The Newcastle 1000 Study

Abstract

Introduction

Research in the developmental origins of health and disease (DOHaD) provides compelling evidence that adverse events during the first 1000 days of life from conception can impact life course health. Despite many decades of research, we are still lacking a complete understanding of the mechanisms underlying some of these associations.

Objectives

The Newcastle 1000 (NEW1000) Study is a comprehensive pregnancy cohort study, based in Newcastle, New South Wales, Australia which will provide detailed data about the first 1000 days of life to investigate the developmental origins of non-communicable disease. NEW1000 is a prospective population-based cohort study which will recruit pregnant women and their partners between 11-14 weeks gestation, with assessments at 20-, 28-, and 36-weeks, birth, 6-weeks and 6-months. The study will provide a longitudinal multisystem approach to phenotyping, supported by robust clinical data and collection of biological samples.

Main outcomes

This manuscript describes in detail the large variety of samples collected within the study, the method of collection, storage, and utility of the samples within the biobank. With a particular focus on incorporation of the samples into emerging and novel large scale “omics” platforms including genome, microbiome, epigenome, transcriptome, fragmentome, metabolome, proteome, exposome and cell free DNA and RNA. Specifically, this manuscript includes the collection of maternal, paternal and fetal blood, microbiome (stool, skin, vaginal, oral), urine, saliva, hair, toenail, placenta, colostrum and breastmilk.

Conclusion

This manuscript provides a comprehensive and transparent overview for all samples to facilitate the utilisation of the NEW1000 as a resource for research.

Introduction

Research in the developmental origins of health and disease (DOHaD) provides compelling evidence that adverse events during the first 1000 days of life from conception can impact life course health; increasing the risk of metabolic risk factors[1,2] such as obesity[3], diabetes[4], hypertension[5], and cardiovascular disease[6], asthma[7,8], allergies[9,10], and adverse neurodevelopmental[11,12] and mental health[13-15] outcomes. Despite decades of research, we are still lacking a complete understanding of the mechanisms underlying some of these associations. Australian pregnancy and birth cohorts have provided excellent contributions to pregnancy health and developmental research over the last three decades, with over 17 pregnancy or birth cohorts to date[16]. Since the inception of these studies, there has been significant advances in technologies increasing our understanding of the underlying biological mechanisms driving the pathogenesis of disease. For example, the rapidly expanding study of '-omics' such as genomics, transcriptomics, proteomics, and metabolomics have contributed to identifying complex traits underlying health outcomes with greater accuracy than standard clinical approaches[17].

The Newcastle 1000 (NEW1000) Study is a comprehensive pregnancy cohort study, based in Newcastle, New South Wales, Australia which will provide detailed data about the first 1000 days of life to investigate the developmental origins of non-communicable disease[18]. The multi-omics design of NEW1000 intends to provide detailed, longitudinal, multi-system phenotyping and serial sample collection for analyses of the genome, microbiome, epigenome, transcriptome, fragmentome, metabolome, proteome, exposome and cell free DNA and RNA. A critical element of biorepositories is standardised collection, handling and storage of samples to ensure reliable results through analyses of high quality samples[19]. In addition, utilisation of a suitable informatics system is recommended to ensure accurate tracking of inventory[20]. This manuscript will describe the methodology used to collect, process and store biosamples within NEW1000 with the intent to facilitate access and utilisation of these resources upon request.

Methods

Study Design

NEW1000 is a prospective, population based, longitudinal pregnancy cohort study. Stage One (2021-2025) will involve recruitment of 500 pregnant participants and their partners per year for five years. The importance of the first 1000 days of life from conception is reflected the number of assessments

during this stage. Each family is seen five times during pregnancy (14-, 20-, 28-, 36- and 40- weeks' gestation), at birth, 1-2 days postnatally, then again at 6 weeks, and 6 months postpartum. Stage Two involves further postnatal follow up of the families at 12, 24 and 36 months of age. Stage Three will involve long term follow up of the cohort through face-to-face appointments, questionnaires and data linkage to establish a deeply phenotyped pregnancy cohort from early gestation through to adulthood. The focus of this manuscript will be the protocol and methodology of biological sample collection and biobanking for Stage One of the study.

NEW1000 has been designed for standardised collection of blood, urine, saliva, buccal swabs, microbiome swabs, toenails, and hair samples from the mother, father (or partner), and infant. In addition, cord blood, placenta, and colostrum samples are collected at birth and breastmilk samples are collected at 6-weeks' and 6-months' postpartum (Figure 1.)

Blood Samples

The use of human blood within biomedical research, particularly biomarkers, is a significant resource and commonplace in discovery and diagnosis of clinical conditions. Biomarkers have been defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention"[21]. In addition to traditional use of biomarkers, there is emerging evidence to support the use of genetic biomarkers for precision medicine – that is, its applications for diagnosis, prognosis, and personalised treatment options based on an individual's genetic profiling[22]. NEW1000 will collect a comprehensive panel of blood and blood products to facilitate all avenues of biomarker research including traditional approaches as well as downstream genetic or multi-omic analyses. Isolating such biomarkers will allow for the prediction of adult disease in the earlier stages of life and thus provide the potential for advancements in precision medicine.

Collection of blood samples will be according to recommended order of draw[23]: Vacuette® Serum tube (silica-coated clot activator and polymer gel), for use in routine clinical chemistry and hormone tests, serology, and immunohaematology; Vacuette® Heparin Plasma tube (lithium heparin-coated), used for clinical biochemistry tests; Vacuette® EDTA tubes (contain K2 EDTA), used for haematological procedures such as complete blood counts and blood typing and plasma isolation to allow for multi-omics analyses; and PAXgene® Blood RNA tube (BD Biosciences, contains 6.9mL RNA stabilisation additive), used to stabilise intracellular RNA to yield accurate and reproducible

gene expression data[24]. Maternal bloods will be collected at 14-, 20-, 28-, 36- and 40-week appointments, as well as 6-weeks postpartum. Partner bloods will be collected at the 20-week appointment and cord blood will be collected at birth. Adult blood samples to be collected include: 8mL in one 8mL Vacuette® Serum tube; 4mL in one 4mL Vacuette® Heparin Plasma tube; 18mL in two 9mL Vacuette® EDTA tubes; and 4mL of cord blood will be collected in one 4mL Vacuette® EDTA tube. The buffy coat, containing white blood cells and platelets, an important source of genomic DNA that can withstand long term storage[25], will be extracted from centrifuged Vacuette® EDTA tubes. In addition to these tubes, 2.5mL (draw volume) in one PAXgene® Blood RNA tube will be collected for maternal blood samples at 14-, 20-, and 28-weeks' gestation. Thus, each family has the potential to donate bloods that total up to 68 aliquots within their first year of the study that can be utilised for traditional and non-traditional "omics" techniques.

Collection of blood samples

Adult blood samples.

Using a tourniquet on the arm above the cubital fossa, a research nurse will locate a suitable vein and clean the site with an alcohol swab. Samples will be collected via a 21G sterile luer and holder with retractable needle, commonly used during venepuncture as the smaller gauge reduces discomfort and prevents haemolysis. Once all tubes are filled, the needle removed and retracted for safe disposable, a sterile IV pressure pad will be applied to the collection site.

Cord blood sample.

The placenta will be placed into a sterile specimen dish post-birth. If umbilical cord blood gas analysis is required as part of clinical care these will be obtained prior to research samples. A large cord vein will be located and cleaned using an alcohol swab to remove any maternal blood. An 18G needle will then be inserted into the vein and a 10mL syringe will be used to draw a blood sample which will be inserted into a 4mL EDTA tube.

The subsequent sections will discuss the methodology of processing, separation and storage of plasma, serum and buffy coat from EDTA, Serum, Lithium Heparin, and PAXgene® blood tubes (Figure 2).

Processing and Storage of blood samples

Plasma.

Samples will be inverted 5-10 times to ensure adequate mixing of blood with the EDTA anticoagulant[26] and centrifuged according to the following conditions: 10min at 500x g at 4˚C, with an acceleration of nine and a deceleration of three. All but approximately 0.2mL of plasma (reserved for buffy coat - Figure 3) will be aliquoted into one 15mL Falcon tube and subjected to a second centrifugation step, for 10 min at 2000x g at 4˚C, with an acceleration of nine and a deceleration of nine. Plasma will be aliquoted into new sterile 15mL Falcon tube and centrifuged again according to the same conditions as the previous spin, and the pellet discarded. The remaining plasma will then be aliquoted: 1.5mL plasma into one 1.5mL low-binding Eppendorf tube, with remaining plasma evenly distributed among four 2mL cryovial tubes. For 36-week appointments, 0.2mL plasma will be transferred into two 2mL cryovial tubes containing 0.6mL DNA/RNA Shield solution prior to the plasma being distributed into the four 2mL cryovial tubes. Cord blood samples are subjected to the same centrifugation conditions; however, a smaller quantity of plasma will be obtained. Approximately 0.5mL of plasma will be placed into one 1.5mL low-binding Eppendorf tube and two 2mL cryovial tubes. When extracting plasma, approximately 0.2mL will be reserved (in each tube) for the buffy coat. Aliquots will be stored at -80˚C until required for analysis, and OpenSpecimen updated to include these aliquots.

Buffy Coat

A sterile 3mL Pasteur pipette will be used to aliquot the buffy coat from both 9mL Vacuette® EDTA tubes, (one 4mL Vacuette® EDTA tube for cord blood), along with the remaining 0.2mL plasma and a small portion of RBCs into one sterile 2mL cryovial tube. Tubes will be stored frozen at -80˚C until required for analysis, and OpenSpecimen updated to include these aliquots.

Serum.

The sample will be inverted 5-10 times to activate the silica clotting agent[26], then kept upright at room temperature for 20 minutes to allow clot formation. Afterwards, the sample will be centrifuged at the following conditions: 10min at 2000x g at 4˚C, with an acceleration of nine and a deceleration of nine. Serum is subsequently aliquoted: 1mL into four 2mL cryovial tubes; and for 14-weeks' gestation only, approximately 0.1mL into one 1.5mL low-binding Eppendorf tube. Aliquots will be stored frozen at -80˚C until required for analysis, and OpenSpecimen updated to include these aliquots.

Lithium Heparin.

Samples will be inverted 5-10 times to ensure adequate mixing of blood with the heparin anticoagulant[26] and centrifuged according to the following conditions: 10 minutes at 2000x g at 4˚C, with an acceleration of nine and a deceleration of nine. All of the plasma will be aliquoted into one 15mL Falcon tube and subjected to a second centrifugation spin at the same conditions as the previous spin. All plasma will then be aliquoted into one 2mL cryovial tube or divided evenly between two 2mL cryovial tubes if there is >2mL plasma. Aliquots will be stored at -80°C until required for analysis, and OpenSpecimen updated to include these aliquots.

PAXgene® Blood RNA

Samples will be kept upright at room temperature for a minimum of two hours before being stored at -30˚C overnight, and OpenSpecimen updated to include the sample. The following morning, the sample will be transferred from -30˚C to permanent storage at -80˚C until required for analysis.

Urine

Urine can be used for the detection and analysis of proteinuria, bacteria, infections, toxicology, renal function and nutrient markers[27-29]. Mid-stream urine samples will be requested at 14-, 20-, 28-, 36-weeks gestation and at 6-weeks postpartum from enrolled women. Partner sample will be collected at 20-weeks. Adult samples will be self-collected in 70mL sterile specimen jars and aliquoted into four 2.0mL sterile cryotubes for storage at -80˚C. Additional urine will be stored in 15mL Falcon Tubes at -80˚C. Infant urine samples will be collected at 6-weeks and 6-months using a cellulose pad insert to collect urine during appointments. The insert will be collected at the end of the appointment and urine extracted using a specialised press device, then aliquoted into 2.0mL sterile cryotubes and stored at -80˚C.

Saliva

Human saliva is a complex biofluid that readily available, non-invasive and can be utilised for a number of diagnostic and biomedical research-based analyses[30]. Saliva has been used to study proteomics, with proteins often closely reflecting those seen in plasma, as approximately 30% of salivary proteins originate in the blood[31]. As such, saliva is emerging as a common alternative to blood in genetic research and PCR-based genotyping[32]. Further, steroid hormone concentrations and fluctuations are often assessed in salivary samples via enzyme-linked immunosorbent assays (ELISA) or liquid-chromatography mass-spectrometry (LC-MS)[33,34]. Salivary cortisol concentrations, routinely studied in animal and human studies, have been validated with serum

cortisol concentrations[35-37]. As stressful experiences in early life are linked to numerous adverse developmental outcomes accurate biospecimen collection for assessment of these pathways within any longitudinal cohort is critical[38,39]. Therefore, the collection of saliva within NEW1000 will be an important forward-looking and versatile sample within the biobank, providing an often-overlooked biological sample for accessing and validating a multitude of biological markers. Investigating response to stressors such as maternal anxiety, depression, familial and sociodemographic adversity[38,40-42], and external and environmental factors such as COVID-19, lockdowns, or natural disasters[43,44] will provide a baseline for future long-term programming studies in children and adults.

Collection Processing and Storage

Maternal saliva samples will be self-collected at home on the morning of the 20- and 36-week appointments. Partners will provide a saliva sample at 28-weeks. Participants will be instructed to collect the sample within 20 minutes of waking up, before food, drink or other substances are consumed and before teeth brushing to enable assessment of baseline levels of biomarkers and stress hormones, such as cortisol. To maximum collection amount, participants will be instructed to let saliva accumulate in the mouth without swallowing for 1 minute, and then to passively drool the accumulated saliva into a sterile 5mL screw cap collection tube (Sarstedt Australia). Samples will be processed by centrifugation in 5mL collection tubes at 2000 x g for 10 minutes at 4° C prior to being placed into two 2mL cryotubes, using a sterile 3 mL Pasteur pipette. Duplicate aliquots will be implemented to minimise the need for freeze/thaw and provide alternative or complimentary examination to blood samples. Samples will be routinely kept at -80°C for long-term storage[15].

Buccal Swabs

Recent studies have highlighted the potential advantages offered by buccal swabs when assessing epigenetics, DNA methylation and genome wide studies[45-47], most notably, the uniformity of the epithelial cell type present compared to other samples[46-47]. Critical to collection of samples for multi-omic analyses, buccal samples have recently been used to assess the predictive nature of epigenetics in preterm birth[46], providing a valuable addition to the biobank.

Collection Processing and Storage

Maternal buccal swabs will be collected at 20- and 36-weeks' gestation, and 6-weeks' postpartum, paternal samples at 20-weeks and infant samples at birth, 6-weeks, and 6-months' post-partum. Prior

to collection participants will be asked to refrain from eating, drinking, chewing gum, brushing teeth or smoking for at least 30 minutes. Participants are instructed to remove a sterile swab from the tube, ensuring the tip does not touch any surfaces, and rub the swab on both sides of their inner cheek for 60 seconds, rotating the end several times to collect skin cells. Once the collection is completed the participant will be instructed to remove the swab, being careful not to touch swab tip against teeth, lips or other surface, then place the swab directly into the collection tube and close the lid firmly. Infant collection of buccal swabs will be completed by a research midwife or nurse.

Microbiome

The microbiome plays a central role in health and disease contributing to the development and regulation of metabolic and immune functions[48,49]. From birth, the microbiome changes in response to host and environmental factors[50]. Disruptions in early life microbiome can negatively impact a wide range of health outcomes[51,52]. Beginning at birth, complex host-microbe-environmental interactions help shape host metabolism, immune and gastrointestinal tract system development and behaviour[48,49], setting the biological foundation for future disease susceptibility[53]. The first colonisers of the infant gut are determined during and immediately after birth when the infant is exposed to microbial communities from the mother and the external environment. The subsequent maturation of the microbiome is dynamic and highly individualised during the first 2 years of life[54]. During this time, multiple factors influence microbiome structure and function including the maternal microbiome across surfaces and fluids the infant is exposed to, such as the vagina, skin, saliva, and breastmilk[55].

The maternal microbiome undergoes substantial remodelling during pregnancy that can promote metabolic changes such as weight gain, insulin insensitivity[56] and promote neonatal growth. Maternal host-microbe interactions contributing to infant health remain poorly understood. Comprehensive studies to address this knowledge gap are critical in order to develop microbiome-targeted strategies to improve pregnancy outcomes and infant health[57]. While recent studies have provided important insights into microbiome development in early life[52,54,58,59], these studies are limited to small samples sizes. This current cohort aims to alleviate these matters and provide a comprehensive and well characterised dataset in which future studies can elucidate the mechanisms and complex interactions behind maternal and infant microbiome on fetal development and the onset of disease later in life.

Collection Processing and Storage

Vaginal microbiome

A self-collected vaginal swab for vaginal microbiome and a swab of the transvaginal probe cover (post-scan) will be requested from each participant and provide assessment of both the lower and upper vaginal microbiome respectively. Low vaginal swabs will be self-collected at 14- and 36-weeks' gestation and during early labour using a standardised health services protocol[60]. Participants will be instructed to wash their hands prior to twisting the cap to break the seal on the sterile swab provided (Copan), being careful not to touch the swab end on any other surfaces. With their other hand they are instructed to gently spread the labia and insert the tip of the swab about 5cm into the vagina opening, pointing the tip of the swab towards their lower back while relaxing their muscles. The swab should be gently rotated and then held against the vagina wall for 10-20 seconds. Participants are to remove the swab without touching the outer skin and return it to its original tube, ensuring the cap is snapped shut. Swabs will be transported immediately to the laboratory and the swab tips cut into 2mL screw cap tubes and snap frozen in liquid nitrogen, before being transferred to a -80 freezer until extraction[61].

Skin microbiome collection process

Skin microbiome samples will be collected by a sterile swab, dipped in 0.15M sodium chloride solution and firmly applied and rotated on the skin for ~60 seconds (chest of mother/father and cheek of the baby)[62]. Swabs will then be transported immediately to the lab and the swab tips cut into 2mL screw cap tubes and snap frozen in liquid nitrogen, before being transferred to a -80°C freezer until extraction[61]. Maternal skin swabs are obtained at 20- and 36-weeks gestation and birth; partner swabs collected at 20-weeks; and infant skin swabs within 24 hours of birth, 6-weeks, and 6-months postpartum.

Oral microbiome collection process

Participants are asked to refrain from eating, drinking, chewing gum, brushing teeth or smoking for at least 30 minutes before sample collection. Participants will be given a sterile swab and asked to collect a saliva sample by rubbing and rotating the swab along the teeth, applying similar pressure as brushing their teeth, then placing the swab under the tongue for 30 seconds to collect saliva. Participants will be instructed to ensure the swab does not touch their lips or other surfaces prior to being placed back in the sterile tube and securing the lid. Swabs tips will be cut into 2mL screw cap tubes and snap frozen in liquid nitrogen, before being transferred to a -80°C freezer until

extraction[61]. Maternal saliva swabs will be obtained at 20- and 36-weeks' gestation and 6-weeks' postpartum; partner swabs will be collected at 20-weeks gestation; and infant saliva swabs obtained at 6-weeks and 6-months postpartum.

Gut microbiome collection process

Collection kits developed by Microba Life Sciences will be used to collect stool samples. This kit consists of an easy-to-use swab and preservation with a liquid-free preservation solution which is temperature stable for up to seven days[63]. A pre-labelled swab collection kit will be given to participants who will use the sterile swab to collect faecal matter from soiled toilet paper after a bowel movement. Instructions on the amount needed for correct sampling are given as part of the self-collection pack. Samples are stable at room temperature for up to seven days, allowing participants to collect their sample several days prior to their next appointment and bring the sample in the provided packaging to their next appointment. Maternal faecal swabs will be obtained at 20- and 36-weeks' gestation; partner swabs at 28-weeks gestation; and infant faecal swabs will be obtained where possible within 24 hours of birth, 6-weeks and 6-months postpartum. Swabs will be stored in a -80°C freezer until analysis.

Measurement of environmental exposure

Substances including endocrine disrupting chemicals, heavy metals, and pesticides are persistent in the environment, leading to human exposure through diet, household, workplace, and ambient surroundings[64]. Maternal exposure to environmental contaminants during pregnancy renders the fetus susceptible to indirect exposure and the associated negative health impacts during critical periods of development[65,66]. These health impacts can be diverse and long lasting, with effects reported including altered hCG secretion, intrauterine growth restriction, reduced birth weight, and alterations of neonatal gut microbiome and metabolome[65-68]. Collection and storage of nail and hair clippings for use in as a biomarker of environmental contaminant exposure is ideal due to the non-invasive nature of sampling, ability to assess cumulative exposure, and ease of long term storage and handling[69]. Additionally, when substances are deposited into keratin rich tissues, their levels remain unchanged, allowing for measurement of long-term cumulative exposure over a time period of 6-12 months[69,70]. This is of critical importance to the value of these samples as human nails develop at ~10 weeks of gestation. Thus, collection and analysis of infant nail samples early postpartum can enable an estimate of exposure over the span of pregnancy as a whole, compared to blood or urine samples that only give insight into a short span of time, usually up to a few days[70].

Toenails

Analysis of nail samples has proven effective for a range of environmental contaminants including heavy metals such as mercury and arsenic, endocrine disrupting chemicals such as bisphenol A, and other industrial chemicals such as chlorinated paraffins and per- and polyfluoroalkyl substances (PFASs)[69-73]. Thus far, studies that have utilised toenail sample analysis in infants have primarily focussed on heavy metal exposure, correlating detected levels with maternal dietary exposure from 8-weeks post-delivery through to 36-weeks from maternal and infant toenails at prenatal and postpartum time points. Collecting toenail samples to analyse environmental contaminant exposure is a promising yet underutilised tool, with the detection of an expansive range of harmful contaminants yet to be addressed in the context of fetal exposure and gestational cohorts.

Collection, Processing and Storage

Participants will be directed to self-collect toenail clippings after a bath or shower, with cleaned clippers or scissors (maternal and partner at 20-weeks gestation and maternal and infant at 6-weeks postpartum). Participants will be instructed to cut toenails as close to the nail bed as comfortable over a clean piece of paper or tissue with at least 2-3mm of clippings from each toenail being obtained. The tissue or paper is to be folded at the edge of each side to enable ease of transfer into a provided self-sealing envelope, to be stored in a dry place at room temperature.

Hair

Similar to toenails, hair samples can provide information on toxicology, exposure to heavy metals, harmful chemicals in the environment, as well as alcohol and illicit substance use. It may also be used as a biomarker in chronic stress and certain pathological conditions[74]. As evidenced by the assessment of cortisol concentrations, 3cm of hair growth from the scalp represents retrospective activity of the HPA-axis in the preceding 3 months[75]. A small amount of hair cut from close to the scalp is an easily accessible, non-invasive sample that is provided with high compliance in many populations[76,77].

Collection, Processing and Storage

Maternal and partner hair samples will be requested at 20-week gestation, and maternal and infants at 6-weeks postpartum. Hair will be parted with a comb horizontally on the crown of the head, a 2-3mm section of hair below sectioned off and, aiming for at least 3cm of length, cut with sharp hair-

cutting shears as close to the scalp as possible. For hair less than 3cm of length, a 4-5mm section of hair will be collected. The cut end of the hair will be fastened with medical tape. Samples will be stored, lying flat, in foil within an envelope and stored in a dry, dark place at room temperature. Hair samples can be stored in this way for several years with little to no degradation[75-77].

Placenta

Placental samples have previously been used in biomedical research using traditional measures such as gene and protein work to interrogate physiology and disease, although rarely in the sample size cohort studies provide. As methodologies facilitate more comprehensive work such as genome wide association studies, proteomics, and metabolomic analysis across more diverse populations, it is critical to maintain high standards of collection and storage to ensure reproducibility. Maintaining accurate and thorough records of placental samples enables the transfer of samples upon request ensuring utilisation of the cohort, independent evaluation and validation and creative approaches to reproductive research. As such, this cohort aims to establish a well characterised and thorough process for consistent and accurate collection to ensure quality. Placentas will be collected as close to birth as possible and taken immediately to the laboratory for processing. Eight individual samples per placenta will be collected, encompassing both the membrane and the villous tissue, in both fixed and frozen format, to provide the utility and quantity to assess multiple measures per sample (Figure 4).

Membrane Collection, Processing and Storage

Placental weight and processing time will be recorded. As the membrane must be repositioned or removed prior to placental villous core sampling, the fetal membrane will be collected first. The site of rupture will be ascertained and a cut made posteriorly to collect a 2x3cm section which will immediately be washed in ice-cold phosphate buffer saline (PBS tablets, Gibco™, pH 7.45, Life Sciences, USA), then dabbed on Kim Wipes to remove excess PBS prior to being snap frozen in 2mL cryotubes (Cryogen Clearline 2mL, biosigma, Italy). For subsequent storage, samples will be frozen at -80˚C until required for analysis. In addition to frozen samples, a section of rolled membrane approximately 2cm by 5cm will be obtained from the rupture site. This membrane strip will be washed in ice-cold PBS prior to placing the amnion face down on a Kim Wipe and forceps will be used to gently roll the sample. Each edge of the membrane roll will then be subjected to trimming with scissors and placed into an immunohistology embedding cassette (TechnoPlas, Australia). The cassette containing the fetal membrane roll will then be placed into a 70mL specimen pot and

subsequently washed three times in PBS and placed on a rocker in formalin at 4°C overnight. Following fixation, formalin will be removed and replaced with a PBS/Sodium Azide solution to prevent bacterial growth for long term storage (0.1M PBS with 0.05% Sodium Azide). Fixed samples will be stored long term at 4°C.

Villous Collection, Processing and Storage

Number of samples obtained from the placenta will be determined by the weight of the placenta and whether clinical histopathology examination has been requested. Placentas <500g will have a maximum of 6 sample cores, while in placentas >500g up to 8 cores may be collected. This is to ensure that all placental integrity can be maintained upon returning the placenta to histopathology for subsequent clinical macro and microscopic evaluation. Cores samples will be obtained numerically from each of the four quadrants (North, South, East and West) of the placenta (Figure 4) in a process adapted from Burton et al[78]. Cores will be gathered by 8mm biopsy punch (Kai) and immediately placed in a petri dish filled with ice-cold PBS ensuring that samples are washed gently and thoroughly to minimize maternal blood carry-over. Samples will be transferred into a new petri dish with ice-cold PBS where scissors will be used to exclude the fetal and maternal membranes. Following this, 4-6 samples (depending on placental weight) will be placed into one screw cap 2mL cryotube (Cryogen Clearline 2mL, biosigma, Itlay) and snap-frozen in liquid nitrogen. Due to limited sample tissue will not be homogenised immediately prior to being frozen, although will form a representative cross-section once frozen within the tubes. Tubes are stored frozen at -80˚C until required for analysis. Remaining core samples will be placed in an immunohistology embedding cassettes and fixed and stored as described above. In addition to whole placental samples, individual biopsies will be obtained from the initial core sites via Klini Tischler (3x7mm jaw) biopsy tool. Samples will be rinsed in PBS, dabbed dry and placed in uniquely labelled and barcoded individual Eppendorf tubes (1.5mL) to represent their respective quarter.

Biobanking

The NEW1000 Study is a large-scale cohort study. Each family will donate dozens of biological samples, thus contributing to an extensive biospecimen dataset. One family could donate up to 150 aliquots of biospecimens within their first year, equating to 75,000 aliquots for the first 500 families. The need to accurately and efficiently store and retrieve samples for subsequent analysis is critical. The NEW1000 Study Biobank will ensure accurate processing, storage and tracking of all specimens. “Biobanking” is a term used to describe large-scale biospecimen collection and storage

within a biorepository[79]. For effective management, biobanking software – known as Laboratory Information Management System (LIMS) – can be implemented. One such platform that uses this software is OpenSpecimen. OpenSpecimen is an open-source software that aims to ensure extensive sample collection, research, and derivation of meaningful data is as streamline as possible, allow researchers to obtain high quality biospecimen data[80]. As OpenSpecimen is highly configurable, it provides a customisable platform that will suit the needs of the study. Each participant will have a deidentified participant identification number, and a profile that contains all relevant medical information pertaining to each gestational timepoint. All biospecimens collected as part of the study will be barcoded and scanned into the NEW1000 biobank according to the participant's visit date. Additionally, OpenSpecimen can be integrated with other applications or instruments such as REDCap[80]. This will ensure that all biospecimens are accounted for and easily retrieved upon request for future studies.

Institutional Review Board Approval

Ethics has been approved by the Hunter New England Local Health District (HNELHD) Human Research Ethics Committee (HREC) on December 8th 2020 (2020/ETH/02881) and included informed written consent by participants. All proposed projects utilising NEW1000 data and/or biological samples will be required to submit an initial application to the NEW1000 Study Executive Committee and have relevant ethics and safety clearances from HNELHD HREC prior to the release of any de-identified data or biological samples.

Conclusion

The NEW1000 Study will generate a multigenerational, deeply phenotyped cohort with a comprehensive biobank of samples relevant to a large variety of analyses including multiple '-omics' platforms.

Authorship Statement

JJF conceptualization (lead), visualization (equal), writing – original draft (equal), writing - review and editing (lead). TG project administration (lead), conceptualization (supporting), visualization (supporting), writing – original draft (supporting), writing – review and editing (equal). NAC visualization (equal), writing – original draft (equal). EJ, SJD, AEP, GKC, ECH, KEW, RGSK, JJH, and KGP: writing – original draft (equal). CEP is the Study Director and was responsible for the Study Protocol on which the manuscript was based and contributed the conceptualization

(supporting), writing – review and editing (equal) of the manuscript.

Competing interest statement

The authors confirm no conflicts of interest.

Funding

The NEW1000 Study is supported by philanthropic donations through the Hunter Medical Research Institute.

References

1. Hoffman DJ, Powell TL, Barrett ES, et al. Developmental origins of metabolic disease. Physiol Rev. 2021;101:739-95.
2. Lurbe E, Ingelfinger J. Developmental and early life origins on cardiometabolic risk factors. Hypertension. 2021;77:308-18.
3. Bouret S, Levin BE, Ozanne SE. Gene-environment interactions controlling energy and glucose homeostasis and the developmental origins of obesity. Physiol Rev. 2015;95:47-82.
4. Qi L, Huang T, and the BIRTH-GENE (BIG) Study Working Group Authors. Association of birth weight with type 2 diabetes and glycemic traits. A mendelian randomization study. JAMA Network Open. 2019;2(9).
5. Davis EF, Lewandowski AJ, Aye C, et al. Clinical cardiovascular risk during young adulthood in offspring of hypertensive pregnancies: insights from 20-year prospective follow-up birth cohort. BMJ Open. 2015;5(6).
6. Rauschert S, Melton PE, Burdge G, et al. Maternal smoking during pregnancy induces persistent epigenetic changes into adolescence, independent of postnatal smoke exposure and is associated with cardiometabolic risk. Frontiers in Genetics. 2019;10.
7. White E, DeKlerk N, Hollams E, et al. Early life risk factors for asthma in young adults in the Western Australian Pregnancy Cohort (Raine Study). Eur Resp Jl. 2014;44.
8. Thorburn AN, McKenzie CI, Shen SJ, et al. Evidence that asthma is a developmental origin disease influenced by maternal diet and bacterial metabolites. Nature Comm. 2015;6:7320.
9. Yabuhara A, Macaubas C, Prescott SL, et al. TH2-polarized immunological memory to inhalant allergens in atopics is establised during infancy and early childhood. Clin Exp Allergy. 1997;27:1261-9.
10. Grieger JA, Clifton VL, Tuck AR, et al. In utero programming of allergic susceptibility. Int

Arch Allergy Imm. 2016;169:80-92.
11. Baumgartel K, Jensen L, White SW, et al. The contributions of fetal growth restriction and gestational age to develomental outcomes at 12 months of age: A cohort study. Early Human Dev. 2020;142(104951).
12. Grace T, Bulsara M, Robinson M, et al. The impact of maternal gestational stress on motor development in late childhood and adolescence: A longitudinal study. Child Dev. 2015;87(1):211-20.
13. Robinson M, Oddy WH, Li J, et al. Pre- and postnatal influences on preschool mental health: a large-scale pregnancy cohort. Child Psych Psych. 2008;49(10):1118-28.
14. Herbison CE, Allen K, Robinson M, et al. The impact of life stress on adult depression and anxiety is dependent on gender and timing of exposure. Dev Psych. 2017;29(4):1443-54.
15. O'Donnell K, Meaney M. Fetal origins of mental health: The developmental origins of health and disease hypothesis Am J Psych. 2017;174:319-28.
16. Borg D, Rae K, Fiveash C, et al. Queensland Family Cohort: a study protocol. BMJ Open. 2021;11(e044463).
17. Akiyama M. Multi-omics study for interpretation of genome-wide association study. J Hum Genet. 2021;66(1):3-10.
18. Grace T, Fisher J, Wang C, Valkenborghs SR, et al. Newcastle 1000 (NEW1000) Study: an Australian population-based prospective pregnancy cohort study design and protocol. BMJ Open. 2023;13(7):e072205.
19. Ferro P, Ortega-Pinazo J, Martinez B, et al. On the use of buffy or whole blood for obtaining DNA of high quality and functionality: What is the best option? Biopres Biob. 2019;17(6):577-82.
20. NSW Health Statewide Biobank. Biobank Inventory Verification Standard Operating Procedure: NSWHSB_Cert.Prog_SOP_5.2.0. In: Health N, editor. https://biobank.health.nsw.gov.au/standard-operating-procedures/2016.
21. Biomarkers Definitions Working Group. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther. 2001;69(3):89-95.
22. Ziegler A, Koch A, Krockenberger K, et al. Personalized medicine using DNA biomarkers: a review. Hum Genet. 2012;131(10):1627-38.
23. Diagnostics B. Order of Draw Chart. In: West P, editor. 2012.
24. Biosciences B. PAXgene ® Blood RNA Tube Instructions for Use.
25. Mychaleckyj JC, Farber EA, Chmielewski J, et al. Buffy coat specimens remain viable as a DNA source for highly multiplexed genome-wide genetic tests after long term storage. J Transl Med. 2011;9:91.
26. Greiner Bio-One. Vacuette Specimen Collection System. Greiner Bio-One | Making a difference (gbo.com).
27. Olsen AK, Li D, Li L. Explore the Dosimetric Relationship between the Intake of Chemical Contaminants and Their Occurrence in Blood and Urine. Environ Sci Technol. 2023.
28. Schrauben SJ, Sapa H, Xie D, et al. Association of urine and plasma ADMA with atherosclerotic cardiovascular disease risk in diabetic kidney disease: findings from the Chronic Renal Insufficiency Cohort (CRIC) study. Nephrol Dial Transplant. 2023.
29. Han L, Holland OJ, Da Silva Costa F, et al. Potential biomarkers for late-onset and term preeclampsia: A scoping review. Front Physiol. 2023;14:1143543.
30. Malamud D, Rodriguez-Chavez IR. Saliva as a diagnostic fluid. Dent Cl Nth Am. 2011;55(1).
31. Lopucki M, Wawrzykowski J, Geca T, et al. Preliminary analysis of the protein profile in saliva
during physiological term and preterm delivery. Mol Med Rep. 2018;17(6):8253-9.
32. Sun F, Reichenberger EJ. Saliva as a source of genomic DNA for genetic studies: review of current methods and applications Oral Health Den Man. 2014;13(2):217-22.
33. Liu J, Qiu X, Wang D, et al. Quantification of 10 steroid hormones in human saliva from Chinese adult volunteers. J Int Med Res. 2018;46(4):1414-27.

34. Wood P. Salivary steroid assays – research or routine? Ann Clin Bio. 2009;46:183-96.
35. Crombie GK, Palliser HK, Shaw JC, et al. Effects of prenatal stress on behavioural and neurodevelopmental outcomes are altered by maternal separation in the neonatal period. Psychoneuroendocrinology. 2021;124(105060).
36. Ahnert L, Gunnar MR, Lamb ME, et al. Transition to child care: associations with infant–mother attachment, infant negative emotion, and cortisol elevations. Child Dev. 2004;75(3):639-50.
37. Dorn LD, Lucke JF, Loucks TL, et al. Salivary cortisol reflects serum cortisol: analysis of circadian profiles Ann Clin Bio. 2007;44(3):281-4.
38. O'Connor TG, Heron J, Golding J, et al. Maternal antenatal anxiety and children's behavioural/emotional problems at 4 years. Report from the Avon Longitudinal Study of Parents and Children. Brit J Psych. 2002;180(6):502-8.
39. Glover V, O'Donnell KJ, O'Connor TG, et al. Prenatal maternal stress, fetal programming, and mechanisms underlying later psychopathology-A global perspective. Dev Psych. 2018;30(3):843-54.
40. Van den Bergh BRH, Alfons Marcoen A. High antenatal maternal anxiety is related to ADHD symptoms, externalizing problems, and anxiety in 8- and 9-year-olds. Child Dev. 2004;75(4):1085-97.
41. O'Connor TG, Heron J, Golding J, Glover V, and the ALSPAC Study Team. Maternal antenatal anxiety and behavioural/ emotional problems in children: a test of a programming hypothesis. J Child Psych Psych. 2003;44(7):1025–36.
42. Laucht M, Esser G, Baving L, et al. Behavioral sequelae of perinatal insults and early family adversity at 8 years of age. J Am Ac Child Adol Psych. 2000;39(10):1229-37.
43. Laplante DP, Barr RG, Brunet A, et al. Stress during pregnancy affects general intellectual and language functioning in human toddlers. Ped Res. 2004;56(3):400-10.
44. Watson JB, Mednick SA, Huttunen M, et al. Prenatal teratogens and the development of adult mental illness. Dev Psych. 1999;11(3):457-66.
45. Eipel M, Mayer F, Arent T, et al. Epigenetic age predictions based on buccal swabs are more precise in combination with cell type-specific DNA methylation signatures Aging. 2016;8(5):1034-44.
46. Winchester P, Nilsson E, Beck D, et al. Preterm birth buccal cell epigenetic biomarkers to facilitate preventative medicine. Sci Reports. 2022;12(3361).
47. van Dongen J, Ehli EA, Jansen R, et al. Genome-wide analysis of DNA methylation in buccal cells: a study of monozygotic twins and mQTLs. Epi Chrom. 2018;11(54).
48. Nash MJ, Frank DN, Friedman JE. Early Microbes Modify Immune System Development and Metabolic Homeostasis-The "Restaurant" Hypothesis Revisited. Front Endocrin. 2017;8(349).
49. Bilbo SD, Levkoff LH, Mahoney JH, et al. Neonatal infection induces memory impairments following an immune challenge in adulthood. Behav Neuro. 2005;119(1):293-301.
50. Milani C, Duranti S, Bottacini F, et al. The first microbial colonizers of the human gut: composition, activities, and health implications of the infant gut microbiota. Micro Molec Biol Rev. 2017;81(4):e00036-17.
51. Uzan-Yulzari A, Turta O, Belogolovski A, et al. Neonatal antibiotic exposure impairs child growth during the first six years of life by perturbing intestinal microbial colonization. Nat Comm. 2021;12(443).
52. Bokulich NA, Chung J, Battaglia T, et al. Antibiotics, birth mode, and diet shape microbiome maturation during early life. Sci Trans Med. 2016;8(343):343ra82.
53. Charbonneau MR, Blanton LV, DiGiulio DB, et al. A microbial perspective of human developmental biology Nat. 2016;535:48-55.
54. Tsukuda N, Yahagi K, Hara T, et al. Key bacterial taxa and metabolic pathways affecting gut short-chain fatty acid profiles in early life. ISME J. 2021(15):2574–90.
55. Stewart CJ, Ajami NJ, O'Brien JL, et al. Temporal development of the gut microbiome in

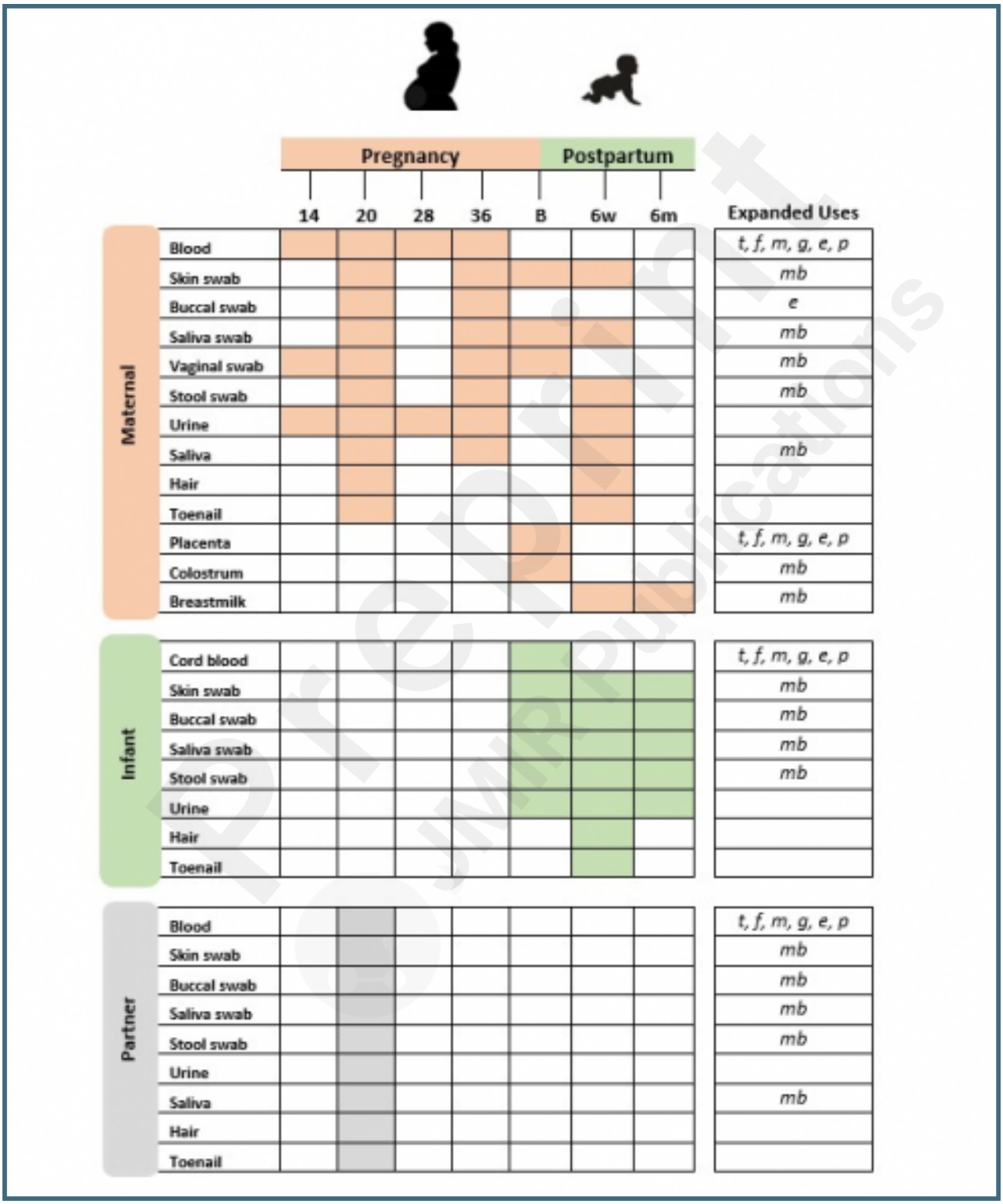
early childhood from the TEDDY study. Nat. 2018;562(7728):583-8.
56. Koren O, Goodrich JK, Cullender TC, et al. Host remodeling of the gut microbiome and metabolic changes during pregnancy. Cell. 2012;150(3):470-80.
57. Grech A, Collins C, Holmes A, et al. Maternal exposures and the infant gut microbiome: a systematic review with meta-analysis. Gut Microbes. 2021;13:1.
58. Bäckhed F, Roswall J, Peng Y, et al. Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host & Microbe. 2015;17(5):690-703.
59. Yassour M, Vatanen T, Siljander H, et al. Natural history of the infant gut microbiome and impact of antibiotic treatments on strain-level diversity and stability Sci Trans Med. 2016;8(343).
60. Koren O, Goodrich JK, Cullender TC, et al. Host remodeling of the gut microbiome and metabolic changes durin pregnancy. Cell. 2012;150(3):470-80.
61. Yu Z, Morrison M. Improved extraction of PCR-quality community DNA from digesta and fecal samples. BioTechniques. 2004;36(5):808-12.
62. Wood DLA, Lachner N, Tan J-M, et al. A natural history of actinic keratosis and cutaneous squamous cell carcinoma microbiomes. mBio. 2018;9(5):e01432-18.
63. Pribyl A, Parks DH, Angel N, et al. Critical evaluation of faecal microbiome preservation using metagenomic analysis. ISME Comm. 2021;1(14).
64. Ma Y, He X, Qi K, et al. Effects of environmental contaminants on fertility and reproductive health. J Environ Sci. 2019;77:210-17.
65. Dai Y, Huo X, Cheng Z, et al. Early-life exposure to widespread environmental toxicants and maternal-fetal health risk: A focus on metabolomic biomarkers. Sci Tot Environ. 2020;739.
66. Windham G, Fenster L. Environmental contaminants and pregnancy outcomes. Fert Ster. 2008;89(2):e111-6.
67. Naspolini NF, Meyer A, Moreira JC, et al. Environmental pollutant exposure associated with altered early-life gut microbiome: Results from a birth cohort study. Environ Res. 2022;205.
68. Paulesu L, Rao CV, Ietta F, et al. hCG and its disruption by environmental contaminants during human pregnancy. Int J Mol Sci. 2018;19(3).
69. Martín-Pozo L, Cantarero-Malagón S, Hidalgo F, et al. Determination of endocrine disrupting chemicals in human nails using an alkaline digestion prior to ultra-high performance liquid chromatography–tandem mass spectrometry. Talanta. 2020;208.
70. Davis MA, Li Z, Gilbert-Diamond D, et al. Infant toenails as a biomarker of in utero arsenic exposure. J Exposure Sci Environ Epid. 2014;24:467-73.
71. Farzan SF, Howe CG, Chen Y, et al. Prenatal and postnatal mercury exposure and blood pressure in childhood. Environ Int. 2021;146.
72. Han X, Chen H, Shen M, et al. Hair and nails as noninvasive bioindicators of human exposure to chlorinated paraffins: Contamination patterns and potential influencing factors. Sci Total Environ. 2021;798.
73. Jian J-M, Chen D, Han F-J, et al. A short review on human exposure to and tissue distribution of per- and polyfluoroalkyl substances (PFASs). Sci Total Environ. 2018;636.
74. Kintz P, Russell E, Baber M, et al. Clinical Applications of Hair Analysis. In: Kintz P, Salomone A, Vincenti M, editors. Hair Analysis in Clinical and Forensic Toxicology2015. p. 141-59.
75. Wright KD, Hickman R, Laudenslager ML. Hair cortisol analysis: A promising biomarker of HPA activation in older adults Gerontol. 2015;55:S140-S5.
76. Tong EK, Fung LC, Stewart SL, et al. Impact of a biospecimen collection seminar on willingness to donate biospecimens among Chinese Americans: results from a randomized, controlled community-based trial Cancer Epid, Bio Prev. 2014;23(3):392-401.
77. Herbertson EC, Lahiri CD, Nwogu JN, et al. High acceptability of donating hair and other biological samples for research among people living with HIV in an outpatient clinic in Lagos, Nigeria. AIDS Res Hum Retroviruses. 2021;37(9):676-82.
78. Burton GJ, Sebire NJ, Myatt L, et al. Optimising sample collection for placental research.

Placenta. 2014;35(1).
79. Annaratone L, De Palma G, Bonizzi G, et al. Basic principles of biobanking: from biological samples to precision medicine for patients. Virchows Archive. 2021;479(2):233-45.
80. OpenSpecimen. Biobanking LIMS Features https://www.openspecimen.org/biobanking-lims-features/ [

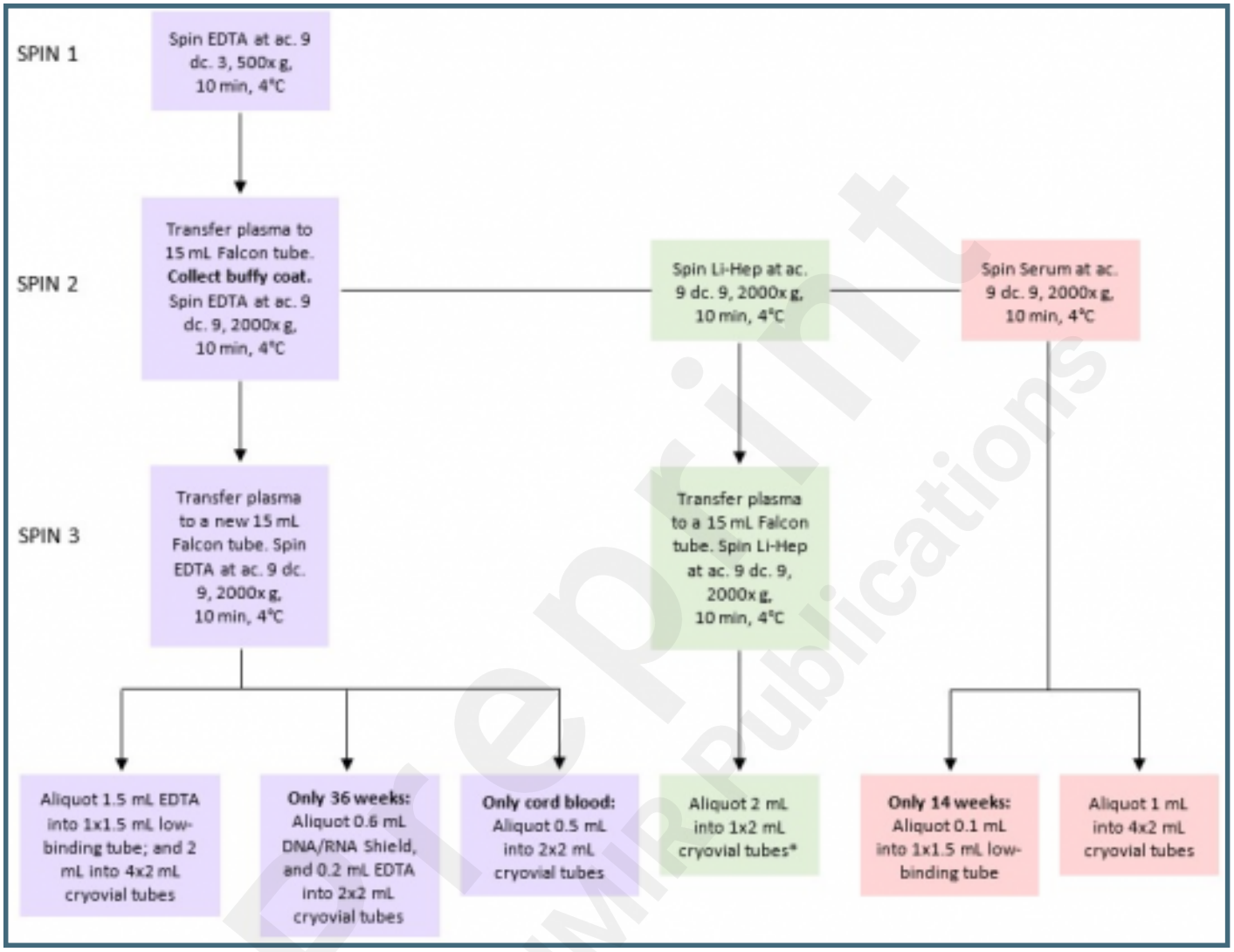
Supplementary Files

Figures

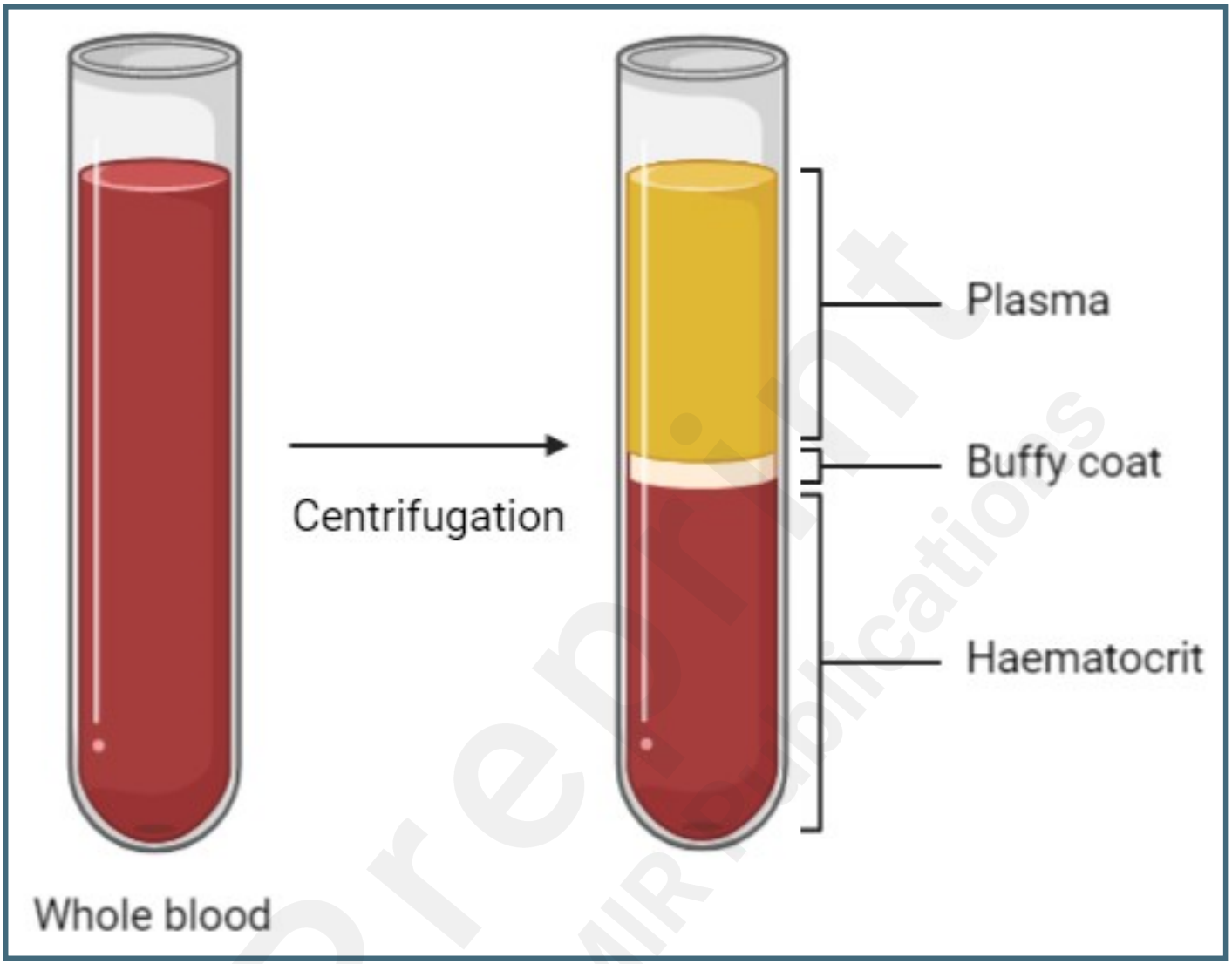
NEW1000 Stage One biological sample collection timepoints. NEW1000 aims to provide a comprehensive database for use in traditional analysis and emerging omics platforms. Expanded uses denotes the intended platforms and appropriate approaches based on the collection methods highlighted within the manuscript; t - transciptomics, f - fragmentome, m – metabolome, g – genome, e – epigenome, p - proteome, mb – microbiome.



Flow diagram for the processing of bloods. Note: * Lithium heparin plasma can be divided evenly between two 2 mL cryovial tubes if there is >2 mL plasma; ac. = acceleration; dc. = deceleration. Purple boxes indicates the processing flow of EDTA tubes, Green depicts Lithium Heprin tubes, and Pink shows Serum tubes.



Centrifugation of whole blood to separate blood components: plasma; buffy coat, which contains white blood cells and platelets; and haematocrit (RBCs). (Figure created in BioRender).



Overview of collection process for placental samples (figure created in Biorender).