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STUDY PROTOCOL

REVISED Study protocol for the Bio-HEAT study: Investigating the Biological pathways from HEAT exposure to preterm birth and other adverse maternal and child health outcomes in South Africa

[version 2; peer review: 2 approved, 1 approved with reservations]

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v2 **First published:** 05 Mar 2025, **10**:121
https://doi.org/10.12688/wellcomeopenres.23616.1
Latest published: 06 Jun 2025, **10**:121
https://doi.org/10.12688/wellcomeopenres.23616.2

Abstract

Introduction

Epidemiological evidence linking heat exposure to adverse maternal and child health outcomes is compelling. However, the biological and social mechanisms underlying these associations remain poorly understood. Understanding the pathways explaining these associations is important given rising global temperatures, and the urgent need for developing and testing adaptive interventions.

Methods

This transdisciplinary study in Johannesburg, South Africa, will monitor a cohort of 200 women from their second trimester until one-year postpartum, alongside their infants. Heat exposure and environmental factors will be tracked using personal, community and facility-level temperature monitors and geospatial data. Data will be collected on social conditions, medical and obstetric history, heat stress and adaptation, hydration, mental wellbeing, and sleep quality. Clinical data includes physical measurements, ultrasound, cardiotocography, and biological specimens (blood, urine, saliva) analysed for inflammatory markers, RNA, metabolic indicators, renal function and hormonal levels. Placental and cord blood analyses will assess foetal stress. Infant data will include medical history, hospital visits, neurodevelopment, anthropometric measurements, vital signs, and urine analysis. Three nested sub-studies (20–50 participants) will explore specific aspects: Sub-study 1 will use wearable devices to monitor sleep, activity, and heart rate in high-risk women; Sub-study 2 will involve qualitative interviews; and Sub-study 3 will assess breastmilk composition and volume.

Planned analyses

Our primary aim is to document linkages between heat exposure and inflammatory pathways that precede preterm birth. The hypothesis that heat exposure triggers maternal inflammation will be tested by analysing epigenetic changes associated with inflammatory cytokine protein and gene expression. We will investigate thermoregulation and hydration during labour. Using isotope techniques, we assess whether heat exposure alters breastmilk composition and volume.

Open Peer Review

Approval Status ✓ ? ✓

	1	2	3
version 2 (revision) 06 Jun 2025			✓ view
version 1 05 Mar 2025	✓ view	? view	

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Any reports and responses or comments on the article can be found at the end of the article.

Conceptual frameworks and graphical causal models will be developed to delineate pathways of vulnerability and protective mechanisms.

PLAIN LANGUAGE SUMMARY

Climate change is causing global temperatures to rise, leading to more frequent and intense heatwaves. This extreme heat can have health risks for pregnant women and their babies such as preterm birth and low birth weight. However, we do not yet fully understand how pregnant women's body's respond to heat, or why these health risks occur. Gaining this knowledge will help us to protect mothers and babies as the world gets warmer.

The Bio-HEAT study will follow 200 pregnant women from an underserved urban community in Johannesburg, South Africa. Heat exposure in the area is worsened by the Urban Heat Island effect where buildings, road and industry trap heat. Many people in this community don't have access to cooling systems like air conditioning, and some use solid fuels like wood and coal for cooking, which makes their homes hotter. These conditions make them especially vulnerable to heat.

Our main goal is to understand how heat exposure might trigger inflammation and other changes in the body and lead to preterm birth. Women will be followed from their second trimester through to one year postpartum, along with their infants. Researchers will collect information through questionnaires, biological samples (like blood, urine and saliva), ultrasound scans, and wearable devices that measure heat exposure. Placenta and cord blood will be assessed for the effects of heat on babies before birth. The babies growth and health will also be monitored.

The study includes three smaller sub-studies: one to monitor activity, sleep and heart rate; another to gather personal experiences, and a third to explore how heat affects breastfeeding.

The findings from the study will help to inform strategies to reduce heat-related health risks for pregnant women and babies in similar urban settings globally.

Keywords

Hot Temperature, Inflammation, Pregnancy, Premature birth, Maternal health, Breastfeeding, Infant health, Epigenetics, Biological markers, South Africa

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Competing interests: IS, DPL, NB and MFC hold investments in the fossil fuel industry through their pension funds. The other authors declare no competing interests.

Grant information: This work was supported by Wellcome [227204]. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.
The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Solarin I, Lakhoo DP, Mc Alpine K *et al.* **Study protocol for the Bio-HEAT study: Investigating the Biological pathways from HEAT exposure to preterm birth and other adverse maternal and child health outcomes in South Africa [version 2; peer review: 2 approved, 1 approved with reservations]** Wellcome Open Research 2025, **10**:121
https://doi.org/10.12688/wellcomeopenres.23616.2

First published: 05 Mar 2025, **10**:121 https://doi.org/10.12688/wellcomeopenres.23616.1

REVISED **Amendments from Version 1**

Summary of changes

We have revised the manuscript in response to reviewer feedback. Key changes include: addition of a supplementary table detailing study measurements and their rationale; clarification of clinical assessments and data collection on pregnancy and birth complications; expanded sections on quality assurance and data management; clearer explanation of public and community involvement; updates to the inflammatory marker threshold; and inclusion of additional references throughout the manuscript.

Any further responses from the reviewers can be found at the end of the article

Introduction

Climate change, driven by anthropogenic greenhouse gas emissions, is one of the greatest threats to global health[1]. Rising global temperatures are one of the most concerning manifestations of climate change, resulting in higher seasonal temperatures and more frequent, intense heatwaves[2]. According to NASA, 2024 was the warmest year on record, approximately 1.47°C above the pre-industrial average[3]. Global temperatures are projected to rise substantially further by 2070, potentially exposing one-third of the global population to mean annual temperatures exceeding 29°C – conditions currently limited to 0.8% of the Earth's land surface[4].

Recently, pregnant women, foetuses and neonates have been identified as particularly vulnerable to the increasing ambient temperatures[5]. Humans regulate their core temperature within a narrow range of 35.5–37.5°C[6], by balancing heat loss through behavioural and physiological mechanisms, with heat gain from metabolic (internal) and environmental (external) sources[7]. Physiological and anatomical changes during pregnancy may impair normal thermoregulation, increasing susceptibility to heat-related stress[5]. Heat exposure during pregnancy has been linked to several adverse pregnancy outcomes including hypertensive disorders of pregnancy[8,9], pre-term birth[8,10], low birthweight[8,10], reduced foetal growth[8,11], stillbirths[8,10], and congenital anomalies[8,12]. Heat exposure in utero has also been associated with a range of detrimental health and social outcomes across the life-course[13]. Underlying biological mechanisms have not yet been characterised, but are hypothesised to include elevated maternal core temperatures[14], endocrine dysfunction[14], inflammation[14], epigenetic changes[7], dehydration[7,14], disrupted breastfeeding[15,16], and compromised placental development[7,10,14]. A recent review provides an overview on hypothetical pathophysiological mechanisms that may underlie the links between heat and preterm birth (PTB)[17]. High ambient temperatures and adverse pregnancy outcomes are of particular concern in climate change hotspots, such as sub-Saharan Africa[1,18]. Despite some progress in recent decades, Africa ranks among the regions with the highest rates of maternal mortality, preterm births and other adverse pregnancy outcomes worldwide[19,20], carrying significant implications for health and development.

Africa is disproportionately vulnerable to the effects of climate change, owing to its warm and dry climate with variable rainfall patterns, and low adaptive capacity[21]. Southern Africa, in particular, is a climate-change hotspot, with temperatures rising at about twice the global rate and more frequent heatwaves of unprecedented intensity[2,22]. In a future with low mitigation efforts, the region is projected to become significantly hotter and drier, with even more variable rainfall[23–25]. Furthermore, sub-Saharan Africa remains a region with persistently high birth rates[26], and projections indicate that the paediatric population will be the largest in the world by 2055, surpassing 1 billion. Rapidly rising temperatures and other climate-related hazards threaten to exacerbate these challenges, further straining maternal and child health programs[27–29].

Current global efforts to reduce emissions fall significantly short of the scale and pace required to avert dangerous climate change[30]. Between 2030 and 2050, climate change is expected to cause approximately 250 000 additional deaths per year from malnutrition, malaria, diarrhoea and heat stress[31]. Implementing adaptation measures alongside mitigation efforts is therefore crucial to reduce climate change-related mortality and morbidity. However, increased understanding of the biological effects of heat in humans is essential for designing effective, targeted adaptive interventions to protect vulnerable populations such as pregnant women, foetuses and neonates[8,14].

Research aims

The Bio-HEAT study aims to explore the underlying associations between heat exposure and the biological and social pathways that contribute to preterm birth, influence intrapartum outcomes, and impact breastfeeding.

Hypotheses

The primary hypothesis of the Bio-HEAT study is that exposure to high ambient temperatures during pregnancy is associated with elevated pro-inflammatory cytokine levels, such as interleukin-6 (IL-6) and other inflammatory markers, driven by heat-induced changes in gene expression, such as transcriptomic and epigenetic signatures. Figure 1 below demonstrates the plausible pathways we are investigating.

We have four secondary hypotheses:

- *Heat and systematic changes*: High ambient temperatures are hypothesised to disrupt endocrine function, enhance oxidative stress and inflammation, and increase the release of heat-shock proteins during pregnancy.

- *Interacting factors:* We hypothesise that multiple interacting factors, such as oxidative stress, altered cytokine profiles and psycho-social stressors contribute to the risk of preterm birth. Our analysis will account for these variables, assessing how they may mediate the relationship between heat exposure, and adverse maternal and neonatal outcomes. Furthermore, as psychosocial factors may evolve over pregnancy, we hypothesize that these fluctuations could influence health outcomes, such

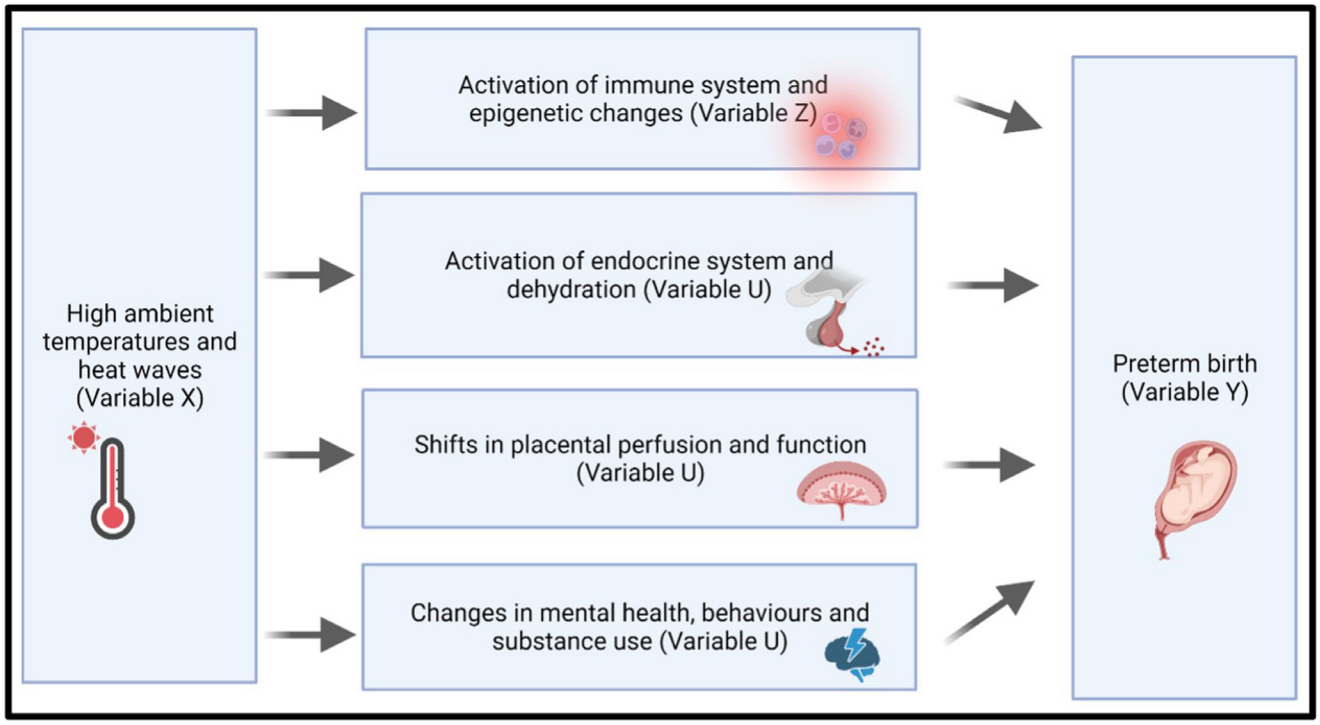


Figure 1. Proposed biological pathways linking heat exposure to preterm birth. Hypothesised mechanisms linking high ambient temperatures and heat waves (Variable X) to adverse maternal and neonatal outcomes, specifically preterm birth (Variable Y).

as preterm birth, and that these links may be accentuated by heat exposure.

- *Pathophysiological mechanisms in labour:* Heat exposure during labour is hypothesised to increase labour duration due to dehydration, and interventions such as hydration and cooling could reduce these effects.

- *Breastfeeding and heat exposure:* We hypothesise that heat exposure will reduce breastfeeding duration and frequency, and increase the sodium content of breastmilk primarily due to dehydration and discomfort experienced by both mother and infant during breastfeeding.

Global health relevance of this study

Understanding the biological mechanisms and social vulnerabilities underpinning heat-health associations in pregnancy is critical in the context of rising global temperatures and the need for adaptive interventions. Adverse pregnancy outcomes, such as preterm birth, significantly influence health and developmental trajectories throughout the life course. While both high and low ambient temperatures have been associated with poor obstetric outcomes[32,33], this study focuses on heat exposure, which is of particular concern in our African context. By elucidating the links between heat exposure, inflammatory pathways, and preterm birth—and identifying actionable factors to mitigate these risks—this research has the potential to yield substantial public health benefits.

Additionally, examining how heat exposure affects hydration status and adverse outcomes during labour, and evaluating interventions to address these concerns, is essential. Breastfeeding, a cornerstone of child health, also warrants investigation to determine how climate-induced heat exposure may impact breastfeeding patterns and breastmilk composition. Figure 2 depicts three mechanistic pathways that we hypothesise contribute to the biological vulnerability of infants to dehydration.

Bio-HEAT will deliver detailed research findings on the relationships between heat exposure and biological vulnerabilities, with a focus on preterm birth, heat stress during labour, and breastfeeding. These insights will inform the development of conceptual frameworks that integrate the perspectives of affected women, ultimately guiding interventional research to address key modifiable factors in these pathways. A list of study measures, along with the rationale for each, is provided in a supplemental file (Appendix 1).

Methods

Study setting

The study will be conducted at a public regional hospital in Johannesburg which offers specialised maternal and paediatric care. The study site is situated in a high-density urban environment characterised by an Urban Heat Island effect, where temperatures can be significantly higher than the surrounding areas. Most residents in this area lack access to air

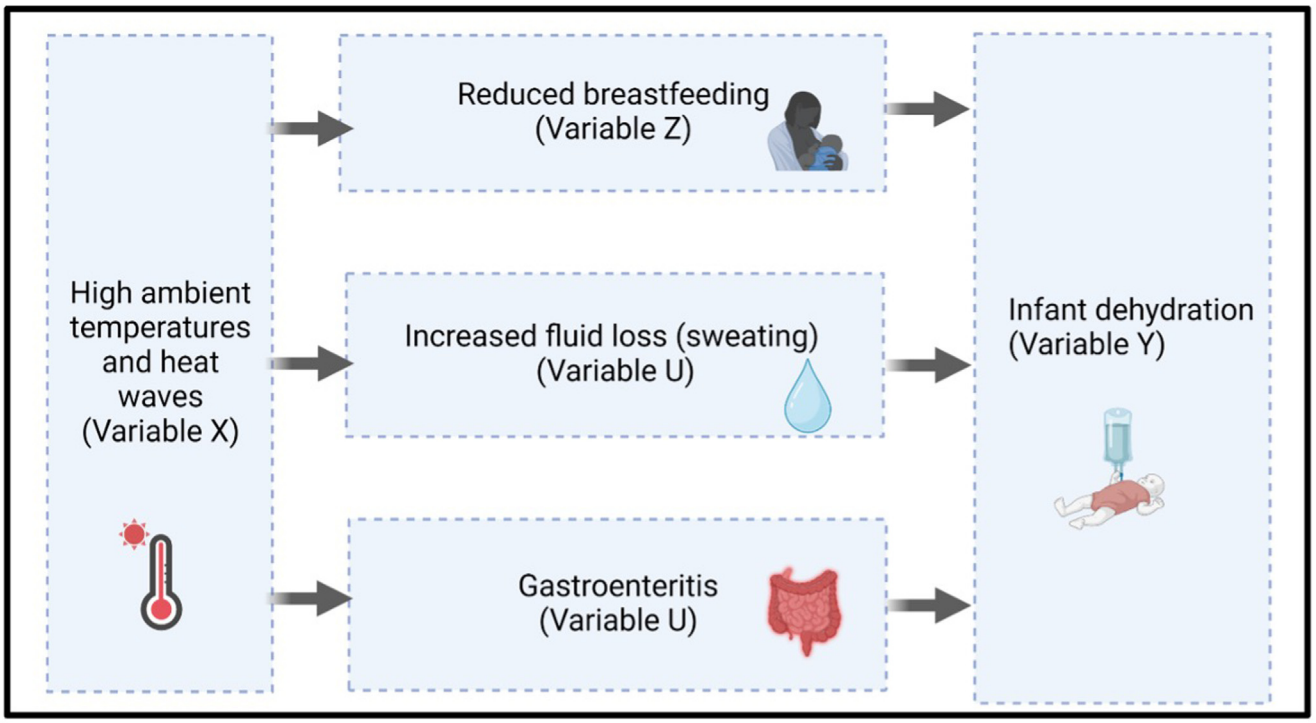


Figure 2. Proposed biological pathways from heat exposure to infant dehydration. Hypothesised mechanisms through which high ambient temperatures and heat waves (Variable X) may contribute to infant dehydration (Variable Y).

conditioning, active or passive cooling systems, and urban greenery, exacerbating their heat vulnerability. Additionally, indoor cooking practices that involve combustion of solid fuels may augment heat exposure and degrade indoor air quality, complicating the health risks associated with high temperatures[30]. Against this backdrop, Bio-HEAT is well-positioned to provide valuable insights into the causal pathways underpinning heat-health impacts in pregnant women and infants that may be generalisable to similar urban environments globally.

Study design and participants

Bio-HEAT is a prospective cohort study designed to collect comprehensive data on heat exposure and its biological impacts on pregnant women and infants. Participants will be recruited from the regional hospital's antenatal clinic, targeting women attending their 12-week nuchal translucency scan. Additional participants may also be recruited from the gynaecology ward, and other antenatal clinics in the catchment area as needed.

Participants will be followed from week 12–16 of pregnancy through to 12 months postpartum. The study comprises an overarching cohort and three nested sub-cohorts, purposively recruited from the overall cohort, each addressing specific research questions. Sample sizes as well as inclusion and exclusion criteria, are detailed below.

Overall cohort: Biological impacts of heat exposure on pregnant and postpartum women and their infants (N=200). Inclusion criteria for the overall cohort are as follows:

- Gestational age of 12–16 weeks at enrolment based on ultrasound assessment of gestation
- Aged 16 years or older
- Resident in the hospital catchment area (hospital surrounds)
- Intending to give birth at the study site and remain in the catchment area for the duration of the study
- Able and willing to give informed consent
- Agrees not to enrol in another study that may interfere with the results and interpretation of findings in this study

Exclusion criteria include:

- Critical illness at enrolment, defined as hospital admission or at the discretion of the research doctor
- Planning to deliver at an alternative maternity facility
- Multiple pregnancy
- Pregnancy with suspected or confirmed congenital anomaly prior to enrolment

Sub-cohort 1: Enhanced individualised activity and heat exposure among the highest-risk women (N=50) will include women with two or more of the following risk factors:

- High heat exposure: Resides in low-cost housing, or has occupational heat exposure (e.g., working in kitchens or outdoor labour)
- Any two of the following:

  Pre-existing maternal health condition (e.g., chronic hypertension, diabetes, renal dysfunction, autoimmune diseases, thyroid dysfunction, obesity (BMI>=30), HIV infection), maternal age–teenage pregnancy (age<20) or primiparous at ≥35 years old

Sub-cohort 2: Psychosocial factors along the pathway from heat exposure to prematurity (N=20–25). Serial qualitative interviews involving two groups of women, with final sample determined by data saturation:

- Antepartum group: 10–15 women between 12–16 weeks gestation
- Postpartum group: Up to 10 women who have had a preterm birth

Sub-cohort 3: Heat exposure and breastfeeding (N=50).

- Participants will be conveniently sampled from the overall cohort with no additional selection criteria applied.

Sample size considerations

Biological pathways analyses involve consideration of a wide range of outcomes. We calculated sample size to test the primary hypothesis using binary outcomes as a conservative approach. Continuous outcomes will offer considerably greater statistical power.

The primary inflammatory outcome interleukin-6 is strongly linked with PTB[34–36]. However, there is currently no universally accepted threshold for IL-6 in predicting PTB, with high levels of variability across studies. We have based our sample size on IL>10pg/ml as a conservative measure in keeping with other studies which identify maternal serum levels around this value as predictive of PTB[37]. With 200 women, two measures per participant, and an assumed 15% specimen loss, the study has >80% power to detect an 8% difference in the primary outcome. This calculation assumes that 5% of women exposed to the lowest quartile of maximum temperatures at a 0–2 day lag will exhibit raised interleukin-6 levels.

Heat exposure monitoring

Monitoring heat exposure involves three components:

- Facility Monitoring: The study site will be equipped with indoor temperature sensors (HOBOnet wireless sensor or similar) to monitor the environments where the participants receive care, including the antenatal clinic and labour ward.
- Community Monitoring: An outdoor weather station (Davis Vantage Pro-2), covering the entire catchment area, will be installed on the premises of the facility to accurately capture temperature exposure in the area.
- Personal Monitoring: Participants will wear wrist-mounted thermometers (ibuttons) throughout the study to unobtrusively and continuously record temperature and humidity exposure, delivering real-time, individual-level environmental data. To supplement this, geolocation of home addresses and pinned locations will be used to confirm housing types. Geolocated addresses will be securely linked with our existing geospatial grid data in Johannesburg, including economic status, vegetation indexes, temperature, air pollution, noise, ozone and other exposure variables.

Study schedule

The study schedule, summarised in Figure 3, includes a screening and enrolment visit, followed by antepartum follow-up visits every four weeks during pregnancy (at 16, 20, 24, 28, 32, 36, and 41 weeks- for those still pregnant) and an intrapartum visit. Postpartum follow-up visits will occur at 1, 3, 6, 9, and 12 months. Whenever possible, study visits will be coordinated with routine antenatal care appointments or baby wellness checks. The research unit is conveniently located near the hospital labour and gynaecology wards, ensuring easy access for participants. A window of +/- 14 days will apply to all scheduled visits, except during heat waves, defined as a maximum temperature >95th percentile for 2 or more consecutive days, when an additional study visit will be scheduled for selected participants within a 2-day window of the heatwave. Participants will be provided with a contact number for the study staff for questions, complaints or to inform the study staff of labour or any complications or adverse events.

Enrolment visit

Overall cohort. Following enrolment, participants will be asked questions on a range of sociodemographic factors, medical and obstetric history, behaviours related to heat adaptation and hydration (e.g., access to water and cool spaces, hydration practices, heat-related behaviours), mental health conditions and habits (substance use and activity levels). We will use validated tools including an Adapted Timeline Alcohol Use Calendar and the Alcohol Use Disorders Identification Test (AUDIT) to assess alcohol use[38], the Whooley antenatal depression and anxiety screening tool[39], the Edinburgh Postnatal Depression Scale (EPDS)[40], the Primary Care Post Traumatic Stress Disorder (PTSD) screening tool[41] to assess mental well-being, and the Pittsburgh Sleep Quality Index[42] to assess sleep. Each of these tools are available for use without further permissions required. Several tools have been validated in the study site or nearby facility. Other relevant data will be extracted from the maternity case record to reduce participant burden.

The participants will be examined clinically, including blood pressure measurements, weight and height, and a general and system-specific examination to assess overall maternal health. A foetal ultrasound will also be conducted to confirm an intrauterine, viable pregnancy, the number of foetuses, and to

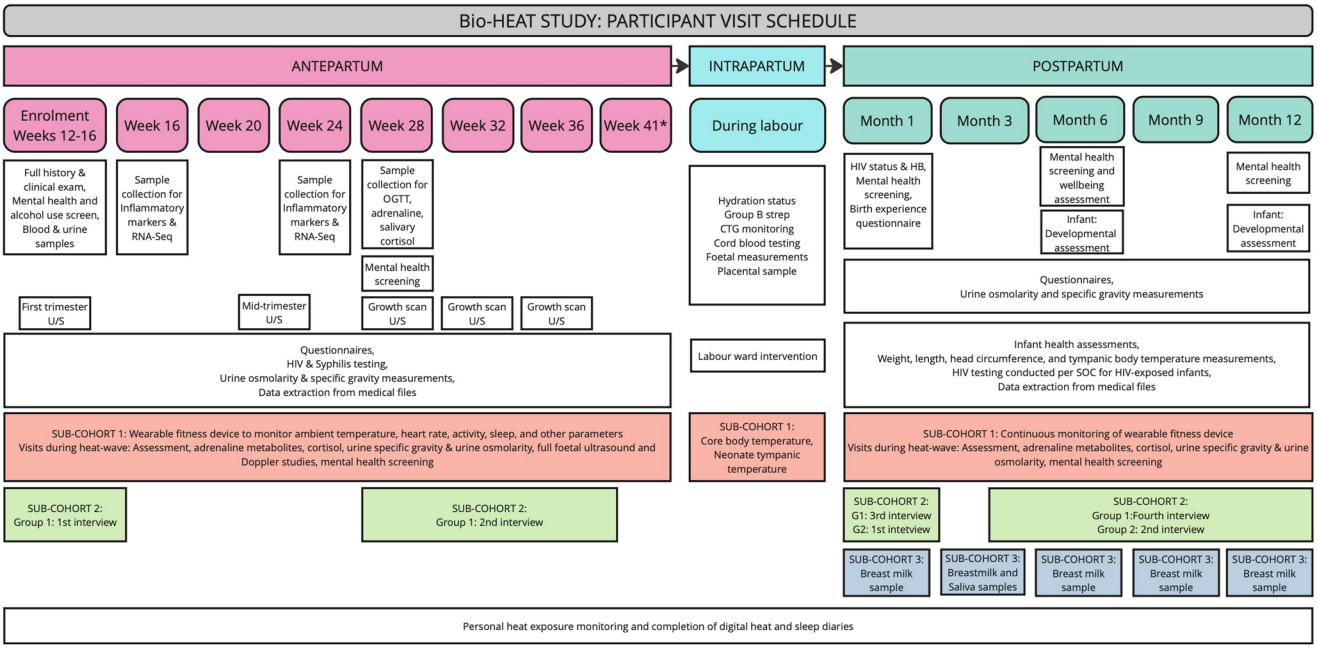


Figure 3. Summary of Participant Visit Schedule. Schedule of visits showing key assessments and unique sample collection across the antepartum, intrapartum, and postpartum periods for the overall cohort and the three sub-cohorts (Overall cohort- white; Sub-cohort 1- orange; Sub-cohort 2- green; Sub-cohort 3- blue).

confirm gestational age. If not already conducted at the antenatal clinic, blood will be drawn to test for renal function, thyroid function, HBA1c, a full blood count, and infections (HIV and syphilis). Cotinine testing will be done to assess nicotine exposure. A mid-stream urine sample will be collected to test for markers of dehydration, specifically osmolarity and urine-specific gravity.

Women will be given a personal wearable thermometer (ibuttons mounted on silicone wrist bands) and will be encouraged to regularly complete a WhatsApp based digital heat and sleep diary on their phones.

Sub-cohort 1: High-risk women with individualised activity and heat exposure monitoring. The participants included in this sub-study will be additionally equipped with a wearable fitness device (Garmin Vivosmart 5) to facilitate continuous measurement of heart rate, activity, sleep, and other metrics.

Sub-cohort 2: Psychosocial factors. Women in this sub-cohort will complete their first in-depth interview at a convenient time after their enrolment visit and before the 2nd study visit. The interviews will be structured around three key areas: 1) socio-economic circumstances, 2) psychological circumstances, and 3) experience of weather and heat. These aspects will be explored initially and reassessed at subsequent interviews, with a focus on changes over time, weather, and the post-birth period. The interview guide is designed to be flexible, enabling questions to be repeated or further explored during each interview. Interviews will be audio-recorded and transcribed continuously for ongoing analysis. The research team will meet weekly to review transcripts and ensure data saturation is reached. Each participant will have a file containing written notes and transcripts for the team to review in advance of each interview.

Antepartum study visits

Overall cohort. As described above, antepartum study visits will take place 4-weekly between 16 and 41 weeks. At each visit we will repeat the questionnaires on heat adaptation and hydration as well as the sleep quality index. Mental health screening will also be conducted at visit 5 through the use of the following publicly available tools: The Patient Health Questionnaire (PHQ-9)[43], the General Anxiety Disorder questionnaire (GAD-7)[44], the World Health Organisation Disability Assessment Schedule (WHODAS-12)[45]. Each of these tools assess different aspects of mental wellbeing, complementing the other tools administered at enrolment, and offer a multidimensional and holistic assessment of mental wellbeing. Study staff will review the data from wearables, and sleep diaries, assessing completeness, accuracy and quality.

Participants will be clinically examined at each visit and blood samples will be taken to test for HIV and syphilis if not already done at the antenatal clinic. At 16-and 28 weeks' gestation, additional blood samples will be collected for inflammatory markers and RNA-Seq gene expression and stored for future analysis. Between 24–28 weeks, an oral glucose

tolerance test will be performed, and samples collected to assess adrenaline metabolites (metanephrine and Vanillylmandelic Acid (VMA). Participants will also provide an early morning saliva sample for cortisol and a midstream urine sample to assess dehydration markers (osmolarity and specific gravity). If dehydration is indicated, participants will be advised to increase fluid intake. A mid-trimester ultrasound will be conducted between weeks 20–24, and follow up growth scans at 28, 32, and 36 weeks. Additional scans may be conducted if clinically indicated.

Sub-cohort 2: Psychosocial factors. Women in this sub-cohort will be interviewed again in their last trimester to provide insights into the changing nature of psychosocial circumstances and relationships, as well as how climate may have influenced these.

Intrapartum study visit

Overall cohort. During labour, the participant will be encouraged to continue wearing their personal wearable temperature monitoring device. Study staff will document the participant's hydration practices and measure hydration status through urine osmolarity, urine specific gravity and blood tests (urea, creatinine and electrolytes). In addition, a vaginal and rectal swab to test for group B streptococcus colonisation will be conducted during labour. Lastly, the women and their foetus will be monitored continuously using a cardiotocograph as part of the routine standard of care and within the confines of the available resources within the labour ward at the hospital. Labour progress, mode of delivery, maternal and foetal morbidity, Apgar scores, foetal weight, length, and head circumference, and additional relevant information will be extracted from the maternity case records. Cardiotocography data will also be documented.

Following labour, placentas will be collected according to site-specific standard operating procedures (SOPs). Placentas will be sent for histological analysis using the Amsterdam classification system which defines four major patterns of placental injury, namely: maternal vascular malperfusion, foetal vascular malperfusion, acute chorioamnionitis, and villitis of unknown aetiology[46]. Placental membranes will also be sent for microscopy, culture and sensitivity analysis (MC&S). Where feasible, a sample of cord blood will be collected to test for biochemical markers of foetal stress.

Sub-cohort 1: High-risk women with individualised activity and heat exposure monitoring. During labour, this sub-cohort will also wear core body thermometers attached to the chest, if clinical care permits. Alternatively, repeated core body temperature measurements will be recorded within the standard of care during labour, for instance, during theatre procedures where all electronic devices must be removed. The neonate will have a core body temperature surrogate measurement (e.g. tympanic temperature) taken immediately post-delivery, and at 15-minute intervals thereafter for an hour after delivery. This will be used to inform the likely intrauterine temperature, due to limitations in measuring intrauterine temperature.

Labour ward intervention

We will collaborate with health workers to explore potential cooling and hydration interventions, guided by data from our ongoing and previous adaptation studies[16]. Intervention selection will involve a literature review and 2–3 consultative meetings between health workers in the maternal child health units, external experts and the study team. Interventions may include increased access to cool water from water coolers installed in the ward, evaporative cooling technologies, such as fans with damp cloths, and installation of shading structures on the windows in the labour ward. Once the interventions have been selected, we will design an intervention evaluation detailing the interventions and the evaluation methodology. As part of the intervention evaluation, we plan to compare labour duration, women's hydration levels and other relevant outcomes in women who received the interventions against historical data of labour outcomes in the facility prior to the study commencement.

Postpartum study visits

Overall cohort. During these visits, which will take place from month 1 and every 3 months post-delivery, the following questionnaires will be repeated: heat adaptation and hydration (every visit), substance use and activity levels (visits 1, 3, 5), and the sleep quality index. A questionnaire on breastfeeding practices will also be completed at every visit. Study staff will clinically examine participants and collect measurements (e.g., height, weight, waist, hip, and mid-upper arm circumference) and vital signs. At month 1, socio-economic conditions, mental health, and medical history will be reassessed and a birth experience questionnaire will be administered. Blood will be collected to check the participants' haemoglobin and HIV status. Mental health screening will be repeated at months 6 and 12. Monitoring of digital heat and sleep diaries will continue throughout. Urine samples will be collected at every visit to check hydration levels.

Infant follow up: History of any infant episodes of gastroenteritis or hospital admissions, as well as milestone achievements will be obtained from the mother. Data on vaccinations, admissions and any other relevant information will be extracted from the *Road to Health Card* (child health record) during each visit. Weight, length and head circumference will be measured at each visit and body temperature measured using a non-invasive tympanic temperature monitoring device. At month 6, the Ages and Stages neurodevelopmental screening questionnaire will be completed. In addition, at month 12 a developmental assessment will be conducted for all children using the Bayley Scales of Infant and Toddler Development (BSID). HIV testing will be conducted as per the standard of care for HIV-exposed infants, and results extracted from the maternity case record. In the case of a foetal/neonatal or infant death,

the postpartum women will be offered to continue her follow-up and referred for counselling, if required.

Sub-cohort 1: High-risk women with individualised activity and heat exposure monitoring. Monitoring of the wearable activity device will continue throughout the postpartum period for these participants.

Sub-cohort 2: Psychosocial factors. Women will be interviewed shortly after delivery and later during the first year of the infant's life. Women can also engage with the study team at their other visits to provide further details about their experiences or if any unforeseen events occur such as admissions, premature delivery or other unexpected pregnancy outcomes, if they wish to.

Sub-cohort 3: Breastfeeding. The dose-to-mother deuterium dilution method described below will be used in this sub-cohort at month 3 postpartum to assess breastmilk volumes and changes over time. Deuterium is a stable isotope of hydrogen that does not emit potentially harmful radiation[47]. Deuterium and other stable isotopes have been used extensively for over half a century in metabolic studies to assess body composition, energy expenditure and protein turnover[47,48]. The deuterium that will be consumed by mothers in this study will enrich body water to a maximum of 0.1% in the mother and less than half of this in her infant[49]. This is the same dose that has been used previously in several studies conducted in at least five African countries, which include Benin, Central African Republic, Morocco, Tanzania and South Africa. No adverse side effects have been reported at these low dose levels, and this method is not considered to be harmful for participating women or their infants.

The dose-to-mother deuterium dilution method involves the participant drinking a 30g dose of deuterium oxide, which mixes rapidly with her body water[50]. The participant will be asked to rotate a small ball of cotton wool around their mouth until it is completely soaked with saliva. The cotton ball will then be placed in a 10-mL sterile disposable syringe and the plunger depressed to transfer the saliva into a 3.6-mL sterile cryovial. Saliva samples from the infant will be collected by a trained technician using a cotton wool swab enhanced with additional cotton wool. The collection process involves allowing the infant to suck on a dummy fitted with a cotton ball until it becomes fully saturated with saliva. Saliva samples will be taken from the mother and infant prior to the dose and self-collected by the mother over a 14 day period thereafter (day 1, 2, 3, 4, 13 and 14)[50]. Women will be given pre-labelled cryovials with their unique participant identification number and dates. Women will be asked to record the time of sample collection on the cryovial.

At each visit, approximately 10 mL of breast milk will be collected from both breasts by maternal manual expression[51]. Samples will be collected into a sterile container and stored in a temperature-controlled environment (between 4 to -20°C) until analysis. Sodium and potassium concentrations will be measured in whole breast milk by flame photometry on a digital flame photometer (IL 943, Analytical Instruments, LLC, USA) using Filteau's or similar method[52]. Briefly, breast milk will be centrifuged to separate the aqueous fraction, which will then be analysed for sodium and potassium levels. Equipment will be calibrated using standard solutions of known concentrations to ensure accuracy. All procedures will adhere to standardised protocols to minimise variability between measurements.

Additional study visits during heatwaves

Sub-cohort 1: High-risk women with individualised activity and heat exposure monitoring. Visits during heatwaves will involve assessment of heat adaptation, access to water and cool spaces, hydration practices, heat-related behaviours, mental health screening and sleep quality. Physical examination will include height, weight, mid-upper arm circumference (MUAC) and foetal ultrasound. Bloods will be drawn for inflammatory markers, RNA gene sequencing, renal function, adrenaline and cortisol, and urine collected for urine osmolarity and urine specific gravity. Temperature and activity data from wearable devices will also be reviewed.

Sub-cohort 3: Breastfeeding. Breast milk will be collected as previously described.

Data analysis

Overall cohort. Analysis will account for clustering from repeated measures where relevant, and adjust for seasonal and sub-season patterns to reduce biases from acclimatisation or acclimation.

Individualised temperature and humidity exposures for women will be modelled from continuous measures of personal temperature exposures as well as more complex climate measures such as wind speed and radiant temperature from household, maternity facility and community weather stations, allowing calculation of cumulative heat stress exposure as well as identification of points of extreme heat stress exposure[53,54]. Heat stress exposure will be calculated using: Wet Bulb Globe Temperature (WBGT), Universal Thermal Climate Index (UTCI), and Apparent temperature (AT) which have been used extensively in Southern Africa[22,55,56], as are appropriate for high incoming solar radiation, higher temperatures and average relative humidity conditions.

Biomarker analysis will be conducted using multivariate regression models to examine the association between heat exposure and inflammatory markers, adjusting for potential confounders. Inferential analysis of longitudinal data will include mixed-effects linear models. Our omics approach, to enhance precision, includes assessing maternal transcriptomic and epigenetic signatures, using serial RNA-Seq data, to track up-and down-regulated genes before-and- after heat extremes. The read-count matrix for differential gene expression and pathway analyses will be produced through the nf-rnaSeqCount pipeline[57], using the raw RNA-Seq data from the different

time points. Similarities and differences between RNA-Seq samples will be assessed through Principal Component Analysis (PCA). Different tools, including DESeq2 and edgeR packages in R, will be used to identify significant gene expression changes related to heat exposure. To visualise the significantly differentiated genes between the different time points, heatmaps and volcano plots will be created. Pathway analyses of the significantly expressed genes will be carried out using gage and pathview packages in R. Analyses will be presented overall, and by prespecified population sub-groups (to identify groups for future targeted interventions). Bonferroni corrections will be applied to counteract potential multiple testing biases.

Sub-cohort 1: High-risk women with individualised activity and heat exposure monitoring. During the analysis, two components related to heat exposure will be explored. Firstly, differential heat stress experienced in different environments, including the community, facility and other areas captured by the personal thermometers will be compared and contrasted using summary statistics as described in the overall cohort above. Wearable activity data will be post-processed to account for no-wear time, including thresholds on inactivity and discarding steps not associated with heart rates. Linear mixed effects models will be used to assess the impact of heat exposure on 1) activity (number of steps and % sedentary time) and 2) sleep (duration and quality)[58,59]. Second, overall heat stress and heat strain experienced by the individual will be modelled based on the integration of multiple data sources, including the various environmental and physiological variables (e.g., using step counts and heart rate as surrogates), as well as the physiological vital signs such as core body temperature and heart rate of the individual. These inputs are vital in assessing common heat strain indices such as the Physiological Strain Index (PSI), which has been utilised in similar studies on biological pathways of heat exposure[60], recorded by wearable devices and weather stations. This index can then be used as a reference in the statistical analysis of the biological end-points which are more detailed physiological responses to heat.

Sub-cohort 2: Psychosocial factors. Data from in-depth interviews will initially be analysed separately, blinded to the quantitative data, using a non-positivist, phenomenological approach to explore the biopsychosocial experiences of pregnant women and construct pathways around pregnancy outcomes. Quantitative data (psychological and socioeconomic) will be analysed to identify associations and causal pathways. The qualitative and quantitative data will then be integrated, comparing interview insights with questionnaire responses to validate the tools and identify gaps or discrepancies. Similarly, discussions on heat experiences will be compared to quantitative thermal recordings to ensure robustness. Key aspects, including mental health, social circumstances, food insecurity, relationships, biological events, and hospital interactions, will be cross-checked. A grounded theory approach will be used with an emphasis on avoiding assumptions about suspected causal pathways. Analysis will take the form of coding the text and using a constant comparative method of analysis.

Sub-cohort 3: Breastfeeding. Deuterium enrichment levels in the saliva of both the mother and the infant will be analysed relative to their pre-dose samples using a Fourier Transform Infrared Spectrometer (FTIR). Model curves will be fitted to these values using the open-source statistical software R to estimate the infant's breastmilk intake and, by extension, the mother's breastmilk output.

Box and whisker plots will be used to give a graphical comparison of the breast milk volume, for all women, but then also by different categories of temperature (e.g. quartiles). Multi-level regression analyses will be conducted to explore the relationship between breast milk volume, and outdoor, household and personal temperature. Breast milk sodium and potassium levels will be assessed for normality. If data are close to symmetric, they will be summarised by measures of central tendency. If they are skewed, they will be log transformed and summarised as geometric means. Multilevel regression models will be used to investigate whether the sodium content of breast milk is affected by ambient and personal temperatures, and to explore the relationship between self-reported breastfeeding and temperature. Analysis will account for potential confounding factors including maternal age and co-morbidities.

Participant safety

Research staff training

All staff will undergo comprehensive training before beginning work on the study, covering ethics, informed consent, protocol-specific procedures, and any qualifications required for their specific roles.

Routine antenatal care and delivery

Study participants will be retained at the hospital site for routine antenatal care and delivery. Participants requiring emergency care or outpatient visits will be referred to the hospital. Continuous communication will be maintained between hospital and study staff throughout.

Risks

Participation in the study involves minimal risks and study participants remain under the care of the research hospital site throughout. Potential risks and mitigating strategies include:

- Psychological risks: Pregnancy and infant related studies have the potential for psychological stress, particularly if there has been an adverse outcome, e.g., preterm birth, miscarriage, stillbirth, or infant death. Research staff will be trained to recognise signs of psychological distress and will be instructed on how to assist participants in obtaining support. A distress protocol, aligned with the current practices in place, will be followed in these instances.

- Risk of vulnerability to theft with wearables: In South Africa, wearing an activity tracker on the wrist may increase the risk of becoming a target for theft. Based on our experience with a similar study outside of Johannesburg, participants did not encounter this issue. However, we appreciate the different study settings in Johannesburg. We will counsel the

pregnant women about the risks and communicate with our Community Advisory Board about mitigating the risk.

- Physical risks: Study activities that involve any physical interventions or procedures (e.g., phlebotomy or ultrasound), will be performed by qualified personnel in a setting equipped to handle and respond to any adverse reactions. Regular monitoring will ensure that any signs of distress or adverse effects are quickly addressed.

Quality assurance

High quality standards will be reinforced through comprehensive training and retraining of staff as required, along with oversight of adherence to SOPs. Several study-specific SOPs will be developed, covering areas such as specimen collection, wearable device data collection and management and qualitative data handling. Any deviations from the protocol or instances of non-compliance with ethical standards or unanticipated problems will be recorded. The PI will be immediately informed and appropriate corrective and preventive actions will be implemented. A corrective and preventive action log will be maintained to track these actions and their outcomes, and these will be reported to our ethics board and the Wellcome Trust as appropriate.

Data management

Data management in the study will follow rigorous protocols across all study components to uphold data accuracy, security, and confidentiality. A wide range of data types will be collected from multiple sources, including questionnaires, physical examinations, radiological procedures, laboratory measurements, and environmental exposure assessments. A detailed Data Management Plan will be developed to guide the handling of data throughout the study. The data types, collection methods and storage locations are summarised in Table 1 below.

We will follow International Council for Harmonisation-Good Clinical Practice (ICH-GCP) guidelines[61] including using unique participant identification numbers, securely storing both hard copies and electronic versions of participant data and restricting access to trained research staff. The study will adhere to the Wellcome Trust regulations on data sharing and relevant local data protection laws, such as the South African Protection of Personal Information Act (POPIA)[62]. External access to the data collected in this study will be partly restricted – i.e., the dataset will be shared only upon written application to the study PIs. Data to be used for other research studies, including studies on a different topic, may be shared subject to ethics and PI approval. This applies to additional research or analyses not mentioned in the study protocol or with collaborators not part of the protocol team.

Data collection

Data will be collected using a combination of electronic data capture systems and paper-based forms. This will include standardised questionnaires, automated logging of wearable

Table 1. Description of data types, collection methods and storage location.

Study component	Data types	Data collection method	Storage location
Overall cohort	Questionnaires	Tablet, paper-based	REDCap, SharePoint
	Clinical and radiological records	Tablet, paper-based	REDCap, SharePoint
	Laboratory data	Computer, paper-based	REDCap, SharePoint
	RNA/DNA sequencing data	Laboratory Interface	Microsoft Azure
	Wearable ambient temperature device	iButton or similar	REDCap
	Environmental data	Davis Vantage Pro-2 weather stations (community and facility), or similar	Vital Weather station, SharePoint
	Heat and sleep diaries	Digital diaries (WhatsApp-based or similar)	Microsoft Azure, SharePoint
Sub-study 1	Wearable activity devices	Garmin watch, or similar	Microsoft Azure, REDCap, SharePoint
	Wearable core temperature devices	TempTraq or similar	REDCap, SharePoint
Sub-study 2	Qualitative interview recordings, transcripts	Digital recorders	SharePoint
Sub-study 3	Questionnaires	Tablet, paper-based	REDCap, SharePoint
	Laboratory data	Computer, paper-based	REDCap, SharePoint
	Data types	Data collection method	Storage location

data, WhatsApp-based diaries and digital recording devices. All equipment used in the study, including data collection and monitoring devices, will be regularly calibrated and maintained according to the manufacturer's specifications to ensure accurate and consistent data collection. Range checks and automated validation rules will be implemented at the point of data entry to minimise errors. Additionally, electronic data will be cross-checked against source documents to ensure accuracy.

Data storage and backup

The electronic data will be stored in a secure, password-protected environment that adheres to international data protection standards, such as REDCap (Research Electronic Data Capture - https://project-redcap.org), Microsoft SharePoint, or Microsoft Azure. Large RNA-Seq data will be stored (and analysed) on the University of the Witwatersrand Compute Cluster. Electronic data will be backed up on both onsite and offsite encrypted servers. Physical forms will be securely stored in locked cabinets within a restricted-access area at the study site. Upon conclusion of the study, all records will be retained for at least ten years per institutional and funding body guidelines. Deidentified data will be archived in a secure, encrypted format in institutional repositories for long-term preservation. At the same time, physical records will be securely shredded through certified destruction services to protect sensitive participant information.

Dissemination and public involvement

We are committed to making the results of this research accessible to all relevant stakeholders, including the scientific community, study participants, the general public, healthcare providers, and policymakers. To support this, findings will be published in open-access, peer-reviewed journals and presented at scientific conferences and expert meetings to advance academic discourse and professional education, as well as public forums. To enhance policy and practice impact, we will actively engage other key stakeholders, including community leaders, advocacy groups, and professional organisations, to ensure the findings inform policy development, advocacy efforts, and intervention strategies at local, national, and international levels.

To reach broader audiences, public communications, such as press releases issued through the University of the Witwatersrand and other media channels–will be used to promote awareness and understanding of the findings. Study participants and local communities will receive simple, non-technical summaries of the findings. These will be translated into appropriate languages and disseminated in accessible formats, including leaflets, posters, and online content.

We will work closely with the Community Advisory Board (CAB), who the research site team has long collaborated with. The CAB will be engaged from the start of the project and provide input on participant information sheets and other participant facing documents. At the dissemination stage, we will collaborate with the CAB to ensure the interpretation and dissemination of findings are grounded in the lived experiences of the communities we aim to serve.

Fully de-identified data will be made available upon publication of the final manuscript planned within Bio-HEAT. These datasets may be placed in repositories and will be made available upon reasonable written request including confirmation of required ethical approval and completion of a Data Transfer Agreement. Code used for analysis will be shared with each manuscript as a supplementary file.

Ethics and consent

The study adheres to ethical guidelines outlined in the Declaration of Helsinki (https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/). Written informed consent will be obtained from all participants. Study staff will emphasise voluntary participation and assure participants that provision of routine antenatal care will not be affected. Participants will be encouraged to discuss their involvement with household members before making a decision. If a participant is unable to speak or understand one of the local languages available for the informed consent process, an impartial witness will be allowed to translate. Separate Informed Consent Forms (ICFs) will be provided for sample storage and genetic testing. For participants under 18, consent will be obtained from a parent or guardian, and the minor will complete an assent form. Participants will have the right to withdraw from the study at any time without any penalty. Further data will not be collected from a withdrawn participant, however data already collected will be retained.

Ethics approval

The study protocol has received ethical approval from the University of the Witwatersrand Human Research Ethics Committee (ethics ref 240804, dated 9th October 2024) as well as facility and district approval through the Hospital review committee (28th January 2025) and the Research Committee of Johannesburg Health District (18th November 2024).

Data availability

No data are associated with this article.

Acknowledgements

During preparation of this manuscript, MMB used OpenAI's ChatGPT (version GPT-4o)to improve readability and reduce word count in certain paragraphs where necessary. The authors reviewed all content and take full scientific responsibility for its accuracy and integrity.

Supplementary Appendix 1. Overview of study measurements and rationale.

	Measurement	What is Measured	Reason for measurement
1.	Full history & clinical exam	Maternal health	Examine and monitor general health status, identify pre-existing or potential risks impacting pregnancy (e.g., pre-eclampsia)
2.	Mental health screening (e.g., EPDS, PHQ-9, GAD-7, WHODAS-12)	Psychological well-being	To assess maternal psychological well-being, as mental health challenges—especially when compounded by heat stress—can influence pregnancy outcomes, maternal functioning, and infant care
3.	Alcohol use screening (Adapted Timeline Alcohol Use Calendar & AUDIT)	Substance exposure	Alcohol use can affect pregnancy outcomes, and may also contribute to dehydration, compounding heat-related fluid loss
4.	Blood tests (e.g., FBC, renal, thyroid, HIV, syphilis)	Maternal health	Identify chronic conditions influencing outcomes
5.	Additional blood tests (urea, creatinine, electrolytes)	Hydration status	Dehydration linked to PTB via thermoregulatory strain triggering uterine contractions, systemic inflammation, and stress responses that disrupt normal pregnancy physiology.
6.	Urine samples (osmolarity, USG)	Hydration status	Dehydration linked to PTB via thermoregulatory strain triggering uterine contractions, systemic inflammation, and stress responses that disrupt normal pregnancy physiology.
7.	Inflammatory markers (e.g., IL-6)	Inflammation	Heat-induced inflammation hypothesised to cause PTB as inflammation can promote cervical ripening, membrane rupture, and uterine contractions that lead to early labour.
8.	RNA-Seq/ Epigenetics	Gene expression & epigenetic changes	To identify heat-induced changes in gene expression and epigenetic regulation that may activate inflammatory, stress, or labour pathways contributing to preterm birth and adverse maternal or foetal outcomes.
9.	OGTT	Metabolic response	Heat may impact glucose metabolism or exacerbate gestational diabetes (GDM). GDM may a potentially compound risks through fluid imbalance, placental strain and/or inflammatory activation.
10.	Adrenaline metabolites	Stress response	To assess physiological stress responses, as elevated stress—potentially triggered by heat exposure—may contribute to preterm labour through activation of the hypothalamic-pituitary-adrenal (HPA) axis and increased uterine contractility.
11.	Salivary cortisol	Stress response	To measure heat-related activation of the stress response via the HPA axis, as elevated cortisol levels are linked to inflammation, altered placental function, and increased risk of preterm birth.
12.	Ultrasound (1st trimester to 36 weeks)	Foetal growth and development	Initial ultrasound to confirm an intrauterine, viable pregnancy, the number of foetuses, and to confirm gestational age. Follow up scans will be used to monitor growth and assess potential impacts of heat stress on intrauterine growth patterns
13.	Vaginal/Rectal swab	Group B streptococcus colonisation	To identify maternal GBS carriage, which poses a risk for neonatal infection and may interact with heat-induced immune changes to increase susceptibility to adverse outcomes like sepsis.
14.	CTG monitoring	Foetal heart rate and uterine activity	Assess foetal distress during labour
15.	Cord blood testing (e.g., acid-base status)	Neonatal stress indicators	To assess foetal stress and immune activation at birth, which may reflect intrauterine exposure to heat-induced maternal inflammation or physiological strain contributing to adverse birth outcomes.
16.	Placental histology (Amsterdam Classification) and MC&S	Placental pathology	To identify patterns of placental injury—such as vascular mal-perfusion or infection-related inflammation—that may result from heat-induced maternal stress or inflammation, helping to explain mechanisms linking heat exposure to foetal stress, impaired growth, and preterm birth.

	Measurement	What is Measured	Reason for measurement
17.	Labour progress, duration, mode of delivery, maternal & foetal morbidity (from Maternal Case Record)	Labour progression and outcomes	To evaluate how heat exposure and related physiological stressors may influence labour dynamics, increase complications, and affect delivery outcomes for both mother and baby. These measures are also used to assess the effectiveness of our labour ward interventions.
18.	Weight, length, head circumference	Infant growth outcomes	To monitor infant growth and general health, assessing whether prenatal heat exposure and related maternal factors have impacted early physical development and thermoregulation.
19.	Tympanic temperature (infant)	Infant thermal regulation	To monitor infant general health
20.	Infant history of gastroenteritis or hospital admissions	Infant morbidity	To monitor infant health outcomes that may potentially be influenced by heat exposure, breastfeeding challenges, or postnatal environmental stressors, helping identify pathways to early-life morbidity.
21.	Developmental assessments (ASQ, BSID, milestone achievements)	Neurodevelopment	To evaluate the potential long-term neurodevelopmental impacts of prenatal heat exposure and related stressors on infants, including those mediated by inflammation, placental dysfunction, or preterm birth.
22.	Wearable fitness tracker (sub-cohort 1)	Personal heat exposure, sleep, heart rate, activity(step count)	Quantify maternal thermal exposure and its effects on related behaviours
23.	Breastmilk samples and deuterium measurements from saliva (sub-cohort 3)	Composition and volume (Na+, K+, deuterium)	To assess how heat exposure affects breastmilk composition and volume, particularly sodium concentration and hydration markers, which may influence infant nutrition and risk of dehydration.
24.	Interviews (sub-cohort 2)	Psychosocial context & heat experience	To explore women's lived experiences of heat exposure, psychosocial stressors, and pregnancy, providing context for quantitative findings and identifying social and emotional pathways.
25.	Heat and sleep diaries	Subjective thermal exposure and rest patterns	To capture daily patterns of heat exposure, sleep quality, and behavioural responses, offering insight into how personal experiences of heat may affect maternal stress and behaviour.

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Open Peer Review

Current Peer Review Status: ✔ ? ✔

Version 2

Reviewer Report 30 June 2025

https://doi.org/10.21956/wellcomeopenres.26873.r126576

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✔ **Santosh Pandipati**
[1] Obstetrix of San Jose, e-Lōvu Health, e-Lōvu Health, USA
[2] Lōvu Health and Silicon Valley Maternal-Fetal Medicine, Los Gatos, California, USA

This is an incredibly exciting research study looking at uncovering mechanisms of excess heat exposure on pregnancy and postpartum outcomes. To my knowledge, such a comprehensive longitudinal study has not been performed. I am especially impressed at the evaluation of both serum markers as well as psychosocial determinants that may contribute to adverse outcomes.

I believe the dataset to be collected will be vast, and I suspect multiple publications spiraling out of this study. I am especially impressed with the comprehensiveness of the data collection, ranging from facility to community to personal monitoring, and looking not just at physical, biochemical, and gene expression parameters, but also at the psychosocial aspects well into postpartum health, including newborn health (vis-à-vis breastfeeding).

It will be good to perform standardized mental health assessments more often throughout pregnancy and postpartum using PHQ-9 or EPDS surveys (not just at 5 months). Additionally, I would highly recommend administering such surveys during heatwave visits. This is not written out in the text, but it seems to be listed in Figure 3, and hence my request for clarification. There is a clear correlation between untreated mental health conditions such as depression and higher rates of preterm birth. There is also clear correlation between climate stressors such as heatwaves and deteriorated mental health. So, it will be interesting to see if there is an interplay between mental health, climate, and outcomes.

Finally, as climate continues to change, it may be good to compare outcomes against a historical comparison if outcome data is available - i.e., compare this study population's overall outcomes to the outcomes of historical controls from the year prior, 5 years prior, and 10 years prior. Of course, prior historical controls will not have all the data these researchers will be collecting in their study, but it will place some historical context for the study population.

Overall, I am excited to see what comes out of this study.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Yes

Are the datasets clearly presented in a useable and accessible format?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Maternal-fetal medicine, climate change impacts to reproductive health, digital health and telemedicine.

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 1

Reviewer Report 24 April 2025

https://doi.org/10.21956/wellcomeopenres.26054.r121795

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?

Claudia Hanson
Karolinska Institutet, Stockholm, Sweden

Thank you very much proving me with the opportunity to review this important protocol. The hypothesis is clearly stated and the sub-hypothesis as well. Understanding physiological mechanisms will be of major importance to develop the best interventions for mitigation.
I am missing a bit a consideration of why which measurements. For example, why which markers in pregnancy, cord blood (which measurement).
Further, I am missing a consideration of pregnancy complications including pre- / eclampsia, vaginal infection other then group B-streptococcus. Also, will you include information of complications at birth?
The protocol is not well referenced, for example, there is no reference to the Amsterdam classification for placenta pathology.
The quality assurance part does not go into sufficient details. You are collecting a large variety of variable, and different quality assurance mechanisms and SOPs will need to be applied. A similar

concern is for the data processes and management. Probably a table on data management for the very different types of data may have been used.
I am also not clear about patient involvement. I am happy to hear about the involvement of the labour room staff in respect to cooling, but I am less clear about patient involvement in the design of the study, eg. patients may raise additional important factors which are not yet included, such as work and commuting.

Is the rationale for, and objectives of, the study clearly described?
Yes

Is the study design appropriate for the research question?
Yes

Are sufficient details of the methods provided to allow replication by others?
Partly

Are the datasets clearly presented in a useable and accessible format?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Obstetrics and women's health, epidemiology

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Author Response 27 May 2025

Ijeoma Solarin

Response to reviewer: **Claudia Hanson, Karolinska Institutet, Stockholm, Sweden 2.1**
Thank you very much proving me with the opportunity to review this important protocol. The hypothesis is clearly stated and the sub-hypothesis as well. Understanding physiological mechanisms will be of major importance to develop the best interventions for mitigation.
Author response: Thank you for these comments.

2.2 *I am missing a bit a consideration of why which measurements. For example, why which markers in pregnancy, cord blood (which measurement).* **Author response:** We thank the reviewer for this thoughtful comment. While we have described the individual measures used throughout the manuscript, we appreciate that we may have overlooked providing specific rational for selecting certain measurements. Our selection of measurements is driven by our primary and secondary hypotheses and in particular focusses on the plausible pathways that we describe in Figure 1. These include several hypothesised pathways linking heat exposure to preterm birth including immune activation, epigenetic changes, endocrine and stress response, dehydration, placental changes and maternal mental health. Measures such as inflammatory markers, RNA Sequencing and gene regulation may help to answer

questions about the effect of heat on the immune system. Urine analysis, including osmolarity and urine specific gravity give us insights into the role of dehydration, while the mental wellbeing tools we use in combination with our qualitative sub-study may help us to understand the role of mental health in the pathways. We have now included a Supplementary Table (Appendix 1) that summarises each measurement, the physiological or psychological process it represents and its relevance to our study. We hope this addition enhances the clarity of our measurement rationale. We have also added the following sentence to the manuscript under the section on Global health relevance to reference the supplementary table. Added text: *A list of study measures, along with the rational for each, is provided in a supplemental appendix (Appendix 1: Overview of study measurements and rationale).*

2.3 *Further, I am missing a consideration of pregnancy complications including pre- / eclampsia, vaginal infection other then group B-streptococcus. Also, will you include information of complications at birth?* **Author response:** Thank you for your comment. In the protocol, we specify that women will be clinically examined at each visit which includes taking blood pressure measurements, checking blood sugar levels and testing for infections, specifically HIV and syphilis. These procedures are designed to detect common antenatal complications including pre-eclampsia and gestational diabetes. We acknowledge that other infections, such as malaria or additional vaginal infections could be relevant however, investigating this goes beyond the scope of our study, and we are limited by available resources. Therefore, we do not plan on testing for other infections and acknowledge this a potential limitation. The relevant text in the manuscript is as follows:

Updated text in section on Enrolment visit: *"The participant will be examined clinically, including blood pressure measurements, weight and height, and a general and system-specific examination to assess overall maternal health. A foetal ultrasound will also be conducted to confirm an intrauterine, viable pregnancy, the number of foetuses, and to confirm gestational age. If not already conducted at the antenatal clinic, blood will be drawn to test for renal function, thyroid function, HBA1c, a full blood count, and infections (HIV and syphilis)."*

We have also clarified that participants will be clinically examined at each antenatal and postnatal visit. Updated text in section on Antepartum study visit: *Participants will be clinically examined at each visit and blood samples will be taken to test for HIV and syphilis if not already done at the antenatal clinic.* Updated text in section on Postpartum study visits: *Study staff will clinically examine participants and collect measurements (e.g., height, weight, waist, hip, and mid-upper arm circumference) and vital signs.* Regarding complications at birth, we do plan to collect this information by extracting data from the maternity records. We hope we have made this clearer in the manuscript. Updated text in section on Intrapartum study visit: *Labour progress, mode of delivery, maternal and foetal morbidity, Apgar scores, foetal weight, length, and head circumference, and additional relevant information will be extracted from the maternity case records. Cardiotocography data will also be documented*. In addition, we hope that the addition of the supplementary table, which lists study measures, helps clarify the scope of data being collected.

2.4 *The protocol is not well referenced, for example, there is no reference to the Amsterdam classification for placenta pathology.* **Author response:** Thank you for this comment. We have included a reference for the Amsterdam classification as suggested and additional references in several other places.

2.5 *The quality assurance part does not go into sufficient details. You are collecting a large variety of variable, and different quality assurance mechanisms and SOPs will need to be applied. A similar concern is for the data processes and management. Probably a table on data management for the very different types of data may have been used.* **Author response:** Thank you for your comment. We agree that we are collecting a large variety of data which needs to be carefully managed. A key aspect of quality assurance is training and the availability of standard operating procedures (SOPs). All staff within our study undergo comprehensive training on all study procedures and re-training will be conducted on a regular basis as required. In addition to site specific SOPs, we have developed several study specific SOPs for specimen collection and sampling, device management, qualitative data management. While we do mention staff training under the section about participant safety, we have now expanded the quality assurance section to also include these details. Updated text is provided below. Updated text: *High quality standards will be reinforced through comprehensive training and retraining of staff as required, along with oversight of adherence to SOPs. Several study-specific SOPs will be developed, covering areas such as specimen collection, wearable device data collection and management and qualitative data handling.* Additionally, as part of our internal data management plan, we have a table which describes data collection methods and storage locations (Table 1). We have updated the data management section to include the following: *Data management in the study will follow rigorous protocols across all study components to uphold data accuracy, security, and confidentiality. A wide range of data types will be collected from multiple sources, including questionnaires, physical examinations, radiological procedures, laboratory measurements, and environmental exposure assessments. A detailed Data Management Plan will be developed to guide the handling of data throughout the study. The data types, collection methods and storage locations are summarised in Table 1 below.* Table 1: Description of data types, collection methods and storage location

2.6 *I am also not clear about patient involvement. I am happy to hear about the involvement of the labour room staff in respect to cooling, but I am less clear about patient involvement in the design of the study, eg. patients may raise additional important factors which are not yet included, such as work and commuting.* **Author response:** Thank you for this important comment. While patients were not directly involved in the initial design of the study, we recognise the value of patient and community research partners. To support meaningful engagement and knowledge translation, we plan to work closely with an established Community Advisory Board (CAB) that the research site team has long collaborated with. The CAB is engaged from the start of the project and provide inputs on components of the study including, for example, the participant information sheets and other participant facing documents. We anticipate that their contributions to this research will ensure that the interpretation and dissemination of findings are grounded in the lived experiences of the communities we aim to serve. In addition, we will draw on insights gained through the qualitative aspect of our study. We have added more detail to the manuscript as follows: ***Dissemination and public involvement*** *We are committed to making the results of this research accessible to all relevant stakeholders, including the scientific community, study participants, the general public, healthcare providers, and policymakers. To support this, findings will be published in open-access, peer-reviewed journals and presented at scientific conferences and expert meetings to advance academic discourse and professional education, as well as public*

forums. To enhance policy and practice impact, we will actively engage other key stakeholders, including community leaders, advocacy groups, and professional organisations, to ensure the findings inform policy development, advocacy efforts, and intervention strategies at local, national, and international levels. To reach broader audiences, public communications, such as press releases issued through the University of the Witwatersrand and other media channels will be used to promote awareness and understanding of the findings. Study participants and local communities will receive simple, non-technical summaries of the findings. These will be translated into appropriate languages and disseminated in accessible formats, including leaflets, posters, and online content. We will work closely with the Community Advisory Board (CAB), who the research site team has long collaborated with. The CAB will be engaged from the start of the project and provide input on participant information sheets and other participant facing documents. At the dissemination stage, we will collaborate with the CAB to ensure the interpretation and dissemination of findings are grounded in the lived experiences of the communities we aim to serve.

Competing Interests: No competing interests were disclosed.

Reviewer Report 23 April 2025

https://doi.org/10.21956/wellcomeopenres.26054.r121797

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Ashish KC
University of Gothenburg, Gothenburg, Sweden

Thank you for inviting me to review this interesting study protocol on biological pathway on heat exposure to preterm birth and other maternal and child health outcomes. This is a very valuable study to provide the epidemiological explanation on heat events to preterm birth
This study aim to investigate the environmental exposure to high ambient temperature on preterm birth and the causal pathway to exposure.
I have some comments on the hypothesis driven for the study
- Non-optimal temperature either lower than 50th centile or higher than 50th centile has been associated to poor obstetric outcomes including preterm birth, still birth and neonatal mortality https://www.nature.com/articles/s41591-024-03245-7 and Ref:1 indicating that non-optimal ambient temperature exposure has a corelation between heat and preterm birth. It would be worth to provide a note to it.

- Even though there is a cumulative effect of non-optimal temperature to preterm birth, the lag effect i.e harvesting effect might be seen in preterm birth, can the researchers discuss about it

-In the abstract researchers mention that "maternal inflammation will be tested by analysis the epigenetic changes associated with inflammatory cytokines protein and gene expression". Based

on the studies from Genome wide association study and Mendenlian law, any genetic variant is always constant since conception, so the expression of cytokine is based on the dysfunction of the cytokine receptor dysfunction. https://doi.org/10.3390/ijms252011332.
further not all peripheral inflammation will cause the trigger preterm labour, until the the inflammation cross the blood brain barrier, the inflammatory chain of reaction is required.
Further, why is the focus on IL-6, there are a range of pro-inflammatory and anti-inflammatory cytokines which can cause BBB inflammation Ref:2

Sample size estimation- I still did not get the basis of the sample size based on IL-6, has there been a study to estimate IL-6 >1000pg/ml is linked with preterm birth.

In hypotheses, the researchers mention that psycho-social stressor is a mediator of preterm birth, but does it not also link with the increase in pro-inflammatory or neuro-hormonal disturbance. Can it be that there is a reversal phenomenon that due to increased pro-inflammatory genetic expression and disturbance in neuro-hormonal gene expression, that the social stress can change the pyschological response and thus preterm birth.
Thank you once again for inviting me to review the protocol

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Is the rationale for, and objectives of, the study clearly described?

Yes

Is the study design appropriate for the research question?

Yes

Are sufficient details of the methods provided to allow replication by others?

Partly

Are the datasets clearly presented in a useable and accessible format?

Not applicable

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Heat event to perinatal health

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Author Response 27 May 2025

Ijeoma Solarin

Reviewer: **Ashish KC,** University of Gothenburg, Gothenburg, Sweden **No Reviewer comment Author response** 1.1 *Thank you for inviting me to review this interesting study protocol on biological pathway on heat exposure to preterm birth and other maternal and child health outcomes. This is a very valuable study to provide the epidemiological explanation on heat events to preterm birth. This study aim to investigate the environmental exposure to high ambient temperature on preterm birth and the causal pathway to exposure.* Thank you for these comments and careful review. 1.2 *I have some comments on the hypothesis driven for the study*
- Non-optimal temperature either lower than 50th centile or higher than 50th centile has been associated to poor obstetric outcomes including preterm birth, still birth and neonatal mortality https://www.nature.com/articles/s41591-024-03245-7 and Ref:1 indicating that non-optimal ambient temperature exposure has a corelation between heat and preterm birth. It would be worth to provide a note to it. Thank you for this helpful comment and for sharing the relevant references. We agree that both high and low ambient temperatures have been associated with adverse obstetric outcomes, including preterm birth, stillbirth, and neonatal mortality. While the primary focus of our study is on high temperature exposures—given their growing relevance in the context of anthropogenic climate change, the particular relevance in our study setting in sub-Saharan Africa and the urgent need to understand underlying biological mechanisms—we recognise the importance of non-optimal low temperature exposure as well. While the references shared by the reviewer (Kc et al. (2024) and Hanson et al (2024)) underscore the importance of considering both cold and hot non-optimal temperatures, even these studies report that heat exposure tends to have a more consistent and pronounced association with adverse outcomes than cold exposure, especially in low-resources contexts. We have nevertheless updated the manuscript to acknowledge this broader evidence base and will consider it when interpreting our findings. Added text in section on Global health relevance: *While both high and low ambient temperatures have been associated with poor obstetric outcomes (Kc et al., 2024,* Nyadanu et al., 2024*), this study focuses on heat exposure, which is of particular concern in our African context.* 1.3 *Even though there is a cumulative effect of non-optimal temperature to preterm birth, the lag effect i.e harvesting effect might be seen in preterm birth, can the researchers discuss about it* Thank you for this comment. We appreciate the suggestion to consider a potential lag or “harvesting” effect. As we understand it, the harvesting effect typically refers to temporal displacement of events that would have occurred soon regardless, and it is most often applied in the context of mortality, particularly among older or frail individuals. We are not certain that this concept directly translates to preterm birth, as a shift from term to preterm delivery due to heat exposure may represent a clinically meaningful outcome that could have been avoided rather than temporal displacement of an outcome that was likely to occur regardless. Nonetheless, we acknowledge that further discussion on lag structures and potential timing effects is warranted and will consider this in our interpretation of findings. 1.4 *In the abstract researchers mention that "maternal inflammation will be tested by analysis the epigenetic changes associated with inflammatory cytokines protein and gene expression". Based on the studies from Genome wide association study and Mendenlian law, any genetic variant is always constant since conception, so the expression of cytokine is based on the dysfunction of the cytokine receptor dysfunction. https://doi.org/10.3390/ijms252011332.* Our approaches to examining heat exposure and inflammation includes assessing maternal

transcriptomic and epigenetic signatures, using serial RNA-Seq data, to track up-and down-regulated genes before-and- after heat extremes. These are not Mendelian changes, but the differential gene expression and pathway analyses assessed using RNA Seq data. We can visualise the significantly differentiated genes between the different time points and see which genes are being expressed. We don't do GWAS or similar genetic testing in this study, as the reviewer points out. 1.5 *2.5. further not all peripheral inflammation will cause the trigger preterm labour, until the inflammation cross the bloodbrain barrier, the inflammatory chain of reaction is required. Further, why is the focus on IL-6, there are a range of pro-inflammatory and anti-inflammatory cytokines which can cause BBB inflammation Ref:2* We appreciate your important point that not all peripheral inflammation will trigger PTB, and that specific inflammatory pathways, including those involving the blood-brain barrier, may play a role. However, our study is primarily focused on inflammatory pathways at the maternal-foetal interface (such as placenta, myometrium), which are more directly implicated in the initiation of labour than the BBB. We also acknowledge that IL-6 is a non-specific marker and that multiple cytokines, both pro- and anti-inflammatory, may contribute to the broader inflammatory response. Our focus on IL-6 is based on several considerations: 1). Evidence supporting its role in PTB; 2). its relative ease of measurement and low cost of testing; and 3). its widespread use in research, which facilitates comparison across studies. Nevertheless, we do plan on conducting a more comprehensive biomarker analysis using a panel of 96 inflammatory markers. This will allow us to investigate a range of cytokines implicated in both peripheral and central inflammation and inform more complex analysis. Biomarker analysis will be conducted using multivariate regression models to examine the association between heat exposure and inflammatory markers, adjusting for potential confounders. Inferential analysis of longitudinal data will include mixed-effects linear models. Much of this analysis is done using visualisations such as heatmaps and volcano plots. Bonferroni corrections will be applied to counteract potential multiple testing biases. We recognise that much remains unknown about the precise pathways - whether systemic, BBB, or at the level of the cervix and membranes - and which pathways are most important. This study will try address some of those questions. We have added reference to a recent review on heat and preterm birth Mushimiyimana 2025, which discusses this in more detail. 1.6 *Sample size estimation- I still did not get the basis of the sample size based on IL-6, has there been a study to estimate IL-6 >1000pg/ml is linked with preterm birth.* **Author response:** Many thanks for this query, the point made here is valid. We should have noted the rationale for selecting Il-6 as the outcome of interest. IL-6 is one of the most widely studied inflammatory markers and indeed, several studies have linked Il-6 with preterm birth, as in the papers we now cite for this: (Prairie et al. 2021, Hatzidaki et al., 2005, Karampitsakos et al., 2025). In addition, IL-6 has been shown to be raised by heat (Maloney & Duffy, 2024). We do however acknowledge that the originally specified IL-6 threshold of >1000 pg/mL was not appropriate for maternal serum. This was an oversight, and we appreciate the opportunity to correct it. We have revised this threshold in the manuscript to reflect more clinically and biologically relevant values for maternal serum which are much lower depending on presence of infection or specific population group being studied. While there is no universally accepted threshold for IL-6 in predicting PTB, and some variability exists across studies, we have revised our threshold to 10pg/ml as a conservative measure based on other studies which identify maternal serum IL-6 levels around this value as predictive of preterm birth (e.g., Murtha et al., 1998) and

which was our original intent. We also acknowledge that we have made some assumptions due to the scarcity of existing environmental exposure literature in this area. Nevertheless, our assumptions are based on conservative and pragmatic estimates to ensure sufficient power. One of our aims is to contribute new evidence on the potential relationship between environmental temperature and maternal systemic inflammation. We have clarified this in the revised manuscript. Updated text: *The primary inflammatory outcome interleukin-6 is strongly linked with PTB (Prairie et al., 2021, Hatzidaki et al., 2005, Karampitsakos et al., 2025). However, there is currently no universally accepted threshold for IL-6 in predicting PTB, with high levels of variability across studies. We have based our sample size on IL>10pg/ml as a conservative measure in keeping with other studies which identify maternal serum levels around this value as predictive of PTB (Murtha et al., 1998).* 1.7 *In hypotheses, the researchers mention that psycho-social stressor is a mediator of preterm birth, but does it not also link with the increase in pro-inflammatory or neuro-hormonal disturbance. Can it be that there is a reversal phenomenon that due to increased pro-inflammatory genetic expression and disturbance in neuro-hormonal gene expression, that the social stress can change the pyschological response and thus preterm birth.* Thank you for this thoughtful comment. We agree that psychosocial stress may not only act as a mediator but may also be shaped by underlying biological processes, such as pro-inflammatory and neuro-hormonal disturbances. We acknowledge the possibility of a bidirectional, feedback loops or reverse relationship, whereby heightened infla*mmatory or neuroendocrine activity—potentially influenced by genetic expression—could amplify psychological vulnerability or stress perception. In our conceptual model, we view psychosocial stress, inflammatory pathways, and neuro-hormonal disturbances as potentially interacting and reinforcing components. While our study is not designed to fully disentangle these causal directions, our analyses will explore how these variables may individually and collectively mediate the association between heat exposure and preterm birth. We will also consider how these relationships may evolve over the course of pregnancy and be modulated by environmental stressors such as heat.* 1.8 *Thank you once again for inviting me to review the protocol* Thank you for your valuable feedback.

Competing Interests: No competing interests were disclosed.

Author Response 27 May 2025

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Apologies, I tried to submit responses in a table format but the layout did not work. I am therefore reposting so the comments and responses display properly. Response to reviewer: **Ashish KC** University of Gothenburg, Gothenburg, Sweden 1.1. *Thank you for inviting me to review this interesting study protocol on biological pathway on heat exposure to preterm birth and other maternal and child health outcomes. This is a very valuable study to provide the epidemiological explanation on heat events to preterm birth. This study aim to investigate the environmental exposure to high ambient temperature on preterm birth and the causal pathway to exposure.* **Author response:** Thank you for these comments and careful review. *1.2. I have some comments on the hypothesis driven for the study*
- Non-optimal temperature either lower than 50th centile or higher than 50th centile has been associated to poor obstetric outcomes including preterm birth, still birth and neonatal mortality https://www.nature.com/articles/s41591-024-03245-7 and Ref:1 indicating that non- optimal

ambient temperature exposure has a corelation between heat and preterm birth. It would be worth to provide a note to it. **Author response:** Thank you for this helpful comment and for sharing the relevant references. We agree that both high and low ambient temperatures have been associated with adverse obstetric outcomes, including preterm birth, stillbirth, and neonatal mortality. While the primary focus of our study is on high temperature exposures—given their growing relevance in the context of anthropogenic climate change, the particular relevance in our study setting in sub-Saharan Africa and the urgent need to understand underlying biological mechanisms—we recognise the importance of non-optimal low temperature exposure as well. While the references shared by the reviewer (Kc et al. (2024) and Hanson et al (2024)) underscore the importance of considering both cold and hot non-optimal temperatures, even these studies report that heat exposure tends to have a more consistent and pronounced association with adverse outcomes than cold exposure, especially in low-resources contexts. We have nevertheless updated the manuscript to acknowledge this broader evidence base and will consider it when interpreting our findings. Added text in section on Global health relevance: *While both high and low ambient temperatures have been associated with poor obstetric outcomes (Kc et al., 2024, Nyadanu et al., 2024), this study focuses on heat exposure, which is of particular concern in our African contex*t. *1.3 Even though there is a cumulative effect of non-optimal temperature to preterm birth, the lag effect i.e harvesting effect might be seen in preterm birth, can the researchers discuss about it* **Author response:** Thank you for this comment. We appreciate the suggestion to consider a potential lag or "harvesting" effect. As we understand it, the harvesting effect typically refers to temporal displacement of events that would have occurred soon regardless, and it is most often applied in the context of mortality, particularly among older or frail individuals. We are not certain that this concept directly translates to preterm birth, as a shift from term to preterm delivery due to heat exposure may represent a clinically meaningful outcome that could have been avoided rather than temporal displacement of an outcome that was likely to occur regardless. Nonetheless, we acknowledge that further discussion on lag structures and potential timing effects is warranted and will consider this in our interpretation of findings. *1.4 In the abstract researchers mention that "maternal inflammation will be tested by analysis the epigenetic changes associated with inflammatory cytokines protein and gene expression". Based on the studies from Genome wide association study and Mendenlian law, any genetic variant is always constant since conception, so the expression of cytokine is based on the dysfunction of the cytokine receptor dysfunction. https://doi.org/10.3390/ijms252011332.* **Author Response:** Our approaches to examining heat exposure and inflammation includes assessing maternal transcriptomic and epigenetic signatures, using serial RNA-Seq data, to track up-and down-regulated genes before-and- after heat extremes. These are not Mendelian changes, but the differential gene expression and pathway analyses assessed using RNA Seq data. We can visualise the significantly differentiated genes between the different time points and see which genes are being expressed. We don't do GWAS or similar genetic testing in this study, as the reviewer points out. 1.5 *further not all peripheral inflammation will cause the trigger preterm labour, until the inflammation cross the bloodbrain barrier, the inflammatory chain of reaction is required. Further, why is the focus on IL-6, there are a range of pro-inflammatory and anti-inflammatory cytokines which can cause BBB inflammation Ref:2* **Author reponse:** We appreciate your important point that not all peripheral inflammation will trigger PTB, and that specific inflammatory pathways, including those involving the blood-brain barrier, may play a role. However, our study is primarily focused on inflammatory pathways at the

maternal-foetal interface (such as placenta, myometrium), which are more directly implicated in the initiation of labour than the BBB. We also acknowledge that IL-6 is a non-specific marker and that multiple cytokines, both pro- and anti-inflammatory, may contribute to the broader inflammatory response. Our focus on IL-6 is based on several considerations: 1). Evidence supporting its role in PTB; 2). its relative ease of measurement and low cost of testing; and 3). its widespread use in research, which facilitates comparison across studies. Nevertheless, we do plan on conducting a more comprehensive biomarker analysis using a panel of 96 inflammatory markers. This will allow us to investigate a range of cytokines implicated in both peripheral and central inflammation and inform more complex analysis. Biomarker analysis will be conducted using multivariate regression models to examine the association between heat exposure and inflammatory markers, adjusting for potential confounders. Inferential analysis of longitudinal data will include mixed-effects linear models. Much of this analysis is done using visualisations such as heatmaps and volcano plots. Bonferroni corrections will be applied to counteract potential multiple testing biases. We recognise that much remains unknown about the precise pathways - whether systemic, BBB, or at the level of the cervix and membranes - and which pathways are most important. This study will try address some of those questions. We have added reference to a recent review on heat and preterm birth Mushimiyimana 2025, which discusses this in more detail. 2.6 *Sample size estimation- I still did not get the basis of the sample size based on IL-6, has there been a study to estimate IL-6 >1000pg/ml is linked with preterm birth.* **Author response:** Many thanks for this query, the point made here is valid. We should have noted the rationale for selecting Il-6 as the outcome of interest. IL-6 is one of the most widely studied inflammatory markers and indeed, several studies have linked Il-6 with preterm birth, as in the papers we now cite for this: (Prairie et al. 2021, Hatzidaki et al., 2005, Karampitsakos et al., 2025). In addition, IL-6 has been shown to be raised by heat (Maloney & Duffy, 2024). We do however acknowledge that the originally specified IL-6 threshold of >1000 pg/mL was not appropriate for maternal serum. This was an oversight, and we appreciate the opportunity to correct it. We have revised this threshold in the manuscript to reflect more clinically and biologically relevant values for maternal serum which are much lower depending on presence of infection or specific population group being studied. While there is no universally accepted threshold for IL-6 in predicting PTB, and some variability exists across studies, we have revised our threshold to 10pg/ml as a conservative measure based on other studies which identify maternal serum IL-6 levels around this value as predictive of preterm birth (e.g., Murtha et al., 1998) and which was our original intent. We also acknowledge that we have made some assumptions due to the scarcity of existing environmental exposure literature in this area. Nevertheless, our assumptions are based on conservative and pragmatic estimates to ensure sufficient power. One of our aims is to contribute new evidence on the potential relationship between environmental temperature and maternal systemic inflammation. We have clarified this in the revised manuscript. Updated text in sample size calculation: *The primary inflammatory outcome interleukin-6 is strongly linked with PTB (Prairie et al., 2021, Hatzidaki et al., 2005, Karampitsakos et al., 2025). However, there is currently no universally accepted threshold for IL-6 in predicting PTB, with high levels of variability across studies. We have based our sample size on IL>10pg/ml as a conservative measure in keeping with other studies which identify maternal serum levels around this value as predictive of PTB (Murtha et al., 1998).* 2.7 *In hypotheses, the researchers mention that psycho-social stressor is a mediator of preterm birth, but does it not also link with the increase in pro-inflammatory or neuro-hormonal disturbance. Can it be that*

there is a reversal phenomenon that due to increased pro-inflammatory genetic expression and disturbance in neuro-hormonal gene expression, that the social stress can change the pyschological response and thus preterm birth. **Author response:** Thank you for this thoughtful comment. We agree that psychosocial stress may not only act as a mediator but may also be shaped by underlying biological processes, such as pro-inflammatory and neuro-hormonal disturbances. We acknowledge the possibility of bidirectional, feedback loops or reverse relationships, whereby heightened inflammatory or neuroendocrine activity—potentially influenced by genetic expression—could amplify psychological vulnerability or stress perception. In our conceptual model, we view psychosocial stress, inflammatory pathways, and neuro-hormonal disturbances as potentially interacting and reinforcing components. While our study is not designed to fully disentangle these causal directions, our analyses will explore how these variables may individually and collectively mediate the association between heat exposure and preterm birth. We will also consider how these relationships may evolve over the course of pregnancy and be modulated by environmental stressors such as heat. 2.8 *Thank you once again for inviting me to review the protocol* **Author response:** Thank you for your valuable feedback.

Competing Interests: No competing interests were disclosed.