

BMJ Open Healthy Foundations Study: a randomised controlled trial to evaluate biological embedding of early-life experiences

Andrea Gonzalez,¹ Nicole Catherine,² Michael Boyle,³ Susan M Jack,⁴ Leslie Atkinson,⁵ Michael Kobor,⁶ Debbie Sheehan,⁷ Lil Tonmyr,⁸ Charlotte Waddell,⁹ Harriet L MacMillan,¹⁰ on behalf of the Healthy Foundations Study Team

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For numbered affiliations see end of article.

Correspondence to
Dr Andrea Gonzalez;
gonzal@mcmaster.ca

ABSTRACT

Introduction Adverse early experiences are associated with long-lasting disruptions in physiology, development and health. These experiences may be ‘biologically embedded’ into molecular and genomic systems that determine later expressions of vulnerability. Most studies to date have not examined whether preventive interventions can potentially reverse biological embedding. The Nurse-Family Partnership (NFP) is an evidence-based intervention with demonstrated efficacy in improving prenatal health, parenting and child functioning. The Healthy Foundations Study is an innovative birth cohort which will evaluate the impact of the NFP on biological outcomes of mothers and their infants.

Methods and analysis Starting in 2013, up to 400 pregnant mothers and their newborns were recruited from the British Columbia Healthy Connections Project—a randomised controlled trial of the NFP, and will be followed to child aged 2 years. Women were recruited prior to 28 weeks’ gestation and then individually randomised to receive existing services (comparison group) or NFP plus existing services (intervention group). Hair samples are collected from mothers at baseline and 2 months post partum to measure physiological stress. Saliva samples are collected from infants during all visits for analyses of stress and immune function. Buccal swabs are collected from infants at 2 and 24 months to assess DNA methylation. Biological samples will be related to child outcome measures at age 2 years.

Ethics and dissemination The study received ethical approval from seven research ethics boards. Findings from this study will be shared broadly with the research community through peer-reviewed publications, and conference presentations, as well as seminars with our policy partners and relevant healthcare providers. The outcomes of this study will provide all stakeholders with important information regarding how early adversity may lead to health and behavioural disparities and how these may be altered through early interventions.

Trial registration number NCT01672060; Pre-results.

INTRODUCTION

Adverse early experiences have enduring effects on cognitive, mental and physical health outcomes,^{1–5} with implications for the

Strengths and limitations of this study

- First biological evaluation in infants of preventive intervention using a randomised controlled trial.
- Assesses multiple biomarkers, including DNA methylation and stress physiology.
- Biomarker collection can be challenging as infants get older.

next generation.⁶ Parallel lines of animal and human research show that early adversity produces long-lasting disruptions in physiology, including alterations in the stress system^{5–7} and changes in immune system function,^{8,9} which in turn adversely affect brain development and health.^{7–9} These experiences may be ‘biologically embedded’ into molecular and genomic systems that determine later expressions of vulnerability^{3–7}; however, the varied mechanisms and their inter-relations are not fully elucidated. These early experiences set developmental trajectories which canalise over time⁸ and become increasingly difficult to reverse.⁷ Recently, 46 pregnancy and birth cohort studies across Canada were documented by the Maternal Infant Child and Youth Research Network.¹⁰ While these studies provide important aetiological, descriptive and surveillance data about early risk factors for subsequent negative outcomes, to our knowledge no study has yet examined the impact of preventive interventions, starting in pregnancy and extending postnatally, on biological outcomes in mothers and their children.

To better understand whether a preventive intervention can potentially reverse the biological embedding, we proposed to recruit and follow prospectively a sample of young

women and their children from within a randomised controlled trial (RCT) evaluating the Nurse-Family Partnership (NFP) programme in a Canadian context—known as the British Columbia Healthy Connections Project (BCHCP). The NFP is an evidence-based nurse home visitation programme aimed at improving the lives of young, first-time mothers and their children who are experiencing socioeconomic disadvantage. The NFP has demonstrated effectiveness in improving prenatal health, parenting and child development and functioning based on trials conducted in the USA and the Netherlands.¹¹ The implementation and delivery of this public health intervention is being evaluated across five unique health authorities (HA) in a mixed-methods process study.¹² The BCHCP seeks to evaluate the impact of the NFP on health and social outcomes, including childhood injuries (primary outcome), early child development and maternal life course to assess its effectiveness for the first time in Canada.¹³ The substudy of the BCHCP, the Healthy Foundations Study (HFS), is examining the impact of the NFP on biological outcomes in women and their infants followed to age 24 months. A greater understanding of the biology of early adversity and the potential to mitigate detrimental effects will provide a powerful framework with which to inform basic and applied research, practice and policy.

Study rationale

Risk factors in young mothers and their children

Young, low-income mothers and their children are at high risk of suffering health, educational and economic disparities. In Canada, young mothers (<24 years) and their children represent about 5% of live births each year and constitute one of the most marginalised groups within society.¹⁴ There is a strong association between young maternal age and socioeconomic deprivation, and higher reported levels of intimate partner violence (IPV),¹⁵ as well as high-risk behaviours such as smoking, street drug use and poor prenatal care.^{15–18} These maternal risk factors can initiate a trajectory for impaired child development that systematically lowers functional ability during the early years. In brief, children born to these young mothers are at increased risk for cognitive difficulties, impairment in educational and social outcomes, including school adjustment problems,^{19–23} and poorer physical and mental health outcomes.^{18 20} There is an urgent need for effective early interventions for at least two reasons: (1) these trajectories are established early and tend to be reinforced over time; and (2) the ability to change behaviour and brain plasticity decreases over time, likely increasing the costs of implementing successful programmes and lessening the opportunity for realising benefits.²⁴

Theoretical and conceptual framework

To conceptualise the early origins of disparities in learning, behaviour and health, this study is informed by a biodevelopmental framework proposed by Shonkoff.²⁵

In this framework, childhood disorders and impairments in learning and behaviour due to early adverse experiences (both prenatal and postnatal) are mediated by alterations in physiological systems, the hypothalamic-pituitary-adrenal (HPA) axis and immune function, and through epigenetic modifications.^{25 26} We focus on these factors for two reasons. First, HPA axis function, inflammatory processes and epigenetic changes are three of four most likely mediators of the biological embedding of early experiences (the other is neural structure and function).²⁷ Second, animal and human research link these mechanisms to prenatal and postnatal risk factors as well as various offspring outcomes.

The most widely studied mechanism explaining associations between prenatal stress and child outcomes involves the HPA axis. Considerable evidence from both animal and human work demonstrates that exposure to prenatal adversity, including maternal stress, maternal substance use and smoking, exposure to IPV and poverty, and postnatal adversity, such as suboptimal maternal care, are all associated with HPA axis dysregulation.^{28–40} Alterations in HPA axis function are one of the hypothesised mediators between early adversity and later mental and physical health outcomes.^{41–43} Various intervention studies in children and adolescents have shown a positive impact on HPA axis functioning in children exposed to early adverse rearing environments including: foster care children,^{44 45} adopted institutionalised children,⁴⁶ maltreated children,^{47 48} children at risk for antisocial behaviour problems,⁴⁹ parentally bereaved children⁵⁰ and infants whose mothers were at risk for antenatal depression.⁵¹ In almost all cases, the impact was reflected in beneficial alterations in the HPA axis (eg, prevention of a blunted cortisol response) in the intervention group compared with the non-intervention group.⁵²

Acute psychological stressors, including prenatal stress, exposure to poverty, child maltreatment, bullying, low-quality child care and maternal distress—all result in increases in proinflammatory cytokines, such as C-reactive protein (CRP) and interleukin-6 (IL-6).^{53–56} Inflammatory markers have also been linked to externalising problems,⁵⁷ negative mood as well as affective and interpersonal problems in adolescents,⁵⁸ as well as executive dysfunction in obese children.⁵⁹ There is emerging evidence that psychological interventions may have the potential to influence inflammatory markers. For example, in a sample of foster care children enrolled in a Cognitively Based Compassion programme, within the intervention group, practice sessions were associated with decreased CRP levels.⁶⁰ No studies to date have examined whether a preventive intervention, starting prenatally, has an impact on child inflammatory markers and child health.

Recent discoveries in the field of epigenetics have led to a greater understanding of how environmental experiences become biologically embedded. Emerging evidence suggests that early-life experiences may cause epigenetic modifications of the genome (such as changes

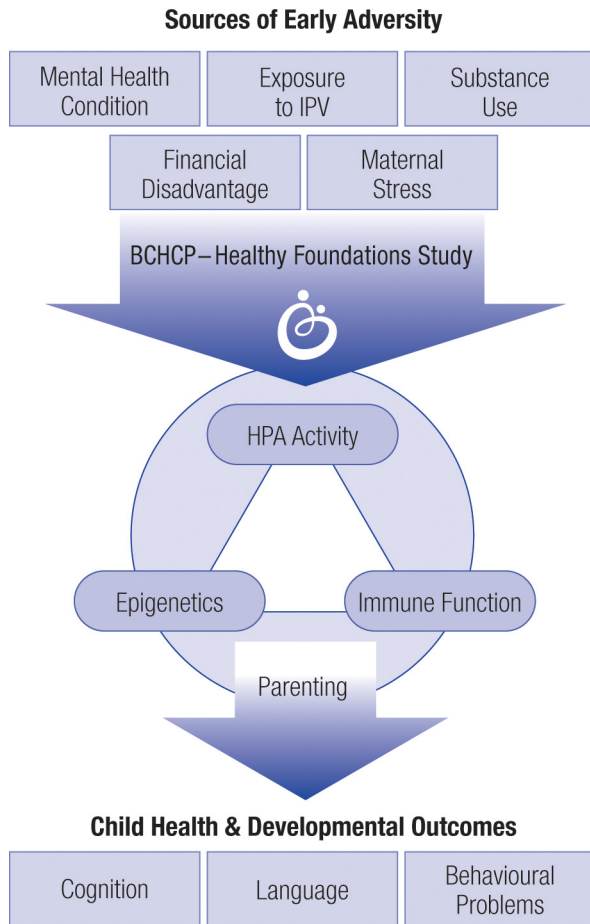


Figure 1 Conceptual model of Healthy Foundations Study (HFS). BCHCP, British Columbia Healthy Connections Project; HPA, hypothalamic-pituitary axis; IPV, intimate partner violence.

in DNA methylation; DNAm), which influence the development of mental and physical health throughout the lifespan.^{61 62} Variations in DNAm patterns have been associated with maternal cigarette smoking during pregnancy,^{63 64} antenatal depression,^{65 66} low socioeconomic status in childhood,^{67 68} institutionalisation⁶⁹ and childhood adversity, including maltreatment.⁷⁰ In addition, differential DNAm is associated with variations in cortisol response in infants,⁷¹ children⁷² and adults,⁷³ across a range of child outcomes.^{74–77} To date, one study has illustrated that a family-centred intervention had a protective effect on epigenetic ageing through enhanced parenting.⁷⁸ However, no study to date has examined this within a preventive intervention context starting prenatally.

Collectively, these findings illustrate that prenatal exposure to maternal stress, postnatal adversity and non-optimal caregiving may exert programming influences reflected in the child's capabilities to regulate stress and may impact inflammation, and DNAm. **Figure 1** depicts a model of key variables in the HFS. We selected risk factors which are most consistently associated with young motherhood and are targeted by NFP. Our risk factors include: maternal cigarette smoking, maternal stress and presence

of mental health condition, exposure to IPV and family financial disadvantage—most of which have been associated with dysregulated HPA function in children, immune function (elevated inflammation levels), alterations in DNAm and various child outcomes. Adopting this model within the context of NFP evaluation provides a unique opportunity to examine associations between these key variables, while assessing the biological impact of this programme.

Nurse-Family Partnership

The NFP is an evidence-based home visitation programme for vulnerable low-income, young mothers and their children developed in the USA.⁷⁹ In Canada, in a few locations where the NFP is being studied, public health nurses (PHN) trained to deliver the NFP provide intensive education and support to mothers during home visits that start in early pregnancy and continue until the child's second birthday.⁸⁰ In one or more of three US RCTs, the NFP has demonstrated improvements in prenatal health behaviours and birth outcomes, reduction in child mortality rates from preventable causes, as well as improvements in sensitive child care. The NFP has also shown consistent effects in improving child functioning.^{81–83} International trials have been conducted in the Netherlands with positive results and England with null findings.^{84–86} Failure to positively replicate the findings in England has been attributed to selection criteria of participants, based solely on age (<20 years), and the frequency and intensity of services received in the control group as 'usual care'.⁸⁵ In the original US trials, programme effects were strongest in families with concentrated disadvantage, and for children whose mothers had few psychological resources to cope with adversity.^{85 86} None of the completed international trials have included biomarkers to date—thus the HFS provides a unique contribution to the literature on the NFP, and preventive interventions more broadly.

Study objectives

1. To determine whether the NFP has an effect on infant biological function, as reflected by alterations in HPA axis, immune function and epigenetic markers. Specifically, whether the NFP will attenuate disruptions in HPA axis function (infant salivary cortisol) and inflammatory markers sensitive to early adverse experiences and whether the NFP influences DNAm as a function of intervention group status.
2. To investigate whether the intervention has an impact on prenatal risk factors, including maternal prenatal cortisol levels, and whether these changes are associated with alterations in infants' biological markers. Specifically, to determine if maternal prenatal stress (maternal prenatal cortisol and prenatal adversity) is associated with infant stress physiology (cortisol reactivity and inflammatory markers) and DNAm and whether the NFP moderates these associations.

3. To examine whether alterations in biological markers explain the impact of the intervention on infant health outcomes. Specifically, we will test a mediation model to examine whether attenuation of postnatal adversity, purported to be induced by the NFP, mediates the association between the NFP on child cortisol levels and health outcomes (cognitive and socioemotional functioning).

Recognising that early adversity does not have the same impact on all children, we consider two additional factors: (1) *Child sex*: Given that epidemiological studies highlight sex differences in the phenotypic expression of health outcomes⁸⁷ and that prenatal programming may be attributed to these observed sex differences in outcomes,^{88–89} sex differences will be considered in all analyses. (2) *Parenting* is an important mechanism for regulating offspring physiology and behaviour,^{90–92} and is specifically targeted in NFP (Dyadic Assessment of Naturalistic Caregiver–Child Experiences—a clinical observation tool to guide clinical implementation of parenting content of the NFP),⁹³ thus we will examine parenting as a potential mediator.

METHODS AND ANALYSIS

Recruitment, ethics and consent: BCHCP

The BCHCP is a prospective, large-scale, public health RCT (NCT 01672060), evaluating NFP's effectiveness compared with BC's existing perinatal and early childhood services (see ref¹³ for full details on the larger trial). Referrals to the RCT are received from PHNs across four participating regional HAs. Participant inclusion and exclusion criteria are outlined in [box](#). Eligible and consenting participants are randomised using an unpredictable randomised sequence protocol to receive either existing services (comparison group) or the NFP plus existing services (intervention group). Existing services within British Columbia's universal healthcare system vary by HA; however, many include primary healthcare services from physicians or midwives; specialist physician services (ie, obstetrics and gynaecology); public health programmes including pregnancy screening, prenatal classes and brief home visitation (non-NFP) by nurses or paraprofessionals; and various targeted and universal parenting and early child programmes. Numerous social services including child benefits, employment and income assistance, child protection services, shelter and housing support and food banks are provided by various levels of government (municipal, provincial and federal) and charitable organisations. Given that availability of services varies across districts and whether mothers and their children access these services, data on health and social service utilisation are collected at each time from all participants within BCHCP.¹³ The field interviewers are masked to participants' treatment group allocation. The BCHCP recruited a sample of 739 participants from four HAs (from October 2012 to November 2017).

Box British Columbia Healthy Connections Project (BCHCP) participant inclusion/exclusion criteria

Interviews

Age 24 years or younger

1. First birth. Women are eligible if a previous pregnancy ended in termination, miscarriage or stillbirth, or if previous parenting involved step-parenting only.
2. Less than 28 weeks' gestation. Women are recruited prior to 28 weeks' gestation to ensure that participants randomised to Nurse–Family Partnership (NFP) receive their first home visit by the end of the 28th week of gestation, according to NFP fidelity requirements.
3. Competent to provide informed consent, including conversational competence in English.
4. Experiencing socioeconomic disadvantage.
 - Age 19 or younger.
 - Ages 20–24: Meets two of three indicators: *Lone parent*, *less than grade 12*; or *low income* which requires one or more of:
 - i. Receiving Medical Services Plan Premium Assistance, disability assistance or other income assistance.
 - ii. Finding it very difficult to live on total household income with respect to food or rent.
 - iii. Homeless, defined as living on the streets, living in a place not meant as a long-term dwelling (eg, car or tent), staying in a shelter, or staying somewhere temporarily with no permanent address (eg, 'couch surfing').

Women are ineligible to participate if they meet any exclusion criteria at time of baseline interviews:

1. Planning to have the child adopted.
2. Planning to leave the BCHCP catchment area (designated Local Health Areas) for 3 months or longer during the trial.

Recruitment for HFS started in April 2013 and was completed in April 2017. Based on estimates from an ongoing evaluation in the USA with a similar population, and our feasibility pilot conducted in Hamilton, Ontario, we estimated that on average, 14% of enlisted participants would be lost to follow-up. To compensate for attrition, the HFS recruited a subsample of 459 women drawn exclusively from two of the four participating HAs: the Fraser Health and the Vancouver Coastal Health. In both HAs, the NFP is delivered by PHNs who have received extensive education in the NFP delivery model. The evaluation follows Consolidated Standards of Reporting Trials guidelines and is registered with the US National Institute of Health clinical trials registry.

Data collection

BCHCP questionnaires: assessment of maternal and environmental factors

Data for the BCHCP being collected are conducted at baseline (prior to 28 weeks' gestation and immediately prior to randomisation) and then at 34–36 weeks' gestation (telephone interview), and postnatally at 2, 10, 18 and 24 months. During the interviews, trained Scientific Field Interviewers (SFI) collect sociodemographic data through questionnaires on age, racial/cultural group, family structure and education.^{94–95} Socioeconomic

factors including employment, income and financial supports, relationship status and housing stability are also being collected. Nicotine, alcohol and drug use is being recorded.^{94 96 97} Anxiety and depression in the mother are assessed using the Kessler Psychological Distress Scale.⁹⁷ Exposure to IPV is assessed using the Composite Abuse Scale.⁹⁸ Working within the existing BCHCP interview schedule, the HFS involves collection of biological measures from consenting mother–infant dyads during in-person visits. Hair cortisol samples are collected from women at baseline and at 2 months post partum. Infant salivary samples are being collected at 2, 10, 18 and 24 months and infant buccal swabs are collected at 2 and 24 months. [Table 1](#) includes the full list of measures administered in the BCHCP and HFS.

BCHCP child outcomes at 24 months

To assess child cognitive development at 24 months the Bayley Test of Infant and Toddler Development (Bayley-III) is being administered. Trained SFI administrators are assessing children using the cognitive and language (expressive and receptive) scales. Standardised scores are derived for each of the cognitive and language domains with a mean of 100 and SD of 15. Standard scores will be used in the mediation model. The Bayley-III has demonstrated adequate validity and reliability.⁹⁹ The ASEBA Child Behavior Checklist (CBCL) for ages 1.5–5 years is administered to assess child socioemotional development. The CBCL consists of 99 items rated by mothers concerning issues, disabilities and descriptions of problems and strengths about the child during the past 6 months. Three primary scales (internalising, externalising, total problems) are also scored. T-scores will be used in the mediation model for Aim 3. Test–retest reliability, inter-rater reliability and predictive validity are high.¹⁰⁰ Child outcome measures at 24 months will be used in the mediation model for the third objective.

Biological samples: primary outcomes for the HFS

Maternal hair cortisol

Hair cortisol analysis is emerging as a biomarker for chronic stress because systemic cortisol is understood to be incorporated into the hair shaft during hair growth.¹⁰¹ To measure maternal physiological stress during pregnancy and early in the post partum, SFIs are collecting hair strands, 3 mm in diameter (≈ 80 strands), taken from the posterior vertex of the head, as close to the scalp as possible. Strands of hair are cut into a 3 cm segment to reflect cortisol production in the last 3 months. Information regarding frequency of hair washes and chemical hair processes (bleaching, dying and permanent waves) is collected in response to the current debate regarding the effect of these techniques on hair cortisol levels.¹⁰²

Infant salivary cortisol

Salivary samples are collected from infants at 2, 10, 18 and 24 months of age. Although there is higher intraindividual variability early in the first year of life,¹⁰³ there

is evidence that cortisol samples are positively associated across time and stressors throughout the latter first year (>6 months) extending to the second year of life.^{104 105} During each of these visits, samples are collected at baseline and then at 20, and 40 min poststressor. Poststressor time points are designed to capture both peak response and recovery.

Infant stress procedures

Given that mild physical stressors (eg, physical examination) are associated with the greatest cortisol reactivity response in infants less than 13 weeks of age (Cohen's d : 0.35–1.69)¹⁰⁵; at 2 months, we administer a mock physical exam as a mild physical stressor and ask the mother to change the infant's diaper and clothing. These procedures have been used successfully in previous studies.^{106 107} To elicit cortisol stress reactivity in infants at 10 months, we use the barrier task,¹⁰⁸ and at 18 months we use a toy frustration task.¹⁰⁹ These procedures have been validated in eliciting emotional reactivity and infant cortisol reactivity.^{103 104 110} At 24 months, samples are collected around the administration of the Bayley cognitive test, as a novel event/task. Although these challenges are not uniformly stressful across infants, ethical considerations preclude the use of extreme stressors in these age groups.¹⁰⁵ Nevertheless, these challenges have been repeatedly shown to demonstrate individual differences in cortisol reactivity based on factors such as maternal sensitivity,^{103 104 111} attachment security,¹¹² maternal antenatal depression,¹¹³ interparental aggression¹¹⁴ and poverty.¹¹⁵

Infant inflammatory markers

CRP and IL-6 will be assayed from baseline, post-20 min and post-40 min salivary samples. Our selection of inflammatory markers was based on a review of the literature and the following criteria: (1) measurable in saliva in children in response to a stressor; (2) associated with prenatal and/or postnatal adversity; and (3) associated with at least one child outcome of interest. Samples will be assayed in duplicate using ELISA with the Salivary ELISA kits from Salimetrics. All markers are detectable in saliva of neonates and older children and can predict abnormal serum thresholds.^{116 117}

Behavioural observations of parenting

The mother and infant are videotaped at 2, 10, 18 and 24 months post partum. Subjects are videotaped interacting during a series of structured interactions. The mother–infant interaction is 4 min without toys, 4 min with toys, followed by a 4 min divided attention task. Mothers are asked to interact as they normally would while remaining within the camera's view. During the last 4 min period, mothers complete a questionnaire while still caring for the infant; the purpose of this task is to place the mother in a divided attention situation thus creating a more ecologically valid situation. The videos will be coded using the Maternal Behaviour Q-Set Revised (MBQS-R)¹¹⁸ consisting of 25 items that are sorted and compared with the sort of

Table 1 Measures and timing of measures in BCHCP and HFS

| Construct | Prenatal | | Postnatal | | | |
|---|-------------------------------|--------------------------------|-----------|-----------|-----------|---|
| | Baseline | 2 months | 10 months | 18 months | 24 months | |
| BCHCP measures | | | | | | |
| Maternal measures | Sociodemographics | | | | | |
| | Age, racial/cultural group | X | | | | |
| | Prenatal substance use | | | | | |
| | Alcohol use | X | X | X | X | X |
| | Other substance use | X | X | X | X | X |
| | Tobacco use | X | X | X | X | X |
| | Postnatal substance use | | | | | |
| | Alcohol use | | X | X | X | X |
| | Other substance use | | X | X | X | X |
| | Tobacco use | | X | X | X | X |
| | Second-hand smoke | | X | X | X | X |
| | Mental health | | | | | |
| | Anxiety and depression | X | X | X | X | X |
| | Financial resources | | | | | |
| | Income and basic needs | X | X | X | X | X |
| | Education, employment | X | X | X | X | X |
| | Exposure to IPV | | | | | |
| Intimate partner violence | X | X | X | X | X | |
| Housing | | | | | | |
| Residential composition and stability | X | X | X | X | X | |
| <i>Secondary outcomes for mediation model</i> | | | | | | |
| Child measures | Child outcome | | | | | |
| | Cognitive development | | | | | X |
| | Child outcome | | | | | |
| | Child behaviour problems | | | | | X |
| Child outcome | | | | | | |
| Language | | | | | X | |
| HFS measures | | | | | | |
| Biological measures | <i>Primary outcomes</i> | | | | | |
| | Maternal physiological stress | Maternal hair cortisol | X | X | | |
| | Infant biological outcomes | Salivary cortisol levels | | X | X | X |
| | | Salivary CRP | | X | X | X |
| | | DNA methylation (buccal swabs) | | X | | X |
| | Parenting | Maternal sensitivity | | X | X | X |

BCHCP, British Columbia Healthy Connections Project; CRP, C-reactive protein; HFS, Healthy Foundations Study; IPV, intimate partner violence.

a prototypically sensitive mother. Ultimately, the MBQS-R yields a single score reflecting the mother's responsiveness and sensitivity to infant signals. Results from a recent study indicate that the MBQS-R is a reliable and valid measure of the quality of maternal interactive behaviour during a short play session at 10 months post partum.¹¹⁹

Epigenetics and DNAm

The plasticity of epigenetic modifications makes it an attractive mechanism at the interface between environment and genome. For example, the best understood epigenetic marker, DNAm, varies with age, gender, tissues and complex disease phenotypes. Importantly, DNAm

is highly changeable during prenatal development and early life and thus provides an excellent potential biological substrate related to intervention effects. At 2 and 24 months, infant buccal epithelial cells will be collected using Simhelix Dri-Capsules (Boca Scientific). We collect samples at 2 months to capture NFP programme effects prenatally, and then at 24 months to capitalise on the impact of the full intervention on DNAm. Similar to the majority of epigenetic studies in the paediatric age range, we will assess methylation in DNA derived from buccal cells. Compared with blood, buccal cells have the following two important features supporting their use in this study. First, on a practical level, and of particular importance when working with young children, their collection is much less invasive than venipuncture necessary to obtain blood, which increases the likelihood of participation and facilitates retention. Second, on a biological level, they originate from the same embryonic germ layer as the brain. However, despite this common origin, DNAm between buccal cells and brain likely is still highly discordant, given the fundamental role of DNAm in specifying tissue identity during development. Despite the highly tissue-specific nature of DNAm, both in terms of interindividual variability and absolute measures, there are specific CpGs that correlate tightly between peripheral tissues and central tissues. For example, concordant DNAm signatures of ageing have been reported between blood and brain. Perhaps more importantly, an increasing body of research is documenting DNAm signatures related to child development and early-life environments in buccal cells. This then provides a broad comparative basis for DNAm measures performed in the HFS, which will be further expanded by ongoing epigenetic studies in buccal cells in major related cohorts.

Statistical power

We focus our power analysis on the latent growth curve (LGC) models with mediation. These analyses are most resource intensive, requiring a larger sample size than other objectives to meet power requirements. Calculation of power and sample size for these models is complex because specialised software does not exist.¹²⁰ In addition, as is often the case for such models,¹²¹ it is difficult to specify the strength of association among the constituent variables. We surveyed articles examining each manifest variable comprising a given latent variable (eg, smoking, maternal mental health, and so on) as they related to the child outcomes of interest here. Ultimately, we decided on a general effect size of $r=0.30-0.40$, what Cohen¹²² considered a medium-to-large association. This figure seemed to reflect the pertinent literature reasonably well and the selection of a narrow range of effect sizes simplified power analyses considerably. A power study by Fan¹²³ demonstrated that a sample size of 200 participants per group is required to detect medium-sized intercept and slope differences between the NFP and control groups using LGC. We then conducted a Monte Carlo simulation to estimate sample size requirements to detect a

significant mediational pathway (parallel process) with latent variables using multiple-group structural equation modelling (SEM), $1 - \beta=0.80$.¹²¹ The simulation was conducted with 10 000 replications using Mplus V.6.11. Given the number of parameters involved in the model, we made a 'best guess' (Thoemmes *et al*, p 515)¹²⁰ at effect size, assuming it to be medium, $R^2=0.13$.¹²¹ Simulation indicated the need for 200 participants per group (NFP and control). We are confident that a sample size of 400 is sufficient to validate all models proposed here.

Statistical analysis

Normality of all variables will be examined prior to analyses and appropriate transformations will be applied if required. The BCHCP collects a wide range of maternal risk variables which can be grouped according to two developmental periods (prenatal and postnatal). Given that multiple risk factors at each time point may be inter-related and that our proposed latent variables have not been reported in the literature, prior to the main analyses we will first examine variables using exploratory factor analysis to examine whether any manifest variables have a low factor loading and can be dropped from the analyses. Measures will be combined into latent factors using confirmatory factor analysis. The analyses used to achieve each study objective appear below.

Analysis 1: assessment of biomarkers (primary outcome)

To evaluate the impact of the NFP on infant biological outcomes (cortisol and inflammatory markers), we will use multilevel growth curve models in MLwiN¹²⁴ to estimate the baseline levels (intercepts) and the trajectories (slopes) of biomarkers over sampling time (baseline, post-20 and post-40) for the four assessment visits (2, 10, 18 and 24 months). Both the model intercept and the slope will be specified as random effects and allowed to covary. Using multilevel growth curve analysis to assess NFP influences on salivary outcomes, we will specify two models: (1) an unconditional model to characterise infant cortisol/inflammatory trajectories (model 1) and (2) a model to compare growth trajectories of NFP infants versus control infants (model 2). Model 2 provides the basis to test whether or not there are statistically significant differences (fixed effects) between infants whose mothers are in the NFP versus standard care. Infant sex will be explored as a moderator (model 3) and parenting will be explored as a mediator (model 4).

Analysis 2: moderation of NFP intervention on prenatal adversity

To examine whether maternal prenatal cortisol levels and prenatal adversity are predictors of infant cortisol reactivity and inflammatory levels we will use LGC models in Mplus V.8.0.¹²⁵ The model will feature the observed construct of maternal prenatal stress (hair cortisol at baseline) and prenatal adversity (measured at baseline). Prenatal cortisol and prenatal adversity will be simultaneously regressed onto the intercept and slope constructs of infant biomarkers. To examine whether group status

(NFP vs control) acts as a potential moderator between prenatal stress (cortisol and adversity) and infant biology, we will use a multiple-group approach where models will be estimated simultaneously for NFP infants and control infants. Measurement model parameters will be constrained to equal each other in the two models (eg, error variance and factor loadings), but will be tested for differences in structural parameters (eg, means, variances and predictor pathways). Significant interactions will be tested and probed.¹²⁶ Finally, to explore whether there is a specific time point in which there are NFP versus control group differences in cortisol levels, we will rerun the LGC models with the intercept located at 10, 18 and 24 months.

Analysis 3: mediational analyses of NFP intervention on biomarkers and child outcome

To test our mediation model, in the absence of data to inform the shape of the growth trajectory, we will investigate prior to the main analyses whether the growth trajectories of both the mediator and outcome measures are linear or non-linear using varying design vectors.¹²⁷⁻¹²⁹

When examining mediation in an LGC model within an intervention context, mediation is supported when the programme changes the level of the mediator, and changes in the mediator then influence the level of the outcome. Testing mediation in a parallel process LGC model involves three steps.¹²⁷ In the first step, growth trajectories of each process (postnatal adversity and cortisol) will be examined to determine whether the trajectory shape fits the data and to examine whether the slopes are different between intervention and control groups. In the second step, LGC models are combined into a single parallel process model in which the mediation effects are tested by relating the intervention condition, the slope of the mediator and the slope of the outcome. In the third step, mediation effects will be tested using the asymmetric CI test.¹²⁹ Because we anticipate a significant shift in the mediation and outcome only within the NFP group, we will examine whether the specification of the trajectory shape is appropriate for both groups. To accomplish this, factor loadings on latent factors and residual variance of the repeated measure at each assessment time point will be specified as equal across both groups. The covariance between the intercept and the slope will be freely estimated and will be allowed to vary between groups. Finally, to examine the association between child outcomes at age 24 months and infant cortisol, postnatal adversity and group status, we will estimate a serial (multiple mediator) model within a SEM framework using Mplus V.8.0. The multiple mediator chain will be specified (intervention → postnatal adversity → infant biomarker → child outcomes), following the logic of our conceptual model (see figure 1). Asymmetric confidence limits will be calculated to test for mediation effects. To test for sex differences, we will examine which regression parameters are substantially different between sexes. We will use SEM to examine the question of whether any

intervention-induced changes in parenting are related to changes in child biological functioning, which is related to child outcomes.

Analysis and interpretation of epigenomic data

The analysis will focus primarily on interrogating DNAm in the context of CpG dinucleotides, as it currently represents the most accessible and reliable marker for quantitative epigenetic measurements. DNAm studies take advantage of bisulfite treatment of genomic DNA that selectively deaminates cytosine residues that are not methylated, while leaving methylated cytosines intact. Thus, this approach converts epigenetic information to sequence-based information that will be measured in an unbiased genome-wide assay, using the Illumina Infinium MethylationEPIC BeadChip array. This enables the quantitative interrogation of more than 850 000 CpG methylation loci per sample, with a throughput of between 96 and 192 samples per week. The array covers all designable RefSeq genes, with CpG island shores, non-island CpGs, CpG islands outside of coding regions and miRNA promoter regions represented. In R statistical software, these data will undergo stringent quality control and normalisation, removal of all low-quality or cross-hybridising probes and, lastly, batch correction using the COMBAT method. Normalised data will then be used for three complimentary analytical approaches. First, in a hypothesis-driven method, we will a priori derive from the array CpG sites in candidate genes and test these for association with demographics (child sex) and group status. Candidate genes will be derived from a combination of literature review and computational pathway predictions. These will include genes involved in dopamine and serotonin metabolism, HPA axis and others. Second, to take advantage of the tremendous information content on the Illumina EPIC array, we will perform epigenome-wide association studies (EWAS). This approach provides an opportunity to identify novel genes and pathways associated with our key variables of interest. In practice, we will use bioinformatics methods such as DMRcate, which uses a Gaussian kernel smoothing of DNAm across the genome, to identify broader regions of CpG loci whose DNAm is associated with NFP status. This analysis will be complemented by CpG site-specific differential methylation analysis using moderated t-statistics with empirical Bayesian variation estimation using the Bioconductor R package 'limma'. In both cases, multiple test correction will be done using the Benjamini-Hochberg method and a false discovery rate (FDR) threshold of ≤ 0.05 and an arbitrary filter of DNAm difference of $\geq 5\%$ for each CpG will be applied to increase the likelihood of biological significance. Candidate gene and EWAS will be repeated with maternal prenatal cortisol as a variable, using a similar set of approaches. For all variables, the lists of CpG resulting from the EWAS approaches will be further examined for enrichment of specific genomic features, including advanced bioinformatics approaches that

incorporate available data such as CpG density, transcription factor binding sites and chromatin features. Furthermore, resultant gene lists will also be interrogated for association with cellular processes, biological pathways and associated functions, using publicly available and validated tools such as ErmineJ. Collectively, this work will enable the identification of DNAm loci and regions associated with NFP status, and reveal whether epigenetic regulation of broader genomic functions underpins the intervention.

Developmental epigenetic clock

A novel tool that offers a fundamentally different window into epigenetic biology will complement the powerful candidate gene and EWAS approaches. Specifically, motivated by the success of epigenetic age and its deviation from chronological age as a marker for health,¹³⁰ we have created a developmental epigenetic clock. This clock is specific for buccal epithelial cells, DNAm, and has a much higher accuracy for the paediatric age range compared with existing epigenetic clocks for adults. Using our predictive algorithm, we will compute DNAm developmental age for each infant in NFP versus control groups and determine age deviation from chronological age. This will allow us to assess whether the developmental epigenetic age is accelerated according to NFP status.

ETHICS AND DISSEMINATION

An independent Data and Safety Monitoring Committee monitors the recruitment, participant safety, protocol compliance and data quality. All data collected (questionnaires, videos and biosamples) are deidentified; labelled only with a unique study code and visit date. Videos are encrypted with 7Zip and stored on password-protected computers within locked facilities. Tissue samples are labelled with unique study codes, and stored in locked cabinets and freezers within locked laboratories. These security provisions are in full compliance with the Tri-Council Policy Statement (TCPS-2, Article 5.3).

Findings from this study will be disseminated in several ways and via multiple platforms, including: publishing in peer-reviewed journals; providing presentations at research conferences and meetings with policy partners and practitioners working with mothers and children; providing regular updates to HFS participants through newsletters and website postings at <http://nfp.mcmaster.ca/>. All results will be reported as analysis of group data.

DISCUSSION

Mounting scientific evidence illustrates that a common foundation for health, learning and behaviour is laid early in life. In parallel, advances in biological sciences have begun to elucidate potential mechanisms for the association between early adversity and later physical and mental health outcomes. A greater understanding of the

biology of early adversity and the potential to reverse its detrimental effects would provide a powerful framework with which to inform basic and applied research, practice and policy. The biodevelopmental approach provides an alluring model of how the above associations are integrated and may be modified through interventions; however, these hypotheses remain relatively unexplored. Prospective studies examining the biological embedding within an intervention context are virtually non-existent. The HFS would be one of the first to examine the associations espoused by the biodevelopmental model within an RCT for a preventive intervention. Using a methodologically rigorous RCT evaluating an evidence-based intervention provides a unique lens through which processes of biological embedding may potentially be averted. Examining HPA function, inflammatory markers and DNAm in infancy also provides us with information on the importance of intervention effects in the short term, which likely have long-term implications. Recognising that some intervention effects may not be apparent until after intervention completion, and that some of the most pronounced positive effects of the NFP were seen at later ages,^{131 132} our long-term plan is to follow this intervention cohort longitudinally during preschool and school-age years. If the NFP can result in a 'healthier biological phenotype', this may suggest a positive impact of intervention at a biological level, and forecast a greater likelihood of future health. Adopting a biodevelopmental approach will provide practitioners and policymakers with a framework for understanding how early adversity may lead to health and behavioural disparities and how these may be altered through early interventions. Finally, discoveries on the impact of the NFP on biological outcomes will provide policymakers with far-reaching evidence on the influence of early developmental processes.

Author affiliations

¹Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, Ontario, Canada

²Children's Health Policy Centre, Simon Fraser University, Vancouver, British Columbia, Canada

³Department of Clinical Epidemiology and Biostatistics, McMaster University, Hamilton, Ontario, Canada

⁴Department of Nursing, McMaster University, Hamilton, Ontario, Canada

⁵Department of Psychology, Ryerson University, Toronto, Ontario, Canada

⁶Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada

⁷Children's Health Policy Centre, Simon Fraser University, Burnaby, British Columbia, Canada

⁸Family Violence Surveillance, Health Promotion and Chronic Disease Prevention Branch, Public Health Agency of Canada, Ottawa, Ontario, Canada

⁹Psychiatry Department, Simon Fraser University, Burnaby, British Columbia, Canada

¹⁰Psychiatry and Behavioural Neurosciences, McMaster University, Hamilton, Ontario, Canada

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Collaborators Members of Healthy Foundations Study Team: Dr Mark Ferro, Dr Pablo Nepomanschy, Dr Ronald Barr, Dr Colleen Varcoe, Dr Lenora Marcellus.

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Competing interests None declared.

Patient consent Not required.

Ethics approval The protocol for recruitment and collection of BCHCP data, and biological samples for the HFS has been approved by seven research ethics boards of all participating institutions: Simon Fraser University, the University of British Columbia, McMaster University, Ryerson University, the Public Health Agency of Canada, and the two participating health authorities (Fraser Health Authority and Vancouver Coastal Health Research Institute). Informed consent was obtained from expectant women (including consent for the baby/babies that the mother is expecting). Women aged 19 years or younger who are competent to provide informed consent are deemed mature minors by the trial's research ethics boards and may consent to participate without parental consent.

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