Predictors and consequences of maternal-origin microchimerism: A study of women in the Philippines

Tiffany D. Pan

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Reading Committee:

Daniel T. A. Eisenberg, Chair

J. Lee Nelson

Patricia A. Kramer

Bettina Shell-Duncan

Program Authorized to Offer Degree:

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University of Washington

Abstract

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Tiffany D. Pan

Chair of the Supervisory Committee:

Daniel T. A. Eisenberg

Department of Anthropology

Microchimerism (Mc) refers to the presence of a small number of cells or DNA from a genetically distinct individual. This phenomenon occurs naturally with bidirectional maternal-fetal exchange during pregnancy, and Mc can persist for decades after delivery. Past research on Mc has largely focused on the long term health of mothers and immune disorders that affect young children. However, each life stage (gestation, infancy, childhood, adolescence, and adulthood) faces different tradeoffs in immune function. This dissertation leverages data from a multigenerational, longitudinal health study and introduces Mc as a mediating factor in immune tradeoffs throughout the life course.

Mc is thought to influence host immune function by: 1) directly contributing stem cells that differentiate and develop into functional immune cells and 2) expressing foreign antigens

that trigger host immune system responses. However, there are considerable gaps in our understanding of the basic biology of Mc, including what determines why some individuals have more Mc than others and how these differences might impact reproductive fitness. This dissertation attempts to fill these gaps by reporting investigations with the following specific aims: 1) evaluate predictors of maternal-origin Mc in young adult women, 2) assess whether Mc is associated with lower risk of early life infection, and 3) determine whether adult women with Mc from their mothers have lower risk of adverse pregnancy outcomes when they have offspring of their own.

Results indicate that participants who were breastfed as infants have diminished maternal-origin Mc compared to those who were never breastfed. There was no evidence of a relationship between Mc and early life infection or third generation offspring birth weight, but financial and technological limitations may have obscured my ability to detect the true relationship.

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ACKNOWLEDGEMENTS

My dissertation focuses on microchimerism, cells and DNA from one individual that become part of another individual. I like to think of microchimerism as tiny traveling companions in life, and it is fitting that on my graduate school journey, I have had so many wonderful traveling companions who have helped me grow as a human and as a human who studies humans.

First, I want to thank my advisor, Dr. Dan Eisenberg, for the detailed, thorough, and prompt feedback on countless drafts of everything from manuscripts and grant applications to emails reaching out to professional colleagues. His diligence, eye for detail, and high scholarly standards pushed me to continuously better my work.

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DEDICATION

To my grandparents, who taught me to value the privilege of education and instilled in me the optimism, bravery, perseverance, and love embodied in their immigrant experiences.

Chapter 1. INTRODUCTION

The classical idea of biological inheritance occurring exclusively via DNA passed on from the egg and sperm has been progressively complicated. Biological anthropologists and allied scientists are increasingly learning how non-classical transmission of epigenetics marks (Thayer and Non, 2015), microbiomes (Martin and Sela, 2013; Amato, 2016), and milk bioactive compounds (Breakey et al., 2015) can have lasting impacts on the immune system, metabolism, and nutritional intake of offspring. These non-genomic modes of biological transmission can shape offspring phenotypes, allowing evolutionary processes and predictive adaptive responses to work on shorter timescales than conventional genetic evolution (West-Eberhard, 2003; Kuzawa, 2005; Gluckman et al., 2009). This dissertation examines the variation and adaptive biology of another such non-classical mode of inheritance: microchimerism.

Cells traffic bidirectionally between mother and fetus during pregnancy, and some of these genetically distinct cells persist in their healthy hosts for decades after delivery (Bianchi et al., 1996; Maloney et al., 1999). The presence of a small quantity of these cells or DNA (<1%) is termed microchimerism (Mc). Mc is a common occurrence in humans, and growing evidence suggests that Mc has important phenotypic effects. Mc is associated with both positive and negative effects on risk of autoimmune disorders, organ transplant outcomes, parasite infections, disorders of pregnancy, and cancer (Nelson, 2009; Boddy et al., 2015; Stevens, 2016; Harrington et al., 2017). Mc is thought to influence host immune function by: 1) directly contributing stem cells that differentiate and develop into functional immune and tissue-specific cells and 2) expressing foreign antigens that trigger host immune system responses (Kinder et al., 2017).

The biomedical focus of the current Mc literature leaves gaps in my understanding of the basic biology and the potential evolutionary significance of Mc. Evolutionary perspectives on the influence of fetal-origin Mc on maternal health and fitness have previously been provided by Haig (2014) and Boddy et al. (2015). However, the retention of cells from one's mother, or maternal-origin microchimerism (MMc), has been little considered by anthropologists and is the focus of this dissertation.

1.1 Human Biological Variation

MMc occurs as commonly as in 10-41% of offspring (Maloney et al., 1999; Reed et al., 2000; Artlett et al., 2001; Nelson et al., 2007), but studies of MMc have almost exclusively been conducted in European-descent populations in high-income, nutritionally abundant, and low infectious disease contexts. Understudied human populations likely have differing infant feeding practices, HLA allele frequencies, infectious disease burdens, and reproductive behaviors, which may affect variation in MMc and its potential broader effects. To improve anthropological understandings of the range of variation in MMc, this dissertation leverages population-based longitudinal data from the Philippines and tests four potential determinants of MMc in young adult women (Aim 1): 1) gestational age as a measure of in utero exposure to MMc, 2) history of being breastfed as a measure of postpartum exposure to MMc, 3) maternal telomere length as a measure of MMc's potential to persist, and 4) parity as a test of possible changes to MMc with the addition of fetal-origin Mc.

1.2 Developmental Plasticity and Maternal Effects on Offspring Survival

Each life stage (gestation, infancy, childhood, adolescence, and adulthood) faces different adaptive tradeoffs to immune function (McDade, 2003). Early life infections are leading causes of infant mortality globally and likely strong selective forces. A mechanism for vertical transmission of maternal immunological experience could advance offspring immune system development and benefit the health of an immunologically naïve infant. Like maternal immunoglobulins transferred to offspring during gestation and via breastmilk, MMc in the form of maternal memory T cells (Kanaan et al., 2017), may be one such adaptive mechanism of transmission. In cases of placental malaria in Tanzania, children with greater MMc were more likely to be infected with malaria parasites but less likely to have symptoms or be hospitalized for malaria symptoms (Harrington et al., 2017). Whether MMc might protect against symptoms of other infectious diseases remains unknown. This dissertation evaluates whether MMc is associated with decreased early life diarrhea and respiratory infection, common causes of childhood mortality in the Philippines (Aim 2).

1.3 Immunological Tradeoffs of Reproduction

Reproduction is another time period during which selective forces are critical. Internal gestation for eutherian mammals, and pregnancy for humans in particular, is a precarious time for both the gestating individual and their future offspring. The maternal immune system must tolerate the genetically distinct embryo or fetus. Inadequate tolerance of pregnancy can inhibit implantation of an embryo or placental development, restricting transfer of nutrients to the growing fetus and producing low birth weight offspring. Persistence of MMc also requires immune tolerance of genetically distinct cells, so an immune system with greater tolerance to

might also better tolerate pregnancy, another source of genetically distinct cells. This dissertation assesses whether women with MMc from their own mothers are better able to immunologically tolerate pregnancy and protect against having low birth weight offspring (Aim 3).

1.4 Specific Aims and Hypotheses

I wrote the following three chapters (Chapter 2-4) with the intention of publishing them as separate, stand-alone manuscripts. Chapter 2 examines predictors of MMc—participants' exposures to MMc and factors that may impact MMc persistence (Aim 1). The next two manuscripts are ordered chronologically by the life stage in which I expect MMc to have impacts on evolutionary fitness. Chapter 3 focuses on early life infections, which have implications for health, growth and development, and survival to reproductive age (Aim 2). Chapter 4 considers reproductive outcomes of the participants in adulthood, specifically offspring birth weight, which is a predictor of offspring survival and long term health (Aim 3). This dissertation addresses these aims by testing the hypotheses below:

Aim 1: Evaluate predictors of MMc in young adult women.

Hypothesis 1.1: Greater gestational age at birth is associated with increased MMc.

Hypothesis 1.2: Longer duration of being breastfed is associated with increased MMc.

Hypothesis 1.3: Maternal cells with longer telomeres are more likely to persist as MMc.

Hypothesis 1.4: Increasing parity is associated with decreased MMc.

Aim 2: Assess whether MMc is associated with lower risk of early life infection.

Hypothesis 2.1: MMc is associated with decreased incidence of diarrheal morbidity in the first two years of life.

Hypothesis 2.2: MMc is associated with decreased symptoms of respiratory infection in the first two years of life.

Aim 3: Determine whether adult women with MMc from their mothers have lower risk of adverse pregnancy outcomes when they have offspring of their own.

Hypothesis 3: MMc in adult women is associated with greater offspring birth weight.

Chapter 2. HISTORY OF BEING BREASTFED PREDICTS DECREASED MATERNAL-ORIGIN MICROCHIMERISM IN YOUNG WOMEN

2.1 INTRODUCTION

Cells traffic bidirectionally between mother and fetus during pregnancy, and some of these genetically distinct cells persist in their healthy hosts for decades after delivery (Bianchi et al., 1996; Maloney et al., 1999). The presence of these cells is known as microchimerism (Mc). Evolutionary perspectives on the influence of fetal-origin Mc on maternal health and fitness have previously been put forward by Haig (2014) and Boddy et al. (2015). However, the retention of cells from one's mother, or maternal-origin microchimerism (MMc), has been little considered by anthropologists. Biomedical researchers have found that MMc is associated with both positive and negative immunological outcomes of clinical importance, such as autoimmune disorders, infectious diseases, tissue transplantation, and reproduction (Gammill et al., 2011, 2014; Stevens, 2016; Harrington et al., 2017; Kanaan et al., 2017). While MMc may impact the immune system throughout the life course, from fetal development to post-reproductive life (recently reviewed in Gammill and Harrington, 2017), little is known about why MMc is detectable at varying levels in different individuals. Here, I examine potential determinants of MMc quanity linked to pregnancy duration, breastfeeding, telomere length, and parity.

During human pregnancy, MMc has been detected in second trimester aborted fetal tissues (Jonsson et al., 2008) and in second and third trimester fetal circulation (Petit et al., 1997; Berry et al., 2004). Fetal-origin Mc in maternal circulation increases in later gestation, and had been suggested to reflect increasing microscopic tears in the placenta, resulting in microtransfusions (Adams Waldorf et al., 2010). These microtransfusions could allow

bidirectional diffusion of cells such that MMc accumulates throughout gestation and increase in transmission rate later in pregnancy. Experiments in rats demonstrate active transport of maternal cells across the placenta that is dependent on a signal protein concentration gradient (Chen et al., 2008). In humans, some converging evidence from an observational study showed a tentative association between MMc and another protein that should accentuate the concentration gradient of the aforementioned signal protein in the third trimester (de la Calle et al., 2016; Harrington et al., 2017) and lead to greater active transport of MMc later in pregnancy. Both proposed routes of *in utero* transfer of maternal cells led to my first hypothesis, that greater gestational age at delivery will positively predict offspring MMc quantity (Hypothesis 1; H1).

MMc is usually assumed to be transmitted during pregnancy and delivery, but breastfeeding could be a postpartum mode for infants to further acquire or maintain MMc. Human breastmilk contains stem cells that have the potential to differentiate (Patki et al., 2010; Hassiotou et al., 2012) and, if able to pass through the gut, could engraft as MMc. Breastfeeding could also promote better immunological tolerance and long-term persistence of MMc. Tolerance of genetically foreign MMc has been studied in organ transplantation as family members are sometimes donors. Transplant recipients with a history of being breastfed have better rates of accepting maternal transplants (Campbell et al., 1984; Kois et al., 1984; Burlingham et al., 1998; Aoyama et al., 2009; Dutta et al., 2009), and this tolerance of maternal antigens might be maintained by MMc (Dutta and Burlingham, 2010). Although the relationship between MMc and history of being breastfed has not previously been examined in humans, an experimental mouse study showed that pups nursed by a foster mother had cells from the foster mother in their livers, demonstrating that breastfeeding is an independent pathway of MMc

transmission (Zhou et al., 2000). Thus, I hypothesize that longer breastfeeding duration will be associated with increased MMc (H2).

The long-term maintenance of MMc relies not only on duration of exposure to maternal cells in fetal development and early life but also on the maternal cells' ability to proliferate over time. One measure of a cell's ability to divide and replicate is telomere length. Telomeres generally shorten with each round of cellular division, and cells can no longer replicate when telomeres are critically short. In hematopoietic cell transplantation, longer donor telomere length is associated with improved survival of recipients with aplastic anemia (Gadalla et al., 2015, 2018a) but no association with acute leukemia (Gadalla et al., 2018b), suggestive of greater persistence or replicative ability of donated cells with longer telomeres. Thus, I predict that MMc from mothers with longer telomeres will be more likely to persist in the offspring and be detectable in adulthood (H3).

A study of post-reproductive women in the United States found decreased MMc in women with higher parity (Gammill et al., 2010). This suggests that MMc in adulthood might fluctuate as fetal-origin Mc is introduced with each pregnancy. I expect a similar pattern of decreased MMc with increasing parity in young women, early in their reproductive careers (H4).

In this study, I test four potential determinants of MMc in young adult women in the Philippines: H1) gestational age as a measure of in utero exposure to MMc, H2) history of being breastfed as a measure of postpartum exposure to MMc, H3) maternal telomere length as a measure of MMc's potential to persist, and H4) parity as a test of possible changes to MMc with the addition of fetal-origin Mc.

2.1 METHODS

2.1.1 *Study Population and Data Collection*

This study included young women (age 20-22) from the larger Cebu Longitudinal Health and Nutrition Survey (CLHNS), a population-based longitudinal cohort study in the Philippines, described in more detail by Adair et al. (2011). Our sampling from the larger CLHNS was designed to maximize statistical power and minimize potential confounding to address H2. Thus, I restricted my sample to 133 participants who were born in 1983-1984 of singleton pregnancies, had matched mother-participant DNA samples available from the 2005 survey wave, and were either 1) never breastfed or 2) breastfed until at least 24 months of age. I further restricted my sample to female participants because of questions outside of the scope of this paper, related to participant reproductive outcomes. Mothers of the participants provided data on infant feeding, demographics, diet, and health by completing surveys soon after the participants' birth and every two months until the participants were 24 months old. CLHNS conducted follow-up surveys every 2-4 years since 1992, including data on participants' reproductive health starting in 2002. Survey data is available for download at https://dataverse.unc.edu/dataverse/cebu. I used venous whole blood collected in EDTA tubes from the participants and their mothers in 2005 as well as 44 maternal specimens from 2016 for all laboratory analyses. All participants provided informed consent at the time of data collection, and the University of North Carolina at Chapel Hill Office of Human Research Ethics, Northwestern Institutional Review Board, or the University of Washington Human Subjects Division reviewed and approved the CLHNS study design and protocols for each round of data collection.

2.1.2 *Laboratory Methods for MMc Assessment*

CLHNS participants and their mothers donated venous whole blood samples from which DNA was extracted (Gentra Puregene, QIAGEN). To measure MMc, I must identify a polymorphism unique to the mother and absent in the participant. Because human leukocyte antigen (HLA) regions are highly polymorphic, they are an ideal and common targets for MMc testing. I genotyped 133 mother-participant pairs using a Luminex-based PCR sequence-specific oligonucleotide probe technique for the following HLA loci: HLA-DRB1, -DQA1, and -DQB1 (One Lambda, Thermo Fisher Scientific). For 66 mother-offspring pairs, an informative HLA polymorphism could not be identified, so I also genotyped them for six non-HLA insertion/deletion/substitution polymorphisms (antithrombin III long and short, thyroglobulin insertion and deletion, tenascin N insertion, and glutathione S-transferase theta 1), as previously described (Kanaan et al., 2017). An informative target for MMc analysis produced a PCR product in a heterozygous mother and was absent in the participant. Of the 133 mother-participant pairs who fit my inclusion criteria, 34 did not produce an informative target polymorphism for which I had a corresponding Mc qPCR assay, and 10 participant samples did not have sufficient DNA to confidently be tested for MMc. I was ultimately able to measure MMc quantity in 89 (67%) participants. The 44 individuals for which MMc could not be analyzed did not significantly differ on a range of demographic and health related variables from the 89 who were analyzed, except those excluded were less likely to have mothers that were smokers at the time of their delivery (Appendix A.1).

Once an appropriately informative polymorphism was identified, I employed the corresponding Mc detection assay from a panel of polymorphism-specific TaqMan qPCR assays (Lambert et al., 2004; Loubière et al., 2006; Gammill et al., 2014; Kanaan et al., 2017). These

assays were previously validated for specificity of the target polymorphism and were able to amplify a single DNA target in a background of up to 60,000 human cell genome equivalent (gEq) per amplification well. I collected all qPCR amplification data on a BioRad C1000 Thermal Cycler with CFX 96 optical reaction module. I used a calibration curve for the polymorphism-specific assay to quantify MMc in participants' DNA. I also assayed each sample for the non-polymorphic housekeeping gene, β-globin, to quantify the total gEq of DNA tested in each reaction. I included only samples with an anticipated total of at least 10,000 gEq (66 ng of genomic DNA) available for MMc testing (range 16,000-258,000 gEq) and ran all samples in 5-6 replicate reactions.

I took stringent precautions against PCR contamination, performing setup of amplification reactions and qPCR in separate locations. I prepared qPCR assays using filter pipette tips under a laminar flow hood equipped with UV light in dedicated clean room facilities consisting of HEPA-filtered, positively pressurized rooms with interlocked doors. I included DNA-free negative controls and no-template controls with non-specific background DNA in duplicate on every experimental plate. One plate resulted in positive amplification in a no-template control. I excluded the data from that plate, and there was not sufficient DNA left to rerun. The remaining MMc quantification experiments passed rigorous quality control checks (i.e., negative and positive control reactions, calibration curves, and assay efficiencies) and were deemed acceptable. I included low-gEq positive controls on each plate to ensure sensitivity, so any positive polymorphism-specific qPCR amplification was considered detectable MMc. I report MMc quantities as the DNA gEq count of microchimeric cells detected using a conversion factor of 6.6 pg of DNA per cell.

2.1.3 *Maternal Telomere Length Measurement*

Maternal telomere length was measured in extracted DNA from venous whole blood collected in 2005. Detailed methods were previously published (Eisenberg et al., 2012, 2015). Briefly, a modified version of the monochrome multiplex qPCR method was used (Cawthon, 2009), and measures show good internal and external validity (Eisenberg et al., 2015).

2.1.4 *Statistical Analysis*

Statistical analysis of MMc count data presents several challenges, including excess zero values due to the detection limits of the assay, rare occurrences of relatively large values that can cause over-dispersion, and variable quantity of DNA tested for each sample. Therefore, I assessed the relationship between MMc quantity (count of MMc gEq detected) and each of my predictors of interest using a negative binomial model with exposure offset to adjust for the total gEq tested and robust standard errors, as recommended by Guthrie et al. (2016). Like a Poisson regression, a negative binomial regression is a log-linear model appropriate for count data, but it relaxes assumptions about variance, allowing for a better fit with the over-dispersion of Mc data. The model output is a detection rate ratio (DRR), which can be interpreted as an X-fold change in MMc quantity for each unit increase in the predictor. I employed separate models for testing the effects of gestational age (range 30-47 weeks of completed gestation), history of being breastfed (never breastfed or breastfed 24+ months), maternal relative telomere length (telomere to single copy gene or T/S ratio range 0.399-0.963), and parity (range 0-3) on MMc quantity in young adulthood (age 20-22). I also tested a model that included all four predictors of interest.

Obstetric outcomes of each participant's birth, such as method of delivery (vaginal/cesarean) and complications of delivery, vary with fetal-origin Mc (Shree et al., 2019) and could also affect the transfer of maternal cells. However, because 98% of my study

participants were delivered vaginally and 87% had uncomplicated deliveries (Table 2.1), there was low power to detect differences across groups. I did not include these obstetric outcomes in my primary analyses, but in exploratory analyses of these variables, results should be regarded with caution.

I also considered the potential effects of socioeconomic status because socioeconomic status is associated with a variety of health-related outcomes in my study population (McDade et al., 2013; Desantis et al., 2015; Tennyson et al., 2018). Although I am not aware of direct pathways by which socioeconomic characteristics might affect MMc transmission to the child, it is conceivable that socioeconomic characteristics might impact health-related behaviors and access to healthcare that may then affect the longer term health of the participant and maintenance of MMc. Health and immune function throughout the life course might influence the detectability of MMc at the time of the present study. To measure socioeconomic status, I conducted a principal components analysis of mother (in 1983-1984) and participant educational attainment (in 2005) and weekly household income, household assets (Desantis et al., 2015), and urbanicity of the participants' barangay/neighborhood (Dahly and Adair, 2007) at two time points—participant's birth (in 1983-1984) and biological specimen collection (in 2005). I retained principal component 1 (PC1), which accounted for 35% of the variance in these variables, as the socioeconomic status (SES) variable in my analyses (Appendix A.2). I conducted all statistical analyses in Stata 14.2.

2.2 RESULTS

I assessed MMc variation in 89 women for this analysis, of whom 26 were never breastfed (29%) and 63 were breastfed at least until they were two years old (71%). MMc was detected in 41 (46%) participants overall. Of those with detectable MMc, the distribution of

MMc concentrations ranged from 0.583 to 354 gEq of MMc per 100,000 gEq of DNA tested among participants who were never breastfed and 0.781 to 86.4 gEq of MMc per 100,000 gEq tested among participants who were breastfed 24+ months (Figure 2.1). Comparing MMc levels across studies is difficult because of differences in Mc detection methods, type of tissue or cells collected, and demographics of participant populations. However, studies of MMc that use qPCR detection methods in peripheral blood from healthy children and adults in Sweden, the United Kingdom, and the United States report prevalences of detectable MMc of 20-40% (Lambert et al., 2004; Loubière et al., 2006; Nelson et al., 2007; Jonsson et al., 2010; Sunku et al., 2010; Suskind et al., 2011; Kanold et al., 2013; Thompson et al., 2013), which is somewhat lower than my study population in the Philippines. MMc concentrations in these studies range from 0.1-153 gEq of MMc per 100,000 gEq tested (ibid), which is similar to the range observed in my study, with the exception of one outlier.

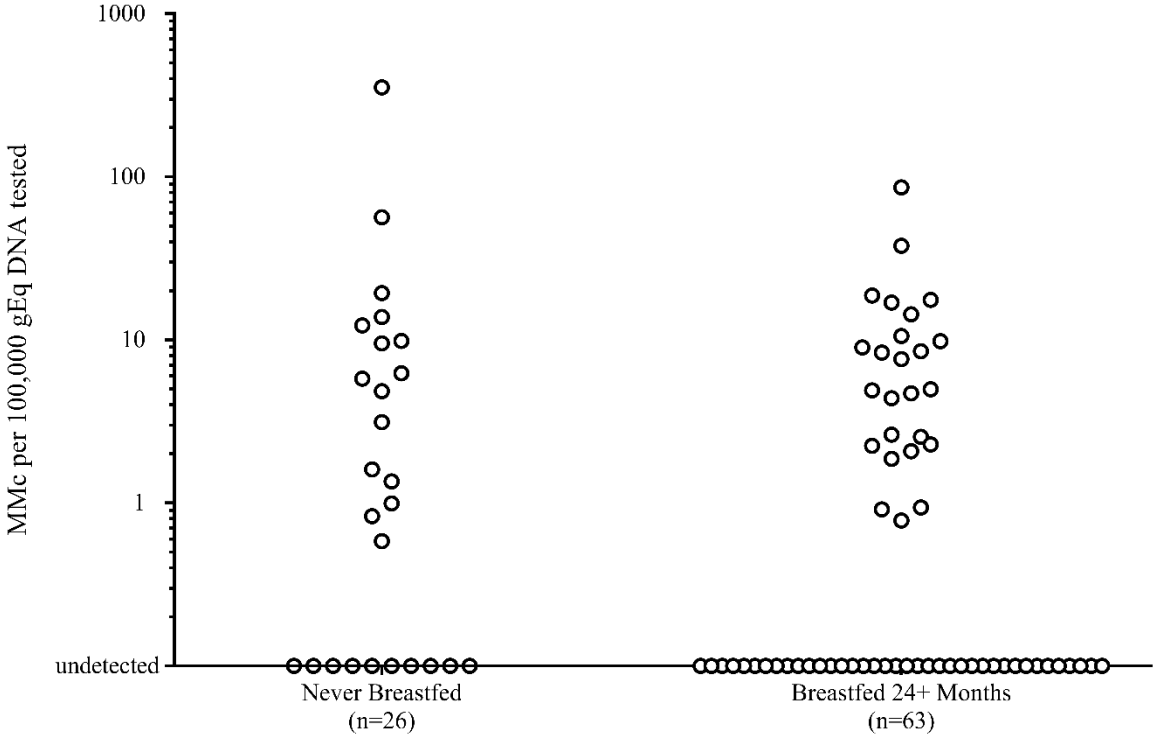

Figure 2.1 Maternal microchimerism detected per 100,000 gEq of DNA tested by history of being breastfed

Table 2.1 summarizes health and demographic characteristics of participants and their mothers from the time of the participant's birth (in 1983-1984) to sample collection (in 2005), grouped by whether they breastfed the participant. The two groups did not differ significantly in maternal age or body mass index (BMI) at participant delivery, completed weeks of gestation at participant's delivery, mother's live births or pregnancies prior to participant's delivery, mother's smoking status, participant's method of delivery, or complications of participant's delivery (Table 2.1). However, participants who were never breastfed had several significantly higher indicators of SES (Table 2.1). Consistent with past analyses in of the larger CLHNS study (Popkin et al., 1990; Zohoori et al., 1993), participants who were never breastfed live in somewhat more urban settings (Appendix A, Table A.1).

Table 2.1 Demographic and health characteristics by history of being breastfed

Characteristic	Never Breastfed (N=26)		Breastfed 24+ Months (N=63)		p-value
	Mean	(SD)	Mean	(SD)	2-sided t-test
Mother's age at participant delivery (years)	27.0	(5.2)	29.6	(6.9)	0.087
Completed weeks of gestation at participant delivery	39.4	(2.5)	39.5	(2.7)	0.816
Mother's live births prior to participant delivery	2.8	(2.5)	3.5	(2.6)	0.213
Mother's pregnancies prior to participant delivery	3.6	(2.5)	4.1	(2.6)	0.479
Mother's body mass index at participant delivery	21.7	(2.8)	22.8	(2.4)	0.087
Mother's relative telomere length (T/S ratio)	0.7	(0.1)	0.6	(0.1)	0.087
Participant's parity (live or still births)	0.8	(1.2)	0.4	(0.6)	0.064
Participant's lifetime socioeconomic status (principal component 1)	0.7	(1.6)	-0.7	(1.5)	<0.001
	n	(%)	n	(%)	Fisher's exact test
Mother was a smoker at participant delivery	6	(23.1)	14	(22.2)	1.000
Participant's delivery method					0.501
Vaginal	25	(96.2)	62	(98.4)	
Cesarean	1	(3.9)	1	(1.6)	
Complications of participant's delivery					0.607
No Complication	23	(88.5)	54	(85.7)	
Heavy Bleeding	1	(3.9)	3	(4.8)	
Breech	1	(3.9)	0		
Cord Coil	1	(3.9)	5	(7.9)	
Other	0		1	(1.6)	

To evaluate my hypotheses that gestational duration, being breastfed, maternal telomere length, and parity influence offspring MMc levels, I first tested each of these variables as predictors in separate models (Table 2.2). I also accounted for the difference in SES by breastfeeding history and the difference in mother's smoking behavior between participants for whom I could test for MMc and those for whom I could not by including SES and mother's smoking behavior as covariates. These models did not substantially alter DRRs or p-values (Appendix A, Tables A.8-A.10) and marginally decreased model quality as evaluated by the Akaike information criterion (AIC) and Bayesian information criterion (BIC) (Appendix A,

Table A.11). We, therefore, reported unadjusted models as my final results. Contrary to my hypotheses, I found non-significant trends for weeks of completed gestation, being breastfed, and longer maternal telomere lengths predicting lower levels of MMc. Specifically, a one-week longer gestation predicted 0.78 times as much MMc detected (DRR 0.78, 95% CI 0.60-1.03, p=0.077; Table 2.2). Those who were breastfed for 24+ months had less than one fourth the MMc of those who were never breastfed (DRR 0.23, 95% CI 0.05-1.05, p=0.058; Table 2.2). Individuals with a relative maternal telomere length of 1 were predicted to have only 2% of the MMc detected in individuals with a relative telomere length of 0 (DRR 0.02, 95% CI 0.00-1.00, p=0.050; Table 2.2). There was no evidence of a relationship between participant's reproductive history, as measured by parity, and MMc (DRR 0.81, 95% CI 0.36-1.81, p=0.605; Table 2.2). My statistical models predict a count of MMc gEq detected, but for ease of visualization, Figures 2.1-2.4 show MMc as a concentration (gEq of MMc +1 per 100,000 gEq of DNA tested) by each of my predictors of interest.

Table 2.2 Bivariate negative binomial regression models predicting MMc quantity

Independent Variable	Hypothesis	N	DRR*	(95% CI)	p
Completed weeks of gestation at participant delivery	1	84	0.78	(0.60-1.03)	0.077
Breastfed	2	89	0.23	(0.05-1.05)	0.058
Mother's telomere length	3	88	0.02	(0.00-1.00)	0.050
Participant's parity (live or still births)	4	89	0.81	(0.36-1.81)	0.605

*Table values are unadjusted Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor. See Appendix A, Tables A.8, A.9, and A.11 for adjusted models.

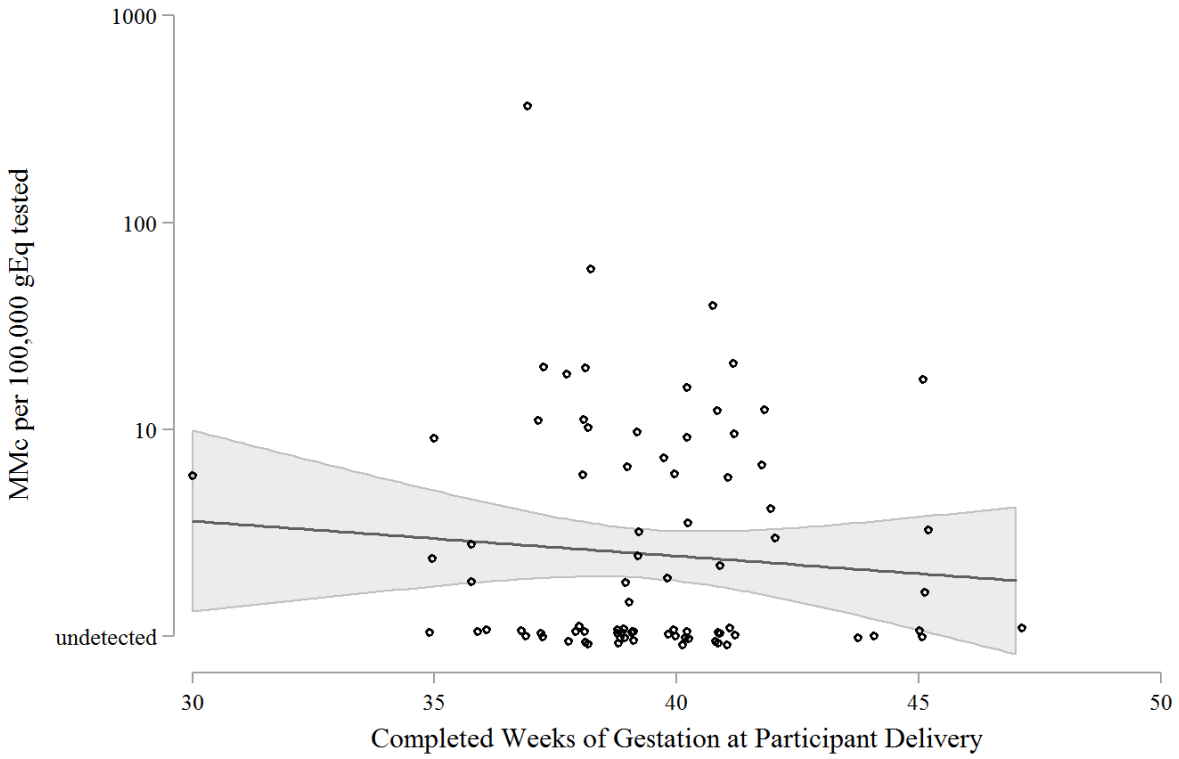

Figure 2.2: Logarithmic-scaled maternal-origin microchimerism detected per 100,000 gEq of DNA tested by completed weeks of gestation at participant's delivery with linear prediction and 95% confidence intervals. Points are jittered for visibility.

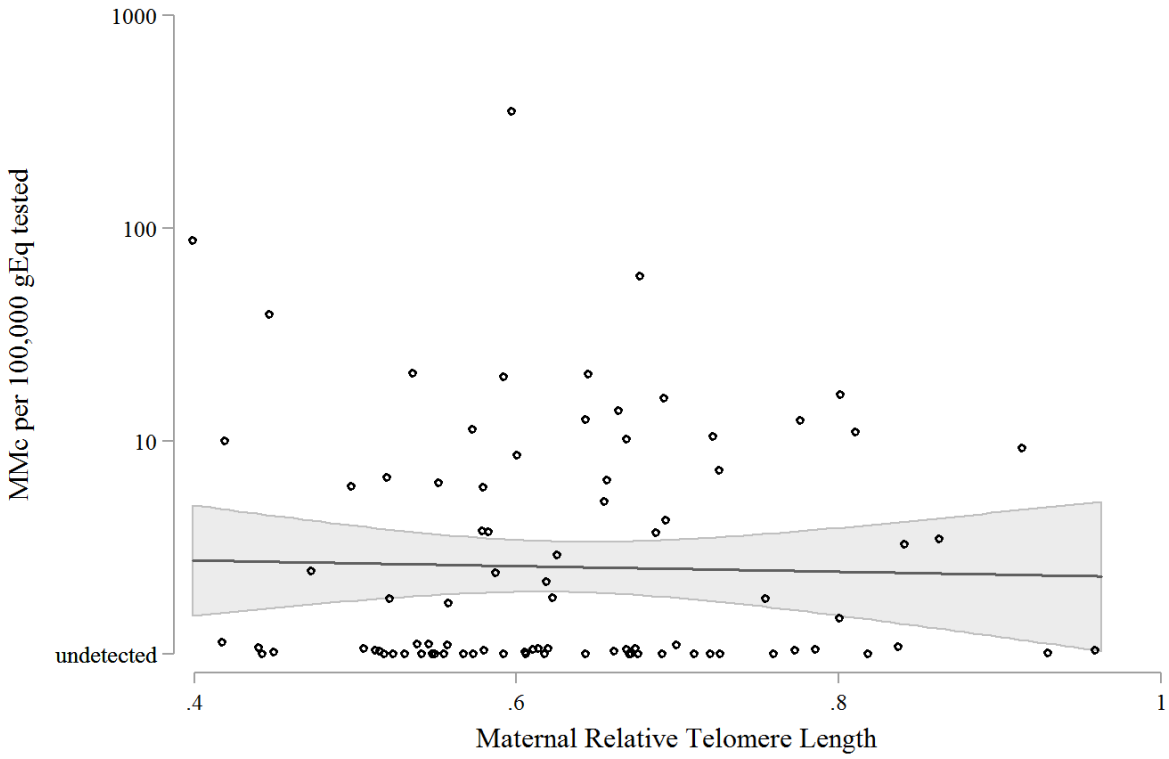

Figure 2.3: Logarithmic-scaled maternal-origin microchimerism detected per 100,000 gEq of DNA tested by maternal relative telomere length (T/S ratio) with linear prediction and 95% confidence intervals. Points are jittered for visibility.

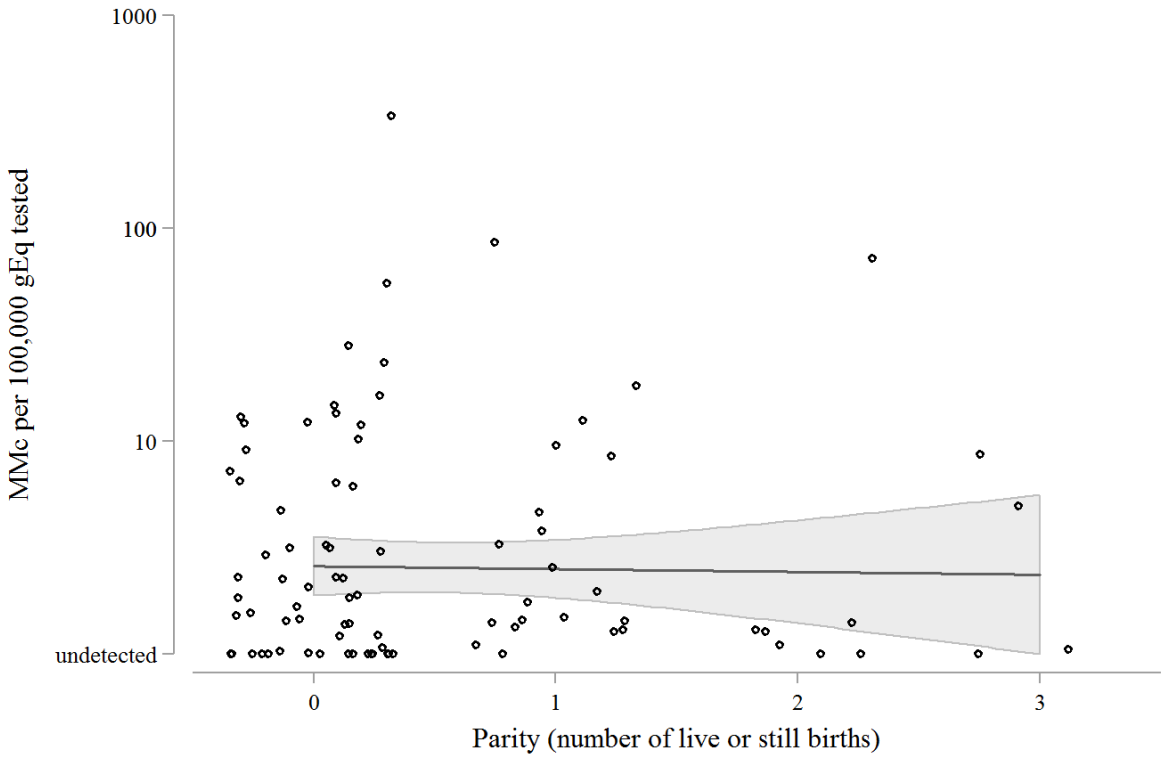

Figure 2.4: Logarithmic-scaled maternal-origin microchimerism detected per 100,000 gEq of DNA tested by participant's parity (number of live or still births) with linear prediction and 95% confidence intervals. Points are jittered for visibility.

When I included all four predictors in the same model, the relationship between history of being breastfed and MMc in adulthood strengthened, but there was no significant or near significant relationships between MMc and weeks of completed gestation, mother's telomere length, or participant's parity. After accounting for these other covariates, individuals who were never breastfed had 7 times the MMc of individuals who were breastfed 24+ months (DRR 0.15, 95% CI 0.04-0.59, p=0.007; Table 2.3).

Table 2.3 Multivariate negative binomial regression models predicting MMc quantity

	Full Model		
Independent Variable	DRR*	(95% CI)	p
Completed weeks of gestation at participant delivery	0.95	(0.80-1.11)	0.497
Breastfed	0.15	(0.04-0.59)	0.007
Mother's telomere length	0.20	(0.00-7.88)	0.388
Participant's parity (live or still births)	0.60	(0.30-1.18)	0.135
N	83		

*Table values are unadjusted Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor. See Appendix A, Tables A.10 and A.11 for adjusted models.

To examine the influence of outliers I did a post-hoc analysis where I truncated the data. One MMc measurement was more than three standard deviations greater than the mean, so I changed this outlier to match the next highest MMc value (from 250 to 45 gEq of MMc detected). Results using the truncated data were similar, but attenuated (Appendix A, Table A.12), but being breastfed remained a significant predictor of lower MMc in the full model (DRR 0.35, 95% CI 0.13-0.97, p=0.043; Appendix A, Table A.13).

I further conducted exploratory analyses of the relationship between obstetric outcomes of each participant's birth and MMc. Bivariate analyses tentatively suggest that MMc is possibly increased in complicated deliveries compared to uncomplicated deliveries, and in contrast to fetal-origin Mc (Shree et al., 2019), is decreased in cesarean section compared to vaginal delivery (Appendix A, Table A.15). However, these findings should be cautiously understood, given the limited sample size.

2.3 Discussion

I examined four potential predictors of MMc: 1) duration of prenatal exposure to MMc measured by gestational age, 2) duration of postpartum exposure to MMc via breastfeeding, 3) the potential for maternal cells to persist long term in offspring indexed by maternal telomere

length, and 4) the number of live and still birth pregnancies. Evaluating each predictor separately demonstrated trends toward decreased MMc with increased gestation duration, among those who were breastfed, and with increased maternal telomere length (Table 2.2). However, when I included all predictors in a multivariate model, only history of being breastfed remained a significant predictor of decreased MMc (Table 2.3).

I hypothesized that greater gestational age at delivery, which indicates a longer duration of exposure to maternal cells *in utero*, would result in more opportunities for MMc transfer. However, our results suggest there is no relationship or a slightly negative relationship between gestational duration and MMc beyond the effects of breastfeeding. Because I only have MMc measurements for individuals who survived to adulthood, I have a restricted range of obstetric outcomes as 90% of my participants were delivered at full term (37+ weeks). Furthermore, in a rat model, active transport of MMc across the placenta is dependent upon a concentration gradient of a signal protein necessary for placental development (Chen et al., 2008). This signal protein is often only measured in maternal circulation as a biomarker for preeclampsia, so concentrations in fetal circulation are unknown but assumed to be constant. If the assumption that the concentration of this signal protein in fetal circulation remains consistent throughout gestation is incorrect, then my prediction for MMc transmission would also be inaccurate.

My results suggest that individuals breastfed beyond 24 months of age have decreased MMc in adulthood compared to individuals who were never breastfed. This relationship is in the opposite direction I predicted based on previous experimental studies in mouse models that found breastmilk to be a route for MMc transmission and important for sustaining immunological tolerance of maternal antigens (Zhou et al., 2000; Dutta et al., 2009). Human infants, however, have a particularly short period of intestinal permeability to large molecules of

about seven days (Molès et al., 2018). By comparison, mouse pups in the aforementioned studies were weaned at 14 days (Zhou et al., 2000) and 21 days (Dutta et al., 2009). Interestingly, breastfeeding is associated with earlier closure of this permeability (Catassi et al., 1995). Therefore, maternal cells in breastmilk may have little opportunity to pass through the gut and into offspring circulation in humans. Instead, breastfeeding-mediated differences in immune development or differential sequestration of MMc in solid organs compared to blood may affect detectability of MMc in adulthood. In my study, I measured MMc by quantifying DNA in whole blood, which includes DNA from the cellular component of blood as well as circulating cell-free DNA. A proinflammatory environment and tissue death can increase release of cell-free DNA into blood (Thierry et al., 2016; Hummel et al., 2018; Meddeb et al., 2019). Breastfeeding is known to lower C-reactive protein (CRP), a biomarker of inflammation, in young adulthood (McDade et al., 2014), so it is possible that the increased MMc in my participants who were never breastfed reflects death of MMc cells in other tissues that might be targeted by the participant's immune system. In this case, the MMc might consist mostly of circulating free DNA shed from other tissues, rather than breast-milk derived hematopoietic cellular MMc.

I measured MMc in adults, so for MMc to persist for decades without conceivable additional sources of MMc after breastfeeding cessation, maternal cells must be able to replicate in the participant. I hypothesized that maternal cells with longer telomeres would be better able to proliferate and be detectable at higher rates in the adult participants. My bivariate results, however, show an effect in the opposite direction. This effect is reduced to non-significance after controlling for history of being breastfed and other covariates. My test of this novel hypothesis was limited by my single time point of biological specimen collection. Because I was unable to directly measure the telomere lengths of MMc cells in the participants, I used the participants'

mother's blood telomere length from ~21 years after delivery to index telomere length of maternal cells that trafficked to the participant during the gestational and early postpartum period. Telomere length is often correlated between mother and offspring, likely because of genomic inheritance and/or shared environmental contexts. Telomere shortening is thought to reflect cellular turnover, so participants with rapid telomere shortening likely also have more cells breaking down in their tissues, resulting in greater circulating cell-free DNA. If mothers of these participants also have rapid shortening of their telomeres, the trend of diminished MMc with increasing maternal telomere length may reflect circulating cell-free DNA from cellular turnover in other tissues, rather than the hypothesized proliferation of cellular MMc. Future research should begin with centrifuging whole blood samples prior to DNA extraction to distinguish cell-free DNA in plasma from peripheral blood leukocytes. Sorting MMc cells from host cells would also be important for directly measuring telomere length in those isolated cells.

A previous study of post-reproductive women found a decrease in MMc with increasing parity, but no difference in MMc between parous and nulliparous women (Gammill et al., 2010). To assess whether MMc was affected by the introduction of fetal-origin Mc with reproduction in adulthood, I tested whether parity was associated with detectable MMc in my population of young adult women. In both bivariate and multivariate analyses, I found no association between MMc and parity. However, my participants were all age 20-22 and early in their reproductive careers; 65% were nulliparous, 22% were primiparous, and only 12% were multiparous. With the small number of parous young women, I did not have sufficient statistical power to detect differences in MMc by additional parity. To better assess changes in MMc with increasing parity, a longitudinal study design following women through their reproductive careers and

accounting for gravidity without parity (e.g., pregnancy loss or elective abortion) would provide a much clearer picture.

This study aimed to assess variation in MMc by participant exposure to maternal cells and the potential for MMc to persist long-term. I designed the sampling criteria for my study to maximize my ability to detect a relationship between MMc and history of being breastfed, and I observed a significant decrease in MMc among participants who were breastfed. After accounting for history of being breastfed, I found no association between MMc detectability in adulthood and gestational duration, maternal telomere length, or participant's parity. However, bivariate analyses of these predictors hint at possible trends toward decreased MMc with increasing gestational duration and increasing maternal telomere length, which are promising areas for further research. Future studies of MMc should consider participant's history of being breastfed and the cell type or source of DNA being analyzed.

Chapter 3. MATERNAL-ORIGIN MICROCHIMERISM AND EARLY LIFE INFECTION

3.1 INTRODUCTION

Anthropologists have played a key role in disrupting simplistic genetic determinist arguments by showing ways the human body develops in response to environmental conditions (e.g. Boas, 1912; Bogin and Loucky, 1997; Frisancho, 2009). Building on this, maternal phenotype, reflecting the mother's cumulative experience of the local environment, is thought to influence offspring developmental trajectories, particularly during critical periods of direct maternal provisioning (Kuzawa and Quinn, 2009; Wells, 2017). For example, mothers can confer specific immunity, which has yet to be attained by an infant's inexperienced immune system, through transfer of antibodies across the placenta during pregnancy (e.g., Immunoglobulin G) or via breastmilk to an infant (e.g., Immunoglobulin A) (McDade, 2003).

Maternal cells and DNA can also traffic across the placenta, and likely during labor/delivery and breastfeeding. Microchimerism is the retention of a small quantity of these cells or DNA from a genetically distinct individual. Maternal-origin microchimerism (MMc) has intriguing parallels to immunoglobulin and microbiome transmission pre-, peri- and postnatally, but they each have different ways of influencing offspring immune function. Maternal immunoglobulins protect infants from infection as their immune systems mature, but maternal immunoglobulins decline after 6-12 months (Niewiesk, 2014). MMc, like maternally derived immunoglobulins, may have short-term phenotypic impacts on offspring, but in contrast, can also persist for decades. Bacterial colonization of a newborn's gut is responsive to the infant's microbial exposure, breastfeeding, and diet, and the quantity and diversity of gut microbiota can influence host metabolism, nutrition, immune function, and behavior (Martin and Sela, 2013;

Hinde and Lewis, 2015; Amato, 2016). Although MMc occurs at much lower quantities than gut microbiota, microchimeric human cells contribute to tissue and may thereby have more direct consequences for organ function (Ye et al., 2014).

There is some evidence that MMc can confer protection against infectious disease morbidity. A recent study found, in cases of placental malaria, children with greater MMc were more likely to be infected with malaria parasites but less likely to have symptoms or be hospitalized for malaria symptoms (Harrington et al., 2017). If the MMc includes regulatory cells, they could directly modulate activity of the child's immune cells to either resist malaria or be anti-inflammatory, minimizing symptoms of collateral damage from an immune response to malaria. Whether MMc might have protective effects against other infectious diseases is currently unknown.

Diarrhea and acute respiratory infections are leading causes of infant and child mortality globally (WHO, 2018) and where my study takes place, in the Philippines (Republic of the Philippines, 2014). Breastfeeding is protective against infectious disease morbidity and mortality, including diarrheal and respiratory mortality, in my study population (Yoon et al., 1996). I build on anthropological literature in breastfeeding ecology and maternal signals that promote offspring fitness by assessing whether MMc is associated with decreased infectious disease morbidity in the first two years of life.

3.2 METHODS

3.2.1 *Study Population and Data Collection*

This study included 89 female infants born in 1983-1984 of singleton pregnancies from the larger Cebu Longitudinal Health and Nutrition Survey (CLHNS), a longitudinal birth cohort study described in more detail by Adair et al., 2011). Mothers of the participants completed

surveys at baseline while pregnant with the participants, soon after the participants' birth, and every two months until the participants were 24 months old for a total of 12 follow-up surveys. Survey data include infant morbidity, demographics, diet, and health and are publicly available at https://dataverse.unc.edu/dataverse/cebu. To address questions from Chapter 2, I only included infants who were either never breastfed or breastfed for the whole 24-month duration of the study. This sampling strategy also allowed for better statistical control of breastfeeding effects on infectious morbidity and eliminate any variability in infections resulting from timing of breastfeeding cessation. I further restricted my study population to females in order to assess reproductive outcomes in adulthood in Chapter 4. For laboratory analyses, I used venous whole blood collected from the participants and their mothers when participants were about 21 years old in 2005 as well as 44 maternal specimens from 2016. All participants or their mothers, when participants were minors, provided informed consent at the time of data collection. The University of North Carolina at Chapel Hill Office of Human Research Ethics, Northwestern Institutional Review Board, or the University of Washington Human Subjects Division reviewed and approved the CLHNS study design and protocols for each round of data collection.

3.2.2 *Laboratory Methods for MMc Assessment*

I previously described MMc data collection methods in detail in Chapter 2. Briefly, I used extracted DNA (Gentra Puregene, QIAGEN) from venous whole blood collected in EDTA tubes. I needed to identify an informative genomic target for MMc testing, a polymorphism that was present in a heterozygous mother and was absent in the participant. To do so, I genotyped mother-participant dyads for three HLA and six non-HLA insertion, deletion, or substitution polymorphisms.

I then employed the quantitative polymorphism-specific assay that corresponded with the target maternal polymorphism from a panel of polymorphism-specific TaqMan qPCR assays (Lambert et al., 2004; Loubière et al., 2006; Gammill et al., 2014; Kanaan et al., 2017). I also assayed each sample for the non-polymorphic housekeeping gene, β-globin, to quantify the total genome equivalents (gEq) of DNA tested in each reaction. I included only samples with an anticipated total of at least 10,000 gEq (66 ng of genomic DNA) available for MMc testing (range 16,000-258,000 gEq) and ran all samples in 5-6 replicate reactions. I report MMc quantities as the DNA gEq of maternal-origin microchimeric cells detected per 100,000 gEq of genomic DNA tested using a conversion factor of 6.6 pg of DNA per cell. For my statistical models, I log-transformed these MMc concentrations by taking the base-10 logarithm (n+1) to account for the exponential growth of cellular replication and reduce the skewedness of the data.

My primary predictor was MMc during the participants' first two years of life, but here, as a proxy, I measured MMc in samples collected when the participants were 20-22 years old. There are presumably no additional sources of MMc transfer after birth and breastfeeding cessation. MMc in early life might diminish in some individuals or increase due to proliferation of MMc over time, but those with detectable MMc as young adults are likely to have had MMc in early life as well.

3.2.3 *Early Life Infection*

In bimonthly surveys from participant's birth to age 24 months, mothers of participants reported whether their baby had diarrhea in the last 7 days or the last 24 hours (yes/no). I created a dichotomous outcome variable that estimated weekly diarrheal prevalence for each two-month period (reported diarrheal episode in either the last 7 days or last 24 hours/no reported diarrheal

episode). I then calculated the mean value for each individual across the 12 survey waves as a measure of the average weekly diarrheal incidence over the participant's first 24 months of life.

During each survey, mothers also provided information about whether their baby had cough, fever, nasal discharge, or sore throat in the last 7 days or the last 24 hours. To estimate weekly respiratory morbidity during each two-month period, I considered maternal report of any 2 or more of the aforementioned symptoms in the same week to indicate presence of respiratory infection and created a dichotomous variable for this respiratory morbidity outcome (reported respiratory infection in either the last 7 days or last 24 hours/no reported respiratory infection). I then averaged across the 12 survey waves as a measure of average weekly respiratory infection incidence over the participant's first 24 months of life.

I had a high response rate over the 12 bimonthly surveys as 81 individuals (91%) had complete data across all 12 surveys (Table 3.1). Of the possible 1068 observations, I had missing data from 20 surveys (2%), and no individual participant had more than 4 missing surveys. Averaging outcome measures across the number of observations available for each individual effectively mean-imputed missing data.

3.2.4 *Statistical Analysis*

I assessed the relationship between MMc and early life infection with ordinary least squares regressions in Stata 14.2. I ran separate unadjusted models for mean diarrheal and respiratory infection outcomes with log-transformed MMc concentration as the primary predictor. I then controlled for breastfeeding (never breastfed/breastfed for 24+ months) as a potential confounding variable in a minimally adjusted model. My fully adjusted models further included household socioeconomic status (SES) in 1983 and microbial exposure as precision variables. To construct an SES measure, I conducted a principle components analysis of

mother's educational attainment, household income, household assets (Desantis et al., 2015), and urbanicity (Dahly and Adair, 2007) for all females from the 1983-1984 birth cohort who completed the follow-up survey in 2005 (n=888; Appendix B, Table B.1). I retained principal component 1, which accounted for 47.5% of the variance in these variables, as the SES variable in my multivariate regressions (Appendix B, Tables B.3 & B.4). Enteric infections that cause diarrheal morbidity and acute respiratory infections have different risk factors and routes of transmission, so I used different measures of microbial exposure for the two outcomes. In the model for diarrheal morbidity, I characterized microbial exposure using a household hygiene score (Tallman et al., 2012) based on domestic animals beneath the house, excrement around the house, an unhygienic food storage area, and type of toilet used (range 0-8, where 0=lowest exposure and 8=highest exposure). Respiratory infections tend to spread among people in crowded conditions, so I characterized microbial exposure for the respiratory morbidity model by the number of people in the household (range 2-13) and the number of primary school-aged children in the household. I defined school-aged as 4-12 years of age at baseline because schooling was compulsory at age 6 years, so the youngest children in this category should have started primary school during the two-year span of this study.

I pre-registered all statistical models at the Open Science Framework (OSF, https://osf.io/qxenf/). I designed and coded models with the primary predictor of interest, log-transformed MMc concentrations, randomly sorted across participants. Replacing my primary predictor with randomly sorted MMc allowed my analysis plan to be attentive to missing data and collinearity issues. Only after I publically posted analysis methods to OSF did I add real MMc data into the analysis script.

3.3 RESULTS

Of the 133 individuals who met my initial inclusion criteria, I was able to test 89 (67%) for MMc. I was unable to test 44 individuals for MMc because an informative genomic marker, for which I had a corresponding assay, was not available, or there was insufficient DNA to be tested. These individuals were excluded from the analyses. Descriptive statistics indicate that individuals for whom I could quantify MMc had a trend towards higher rates of diarrhea (weekly incidence of 0.18 vs. 0.14, p=0.060) and significantly higher rates of respiratory infection (weekly incidence of 0.54 vs. 0.46, p=0.032) in their first two years of life than those excluded (Table 3.1). However, participants who could or could not be tested for MMc did not differ by other key characteristics that may impact early life infection (Table 3.1).

Table 3.1 Demographic and household characteristics

Characteristic	Included (N=89)		Excluded (N=44)		p-value
	Mean	(SD)	Mean	(SD)	2-sided t-test
Maternal-origin microchimerism *(MMc per 100,000 gEq tested)*	8.78	(38.91)			
Socioeconomic index *(principal component 1)*	-0.23	(1.22)	0.09	(1.57)	0.207
Hygiene score *(pathogen exposure range 0-8)*	4.91	(1.99)	5.36	(1.82)	0.206
Number of people in household *(range 2-13)*	6.00	(2.58)	5.27	(2.35)	0.118
Number of primary school-aged children in household *(range 0-2)*	0.10	(0.37)	0.07	(0.25)	0.597
Average probability of weekly diarrheal incidence *(range 0-1)*	0.18	(0.14)	0.14	(0.13)	0.060
Average probability of weekly respiratory infection incidence in the first 24 months *(range 0-1)*	0.54	(0.20)	0.46	(0.19)	0.032
	n	(%)	n	(%)	Fisher's exact test
Breastfeeding					0.684
Never breastfed	26	(29.2)	11	(25.0)	
Breastfed 24+ months	63	(70.8)	33	(75.0)	
Survey response rate					0.758
Completed all 12 surveys	81	(91.0)	39	(88.6)	
Missing 1 or more surveys	8	(9.0)	5	(11.4)	

Table 3.2 presents results of the regression models assessing the relationship between MMc concentration and incidence of diarrhea. The unadjusted model estimates that a 10-fold increase in MMc predicted an increase of 2.4 percentage points in weekly risk of diarrhea during the first 24 months of life, but this relationship was not statistically significant (p=0.337, Figure 3.1). Adjusting for breastfeeding or breastfeeding, SES, and household hygiene did not alter the relationship between MMc and diarrheal incidence.

Table 3.2 Ordinary least squares regression models predicting average weekly diarrheal incidence in the first 24 months of life

	Unadjusted		Minimally Adjusted		Maximally Adjusted	
Independent variables	β ± SE	p	β ± SE	p	β ± SE	p
Log-transformed MMc concentration *(log$_{10}$ [MMc per 100,000 gEq tested +1])*	0.024 ± 0.027	0.337	0.022 ± 0.027	0.428	0.021 ± 0.028	0.451
Breastfeeding *(never breastfed=0, breastfed 24+ months=1)*			-0.013 ± 0.033	0.695	-0.005 ± 0.038	0.885
Socioeconomic index *(principal component 1)*					0.007 ± 0.015	0.636
Hygiene score *(pathogen exposure; lowest =0, highest=8)*					-0.003 ± 0.008	0.672

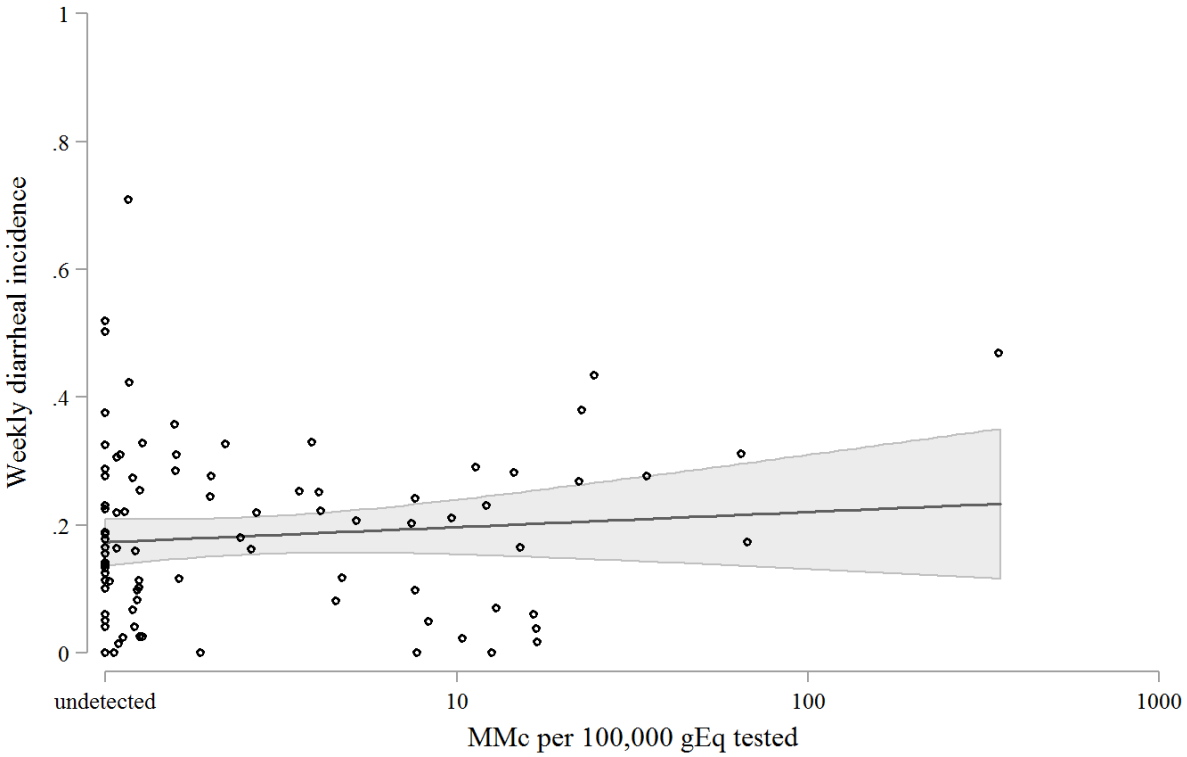

Figure 3.1 Average weekly diarrheal incidence in the first 24 months of life by log-transformed maternal-origin microchimerism detected per 100,000 gEq of DNA tested with linear prediction and 95% confidence intervals. Points are jittered for visibility.

I analyzed the relationship between MMc concentration and weekly report of respiratory infection (Table 3.3). The unadjusted model estimates a decrease in probability of the infant experiencing a respiratory infection during a given week in the first 24 months of life by 2.2 percentage points with each 10-fold increase in MMc, but again, this relationship was not statistically signification (p=0.573, Figure 3.2). Adjusting for breastfeeding or breastfeeding, SES, household size, and number of school-aged children in the household attenuated the relationship between MMc and respiratory infection incidence.

Table 3.3 Ordinary least squares regression models predicting average weekly respiratory infection incidence in the first 24 months of life

	Unadjusted		Minimally Adjusted		Maximally Adjusted	
Independent variables	β ± SE	p	β ± SE	p	β ± SE	p
Log-transformed MMc concentration *(log$_{10}$ [MMc per 100,000 gEq tested +1])*	-0.022 ± 0.039	0.573	-0.017 ± 0.040	0.666	-0.009 ± 0.040	0.824
Breastfeeding *(never breastfed=0, breastfed 24+ months=1)*			0.032 ± 0.048	0.506	0.050 ± 0.055	0.371
Socioeconomic index *(principal component 1)*					-0.003 ± 0.020	0.870
Number of people in the household *(range 2-13)*					0.007 ± 0.009	0.437
Number of school-aged children in the household *(range 0-2)*					0.084 ± 0.065	0.199

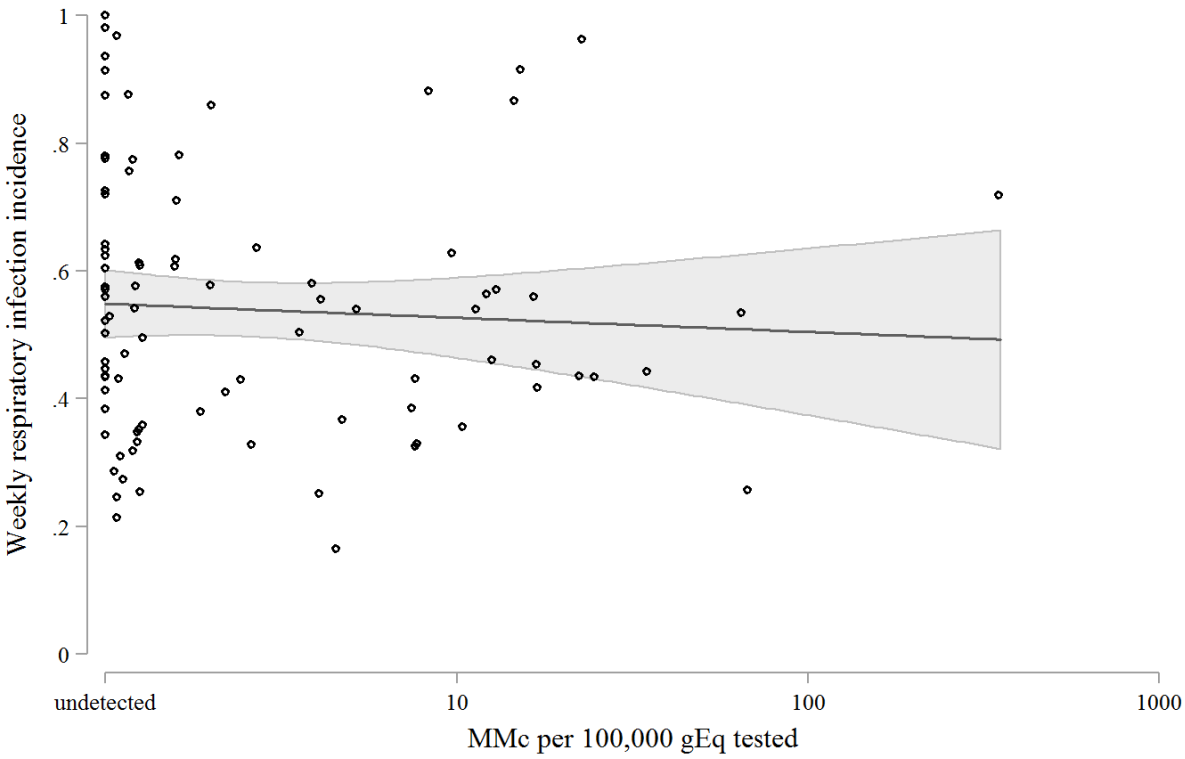

Figure 3.2 Average weekly respiratory infection incidence in the first 24 months of life by log-transformed maternal-origin microchimerism detected per 100,000 gEq of DNA tested with linear prediction and 95% confidence intervals. Points are jittered for visibility.

3.4 Discussion

I evaluated whether having MMc in peripheral blood conferred protection against symptoms of common early life infections. My results showed no evidence of a relationship between MMc and diarrheal or respiratory infection incidence in the first two years of life. Though not statistically significant, the unadjusted models estimated that each 10-fold increase in MMc predicted an increase in weekly risk of diarrhea by 2.4 percentage points and a decrease in weekly risk of respiratory infection by 2.2 percentage points in the first two years of life (Table 3.2 & 3.3). My inability to complete MMc assessment for all eligible participants may have obscured the true relationship between MMc and early life infection.

Of the participants I could not test for MMc, some did not have sufficient DNA for testing, but most were excluded because I could not identify a target polymorphism, for which I had a corresponding TaqMan qPCR assay. Target polymorphisms had to distinguish maternal cells from the participant sample, so excluded participants were all HLA-compatible with their mothers at HLA-DRB1, -DQA1, and -DQB1. In the first study of microchimerism in human disease, female scleroderma patients had more male Mc (of presumed fetal origin) compared to healthy controls, and they were more likely to be HLA-compatible with their offspring (Nelson et al., 1998). This supports the idea that, like immune tolerance of organ or tissue transplantation, HLA compatibility is more likely to permit Mc persistence and proliferation. If this pattern holds true in my study, I would expect participants who could not be tested for MMc and were more commonly HLA compatible with their mothers to have higher levels of MMc than those included in the analyses. These excluded participants also had lower rates of both diarrheal and respiratory infections (Table 3.1). Being able to test all eligible participants for MMc may increase my ability to detect a negative relationship between MMc and early life infection. Development of additional MMc quantification assays that do not depend on HLA discordance is necessary.

Adjusting for breastfeeding, SES, and microbial exposure in my analyses attenuated model estimates toward the null (Table 3.2 & 3.3), suggesting possible collinearity between these predictors of infection and MMc. Because I used measurements of MMc in whole blood collected in 2005, when participants were 20-22 years old, as a proxy for MMc in the first 24 months of life, my interpretation of results depends on the assumption that MMc quantities in early life correlate with MMc quantities in later life. However, if MMc in young adulthood is responsive to early life immunological experiences, causality may be reversed. It is possible that immune activation in response to early life infection, oral exposure to maternal cells via

breastmilk, access or knowledge of health care indexed by SES, or immunological development due to microbial exposure could incidentally impact whether MMc persists into adulthood. Future research with longitudinal sample collection that clarifies how MMc concentration might fluctuate over time is warranted.

The current paper is only the second study to empirically examine MMc in relation to human infectious disease (see Harrington et al., 2017 for the first, a study of malaria). Although my analysis showed little evidence of an association between MMc concentration and symptoms of life diarrhea or respiratory infection in the first 2 years of life, the relationship between MMc and infections merits further inquiry. Distinguishing between infectious agents and the various mechanism that may produce similar common symptoms, expanding Mc assay development to ensure all participants can be tested for Mc, focusing on the cellular component of peripheral blood, and longitudinal study design can all improve my ability assess how Mc may impact immune response to infection or how microbial exposure may impact MMc persistence.

Chapter 4. MATERNAL-ORIGIN MICROCHIMERISM AND OFFSPRING BIRTH WEIGHT

4.1 INTRODUCTION

Placental mammals face an immunological conundrum during gestation as *in utero* maturation requires that the maternal immune system must not reject fetal tissues that are partially foreign due to the contribution of paternal genes (Medawar, 1953). The immunological risks of internal gestation are thought of as tradeoffs for more efficient, continuous nutrient transfer to the fetus, and species vary in the degree of connection at the maternal-fetal interface as a result of differential placental forms and invasiveness. Species with more invasive placentas are at greater risk of prenatal bacterial infection (Capellini et al., 2015) and immunological rejection of the fetus due to exposure to maternal allogeneic immune response (Abrams and Miller, 2011). However, species with more invasive placentas also generally have greater prenatal brain growth relative to body size (Elliot and Crespi, 2008) and shorter gestation times for their body and brain size (Capellini et al., 2011). Human placental invasion is especially aggressive compared to other mammals, implanting deep into the uterus and with only a single fetal tissue layer separating maternal blood from fetal blood, so immune regulation during pregnancy for humans is especially precarious (Abrams and Rutherford, 2011; Carter et al., 2015). During pregnancy, maternal immune function adjusts to maintain fetal tolerance, both locally at the uterine-placental interface and systemically with the expansion of immunosuppressive regulatory T (Treg) cells. Lower levels of maternal Tregs indicate inadequate fetal tolerance and are associated with pregnancy complications such as preeclampsia, miscarriage, and premature birth (reviewed by Jiang et al., 2014).

Prior to a woman becoming pregnant, there is another period of obligatory exposure to and tolerance of genetically foreign antigens—exposure to non-inherited maternal antigens (NIMAs) during her own fetal life and early development. In fact, tolerance of NIMAs can be maintained into adulthood by NIMA-specific Tregs that develop in response to the presence of maternal cells in offspring lymph nodes (Mold et al., 2008). These maternal cells harbored by the offspring are termed maternal-origin microchimerism (MMc), where microchimerism refers to the presence of a small quantity of cells or DNA from a genetically distinct individual. Microchimeric cells are naturally exchanged bidirectionally during pregnancy and can persist indefinitely. MMc presence extends what the offspring's immune system recognizes as "self" and can help prevent immune responses against NIMAs.

In addition to sustained tolerance of NIMAs into adulthood, this MMc induced "extended self" may promote additional tolerance of other half-foreign cells in next-generation pregnancies, protecting against immune-related disorders of pregnancy. In humans, this idea is supported by studies showing that women who have increased MMc from their own mothers have decreased risk of pregnancy disorders with immune-related etiologies, such as preeclampsia (Gammill et al., 2011) and a suggested similar trend in recurrent miscarriage (Gammill et al., 2014). A mouse model study also demonstrated MMc mediated protection against fetal loss following experimentally induced disruptions in pregnancy tolerance when there was commonality between NIMA of the pregnant female and paternal-fetal antigens (Kinder et al., 2015).

These above reviewed studies suggest that, when a female grows up and reproduces, MMc may contribute to enforcing fetal tolerance and promote favorable pregnancy outcomes. However, less is known about how MMc might affect outcomes of pregnancies that result in live birth. Insufficient immune tolerance of pregnancy may result in preterm delivery or inhibit

placental development, restricting nutrient transfer to a growing fetus. Both cases result in lower birth weight, which is a leading cause of neonatal mortality and morbidity and a predictor of chronic diseases in adulthood such as type 2 diabetes, hypertension, and heart disease. Here, I examine whether MMc might be a mechanistic link between maternal immune tolerance of pregnancy and reproductive outcomes by testing the relationships of offspring birth weight and pregnancy loss with MMc.

4.2 METHODS

4.2.1 *Study Population and Data Collection*

This study included 65 women from the larger Cebu Longitudinal Health and Nutrition Survey (CLHNS), a longitudinal birth cohort study described in more detail by Adair et al. (2011). Participants were born in 1983-1984 of singleton pregnancies and had at least one live birth delivery of a child of her own with known birth weight by 2013. To address questions outside the scope of this paper (Chapter 2), I restricted my study population to participants with a history of either 1) never having been breastfed or 2) being breastfed until at least 24 months of age. For laboratory analyses, I used venous whole blood collected from the participants and their mothers in 2005 as well as 44 maternal specimens from 2016. All participants provided informed consent at the time of data collection, and the University of North Carolina at Chapel Hill Office of Human Research Ethics, Northwestern Institutional Review Board, or the University of Washington Human Subjects Division reviewed and approved the CLHNS study design and protocols for each round of data collection.

4.2.2 *Laboratory Methods for MMc Assessment*

I previously described MMc data collection methods in detail (Chapter 2). Briefly, I used extracted DNA (Gentra Puregene, QIAGEN) from venous whole blood collected in EDTA tubes. I genotyped mother-participant dyads for three HLA and six non-HLA insertion/deletion/substitution polymorphisms to identify an informative target for MMc analysis. An informative target produced a PCR product in a heterozygous mother and was absent in the participant.

To quantify MMc in the participant's sample, I employed the Mc detection assay that corresponded with the target maternal polymorphism from a panel of polymorphism-specific qPCR assays using TaqMan probes (Lambert et al., 2004; Loubière et al., 2006; Gammill et al., 2014; Kanaan et al., 2017). I also assayed each sample for the non-polymorphic housekeeping gene, β-globin, to quantify the total genome equivalents (gEq) of DNA tested in each reaction. I included only samples with an anticipated total of at least 10,000 gEq (66 ng of genomic DNA) available for MMc testing (range 16,000-258,000 gEq) and ran all samples in 5-6 replicate reactions.

I calculated MMc concentrations as the DNA gEq of maternal DNA expressed as microchimeric cell equivalents detected per 100,000 gEq of participant DNA tested, using a conversion factor of 6.6 pg of DNA per cell. I then log transformed these MMc concentrations by taking the common logarithm (n+1) to account for the exponential growth of cellular replication and reduce the skewedness of the data. These log-transformed MMc concentrations are my primary predictor.

4.2.3 *Outcome*

Offspring birth weight is my primary outcome, and it was reported by the participant either from recall or from birth records. No participants in my study reported having twins or other multiple births. For ease of interpretation, I centered offspring birth weights at 2500 grams, the threshold for low birth weight used by the World Health Organization (Wardlaw, 2004).

4.2.4 *Participant-level variables*

Participant-level demographic and anthropometric data from the time of blood draw (in 2005) included measures known to influence offspring birth weight participant's adult height (in centimeters, mean-centered at 150 cm), participant's smoking status (never-smoker=0/ever-smoker=1), marital status (never married=0/ever married=1), and participant's socioeconomic status (SES). To measure SES, I conducted a principle components analysis of participant educational attainment, weekly household income, household assets (Desantis et al., 2015), and urbanicity of the participants' barangay/ neighborhood (Dahly and Adair, 2007) for all female participants of the birth cohort who reported ever having been pregnant by 2009 and had matched mother-participant blood samples, regardless of breastfeeding history (n=681) (Appendix C, Table C.1). I retained principle component 1, which accounted for 44% of the variance in these variables, as the SES variable in my analyses (Appendix C, Table C.3). More positive SES indicates greater educational attainment, greater income, greater assets, and greater urbanicity (Appendix C, Table C.4).

4.2.5 *Offspring-level variables*

Participants self-reported on reproductive outcomes of each pregnancy in surveys from 2002-2013 (age 18-30). I included measures known to influence offspring birth weight as

potential precision variables. Offspring-level variables include maternal age (participant's age at offspring delivery, centered at 16 years of age), offspring birth order (later-born=0/first-born=1), and sex of the offspring (male=0/female=1).

4.2.6 *Statistical Analysis*

I employed two-level mixed-effects linear regressions using maximum likelihood estimates, modeled by the MIXED command in Stata 14.2. This approach addressed the non-independence in birth weight among siblings (each participant had a variable number of offspring and birth weight is known to vary with maternal age and/or birth order) by 1) estimating how much variation in birth weight lies between and among offspring with the same mother and 2) adjusting standard errors to take into account the hierarchical structure of the data.

To assess the effect of MMc on offspring birth weight, I used four different models. Model 1 was a random effects ANOVA, an unconditional null model that estimated the grand mean offspring birth weight and intra-class correlation (ICC). The ICC indicated what portion of the variance in offspring birth weight was due to differences across participants, as opposed to differences among offspring of the same participant. Model 2 adds offspring-level variables as fixed effects but includes a random intercept that is allowed to vary across participants. From Model 2 estimates, I calculated how much of the variance in offspring birth weight was explained by the three offspring-level covariates (whether offspring was first-born, offspring sex, and participant's age at offspring delivery). Model 3 added participant-level covariates as fixed effects, and Model 4 further added my primary predictor of interest, MMc, which is also a participant-level variable. Comparing Models 3 & 4 allowed me to calculate how much of the participant-level variance in offspring birth weight was explained by MMc concentration.

I also conducted a sensitivity analysis to see if results differed by source of offspring birth weight data (participant's recall or birth records). I did so by stratifying my models by data source, running separate models for offspring birth weight reported by participant recall and those from birth records (Appendix C.2).

Except as otherwise noted, I pre-registered all statistical models at the Open Science Framework (OSF, https://osf.io/9x3hm/). I designed and coded models with the primary predictor of interest, log-transformed MMc concentration, randomly sorted across participants. Replacing my primary predictor with randomly sorted MMc allowed my analysis plan to be attentive to missing data and collinearity issues. Only after I posted analysis methods to OSF did I add real MMc data into the analysis script.

4.3 RESULTS

I assessed the birth weights of 141 offspring of 65 women (mean 2.2 offspring per woman; range 1-6 offspring). 19 offspring of my participants (12%) were missing data on birth weights and not included in the analysis. Offspring birth weight data were either from participant recall (n=104) or birth records (n=37). Sensitivity analyses showed that data from differing sources did not differ substantially, so these data could reasonably be combined in my primary analyses (Appendix C, Tables C.5 & C.6). I was unable to test for MMc in 26 women because I either could not identify an informative target polymorphism for which I had a corresponding Mc assay or I did not have sufficient DNA to confidently test for MMc.

Table 4.1 summarizes relevant characteristics and pregnancy outcomes of women who fit my inclusion criteria and compares those for whom MMc could or could not be analyzed. Women who were excluded because I could not test for MMc reported lower offspring birth weights and were taller (Table 4.1). Women excluded also had slightly more adverse pregnancy

outcomes and reported on birth weights of slightly fewer later-born offspring (non-first-born) (Table 4.1). However, women for whom MMc could not be tested did not differ from those who could be tested in their age at pregnancy termination, sex of their offspring, their marital status, or their SES (Table 4.1). This suggests that the sample of individuals I could measure MMc in might be systematically different from those I could not test for MMc.

Table 4.1 Participant characteristics for each pregnancy by whether MMc could be tested

	Tested for MMc			Not tested for MMc			p-value
Characteristic	N	Mean	(SD)	N	Mean	(SD)	2-sided t-test
Singleton offspring birth weight (grams)	141	3078	(529)	48	2889	(657)	0.046
Participant's age at delivery (years)	141	24.2	(3.6)	48	24.3	(3.0)	0.926
Participant's adult height in 2005	65	150	(5.5)	28	153	(4.9)	0.025
Participant's socioeconomic index in 2005	65	-0.5	(1.4)	28	-0.5	(1.3)	0.845
		n	(%)		n	(%)	Fisher's exact test
Pregnancy outcome							0.184
Singleton live birth, still living		160	(89.9)		50	(80.7)	
Singleton live birth, now dead		2	(1.1)		0	(0.0)	
Still birth		5	(2.8)		3	(4.8)	
Miscarriage		9	(5.1)		7	(11.3)	
Multiple deliveries (twins)		0	(0.0)		0	(0.0)	
Pregnant during last survey		2	(1.1)		2	(3.2)	
Birth order							0.090
First-born		57	(40.4)		26	(55.3)	
Later-born		84	(59.6)		21	(44.7)	
Sex of living singleton offspring							0.407
Female		75	(53.2)		22	(45.8)	
Male		66	(46.8)		26	(54.2)	
Participant's smoking status in 2005							0.815
Ever-smoker		23	(35.4)		11	(39.3)	
Never-smoker		42	(64.6)		17	(60.7)	
Participant's marital status in 2005							1.000
Ever married		28	(43.1)		12	(42.9)	
Never married		37	(56.9)		16	(57.1)	

Birth weight data were centered at 2500 grams, so for Model 1 (Table 4.2), the fixed intercept of 571.1 indicates an estimated grand mean offspring birth weight of 3071.1 grams. That this estimate is significantly different from zero ($p<0.001$) is to be expected because only 11% of the offspring in this study had low birth weight, <2500 grams (Figure 4.1). The intra-class correlation (ICC) estimate for Model 1 is 0.061, which implies that about 6% of the variance in offspring birth weight is due to differences across participants/mothers, with the remaining 94% attributable to offspring-level differences (Table 4.2).

Figure 4.1 Histogram of offspring birth weight

For Models 2-4, β coefficients can be interpreted as estimated change in offspring birth weight (in grams) per unit increase in the predictor. Model 2 included three offspring-level covariates. Although not statistically significant, β coefficients were in the expected direction. Offspring birth weight tended to be lower among first-born offspring compared to later-born offspring, lower among female offspring compared to males, and increased with maternal age

(16-30 years). Together, these three offspring-level variables accounted for about 3% of the variance in non-smokers' offspring birth weight. (Table 4.2)

Model 3 added four participant-level covariates, which attenuated the estimated effect of offspring birth order, did not alter the estimated effect of offspring sex, and increased the effect of participant's age at offspring delivery. The attenuation of offspring birth order β-coefficient likely reflects some collinearity with participant level variables as each participant can only have one first-born offspring. After accounting for participant-level characteristics, the estimated effect of participant's age at offspring delivery was nearly significant, suggesting a possible increase in offspring birth weight with increasing maternal age (from 16-30 years of age). Participant's adult height and SES in 2005 did not predict offspring birth weight. However, there may be a trend toward increased offspring birth weight among participants who were ever smokers compared to those who were never smokers in 2005, and participants who had ever been married by 2005 had offspring with significantly greater birth weights than participants who had never been married. Model 3 had an expected decrease in the ICC compared to Model 1 as participant-level predictors were introduced to the model, reducing the unexplained variability between participants. Together, participant's adult height, smoking status, marital status, and SES in 2005 explain nearly all of the variance in offspring birth weight between participants.

Finally, in Model 4, I added log-transformed MMc concentration, my predictor of interest. There is no relationship between log-transformed MMc concentration and offspring birth weight, and including MMc in the model did not alter estimates of any other predictors of birth weight (Table 4.2). In addition to the model estimates, comparing the R^2 between Models 3 & 4 gives an estimate of the explanatory power of MMc as a predictor of offspring birth weight.

However, because the R^2 for Model 3 is already 1, there is negligible variance in offspring birth weight at the participant level left that can be attributed to MMc. The Bayesian information criteria (BIC) allows comparisons of model fit with the additional covariates, where lower BIC indicating better model fit. Here, inclusion of each additional predictor of offspring birth weight increased the BIC, suggesting that these additional covariates did not improve the explanatory power of the model beyond the unconditional null model, which accounted for the hierarchical structure of the data.

Table 4.2 Mixed-effects linear regression models predicting singleton offspring birth weight (grams, centered at 2500)

	Model 1*		Model 2*		Model 3*		Model 4*	
	β ± SE	p	β ± SE	p	β ± SE	p	β ± SE	p
Log-transformed MMc concentration							18.3	
(log_{10} [MMc per 100,000 gEq tested +1])							± 89.8	0.839
Offspring-level covariates								
Offspring birth order			-108.7		6.2		8.2	
(later-born=0, first-born=1)			± 98.7	0.271	± 110.3	0.955	± 110.7	0.941
Offspring sex			-90.0		-75.2		-76.0	
(male=0, female=1)			± 88.8	0.311	± 87.3	0.389	± 87.4	0.385
Participant's age at offspring delivery			10.8		25.7		25.7	
(years, centered at 16)			± 13.4	0.419	± 14.5	0.077	± 14.5	0.077
Participant-level covariates								
Participant's adult height					2.7		2.6	
(cm, mean centered at 150)					± 8.2	0.744	± 8.2	0.755
Participant's smoking status					121.6		117.0	
(never-smokers=0, ever-smokers=1)					± 89.2	0.173	± 91.9	0.203
Participant's marital status					208.5		215.1	
(never married =0, ever married=1)					± 105.8	0.049	± 110.7	0.052
Participant's socioeconomic status					11.8		12.4	
(principal component 1)					± 38.8	0.760	± 38.9	0.749
Intercept	571.1 ± 47.0	<0.001	577.8 ± 148.1	<0.001	439.2 ± 121.4	<0.001	224.5 ± 207.4	0.279
Model post-estimation								
R^2			0.027		1		1	
Intra-class correlation (ICC)	0.061		0.033		6.94×10^{-14}		1.62×10^{-14}	
Bayesian information criteria (BIC)	2182		2193		2207		2211	

*Models include random intercepts for participants and offspring

4.4 DISCUSSION

I tested whether participants with more MMc might be better able to immunologically tolerate pregnancy and have offspring with higher birth weights. My analyses do not show evidence of a relationship between MMc concentration in whole blood and offspring birth weight, even after adjusting for other predictors of offspring birth weight including offspring birth order, offspring sex, maternal age at delivery, maternal adult height, maternal smoker status, maternal marital status, and SES (Table 4.2).

Although I had birth weight data for 141 offspring, I only had a single measure of MMc for each participant/mother, some of whom had more than one offspring. Only 6% of the variation in offspring birth weight could be accounted for by differences across participants/mothers, with the remaining 94% attributable to offspring-level differences (Table 4.2). In a cross-sectional study, women with increasing parity had decreased MMc (Gammill et al., 2010), so obtaining MMc measurements that vary with each progressing offspring might have allowed my model to explain more of the variation in offspring birth weight. Future research on offspring birth weight should collect longitudinal measures of MMc prior to and during each pregnancy as offspring-level predictors.

A limitation to this study was my inability to test some participants for MMc. Of the 93 eligible participants, 24 did not have an informative maker among the polymorphisms for which I had a corresponding assay, and 4 did not have sufficient DNA to be confidently tested for MMc. Most of the assays I have target polymorphisms at HLA Class II loci, so if the participant's mother is homozygous at HLA-DRB1, -DQA1, or -DQB1 or if the participant and her mother are concordant at these HLA loci, an informative HLA marker for MMc testing is not available. If HLA concordance between participants and their mothers causally impacts MMc

persistence or participant health, that might explain some of the discrepancy in participant's adult height and offspring birth weight by whether I could test individuals for MMc (Table 4.1). However, a strength of my study is the use of assays that target polymorphisms in non-HLA loci. These non-HLA assays allowed me to test for MMc in another 17 individuals, so some of the participants included in this study are HLA concordant with their mothers at the aforementioned Class II loci. Because HLA likely affects the immunological outcomes of interest in Mc research, future Mc assay development should not rely on HLA polymorphisms.

This study aimed to assess whether MMc in young women might affect immune tolerance of pregnancy and, in turn, offspring birth weight. I found no relationship between MMc concentration measured from whole blood in women at age 20-22 and the birth weight(s) of their offspring. It is possible that changes in MMc concentration over time or with increasing parity, type and function of MMc cells, availability of MMc assays, and other predictors of birth weight that I was unable to account for might be obscuring a relationship between MMc and offspring birth weight and warrants additional research.

Chapter 5. CONCLUSIONS

Biological inheritance of traits, on which natural selection can act, does not occur exclusively by conventional genomic transmission. The focus of this dissertation was on a lesser-studied mode of inheritance, microchimerism (Mc). Past research on Mc has largely focused on the long term health of mothers and immune disorders that affect young children. However, individuals at each life stage (gestation, infancy, childhood, adolescence, and adulthood) face different tradeoffs to immune function. This dissertation leveraged data from a multigenerational, longitudinal health and nutrition study to test three novel research aims regarding maternal-origin Mc (MMc): 1) to evaluate potential predictors of MMc, 2) to assess whether MMc is associated with risk of early life infection, and 3) to determine whether women with MMc from their mothers have different adverse pregnancy outcomes as they have offspring of their own. My findings failed to support any of my initial hypotheses.

In Chapter 2, I assessed four potential predictors of MMc. Contrary to my expectations, bivariate analyses evaluating each predictor separately showed near-significant trends of decreased MMc with increased duration of exposure to maternal cells (both in utero and via breastmilk) and with longer maternal telomere lengths. In multivariate analyses that included all predictors in the same model, only being breastfed remained as a significant predictor of decreased MMc. Chapters 3 and 4 examined possible fitness consequences of MMc, but results showed no evidence of relationships between MMc and early life infection symptoms nor third-generation offspring birth weights. However, these studies have several limitations that may have hampered my ability to detect associations between MMc and fitness outcomes.

Additionally, I employed participant inclusion/exclusion criteria that would allow me to address all three aims of this dissertation with the same limited sample of participants. With

more funds, I could increase the sample size for addressing Aims 2 and 3 and reduce the likelihood of bias introduced by my inclusion criteria.

5.1 LESSONS LEARNED

Due to the novelty of my research aims, the questions and methods used in this dissertation have plenty of room for refinement. First, I only had DNA samples from a single time point (2005, when participants were 20-22 years old), so making inferences about fitness outcomes at various time points across the life course relied on the assumption that MMc quantity remained stable in this population. Longitudinally collected samples can build on this work to clarify when fluctuations in MMc quantity might occur or ensure the reliability of MMc quantification with a cross-sectional data collection plan. Second, I conducted all lab work on previously extracted DNA from whole blood, so I could not determine cell type or source of the DNA. Separating out the cellular component of blood from plasma or even sorting cells prior to extracting the DNA would have allowed me to distinguish between cellular DNA from white blood cells and cell-free DNA shed into circulation as cells from other tissues died, which have very different functional implications. Third, whether I could test a participant for MMc was largely dependent upon the HLA compatibility between the participant and her mother. However, HLA compatibility likely has a direct impact on immune tolerance of MMc as well as the fitness outcomes I focused on in this dissertation, possibly introducing bias in my study sample. Developing additional Mc assays that are not dependent on HLA discordance is crucial for differentiating between the immune effects of Mc and HLA. Fourth, the outcome measures I used focused on symptoms with non-specific causes, rather than mechanistic drivers of health and fitness. Diarrhea, fever, cough, nasal discharge, and sore throat can be caused by various infectious agents, and they can have non-infectious causes such as allergic reactions or

inflammation. Low birth weight can be the result of prematurity or intrauterine growth restriction, which may or may not have the same etiology. The relationship between MMc and evolutionary fitness likely depends on the causative pathway. All four of these limitations can be addressed with input in future study design phases, which was not possible with the secondary data analysis in this dissertation.

5.2 FUTURE DIRECTIONS

The data from this dissertation may be used to begin to address the question of whether HLA compatibility between participants and their mothers may impact MMc. I have 24 participants whose MMc levels were assayed with a non-HLA target. I can compare these individuals to the 65 whose samples were assayed with HLA polymorphism-specific assays. Although these numbers are small, they can give me a sense of the likelihood of systematic bias by assay availability. Mc assays that target non-HLA markers are also more broadly necessary for understanding the role of HLA compatibility in the longer term immune tolerance of Mc, particularly if I want to test for Mc from multiple sources.

The potential for an individual to harbor Mc from multiple origins in a diverse "microchiome" is fascinating to me. Human behavior around reproduction and care of offspring is extremely flexible, so even in the context of fetal-maternal microchimerism, there can be great variability. A person who has had multiple pregnancies by different partners can have distinct fetal-origin Mc from each of those pregnancies. If maternal cells in breastmilk can engraft in infants, then babies who have been wet-nursed or fed donor milk can have multiple sources of MMc. Now with the advent and growing demand for assisted reproductive technologies, an individual may have genomic DNA from the egg and sperm of two individuals but be gestated by a third person, a surrogate. Often times, multiple embryos are implanted, creating

opportunities for horizontal transmission of Mc in utero, and the embryos may not be from the same egg or sperm donors. There are endless scenarios that can create a diverse microchiome without even considering the possibility of multigenerational transfer of Mc. How cells with different genotypes and phenotypes might interact within a host, whether diversity or quantity of Mc affects health and reproductive fitness, and the malleability of ideas of kinship and relatedness in light of Mc are all interesting research topics for biological anthropologists.

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APPENDIX A: CHAPTER 2 SUPPORTING INFORMATION

A.1 ASSESSING POTENTIAL BIAS OF EXCLUDING PARTICIPANTS FOR WHOM MMC COULD NOT BE MEASURED

I was unable to identify an informative marker or did not have sufficient quantities of DNA to measure MMc in 33% of the participants who met my initial inclusion criteria. Participants for whom I was able to measure MMc did not significantly differ in key demographic, health, and socioeconomic characteristics from those for whom MMc could not be measured, except in the case of mother's smoking behavior (Table A.1). The excluded participants were significantly less likely to have mothers who were smokers. Mother's smoking behavior is unlikely to be causally associated with the participant's and mother's genotypes used for identifying an informative marker for Mc detection. However, mother's smoking behavior may influence the participant's health downstream, so I assessed whether each of my MMc predictors of interest differed by mother's smoking behavior. If their mother was a smoker at the time of their delivery, participants were more likely to have higher parity and lower lifetime socioeconomic status at age 20-22 (Table A.2).

Table A.5.1 Demographic and health characteristics

Characteristic	Included (N=89)		Excluded (N=44)		p-value
	Mean	(SD)	Mean	(SD)	2-sided t-test
Mother's age at participant delivery	28.8	(6.5)	28.4	(6.4)	0.689
Completed weeks of gestation at participant delivery	39.5	(2.7)	39.6	(1.7)	0.815
Mother's live births prior to participant delivery	3.3	(2.6)	2.9	(2.7)	0.395
Mother's pregnancies prior to participant delivery	3.9	(2.6)	3.8	(2.8)	0.748
Mother's body mass index at participant delivery	22.5	(2.5)	22.0	(2.2)	0.283
Mother's telomere length	0.6	(0.1)	0.6	(0.1)	0.932
Participant's lifetime socioeconomic status	-0.3	(1.6)	-0.1	(1.9)	0.286
Participant's parity (live or still births)	0.5	(0.8)	0.3	(0.5)	0.106
	n	(%)	n	(%)	Fisher's exact test
Mother was a smoker at participant delivery	20	(22.5)	3	(6.8)	0.028
Participant's delivery method					0.331
Vaginal	87	(97.8)	41	(93.2)	
Cesarean	2	(2.3)	3	(6.8)	
Complications of participant's delivery					0.398
No complication	77	(86.5)	37	(84.1)	
Heavy bleeding	4	(4.5)	1	(2.3)	
Prolonged labor (24+ hours)	0		2	(4.6)	
Breech	1	(1.1)	1	(2.3)	
Cord coil	6	(6.7)	2	(4.6)	
Other	1	(1.1)	1	(2.3)	
Participant's history of being breastfed					0.684
Never breastfed	26	(29.2)	11	(25.0)	
Breastfed 24+ months	63	(70.8)	33	(75.0)	

Table A.5.2 Predictors of interest and covariates by mother's smoker status at participant's delivery

Characteristic	Smoker (N = 23)		Non-smoker (N=110)		2-sided t-test
	Mean	(SD)	Mean	(SD)	p-value
Completed weeks of gestation at participant delivery	39.2	(2.1)	39.6	(2.4)	0.555
Mother's telomere length	0.6	(0.1)	0.6	(0.1)	0.613
Participant's parity (live or still births)	0.7	(1.0)	0.4	(0.7)	0.035
Participant's lifetime socioeconomic status	-1.0	(1.3)	0.0	(1.8)	0.011
	n	(%)	n	(%)	Fisher's exact test
Participant's history of being breastfed					1.000
Never breastfed	6	(26.1)	31	(28.2)	
Breastfed 24+ months	17	(73.9)	79	(71.8)	
Participant's delivery method					1.000
Vaginal	22	(95.7)	106	(96.4)	
Cesarean	1	(4.4)	4	(3.6)	
Complications of participant's delivery					1.000
No complication	21	(91.3)	93	(84.6)	
Heavy bleeding	1	(4.4)	4	(3.6)	
Prolonged labor (24+ hours)	0		2	(1.8)	
Breech	0		2	(1.8)	
Cord coil	1	(4.4)	7	(6.4)	
Other	0		2	(1.8)	

A.2 Principal Components Analysis of Socioeconomic Measures

Table A.5.3 Definition of socioeconomic measures

A) Mother's Education	Highest grade completed in 1983 (range 0-15)
B) Participant's Education	Highest grade completed in 2005 (range 2-16)
C) Household Income at Birth	Weekly household income in 1983 (range 1-3592 Philippine Pesos)
D) Household Income at Specimen Collection	Weekly household income in 2005, deflated to 1983 levels (range 76-3420 Philippine Pesos)
E) Household Assets at Birth	Scale described in detail by Desantis et al., 2015 of assets in 1983 that reflect population-relevant aspects of social class, including electricity, televisions, refrigerators, air conditioners, tape recorders, electric fans, jeepneys, cars, houses, and house construction type (range 0-9 of 11 possible)
F) Household Assets at Specimen Collection	Same as above for 2005 (range 0-10)
G) Urbanicity at Birth	Scale described in detail by Dahly and Adair, 2007 that accounts for population size, population density, communications, transportation, educational facilities, health services, and markets in 1983 (range 5-49)
H) Urbanicity at Specimen Collection	Same as above for 2005 (8-61)

Table A.5.4 Socioeconomic characteristics by history of being breastfed

	Never Breastfed (N=26)		Breastfed 24+ Months (N=63)		p-value
Characteristic	Mean	(SD)	Mean	(SD)	2-sided t-test
A) Mother's Education	8.8	(3.8)	5.3	(2.8)	<0.001
B) Participant's Education	11.0	(3.5)	10.3	(3.4)	0.410
C) Household Income at Birth	350	(196)	156	(134)	<0.001
D) Household Income at Specimen Collection	808	(874)	539	(470)	0.063
E) Household Assets at Birth	3.2	(2.0)	1.9	(1.6)	0.002
F) Household Assets at Specimen Collection	5.6	(1.8)	4.7	(1.9)	0.047
G) Urbanicity at Birth	30.4	(13.1)	29.6	(13.0)	0.785
H) Urbanicity at Specimen Collection	42.7	(9.5)	38.5	(13.8)	0.166

Table A.5.5 Correlation matrix of socioeconomic measures

	A	B	C	D	E	F	G	H
A	1.000							
B	0.451	1.000						
C	0.320	0.184	1.000					
D	0.203	0.151	0.090	1.000				
E	0.392	0.280	0.393	0.170	1.000			
F	0.427	0.509	0.193	0.283	0.359	1.000		
G	0.296	0.146	0.066	0.063	0.206	0.192	1.000	
H	0.230	0.125	0.095	0.109	0.202	0.199	0.647	1.000

Table A.5.6 Principal components

Component	Eigenvalue	Proportion of variance	Cumulative variance
Comp1	2.823	0.353	0.353
Comp2	1.379	0.172	0.525
Comp3	1.000	0.125	0.650
Comp4	0.877	0.110	0.760
Comp5	0.594	0.074	0.834
Comp6	0.537	0.067	0.901
Comp7	0.450	0.056	0.958
Comp8	0.339	0.042	1.000

Table A.5.7 Principal components eigenvectors

Socioeconomic Measure	Comp1	Comp2	Comp3
A) Mother's Education	0.443	-0.111	-0.035
B) Participant's Education	0.381	-0.252	0.257
C) Household Income at Birth	0.283	-0.247	-0.657
D) Household Income at Specimen Collection	0.226	-0.157	0.515
E) Household Assets at Birth	0.389	-0.149	-0.366
F) Household Assets at Specimen Collection	0.420	-0.204	0.321
G) Urbanicity at Birth	0.320	0.624	-0.012
H) Urbanicity at Specimen Collection	0.313	0.621	-0.004

A.3 ASSESSMENT OF MODEL QUALITY

Table A.5.8 Bivariate negative binomial regression models predicting MMc quantity, adjusted for mother's smoking status

Independent Variable	Hypothesis	N	DRR*	(95% CI)	p
Completed weeks of gestation at participant delivery	1	84	0.78	(0.60-1.01)	0.063
Breastfed	2	89	0.14	(0.03-0.65)	0.013
Mother's telomere length	3	88	0.02	(0.00-1.98)	0.095
Participant's parity (live or still births)	4	89	0.76	(0.34-1.70)	0.509

*Table values are Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor, adjusted for mother's smoking status

Table A.5.9 Bivariate negative binomial regression models predicting MMc quantity, adjusted for socioeconomic status

Independent Variable	Hypothesis	N	DRR*	(95% CI)	p
Completed weeks of gestation at participant delivery	1	84	0.79	(0.61-1.01)	0.062
Breastfed	2	89	0.23	(0.05-0.98)	0.047
Mother's telomere length	3	88	0.02	(0.00-0.86)	0.042
Participant's parity (live or still births)	4	89	0.91	(0.45-1.83)	0.794

*Table values are Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor, adjusted for socioeconomic status

Table A.5.10 Multivariate negative binomial regression models predicting MMc quantity

	Full Model, adjusted for mother's smoking status*			Full Model, adjusted for socioeconomic status*		
Independent Variable	DRR	(95% CI)	p	DRR	(95% CI)	p
Completed weeks of gestation at participant delivery	0.94	(0.79-1.11)	0.475	0.94	(0.79-1.11)	0.483
Breastfed	0.13	(0.03-0.56)	0.006	0.13	(0.03-0.55)	0.005
Mother's telomere length	0.35	(0.01-12.68)	0.568	0.29	(0.01-10.86)	0.502
Participant's parity (live or still births)	0.54	(0.25-1.17)	0.118	0.54	(0.26-1.10)	0.091
N	83			83		

*Table values are adjusted Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor

Table A.5.11 Assessing quality of negative binomial models

	Hypotheses									
	1		2		3		4		Full Model	
Model Adjustment	AIC	BIC	AIC	BIC	AIC	BIC	AIC	BIC	AIC	BIC
Unadjusted	341	349	363	370	367	374	370	377	337	352
Adjusted for mother's smoking	343	353	361	371	369	378	371	381	338	355
Adjusted for socioeconomic status	343	353	365	375	368	377	371	381	339	356
N	84		89		88		89		83	

*Table values are Akaike's information criteria (AIC) and Bayesian information criteria (BIC). Lower values indicate better model quality.

Table A.5.12 Bivariate negative binomial regression models predicting truncated MMc quantity

Independent Variable	Hypothesis	N	DRR*	(95% CI)	p
Completed weeks of gestation at participant delivery	1	84	0.91	(0.76-1.09)	0.311
Breastfed	2	89	0.55	(0.19-1.55)	0.257
Mother's telomere length	3	88	0.07	(0.00-1.95)	0.116
Participant's parity (live or still births)	4	89	1.21	(0.64-2.30)	0.558

*Table values are unadjusted Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor

Table A.5.13 Multivariate negative binomial regression models predicting truncated MMc quantity

	Full Model		
Independent Variable	DRR*	(95% CI)	p
Completed weeks of gestation at participant delivery	0.97	(0.84-1.12)	0.726
Breastfed	0.35	(0.13-0.97)	0.043
Mother's telomere length	0.32	(0.01-13.03)	0.548
Participant's parity (live or still births)	0.86	(0.46-1.58)	0.623
N	83		

*Table values are unadjusted Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor

A.4 EXPLORATORY ANALYSIS OF THE RELATIONSHIP BETWEEN OBSTETRIC OUTCOMES OF PARTICIPANTS' BIRTH AND MMC

Table A.5.14 Bivariate negative binomial regression models predicting MMc quantity

Independent Variable	N	DRR	(95% CI)	p
Vaginal delivery	87	Ref		
Cesarean section	2	0.20	(0.06-0.61)	0.005
Uncomplicated delivery	77	Ref		
Complicated delivery	12	5.81	(0.88-38.39)	0.068
Full term birth	76	Ref		
Preterm birth	8	0.24	(0.06-0.99)	0.049

Table values are unadjusted Detection Rate Ratios (DRR), 95% Confidence Intervals (CI), and p-values for each predictor

APPENDIX B: CHAPTER 3 SUPPORTING INFORMATION

B.1 PRINCIPAL COMPONENTS ANALYSIS OF SOCIOECONOMIC MEASURES

Table B.1 Definition of socioeconomic measures in 1983

A) Mother's Education	Highest grade completed (range 0 – 15)
B) Household Income	Weekly household income (range -44 – 5779 Philippine Pesos)
C) Household Assets	Scale described in detail by Desantis et al., 2015 of assets that reflect population-relevant aspects of social class, including electricity, televisions, refrigerators, air conditioners, tape recorders, electric fans, jeepneys, cars, houses, and house construction type (range 0 – 9 of 11 possible)
D) Urbanicity	Scale described in detail by Dahly and Adair, 2007 that accounts for population size, population density, communications, transportation, educational facilities, health services, and markets (range 5-49)

Table B.2 Correlation matrix of socioeconomic measures

	A	B	C	D
A	1.000			
B	0.299	1.000		
C	0.441	0.385	1.000	
D	0.302	0.092	0.224	1.000

Table B.3 Principal components

Component	Eigenvalue	Proportion of variance	Cumulative variance
Comp1	1.900	0.475	0.475
Comp2	0.930	0.232	0.707
Comp3	0.632	0.158	0.865
Comp4	0.538	0.135	1.000

Table B.4 Principal components eigenvectors

Socioeconomic Measure	Comp1	Comp2	Comp3	Comp4
A) Mother's Education	0.561	0.121	-0.545	0.612
B) Household Income	0.468	-0.567	0.632	0.245
C) Household Assets	0.569	-0.180	-0.293	-0.747
D) Urbanicity	0.377	0.795	0.467	-0.087

APPENDIX C: CHAPTER 4 SUPPORTING INFORMATION

C.1 PRINCIPAL COMPONENTS ANALYSIS OF SOCIOECONOMIC MEASURES

Table C.1 Definition of socioeconomic measures

A) Participant's Education	Highest grade completed in 2005 (range 2-16)
B) Household Income	Weekly household income in 2005, deflated to 1983 levels (range 76-3420 Philippine Pesos)
C) Household Assets	Scale described in detail by Desantis et al., 2015 of assets in 2005 that reflect population-relevant aspects of social class, including electricity, televisions, refrigerators, air conditioners, tape recorders, electric fans, jeepneys, cars, houses, and house construction type (range 0-10 of 11 possible)
D) Urbanicity	Scale described in detail by Dahly and Adair, 2007 that accounts for population size, population density, communications, transportation, educational facilities, health services, and markets in 1983 (range 8-61)

Table C.2 Correlation matrix of socioeconomic measures

	A	B	C	D
A	1.000			
B	0.157	1.000		
C	0.511	0.286	1.000	
D	0.135	0.112	0.205	1.000

Table C.3 Principal components

Component	Eigenvalue	Proportion of variance	Cumulative variance
Comp1	1.755	0.439	0.439
Comp2	0.910	0.228	0.666
Comp3	0.869	0.217	0.884
Comp4	0.466	0.117	1.000

Table C.4 Principal components eigenvectors

Socioeconomic Measure	Comp1	Comp2	Comp3
A) Participant's Education	0.571	-0.317	-0.415
B) Household Income	0.406	-0.046	0.892
C) Household Assets	0.631	-0.175	-0.135
D) Urbanicity	0.333	0.931	-0.123

C.2 Sensitivity Analyses

Offspring birth weight data, my primary outcome, came from two sources. To ensure that data from participant recall did not differ from data from birth records and could reasonably be combined in my analyses, I conducted a sensitivity analysis. I stratified my models by offspring birth weight data source. Birth weights for 104 offspring reported by 52 participants came from participant recall (mean 2.0 offspring per woman; range 1-6 offspring), and data for 37 offspring of 31 participants came from birth records (mean 1.2 offspring per woman; range 1-3 offspring. β-coefficients differed by birth weight data source for offspring birth order, participant's age at offspring delivery, and SES, but the large standard errors indicate that these differences are unlikely to have impacted combined estimates.

Table C.5 Mixed-effects linear regression models predicting singleton offspring birth weight (grams, centered at 2500) from participant recall

	Model 1*		Model 2*		Model 3*		Model 4*	
	β ± SE	p	β ± SE	p	β ± SE	p	β ± SE	p
Log-transformed MMc concentration							-21.7	
(log_{10} [MMc per 100,000 gEq tested +1])							± 120.0	0.856
Offspring-level covariates								
Offspring birth order			-94.3		-1.4		-4.3	
(later-born=0, first-born=1)			± 132.1	0.476	± 143.9	0.992	± 144.7	0.976
Offspring sex			-122.2		-143.0		-143.3	
(male=0, female=1)			± 111.7	0.274	± 110.3	0.295	± 110.3	0.194
Participant's age at offspring delivery			9.6		26.5		26.4	
(years, centered at 16)			± 16.5	0.560	± 17.8	0.136	± 17.8	0.137
Participant-level covariates								
Participant's adult height					5.6		5.6	
(cm, mean centered at 150)					± 10.4	0.590	± 10.4	0.591
Participant's smoking status					127.7		131.7	
(never-smokers=0, ever-smokers=1)					± 113.8	0.262	± 116.3	0.257
Participant's marital status					277.4		270.8	
(never married=0, ever married=1)					± 139.0	0.046	± 143.3	0.059
Participant's socioeconomic status					29.1		29.3	
(principal component 1)					± 49.1	0.554	± 49.2	0.552
Intercept	587.9 ± 64.5	<0.001	621.9 ± 184.1	0.001	469.9 ± 154.2	0.002	270.5 ± 256.7	0.292
Model post-estimation								
R^2			0.034		0.768		0.761	
Intra-class correlation (ICC)	0.203		0.152		0.038		0.039	
Bayesian information criteria (BIC)	1627		1638		1652		1656	

*Models include random intercepts for participants and offspring

Table C.6 Mixed-effects linear regression models predicting singleton offspring birth weight (grams, centered at 2500) from birth records

	Model 1*		Model 2*		Model 3*		Model 4*	
	β ± SE	p	β ± SE	p	β ± SE	p	β ± SE	p
Log-transformed MMc concentration							-6.4	
(log_{10} [MMc per 100,000 gEq tested +1])							± 140.8	0.964
Offspring-level covariates								
Offspring birth order			33.5		60.6		62.1	
(later-born=0, first-born=1)			± 120.0	0.780	± 133.0	0.649	± 135.4	0.647
Offspring sex			-58.2		-22.5		-21.2	
(male=0, female=1)			± 114.1	0.610	± 132.7	0.865	± 134.8	0.875
Participant's age at offspring delivery			50.7		58.9		59.1	
(years, centered at 16)			± 26.2	0.053	± 27.5	0.032	± 28.7	0.039
Participant-level covariates								
Participant's adult height					10.3		10.7	
(cm, mean centered at 150)					± 13.6	0.447	± 16.4	0.514
Participant's smoking status					148.5		151.6	
(never-smokers=0, ever-smokers=1)					± 146.5	0.311	± 159.3	0.341
Participant's marital status					-71.3		-74.0	
(never married=0, ever married=1)					± 145.7	0.625	± 161.4	0.647
Participant's socioeconomic status					-25.2		-27.0	
(principal component 1)					± 74.0	0.734	± 85.1	0.751
Intercept	492.5 ± 58.5	<0.001	3.6 ± 293.3	0.990	317.6 ± 167.2	0.058	-155.3 ± 343.8	0.652
Model post-estimation								
R^2			0.071		0.156		0.172	
Intra-class correlation (ICC)	7.51×10^{-19}		0.208		0.185		0.182	
Bayesian information criteria (BIC)	551		558		571		574	

*Models include random intercepts for participants and offspring