University of Alberta

Chronic Maternal Stress and Genetic Variants in the Etiology of Spontaneous Preterm Birth

by

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A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Physiology

Fall 2012
Edmonton, Alberta

To Rafał

For always believing in me

ABSTRACT

Preterm birth is the leading cause of mortality and morbidity in newborn infants. With an estimated 15 million preterm births annually worldwide, the global burden of preterm birth is enormous. Despite decades of research, its etiology remains elusive. Preterm birth is a complex phenomenon with genes and environmental factors contributing to its risk, both in the mother and the fetus. For this dissertation, we explored the role of chronic maternal stress and genetic variants in the etiology of spontaneous preterm birth. We conducted a case-control study in 622 women in Edmonton. First, we examined the association between lifetime chronic stress and preterm birth. Exposure to two or more adverse childhood experiences was associated with a two-fold risk of preterm birth, regardless of maternal age, smoking status, educational status and a history of miscarriage (OR 2.09, p=0.024) Lifetime physical and emotional abuse was also associated with spontaneous preterm in our study population (OR 1.3, p=0.033) Second, we conducted genomic studies for preterm birth in collaboration with the Preterm Birth Genome Project, the World Health Organization and the March of Dimes. Using a candidate gene approach, we discovered two novel single nucleotide polymorphisms (SNPs), both located in the mineralocorticoid receptor gene that associate with spontaneous preterm birth: rs17484063 (OR 0.50, p=0.038) and rs2883929 (OR 0.49,

p=0.017). For each additional effect allele, the risk of preterm birth was halved. In women with ≤1 adverse childhood experiences, the odds ratios of rs17484063 and rs2883929 for preterm birth were further lowered (OR 0.37; *p*=0.024 and OR 0.37; *p*=0.013, respectively). Via activation of the maternal-fetal HPA axis, a biological plausibility of the role of these SNPs in the etiology of preterm birth exists.

This is the first evidence of genetic variations in the mineralocorticoid receptor gene that associate with spontaneous preterm birth. In addition, we discovered a strong relationship between adverse childhood experiences and preterm birth. Taken together, this research confirmed that chronic maternal stress and genes involved in the stress response likely have an impact on the risk of spontaneous preterm birth.

ACKNOWLEDGEMENT

It is a great pleasure to thank the many people who made this thesis possible.

Foremost, I would like to express my sincere gratitude to my supervisor Dr. David Olson for his guidance, support and encouragement throughout my PhD studies. Without his help, I would never have had the opportunity to collaborate with some of the best preterm birth researchers in the field.

I would like to thank all my collaborators from PREBIC, the PGP Consortium and PreHOT for making this project possible. I feel very fortunate to be part of such an excellent network of researchers.

I am extremely grateful to Dr. Craig Pennell for his support and mentorship concerning the genetic studies. He is one of the driving forces behind the PGP and his help with setting up the candidate gene studies has been invaluable. A special thanks goes to Wei Ang for his instrumental advice on the statistical analyses.

I would like to thank the members of my supervising committee for their assistance, advice and critical questions: Dr. Kathleen Hegadoren, Dr. Martin Somerville and Dr. Radha Chari.

This dissertation would not have been possible without the support of my wonderful colleagues. I wish to thank Sheena Fang, Dean Zaragoza, Yuka Yamamoto, and all the past and present members of the Olson lab with whom I had the pleasure working with.

I am grateful for the help I have received from the research nurses and students throughout this project. Their help with the recruitment of subjects and data collection has been vital.

I would like to thank all the women who participated in this study. Without them, this dissertation would not have been possible.

A big thank-you goes to all my friends in Canada and the Netherlands for their friendship, joy, laughter, dinners, hiking trips and nights out on the town. They have kept me sane throughout this PhD and I am forever grateful for having such wonderful people in my life. A very special thank-you goes to Yvonne Coors: dankjewel, lieve Von.

I would like to thank my family for their love and never-ending support, especially the two most important people in the world, my parents, Theo and Marlies Christiaens. Without their encouragement and belief in me, I would never have completed this PhD. Dankjewel, lieve papa en mama. To my sister Anke De La Flor for her support from afar and to my little nephew Liam for always bringing a smile to my face.

Finally, I thank Rafał, for his instrumental support, advice and my database, and, most importantly, for always being there for me. He is my rock, my best friend and my love. To him I dedicate this thesis.

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LIST OF ABBREVIATIONS

5HT	Serotonin
5HTT	Serotonin transporter – also known as SLC6A4
95% CI	95 Percent confidence interval
A	Adenine
AAS	Abuse Assessment Screen
ACE	Adverse Childhood Experiences
ACTH	Adrenocorticotropic hormone
ADRB2	Adrenergic beta-2 receptor
AIDS	Acquired immunodeficiency syndrome
ARL9	ADP-ribosylation factor-like 9
ART	Assisted reproductive technology
BMI	Body mass index
BUD13	BUD13 homolog (S. cerevisiae)
C	Cytosine
cAMP	Cyclic adenosine monophosphate
CAPS	Clinician Administered Posttraumatic Stress Disorder Scale
CCDC77	Coiled-coil domain containing 77
CIS	Canadian Incidence Study of Reported Child Abuse and Neglect
CNTNAP5	Contactin associated protein-like 5
COMT	Catecholoxymethyltransferase
COX	Cyclooxygenase – also known as prostaglandin synthase
CRH	Corticotropin-releasing hormone

CRHR1	Corticotropin-releasing hormone receptor 1
CX-43	Connexin 43
DNA	Deoxyribonucleic acid
F1-F15	Fetal samples number 1 to 15
F2	Coagulation factor II precursor
FKBP5	FK505 binding protein 5
FOIPP Act	Freedom of Information and Protection of Privacy Act
G	Guanine
GAS	General adaptation syndrome
GR	Glucocorticoid receptor – also known as NR3C1
GRIA2	Glutamate receptor, ionotropic, AMPA 2
GWAS	Genome-wide association study
H1N1	Influenza A virus subtype H1N1
HIV	Human immunodeficiency virus
HPA axis	Hypothalamic-pituitary-adrenal axis
HREB	Health Research Ethics Board
HWE	Hardy-Weinberg equilibrium
IFNG	Interferon gamma
IL	Interleukin
ISEL	Interpersonal Support Evaluation List
IUGR	Intrauterine growth restriction
KANK1	KN motif and ankyrin repeat domains 1
LD	Linkage disequilibrium
LOC	Locus (location)
M1-M15	Maternal samples number 1 to 15
MAF	Minor allele frequency

MAOA	Monoamine oxidase A
MDG	Millennium Development Goal
M.I.N.I.	Mini International Neuropsychiatric Interview
MLCK	Myosin light-chain kinase
MLCP	Myosin light-chain phosphatase
MMP	Matrix metalloproteinase
MR	Mineralocorticoid receptor – also known as NR3C2
mRNA	Messenger RNA
N	Number
NR3C1	Nuclear receptor subfamily 3, group C, member 1 – also known as glucocorticoid receptor or GR
NR3C2	Nuclear receptor subfamily 3, group C, member 2 – also known as mineralocorticoid receptor or MR
nsSNP	Nonsynonymous single nucleotide polymorphism
OR	Odds ratio
OT	Oxytocin
OTR	Oxytocin receptor
P_4-R_{ABC}	Progesterone receptor A, B and C
PCR	Polymerase chain reaction
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGP	Preterm birth Genome Project
PPROM	Preterm premature rupture of membranes
PREBIC	Preterm Birth International Collaborative
PTGFR	Prostaglandin F receptor
PTGS	Prostaglandin-endoperoxide synthase
PTSD	Posttraumatic stress disorder

QC	Quality control
RANBP6	RAN binding protein 6
SAM axis	Sympathetic-adrenal-medullary axis
SD	Standard deviation
SES	Socioeconomic status
SLC6A4	Serotonin transporter – also known as 5HTT
SNP	Single nucleotide polymorphism
STI	Sexually transmitted infection
T	Thymine
TIMP-1	TIMP metallopeptidase inhibitor 1
TNF	Tumor necrosis factor
UAP	Uterine activation protein
UBE2W	Ubiquitin-conjugating enzyme E2W
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

1. INTRODUCTION

1.1 PRETERM BIRTH

Preterm birth, defined by the World Health Organization (WHO) as birth at less than 37 weeks of gestation, is the leading cause of mortality in newborn infants.[1] Of the annual 4 million neonatal deaths (less than 28 days of life) worldwide, 28 percent are directly attributed to preterm birth.[2] It is estimated however, that prematurity and its consequences, such as sepsis and asphyxia, are responsible for more than half of all neonatal deaths. Preterm birth also leads to significant neonatal morbidities.[3] It is associated with chronic respiratory disease, necrotizing enterocolitis, retinopathy of prematurity, brain haemorrhage and various neurodevelopmental problems, such as cerebral palsy, mental retardation and sensory impairments.[4-8] Long-term sequelae of preterm birth include behavioural and learning problems, developmental delay and adult diseases, for example diabetes and cardiovascular disease.[9-12] While the risk for mortality and morbidity is substantially higher in very preterm births at <32 weeks' gestation, late preterm births (34 to 36 weeks' gestation) represent the vast majority of all preterm births.[13] It was long thought that these late preterm infants would have a low risk of developing morbidities. However, it is now widely accepted that they have a significantly increased risk of complications

compared to infants born at term.[14] Respiratory distress, periventricular leukomalacia, hypoglycaemia, sepsis and jaundice are more common in late preterm infants than term infants.[15,16] Being born late preterm is also associated with long-term effects such as learning difficulties, decreased motor skills and behavioural problems.[17] The data clearly show that late preterm infants are by no means the same as full term babies.

1.2 TRENDS IN PRETERM BIRTH RATES

The global burden of preterm birth is an estimated 15 million preterm deliveries per year representing a prevalence of more than 10 percent.[18] Africa and southern Asia share 85 percent of this burden, mainly due to poverty, poor nutrition, co-morbidities and poor access to reproductive health care.[19] However, preterm birth in developing countries is not the only major jurisdiction of concern. After Africa, North America (United States and Canada combined) has the highest preterm birth rate of all United Nations regions. In the United States, 1 in 8 infants is born premature,[20,21] and in the past 25 years, its preterm birth rate has increased by 36 percent.[22] After peaking at 12.8 percent in 2006, the United States' preterm rate dipped slightly to 12.2 percent in 2009, the most recent year in which data are available (FIGURE 1).

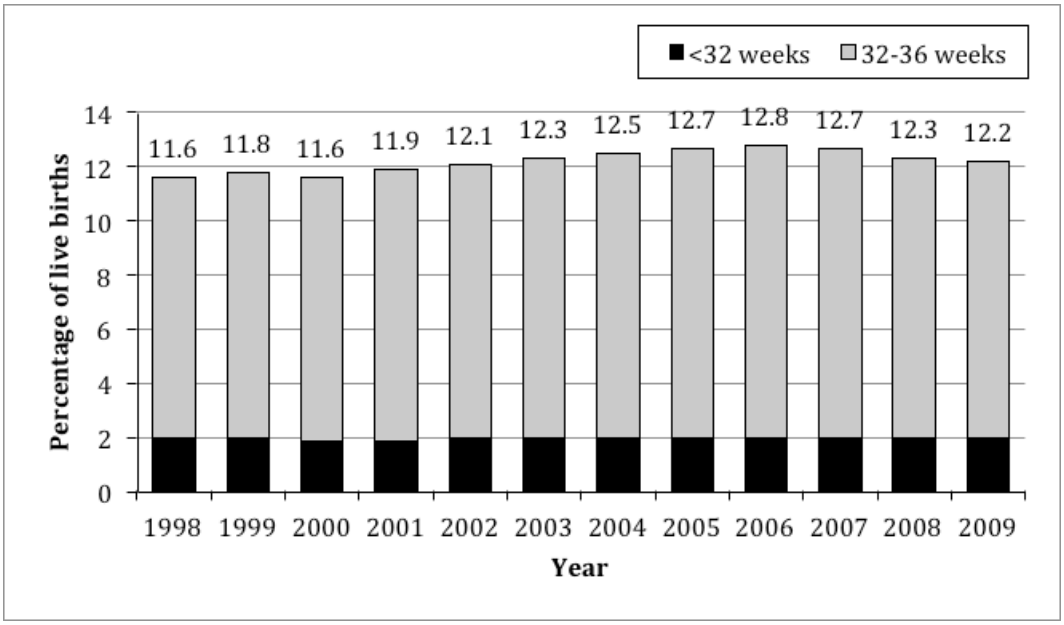

FIGURE 1. PRETERM BIRTH RATES BY GESTATIONAL AGE AS A PERCENTAGE OF LIVE BIRTHS IN THE UNITED STATES FROM 1998 TO 2009. Source: National Centre for Health Statistics, retrieved from www.marchofdimes.com/peristats.[20]

Among African Americans, the preterm birth rate in 2008 was 17.5 percent,[23] showing a large racial-ethnic disparity in the proportion of preterm births. The rise in preterm births of the past decades is entirely attributable to an increase in moderate and late preterm births (32-36 completed weeks of gestation), whereas the rate of very and extreme preterm infants (less than 32 weeks) has been stable (FIGURE 1).

In Canada, the preterm birth rate is lower than in the United States,[24] although the proportion of preterm deliveries has steadily risen from 6.8 to almost 8 percent of all live births in recent years (FIGURE 2).

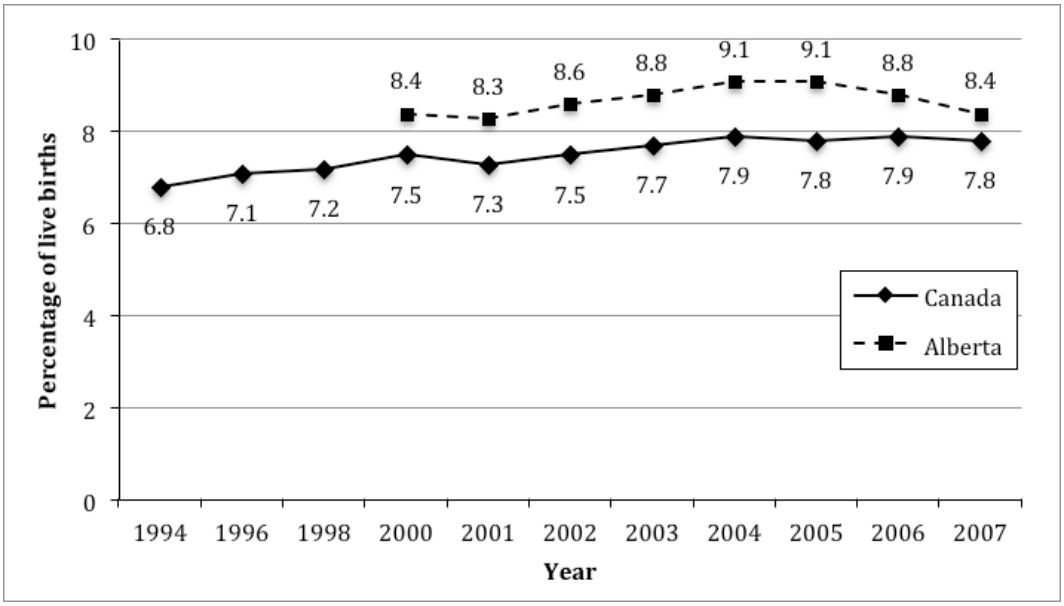

FIGURE 2. PRETERM BIRTH RATES AS A PERCENTAGE OF ALL LIVE BIRTHS IN CANADA AND ALBERTA FROM 1994 TO 2007.

Source: Statistics Canada.[24]

Interestingly, the percentage of preterm births in the province of Alberta is one percent above the national average, and with the two largest cities, Edmonton and Calgary, having preterm birth rates of 9.1 and 9.3 percent, respectively, in 2007.[25]

1.3 THE COST OF PRETERM BIRTH

Despite major advancements in neonatal medicine, the incidence of both acute and chronic complications in surviving preterm infants has not decreased, and combined with the rising preterm birth rates, this has resulted in escalating health care expenditures.[26,27] Canadian data from 1998 reported that over $13 billion are spent annually on the total costs of prematurity.[28] A more recent report in the United States estimated the annual societal economic cost – medical, educational and lost productivity – conservatively at $26 billion.[29] As one would expect, hospital costs increase as gestational age decreases. The average hospital cost for an extremely preterm infant is roughly $85,000, while a full term infant 'only' costs $1100.[30] Similarly, the length of hospital stay increases with decreasing gestational age. Furthermore, prematurity is associated with increased costs for long-term care and remediation of developmental delay or disabilities, and perhaps most importantly, the emotional and social costs to parents, families and communities due to preterm birth are immeasurable.[31,32]

1.4 GLOBAL AWARENESS

In high-income countries, the impact of preterm birth on public health has been recognized for many years. However, it is now widely accepted that preterm birth is foremost a global perinatal health problem. In 2000, world

leaders adopted the eight United Nations Millennium Development Goals (MDGs) to reduce extreme poverty and improve health before the deadline of 2015.[33] More specifically, the targets of MDGs 4 and 5 are to reduce the under-five mortality rate by two-thirds (between 1990 and 2015) and to reduce the maternal mortality rate by 75 percent respectively. In addition, MDG 5 targets universal access to reproductive health. To achieve Goal 4, it is critical to develop better intervention strategies for preterm birth and to improve survival of preterm infants. At the same time, this will contribute to MDG 5 and improve maternal health.

1.5 CAUSES OF PRETERM BIRTH

Overall, there are three different obstetric precursors leading to preterm birth.[34] In high-income countries, around 30 percent of preterm births are indicated, for either maternal or fetal indications. Another 45 percent are due to spontaneous preterm labour with intact membranes, and lastly, 25 percent of preterm births follow preterm premature rupture of the membranes (PPROM). PPROM is defined as spontaneous rupture of the membranes at less than 37 weeks of gestation at least one hour before the onset of any contractions. Similar to spontaneous preterm labour, the cause of PPROM is in most cases unknown, however PPROM is frequently preceded by asymptomatic intrauterine infection.[35]

An important reason for the increase in the singleton preterm birth rates is the increasing number of medically indicated preterm births.[36] Other explanations for the increasing preterm birth rates include a rise in the proportion of births to women over 35 years of age, the use of assisted reproductive technologies (ARTs), for instance *in vitro* fertilization, an increase in the number of multiple births – often associated with ARTs, and better surveillance due to improved dating technologies.[23,37-41] Most notably in the United States, there has been a change of clinical practice such as the early induction of labour or performance of Caesarean sections close to term leading to an increase in indicated preterm births.[42,43] However, all these factors alone fail to explain the trend.[44]

Various maternal sociodemographic factors contribute to the risk of preterm birth. Not only is advanced maternal age (>35 years) an important factor as mentioned above, young maternal age (<18 years) is associated with preterm birth as well.[45,46] Marital status, more specifically being unmarried, and low socioeconomic status (SES) are also risk factors for prematurity.[47-50] Smoking, alcohol use, drug use and both a low and high body mass index (BMI) have all been linked to adverse pregnancy outcomes, including preterm birth.[51-56] In contrast, favourable lifestyle choices can reduce the risk of preterm birth. Especially evident in the United States, there is a racial-ethnic disparity in the incidence of preterm birth. The largest

disparity is seen between non-Hispanic white and black women, with the highest rate of preterm birth occurring in the African American population.[23] This disparity has remained largely unexplained over the past decades, however factors including SES, maternal behaviour, infections and notably, genetics are partly responsible for this disparity.

For decades, scientists and clinicians have attempted to elucidate the etiology of preterm birth, yet the majority of preterm deliveries are still unexplained.[44] In the literature, preterm birth is often described as a single entity, ignoring different phenotypes, such as idiopathic spontaneous preterm delivery, infection-associated preterm delivery and PPROM eventually leading to preterm birth. This 'simple' approach makes an easier model for research and statistical purposes, but it also wrongly suggests that there is only one pathway leading to preterm birth.

1.6 THE PHYSIOLOGY OF PARTURITION

Pregnancy and parturition involve complex pathways in both mother and fetus, which to date are not well understood. Parturition is a natural continuum of processes that can be divided into four distinct phases: Phase 0 (quiescent phase), Phase 1 (myometrial activation), Phase 2 (myometrial stimulation) and Phase 3 (uterine involution), as shown in FIGURE 3.[57,58]

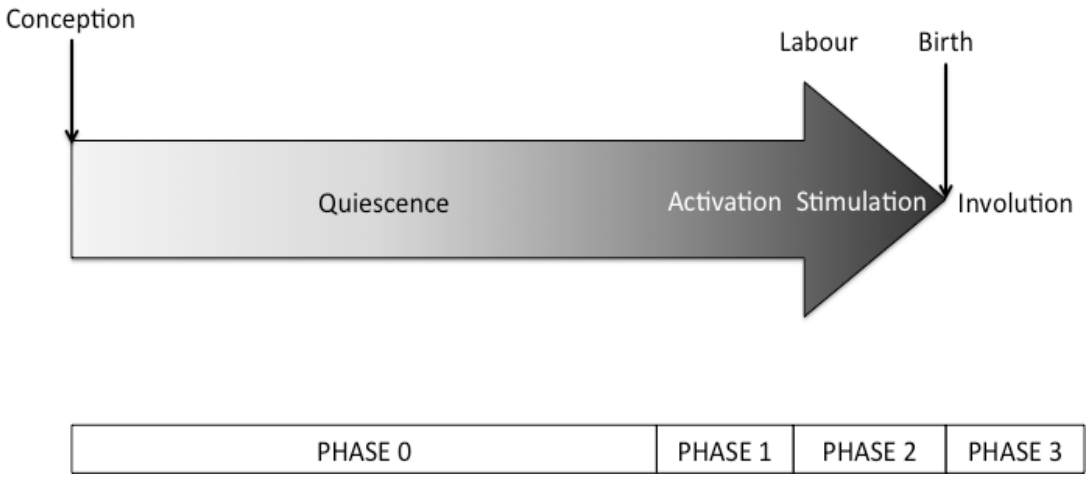

FIGURE 3. THE PHASES OF PARTURITION.

1.6.1 FOUR PHASES OF PARTURITION

Phase 0 is the quiescent phase that accounts for roughly 95 percent of pregnancy, during which myometrial activity is inhibited, most importantly by progesterone. Csapo was the first to describe the progesterone block hypothesis.[59] In pregnancy, progesterone blocks the gene expression of various uterine activation proteins (UAPs). These UAPs promote the ability of the tissues to carry out the processes of parturition and they include the oxytocin receptor (OTR) and the prostaglandin $F_{2\alpha}$ receptor (PTGFR).[60] Progesterone also inhibits gap-junction formation, of which the major protein is connexion-43 (CX-43), within the myometrium, while it up regulates various substances, such as nitric oxide, that are important for myometrial relaxation.[61,62] In addition to progesterone, other inhibitory

agents of myometrial contractility include nitric oxide, relaxin, prostacyclin, and parathyroid hormone-related protein.[63-66] In general, intracellular levels of cyclic nucleotides, including cyclic adenosine monophosphate (cAMP), are increased.[67] As a result, the release of calcium is inactivated and the enzyme myosin light-chain kinase (MLCK) is inactivated, both of which are central to smooth muscle contraction as described below.

During transition from quiescence to activation of the myometrium (phase 1), the levels of the UAPs CX-43, cyclooxygenase-2 (COX-2), OTR and PTGFR increase. The signals for myometrial activation can come from uterine stretch and activation of the inflammatory pathway.[68-71] Parturition is an inflammatory response whereby an influx of inflammatory mediators, such as chemokines and cytokines stimulate the activation of the myometrium.[72,73] In addition, signals also come from the fetal hypothalamic-pituitary-adrenal (HPA) axis. This was first shown in sheep by Liggins and Thorburn.[74,75] Through stimulation of cortisol and estrogens, and possibly induced by a functional progesterone withdrawal, a biological cascade of the various uterotonins, such as oxytocin and prostaglandins, is initiated, leading to strong and regular uterine contractions in phase 2 – myometrial stimulation.[61,76] Also during this phase, the cervix undergoes ripening and dilation with decreasing collagen content and remodelling of the extracellular matrix. Matrix metalloproteinases (MMPs), such as MMP-2 and

MMP-9, are involved in the activation of the cervix, decidua and fetal membranes eventually leading to the rupture of the membranes.[77,78] Phase 2 concludes with the birth of the fetus.

The last phase of parturition (phase 3) involves separation of the placenta and contraction and involution of the uterus. The uterine contraction after delivery, induced by oxytocin, is essential to prevent post-partum haemorrhage, which is the most common cause of perinatal maternal death in developing countries.

1.6.2 MYOMETRIAL CONTRACTILITY

Myometrial contractility, like all smooth muscle contractility involves the interaction of actin and myosin leading to muscle contraction. Firstly, there is an increase in intracellular calcium, mainly generated by the act of the uterotonic stimulators, oxytocin and prostaglandins.[79,80] Calcium binds to calmodulin and this activates MLCK. The 20-kDa light chain of myosin is then phosphorylated by MLCK and this in turn induces ATPase activity and enables the formation of cross-bridges with actin. As a result, the muscle cell shortens.[81] Inversely, smooth muscle relaxation requires a decrease in intracellular calcium and MLCK activity. Subsequently, the activity of myosin light-chain phosphatase (MLCP) is increased, the myosin light chains are dephosphorylated and the muscle relaxes.

1.6.3 PROSTAGLANDINS

Prostaglandins, produced by the myometrium and the intrauterine tissues of pregnancy, are involved in all the different physiological events of parturition.[57,82] The key regulatory steps in prostaglandin synthesis involve the release of the precursor arachidonic acid from membrane phospholipids and its conversion to an endoperoxide intermediate by prostaglandin-endoperoxide synthase (PTGS), also known as cyclooxygenase (COX). COX-1 is expressed in many tissues and is responsible for constitutive prostaglandin production; COX-2 is an inducible form of the enzyme. The increase in the production of prostaglandins before parturition is predominantly due to increased COX-2.[83,84] Prostaglandins are most commonly associated with stimulation of the myometrium. Indeed, treatment of pregnant women with prostaglandins induces labour,[58] while inhibitors of COX delay the time of onset of labour.[85]

1.7 PATHWAYS OF PRETERM BIRTH

Preterm birth is an adverse outcome of the parturition process, and it results from either iatrogenic preterm delivery for maternal or fetal indications, or from spontaneous preterm labour with or without intact membranes. The etiology of spontaneous preterm birth is multifactorial with various common pathways that exist.[29,86] Currently, four common pathways

are recognized and these include stress (maternal and fetal), inflammation, haemorrhage and uterine distension (FIGURE4).

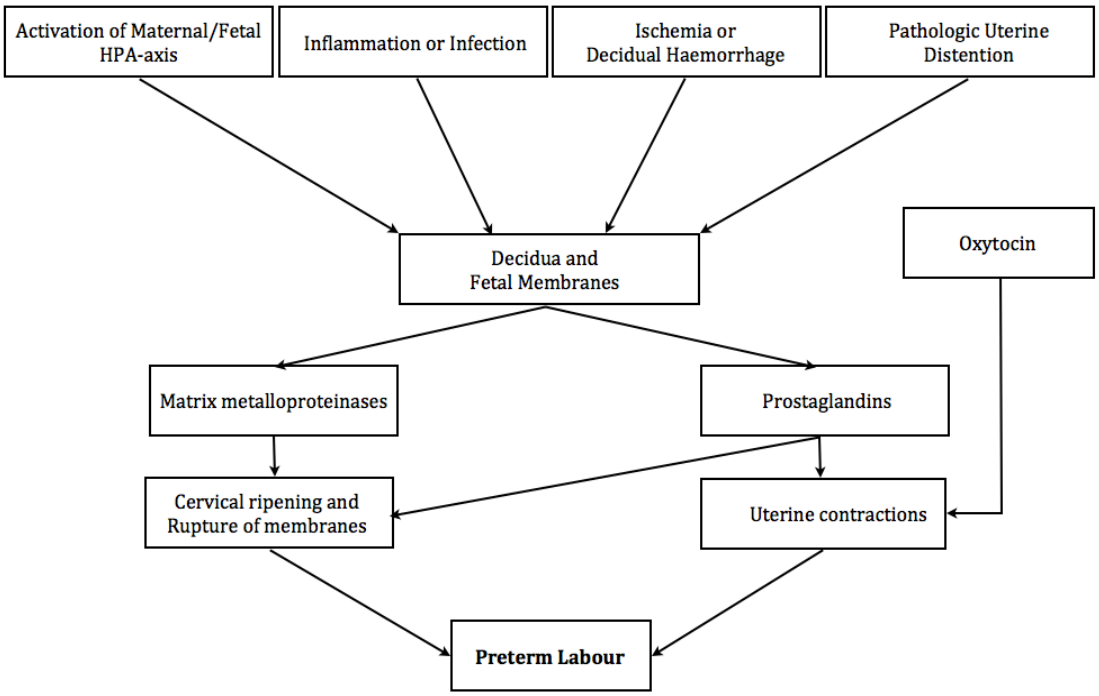

FIGURE 4. COMMON PATHWAYS LEADING TO PRETERM LABOUR AND DELIVERY.

Adapted from Behrman *et al.*[29]

1.7.1 Activation of the Maternal-Fetal HPA axis

Maternal stress is increasingly recognized as an important risk factor for preterm birth. Stress can be defined as any challenge – psychological or physical – that threatens or that is perceived to threaten, homeostasis.[29]

Many epidemiologic studies have reported that pregnant women who experience high levels of psychosocial stress before or during pregnancy are at significant risk for preterm labour.[87-93] Stress can result in preterm activation of the maternal and fetal HPA axes. The actions of the HPA axis in the body are described in detail in Chapter 3 (Section 3.1.2). Corticotrophin releasing hormone (CRH) is a central factor in both fetal maturation and parturition. This neuropeptide predominantly originates in the hypothalamus,[94] but in pregnancy CRH is also expressed in the human placenta, fetal membranes and myometrium.[95,96] Throughout gestation, it is released in increasing amounts into the maternal and fetal compartments.[97] This rise in CRH levels has been associated with the length of gestation and the timing of birth. McLean *et al.* were the first to report that women destined to go into preterm labour have higher concentrations of maternal CRH in their plasma as early as 16 weeks of gestation.[98] These women also show a more rapid rise in CRH levels compared to women with term labour. Thus, it was proposed that placental CRH acts as a 'placental clock' and as such can regulate the length of gestation.[98,99] In contrast to hypothalamic CRH, which is suppressed by glucocorticoids, placental CRH is stimulated by glucocorticoids.[100,101] This suggests a positive-feedback loop between the fetal HPA-axis and CRH produced by the trophoblast cells, which can then drive the process of parturition.

Various reports confirmed the placental clock hypothesis and the premise that placental CRH is potentially implicated in the timing of human parturition. They showed that preterm birth can be predicted by measuring CRH levels during pregnancy and therefore that CRH levels could be used as potential biomarkers for the prediction of preterm birth.[99,102-104] As promising as this may sound, reality learns from numerous other studies that did not find a clear association between CRH levels and preterm birth.[105,106] In addition, women who present themselves with threatened preterm labour will receive a single course of antenatal glucocorticoids to improve fetal lung maturation. However, the majority of these women do not progress to preterm labour. The theory of the placental clock therefore remains controversial.

1.7.2 INFLAMMATORY PATHWAY

Worldwide, inflammation and infection are the most frequent causes of preterm birth.[107] Especially extreme preterm births (<28 weeks) result from intrauterine infections and the morbidity and mortality rate in these infants are very high. Around 20 to 40 percent of spontaneous preterm deliveries and PPROM are associated with intrauterine infection.[108,109] Studies have also shown an association between various genital tract infections, most notably bacterial vaginosis, sexually transmitted infections, and, although controversial, periodontal disease and preterm birth.[110-115]

Infection leads to activation of the innate immune system and the release of many pro-inflammatory cytokines, including interleukin (IL)1β, IL6 and tumor necrosis factor alpha (TNF).[108,116-119] These cytokines in turn stimulate the production of prostaglandins, largely via an increase in COX-2.[83,84] In addition, the levels of all UAPs increase,[120-124] vascular endothelial growth factor (VEGF) is activated, activity of matrix metalloproteinases is increased and the production of inflammatory mediators, including cytokines is further stimulated, causing a feed-forward loop eventually leading to myometrial stimulation.[71] These events are summarized in FIGURE 5.

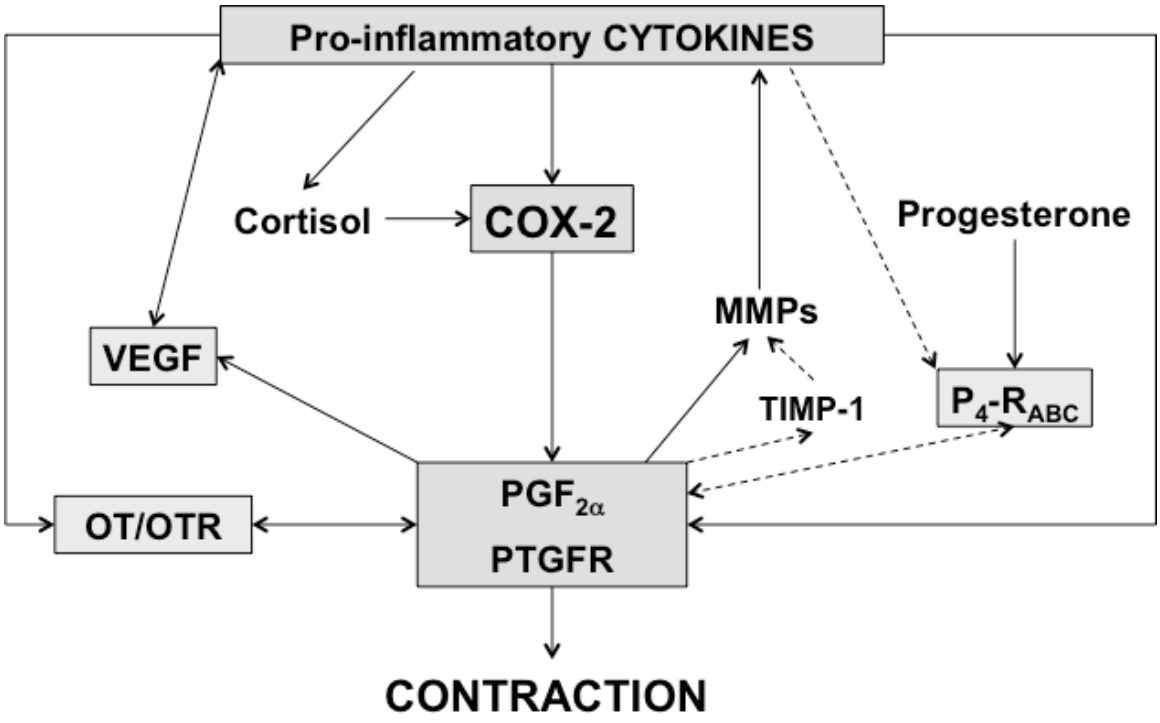

FIGURE 5. INFLAMMATORY ACTIONS ON THE ACTIVATION OF THE DECIDUA AND MYOMETRIUM.

Solid arrows represent stimulatory effects; dashed arrows represent inhibitory effects. Adapted from Christiaens *et al.*[71]

COX-2: cyclooxygenase-2; MMPs: matrix metalloproteinases; OT: oxytocin; OTR: oxytocin receptor; $PGF_{2\alpha}$: prostaglandin $F_{2\alpha}$; PTGFR: prostaglandin $F_{2\alpha}$ receptor; P_4-R_{ABC}: progesterone receptor A, B and C; TIMP-1: TIMP metallopeptidase inhibitor 1; VEGF: vascular endothelial growth factor.

1.7.3 HAEMORRHAGE AND UTERINE DISTENTION

Furthermore, placental abruption or decidual haemorrhage may cause preterm labour. Thrombin induces protease expression and subsequently disturbances in uterine tone.[125] Abnormal uterine distention, most frequently due to a multifetal pregnancy or polyhydramnios, increases the mechanical stretch on the uterus. Uterine stretch stimulates the expression of CX-43, the major gap-junction protein in the myometrium, and the expression of other UAPs such as the oxytocin receptor.[69] This can lead to preterm contractions and eventually result in preterm birth.

The pathways may occur independently but more commonly, they are present in various degrees of combination. Moreover, studies have shown that there is a strong genetic component to the risk of preterm birth (Chapter 4). Each pathway is therefore most likely influenced by genetic variability and gene-environment interactions, adding to the complexity of preterm birth.

1.8 RATIONALE

It is clear that preterm birth is an important health problem worldwide. Encouragingly, there is more global awareness of the problems of prematurity than ever before. Indeed, the March of Dimes has declared

November 17 has as 'World Prematurity Day'. However, in spite of decades of research, the etiology of spontaneous preterm birth remains largely elusive. Understanding the various pathways leading to preterm labour would aid significantly in the development of better predictive methods and more specifically targeted therapeutics. Successful therapies for the treatment of spontaneous preterm birth have yet to be developed. At present, the major outstanding issue is that we are unable to predict whether a woman will deliver her newborn preterm. Tocolytics currently used are only able to prolong gestational length in symptomatic women, i.e. after contractions have already started, as they target the mechanisms responsible for myometrial contractions specifically.[126] Since tocolytics are administered only in patients who are already in active preterm labour (Phase 2 of parturition), they are often not effective at all in delaying birth. In order to be able to delay or prevent a preterm delivery, it is key to identify women at risk of delivering early, i.e. weeks or months prior to labour. A recent systematic review of the literature has demonstrated that more than a hundred biomarkers for preterm birth have been identified.[127] Some of the best, although by no means perfect, predictive factors that have been found are cervical length and fetal fibronectin.[128] However, the sensitivity of cervical length as a predictive tool is low (up to 40 percent using endovaginal ultrasound).[129] Fetal fibronectin has a very poor positive predictive value, but a negative fetal fibronectin test can accurately identify women at low risk of

preterm labour (negative predictive value of 95 percent).[130] At present, the prediction of preterm birth remains elusive. Recent advancements in genetic technologies, including the work of the Human Genome Project and the International HapMap Project,[131-133] provide an excellent opportunity for further research into the prevention of spontaneous preterm birth. The knowledge gained from these studies could ultimately be used for the development of new prediction and prevention strategies of preterm birth.

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2. STUDY DESIGN AND DEMOGRAPHIC CHARACTERISTICS

2.1 OVERALL OBJECTIVES AND HYPOTHESES

The purpose of this research study was to expand our knowledge related to the causes of spontaneous preterm birth through the analysis of genetic and environmental factors. More specifically, we examined the contribution of single nucleotide polymorphisms (SNPs) and chronic, or lifelong, maternal stress in the etiology of spontaneous preterm birth.

2.1.1 HYPOTHESIS 1: CHRONIC MATERNAL STRESS INCREASES THE RISK OF SPONTANEOUS PRETERM BIRTH

Maternal stress is increasingly recognized as a variable of interest in the etiology of preterm birth. The effect of stress during pregnancy on the risk of spontaneous preterm birth continues to be of debate. Little is known about the role of chronic stress, therefore examining the exposure to stressors over a mother's life course might give a better perspective on the contribution of stress to preterm birth. Our aim was to examine the association between chronic maternal stress, determined by a postpartum

questionnaire, and preterm labour. These studies are described fully in Chapter 3 'Chronic Maternal Stress and Spontaneous Preterm Birth.'

2.1.2 Hypothesis 2: Genetic variations increase the risk of spontaneous preterm birth

A strong genetic disposition for spontaneous preterm birth exists. Single nucleotide polymorphisms, or SNPs, are the most common genetic variations in the human genome.[132] A SNP is simply a single base pair substitution at a particular locus, or location, in the genome. The specific aims of our genomic studies were to identify genetic variants (single nucleotide polymorphisms) that are associated with spontaneous preterm labour using two different approaches:

1. Genome-wide association studies in collaboration with the Preterm Birth Genome Project
2. Candidate gene studies

The genomic studies are described in detail in Chapter 4 'Genomics and Spontaneous Preterm birth.'

2.1.3 Hypothesis 3: The preterm birth risk is further increased when both the risk genotype and maternal stress are present

Preterm birth is considered a complex phenomenon with various pathways recognized in its etiology. In complex diseases or conditions, there are interactions between an individual's genetic make-up and environmental factors. Our third aim therefore was to explore the possible interactions between genetic variants and chronic maternal stress and spontaneous preterm birth. This is described fully in Section 4.5. 'Gene-environment interactions.'

2.2 Study Design

This was a retrospective case-control study based in Edmonton, Alberta. Cases were defined as mothers with a preterm birth (<37 weeks of gestation) of a singleton as a result of spontaneous idiopathic preterm labour with intact membranes. Women with uncomplicated preterm caesarean sections were included when contractions had started spontaneously. Controls were mothers with either a spontaneous uncomplicated birth of a singleton at 38 to 41 weeks of gestation or an (elective) uncomplicated caesarean section between 38 and 41 gestational weeks. We excluded women with a delivery between 37 and 38 weeks. We made this decision because we wanted a clean separation between cases and controls. Also, due

to inaccuracies of gestational age calculations (+/- 7 days) from early ultrasound scans, a gestation of 38 weeks was chosen as the lower limit of control deliveries. Since we only wanted to include 'true controls,' women with a history of preterm delivery were excluded in the control group as well. Exclusion criteria for all subjects are listed in TABLE 1.

Multiple gestation (twins, triplets)	Minor and major fetal malformations
PPROM	HIV or AIDS
Pre-eclampsia	H1N1
Placental abruption	Cancer (current)
Uterine malformations	Non-English speaking
Delivery between 37 and 38 weeks	History of preterm birth *(controls only)*

TABLE 1. EXCLUSION CRITERIA

The study was approved by the Human Research Ethical Board (HREB) of the University of Alberta. Both ethical and administrative approvals were obtained from Alberta Health Services (formerly Capital Health) for the Royal Alexandra Hospital, and Covenant Health (formerly Caritas Health Group) for the Grey Nuns Hospital and Misericordia Hospital. Between January 2009 and August 2010, four trained research nurses screened women who delivered in one of the three Edmonton hospitals for eligibility. In addition, women who had their newborn infant taken to the

Neonatal Intensive Care Unit in Edmonton from other cities in Alberta and the Northwest Territories were also screened for possible enrolment. Written informed consent was obtained from all subjects before participating in the study.

2.3 COLLECTION OF DNA

Directly after enrolment, a saliva sample was taken using the Oragene DNA Self Collection Kit (DNA Genotek) for DNA analysis. The choice for this method of DNA collection was made for the following reasons:

- It is an easy collection method for DNA,
- Saliva is very accessible,
- Collecting DNA via saliva pots is a completely non-invasive method and therefore patient friendly,
- The DNA collected is of high quality and yield and comparable to DNA collected via blood samples,[134]
- Non-processed saliva pots can be stored at room temperature for years while DNA remains stable,[135]
- It provides a reliable method for many downstream applications, including genome-wide association studies (GWAS) (Section 4.3),[136]

- The kit is a 'All-in-one' system for collection, transport, storage and purification of DNA,
- Saliva pots are a cost-effective method for DNA collection as there is no need for specialised phlebotomists, and
- Other non-invasive methods, such as buccal swabs, provide significant lower DNA yields and lower DNA quality and were also found to be unsuitable for GWAS in our validation study (Section 4.3).[137]

In summary, the saliva pots provide an easy, non-invasive method for the collection and processing of high quality DNA.

After collection, saliva samples were stored at room temperature for up to two years until further DNA analysis. DNA collection, extraction and analysis are described in detail in Chapter 4.

2.4 COLLECTION OF MEDICAL AND DEMOGRAPHIC DATA

2.4.1 ENROLMENT DATASHEET

During the postpartum enrolment procedure, the research nurses filled out a data sheet for all participating study subjects. Patients were given a subject number and several demographic parameters were obtained for early assessment. These parameters included collection date, maternal age

and ethnicity, parity, gestational age and delivery method, fetal sex, birth weight and Apgar scores. Also noted, when possible, was maternal height, pre-pregnant weight, smoking status, alcohol and drug use, medications and any important information from the obstetrical history. In addition, the subject's telephone number was recorded for later follow-up.

2.4.2 MEDICAL CHART EXTRACTION

Participants in the study had given informed consent to have their medical records assessed. Medical charts were extracted manually by four trained research assistants and entered into a password-protected online database, developed by an academically trained computer scientist and software engineer. This database was populated using a web-based chart extraction form. The form allowed for multi-user, collaborative addition of subject medical data and automatic validation of input parameters. Since the database was stored in and accessed from a central server, no software needed to be installed on personal computers and the database could be accessed from anywhere provided that a user had been granted access. We opted for a secure online server, as the medical charts were located at four different hospital sites and multi-user access at the same time needed to be possible with all access-granted users having private login credentials.

Given the complex etiology of preterm birth, it was essential to collect sufficient phenotype information for all retrospective (genetic) epidemiology studies of preterm birth. Pennell *et al.* described the use of a minimum and optimal dataset for genetic epidemiology studies into preterm birth.[138] Our medical chart extraction form followed the guidelines for the optimal data set, containing key medical variables and risk factors for preterm birth within six categories:

1. Maternal history
2. Medical history
3. Pregnancy
4. Complications during pregnancy
5. Labour and delivery
6. Fetal data

The full medical extraction form is shown in APPENDIX 2. In short, maternal data included maternal age, parity, height and pre-pregnant weight for body-mass index (BMI) calculation, smoking and alcohol and drug use. A history of uterine malformations, cervical procedures, medication use and pre-existing medical conditions, such as hypertension, diabetes mellitus and autoimmune diseases, were recorded. Further, we extracted information regarding mode of conception, gestational age determination, blood pressure (both early gestation and at term), cervical cerclage and any medication use

throughout pregnancy. We also extracted data concerning common complications during pregnancy, including genital tract infections and sexually transmitted infections (STIs), hypertension, gestational diabetes, polyhydramnios and placental complications. Labour and delivery records were extracted for data regarding gestational age at delivery, type of labour and mode of delivery, medication, evidence of maternal infection and placental histopathology. Last, extracted fetal data included sex of the infant, Apgar scores, cord pH, congenital malformations and the evidence of infection in the first 48 hours.

Data were stored in the database and subsequently downloaded into spreadsheets for analysis.

2.4.3 COLLECTION OF OTHER DEMOGRAPHIC AND ENVIRONMENTAL DATA

Not all desired demographic variables could be obtained from the medical chart extractions, as medical records were often incomplete. Between three months and one year postpartum, we attempted to contact all participating subjects by telephone where possible for follow-up and administration of the 'Well-being and Pregnancy Questionnaire.' At least three attempts were made for each subject. Details regarding this questionnaire and its development are described fully in Chapter 3.

During the follow-up telephone interviews, various demographic and environmental variables were also obtained and entered into the web-based form. The self-report variables were:

1. Maternal age
2. Height and pre-pregnancy weight
3. Ethnicity (Caucasian/African American/Hispanic/Asian/Aboriginal)
    a. Mother
    b. Father
    c. Maternal grandparents
    d. Paternal grandparents
4. Determinants of socio-economic status
    a. Marital status
    b. Neighbourhood
    c. Educational level
    d. Annual income of the household
    e. Occupation
5. Substance use
6. Self-report medical and obstetric history
7. Previous preterm births and/or miscarriages
8. Family history of preterm birth

These socio-demographic data were collected following the aforementioned guidelines for an optimal dataset and given their importance in the etiology of spontaneous preterm birth. As described in Chapter 1, there is extensive literature on the association between socio-economic status, substance use, pre-pregnancy BMI and preterm birth.[50,52-56] Further, it was essential to collect data on ethnicity, previous preterm births and a family history of preterm birth, as they are strongly correlated with an increased risk of preterm birth (see Chapter 4). Ethnicity was reported by self-identification back to three generations from both the maternal and paternal side where possible. Data were subsequently stored in the online database and downloaded into spreadsheets for analysis.

2.4.4 Analysis of demographic and medical variables

All data were analyzed using SPSS 19.0 statistical software. Before analysis, the data set was cleaned and data from different sources were merged. Data were coded or recoded for analysis when required. Demographic and medical variables were compared between case and control subjects. For this univariate analysis, variables were compared using chi-square or binominal logistic regression, and odds ratios (OR) and 95 percent intervals (95% CI) were given. A p-value < 0.05 was considered significant.

2.5 RESULTS OF DEMOGRAPHIC AND MEDICAL DATA ANALYSIS

2.5.1 STUDY POPULATION

In total, 680 women were recruited into the study. 439 Subjects had a term delivery and 241 women delivered their infant prematurely. Of these women, 58 subjects (27 controls and 31 cases) were excluded from the study after examining the medical and demographic data. The reasons for exclusions and the number of participants excluded per exclusion criterion are summarized in TABLE 2.

Exclusion criterion	**Number of study subjects**
Uterine malformation	2
Delivery between 37 and 38 gestational weeks	18
No knowledge of English	1
PPROM	6
Placental Abruption	1
Within control group:	
History of preterm birth in controls	7
Within case group:	
No spontaneous preterm labour	23
Total	**58**

TABLE 2. SECONDARY EXCLUSION AFTER INFORMED CONSENT.

As a result, a total of 622 women were included in the study. The case group consisted of 88 subjects with a delivery at less than 34 weeks of

gestation and 122 subjects with a preterm delivery between 34 and 37 gestational weeks, comprising a total of 210 cases that fit the inclusion criteria. Our final control group consisted of 412 participants.

2.5.2 DEMOGRAPHICS

All socio-demographic and medical variables were compared between the case and control group and their possible relationship with spontaneous preterm birth was assessed. TABLE 3 shows the main socio-demographic characteristics of our study population.

Characteristic	**Cases N=210**	**Controls N=412**	**OR[1]**	**95% CI**	***p***
Maternal age, yr[2]	28.3 ± 5.6	29.6 ± 5.2	0.96	0.93-0.99	0.004
Caucasian, n (%)	177 (84)	341 (83)	1.12	0.71-1.75	0.63
Smoking, n (%)	62 (30)	69 (17)	2.08	1.41-3.09	<0.001
Alcohol, n (%)	12 (6)	7 (2)	3.51	1.36-9.04	0.009
Street drugs, n (%)	15 (7)	8 (2)	3.89	1.12-9.32	0.002
Educational status					0.008[#]
High school diploma or less, n (% of known status)	34 (45)	37 (25)	Reference		
Undergraduate degree, n (% of known status)	35 (46)	99 (66)	0.39[$]	0.21-0.70	0.002
Graduate degree, n (% of known status)	7 (9)	14 (9)	0.54[$]	0.19-1.51	0.24

Characteristic	**Cases N=210**	**Controls N=412**	**OR[1]**	**95% CI**	***p***
Marital status					0.43[#]
Pre-pregnant BMI[2]	26 ± 6.7	26 ± 6.2	1.00	0.97-1.03	0.93
Parity	0.78 ± 1	0.68 ± 0.89	1.12	0.93-1.33	0.21
Previous miscarriage, n (%)	68 (32)	96 (23)	1.58	1.09-2.28	0.015
ART, n (%)	6 (3%)	13 (3%)	0.79	0.30-2.09	0.63
Gestational age, wks[2]	33.7 ± 2.5	39.7 ± 1.0			<0.001
Birth weight, g[2]	2269 ± 584	3531 ± 461			<0.001

TABLE 3. MAIN DEMOGRAPHIC CHARACTERISTICS OF THE STUDY POPULATION.

Variables were analyzed using Chi-squared test or univariate logistic regression. [1]Odds ratio for spontaneous preterm birth; [2]Mean ± standard deviation; [#]Analyzed as continuous variable; [$]Compared to reference group.

Naturally, gestational age and birth weight were significantly different between cases and controls (p<0.001). Maternal age – on a continuous scale – was significantly associated with spontaneous preterm birth (OR 0.96; 95% CI 0.93-0.99). Overall, mothers in our case group were younger than controls (mean age 28.3 years versus 29.6 years). Not surprisingly, substance use was also associated with spontaneous preterm birth. The odds ratios of smoking, alcohol use and street drug use were 2.08 (1.41-3.09), 3.51 (1.36-9.04), and 3.89 (1.12-9.32) respectively. Thirty percent of women with a preterm birth

smoked during their pregnancy. In our control group, only 17 percent of the women smoked. The use of alcohol and street drugs, in our study mainly marihuana, was also more present in cases than controls. In addition, educational status had a significant relationship with preterm birth in our population. In the case group, 45 percent of the women achieved a high school diploma or less, compared to 25 percent in controls. In other words, of the women in the control group, 75 percent completed education beyond high school, whereas as only 55 percent of the women in the case group completed (under)graduate education. Other factors of socio-economic status, such as marital status and income, were not different between cases and controls. The percentage of Caucasian ethnicity, as reported by self-identification back to three generations, was 84 percent in cases and 83 percent in controls. Other ethnicities reported were Asian, African (or Black), Hispanic and Aboriginal. None of these ethnicities were significantly different (SUPPLEMENTARY TABLE 1). Mean pre-pregnant BMI was 26 kg/m^2 in both groups, and also parity and the use of assisted reproductive technologies were similar in both groups. Notably, a history of one or more spontaneous abortions in previous pregnancies was significantly associated with preterm birth (OR 1.58; 95% CI 1.09-2.28). Most medical variables were not associated with spontaneous preterm birth in our study population. Several medical variables, although only present in very small numbers, were differently distributed between both study groups. These included pre-

existing hypertension, polyhydramnios and intrauterine growth restriction (IUGR). The complete demographic and medical characteristics of the study population are listed in SUPPLEMENTARY TABLE 1.

2.6 DISCUSSION

Over a time span of 20 months, we recruited 622 women into our study, of whom 210 delivered their infants preterm and 412 had a term delivery. Univariate analyses of all socio-demographic and medical variables demonstrated that in our study population, maternal age, smoking, alcohol use, street drug use, educational status and a previous miscarriage were significantly associated with preterm birth. All of these factors were known to be risk factors for preterm birth and it was therefore no surprise that they turned out to be significant in our study. Mothers in our case group were slightly younger than controls and this difference was statistically significant. However, this was only the case overall and not in the high-risk groups. Literature has shown that both young maternal age (<20 years) and advanced maternal age (>35 years) are associated with preterm birth.[37,38,45,46,139] In our study, women of young maternal age had an almost two-fold increased risk of preterm birth compared to women between 20 and 34 years of age, however this did not reach statistical significance, likely due to the small sample size in this age group.

Smoking doubled the risk of spontaneous preterm birth in our study. Not only does smoking increase the risk of preterm birth, the literature suggests that smokers also have higher rates of other pregnancy complications including hypertension, diabetes, haemorrhage and placental abruption.[52,140-142] Of the women that smoked at the start of pregnancy (N=131), only ten women quit during pregnancy. The average cigarette consumption per week during pregnancy was 46 cigarettes and the majority of study subjects had reduced their weekly cigarette consumption during pregnancy. The percentage of women smoking in our case group was considerably higher at 30 percent, especially when compared to the general population. According to the Canadian Tobacco Use Monitoring Survey of Health Canada, the smoking rate among Canadians of 15 years and older was 17% in 2010, with the highest rate in the age group 20-24 years at 22 percent.[143] In Alberta specifically, the prevalence of smoking among all people above 15 years was 19 percent. This rate was roughly the same in all age groups. The smoking rate in cases in our study population was at least 10 percent higher than the Albertan smoking rate. An explanation for this occurrence could be the close relationship of smoking with low socio-economic status. One of the measures of SES is education. Indeed, in our study the number of women with low educational status – no completion of a degree beyond high school – was significantly higher in women with a preterm birth compared to women with a term delivery (45 and 25 percent

respectively). In the general Canadian population, the percentage of people with the highest completed education being a high school diploma or less was 39 percent, according to Census data of 2006.[144] Educational attainment rates in Alberta were on par with the national averages, with 39 percent of the Albertan population having a high school diploma or less as the highest completed education. Compared to the general population, subjects in our case group had a higher prevalence of low educational status, whereas among controls there were a considerably lower percentage of women with a high school diploma or less. These data correlated perfectly with the smoking rates in our study population. Education is both linked to occupation and income, the other measures of SES.[145] These measures however, were found not to be significantly different between cases and controls. The rates of alcohol use and street drug use were also significantly higher in the preterm group. The street drug of 'preference' was mainly marihuana, though three women also used (crack) cocaine. Alcohol and drug use during pregnancy increased the risk of preterm birth a significant 3.5 and almost 4 fold respectively. Again, this related perfectly with high smoking rates and low educational status, i.e. low socio-economical status.

The mean pre-pregnant BMI was 26 kg/m^2 in both the case and control group, meaning that on average, the study population was overweight.[146] According to the latest Statistics Canada data, the average BMI

in the Canadian population aged 20 to 39 in 2009 was 26.2 kg/m^2, demonstrating that body composition in our study population was comparable to the general population.[147]

It is well established that previous spontaneous abortions, defined as spontaneous pregnancy loss before 20 weeks of gestation,[148] are associated with adverse pregnancy outcomes in subsequent pregnancies.[149-152] A history of miscarriage is a significant risk factor for preterm birth. Indeed, we found that in our study, the proportion of women with a previous miscarriage was significantly higher in the case group. A history of spontaneous abortion was associated with an odds ratio of 1.58 (95% CI 1.09-2.28) on the risk of preterm birth. The proportion of women with one or more spontaneous abortions was relatively high in both our case and control group (32 and 23 percent, respectively), as the incidence of spontaneous abortion is estimated at 10 to 15 percent of all pregnancies.[153] One possible explanation is that our data were collected retrospectively introducing selection bias. Most subjects were collected from tertiary referral centres, which could have influenced the prevalence of miscarriage as it is known to be associated with adverse outcomes in subsequent pregnancies and thus the need exists for referral to a tertiary hospital. Unfortunately, we were unable to stratify between first and second trimester loss, due to unavailability of the data. Most early pregnancy losses occur before 12 weeks of gestation as a result of implantation defects

or fetal anomalies.[150,151] Other possible pathways leading to miscarriage include maternal immunologic abnormalities, uterine malformations and incompetent cervix. These pathological mechanisms are also associated with preterm birth and can explain, at least in part, the association between previous miscarriage and spontaneous preterm birth.

Established medical risk factors for preterm birth such as the use of assisted reproductive technologies, cervical procedures, diabetes, sexually transmitted infections, and pregnancy-induced hypertension, were not associated with preterm birth. We expect this was simply due to the fact that the number of women with these risk factors were too low in our study. In contrast, several medical conditions were more present in the case group compared to the control group. Not unexpectedly, these included pre-existing hypertension, placenta praevia, IUGR, and polyhydramnios. Yet the number of women with these conditions was too low, i.e. a count of less than five in one or both groups, to draw any definite conclusions regarding their possible significant relationship with preterm birth in our study. These factors were therefore not incorporated as covariates in subsequent modelling.

Delivery records showed that all babies in our study population were born alive and fetal sex was balanced between cases and controls. Interestingly, several studies previously have shown that the proportion of males among preterm births is greater than among term births and that male

fetuses are more susceptible to preterm labour.[154-156] We did not find such a relationship in our study, as the proportion of male infants was 53 percent in both groups. Gestational age and birth weight were obviously significantly lower in the case group, and as a result, Apgar scores at both 1 and 5 minutes postpartum were also significantly lower in preterm infants.

Placenta pathology was performed for 110 cases, but only for 33 controls. Evidence of placental infection was found in 42 preterm placentas, that is 38 percent of placentas sent for pathology and 20 percent of all preterm births in the study. As described in Chapter 1, parturition – both at term and preterm – is largely an inflammatory response. Intrauterine infection and inflammation are associated with preterm birth. Research has shown that up to 40 percent of preterm births have positive cultures showing evidence of intrauterine infection that is mainly subclinical.[157] In controls, evidence of placental infection was found in only 12 women meaning that in 36 percent of the circumstances a term placenta was sent for pathology, a positive histology was found. Again, this suggested that inflammation plays a role in both term and preterm parturition.

As mentioned in TABLE 1, we incorporated several exclusion criteria. These included causes of preterm birth, such as PPROM, placental abruption, multifetal gestation, pre-eclampsia, fetal malformations and uterine malformations. Our primary outcome was spontaneous, idiopathic preterm

birth, and therefore women with any of these other possible causes had to be excluded from the study. In addition, women with one of the following serious illnesses were excluded: HIV or AIDS, current cancer and H1N1. In 2009, there was an H1N1 influenza pandemic worldwide. At the time, not much was known about the risk of H1N1 on pregnancy and preterm birth. We therefore decided to incorporate H1N1 as one of our exclusion criteria. Several reports have now demonstrated that infection with H1N1 during pregnancy was associated with high rates of preterm birth up to 63 percent in women with severe illness.[158] A published systematic review of the literature showed that preterm birth rates after H1N1 infection were approximately 30 percent.[159] This is much higher than the baseline preterm prevalence of 9.6 percent.[19]

In summary, maternal age, smoking, alcohol use, street drug use, educational status and a history of spontaneous abortion were significantly associated with spontaneous preterm birth. These variables were therefore incorporated in the multivariate statistical models that were used in our maternal stress studies (Chapter 3) and genomic studies (Chapter 4).

2.7 DATA INTEGRITY

This study complied fully with the research policies set by the Tri-Council, the University of Alberta and its Human Ethics and Research Board

(HREB). In addition, approvals were obtained from Alberta Health Services and Covenant Health. We ensured that the privacy and confidentiality of all participants was maintained at all times in compliance with the Freedom of Information and Protection of Privacy Act (FOIPP Act) and the Health Information Act. For all study subjects, written consent was obtained and participants have the right to withdraw from the study at any time. We did not in any way collect, use or disclose personal or health information that was not in agreement with the FOIPP Act or the Health Information Act. No persons other than the principal researchers and research assistants directly involved in the study had access to the data. All researchers in the research team had signed confidentiality agreements conforming the University of Alberta standards of data privacy and confidentiality.

Upon enrolment, all study participants were given a coded study number. Saliva specimens and subsequently extracted DNA samples were only identified by this study number. Aliquots of DNA samples were kept frozen in the laboratory. In addition, DNA aliquots were sent to Vanderbilt University in Nashville, TN and the University of Western Australia in Perth, Australia for genotyping (Chapter 4). Again, these samples were only identified by subject number. DNA samples are stored for five years according to HREB regulation. These samples were only used for the purpose

of this study. There will be no secondary use of the DNA without additional consent of the study participants.

The raw data that were collected contained directly and indirectly identifiable information, including name, hospital number, date of birth, and telephone number. This was needed to contact study subjects at home for follow-up and stress assessment via telephone questionnaire in the post partum period, and to link medical chart data with questionnaire data. Consent forms and collection sheets containing directly identifying information were securely stored in a locked file cabinet in the main research office and only the principal investigators had access to these documents.

The database created for the medical chart extractions and telephone questionnaires was stored on a central server with a password-protected web-based interface (a website) to edit the data. The website was only available to the principal researchers and research assistants who each have their own login credentials. For added security, and to prevent accidental or malicious copying of the data, the website would show only a single section of the extraction form for a single patient at a time, i.e., the users were not able to see complete charts for a single or multiple patients; instead they had to complete a section before moving to the next one. Each research assistant was able to access only the data (charts) of patients he/she entered into the database. Only the principal researchers had the ability to download a

complete database onto their personal computers and thus see the work of all of the assistants. Data were downloaded into spreadsheets for analysis. Prior to analysis, all personal identifiers were removed and subjects could only be identified by their specific subject number.

After the study was completed, the website together with the database was removed from the central server. An encrypted copy of the data is stored on an external hard disk in the laboratory for five years according to HREB policies. Only the principal researchers have access to the data. Only if law requires it, will funding agencies and other agencies be given access to the data.

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3. CHRONIC MATERNAL STRESS AND SPONTANEOUS PRETERM BIRTH

3.1 BACKGROUND

Stress is a word that is heard everywhere and every day. Yet, it is a very ambiguous term as it means something different to everyone. It is often used in a negative sense, but is stress always bad for you? In general terms, stress can be defined as any challenge – psychological or physical – that threatens or that is perceived to threaten homeostasis.[29] It encompasses both environmental demands and cognitive and emotional responses, such as stress perception, to those demands. Over the years, scientists have developed various biological and psychological concepts to define stress and the stress response.

3.1.1 EARLY PIONEERS – CANNON AND SELYE

Walter Cannon (1871-1945) developed the concept of homeostasis, based on the *milieu interieur* (Claude Bernard) and he used the following concept in defining homeostasis: physiological reactions are coordinated to maintain a steady state or equilibrium in the body.[160,161] This is required to sustain life. Homeostasis includes the regulation of body temperature, pH,

blood glucose and oxygen tension. Cannon stressed the importance of the autonomic nervous system as a homeostatic control mechanism. Hans Selye (1907-1982) was an endocrinologist and a pioneer in the field of biological stress. He introduced the well-known 'General Adaptation Syndrome' (GAS) model in 1936, after endocrinological experiments in mice. The general adaptation syndrome described the physiological adaptive reactions in the body in response to a stressor:[162,163]

1. Alarm reaction – 'Fight or flight' response: initial reaction stage in the body with activation of the autonomous nervous system and hormonal systems to prepare for 'action'
2. Stage of resistance: adaptation stage in which the body actively copes with the stressor and attempts to return to a homeostatic state
3. Stage of exhaustion: when stress persists beyond the capability of the body to cope, the body becomes exhausted resulting in (permanent) damage to internal organs and increased susceptibility to disease

According to Selye, GAS was largely dependent on the function of the autonomic nervous system. In his model, a stressor – whether an injury, damaging agent or psychological – always influenced certain tissues directly, followed by systemic damage and defense due to nervous and hormonal

mediation. In his search to identify the hormonal mediators of GAS, Selye was the first describe the role of the hypothalamic-pituitary-adrenocortical axis, or HPA axis, in response to a stressor.[163]

3.1.2 THE HPA AXIS

Any type of physical or mental stress can elicit a rapid and greatly enhanced secretion of the stress hormone, cortisol. The physiologic stress response involves the autonomic nervous system, in particular the sympathetic-adrenal-medullary (SAM) axis and the HPA axis.[164,165] Both systems originate in the brain. The hypothalamus, located in the middle of the base of the brain, is the 'control center' of the neuroendocrine systems in the body and plays a vital role in maintaining homeostasis. The paraventricular nucleus of the hypothalamus contains neuroendocrine neurons that synthesize and secrete corticotropin-releasing hormone (CRH).[94] CRH release from the hypothalamus is under nervous control and influenced by cortisol levels in the blood – mainly inhibitory - and by stress. In addition, CRH mRNA and cortisol levels are under circadian influence.[166] After secretion, CRH is transported through the portal blood vessel system of the pituitary stalk to the anterior pituitary gland, where it binds to CRHR1 receptors on the corticotrope cells. This stimulates the anterior pituitary to secrete adrenocorticotropic hormone (ACTH) stored in corticotrope cells. ACTH is then released into the blood stream and transported to the adrenal

cortex of the adrenal glands. ACTH stimulates the synthesis of glucocorticoids, mainly cortisol, from cholesterol via activation of adenylyl cyclase and formation of cAMP in the zona fasciculata of the adrenal cortex.[167]

Cortisol, or hydrocortisone, is a steroid hormone and acts on the glucocorticoid receptor (GR) and the mineralocorticoid receptor (MR).[168] The affinity of cortisol for the MR is much greater than for GR, and binding of cortisol to MR is maintained at basal levels while the GR becomes activated in response to a stressor. The GR is present in almost every cell in the body.[169] When bound to the GR, cortisol has important effects on carbohydrate metabolism, protein metabolism, fat metabolism, and on stress and inflammatory responses.[164] Cortisol stimulates gluconeogenesis and glycogenesis in the liver while reducing the utilization of glucose by cells, resulting in increased blood glucose level in the body. It has catabolic effects, such as proteolysis and lipolysis, in tissues. The resulting mobilization of amino acids and fatty acids, together with higher blood glucose levels, can be considered as adaptive processes to provide energy substrates for the body, in particular the heart, brain and skeletal muscles, to cope with demands. Cortisol also exhibits anti-inflammatory effects. It can suppress leukocyte proliferation and migration, and it can inhibit the synthesis of pro-inflammatory cytokines resulting in a suppressed inflammatory response.[89,170] Circulating cortisol inhibits the secretion of ACTH and CRH. It

exerts its negative feedback effect both at the level of the anterior pituitary gland and the hypothalamus. A schematic overview of the HPA axis is illustrated in FIGURE 6.

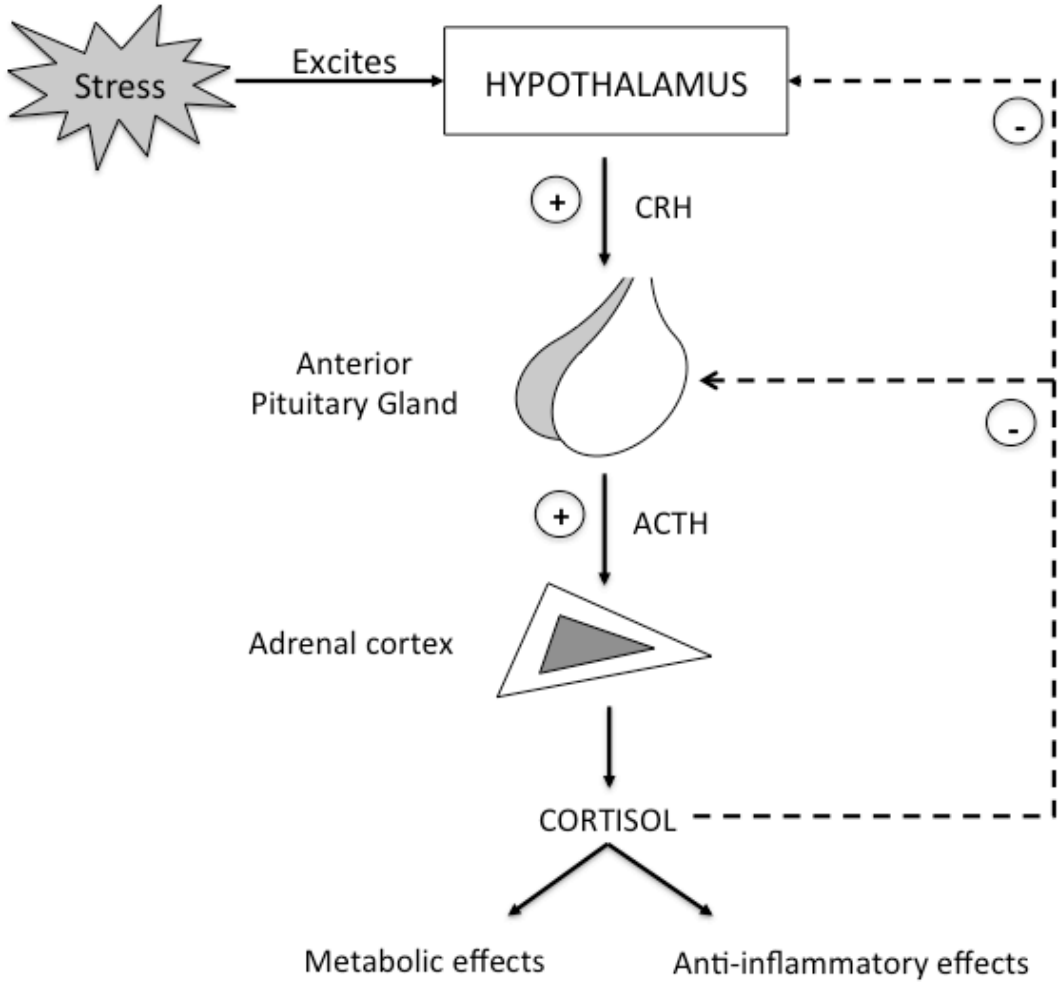

FIGURE 6. OVERVIEW OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS.

In chronic or repetitive stress, the HPA axis is repetitively activated. This in turn can cause wear and tear on the body. The effects of chronic stress are described in Section 3.1.4.

The autonomic nervous system via the SAM axis also plays a vital role in the stress response. The catecholamines, epinephrine and, in much lesser degree, norepinephrine, are produced by the adrenal medulla through sympathetic stimulation and indirectly through cortisol. Epinephrine is especially important in acute or emergency situations when the sympathetic nervous system is activated – the fight or flight response. Circulating (nor)epinephrine causes vasoconstriction in essentially all blood vessels and stimulates the heart. In addition, epinephrine has a metabolic effect by increasing both plasma glucose and plasma fatty acid concentrations due to enhanced utilization of fat during acute stress.

Some studies have found sex differences in HPA axis responses to psychological stress. The evidence of HPA response with respect to gender is, however, mixed. Cortisol responses seem to be higher in men compared to women.[171] Men also have higher ACTH levels in response to stressful situations.[172] In contrast, Seeman *et al.* reported increased ACTH and cortisol levels in elderly women compared to men, while a recent study from Hatzinger *et al.* did not find any gender differences in HPA responses at all.[173,174]

3.1.3 COGNITIVE APPRAISAL AND COPING

Whereas Cannon and Selye mainly measured the physiological responses to external stressors, it was later thought that external stressors are mediated by the perception of the individual. Therefore Lazarus and Cohen defined a biobehavioural model of the stress response.[175] They stated that when 'environmental demands, such as chronic stressors, exceed the adaptive capacity of an organism this can result in psychological and biological changes that may place persons at risk of disease.' Adding to the complexity of their model, they argued that cognitive appraisal plays a central role.[176,177] Each individual evaluates whether a particular encounter with the environment is relevant to his or her well-being and whether it will be perceived as stressful. In addition, individual coping skills – cognitive and behavioural efforts to manage the internal and external demands – and modifiers of the stress response, such as social support, are important elements in the stress response. For instance, someone with an extensive and adequate support network may well appraise stressors more optimistically or use adaptive coping styles to solve a problem since friends and family are available for support. In contrast, people in an abusive environment may have a more negative outlook on the world and this affects their cognitive appraisal of stressors. Adequate social support, adaptive coping skills, resilience and optimism are all moderators of stress and can be seen as

'protective,' resulting in decreasing perceived stress and better health outcomes. Conversely, maladaptive coping styles and risky behaviour and lifestyle, such as smoking and alcohol use, may reduce stress for many people, however they can be harmful and lead to negative health outcomes. Overall, men and women show different coping styles. Where men use relatively more problem-focused coping, women tend to focus more on emotional coping and seeking social support.[178,179]

3.1.4 ALLOSTASIS AND ALLOSTATIC LOAD

In the late 1980s, a new term was introduced in the stress literature as an alternative to the homeostatic model: allostasis. Originally proposed by Sterling and Eyer, allostasis is the adaptive processes for actively maintaining stability through change.[180] Allostasis is derived from the Greek 'allo,' meaning 'variable,' while 'stasis' means 'stand.' Therefore, allostasis means 'stable by being variable' and it is a fundamental process supporting homeostasis through which the body can adjust to stressors. Allostasis – extensively described by McEwen – is achieved through the production of mediators of the stress response such as adrenal hormones, inflammatory cytokines and neurotransmitters that help us adapt to new situations and challenges.[181-184] The brain plays a central role, by controlling various mechanisms simultaneously. In acute situations, allostasis is beneficial for the body, as it is essential for the body to respond and adapt to stressors. As a

result, an effective solution to the threat is achieved. However, when allostasis is prolonged – in chronic or repetitive stress – the autonomic nervous system and HPA axis are repetitively activated and the neuroendocrine and inflammatory adaptive processes now become damaging to the body. For this, McEwen coined the term 'allostatic load' and this is defined as the cumulative results of allostasis. In other words, it comprises the 'wear and tear' of allostasis over a lifetime on the body and the brain. In chronic stress, the allostatic load increases as the body attempts to cope with stressors. Thus, the main hormonal mediators cortisol and epinephrine, that normally maintain homeostasis, now have a negative effect on the body, resulting in the acceleration of disease processes such as cardiovascular disease. In addition, allostatic load over a long period of time might cause the allostatic systems to be come exhausted leading to dysregulation of the HPA axis and compensatory responses in other systems. Chronic stress can therefore result in an increase in inflammatory cells and cytokines and increased susceptibility to infection and inflammation.[170,182,185] The development of allostatic load is illustrated in FIGURE 7.

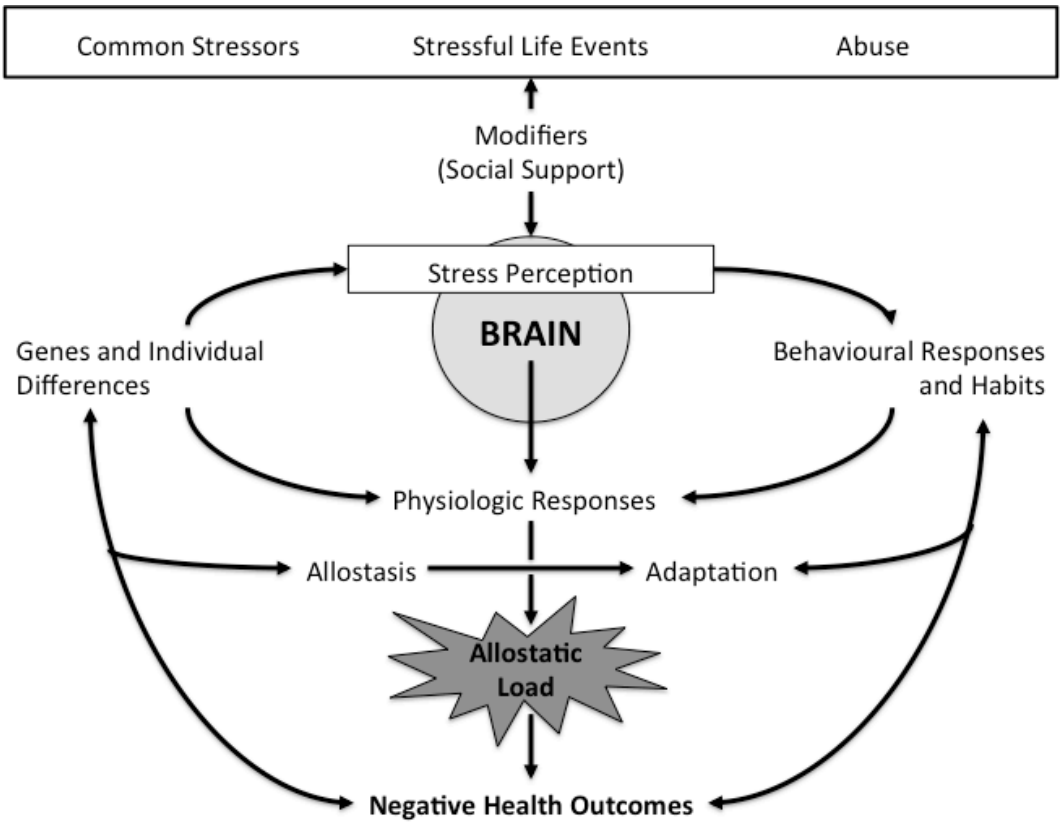

FIGURE 7. DEVELOPMENT OF ALLOSTATIC LOAD.

Adapted from McEwen.[182]

3.1.5 STRESS AND PRETERM BIRTH

The framework of allostatic load can certainly be applied to the etiology of preterm birth. As mentioned in Chapter 1, the HPA axis plays a central role in pregnancy. During pregnancy, CRH is not only secreted by the hypothalamus, but it is expressed in increasing amounts in the placenta, fetal membranes and myometrium throughout gestation.[97] Whereas cortisol normally inhibits hypothalamic CRH secretion, in pregnancy a positive

feedback loop exists: both maternal and fetal cortisol stimulate the production of placental CRH resulting in increasing levels of placental CRH in the maternal circulation.[100,101] In addition, other mediators of the stress response, including ACTH and catecholamines can also stimulate CRH production in the placenta.[186] This rise in CRH levels has been associated with the length of gestation and the timing of birth, the so-called 'placental clock.'[98] Increased perceived stress during pregnancy and higher levels of maternal CRH are associated with preterm birth.[102-104] In addition, one study found that perceived stress levels in early pregnancy could predict CRH levels later in pregnancy.[99] Thus, maternal stress may prematurely activate the physiologic mechanisms leading to preterm labour.

Inflammation and infection are the most frequent causes of preterm birth.[107] Consistent with the concept of allostatic load, it is very plausible that chronic stress can increase the risk of preterm birth via the neuroendocrine and inflammatory pathways. Physiologically, glucocorticoids exert anti-inflammatory effects. However, during chronic or repetitive stress, the adaptive systems of the body fail to respond properly.[170,182,185] As a result, the HPA axis may fail to shut down or inadequate responses may trigger other compensatory mechanisms in the body such as the immune system. This can lead to increased production of certain pro-inflammatory cytokines and increased trafficking of leukocytes. Thus, the body is more susceptible to

inflammation and infection. Chronic stress can therefore lead to chronic activation of the inflammatory response.[187]

Many epidemiological studies have also shown that pregnant women who experience high levels of psychosocial stress before or during pregnancy are at significant risk for preterm labour despite their ethnicity and socioeconomic status.[87-90,133,188] Women who experienced major and traumatic life events early in pregnancy were also found to have an increased risk of preterm birth. Examples include Hurricane Katrina,[189] the war in Bosnia-Herzegovina[190] and World Trace Center disaster.[191] Hedegaard *et al.* found that major life events during pregnancy were only associated with preterm when they were perceived to be stressful.[192] Indeed, women who have increased perceptions of stress also have a higher risk of preterm delivery.[91-93] In addition, physically demanding work, prolonged standing, shift and night work, and a high cumulative work fatigue score have been associated with preterm birth.[193,194] Perceived racial discrimination can increase the risk of preterm birth. In Canada, Heaman *et al*. indicated that spontaneous preterm birth among Aboriginal women was related to a high level of perceived stress.[195] Research suggests that physical and emotional abuse or domestic violence prior to or during pregnancy is associated with preterm birth.[196-199] Distressed states such as major depressive disorder and anxiety can play a role in the onset of preterm labour.[91,200] In addition,

depression and anxiety can increase a woman's stress levels. Conversely, high levels of stress can result in the development of depression and anxiety.[165] In posttraumatic stress disorder (PTSD), an anxiety disorder that can develop after exposure to a traumatic event, CRH levels are increased and negative feedback control is suppressed.[201,202] As previously mentioned, low socio-economical status is believed to be an important risk factor for preterm birth.[49,203,204] Socio-economic disadvantage is associated with unhealthy or risky behaviours, including smoking, alcohol abuse and poor eating habits, exposure to stress, and psychological reactions that influence gestation negatively.[204] Indeed, behavioural risk factors, like cigarette smoking, alcohol and drug use, sexually transmitted infections, poor food intake and obesity are all associated with preterm birth. [51-56,112]

Maternal stress is a complex entity with many different environmental and psychosocial components, as is preterm birth. Joint examination of common stressors and individual socioeconomic, psychosocial and behavioural risk factors would provide a better strategy for research and may increase our understanding of the complex causes of preterm delivery.[205-207] Returning to the concept of allostatic load, stressors throughout life can have a significant effect on pregnancy outcomes. A healthy pregnancy therefore starts long before conception.

3.2 RATIONALE AND OBJECTIVE

Maternal stress during pregnancy is increasingly recognized as a variable of interest in the etiology of spontaneous preterm birth, however its contribution to the risk of preterm birth remains controversial. Studies examining the effect of maternal stress during pregnancy on preterm birth have shown varied results, partly due to the fact that they have only explored separate stressors and their relationship with preterm birth. Often, cognitive appraisal of stressors or individual responses were not considered in the studies. Moreover, there is a lack of the use of a comprehensive measure of chronic stress. The concept of allostatic load provides a compelling rationale for the contribution of chronic stress to spontaneous preterm birth. Therefore, examining the exposure to stressors over a mother's life course might give a better perspective on the role of maternal stress in the etiology of spontaneous preterm birth.

The objective was to retrospectively explore the associations between chronic, lifelong stressors and protective factors and spontaneous preterm birth in our case-control study. We hypothesized that the stress scores would be higher in our case group compared to controls, meaning that higher levels of chronic maternal stress increase the risk of spontaneous preterm birth.

3.3 COLLECTION OF STRESS DATA

3.3.1 DEVELOPMENT OF THE WELL-BEING AND PREGNANCY QUESTIONNAIRE

For the assessment of chronic, lifelong stressors, we have designed the 'Well-being and Pregnancy Questionnaire' (Appendix 3). Using this questionnaire, both individual and contextual variables that influence the stress response were examined for all subjects. It incorporated several checklists designed for this study and validated research instruments to measure concepts related to stress and personal resources. They included perceived stress, common stressors during pregnancy, social support, life events, coping, childhood adverse experiences, adult abuse and depression. Instruments were chosen after review of the literature and based on their possible direct and/or indirect association with spontaneous preterm birth. Where possible, we used validated tools that are available in the public domain. The instruments that were incorporated in the questionnaire are described below. The complete 'Well-being and Pregnancy Questionnaire' can be found in Appendix 3.

3.3.1.1 Perceived Stress

The first instrument measured a global level of perceived stress before and during pregnancy. Women were asked to indicate how stressed they felt six months prior to their pregnancy – as a reference score –, in their first trimester and in their second trimester. Scores were given on a scale from 0 to 100, where 0 meant that they did not feel any stress and 100 meant that they felt very stressed. This instrument was designed for this study and was loosely based on the validated Perceived Stress Scale.[208]

3.3.1.2 Common Stressors

The second instrument was a checklist designed to assess the presence of common stressors during pregnancy via eleven yes/no questions and is intended to measure stress load during pregnancy. The common stressors assessed included high workload, financial problems, personal conflicts at home and at work, parenting problems, perceived racial discrimination and unfavourable neighbourhood.

3.3.1.3 Interpersonal Support Evaluation List

Strong support networks are associated with a person's well-being.[209] To assess social support, we used the validated Interpersonal Support Evaluation List (ISEL) – Short Form. This is a 15-item measure of perceived

social support.[210] This short version was derived from the 48-item ISEL, designed by Cohen.[211] The scale was made up of a list of statements and women were asked to indicate how true or false that statement is about them *in general*. This could range from completely false to completely true. The three areas assessed were tangible support or material aid, appraisal support or the availability of a confidant, and belonging support or the availability of someone with whom the respondent can socialize or relax. In a study in 2000, Widows *et al*. found adequate internal consistency of the ISEL.[212]

3.3.1.4 Life Events Checklist

The fourth instrument, which assessed historical exposure to stressors, was the Life Events Checklist (Page 1) of the Clinician Administered Posttraumatic Stress Disorder Scale (CAPS 1) (National Center for Posttraumatic Stress Disorder).[213,214] This scale was originally developed by the National Center for Posttraumatic Stress Disorder as part of a diagnostic interview to assess presence of posttraumatic stress disorder diagnostic status and symptom severity. Listed were several stressful life events that have all been shown to increase risk of stress-related disorders. Women were asked to indicate whether these events happened to them personally in their lifetime, whether they witnessed them happen to someone else or whether they do not apply to them. In a review, Weathers *et*

al. found excellent reliability rating and convergent and discriminant validity were also shown to be strong.[215]

3.3.1.5 Coping

Adaptive coping styles are also associated with well-being. The Brief COPE is a 28-item measure shortened from the original 60-item COPE.[216,217] It was used to assess situational reports of coping as well as dispositional coping styles. Women were asked to indicate what they *usually* do and feel when they experience stressful events or times in their life. The different coping strategies assessed were passive/avoidant behaviour, action oriented behaviour, and emotional coping. The Brief COPE scales all met the criteria for internal reliability.[217]

3.3.1.6 Adverse Childhood Experiences

Our sixth tool was the Adverse Childhood Experiences (ACE) Score. This questionnaire investigated the connection between adult health problems, including preterm birth, and adverse childhood experiences. It has long been known that significant life experiences can affect health in later life. Childhood abuse, neglect and dysfunctional household are associated with increased health risks, such as smoking, alcoholism and drug abuse, and many adult diseases, such as cardiovascular disease and depression.[218] The ACE Score is a 10-item measure of yes/no questions to identify childhood

abuse, neglect and household dysfunction. All questions pertained to the respondent's first 18 years of life. The test-retest reliability was found to be in the good range with kappa coefficients between 0.6 and 0.7.[219]

3.3.1.7 Abuse Assessment Screen

The instrument used for the assessment of abuse as an adult was the Abuse Assessment Screen (AAS).[220] The AAS has been widely used to identify abuse during pregnancy in health settings. Women were asked questions about emotional and physical abuse during life and during their pregnancy. Content and criterion validity and test-retest reliability have been established.[221,222]

3.3.1.8 Depression and suicidality

Depression is a contributor to the stress response. It can activate the stress response directly and it can affect a person's cognitive appraisal of stressors.[223] In addition, it can also be an outcome of chronic stress as high levels of stressors can cause the development of depression and anxiety. Depression prior and during pregnancy is associated with preterm birth.[91,200] For the assessment of depression and suicidality, we used sections A and C of the validated Mini International Neuropsychiatric Interview (M.I.N.I.).[224] We slightly modified the scales for our questionnaire and both sections include questions about depressive episodes and thoughts of suicidality both before

and during pregnancy. In addition, the presence of post-partum depression was assessed. Since the questionnaire was administered in the year post-partum, it was essential to control for the effect of postpartum depression as its presence might introduce recall bias and affect stress appraisal.

3.3.2 ADMINISTERING OF THE QUESTIONNAIRE

Between three months and one year postpartum, participating subjects were contacted by telephone where possible for follow-up and administration of the 'Well-being and Pregnancy Questionnaire.' To maximise the number of respondents, we attempted to contact each participant at least three times at different times during days and evenings. The questionnaire was administered during the telephone interview and answers were entered into our secure online database. This database is an extension of the database developed for the medical chart extractions (Chapter 2). Similarly, it allowed for multi-user, collaborative addition of subject questionnaire data and automatic validation of input parameters. Data were subsequently downloaded in spreadsheets to be used for analysis. Screenshots of the questionnaire database are shown in Appendix 4.

3.3.3 ANALYSIS OF STRESS DATA

All questionnaire data were analyzed using SPSS 19.0 statistical software. Before analysis, the data set was cleaned and detected

inconsistencies were replaced, modified or removed. Data was coded or recoded for analysis when required and missing data were indicated. Scores for all questionnaire tools separately were calculated using predefined scoring keys (SUPPLEMENTARY TABLE 2). We also calculated a combined childhood and adult abuse score. For this score, the separate scores of childhood abuse, childhood neglect and adult physical and emotional abuse were added. In addition, a total combined stress score was computed using the following formula:

$$\textbf{\textit{Total Stress}} = (Perceived\ stress\ 6\ months\ prior/2 + Perceived\ stress\ first\ trimester + Perceived\ stress\ second\ trimester)/250 + Common\ Stressors/11 + Life\ Events/17 + Passive-avoidance\ COPE/48 + ACE/10 + AAS + Depression/3 + (1 - ISEL/15) + (1 - Action\ oriented\ COPE/24) + (1 - Emotional\ support\ COPE/40)$$

In this formula, tools that represent stressors are added, while tools that represent modifiers of the stress response – social support and adaptive coping- are subtracted. In addition, the formula is build so that all stressors have the same weight. Univariate analysis was performed on all separate questionnaire tools and the total stress score to assess the relationship with spontaneous preterm birth. Some, but not all, scores were also dichotomised

based on their median split and subsequently analysed. Variables were compared using binominal logistic regression and odds ratios (OR) and 95 percent intervals (95% CI) were given. A p-value < 0.05 was considered significant. Last of all, multivariate logistic regression was performed. A multivariate model was created including those demographic variables that were significantly different between cases and controls in our population. Adjusted ORs and 95% CI were given.

3.4 RESULTS

3.4.1 CALL RATE

In total, 234 telephone questionnaires were administered. However, 11 study subjects that completed the questionnaire were later excluded from the study after secondary exclusion as described in Chapter 2. Reasons for exclusion were the following: uterine malformation (1 respondent), delivery between 37 and 38 gestational weeks (4 respondents), PPROM (1 respondent), placental abruption (1 respondent) history of preterm birth in control (1 respondent) and no spontaneous preterm labour (3 respondents). As a result, 223 completed telephone questionnaires were included in the study. The respondents were 148 controls and 75 cases. The case group could not be stratified into early and late preterm birth, as the numbers were simply too low. Our call rate was 36 percent for controls (148 women out of

412), and 36 percent for cases (75 women out of 210). The respondent rate for the total study population was also 36 percent (223 women out of 622).

3.4.2 UNIVARIATE ANALYSIS

Of all separate questionnaire instruments, only the Adverse Childhood Experiences score was significantly associated with spontaneous preterm birth in univariate analyses (TABLE 4).

Questionnaire Tool	**Crude OR**	**95% Confidence Interval**
Perceived Stress	1.01	1.00-1.02
Common Stressors	1.09	0.92-1.30
ISEL Social Support	0.91	0.78-1.06
Life Events Checklist	1.04	0.91-1.20
COPE Adaptive Coping	1.02	0.97-1.06
ACE Adverse Childhood Events	1.26 *	1.08-1.48
High ACE Score (≥2 ACE) [1]	2.45 *	1.37-4.38
Abuse Assessment Screen	1.75	0.96-3.20
Childhood and Adult Abuse	1.40 *	1.13-1.74
Depression during pregnancy	1.53 *	1.01-2.33
Lifetime history of depression	1.70	0.90-3.24

Questionnaire Tool	Crude OR	95% Confidence Interval
Total Stress	1.46 *	1.08-1.96
High Stress [1]	1.86 *	1.06-3.28

TABLE 4. UNIVARIATE ANALYSIS OF ALL STRESS QUESTIONNAIRE TOOLS AND COMPUTED TOTAL STRESS SCORE.

[1]Based on median split; *p<0.05.

The crude odds ratio of ACE score on a continuous scale was 1.26 (95% CI 1.08-1.48). We also dichotomized the ACE score into high (≥2 ACEs) versus low ACE, based on median split, showing a crude OR on the risk of preterm birth of 2.45 (95% CI 1.37-4.38). Crude odds ratios for perceived stress, common stressors, ISEL, and COPE were all very close to 1, with ORs of 1.01 (95% CI 1.00-1.02), 1.09 (95% CI 0.92-1.30), 0.91 (95% CI 0.78-1.06), 1.04 (95% CI 0.91-1.20), and 1.02 (95% CI 0.97-1.06) respectively.

Physical and emotional abuse as an adult, assessed with the AAS, on its own was not associated with preterm birth in our study. However, the combined abuse score of childhood and adult abuse was significantly associated with preterm birth (crude OR 1.40; 95% CI 1.13-1.74). We found a significant relationship between the computed Total Stress score and spontaneous preterm birth after univariate logistic regression, showing a crude OR on the risk of preterm birth of 1.46 (95% CI 1.08-1.96). After

dichotomization, a high stress score had an even greater crude OR of 1.86 (95% CI 1.06-3.328). The score for depressive symptoms during pregnancy was significantly associated with preterm birth in our univariate analysis (crude OR 15.3; 95% CI 1.01-2.33). A history of major depression in a lifetime had a fairly high crude OR of 1.70, however this was not significant (95% CI 0.90-3.24).

When looking more specifically at the relationship between ACE score and spontaneous preterm birth, we found that the proportion of women with preterm birth gradually increased with increasing number of adverse childhood experiences. Inversely, the percentage of women with a term birth decreased as the number of ACEs increased (FIGURE 8). The Chi-square test for trend confirmed there was a linear trend (p=0.003).

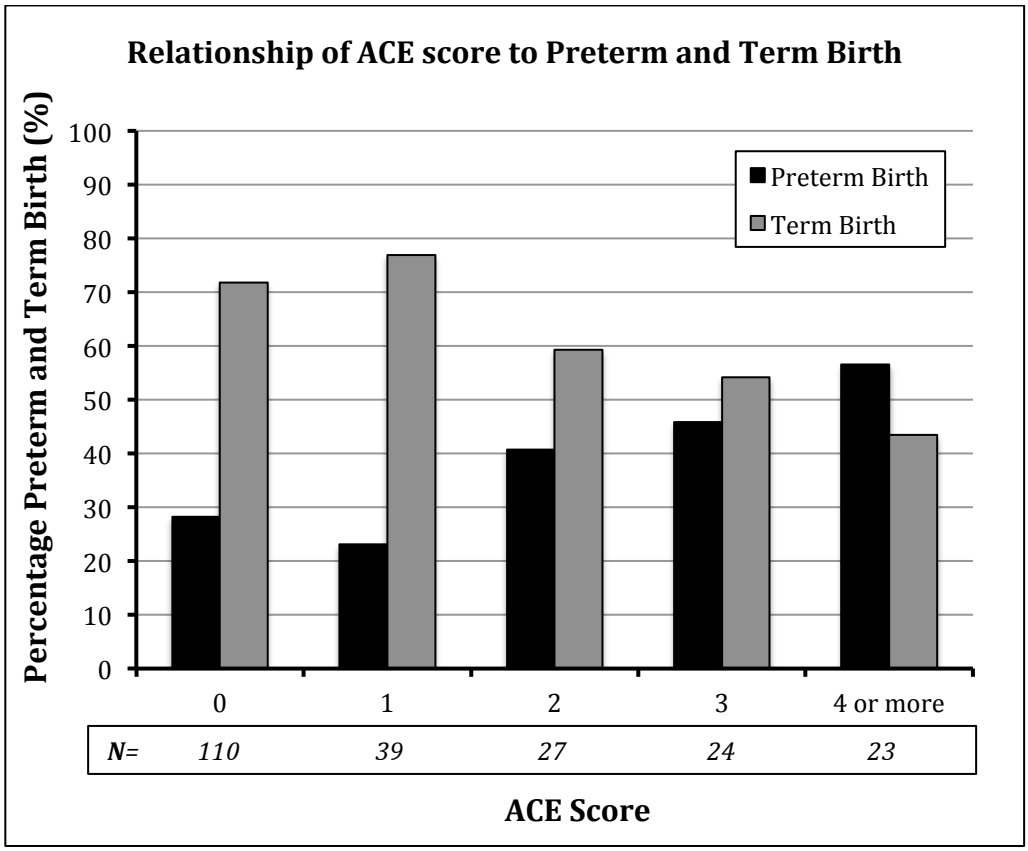

FIGURE 8. THE RELATIONSHIP BETWEEN ACE SCORE AND PRETERM BIRTH. χ^2 for linear trend p=0.003.

3.4.3 MULTIVARIATE ANALYSIS

Univariate analyses of the socio-demographic and medical variables demonstrated that maternal age, smoking, alcohol use, educational status, and a history of miscarriage were significantly associated with preterm birth in our study population. In our multivariate model we included maternal age, smoking, education, and history of spontaneous abortion as covariates. We

excluded alcohol use since the number of respondents with alcohol use was very small (n=6).

The adverse childhood experiences score was almost significantly associated with spontaneous preterm birth after adjusting for maternal age, smoking and educational status (TABLE 5).

Questionnaire Tool	**Adjusted Odds Ratio [1]**	**95% Confidence Interval**
Total Stress	1.26	0.90-1.76
High Stress [2]	1.61	0.88-2.94
ACE Score	1.18	0.99-1.40
High ACE Score (≥2 ACEs) [2]	2.09 *	1.10-3.98
Childhood and Adult Abuse	1.30 *	1.02-1.65
Depression during pregnancy	1.42	0.91-2.22

TABLE 5. MULTIVARIATE ANALYSES OF TOTAL STRESS, ACE SCORE, LIFETIME ABUSE AND DEPRESSION.

[1]Adjusted for maternal age, educational status, smoking and history of miscarriage; [2]Based on median split; *p<0.05.

The adjusted odds ratio for ACE score was 1.18 (95% CI 0.99-1.40), showing that for every increase in childhood adverse event endorsed, the risk of preterm birth increased with 18 percent. Notably, a high ACE score of two or

more adverse childhood experiences was associated with more than a two-fold increase in the risk of spontaneous preterm birth (adjusted OR 2.09; 95% CI 1.10-3.98).

When exploring the effect of lifetime abuse – combining childhood and adult abuse scores – we found that with each additional increment of 1 on the abuse score scale, the risk of spontaneous preterm birth increased by 34 percent (adjusted OR 1.30; 95% CI 1.02-1.65). Although the odds ratios of total stress score and high stress score for preterm birth remained high in our multivariate model, neither total stress nor a high stress score was significantly associated with spontaneous preterm birth (adjusted OR 1.26; 95% CI 0.90-1.76 and adjusted OR 1.61; 95% CI 0.88-2.94 respectively). The same was true for the depressive symptoms score during pregnancy (adjusted OR 142; 95% CI 0.91-2.22).

3.5 DISCUSSION

This study is the first to demonstrate that there is a strong relationship between adverse childhood experiences – assessed with the ACE measure – and spontaneous preterm birth in later life. Every additional ACE increased the risk of spontaneous preterm birth by 18 percent. This was after adjustment for maternal age, smoking, educational status and history of miscarriage, all of which were found to be confounding factors in our study.

More importantly, being exposed to two or more ACEs prior to one's 18th birthday was associated with a highly significant two-fold increase in the risk of delivering an infant preterm. Given the baseline risk of spontaneous preterm birth of around nine to ten percent (see Chapter 2), this meant that having experienced two or more adverse events during childhood increased the risk of preterm birth to 20 percent, regardless of age, smoking, educational status and a history of spontaneous abortion. In addition, our study showed that with increasing number of ACEs, the proportion of women with a term birth decreased, whereas the proportion of women with a preterm birth increased (FIGURE 6).

An important conclusion that can be drawn is that adverse childhood experiences were very common in our study population. All adverse events in childhood were more prevalent in cases than controls (SUPPLEMENTARY TABLE 3). For instance, 18 percent of the women in our case group experienced sexual abuse as a child compared to eight percent of the control women. A similar difference was seen in the prevalence of emotional neglect: 20 and 6 percent of the women were emotionally neglected during childhood in the case and control group respectively. Apart from criminal behaviour, i.e. a household member imprisoned, all ACEs regarding household dysfunction were very common in both groups of women with percentages up to 23 percent. In addition, almost a quarter of all women with a preterm birth

admitted to be physically abused during childhood, while 15 percent of the controls experienced physical abuse as a child.

It is very difficult to compare the frequencies of the various forms of childhood abuse and neglect to national and provincial data. The major issue is that a large percentage of child maltreatment is not reported and therefore the official data underestimate the incidence of childhood abuse. Presumably, the reported statistics on the rate of child abuse therefore only represent the tip of the iceberg. In Canada, every five years data are collected on child maltreatment that are reported to and investigated by the child welfare agencies in the country. Data from the 2008 Canadian Incidence Study of Reported Child Abuse and Neglect (CIS) showed that of all investigations of child maltreatment that were carried out, over 85,000 (14 per 1000 children) were substantiated.[225] Another 18,000 investigations remained suspect, but not proven. A prevalence of 1.4 percent is much lower than the rates of abuse in our study, however the reported rates of the CIS likely represent only a small fraction of total cases. More comparable are the childhood abuse rates reported in the Adolescent Health Survey. This survey among students in British Columbia in 2003 reported that 18 percent of the females were physically abused as a child and 13 percent of the females were sexually abused.[226] In addition, data published by authors from the original Adverse Childhood Experience study in the United States showed prevalence rates of

the different ACEs that were similar or slightly higher than in our study.[227,228] For example, they found that 28 percent of all participants in the study, both men and women, experienced physical abuse during their childhood.

The Adverse Childhood Experiences study was first described by Felitti *et al.* in 1998.[218] The study was developed to assess the long-term impact of adverse childhood experiences – abuse, neglect and household dysfunction – on negative health outcomes in adults. The measure of childhood exposure that was utilized was to simply add up the number of exposures. The ACE study is an ongoing study of the Center for Disease Control and Prevention and Kaiser Permanente with over 17,000 study participants. ACEs have been found to be associated with a scale of negative health outcomes and risky behaviour, including depression,[229] ischemic heart disease,[230] obesity,[231] liver disease,[232] fetal death,[233] sexually transmitted infections,[234] alcohol abuse,[235,236] smoking,[237,238] drug use[239] and adolescent pregnancy.[233,240]

Many of the adverse health outcomes and health risk behaviours associated with ACEs are associated with preterm birth as well, demonstrating an indirect relationship between adverse childhood experiences and preterm birth. Smoking, alcohol use, obesity, adolescent pregnancy and depression have all found to be associated with both preterm birth and adverse childhood events. Indeed, several of these factors were

significantly associated with spontaneous preterm birth in our study population. It is very likely that ACEs interact with the various sociodemographic and medical risk factors for preterm birth resulting in increased risks of preterm birth. Our study sample was however not adequately powered to test for these possible interactions. Adult abuse on its own was not associated with spontaneous preterm birth. Yet when we combined the scores of childhood abuse and neglect for the ACE score and adult abuse from the AAS, we discovered a significant relationship between lifetime abuse and preterm birth. We found that with each additional increment of 1 on the abuse score scale, the risk of spontaneous preterm birth increased by 30 percent. That is much higher than the 18 percent increase of risk found with each additional increment on the ACE score. These data proposed that when measures of childhood and abuse are taken together, an almost synergistic effect was seen for the risk of spontaneous preterm birth.

Evidence suggests that adverse childhood experiences can lead to hyper reactivity of the HPA and SAM axes in response to stress in adulthood.[241] This effect was even stronger in women with symptoms of depression. It is believed that ACEs can induce persistent changes in the systems involved in the stress response leading to negative health outcomes such as depression.[242] This is in complete agreement with the concept of

allostatic load as described earlier in this chapter. It is biologically very plausible that chronic stress, and more specifically, adverse childhood experiences, can increase the risk of preterm birth via the neuroendocrine and inflammatory pathways. We believe therefore that a healthy pregnancy starts long before conception.

The ACE score would provide a perfect screening tool in the prenatal care setting to identify women who were exposed to adverse childhood events. It is a quick and straightforward tool that could be easily used in the antenatal clinic. If administered early in pregnancy, women with a high ACE score (exposure to two or more ACEs) could be monitored more closely during their pregnancies. Due to close monitoring, other potential risk factors would likely be recognized at an earlier stage or might even be prevented. Adequate prevention and treatment strategies could then lead to a reduction in the risk of adverse pregnancy outcomes such as preterm birth.

One of most the difficult aspects of the study design was the composition of the 'Well-being & Pregnancy' questionnaire. First of all, how can one define chronic stress? Secondly, we had to decide which instruments would adequately represent a measure of chronic stress. For the assessment of chronic lifelong stressors we used both individual and contextual variables that influence the stress response. The chosen concepts related to stress and personal resources were perceived stress, common stressors during

pregnancy, social support, life events, coping, childhood adverse experiences, adult abuse and depression. These specific concepts were chosen based on review of the literature and their possible direct and/or indirect association with spontaneous preterm birth. As our study was a retrospective case-control study, instruments that were chosen were designed for retrospective assessment. We used validated tools that are available in the public domain where possible.

We decided that, for our purpose, the preferred method of stress assessment was via telephone. As the questionnaire was administered several months postpartum, there would be no convenient time point, such as a clinic appointment, to administer the questionnaire in person. Another option would be to mail out the questionnaires to the participant's homes. It was thought however, that the response rate would be too low utilizing mail questionnaires. In addition, some of the questions in the questionnaire might be upsetting. With direct contact with the study subjects over the telephone, this could be acknowledged and if needed, additional help could be offered. One important criterion used for the development the questionnaire was that the length of the telephone questionnaire should be less than thirty minutes long. It was believed that beyond 20 to 30 minutes of time, the compliance of participants would decline for the last part of the questionnaire. There are dozens of different tools published and available in the public domain and

including them all would have been impossible, as it would simply make the length of the telephone questionnaire too long. Aside from the eight chosen instruments, other measures of chronic stress, such as resilience and anxiety, were therefore not incorporated in the questionnaire.

After adjusting for confounding variables, a high total stress score – comprising all measures of chronic stress – was not associated with spontaneous preterm birth. One explanation for this could be our small sample size. It could also be explained by our method of calculating this score. No composite measure of chronic stress including perceived stress, common stressors, social support, life events, coping, adverse childhood experiences, adult abuse and depression exists in the literature. In addition, no standardized calculation of chronic stress exists. To calculate a score of total stress, we used a formula in which tools that represent stressors are added, while tools that represent modifiers of the stress response – social support and adaptive coping- are subtracted. As there is no consensus on the 'importance' of each stressor, the formula was built with all stressors having the same weight. However, when exploring the odds ratios and associations of all separate measures of stress and preterm birth, it was clear that they do not contribute equally to the risk of preterm birth in our study population. Adverse childhood experiences and depression had the highest odds ratios for preterm birth and therefore they might play a larger role in the etiology

of preterm birth than other non-interpersonal life events or adaptive coping methods. The development of better and most importantly standardized composite measures of chronic stress will aid in the assessment of chronic stress and might reveal different results in the future.

We are aware that retrospective assessment of maternal stress has its limitations. Women who deliver preterm will be more likely to report stress as part of the recall and rationalization process. However, all the instruments used in the questionnaire only contained questions about lifetime events, about how women feel and respond in general and about specific time points in life. Moreover, the majority of instruments, such as the Adverse Childhood Experiences score, incorporated in the questionnaire were designed and validated for retrospective assessment and therefore suitable for our study design.

For future directions, a large prospective cohort would be the preferred study population. This study design does not have some of the limitations that are associated with case-control studies, such as selection bias and confounding. Using a prospective design, childhood and life time measures of stress could be assessed retrospectively early in pregnancy and specific measures of pregnancy and perceived stress can be measured prospectively. In addition, one would have the opportunity to measure biological factors involved in the stress response, such as cortisol,

simultaneously. Due to our sample size, it was not possible to stratify between early and late preterm birth. It is likely that they involve different etiologies. It would therefore be of interest to explore the contribution of adverse childhood experiences and other measures of chronic stress in the specific phenotypes of spontaneous preterm birth.

In summary, adverse child experiences are associated with spontaneous preterm birth. After adjustment for confounding variables, we found that women who were exposed to two or more adverse childhood events have a notable two-fold increase in the risk of preterm birth. In addition, lifetime abuse was also linked to preterm birth. The data demonstrate that stressors throughout life can have a significant effect on pregnancy outcomes, including preterm birth. A healthy pregnancy therefore starts long before conception.

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4. GENOMICS AND PRETERM BIRTH

4.1. BACKGROUND

4.1.1 GENETIC BASIS FOR PRETERM BIRTH

Genetic predisposition plays an important role in the etiology of many common multifactorial diseases. Preterm birth can be considered a multifactorial 'disease' or phenomenon. Indeed, a strong genetic basis for preterm birth is widely recognized.[243-248] Twin studies estimate the heritability of preterm birth at 20 to 40 percent.[244,248] In addition, those who were born preterm themselves have an increased risk of delivering their infants preterm.[247] The data show that there is both a familial tendency towards the risk of preterm birth and that the risk persists across generations.[249] At present, one of the best predictors that exist for preterm birth in multiparous women is a previous preterm birth.[138] A woman with one prior preterm birth has a recurrence risk of 15 percent while women with two or more prior preterm births have a recurrence risk of 30 percent.[243] Preterm birth rates also significantly differ between ethnic groups. There is a large black-white racial disparity in the risk of preterm birth.[250,251] This racial disparity in preterm birth rates persists even if corrected for lifestyle factors and other demographic risk factors, such as age, socio-

economic status and proper access to health care. The ethnic disparity in preterm birth rates is particularly prominent in the United States, as clearly shown in FIGURE 7.

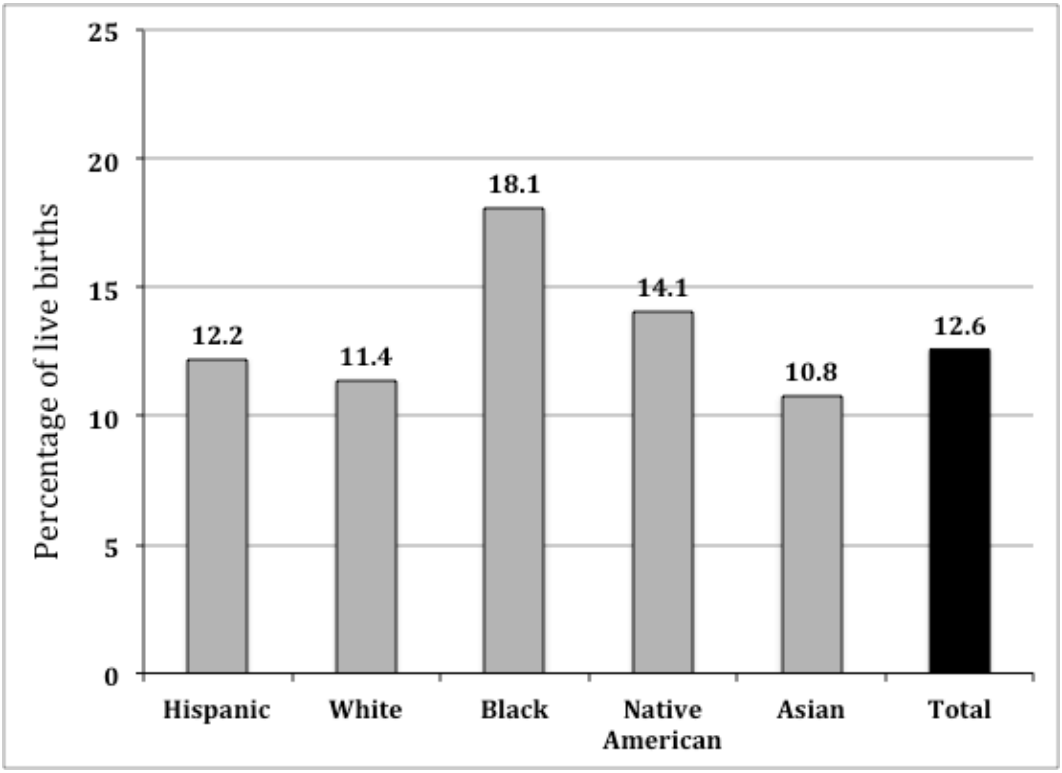

FIGURE 9. ETHNIC DISPARITY OF PRETERM BIRTH IN THE UNITED STATES (2006-2008).

Source: National Centre for Health Statistics, retrieved from www.marchofdimes.com/peristats.[20]

Taken all together, these findings strongly suggest that there is a genetic component to the risk of preterm birth.

4.1.2 SINGLE NUCLEOTIDE POLYMORPHISMS AND COMPLEX DISEASES

The haploid human genome consists of roughly three billion base pairs.[252] Considering the fact that any two genomes are 99.9 percent identical, several million base pairs contain individual DNA sequence variants also known as alleles.[253] Common alleles – present in more than one percent of the population – are called genetic polymorphisms. Single nucleotide polymorphisms (SNPs) are the most common variations in the genome and roughly make up 90 percent of all human genetic variation. A SNP is simply a single base pair substitution at a particular locus (location) in the DNA.[254] SNPs can occur within coding regions of genes and cause amino acid changes (nonsynonymous SNPs).[255] Noncoding SNPs can be located in regulatory regions affecting transcription or mRNA stability. In addition, many noncoding SNPs are located between genes or within introns, often without any consequence to genetic function. At present, over 12 million SNPs have been identified in the human genome and are catalogued in the dbSNP database of the National Center for Biotechnology Information (NCBI).[256] SNPs are identified by their reference SNP ID number (rs#), which is a unique and stable identifier. As SNPs are responsible for ninety percent of genetic variation, they can therefore influence a person's susceptibility to diseases or health outcomes like preterm birth. Nonsynonymous SNPs (nsSNPs) and SNPs in regulatory regions most likely have highest impact on

phenotype and disease susceptibility because they can directly affect protein structure or function.[257]

4.1.3 CANDIDATE GENE STUDIES AND PRETERM BIRTH

The relationship between SNPs and preterm birth has been extensively studied over the past decade.[258-268] Most, if not all, reports published to date examined the association between a certain SNP and preterm birth using a candidate gene approach. This means that SNPs are selected from genes based on biologically plausible pathways in the etiology of preterm birth. Many studies have explored the role of SNPs in genes involved in inflammatory pathways. Indeed, given the importance of inflammation and infection in the etiology of preterm birth, genetically controlled variations between women may be related to preterm birth. Several polymorphisms within the TNF gene are known and especially the association between the TNF(-308)allele, or rs1800629, and preterm birth has been studied to a great extent. Unfortunately, positive results from earlier studies could not be replicated in others. Menon *et al.* performed a meta-analysis of seven studies and genetic data from their own lab and found no association between prematurity and rs1800629.[268] Other polymorphisms that were found to be associated with preterm birth in some, but not all studies, are located in the genes encoding for IL6, IL1β, IL10, MMP9, and beta-2 adrenergic receptor.[260,261,265-267,269,270] Dolan *et al.* created

an online database, PTBGene, containing a synopsis of all genetic association studies that are published in the field of preterm birth.[271] To date, more than 1600 SNPs in 80 candidate genes in the putative preterm birth pathways having been analyzed in both maternal and fetal DNA samples. The knowledge base PTBGene shows that of all these SNPs only 3 variants were found to be significantly associated with preterm birth in meta-analyses:

1. rs1042713 (adrenergic beta-2 receptor; ADRB2): overall OR of 0.6 (95% CI 0.41-0.88) for preterm birth
2. rs1799963 (coagulation factor II precursor; F2): overall OR of 1.84 (95% CI 1.04-3.28) for preterm birth
3. rs2430561 (interferon gamma; IFNG): overall OR of 1.43 (95% CI 1.08-1.91) for preterm birth

Unfortunately, many of the candidate gene studies were hindered by small sample sizes and failure of replication. In addition, researchers have been using various definitions of preterm birth. Given the fact that preterm birth is multifactorial in origin, it has been problematic to find a robust association between one SNP and preterm birth.

4.1.4 Genome-wide Association Studies

With the completion of the Human Genome Project followed by the HapMap project, and the wide availability of high throughput genotyping,

acquiring genetic data has become much more effortless.[131-133,252] High throughput genotyping platforms (Affymetrix and Illumina) allow for fast and less expensive genotyping of large numbers of samples. Over a million SNPs and other genetic variants, such as copy number variants can be assayed up simultaneously. The availability of these platforms initiated GWAS of a scale of complex diseases.[272-278] GWAS are designed to identify common genetic variants – most commonly SNPs – that are associated with complex diseases or other health outcomes. In contrast with the candidate gene approach, a GWAS is discovery driven and therefore hypothesis free. In diseases or conditions where the etiology is largely elusive, such as preterm birth, a GWAS can provide useful new evidence and point towards genes and pathways that were previously not known to be involved, or not thought to be important, in the etiology. A major challenge, however, is that genome wide studies require large sample sizes to be adequately powered as millions of genetic variants are analyzed simultaneously. In addition, the results of the initial discovery phase must be replicated in independent cohorts in order to assure that the found genetic associations are actually robust. Successful GWAS have been conducted for age-related macular degeneration, diabetes, rheumatoid arthritis, height, obesity, and Crohn's disease among many others. A complete catalogue of published genome wide studies is found on the website of the National Human Genome Research Institute.[279] Despite the fact that in some diseases, such as macular degeneration and Alzheimer's

disease, the associated SNPs explained a substantial fraction of the heritability, it is thought that in most cases GWAS only discover rare variants and therefore these discoveries will not be useful in the development of targeted treatments or risk prediction.[280] In these cases, GWAS may, however, still provide important new evidence regarding the etiology and direct researchers towards novel hypotheses.

An important concept in genetic studies is linkage disequilibrium (LD), the non-random association of alleles at two or more loci. In other words, combinations of alleles or genetic markers occur more or less frequently together in a population than would be expected from a random formation of haplotypes (series of SNPs close together in the genome) from alleles based on their frequencies. SNPs that are in strong LD may serve as proxies for one another. Genotyping one of the SNPs, a tag SNP, gives nearly complete information regarding the genotype of the other SNPs with which it is in strong LD. The current genotyping arrays are specifically designed to detect SNPs that correlate with, or tag, a large number of other SNPs in the human genome. This concept was largely facilitated by the efforts of the International HapMap Project.[132,133,281]

To date, a successful GWAS in the field of preterm birth has not been published. Genome wide association studies would be extremely helpful to identify areas of the genome associated with preterm birth and they may

discover new pathophysiological mechanisms in the etiology of preterm. This would help researchers elucidate the various pathways leading to spontaneous preterm birth.

4.2 OVERVIEW OF THE GENOMIC STUDIES

Genetics are known to be important in the etiology of preterm birth. The aim of our genomic studies therefore was to identify genetic variants (single nucleotide polymorphisms) that are associated with spontaneous preterm labour. For this, we used two different approaches:

1. Genome-wide association studies in collaboration with the Preterm Birth Genome Project (PGP)
2. Candidate gene studies

The completed phases of the Preterm Birth Genome Project are described in Section 4.3. However, the results of the GWAS are currently still privileged since the initial PGP consortium report is not published yet. The specific outcomes of the GWAS are therefore beyond the scope of this thesis. The candidate studies were designed following the GWAS and incorporated data resulting from the PGP. The candidate gene study is described in detail in Section 4.4. In addition, gene-environment interaction analyses were performed including data from both the candidate gene studies and the

maternal stress studies. Section 4.5 provides a detailed overview of the gene-environment interaction analyses.

4.3 PRETERM BIRTH GENOME PROJECT

The Preterm Birth Genome Project (PGP) Consortium was established at the World Organization (WHO) Headquarters in Geneva in 2007 as a project of the genetics group of the Preterm Birth International Collaborative (PREBIC), of which I am a member. It was proposed that a consortium of researchers be formed to perform a GWAS study on preterm birth using existing samples in the research community and combining them to adequately power the study. The PGP Consortium was then initiated by PREBIC, the March of Dimes and WHO. The goals of the PGP were to create a community of investigators to identify susceptibility genes of preterm birth using a genome wide approach. To reach an adequate sample size needed for GWAS, the purpose was to pool established resources of DNA from multiple geographic populations and to organize samples for the necessary replication. In order to attract researchers and the needed resources for GWAS, we published 'A call for an international consortium on the genetics of preterm birth' in 2008.[282]

4.3.1 OBJECTIVES

The main objective of the PGP was to identify genetic variants that affect the susceptibility to spontaneous preterm birth and as a result we aimed to expand our knowledge related to the causes preterm birth. To achieve this aim, five phases of the PGP were developed:

1. Validation of existing resources
2. Proof of principle
3. Global evaluation of preterm birth risk genes
4. Evaluation of interactions between maternal and fetal genomes
5. Evaluation of gene-environment interactions predisposing to preterm birth

Phase 1 has been completed and is described in detail in this dissertation. Phase 2 of the PGP has also been finalized and the methods of this phase are outlined in this chapter as the results are still privileged. Phase 3 is planned to commence in the upcoming year. In the future, this work could lead to the development of better strategies in the screening, intervention and prevention of spontaneous preterm birth.

4.3.1.1 Phase 1 – Validation of existing resources

The PGP aimed to utilize DNA samples from multiple preterm birth studies, from multiple countries, from multiple types of samples and multiple types of DNA extraction methods. It was therefore of vital importance to perform quality control across recruitment sites and extraction methods prior to GWAS. Thus a quality control phase was proposed – Phase 1.

The main objective of Phase 1 was to assess the usability and quality of the DNA collected and processed from four different countries. These four countries were Korea, Mexico, Denmark and Canada. All countries utilized a different DNA extraction technique from different biological samples. The four countries and DNA extraction techniques are listed in TABLE 7.

Source	**DNA Extraction Technique**
Korea	Blood
Mexico	Salivette®
Denmark	Blood spot
Canada	Buccal swab

TABLE 6. COUNTRIES PARTICIPATING IN PHASE 1 AND DNA EXTRACTION TECHNIQUES USED.

Genotyping was to be performed at a single site in Perth, Australia. Shipping can be very costly, especially when samples need to be sent on dry

ice. Therefore, the second objective of Phase 1 was to evaluate the effect of shipping at different temperatures on down-stream array performances.

4.3.1.2 Phase 2 – Proof of principle

Most candidate gene studies in the field of preterm birth were inconclusive and failed to replicate in independent study populations.[271] The heterogeneity of the phenotype preterm birth, small study samples and selection bias were most likely the main reasons for the inconsistency of findings. To identify genetic variants that affect the susceptibility to spontaneous preterm birth a genome wide association study was designed: Phase 2 of the PGP.

4.3.2 DNA COLLECTION AND EXTRACTION

4.3.2.1 Phase 1

Initially, we had opted for buccal swabs as our method for DNA collection, using the BuccalAmp™ DNA Extraction kit (Epicentre Biotechnologies). At the time, our aim was to collect and analyze DNA from both the mother and the newborn. Using buccal swabs is a simple and rapid method for collection DNA, suitable for newborns, that is widely used in many genomic studies.[283-287] The median DNA yield expected was 2-14 ng/µl. DNA was collected and extracted as per the manufacturer's standard protocol.

First, tissue was collected by rolling the Catch-All™ buccal swab firmly 20 times on the inside of each cheek. The swab was air dried for 10 min before stored at -20°C until the time of extraction. Samples could be safely stored up to 6 months, according to the manufacturer's guidelines. For extraction, the swab end was place into a tube containing QuickExtract™ DNA extraction solution and rotated a minimum of five times. Tubes were vortexed and then incubated at 65°C for 1 min. After vortexing, the tubes were subsequently incubated at 98°C for 2 min and mixed again. DNA samples were stored at -20°C.

For phase 1, 30 samples – 15 mother-infant pairs – were sent to Perth, Australia. DNA concentrations were quantified based on optical density measures and quality control checks were performed using 1% agarose gel. DNA samples were normalized to 50 ng/µl in reduced EDTA TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). All samples had OD260:280 ratios between 1.8 and 2.0.

DNA samples via buccal swabs and newborn samples were only collected for phase 1 of the PGP.

4.3.2.2 Phase 2

For the GWAS in phase 2, we decided to only include early spontaneous preterm births at less than 34 weeks of gestation without

PPROM to partly overcome the phenotypic heterogeneity of preterm birth. As described in Chapter 1, the rate of early preterm births has largely been steady over the past decade while the rate of late preterm births (34-36 gestational weeks) has shown dramatic increases and is more likely influenced by environmental factors. In a further attempt to tighten the phenotype, only Caucasian samples were included. We believed this would increase our chances of finding positive results since there would be less genetic variability based on ethnicity.

Maternal DNA samples were accumulated from preterm birth studies in countries worldwide, including Australia, Denmark, the United States and our study in Canada. All samples were accompanied by detailed phenotype information based on the minimal and optimal phenotype data sets described by Pennell *et al*.[138] Samples from Australia and Denmark were utilized for the discovery phase. During this phase, 456 cases and 526 controls were genotyped at a centralized genotyping facility at the University of Western Australia in Perth using the Affymetrix Genome-Wide human SNP 6.0 Assay, as described in Section 4.3.4. Three independent populations from Canada, United States and Australia were used for replication. This PGP Replication Cohort consisted of 222 cases and 431 controls. In addition, testing for association was performed insilico on data from Norway and Denmark (229 cases and 1746 controls).

4.3.2.3 DNA extraction of Canadian saliva samples

DNA was collected via saliva using the Oragene DNA Self-Collection kit (OG-500, DNA Genotek). As mentioned in Chapter 2, this method is equivalent to blood – often seen as the 'gold standard' – regarding the yield and quality of DNA for downstream applications. It is easy, non-invasive, stable and cost effective. The Oragene DNA-saliva samples are stable at room temperature without processing for years. More importantly, the DNA quality and yield collected with saliva pots is far superior to DNA collected via buccal swabs. According to the manufacturer, the median yield of DNA is 110 μg/2 ml saliva.

The collection process of DNA from subjects was as follows: approximately 2 ml of saliva was delivered into the saliva funnel collector after at least 30 minutes of not eating, drinking, smoking or chewing gum; the funnel lid was then closed releasing the DNA-stabilizing fluid; the funnel was removed and the collector tube closed and shaken for several seconds to mix the saliva with the preserving fluid. DNA was then transported to the laboratory and stored at room temperature until the time of extraction. Proper mixing of the saliva with the DNA-preserving fluid is necessary for DNA stabilization and inhibition of bacterial growth.

DNA purification and extraction was performed manually using the manufacturer's protocol. Before purification, DNA samples were first transferred into 15 ml centrifuge tubes and incubated in a water bath at 50°C for one hour. To remove impurities, a 1/25th volume of the Oragene DNA Purifier (DNA Genotek) was then added to each sample and samples were incubated for 10 minutes on ice. After 10 minutes of centrifugation at 3,500 x g, the supernatant of each sample was transferred in to a fresh centrifuge tube. An equal volume of 100% ethanol at room temperature was then added for the precipitation of DNA. Following 10 minutes of incubation at room temperature, samples were centrifuged for 10 minutes at the highest speed possible. The supernatant containing impurities was discarded without disturbing the DNA pellet. Samples were washed with 1 ml of 70% ethanol at room temperature and subsequently rehydrated in reduced EDTA TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0). To ensure complete rehydration of the DNA, samples were incubated at room temperature for 2 days, before aliquots were transferred into 1.5 ml microcentrifuge tubes for storage at -20°C.

The concentration of 1 µl DNA of each sample was determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). One set of aliquots was normalized to 20 ng/µl with a total of 750 ng DNA. These samples were shipped at room temperature to Vanderbilt University

(Nashville, TN) for genotyping. Samples with concentrations below the necessary threshold (20 ng/µl) were excluded and therefore not shipped. A subset of the collected DNA samples – all Caucasian case samples of <34 gestational weeks and 165 Caucasian control samples – were used as part of the PGP Replication cohort. All included DNA samples were used in the candidate gene study (Section 4.4).

4.3.3 GENOTYPING

4.3.3.1 Phase 1

From each country, fifteen maternal-fetal pairs were identified that met the criteria set by the PGP for GWAS. One set of aliquots of the fifteen maternal samples (M1-15) was sent to Perth on dry ice. A duplicate set of aliquots of M1-M15 was sent at room temperature. Fetal samples (F1-F15) were all sent to Perth on dry ice. All samples reached the genotyping facility within 3 days from the date of shipping. Fifty arrays were performed on samples from each country:

- M1-M15 shipped on dry ice
- M1-M15 shipped at room temperature
- M1-M5 shipped on dry ice (replicates)
- F1-F15 shipped on dry ice

High-throughput genotyping was performed at a centralized genotyping facility at the University of Western Australia in Perth using the Affymetrix Genome-Wide human SNP 6.0 Assay utilizing standard protocols and validated reagents and equipment. The protocol incorporated the following stages:

1. Sty I restriction enzyme digestion
2. Sty I ligation
3. Sty I Polymerase Chain Reaction (PCR)
4. Nsp restriction enzyme digestion
5. Nsp ligation
6. Nsp PCR
7. PCR product purification using filter plates
8. Quantitation
9. Fragmentation
10. Labeling
11. Target hybridization

In summary, genomic DNA was aliquoted into corresponding wells of two 96-well plates (250 ng DNA per well). One set of DNA was digested by the Sty I restriction enzyme using 14.75 µl of Sty I Digestion Master Mix (AccuGENE water, 10X NE Buffer 3, 100X BSA, 10U/µl Sty I) for each well and subsequently ligated by adding 5.25 µl Sty Ligation Master Mix (10X T4

Ligase Buffer, 50µM Adaptor Sty I, 400U/µl T4 DNA Ligase). Prior to PCR, samples were diluted 4x and aliquoted into three corresponding 96-well plates. To each sample, 90 µl of PCR Master Mix (AccuGENE water, 10X TITANIUM *Taq* PCR Buffer, 5M GC-Melt, 2.5mM dNTP, 100µM PCR Primer 002, 50X TITANIUM *Taq* DNA Polymerase) was added and PCR was performed. 2% Agarose E-gels were run from each PCR to confirm the PCR reactions.

The second set of DNA was digested by the Nsp I restriction enzyme, similarly to the Sty 1 digestion step earlier. Following digestion, samples were ligated using 5.25 µl of Nsp Ligation Master Mix (10X T4 Ligase Buffer, 50µM Adaptor Nsp I, 400U/µl T4 DNA Ligase) and PCR was performed. Again, 2% agarose E-gels were run for quality control.

Sty and Nsp PCR products (100 µl of each well) were pooled into corresponding wells of a deep well pooling plate. Then, 1 ml of magnetic beads (AMPure) was added to each well, and after incubation, each reaction was transferred to a filter plate. Reactions were purified and then elated by adding 55 µl of Elution Buffer to each sample. After additional vacuuming and centrifugation, samples (45 µl per eluate) were transferred to two fresh PCR plates. Quantitation of the purified DNA samples was performed via optical density measurements using UV spectrophotometry.

The last stages of the genotyping protocol were fragmentation, labeling and hybridization. For fragmentation, 5 µl of 10X Fragmentation Buffer and 5 µl of 0.1U/µl Fragmentation Reagent per well were used. E-gel 4% agarose gels were run to ensure that fragmentation was successful. Labelling was performed by adding 19.5 µl of Labelling Master Mix (5X TdT Buffer, 30mM DNA Labeling Agent, 30U/µl TdT enzyme) to each well. During the target hybridization stage, 190 µl of Hybridization Master Mix (12X 1.25M MES, 50X Denhardt's solution, 0.5M EDTA, 10mg/ml Herring Sperm DNA, Oligo Control Reagent 0100, 1mg/ml Human Cot-1 DNA®, 3% Tween-20, 100% DMSO, 5M Tetramethyl Ammonium Chloride) was added to each sample. Samples were loaded onto arrays and then swiftly placed into the hybridization oven. The arrays were left in the oven for 16 to 18 hours at 50°C rotating at 60 rpm.

After hybridization, the hybridization mix from each array was transferred to a 96-well plate. Arrays were then filled with 270 µl of Array Holding Buffer (12X MES Stock Buffer, 5F NaCl, 10% Tween-20, water), before washing and staining. To wash and stain the arrays, a specified protocol was used for the Fluidics Station 450. Lastly, arrays were inserted into the GeneChip Scanner 3000 for scanning and raw data was generated.

All quality control (QC) and data analyses were conducted using Genotyping Console and PLINK software package. QC of sample and SNP data

was performed before final genotyping. The QC procedures included minor allele frequency > 0.01, maximum missing in genotype calls < 0.05 and tests for Hardy Weinberg equilibrium. QC metrics were compared using Chi-Square tests. Extraction techniques and the effect of shipping were compared. The accuracy of genotyping calls was assessed and call rates are presented as mean ± SD. Call rates between countries were compared using Kruskal-Wallis nonparametric analyses of variance. A *p*-value of < 0.05 was considered significant.[i]

4.3.3.2 Phase 2 - SNP Selection for replication and genotyping

For the discovery phase of the GWAS, high-throughput genotyping of Danish and Australian DNA samples was performed at the University of Western Australia in Perth using the Affymetrix Genome-Wide human SNP 6.0 Assay as described in the previous section. For replication, DNA samples were genotyped and analysed for 14 SNPs that were initially selected for replication based on the discovery phase. The selection was later adjusted to eleven SNPs. In the discovery phase, 53 unique SNPs in six chromosomes were identified with *p*-values between 1.95×10^{-6} and 1.99×10^{-5}. From these

[i] Genotyping of Canadian DNA samples was carried out by I. Christiaens and laboratory technicians at the University of Western Australia (UWA) in Perth, Australia. Genotyping for the remaining DNA samples was performed by technicians at UWA.

53 SNPs, eleven SNPs were selected for replication base on the following criteria:

1. If there is only one SNP in a haplotype block then that SNP was selected for replication
2. If multiple SNPS were in the same haplotype block with similar *p*-values, the tag SNP was selected for replication
3. If multiple SNPs were in the haplotype block and one SNP had a *p*-value that was 10-fold lower, that SNP was selected for replication

The Canadian samples were all genotyped for the originally selected 14 SNPs. Genotyping and sequencing of the DNA were performed at Vanderbilt University in Nashville, TN. Genotyping was performed using the MassARRAY system from Sequenom (Sequenom proprietary sequences) following the manufacturer's standard protocol. Prior to genotyping, genomic DNA samples were amplified using the polymerase chain reaction (PCR). Using a DNA polymerase, the iPLEX Gold reaction (Sequenom) produces allele-specific extension products of different masses depending on the sequence. Following PCR amplification, products were dispensed onto a

SpectroCHIP array, and the arrays were then analyzed by the MassARRAY analyzer. [ii]

4.3.4 RESULTS OF PGP PHASE 1

The validation phase of the PGP showed that DNA extracted from buccal swabs was found to be a poor source for GWAS. Only 56 percent of the SNPs passed the quality control and this was significantly less when compared to DNA derived from blood or Salivette® (SUPPLEMENTARY TABLE 4). Using DNA from buccal swabs, only 11 of the 35 arrays could be processed to completion as samples failed the Affymetrix QC metrics due to poor DNA quality. Moreover, the DNA that we obtained from buccal swabs had significantly lower call rates when compared to the Korean and Mexican samples, demonstrating that buccal DNA performed the worst of the four extraction techniques in the study.

Reassuring was the fact that shipping DNA at room temperature did not affect the call rates between samples from any country. Temperature comparisons between dry ice and room temperature call rates showed *p*-values of 0.19, 0.95, 0.2 and 0.4 for Korean, Mexican, Danish and Canadian

[ii] Genotyping and sequencing was carried out by laboratory technicians at Vanderbilt University, Nashville, TN.

samples respectively. These data are summarized in SUPPLEMENTARY TABLE 5.

The call rate accuracy was significantly lower in arrays using DNA from buccal swabs (SUPPLEMENTARY TABLE 6). The percentage of inconsistency in genotyping replication was more than four percent (p=0.004), compared to 0.26, 0.48, and 0.47 percent in arrays using DNA from blood, Salivette® and blood spots respectively.

4.3.5 Discussion

Phase 1 of the PGP showed that blood, saliva, and blood spots with whole genome amplification all provide a good source of DNA for genome wide association studies. However, it also demonstrated that DNA extracted from buccal swabs was an unsuitable substrate for GWAS. Arrays utilizing DNA from buccal swabs had significantly lower call rates and higher rates of inaccuracy compared to arrays using DNA from blood, Salivette® and blood spots with whole genome amplification. Moreover, less than half of the arrays with the Canadian samples could be processed to completion as the majority failed to pass the quality control measures. DNA from buccal swabs was found to be of poor quality, suggesting that samples may have been contaminated or degraded and therefore the ability to either label DNA or hybridize it was impaired. Buccal swabs are used in many genomic studies

examining candidate SNPs.[283-287] In contrast to our findings, they were found to be suitable sources for DNA in other GWAS.[288,289] Comparable to our results, a research group in the UK found that using a DNA pooling GWAS approach, pooled buccal swab DNA could not accurately predict allele frequencies whereas the frequency estimates from blood DNA pools were acceptable.[290] Taken in consideration cost, call rates, accuracy and reproducibility, blood would be the preferred method for DNA collection for GWAS. DNA extracted from saliva obtained with Salivette® was found to be of equal quality to blood DNA. Although sample containers can be costly, saliva pots provide an easy, non-invasive method for the collection and processing of high quality DNA. (Section 2.3) As a result, the decision was made to no longer use buccal swabs for DNA collection, and to switch to saliva pots as described previously in Chapter 2.

The validation phase of the PGP also demonstrated that shipment temperature has no impact on the outcome of GWAS. Call rates between DNA shipped at room temperature and DNA shipped on dry ice did not differ. This was the case for all samples regardless of DNA source and extraction method. Our data therefore indicated that there was no need to ship DNA on dry ice. Shipping at room temperature provided an easier and above all more cost-effective method of shipment of DNA.

Overall, Phase 1 has shown that existing resources of DNA can be successfully pooled for GWAS and shipped to utilize common genotyping facilities providing high quality DNA is used.

4.4 CANDIDATE GENE STUDIES

The alternative to a genome wide analysis is a candidate gene approach based on the pathophysiologic pathways of preterm birth. Although more than 1600 SNPs in 80 candidate genes in the putative preterm birth pathways have been studied, using a candidate gene approach does not guarantee any associations. It is however a more affordable method to explore genetic associations of preterm birth without the need of large sample sizes. With adequate phenotyping and a suitable strategy for candidate SNP selection, this approach could provide very informative results regarding genetic variations associated with preterm birth.

From Phase 2 of the PGP we learned that certain SNPs are associated with early spontaneous preterm birth (unpublished data). In addition, many SNPs were found to be associated with preterm birth in candidate gene studies. While the majority of these studies remained inconclusive, to date three maternal SNPs are significantly associated with preterm birth in meta-analyses performed by the PTBGene research team.

Numerous studies, including our own, have demonstrated that maternal stress – chronic or prenatal – can increase the risk of preterm birth. Common mental disorders such as major depressive disorder and anxiety disorders, such as panic disorders, are often associated with stress and have a heritability of 40 to 50 percent.[291] It would therefore be interesting to investigate whether genetic variations in genes involved in the stress response or mood regulation would also be associated with spontaneous preterm birth in our study population.

4.4.1 OBJECTIVES

The specific aims of the candidate gene study were therefore the following:

1. To replicate the positive findings of the discovery phase of PGP Phase 2 in all preterm and term Canadian samples,
2. To replicate the three significant candidate SNPs outlined in the PTBGene knowledge base in our study population,
3. To explore whether candidate SNPs found to be associated with adverse mental health outcomes, such as major depressive disorder and anxiety disorders, in the published literature are associated with spontaneous preterm birth as well, and

4. To identify tag SNPs located in genes involved in the HPA axis and serotonin pathway that were associated with spontaneous preterm birth in the PGP discovery phase (at $p<0.05$) and to examine their association with preterm birth in our study population.

4.4.2 SELECTION OF CANDIDATE GENES

A total of 33 SNPs were selected for genotyping. The complete list of selected SNPs, including information regarding the associated gene and allele, is outlined in TABLE 7.

SNP	Gene Name	Candidate SNP based on	Allele
rs30103	CXCR7||COPS8	PGP Replication	A/G
rs1144035	LOC100128347||BUD13	PGP Replication	T/C
rs1145211	LOC100128347||BUD13	PGP Replication	C/A
rs1240776	LOC100128347||BUD13	PGP Replication	C/T
rs1268832	LOC100128347||BUD13	PGP Replication	T/G
rs1891385	RANBP6||IL33	PGP Replication	A/C
rs2041298	CCDC77	PGP Replication	C/G
rs2240183	MORC2	PGP Replication	C/T
rs2286606	CCDC77	PGP Replication	C/T
rs4131086	ARL9	PGP Replication	A/G

SNP	Gene Name	Candidate SNP based on	Allele
rs6838375	GRIA2||LOC100132922	PGP Replication	G/T
rs7860996	KANK1	PGP Replication	C/T
rs12541626	LOC729696||UBE2W	PGP Replication	C/G
rs17394205	CNTNAP5	PGP Replication	A/T
rs1042713	ADRB2	PTB Gene	A/G
rs1799963	F2	PTB Gene	A/G
rs2430561 Proxy: rs2069727	IFNG	PTB Gene	T/C
rs9470080	FKBP5	Literature	C/T
rs10482605 Proxy: rs4128428	NR3C1	Literature – TagSNP PGP	C/T
rs6190	NR3C1	Literature	T/C
rs2070951	NR3C2	Literature	G/C
rs5522	NR3C2	Literature	T/C
rs4680	COMT	Literature	A/G
rs110402	CRHR1	Literature	G/A
rs6323	MAOA	Literature	G/T
rs852978	NR3C1	TagSNP PGP database	C/T
rs2963155	NR3C1	TagSNP PGP database	A/G
rs17484063	NR3C2	TagSNP PGP database	C/T
rs2883929	NR3C2	TagSNP PGP database	A/G
rs4835136	NR3C2	TagSNP PGP database	C/T
rs6826213 Proxy: rs7680420	NR3C2	TagSNP PGP database	C/T

SNP	Gene Name	Candidate SNP based on	Allele
rs173365	CRHR1	TagSNP PGP database	G/A
rs1912151	CRHR1	TagSNP PGP database	T/C

TABLE 7. LIST OF SELECTED SNPS FOR GENOTYPING IN CANDIDATE GENE STUDIES

For our first specific aim, 14 SNPs were included that were initially selected for replication based on the discovery phase as described earlier in this chapter. For aim 2, the three significant maternal genetic variants from the PTBGene Knowledge base were selected. These were rs1042713 (adrenergic beta-2 receptor; ADRB2), rs1799963 (coagulation factor II precursor; F2), and rs2430561 (interferon gamma; IFNG).

Various pathways are involved in the stress response and mood regulation. These include the HPA and SAM axes as described in Chapter 3. In the brain, these pathways are connected to other regulatory systems such as the meso-corticolimbic system encompassing serotonin and dopamine pathways.

Important mediators of the HPA axis are CRH, ACTH and cortisol. In short, CRH released from the hypothalamus binds to its CRHR1 receptor in the anterior pituitary gland. In turn, ACTH is secreted by the pituitary, released into the blood stream and elicits the secretion of cortisol by the

adrenal cortex. Cortisol acts on two receptors, the glucocorticoid receptor (GR or NR3C1) and the mineralocorticoid receptor (MR or NR3C2). There is evidence of genetic associations between SNPs in genes involved in the HPA axis and adverse mental health outcomes. Several reports have been published that demonstrate the association between polymorphisms in CHR1 and depression and suicidality.[292-295] In addition, various polymorphisms in the GR gene have been associated with depression and HPA axis regulation.[296,297] A recent Dutch study found two functional haplotypes in the NR3C2 gene to be associated with perceived chronic stress and increased levels of salivary and plasma cortisol, plasma ACTH and heart rate.[298] FK506 binding protein 5 (FKBP5) is part of a receptor complex regulating the sensitivity of the glucocorticoid receptor.[299] Several SNPs of the FKBP5 gene were found to interact with child abuse and adult post-traumatic stress disorder,[292,300,301] and were found to increase the risk of depression.[302]

The serotonin (5HT) pathway is, together with the dopamine pathway, part of the meso-corticolimbic system in the brain and involved in the regulation of stress sensitive mood states. Reuptake and availability of 5HT is controlled by the serotonin transporter (5HTT or SLC6A4) and the importance of this transporter in the regulation of 5HT has long been recognized.[303] A gene-environment interaction study linking childhood abuse, SLC6A4 risk allele and depression, found that those individuals who were

homozygous for the effect allele and who were abused during childhood, had a three-fold increase in their risk of developing major depression.[304] The enzymes monoamine oxidase A (MAOA) and catechol-oxymethyltransferase (COMT) control the metabolism of serotonin, dopamine and norepinephrine in the brain. Polymorphisms in the genes encoding for these enzymes have been linked to major depressive disorder and anxiety disorders including PTSD.[292,305-307]

SNPs in genes involved in the stress response or mental health were selected using two different strategies. First, a literature search was conducted to find published reports regarding genetic variants in genes involved in the regulation of the HPA axis and the meso-corticolimbic system and the associations with adverse mental health outcomes such as mainly depression. Candidate SNPs were then selected based on the literature search to explore whether these SNPs would also be associated with spontaneous preterm birth (Aim 3). The selected SNPs are listed in TABLE 7.

For our final specific aim, we selected seven key genes that are involved in the stress response and mood regulation. These genes code for the corticotropin releasing hormone receptor (CRHR1), the glucocorticoid receptor (NR3C1), the mineralocorticoid receptor (NR3C2), FK506 binding protein 5 (FKBP5), the serotonin transporter (SLC6A4) and the enzymes MAOA and COMT. It would not be feasible to select all known SNPs from

these seven genes since they all contain hundreds of SNPs. This is where the concept of linkage disequilibrium, or LD, becomes relevant. Using a haplotype approach provides a broad coverage of functional and non-functional SNPs in these genes. Therefore, tag SNPs for all genes were identified using the TagSNP function on the SNPinfo web server.[308] All identified tag SNPs were then compared to the PGP database (PGP phase 2) and SNPs that were found to be associated with preterm birth in this database at $p<0.05$ were selected for the candidate gene study. As a result, 9 tag SNPs in three different genes – NR3C1, NR3C2 and CRHR1 – were included (TABLE 7).

4.4.3 GENOTYPING

Replication genotyping of the PGP phase 2 SNPs was performed at Vanderbilt University as described previously. A duplicate set of aliquots was normalized to 50 ng/µl with a volume of 170 µl and shipped to the University of Western Australia in Perth, Australia for genotyping of the mental health SNPs and SNPs selected from PTBGene. Samples below the necessary threshold of 50 ng/µl were excluded from genotyping. A total of 190 case samples and 369 control samples were included.

SNP genotyping and sequencing were performed by the PathWest Molecular Genetics Service in Perth, Australia. SNPs were genotyped using *Taqman*® SNP genotyping assays, designed and supplied by Applied

Biosystems (ABI proprietary sequences). Initially, for 5 SNPs no *Taqman*® probe could be designed as they all had another SNP within 2 base pairs. Therefore, utilizing the web-based tool SNAP,[309] perfect proxies, based on linkage disequilibrium, were identified for three SNPs: rs2069727 for rs2430561 (IFNG; r^2=1.00), rs7680420 for rs6826213 (NR3C2; r^2=1.00), and rs4128428 for rs10482605 (NR3C1; r^2=0.95). The SNPs rs6190 (NR3C1) and rs6323 (MAOA) were excluded from further analyses as no proxy SNPs could be identified.

DNA samples were pipetted into 384-well PCR plates using a PerkinElmer Janus™ robot (4ng DNA per well). The plates were allowed to dry at room temperature prior to use. Taqman genotyping reactions were carried out in a total volume of 3ul, containing 4ng of dried template DNA, 1x *Taqman*® SNP genotyping assay, 200μM dNTP (Promega), 3.5mM $MgCl_2$ (Applied Biosystems), 1X PCR buffer II (Applied Biosystems) and 0.18U AmpliTaq Gold® DNA Polymerase (Applied Biosystems). The reaction plates were incubated on a PCR thermal cycler for 10 min at 95°C, followed by 40 cycles of 15 sec at 92°C and 1 min at 60°C. Following PCR amplification, an allelic discrimination plate read was performed using an Applied Biosystems 7900HT Fast System.

For all SNPs with a minor allele frequency of greater or equal to 40 percent, samples of each genotype were sequenced to ensure that genotypes

were called correctly. Samples of each genotype were used as controls in each experiment. To ensure that the data were reproducible, approximately 10 percent of the samples were genotyped in duplicate. [iii]

4.4.4 STATISTICAL ANALYSIS

All genomic data were analyzed using SPSS 19.0 statistical software. Before analysis, the data set was cleaned and data from different sources were merged. Data were coded or recoded for analysis when required and missing data were indicated. For all SNPs, minor allele frequencies were calculated and tested for Hardy-Weinberg equilibrium (HWE) by Chi-squared test. SNPs that failed the HWE tests were excluded from further analyses. To assess the relationship between each SNP and spontaneous preterm birth, univariate logistic regression was performed using an additive model. Odds ratios (OR) and 95 percent intervals (95% CI) or *p*-values were given. A *p*-value < 0.05 was considered significant. Next, a multivariate model was used including maternal age, smoking, alcohol use, history of miscarriage and educational status as covariates, as these factors were found to be significantly different between cases and controls in our population. Adjusted ORs and 95% CI were given and a *p*-value < 0.05 was considered significant.

[iii] Genotyping and sequencing were carried out by laboratory technicians at PathWest Molecular Genetics Service in Perth, Australia.

4.4.5 RESULTS

For the candidate gene studies, a total of 190 case samples and 369 control samples were analyzed. Of the 14 SNPs that were selected based on the top hits of the PGP discovery phase, two SNPs – rs2240183 in MORC2 gene and rs4131086 in the ARL9 gene – failed the HWE tests and were therefore not further analysed. Of the 15 SNPs that were genotyped based on selection from PTBGene and genes involved in the regulation of the HPA and serotonin pathways, 2 SNPs did not pass the HWE test. Rs9470080, located in the FKBP5 gene, and rs110402, located in the CRHR1 gene were therefore excluded.

None of the remaining 12 SNPs selected for replication for the PGP GWAS study were significantly associated with spontaneous preterm birth in our study population (SUPPLEMENTARY TABLE 7). In addition, no associations were found between any of the twelve SNPs and preterm birth when only Caucasian samples were included or when late preterm births were excluded. Similarly, none of the three SNPs that were selected from the PTBGene knowledge base were found to be associated with spontaneous preterm birth in our univariate analyses (TABLE 8).

SNP	Gene	Crude OR	*p*-value
rs1042713	ADRB2	0.91	0.47
rs1799963	F2	1.98	0.18
rs2069727 (proxy)	IFNG	1.00	0.98
rs4128428	NR3C1	1.18	0.35
rs2070951	NR3C2	1.09	0.52
rs5522	NR3C2	1.33	0.14
rs4680	COMT	1.01	0.95
rs852978	NR3C1	0.70	0.08
rs2963155	NR3C1	0.75	0.055
rs17484063	NR3C2	0.71	0.047
rs2883929	NR3C2	0.63	0.005
rs4835136	NR3C2	0.66	0.004
rs7680420 (proxy)	NR3C2	1.60	0.017
rs173365	CRHR1	0.94	0.61
rs1912151	CRHR1	0.91	0.56

TABLE 8. UNIVARIATE ANALYSES OF CANDIDATE SNPS SELECTED BASED ON PTBGENE AND LITERATURE AND TAG SNPS IN MENTAL HEALTH GENES

For the genes involved in the stress response, we found that four tag SNPs, all located in the mineralocorticoid receptor gene, were all associated with spontaneous preterm birth in our study. These SNPs were rs17484063, rs2883929, rs4835136, and rs7680420 (crude ORs 0.71, 0.71, 0.63, and 1.60

respectively). None of the other selected tag SNPs and candidate SNPs based on the literature showed a significant association with preterm birth.

Univariate analysis of the sociodemographic and medical variables showed that maternal age, smoking, alcohol use, educational status, and a history of spontaneous abortion were all found to be associated with preterm birth in our study sample (Chapter 2). In our multivariate model, we therefore included maternal age, smoking, alcohol use, education, and history of spontaneous abortion as covariates. The NR3C2 polymorphisms rs17484063, rs2883929, rs4835136, and rs7680420 were all analyzed utilizing the multivariate model.

After adjustment, two SNPs, rs1784063 and rs2883929 remained significantly associated with spontaneous preterm birth (OR 0.50; 95% CI 0.26-0.96 and OR 0.49; 95% CI 0.27-0.88). The results are summarized in TABLE 9.

SNP	Adjusted OR[1]	95% CI	*p*-value
rs17484063	0.50	0.26-0.96	0.038
rs2883929	0.49	0.27-0.88	0.017
rs4835136	0.62	0.38-1.02	0.060
rs7680420	1.73	0.90-3.31	0.099

TABLE 9. MULTIVARIATE ANALYSIS OF 4 SNPS LOCATED IN THE MR GENE

[1]Adjusted for maternal age, educational status, smoking, alcohol use and history of miscarriage.

In both cases, the effect allele was found to be protective of the risk of spontaneous preterm birth. For each additional effect allele, the risk of preterm birth was reduced by an odds of 2.00 and 2.04 for rs1784063 and rs2883929, respectively. In contrast, rs4835136 and rs7680420 were not significantly associated with spontaneous preterm birth after adjustment (OR 0.62; 95% CI 0.38-1.02 and OR 1.73; 95% CI 0.90-3.31).

4.4.6 DISCUSSION

Our candidate gene studies have demonstrated that the polymorphisms rs17484063 and rs2883929, both located in NR3C2 gene coding for the mineralocorticoid receptor are significantly associated with spontaneous preterm birth in a protective manner. This association was independent of the known preterm birth risk factors maternal age, smoking

status, alcohol use, educational status and history of miscarriage. For each additional effect allele, the risk of preterm birth was reduced by an odds of 2.00 for rs17484063 and 2.04 for rs2883929. In other words, for women who are heterozygous for the rs17484063 or the rs2883929 effect allele, the risk of spontaneous preterm birth was halved. If a woman is homozygous for either risk allele, the risk of delivering preterm is further halved. This is the first report ever published demonstrating the association between these polymorphisms and preterm birth.

No studies have been published at all for either rs17484063 or rs2883929 and therefore little is known about them. Rs17484063 is a noncoding SNP located on Chromosome 4 in the intron region of NR3C2, which is the gene for the mineralocorticoid receptor. The alleles for rs17484063 are C/T, with T being the minor or effect allele. The minor allele frequency (MAF; frequency at which the less common allele occurs in a given population) in our population was 18 percent. The SNP function prediction tool on the SNPinfo web server shows that rs17484063 is non-functional.[308] This was to be expected, as it is located in the intron region of NR3C2. Rs17484063 is a tag SNP representing a haplotype block of five SNPs all in close LD with rs17484063 (r^2>0.8): rs10519951, rs17484063, rs17484118, rs17484259, and rs17581262. Two SNPs - rs17581262 and rs17484118 –

are also in close LD with rs2883929. All SNPs in this haplotype block are located in the intron region of NR3C2.

Rs2883929 is also a noncoding SNP located in the intron region of NR3C2. Its alleles are A and G, with G being the minor allele. The MAF in our population was 20 percent. Similar to rs17484063, rs2883929 is non-functional. It is a tag SNP representing a haplotype block of 8 SNPs: rs11936376, rs17484118, rs17581262, rs17581570, rs2356210, rs2883929, rs3846326, and rs7689925. All SNPs of this haplotype block are in close LD with rs2883929 (r^2>0.8) and are located in the intron region of NR3C2. Two SNPs - rs17581262 and rs17484118 – were found to be in close LD with both tag SNPs rs17484063 and rs288392.

As the two SNPs associated with spontaneous preterm birth are both intronic, it is unclear what their functional significance might be. When exploring the SNP function potential on the SNPinfo web server of all SNPs in the two haplotype blocks, the polymorphisms rs17581262 and rs3846326 showed a low regulatory potential score of 0.09 and 0.01, respectively. The regulatory potential score is calculated for every SNP that is not in an exonic region.[310,311] It is a computed method to predict and identify potential functional elements in the genome sequence. The score is based on the SNP location within conserved regions, conserved transcription factor binding sites, CpG islands (genomic regions that contain a high frequency of CG

dinucleotides which are often associated with promoters and transcription start sites) and microRNA gene. A high combined score implies a high regulatory potential of the SNP. Rs17581262 is in close LD with both the genotyped tag SNPs rs17484063 and rs288392, whereas rs3846326 is in close LD with rs288392. It is not known whether the LD blocks of the SNPs that were associated with preterm birth contain any functional variants that could play a biological role. Sequencing of these regions in combination with functional studies may give more insight into the biological role of rs17484063 and rs2883929 and preterm birth.

Cortisol is the main effector of HPA axis and the stress response (Chapter 3). Its actions are exerted when cortisol is bound to the glucocorticoid (GR) and mineralocorticoid (MR) receptors. While the GR is present in almost every cell in the body,[169] the MR occurs mainly in brain areas of the limbic system and the hippocampus.[298] The affinity of cortisol for the MR is much greater than for GR, and binding of cortisol to MR is maintained at basal levels while the GR becomes activated in response to a stressor. It is thought that the MR mainly regulates the onset of the HPA axis, while the GR regulates the termination.[312] Functional polymorphisms in the human NR3C2 gene have been identified. [298,313,314] These were found to be associated with increased levels of cortisol and psychosocial stress.

To date, no reports on a possible link between polymorphisms in NR3C2 and preterm birth have been published. One biological pathway in the etiology of spontaneous preterm birth is the activation of the HPA axis. As described in detail in the previous chapter, maternal stress is associated with preterm birth. As genetic variants in the MR gene were found to upregulate the expression and activity of MR, and are associated with higher levels of cortisol and perceived chronic stress, it is biologically plausible that SNPs in NR3C2 are associated with preterm birth. Although the functional role of rs17484063 and rs2883929 remains unclear, it was shown in our study that these polymorphisms might play a protective role in the etiology of spontaneous preterm birth.

None of the SNPs selected for replication for the PGP GWAS study were significantly associated with spontaneous preterm birth in our study population, neither were the candidate SNPs based on the PTBGene knowledge base. This might be due to the sample size resulting in inadequate power. In addition, the phenotype criteria for the candidate gene studies were less strict than those for the PGP. Here we included all preterm births – both early and late preterm birth – and all ethnic groups, even though the majority of our study sample was Caucasian. Taken together, this might explain why we could not replicate any of the positive findings of the PGP discovery phase. In contrast, five of the eleven top SNPs from the discovery

analysis were successfully replicated in the PGP cohort that included a subset of our samples (unpublished data). This shows that for these genomic studies in complex conditions like preterm birth, adequate sample size and extremely careful phenotyping to reduce etiological heterogeneity are important.

4.5 GENE-ENVIRONMENT INTERACTION STUDIES

The etiology of preterm birth is multifactorial. Only 20 to 40 percent of its risk can be contributed to genetics. Although more than one hundred possible genetic associations with preterm birth are identified, the reports remain inconclusive. In complex diseases and conditions however, there are interactions between an individual's genetic make-up and environmental factors. It is therefore very likely that the relationships between genetic variants and preterm birth depend on environmental triggers.

Many medical and sociodemographic risk factors have been identified for preterm birth, including maternal stress.[34] When exploring the relationship between chronic maternal stress and preterm birth, we have found that adverse childhood experiences (ACEs) were significantly associated with spontaneous preterm birth, regardless of maternal age, smoking status, educational status and history of spontaneous abortion. Exposure to two or more adverse childhood experiences, such as abuse,

neglect and household dysfunction, was associated with a two-fold increase in the risk of preterm birth later in life.

In a very recent study, Bogdan *et al*. observed a significant gene-by-childhood neglect interaction when studying the effect of a functional NR3C2 polymorphism (rs5522) on amygdala reactivity.[315] A gene-environment interaction was also found when linking childhood abuse, SLC6A4 risk allele and depression.[304] Individuals who were homozygous for the effect allele and who were abused during childhood had a three-fold increase in their risk of developing major depressive disorder. The possibility exists that any associated SNPs with spontaneous preterm birth found in the candidate studies show an interaction with adverse childhood experiences as well. Our objective therefore, was to explore the possible relationship between any of the possible associated polymorphisms, adverse childhood experiences and spontaneous preterm birth.

4.5.1 GENE-ENVIRONMENT INTERACTION ANALYSES

Since both the SNPs rs17484063 and rs2883929 have a protective association with preterm birth, high ACE score (≥2 adverse childhood experiences) was first recoded into low ACE score (<2 ACEs) to make the analyses easier to interpret. Logistic regression analyses with SNP, low ACE score and the interaction term in the model were performed using SPSS 19.0

statistical software. In addition, conditional multivariate logistic regression with the SNP, maternal age, smoking, alcohol use, history of miscarriage and educational status as covariates and using low ACE score as a conditional variable. Adjusted ORs and 95% CI were given and a p-value < 0.05 was considered significant.

4.5.2 RESULTS

We found no significant interaction between rs17484063 and low ACE and the association with spontaneous preterm birth (OR of the interaction term 0.69; 95% CI 0.19-2.58). Similarly, no interaction was found between rs2883929 and low ACE and the association with preterm birth (OR of the interaction term 0.65; 95% CI 0.20-2.14).

We therefore decided to explore whether the associations between either rs17484063 or rs2883929 and spontaneous preterm birth would differ when examining women with low ACE scores and women with high ACE scores separately. Indeed, we found that when only women with low ACE scores were included, rs17484063 showed an adjusted OR of 0.37 (95% CI 0.16-0.87) for the risk of preterm birth (TABLE 10).

SNP	Gene	Low ACE[#]	Adjusted OR[1]	95% CI	*p*-value
rs17484063	NR3C2		0.50	0.26-0.96	0.038
		Yes	0.37	0.16-0.87	0.024
		No	0.68	0.23-2.02	0.49
rs2883929	NR3C2		0.49	0.27-0.88	0.017
		Yes	0.37	0.17-0.81	0.013
		No	0.82	0.28-2.40	0.72

TABLE 10. CONDITIONAL MULTIVARIATE LOGISTIC REGRESSION OF MR POLYMORPHISMS FOR WOMEN WITH AND WITHOUT HIGH ACE SCORE.

[#]Reverse of high ACE score as defined in Chapter 3; [1]Adjusted for maternal age, smoking, alcohol use, educational status and history of miscarriage.

This odds ratio was lower, showing a more protective effect, compared to the adjusted OR of rs17484063 in all included subjects (OR 0.50; 95% CI 0.26-0.96). When only women with high ACE score were included, rs17484063 was no longer associated with spontaneous preterm birth (p=0.49). The analyses of rs2883929 showed comparable results. The adjusted OR of rs2883929 for spontaneous preterm birth was decreased when only women with a low ACE score were analyzed, compared to the OR of rs2883929 when all subjects were included (adjusted OR 0.37; 95% CI 0.17-0.81 versus adjusted OR 0.49; 95% CI 0.27-0.88). Again, rs2883929 was no longer

significantly associated with spontaneous preterm birth when only women with a high score were included.

4.5.3 Discussion

In contrast to our hypothesis, we did not find a significant gene-environment interaction between either NR3C2 polymorphism, adverse childhood experiences and spontaneous preterm birth. Sample size may have limited our ability to detect any interactions. Although our sample size for the candidate gene study was reasonable (190 cases and 369 controls), only 75 cases and 148 controls were included in the studies examining the risk of chronic stress and preterm birth. Conditional multivariate regression however did show that when only women with a low ACE score were included, the adjusted odds ratios for preterm birth of rs17484063 and rs2883929 were lower compared to the overall ORs. We found that in women who experienced 0 or 1 adverse childhood events, each additional risk allele of either rs17484063 or rs2883929 was associated with a 2.7-fold decrease in the risk of spontaneous preterm birth. These data demonstrate that adverse childhood experiences can influence the genetic association between SNPs of the mineralocorticoid receptor and preterm birth.

The role of gene-environment interactions in the etiology of preterm birth is relatively unexplored. Only a few reports examining the role of gene-

environment interactions on preterm have been published and the results have been inconsistent. Several studies have found evidence that the presence of the environmental factor, bacterial vaginosis, modifies the genetic association with preterm birth.[316,317] There is evidence in the literature that gene-environment interactions also play a role in the development of adverse mental health outcomes, such as post-traumatic stress disorder and depression.[318,319] Several studies have shown an interaction of childhood adverse experience with polymorphisms in predicting major depressive disorder, post-traumatic stress disorder and alcohol dependence.[294,304,320,321]

We found a significant direct relationship between adverse childhood experiences and spontaneous preterm birth. In addition, a strong genetic basis for preterm birth exists, including a possible role for genetic variations in the mineralocorticoid receptor. It is thus likely that genes and adverse childhood experiences or other measures of chronic stress can moderate each of the other effects for risk of preterm birth. However, larger studies are needed to test the possible interactions between genetic variants, chronic maternal stress and spontaneous preterm birth.

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5. OVERALL SUMMARY

Preterm birth is a major health problem worldwide as it is the leading cause of mortality and morbidity in newborn infants. The global burden of preterm birth is enormous as 15 million babies are born preterm each year. Despite all research efforts, the preterm birth rate remains high. Though various biological pathways and risk factors for preterm birth have been identified, the majority of preterm births remain unexplained. There is not one single phenotype describing preterm birth, yet it is a complex condition with genes and environmental factors contributing to its risk, both in the mother and the fetus.

Women who experience high levels of psychosocial stress prenatally have an increased risk of preterm birth. Stress, via activation of the maternal and fetal HPA axis, is recognized as one of the pathophysiological mechanisms leading to preterm birth. The concepts of allostasis – the adaptive processes in the body to maintain stability through change – and allostatic load – the cumulative results of allostasis – provide a compelling rationale for the role of chronic stress in the etiology of preterm birth (Chapter 3)

A strong genetic basis for preterm birth also exists. The heritability of preterm birth is estimated at 20 to 40 percent. Currently, one of the best

predictors of preterm birth in multiparous women is a previous preterm birth. Single nucleotide polymorphisms are the most common genetic variations in the DNA and a relationship between SNPs and preterm birth has been established. To date, more than 1600 SNPs have been linked to preterm birth (Chapter 4).

Our research purpose was to expand our knowledge related to the causes of preterm birth through the analysis of genetic and environmental factors. For this dissertation, the aim was to explore the role of chronic maternal stress and genetic variants in the etiology of spontaneous preterm birth. We conducted a retrospective case-control study in 622 women in Edmonton. The specific aims of this study were:

1. To examine the association between lifetime chronic stress – determined by a postpartum questionnaire – and preterm birth.
2. To identify genetic variants that are associated with spontaneous preterm birth using two different approaches:
    a. Genome-wide association studies (Preterm Birth Genome Project)
    b. Candidate gene studies
3. To explore the possible interactions between genetic variants and chronic maternal stress and spontaneous preterm birth

5.1 MAJOR FINDINGS

The demographic analyses, chronic stress analyses and genomic studies resulted in many significant findings. The most important findings of this dissertation are summarized below:

- Analyses of the sociodemographic characteristics of the study population demonstrated that maternal age, smoking, alcohol use, use of street drugs, educational status and a previous miscarriage were significantly associated with preterm birth
- Our stress studies showed that there is a strong relationship between adverse childhood experiences and spontaneous preterm birth, regardless of maternal age, smoking status, educational status and history of miscarriage
- Women who were exposed to 2 or more adverse childhood events had a two-fold increase in the risk of preterm birth
- Being exposed to physical or emotional abuse during life time was also associated with an increased risk of preterm birth
- Phase 1 of the Preterm Birth Genome Project demonstrated that existing resources of DNA could be successfully pooled and shipped to utilize common genotyping facilities for GWAS providing that high quality DNA is used.

- DNA extracted from buccal swabs was found to be a poor source for GWAS
- PGP Phase 2 successfully discovered two novel genetic regions that are associated with early spontaneous preterm birth in a GWAS
- Using a candidate gene approach, we identified two novel in the mineralocorticoid receptor gene, that are associated with spontaneous preterm birth: rs17484063 and rs2883929
- Both rs17484063 and rs2883929 associated with preterm birth in a protective manner: for each additional effect allele, the risk of preterm birth was halved
- In women with ≤1 adverse childhood experiences, the odds ratios of rs17484063 and rs2883929 for preterm birth were further lowered
- Our study was inadequately powered to evaluate any gene-environment interactions

The potential role of chronic stress in preterm birth requires a multidimensional approach across the life span, including current and past adverse events, a measure of constricts/factors that attenuate the potential impact of chronic stress on biological systems and psychological schemas. Both adverse events and protective factors may act synergistically to increase or decrease risk of preterm birth. Overall, our research has shown

that chronic maternal stress and genes involved in the stress response likely play a role in the etiology of spontaneous preterm birth.

APPENDIX 1. SUPPLEMENTARY TABLES

Characteristic	**Cases N=210**	**Controls N=412**	**OR[1]**	**95% CI**	***p*-value**
Maternal age, yr[2]	28.3 ± 5.6	29.6 ± 5.2	0.96	0.93-0.99	0.004
Maternal age groups					0.09
20-34 yr, n (%)	167 (80)	326 (79)	Reference		
<20 yr, n (%)	15 (7)	15 (4)	1.95[$]	0.93-4.09	0.07
≥ 35 yr, n (%)	28 (13)	71 (17)	0.77[$]	0.48-1.24	0.28
Ethnicity					0.61
Caucasian, n (%)	177 (84)	341 (83)	1.12	0.71-1.75	0.63
Black, n (%)	5 (2)	4 (1)	2.49	0.66-9.36	0.18
Asian, n (%)	18 (9)	42 (10)	0.82	0.46-1.47	0.52
Hispanic, n (%)	2 (1)	6 (1)	0.65	0.13-3.25	0.60
Aboriginal, n (%)	8 (4)	19 (5)	0.82	0.35-1.90	0.64
Smoking, n (%)	62 (30)	69 (17)	2.08	1.41-3.09	<0.001
Alcohol, n (%)	12 (6)	7 (2)	3.51	1.36-9.04	0.009
Street drugs, n (%)	15 (7)	8 (2)	3.89	1.12-9.32	0.002
Educational status					0.008[#]
High school diploma or less, n (%)[3]	34 (45)	37 (25)	Reference		
Undergraduate degree, n (%)[3]	35 (46)	99 (66)	0.39[$]	0.21-0.70	0.002
Graduate degree, n (%)[3]	7 (9)	14 (9)	0.54[$]	0.19-1.51	0.24
Marital status					0.43[#]
Single, n (%)[3]	7 (8)	6 (4)	Reference		

Characteristic	**Cases N=210**	**Controls N=412**	**OR[1]**	**95% CI**	***p*-value**
Common-law, n (%)[3]	15 (19)	26 (16)	0.50[$]	0.14-1.75	0.27
Married, n (%)[3]	59 (73)	128 (80)	0.40[$]	0.13-1.23	0.11
Divorced, n (%)[3]	0 (0)	1 (0.6)	N/A		
Annual household income					0.42[#]
< $20,000, n (%)[3]	5 (7)	6 (4)	Reference		
$20,000-49,999, n (%)[3]	11 (15)	19 (13)	0.70[$]	0.17-2.82	0.61
$50,000-99,999, n (%)[3]	34 (46)	57 (39)	0.72[$]	0.20-2.53	0.60
> $100,000, n (%)[3]	24 (32)	63 (43)	0.46[$]	0.13-1.64	0.23
Pre-pregnant BMI[2]	26 ± 6.7	26 ± 6.2	1.00	0.97-1.03	0.93
Parity[2]	0.78 ± 1	0.68 ± 0.89	1.12	0.93-1.33	0.21
History of preterm birth, n (%)	53 (25)	0 (0)	N/A		
Previous miscarriage, n (%)	68 (32)	96 (23)	1.58	1.09-2.28	0.015
ART, n (%)	6 (3)	13 (3)	0.79	0.30-2.09	0.63
Pre-existing hypertension	5 (2)	1 (0.2)	10.02	1.16-86.37	0.036
Pre-existing maternal diabetes	6 (3)	3 (1)	4.01	0.99-16.20	0.051
Pre-existing medication use	47 (22)	77 (19)	1.32	0.88-2.00	0.18
Cervical procedures	4 (2)	13 (3)	0.60	0.19-1.85	
Sexually transmitted infection	6 (3)	15 (4)	0.78	0.28-2.06	0.62
Pregnancy-induced hypertension	13 (6)	14 (3)	2.07	0.95-4.50	0.06
Gestational diabetes	16 (8)	27 (7)	1.30	0.68-2.47	0.43

Characteristic	**Cases N=210**	**Controls N=412**	**OR[1]**	**95% CI**	***p*-value**
Placenta praevia	3 (1)	0 (0)	N/A		
Polyhydramnios	10 (5)	3 (1)	7.34	2.00-27.01	0.003
IUGR	10 (5)	2 (0.5)	11.41	2.47-52.64	0.002
Gestational age, wks[2]	33.7 ± 2.5	39.7 ± 1.0			<0.001
Birth weight, g[2]	2269.7 ± 584.5	3531.0 ± 461.7			<0.001
Fetal sex					0.99
Male, n (%)	112 (53)	220 (53)	1.00	0.72-1.39	0.99
Female, n (%)	98 (47)	192 (47)	N/A		
Apgar score at 1 min[2]	7.0 ± 2.3	8.3 ± 1.4	0.68	0.62-0.76	<0.001
Apgar score at 5 min[2]	8.3 ± 1.4	9.0 ± 0.5	0.36	0.28-0.48	<0.001
Placenta pathology performed, n (%)	110 (52)	33 (8)	N/A		
Placental infection, n (% of placentas sent for pathology)	42 (38)	12 (36)	4.62	2.30-9.27	<0.001

SUPPLEMENTARY TABLE 1. SOCIODEMOGRAPHIC AND MEDICAL CHARACTERISTICS AND FETAL OUTCOMES OF THE STUDY POPULATION.

Variables were analyzed using Chi-squared test or univariate logistic regression.

[1]Odds ratio for spontaneous preterm birth; [2]Mean ± standard deviation; [3]Percentage of known status; [#]Analyzed as continuous variable; [$]Compared to reference group.

Questionnaire Tool	**Scoring**	**Maximum Score**
Perceived Stress	½ x Scale 1 + Scale 2 + Scale 3	250
1. 6 months prior 2. First trimester 3. Second trimester	0-100 0-100 0-100	100 100 100
Common Stressors	Yes = 1 No = 0 Add scores of question 2-3-4-5-6-7-8-10-11-12-13	13
ISEL	If equal to score key = 0 If not equal to score key = 1	15
1. Tangible support 2. Appraisal support 3. Belonging support	Questions 1-5 Questions 6-10 Questions 11-15	5 5 5
Life Events Checklist	Happened to me = 1 Witnessed or doesn't apply = 0	17
Brief COPE	4-point scale per question	
1. Action oriented 2. Emotional	Q 2-7-10-13-22-24 Q 5-12-14-16-17-19-21-23-	24 40

Questionnaire Tool	**Scoring**	**Maximum Score**
Adaptive 3. Passive/avoidant	26-27 *Combine Scale 1 + Scale 2* Q 1-3-4-6-8-9-11-15-18-20-25-28	*64* 48
ACE Score	Yes = 1 No = 0	10
Abuse Assessment Screen	Affirmative for abuse if yes to any question	1
Childhood and adult abuse	Combined scores of childhood abuse, neglect and AAS	6
Depression during pregnancy	Yes = 1 No = 0 Add scores of A1b + A2b + C8	3
Lifetime history of depression	If A8 yes = 1	1

SUPPLEMENTARY TABLE 2. SCORING OF THE QUESTIONNAIRE INSTRUMENTS.

Adverse Childhood Experience	**Cases** **N (%)**	**Controls** **N (%)**
Psychological abuse	17 (23)	22 (15)
Physical abuse	11 (15)	12 (8)
Sexual abuse	14 (19)	12 (8)
Emotional neglect	15 (20)	9 (6)
Physical neglect	8 (11)	4 (3)
Witnessing domestic violence	13 (17)	13 (9)
Alcohol or substance abuse in the home	17 (23)	25 (17)
Mentally ill household member	20 (27)	31 (21)
Parental marital discord	17 (23)	16 (11)
Household member imprisoned	6 (8)	9 (6)

SUPPLEMENTARY TABLE 3. OVERVIEW OF ALL ADVERSE CHILDHOOD EXPERIENCES IN CASES AND CONTROLS.

Country	DNA Extraction Technique	Arrays processed to completion	SNPs passing QC (%)	Call Rate		Call Rate %[1]
				>98%	>99%	
Korea	Blood	33/35	638981 (71)	33	34	99.21 ± 0.36
Mexico	Salivette®	34/35	757863 (84)	41	17	99.32 ± 0.23
Denmark	Blood spot + WGA[#]	35/35	694584 (77)	33	13	98.89 ± 0.40
Canada	Buccal swab	11/35*	506607 (56)	0	0	96.14 ± 1.19

SUPPLEMENTARY TABLE 4. PHASE 1 – COMPARISON OF DNA EXTRACTION TECHNIQUES

[1]Mean ± SD; [#]WGA, whole genome amplification.

*Only 11 of the 35 arrays were processed to completion due to poor DNA quality.

Country	Pairs available for comparison	Dry Ice Call Rate %	Room Temp Call Rate %	Temperature Comparison *p*-value
Korea	15/15	99.14 ± 0.41	99.32 ± 0.30	0.191
Mexico	15/15	99.32 ± 0.22	99.35 ± 0.16	0.945
Denmark	15/15	98.78 ± 0.44	98.54 ± 0.62	0.198
Canada	3/15*	96.91 ± 0.43	96.45 ± 0.43	0.400

SUPPLEMENTARY TABLE 5. PHASE 1 – COMPARISON OF SHIPPING DNA ON DRY ICE VERSUS ROOM TEMPERATURE

[1]Mean ± SD.

*Only 3 of the 15 arrays were processed to completion due to poor DNA quality.

Country	Pairs available for comparison	Call Rate %	Inconsistency Replication %
Korea	5/5	99.21 ± 0.36	0.26 ± 0.22
Mexico	5/5	99.32 ± 0.23	0.48 ± 0.18
Denmark	5/5	98.89 ± 0.40	0.47 ± 0.55
Canada	4/5*	96.14 ± 1.19	4.37 ± 2.27§

SUPPLEMENTARY TABLE 6. PHASE 1 – ASSESSMENT OF ACCURACY USING REPLICATION OF GENOTYPING FOR SAMPLES SHIPPED ON ICE

[1]Mean ± SD; §p=0.004.

*Only 4 of the 5 arrays were processed to completion due to poor DNA quality.

SNP	Gene	Crude OR	*p*-value
rs30103	CXCR7||COPS8	0.96	0.78
rs1144035	LOC100128347||BUD13	1.05	0.71
rs1145211	LOC100128347||BUD13	1.09	0.50
rs1240776	LOC100128347||BUD13	1.12	0.90
rs1268832	LOC100128347||BUD13	1.07	0.61
rs1891385	RANBP6||IL33	1.03	0.86
rs2041298	CCDC77	1.08	0.59
rs2286606	CCDC77	1.01	0.93
rs6838375	GRIA2||LOC100132922	1.01	0.94
rs7860996[$]	KANK1	N/A	
rs12541626	LOC729696||UBE2W	1.10	0.46
rs17394205	CNTNAP5	0.73	0.11

SUPPLEMENTARY TABLE 7. UNIVARIATE ANALYSIS OF CANDIDATE SNPS SELECTED FOR REPLICATION FROM PGP PHASE 2 DISCOVERY ANALYSES.

[$]No risk allele in the study population.

APPENDIX 2. MEDICAL EXTRACTION FORM

Single Nucleotide Polymorphism and Chronic Maternal Stress in the Etiology of Spontaneous

MEDICAL EXTRACTION FORM

MATERNAL ID: BABY ID:

Maternal Surname: Baby Surname:

1. Maternal History

Maternal Age _____yr

Gravidity _____

Parity_____

History of previous preterm birth YES / NO

Number _____

History of miscarriage YES / NO

Number _____

Height _____

Pre-pregnancy weight _____

BMI _____

Weight gain in pregnancy _____

Smoking in pregnancy YES / NO

Alcohol use in pregnancy YES / NO

Street drug use in pregnancy YES / NO

Drugs _____

2. Medical History

Uterine anomalies YES / NO

Previous cervical conisation YES / NO

Previous cervical loop excision YES / NO

Pre-existing medical conditions YES / NO

Hypertension □

Diabetes Mellitus □ Type 1 □ Type 2 □

Autoimmune disease □

Other _____

Medications YES / NO

Name _____

3. Pregnancy

Mode of conception:

Natural YES / NO

Assisted YES / NO

Ovulation Induction □

In Vitro Fertilization □

Intracytoplasmic sperm injection □

Donor sperm □

Donor egg □

Gestational age determination:

LMP YES / NO

Ultrasound YES / NO

Cervical cerclage YES / NO GA _____

Elective □

Emergency □

Medication during pregnancy YES / NO

Name _____

Blood pressure: Early GA _____

At term (highest) _____

Fetal Fibronectin (FFN) Positive / Negative / Not Done

4. Complications during Pregnancy

Genital tract infection	YES / NO	
STI	YES / NO	
Pre-eclampsia/eclampsia	YES / NO	
Abruption	YES / NO	
PPROM	YES / NO	
Recurrent haemorrhage	YES / NO	GA _____
Placenta praevia	YES / NO	
Pregnancy induced hypertension	YES / NO	
Gestational diabetes	YES / NO	
Intrauterine growth restriction	YES / NO	
Polyhydramnios	YES / NO	

5. Labour and Delivery

Gestation at delivery _____

Spontaneous labour YES / NO

Medication YES / NO

Celestone □ GA _____

Nifedipine □ GA _____

Other tocolytic □ GA _____

Name _____

Antibiotics □ GA _____

Name _____

Antenatal corticosteroids □ GA _____

Induction of labour YES / NO

Drug _____

Mode of delivery:

Vaginally □

Labour and emergency C/S □

No labour and emergency C/S □

Elective C/S □

Why? _____

Evidence maternal infection YES / NO

Fever >38°C □

Tachycardia □

Positive blood cultures □

Urinary tract infection □

Placental histopathology YES / NO

Presence of infection YES / NO Chorioamnionitis □

Uteroplacental ischemia YES / NO

Clot(s) YES / NO

6. Fetus

Living fetus YES / NO

Sex M / F

Birth weight _____

APGAR 1 min _____

APGAR 5 min _____

Cord pH _____

Congenital anomalies YES / NO _____

Evidence infection 1st 48 hrs YES / NO

- Fever >38°C □
- Tachycardia □
- Positive swabs □
- Amniotic fluid assessment □

APPENDIX 3. 'WELL-BEING & PREGNANCY QUESTIONNAIRE'

Well-being
&
Pregnancy

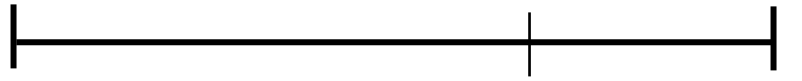
Questionnaire

Funded by the ALBERTA HERITAGE FOUNDATION
FOR MEDICAL RESEARCH Endowment Fund
Government of Alberta

PERCEIVED STRESS

We want to know how you would rate your overall stress level at pregnancy. Please indicate how stressed you felt before and during your pregnancy by giving a number between 0 and 100, where 0 means that you did not feel any stress and 100 means that you felt very stressed.

Examples:



0 100

I felt stressed during pregnancy

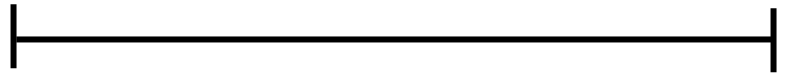
0 100

I did not feel stressed during pregnancy

Scale 1:
Indicate how stressful you felt **6 months prior** to your pregnancy:

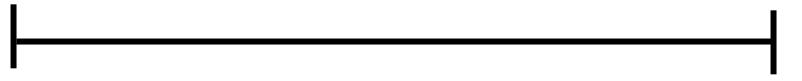
0 100

Scale 2:
Indicate how stressful you felt during your **first trimester** (0-3 months) of pregnancy:



0 100

Scale 3:
Indicate how stressful you felt during your **second trimester** (4-6 months) of pregnancy:



0 100

COMMON STRESSORS DURING PREGNANCY

1. Did you have a job during your pregnancy?
   - ☐ Yes
   - ☐ No → *go directly to question 5.*

2. Did you feel your workload was too high during your pregnancy?
   - ☐ Yes
   - ☐ No

3. Were there high physical demands at work during your pregnancy?
   - ☐ Yes
   - ☐ No

4. Were there any personal conflicts at work during your pregnancy?
   - ☐ Yes
   - ☐ No

5. Have you changed jobs *in the last year?*
   - ☐ Yes
   - ☐ No

6. Did you have financial problems during your pregnancy?
   - ☐ Yes
   - ☐ No

7. Were there times that there was not enough food in the house during your pregnancy?
   - ☐ Yes
   - ☐ No

8. Did you have any family problems during your pregnancy?
   - ☐ Yes

☐ No

9. Do you have other children than this newborn baby?

☐ Yes

☐ No → *go directly to question 11.*

10. Did you have parenting problems during your pregnancy?

☐ Yes

☐ No

11. Have you moved to another house or apartment *in the past year*?

☐ Yes

☐ No

12. Did you live in an unsafe neighbourhood during your pregnancy?

☐ Yes

☐ No

13. Did you feel you were discriminated against, or hassled, or made to feel inferior because of your race, colour or religion during your pregnancy?

☐ Yes

☐ No

INTERPERSONAL SUPPORT EVALUATION LIST

*This scale is made up of a list of statements, each of which may or may not be true about you. Please give the answer that best describes how true or false that statement is about you **in general**.*

		Completely False	Somewhat False	Somewhat True	Completely True
1.	If I had to go out of town for a few weeks, someone I know would look after my home, such as watering the plants or taking care of the pets.	**1**	**2**	**3**	**4**
2.	If I were sick and needed someone to drive me to the doctor, I would have trouble finding someone.	**1**	**2**	**3**	**4**
3.	If I were sick, I would have trouble finding someone to help me with my daily chores.	**1**	**2**	**3**	**4**
4.	If I needed help moving, I would be able to find someone to help me.	**1**	**2**	**3**	**4**
5.	If I needed a place to stay for a week because of an emergency, such as the water or electricity being out in my home, I could easily find someone who would put me up.	**1**	**2**	**3**	**4**
6.	There is at least one person I know whose advice I really trust.	**1**	**2**	**3**	**4**
7.	There is no one I know who will tell me honestly how I am handling my problems.	**1**	**2**	**3**	**4**
8.	When I need suggestions about how to deal with a personal problem, I know there is someone I can turn to.	**1**	**2**	**3**	**4**
9.	There isn't anyone I feel comfortable talking to about intimate personal problems.	**1**	**2**	**3**	**4**
10.	There is no one I trust to give me good advice about money matters.	**1**	**2**	**3**	**4**
11.	I am usually invited to do things with others.	**1**	**2**	**3**	**4**

12.	When I feel lonely, there are several people I could talk to.	**1**	**2**	**3**	**4**
13.	I regularly meet or talk with my friends or members of my family.	**1**	**2**	**3**	**4**
14.	I often feel left out by my circle of friends.	**1**	**2**	**3**	**4**
15.	There are several different people I enjoy spending time with.	**1**	**2**	**3**	**4**

CAPS 1 – LIFE EVENTS CHECKLIST

Listed in this checklist are a number of difficult or stressful things that sometimes happen to people during their lives. For each event, please indicate that:

*a) it **happened to you** personally*
*b) you **witnessed it** happen to someone else*
*c) it **doesn't apply** to you*

	Event	Happened to me	Witnessed it	Doesn't apply
1.	Natural disaster (for example flood, hurricane, tornado, earthquake)	**1**	**2**	**3**
2.	Fire or explosion	**1**	**2**	**3**
3.	Transportation accident (for example car accident, boat accident, train wreck, plane crash)	**1**	**2**	**3**
4.	Serious accident at work, home or during recreational activity	**1**	**2**	**3**
5.	Exposure to toxic substance (for example dangerous chemicals, radiation)	**1**	**2**	**3**
6.	Physical assault (for example being attacked, hit, stabbed, kicked, beaten up)	**1**	**2**	**3**
7.	Assault with a weapon (for example being shot, stabbed, threatened with a knife, gun or bomb)	**1**	**2**	**3**
8.	Sexual assault (rape, attempted rape, made to perform any type of sexual act through force or threat of harm)	**1**	**2**	**3**
9.	Other unwanted or uncomfortable sexual experience	**1**	**2**	**3**
10.	Combat or exposure to a war-zone (in the military or as a civilian)	**1**	**2**	**3**
11.	Captivity (for example being kidnapped, abducted, held hostage, prisoner of war)	**1**	**2**	**3**
12.	Life-threatening illness or injury	**1**	**2**	**3**
13.	Severe human suffering	**1**	**2**	**3**

14.	Sudden, violent death (for example, homicide, suicide)	**1**	**2**	**3**
15.	Sudden, unexpected death of someone close to you	**1**	**2**	**3**
16.	Serious injury, harm, or death you caused to someone else	**1**	**2**	**3**
17.	Any other very stressful event or experience	**1**	**2**	**3**

Please specify

_____

_____

COPE

*We are interested in how people respond when they are confronted with difficult or stressful events in their lives. There are lots of ways to try to deal with stress. This questionnaire asks you to indicate what you **generally** do and feel when you experience stressful events. Obviously, different events bring out somewhat different response, but think about what you **usually** do when you are under a lot of stress.*

	Event	I usually don't do this at all	I usually do this a little bit	I usually do this a medium amount	I usually do this a lot
1.	I turn to work or other activities to take my mind off things.	**1**	**2**	**3**	**4**
2.	I concentrate my efforts on doing something about the situation I'm in.	**1**	**2**	**3**	**4**
3.	I say to myself "this isn't real".	**1**	**2**	**3**	**4**
4.	I use alcohol or other drugs to make myself feel better.	**1**	**2**	**3**	**4**
5.	I get emotional support from others.	**1**	**2**	**3**	**4**
6.	I give up trying to deal with it.	**1**	**2**	**3**	**4**
7.	I take action to try and make the situation better.	**1**	**2**	**3**	**4**
8.	I refuse to believe that it has happened.	**1**	**2**	**3**	**4**
9.	I say things to let my unpleasant feelings escape.	**1**	**2**	**3**	**4**
10.	I get help and advice from other people	**1**	**2**	**3**	**4**
11.	I use alcohol or other drugs to help me get through it.	**1**	**2**	**3**	**4**

12.	I try to see it in a different light, to make it seem more positive.	**1**	**2**	**3**	**4**
13.	I try to come up with a strategy about what to do.	**1**	**2**	**3**	**4**
14.	I get comfort and understanding from someone.	**1**	**2**	**3**	**4**
15.	I give up the attempt to cope.	**1**	**2**	**3**	**4**
16.	I look for something good in what is happening.	**1**	**2**	**3**	**4**
17.	I make jokes about it.	**1**	**2**	**3**	**4**
18.	I do something to think about it less, such as going to the movies, watching TV, reading, or shopping.	**1**	**2**	**3**	**4**
19.	I accept the reality of the fact that it has happened.	**1**	**2**	**3**	**4**
20.	I express my negative feelings.	**1**	**2**	**3**	**4**
21.	I try to find comfort in my religion or spiritual beliefs.	**1**	**2**	**3**	**4**
22.	I try to get advise or help from other people about what to do.	**1**	**2**	**3**	**4**
23.	I learn to live with it.	**1**	**2**	**3**	**4**
24.	I think hard about what steps to take.	**1**	**2**	**3**	**4**
25.	I blame myself for things that happened.	**1**	**2**	**3**	**4**
26.	I pray or meditate.	**1**	**2**	**3**	**4**
27.	I make fun of the situation.	**1**	**2**	**3**	**4**

28. I criticize myself. **1** **2** **3** **4**

ADVERSE CHILDHOOD EXPERIENCES – ACE SCORE

It has long been known that significant life experiences can affect our health in later life. This questionnaire investigates the connection between adult health problems, including preterm birth, and adverse childhood events.

Prior to your 18th birthday:

1. Did a parent or other adult in the household **often or very often**…
   Swear at you, insult you, put you down, or humiliate you?
   or
   Act in a way that made you afraid that you might be physically hurt?
   - ☐ Yes
   - ☐ No

2. Did a parent or other adult in the household **often or very often**…
   Push, grab, slap, or throw something at you?
   or
   Ever hit you so hard that you had marks or were injured?
   - ☐ Yes
   - ☐ No

3. Did an adult or person at least 5 years older than you **ever**…
   Touch or fondle you or have you touch their body in a sexual way?
   or
   Attempt or actually have oral, anal, or vaginal intercourse with you?
   - ☐ Yes
   - ☐ No

4. Did you **often or very often** feel that…
   No one in your family loved you or thought you were important or special?
   or
   Your family didn't look out for each other, feel close to each other, or support each other?
   - ☐ Yes
   - ☐ No

5. Did you **often or very often** feel that…
   You didn't have enough to eat, had to wear dirty clothes, and had no one to protect you?

   or

   Your parents were too drunk or high to take care of you or to take you to the doctor if you doctor if you needed it?

   ☐ Yes

   ☐ No

6. Was a biological parent **ever** lost to you through divorce, abandonment, or other reason?

   ☐ Yes

   ☐ No

7. Was your mother (or stepmother)…
   Often or very often pushed, grabbed, slapped, or had something thrown at her?

   or

   Sometimes, often or very often kicked, bitten, hit with a fist, or hit with something hard?

   or

   Ever repeatedly hit over at least a few minutes or threatened with a gun or knife?

   ☐ Yes

   ☐ No

8. Did you live with anyone who was a problem drinker or alcoholic or who used street drugs?

   ☐ Yes

   ☐ No

9. Was a household member depressed or mentally ill or did a household member attempt suicide?

   ☐ Yes

   ☐ No

10. Did a household member go to prison?

- ☐ Yes
- ☐ No

ABUSE ASSESSMENT SCREEN

We would like to ask you some questions about emotional and physical abuse as an adult. We know that the incidence of abuse increases during pregnancy, and some studies have linked abuse to preterm birth.

1. *As an adult*, have you ever been emotionally or physically abused by your partner or someone important to you?

   ☐ Yes

   ☐ No

2. *Within the last year*, have you been hit, slapped, kicked, or otherwise physically hurt by someone?

   ☐ Yes

   ☐ No

   If yes, by whom (circle all that apply):
   Husband Ex-husband Boyfriend Stranger Other Multiple
   Total number of times? _____

3. *When you were pregnant*, have you been hit, slapped, kicked, or otherwise physically hurt by someone?

   ☐ Yes

   ☐ No

   If yes, by whom (circle all that apply):
   Husband Ex-husband Boyfriend Stranger Other Multiple
   Total number of times? _____

4. *Within the last year*, has anyone forced you to have sexual activities?

   ☐ Yes

   ☐ No

   If yes, by whom (circle all that apply):
   Husband Ex-husband Boyfriend Stranger Other Multiple
   Total number of times? _____

5. Are you afraid of your partner or anyone you listed above?

   ☐ Yes

   ☐ No

M.I.N.I
MINI INTERNATIONAL NEUROPSYCHIATRIC INTERVIEW

A. MAJOR DEPRESSIVE EPISODE

FOR PATIENTS WHO APPEAR PSYCHOTIC BEFORE STARTING THE INTERVIEW, OR WHO ARE SUSPECTED TO HAVE SCHIZOPHRENIA, PLEASE ADOPT THE FOLLOWING ORDER OF ADMINISTRATION OF MODULES:

1) PART 1 OF MODULE M (PSYCHOTIC DISORDERS M1-M18).
2) SECTIONS A-D (DEPRESSION TO (HYPO)MANIC EPISODE).
3) PART 2 OF MODULE M (PSYCHOTIC DISORDERS M19-M23).
4) OTHER MODULES IN THEIR USUAL SEQUENCE.

IF MODULE M HAS ALREADY BEEN EXPLORED AND PSYCHOTIC SYMPTOMS HAVE BEEN IDENTIFIED (M1 TO M10b), EXAMINE FOR EACH POSITIVE RESPONSE TO THE FOLLOWING QUESTIONS IF THE DEPRESSIVE SYMPTOMS ARE NOT BETTER EXPLAINED BY THE PRESENCE OF A PSYCHOTIC DISORDER AND CODE ACCORDINGLY.

A1	a	Have you **ever** been consistently depressed or down, most of the day, nearly every day, for at least two weeks?	NO YES
		IF A1a = YES:	
	b	Have you been consistently depressed or down, most of the day, nearly every day, during your pregnancy?	NO YES
A2	a	Have you ever been much less interested in most things or much less able to enjoy the things you used to enjoy most of the time over at least 2 weeks?	NO YES
		IF A2a = YES:	
	b	During your pregnancy, have you been much less interested in most things or much less able to enjoy the things you used to enjoy most of the time.	NO YES
		IS **A1a** OR **A2a** CODED **YES**?	NO YES

IF CURRENTLY DEPRESSED (**A1b** OR **A2b = YES**), EXPLORE THE CURRENT EPISODE AND THE MOST SYMPTOMATIC PAST EPISODE.
OTHERWISE EXPLORE THE MOST SYMPTOMATIC PAST EPISODE.

A3		**Over the two week period when you felt depressed or uninterested**		
			Current	Past Episode
	a	Was your appetite decreased or increased nearly every day? Did your weight decrease or increase without trying intentionally (I.E., BY ±5% OF BODY WEIGHT OR ±8 LBS. OR ±3.5 KGS. FOR A 160 LB./70 KGS. PERSON IN A MONTH)? IF **YES** TO EITHER, CODE **YES**.	NO YES	NO YES

	b	Did you have trouble sleeping nearly every night (difficulty falling asleep waking up in the middle of the night, early morning wakening or sleeping excessively)?	NO YES	NO YES
	c	Did you talk or move more slowly than normal or were you fidgety, restless or having trouble sitting still almost every day?	NO YES	NO YES
	d	Did you feel tired or without energy almost every day?	NO YES	NO YES
	e	Did you feel worthless or guilty almost every day?	NO YES	NO YES
		IF **A3e = YES**: ASK FOR AN EXAMPLE. THE EXAMPLE IS CONSISTENT WITH A DELUSIONAL IDEA. o NO o YES		
	f	Did you have difficulty concentrating or making decisions almost every day?	NO YES	NO YES
	g	Did you repeatedly consider hurting yourself, feel suicidal, or wish that you were dead?	NO YES	NO YES
A4		ARE **3** OR MORE **A3** ANSWERS CODED **YES** (OR **4 A3** ANSWERS, IF **A1a** OR **A2a** ARE CODED **NO** FOR PAST EPISODE OR IF **A1b** OR **A2b** ARE CODED **NO** FOR CURRENT EPISODE)?	NO YES	NO YES
		VERIFY IF THE POSITIVE SYMPTOMS OCCURRED DURING THE SAME 2 WEEK TIME FRAME.		
		IF **A4** IS CODED **NO** FOR CURRENT EPISODE THEN EXPLORE **A3a** - **A3g** FOR MOST SYMPTOMATIC PAST EPISODE.		

A5		Did the symptoms of depression cause you significant distress or impair your ability to function at work, socially, or in some other important way?	NO YES
A6		Are the symptoms due entirely to the loss of a loved one (bereavement) and are they similar in severity, level of impairment, and duration to what most others would suffer under similar circumstances? If so, this is uncomplicated bereavement.	
		HAS UNCOMPLICATED BEREAVEMENT BEEN RULED OUT?	NO YES
A7	a	Were you taking any drugs or medicines just before these symptoms began?	NO YES
	b	Did you have any medical illness just before these symptoms began?	NO YES
		IN THE CLINICIAN'S JUDGMENT: ARE EITHER OF THESE LIKELY TO BE DIRECT CAUSES OF THE PATIENT'S DEPRESSION? IF NECESSARY ASK ADDITIONAL OPEN-ENDED QUESTIONS.	
		A7 (SUMMARY): HAS AN ORGANIC CAUSE BEEN RULED OUT?	NO YES UNCERTAIN

A8	CODE **YES** IF **A7(SUMMARY) = YES** OR **UNCERTAIN**. SPECIFY IF THE EPISODE IS CURRENT AND/ OR PAST OR BOTH (RECURRENT).	**NO YES** ***Major Depressive Episode*** Current o Past o
A9	CODE **YES** IF **A7b = YES** AND **A7 (SUMMARY) = NO**. SPECIFY IF THE EPISODE IS CURRENT AND/ OR PAST OR BOTH (RECURRENT).	**NO YES** ***Mood Disorder Due to a General Medical Condition*** Current o Past o
A10	CODE **YES** IF **A7a = YES** AND **A7 (SUMMARY) = NO**. SPECIFY IF THE EPISODE IS CURRENT AND/ OR PAST OR BOTH (RECURRENT)	**NO YES** ***Substance Induced Mood Disorder*** Current o Past o

CHRONOLOGY

A11	How old were you when you first began having symptoms of depression?	___ age
A12	During your lifetime, how many distinct times did you have these symptoms of depression (daily for at least 2 weeks)?	___
A13	Is there any family history of bipolar disorder or any relative ever treated with a mood stabilizer?	NO YES

C. SUICIDALITY

During your pregnancy did you:

C1	Suffer any accident?	NO YES	 0
	IF NO TO C1, SKIP TO C2; IF YES, ASK C1a:		
C1a	Plan or intend to hurt yourself in that accident either passively or actively?	NO YES	 0
	IF NO TO C1a, SKIP TO C2: IF YES, ASK C1b:		
C1b	Did you intend to die as a result of this accident?	NO YES	 0
C2	Think that you would be better off dead or wish you were dead?	NO YES	 1
C3	Want to harm yourself or to hurt or to injure yourself?	NO YES	 2
C4	Think about suicide?	NO YES	 6

IF YES, ASK ABOUT THE INTENSITY AND FREQUENCY OF THE SUICIDAL IDEATION:

Frequency		Intensity	
Occasionally	o	Mild	o
Often	o	Moderate	o
Very often	o	Severe	o

	Can you control these impulses and state that you will not act on them while in this program?	NO YES	8
C5	Have a suicide plan?	NO YES	 8
C6	Take any active steps to prepare to injure yourself or to prepare for a suicide attempt in which you expected or intended to die?	NO YES	 9
C7	Deliberately injure yourself without intending to kill yourself?	NO YES	 4
C8	Attempt suicide?	NO YES	 10

Hoped to be rescued / survive o
Expected / intended to die o

In your lifetime:

C9	Did you ever make a suicide attempt?	NO YES	 4

IS AT LEAST **1** OF THE ABOVE (EXCEPT C1) CODED **YES**?

IF YES, ADD THE TOTAL NUMBER OF POINTS FOR THE ANSWERS (C1-C9) CHECKED 'YES' AND SPECIFY THE LEVEL OF SUICIDE RISK AS INDICATED IN THE DIAGNOSTIC BOX:

MAKE ANY ADDITIONAL COMMENTS ABOUT YOUR ASSESSMENT OF THIS PATIENT'S CURRENT AND NEAR FUTURE SUICIDE RISK IN THE SPACE BELOW:

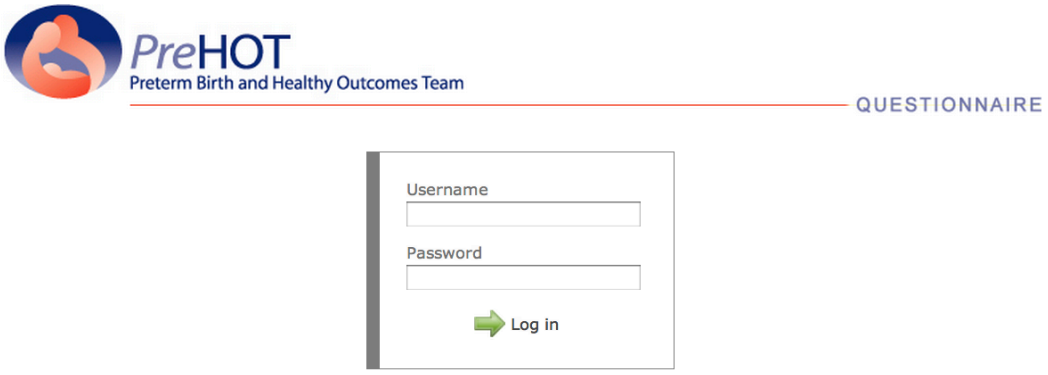
NO		**YES**
SUICIDE RISK CURRENT		
1-8 points	Low	o
9-16 points	Moderate	o
≥ 17 points	High	o

APPENDIX 4 DATABASE SCREENSHOTS

Access to the database is provided through a website. The website consists of self-contained interface to add, edit, delete, and download patients' data. The following sections demonstrate a few of the website's capabilities.

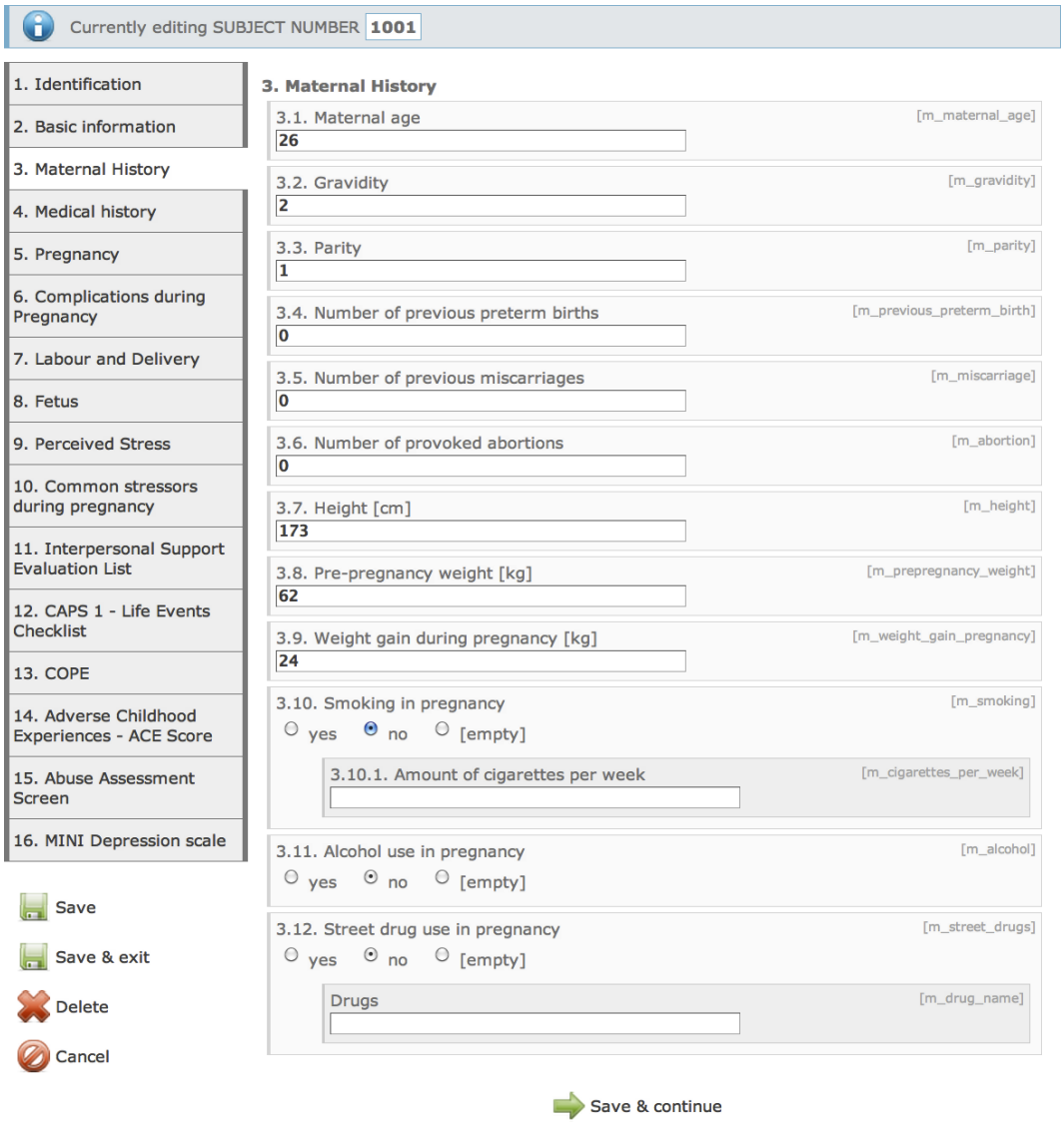
AUTHENTICATION

The database website is password-protected and each eligible user is given a username and password, which are then asked for by the system, as shown below.



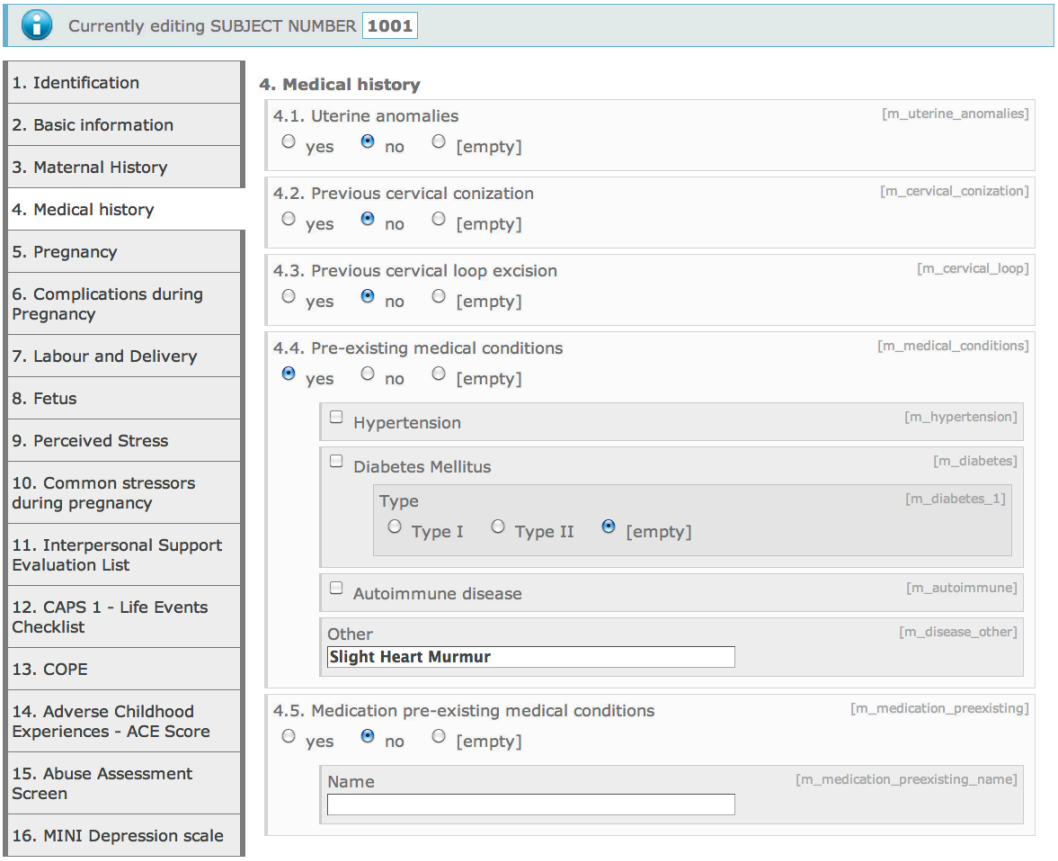
EDITING PATIENT'S DATA

Once a new subject ID is entered to the system, the user may edit the patient's data using the form presented below.



The left-hand panel of the form shows the questionnaire's sections. The above screenshot shows the view for editing Section 3 'Maternal History.' The middle panel is an editing panel. Each field of the form is labeled in two ways: verbose (labels right above the text boxes) and coded as found in the downloadable Excel sheet (gray labels in brackets).

The form incorporates several graphical elements for entering the values for the different fields, which are categorized into text boxes (for textual or numerical values), check boxes (for yes/no or true/false values), and radio buttons (for choice values). These elements can also appear nested (one inside another) if they refer to a previous question, as shown in the following screenshot.



Moving to the next section, by either clicking the *Save & continue* button or clicking on any other section label in the left-hand panel, automatically saves the changes to the database.

The user may also cancel the changes or delete all the data related to the currently edited subject by clicking on the appropriate buttons in the bottom-left corner.

FORM VALIDATION

One of the main capabilities of the database website is the automatic validation of values entered by the user. If the user enters incorrect values, the application will display error messages prompting the user for correcting the indicated fields. The incorrect values will not be saved into the database.

The screenshot below shows the automatic validation in action: the user entered incorrect values for age and height.