PLACENTAL MICROBES AS AN INDICATOR OF NEUROCOGNITIVE OUTCOMES IN
CHILDREN BORN PRETERM

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ABSTRACT

Martha Scott Tomlinson: Placental Microbes as an Indicator of Neurocognitive Outcomes in
Children Born Preterm
(Under the direction of Rebecca C. Fry)

Prenatal exposure to various stressors can influence both early and later childhood health.

Microbial infection of the intrauterine environment, and specifically within the placenta, has

been associated with deleterious pregnancy outcomes, such as preterm birth. Children that are

born prematurely experience a higher rate of developmental problems throughout their lives,

including neurocognitive impairment which persists into school-age and manifests as poor

performance in school. The relationships among microorganisms in the placenta, placental

function and fetal development are not well understood. Microorganisms have been associated

with epigenetic modifications in other tissue types and are known to trigger an inflammatory

response. Inflammatory proteins can damage the developing fetal brain.

This goal of this research was to explore microorganisms in preterm placentas, their

association with inflammation and DNA methylation, as well as later-life neurocognitive

function. Using data from the Extremely Low Gestational Age Newborn (ELGAN) cohort we

assessed the relationship between 15 microorganisms and three outcomes: (1) neurocognitive

and social-communicative outcomes at age 10, (2) genome-wide DNA CpG methylation of the

placenta, and (3) placental mRNA expression of inflammation-related genes. Through these

studies we demonstrated that different bacterial species have differential effects on the placenta

and the child. The presence of certain microorganisms in the placenta were associated with

neurocognitive delays at age 10. In contrast, the presence of *Lactobacillus* sp. was associated

with a lower risk of impaired neurocognitive functions. In the placenta, we found that the

presence of bacteria led to differential methylation of 1,789 CpG sites, corresponding to 1,079

genes. The altered genes encode for proteins that are involved in immune/inflammatory

responses, specifically the NF-κB signaling pathway. Through evaluation of mRNA expression,

we discovered that in the presence of certain bacteria there was an upregulation of inflammation-

related genes. Taken together, these findings increase the understanding of mechanisms by which

microbial presence in the placenta contributes to the outcomes of the children later in life.

To my grandmother and namesake, Martha Vardeman, for showing me the importance of education and teaching me to be dedicated to my schoolwork.

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LIST OF ABBREVIATIONS

ANCOVA   Analysis of Covariance

ANKRD11   Ankyrin Repeat Domain 11

ASD   Autism Spectrum Disorder

BMIQ   Beta-Mixture Quantile

BMPR1A   Bone Morphogenetic Protein Receptor Type 1A

C2   Complement C2

C4A   Complement C4A

CBCL   Child Behavior Check List

CBFB   Core-binding Factor Beta Subunity

CCC-2   Children's Communication Checklist-2

CCL   C-C Motif Chemokine Ligand

CCR3   C-C Motif Chemokine Receptor 3

CD163   Cluster of Differentiation

CD4   CD4 Molecule

CI   Confidence interval

CP   Cerebral Palsy

CpG   Cytosine proximal to Guanine

CRP   C-reactive protein

CXCL   C-X-C Motif Chemokine Ligand

CYSLTR2   Cysteinyl Leukotriene Receptor 2

DAS-II   Differential Ability Scales-II

DNMT   DNA Methyltransferase

DOHaD	Developmental Origins of Health and Disease
ELGAN	Extremely Low Gestational Age Newborn
FHIT	Fragile Histidine Triad
GNGT1	G Protein Subunit Gamma Trasducin 1
HDAC4	Histone Deacetylase 4
HLA-DRB1	Major Histocompatibility Complex, Class II, DR Beta 1
HPA	Hypothalamic-pituitary-adrenocortical
HSD11B2	Hydroxysteroid 11-β Dehydrogenase 2
ICAM	Intercellular Adhesion Molecule
IGF1R	Insulin Like Growth Factor 1 Receptor
IGF2	Insulin Like Growth Factor 2
IL	Interleukin
IQ	Intelligence Quotient
IRF7	Interferon Regulatory Factor 7
LMP	Last Menstrual Period
LPA	Latent Profile Analysis
LPS	Lipopolysaccharide
MDI	Mental Development Index
mRNA	Messenger RNA
NEPSY-II	A Developmental NEuroPSYchological Assessment-II
NF-κB	Nuclear Factor Kappa-light-chain enhancer of activated B cells
NNNS	NICU Network Neurobehavioral Scales
NR3C1	Nuclear Receptor Subfamily 3 Group C Member 1

OR	Odds Ratio
OWLS	Oral and Written Language Scales
PBS	Phosphate Buffered Saline
POU5F1	POU Class 5 Homeobox
PRKCZ	Protein Kinzse C Zeta
PTB	Preterm Birth
RELA	RELA Proto-Oncogene NF-κB Subunit (gene)
RelA	RELA Proto-Oncogene NF-κB Subunit (protein)
RICHS	Rhode Island Child Health Study
RNA-Seq	RNA sequencing
ROS	Reactive Oxygen Species
rRNA	Ribosomal RNA
RT-PCR	Real Time-Polymerase Chain Reaction
SHC1	SHC Adaptor Protein 1
SMAD7	SMAD Family Member 7
SNP	Single Nucleotide Polymorphism
SRS	Social Responsiveness Scale
TGFB3	Transforming Growth Factor Beta 3
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TNFAIP3	TNF Alpha Induced Protein 3
TOLLIP	Toll Interacting Protein
TP53	Tumor Protein 53

TRIM24	Tripartite Motif Containing 24
TSLP	Thymic Stromal Lymphopoietin
UBE2N	Ubiquitin Conjugating Enzyme E2 N
UTI	Urinary Tract Infection
WIAT-III	Wechsler Individual Achievement Test-III

INTRODUCTION

The developmental origins of health and disease (DOHaD) hypothesis proposes that the prenatal environment, through fetal reprogramming, can influence adult disease and later-life outcomes [1, 2] including neurocognitive and mental health [3-5]. Epigenetic modifications are one proposed biological mechanism that controls early life programming of long-term health [6]. Supporting the DOHaD hypothesis, intrauterine infection and inflammation are associated with adverse birth outcomes, such as preterm birth (PTB), and neurodevelopmental outcomes later in life [5, 7-9]. Any attempt to understand the mechanisms underlying the association between prenatal exposures and adult disease must closely consider the placenta: a crucial organ at the interface of mother and fetus. The placenta also plays a critical role in the regulation of the intrauterine environment and providing the appropriate hormones necessary for fetal growth and development.

The presence of microorganisms in the human placenta and the potential of a placental microbiome is currently a contested topic, as the placenta has been considered a sterile organ [7]. However, a recent study suggests that the placenta harbors a unique microbiome [10]. This discovery could be due to a shift in the methods and technologies available for detecting bacteria. Traditional culture techniques focus on pathogenic organisms that are isolated, grown, and identified, a time-consuming process and one which can only detect those organisms amenable to culture [11]. However, new culture-independent techniques, including 16S ribosomal RNA (rRNA) gene sequencing, allow for the full microbiological biodiversity to be detected. Importantly, this method does not differentiate between living, dead or ruptured bacteria [12] but

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can detect organisms of low-abundance [13], as is typical in the placenta [10]. Since the

presence of microorganisms in the placenta can lead to PTB and other deleterious birth outcomes

[7, 14] it is relevant to identify and study these bacterial species and how they affect the placenta.

In this review, we focus on the association between placental microorganisms and

neurocognitive outcomes and examine potential biological mechanisms contributing to this

association (**Figure 1**). There are well-established associations between intrauterine infection

and inflammation [7, 15] and prenatal inflammation and neurodevelopment [16-20]. Here, we

summarize evidence that bacteria in the placenta induce an inflammatory response through

epigenetic modifications, specifically DNA methylation, that is associated with

neurodevelopment and neurological functioning later in life.
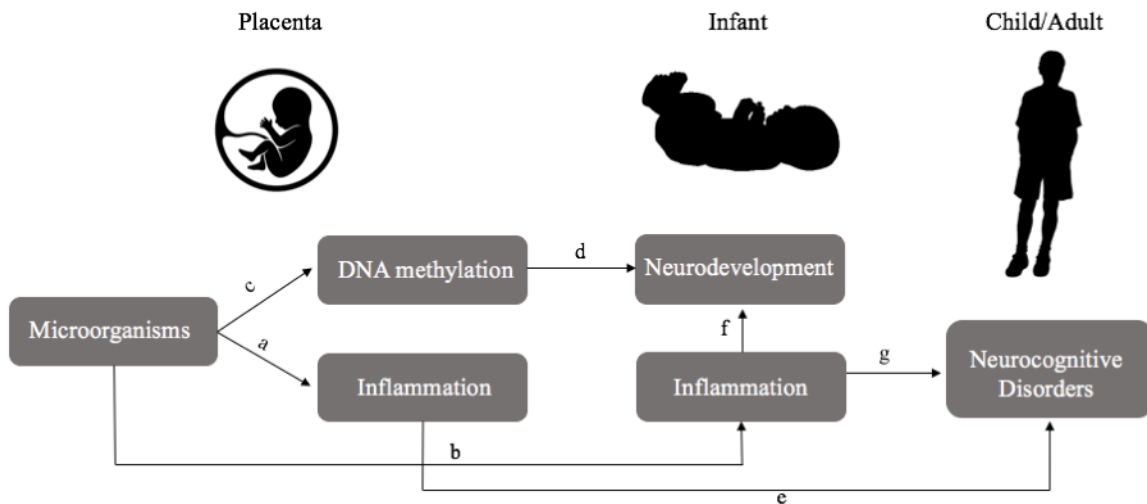
Figure 1. Schematic of the biological mechanisms underlying the association between placental microorganism and later life neurological outcomes. The arrows indicate the different associations that are discussed in the article. The presence of microorganisms in the placenta are associated with a) the production of inflammatory-proteins in placental cells [21, 22]; b) a sustained systemic inflammatory response in newborns [23]; c) and the placental methylome [24]; d) the DNA methylation profile of the placenta has been associated with different neurodevelopmental outcomes in infants [25]; e) exposure to *in utero* inflammation leads to a greater risk of neurocognitive disorders [26, 27]. The presence of inflammatory

proteins in newborn blood has been associated with a variety of neurodevelopmental outcomes at; f) 2-years of age [28-31] and ; g) 10-years of age [32].

i. Microorganisms in the Placenta

The presence of pathogenic bacteria in the placenta is associated with adverse birth outcomes, including prematurity [7, 14], stillbirth [14, 33], and fetal inflammatory response syndrome [34, 35]. At one time the placenta and intrauterine environment were considered sterile and any bacterial presence was assumed to have originated from the lower genital tract [7]. Initially many of the bacteria identified in the uterus were of vaginal origin [36-38]. However, as techniques for identifying bacteria improved, species that were not part of the vaginal microflora were detected in the intrauterine environment, including the placenta. Recently, a metagenomics technique was used to characterize placental specimens and provided one of the first reports of a placental microbiome [10]. The studies demonstrated that the placenta harbors a microbiome that is of low-abundance but is metabolically rich and resembles the oral microbiome [10]. An association was also found between the placental microbiome and PTB [10]. This is of interest because many studies have documented the association between periodontal disease and PTB [39-43] and there is evidence that oral bacteria can translocate to the placenta [44]. However, the data on a placental microbiome are still contested, and more research is needed to confirm these findings [12, 45].

Bacterial colonization of the human placenta not only occurs in preterm or at-risk pregnancies but also in the placentas of patients with normal, term pregnancies [10, 46]. The basal plate of 195 placentas have been analyzed for bacterial presence using staining methods [46]. A little over 25% of the placentas showed evidence of bacterial presence and no difference was found between preterm and term placentas [46]. These results suggest that there are potentially bacteria whose presence in the placenta exert no pathogenic effect and could even

promote normal development of the fetal immune system [47-50], whereas the presence of other microbes may activate an inflammatory response. Since this study used staining methods, bacterial species and their viability were not determined [46]. Conducting a deep sequencing analysis of the placentas of both term and preterm births could better characterize the placental microbiome, identify organisms of low prevalence, and determine which bacterial species may contribute to PTB [51].

Hematogenous transmission, or transmission through the bloodstream, is the leading explanation for how bacteria from the oral cavity colonize the placenta. Isolates of *Enterococcus* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Propionibacterium* sp. have been isolated from umbilical cord blood, supporting the ability of these microorganisms to infect the bloodstream [52]. Hematogenous infection of *Fusobacterium nucleatum*, a bacterium present in the oral cavity, has been associated with colonization of the mouse placenta and was associated with preterm and stillbirth [53]. In fact a variety of oral bacteria have the ability to translocate to the placenta following hematogenous infection [44].

Together these studies present evidence of a placental microbiome that is similar to the oral microbiome [10, 46]. While the presence of microorganisms in the placenta is associated with PTB, [10, 53] microorganisms have been detected also in placentas from term pregnancies [46]. These observations indicate that there may be microorganisms that are commensal and nonpathogenic in the placenta. However, there is evidence that the presence of certain bacterial types or species can have deleterious effects on placental function and birth outcomes.

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ii. Placental Microorganisms and Inflammation

Microorganisms in the placenta have been shown to induce an inflammatory response (**Figure 1**) that could trigger preterm labor and delivery [15]. Two studies have examined how trophoblasts, cells that form the outer layer of the blastocyst and develop into the placenta, respond to specific microorganisms. First, in cell culture, exposure of trophoblasts isolated from human placentas to a variety of microorganisms induced a release of interleukin (IL)-1β, IL-6, IL-8, and IL-10, all of which are pro-inflammatory, except for IL-10 [21]. Separately, in a mouse model, placental trophoblasts were shown to play a role in the innate immune response to placental infection with *Listeria monocytogenes* and IL-12, IL-18, tumor necrosis factor (TNF)-α, and interferon-γ production increased following infection [22].

In order for inflammatory proteins to be produced, the initiating stimulus (e.g. microorganisms) must be identified by the host. Toll-like receptors (TLR) recognize molecules derived from microbes and are important to the innate immune response, which includes inflammation [54]. The TLR signaling pathway triggers NF-κB [55], which controls transcription for IL-1β, TNF-α, and other pro-inflammatory cytokines [8]. Researchers have analyzed the human placenta for the presence and regulation of TLR2 and TLR4, specifically. TLR4 recognizes Gram-negative bacteria by its endotoxin lipopolysaccharide (LPS) [56, 57]. TLR2 recognizes a wide variety of pathogens including yeast [58], mycobacteria [59], and Gram-positive bacteria [60]. Placentas were analyzed from normal, term pregnancies and analyzed by immunohistochemical staining and found a presence of TLR2 and TLR4 in the trophoblasts of the placenta [61].

Microbes in the placenta not only induce a localized inflammatory response but have also been associated with a sustained systemic inflammatory response in newborns (**Figure 1**). Using

527 placentas from the Extremely Low Gestational Age Newborn (ELGAN) study and newborn blood for microorganisms and inflammatory proteins, it was shown that newborns from whose placentas bacteria were recovered also had an increased expression of inflammatory proteins in their blood [23]. One exception to this was the presence of *Lactobacillus* sp. which was associated with a suppressed inflammatory response [23]. This finding supports that not all microbes in the placenta are pro-inflammatory.

Taken together, there is evidence that bacterial colonization of the placenta induces an inflammatory response that could potentially be sustained and impact birth outcomes and the long-term health of the infant. However, there are few studies that focus specifically on microbes in the placenta and an inflammatory response. One of these studies shows that the presence of bacteria in the fetal membranes does not always induce an inflammatory response [62]. The type of bacteria in the placenta could determine whether an inflammatory response is induced. TLR4 is an active component of the inflammatory response in the placenta so Gram-negative bacteria could be of more consequence then other types of bacteria. Also, the expression of TLR4 could impact the magnitude of the inflammatory response. response.

iii. Microorganisms and DNA methylation

Epigenetic mechanisms control gene expression but do not change base pair sequences [63]. There are different types of epigenetic modifications, including DNA methylation, histone modification, and microRNAs. DNA methylation is a process in which methyl groups are added to a base pair, specifically cytosine. The methyl group is added to DNA by the enzyme DNA methyltransferase (DNMT). Hypermethylation reflects an increase in methyl groups at a specific site while hypomethylation means a decrease in methyl group. Hypomethylation in promoter regions of DNA often leads to gene upregulation, with some exceptions [64].

Our current working hypothesis is that bacteria can lead to altered DNA methylation in a gene-specific or non-gene specific pattern (**Figure 2**). The presence of bacteria can induce the production of reactive oxygen species (ROS) by phagocytes [65] and ROS-induced oxidative stress has been shown to modulate DNA methylation that alters gene expression [66]. ROS can damage DNA and cause structural modifications through base modifications, base deletions and chromosomal breakages [67]. These structural modifications interfere with the activity of DNMT and lead to genome-wide, non-gene specific hypomethylation [66]. On the other hand, the transcription factor occupancy theory proposes that transcription factors are drivers of gene-specific DNA methylation patterns [68]. The transcription factor binding to the DNA either prevents or allows the DNA methylation machinery access to the DNA sequences and therefore this binding influences gene-specific methylation. In this case the presence of bacteria induces an inflammatory response, therefore inflammation-related transcription factors are active, which leads to altered methylation of inflammatory genes.
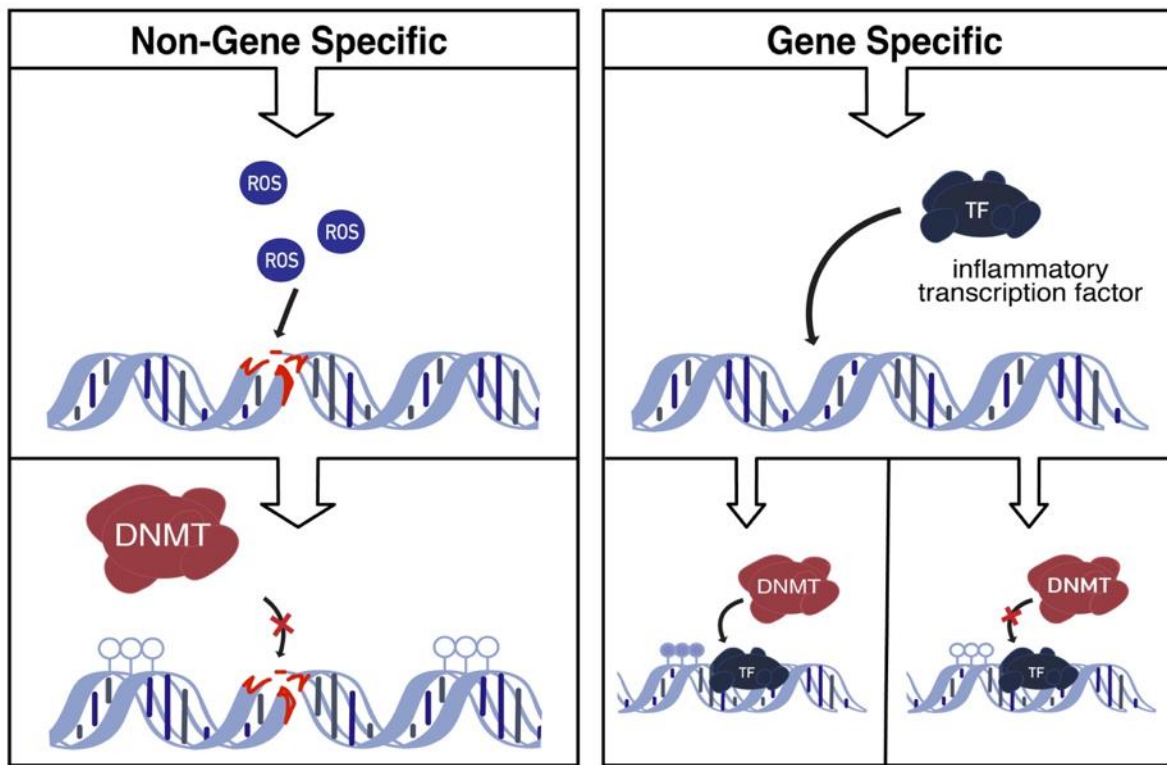
Figure 2. Proposed mechanisms for non-gene specific and gene specific mechanisms of altered DNA methylation. Microbial presence may trigger the production of reactive oxygen species (ROS) and inflammation-associated transcription factors as a mechanism of cellular defense. ROS damages DNA which may render DNMT unable to access the DNA leading to genome-wide hypomethylation. On the other hand, transcription factors may bind to the DNA at specific inflammatory-related promoter regions. This binding may prevent or allow DNMT access to the DNA and leading to gene-specific differential methylation.

Multiple studies have examined the presence of microorganisms in a variety of tissue types in relation to alterations of epigenetic mechanisms. The presence of *E. coli* in uroepithelial cells significantly increased the expression of DNMT and the promoter region of Cyclin Dependent Kinase Inhibitor 2A, a tumor suppressor, was hypermethylated while its expression was downregulated [69]. There is also evidence that the presence of *Helicobacter pylori*, which is associated with ulcers and stomach cancer, effects methylation patterns in the gastric mucosa. Eight sites that are known to be methylated in gastric cancer were found to be hypermethylated in the presence of *H. pylori* [70]. This is evidence that the presence of microorganisms can

influence the methylation state of DNA and alter epigenetic machinery, thereby contributing to disease.

In examining the effects of bacteria on placental tissue methylation, a mouse model was used to determine the association between *Campylobacter rectus* and methylation of the Insulin Like Growth Factor 2 (*IGF2*) gene in the placenta. The promoter region of *IGF2* was hypermethylated when *C. rectus* was present in the placenta [24]. *IGF2* plays a critical role in placental growth as well as growth of the developing fetus [71]. In one of our recent studies we analyzed placental microorganisms in relation to genome-wide DNA methylation in the human placenta (**Figure 1**). Placentas in the ELGAN cohort were examined for the presence of 16 different microbial species, of which 14 were associated with differential methylation of unique CpG probes. In total 1,789 probes were differentially methylated, corresponding to 1,080 genes. These genes were enriched for growth and transcription factors, the immune response, and the inflammatory response, specifically the NF-κB pathway [72]. All of the probes associated with the genes were hypomethylated, which most likely leads to increased gene expression [72]. NF-κB has also been shown to increase with the onset of labour [73, 74] and also plays a role in inflammation-related disease [18].   This could be evidence that microorganisms in the placenta affect fetal development, birth outcomes, and later life disease through epigenetic changes in the placenta. However, in order to confirm this, it is necessary to examine gene expression of inflammatory-associated proteins in placental tissues in the presence of microorganisms. As discussed in the previous section this has been studied in cell culture and mouse model but not in the human placenta.

iv. Placental Methylome and Neurodevelopment

Since the DOHaD hypothesis proposes epigenetic modifications as a mechanism by which prenatal exposures can contribute to later life disease [6], the placental methylome, as it relates to later life disease, including neurological function, has been of interest. Similarities have been shown between neuronal and placental DNA methylation profiles in genes that are associated with neuronal development [75], meaning DNA methylation in the placenta could contribute to neurodevelopment and neurocognitive outcomes later in life. Multiple studies have examined the association between the DNA methylation patterns of specific genes in the placenta and neurological outcomes in infants, primarily using the NICU Network Neurobehavioral Scales (NNNS) (**Figure 1**). The NNNS evaluates neurologic measures, behavioral measures, and signs of stress in infants to determine neurobehavioral performance [76]. In addition to its value as an early-life assessment, the NNNS is predictive of neurodevelopmental and cognitive performance as well as school readiness in children [77, 78].

Multiple epigenetic studies have been conducted using the Rhode Island Child Health Study (RICHS), a birth cohort of term pregnancies delivered at Women and Infants' Hospital in Providence, Rhode Island and the findings have been consistent across the different approaches and neurobehavioral outcomes [25]. The methylation status of two target genes, Hydroxysteroid 11-β Dehydrogenase 2 (*HSD11B2*) and Nuclear Receptor Subfamily 3 Group C Member 1 (*NR3C1*), have been analyzed in relation to NNNS measures. These genes were selected because *HSD11B2* is involved in cortisol regulation in the placenta and in the hypothalamic-pituitary-adrenocortical (HPA) axis. Previous studies have shown that reduced *HSD11B2* expression in the placenta leads to the fetus being exposed to increased levels of cortisol and dysregulation of the infant's HPA axis and neurodevelopment [79, 80]. *NR3C1* is the glucocorticoid receptor

gene, which binds cortisol. Placentas of 185 newborn infants were analyzed and showed that the promoter region of *HSD11B1* was hypermethylated in infants who had reduced scores for quality of movement [81]. Similar analyses showed an association between the methylation pattern in the promoter region of *NR3C1* and the quality of infant movement [82], infant attention [82, 83], self-regulation [83], and lethargy [83] . The interaction of DNA methylation of *HSD11B2* and *NR3C1* leads to distinct neurobehavioral phenotypes [84]. For example, when methylation was high for both genes the children had higher habituation scores [84].

In more recent studies, genome-wide methylation of the placenta was used instead of examining specific candidate genes. An epigenome-wide study of 335 infants found that an increase in methylation of a CpG site in the Fragile Histidine Triad (*FHIT*) gene was associated with increased infant attention whereas the inverse was true for a CpG site in the Ankyrin Repeat Domain 11 (*ANKRD11*) gene [85]. *FHIT* is a tumor suppressor gene that has been linked to autism spectrum disorder [86] and *ANKRD11* acts as a nuclear co-regulator in the developing brain [87] and has been associated with KBG syndrome [88], which is associated with developmental delays and intellectual deficiencies [89]. In addition to the methylation analysis, a gene ontology analysis was conducted on genes represented by CpGs associated with NNNS outcomes. The genes with variable methylation were enriched for biological pathways involved in both brain development and placental physiology [85].

Recently, we analyzed genome-wide placental methylation in relation to neurocognitive function at 10-years of age. Genes that were associated with neuronal development and function displayed hypermethylated in extremely preterm births [90]. This hypermethylation could lead to suppressed expression of these genes which are critical for neurodevelopment. There is also an association between hypermethylation of 16 genes involved neuronal development and function

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and moderate to severe cognitive impairment at ten years of age [90]. This is one of the first studies that directly analyzes the association of placental methylation and neurocognitive outcomes later in life. More studies like this are necessary in order to confirm this association.

v. Inflammation and Neurodevelopment

Many studies have shown that inflammation and cytokines can damage the developing fetal brain [9, 16-20, 91-93]. Specifically in the placenta, an inflammatory response activates a cytokine cascade that can transmit inflammatory signals between maternal and fetal tissues [94]. Inflammation of fetal membranes is associated with the upregulation and shedding of cell adhesion molecules [95, 96] and elevation of IL-6 levels [97, 98]. These proteins activate fetal leukocytes; [99, 100] this activation is associated with white matter damage [101]. Cytokines and other large molecules have access to the brain through circumventricular organs, areas in the brain that are devoid of the blood-brain barrier [9]. It has been shown that cells in the circumventricular organs contain TLR4 and IL-1 receptors [102, 103]. These receptors trigger the NF-κB signaling cascade that releases multiple cytokine and chemokines that can send inflammatory signals to other cells in the central nervous system and cause neuroinflammation [104, 105].

The effects of both prenatal and postnatal inflammation on neurodevelopment have been studied. Children exposed to inflammation *in utero* are at greater risk for developing neurological, emotional, and learning disorders, including autism [26, 27, 106-108] (**Figure 1**). The Providence cohort in the Collaborative Perinatal Project was studied to examine the association between maternal cytokine levels and adult psychosis. Increased levels of TNF-α in the maternal serum led to increased odds of schizophrenia and other psychoses in the offspring [109]. TNF-α has been shown to irreversibly alter synaptic transmission and impair cognition in

adult inflammation models [110-112]. Interestingly, in a mouse model study, maternal inflammation lead to an increase in placental serotonin output [113]. Maternal serotonin from the placenta reaches the fetal brain and modulates key neurodevelopmental processes [114-116].

The ELGAN study has resulted in multiple analyses of the relationship between the concentrations of 25 inflammation-related proteins in the blood of newborns and neurodevelopmental outcomes at 2 years of age, including the Bayley Scales of Infant and Toddler Development-II and the Child Behavior Check List (CBCL) [28-31] (**Figure 1**). Out of the 25 inflammation-related proteins 17 of them were associated with developmental impairment [28]. The risk of abnormal brain structure and function was found to be increased when at least four or more of the inflammation-related proteins were present in the blood [29]. The risk of low Mental Development Index (MDI), microcephaly, and attention problems defined by CBCL was increased in children whose blood persistently had elevated proteins [29]. The ELGAN researchers also followed up with the participants at 10 years of age and conducted another neurocognitive assessment (**Figure 1**). They showed that early elevation of C-reactive protein (CRP), TNF-α, IL-8, intercellular adhesion molecule (ICAM-1) and erythropoietin were associated with low IQ values and moderate to severe cognitive impairment [32]. As was true at 2 years, the presence of four or more inflammatory proteins in the newborn blood was associated with an increased risk of neurocognitive deficiencies, [29], including intellectual deficit and impaired cognitive ability. [32].

These studies demonstrate the impacts that inflammation can have on neurological outcomes later in life. The placenta plays a functional role in fetal neurodevelopment and can pass inflammatory signals from mother to fetus; hence inflammation in the placenta can be deleterious. Postnatal inflammation can also contribute to neurocognitive delays later in life, as

show in in the ELGAN cohort. However, this postnatal inflammation could be sustained from a prenatal inflammatory response. Even though there is a lot of research on this subject both inflammation and neurodevelopment are extremely complex systems that are not fully understood.

vi. Placental Microorganisms and Neurodevelopment

Infection of the placenta has associated with adverse neurological outcomes, especially in those born preterm [117-119]. The majority of the studies have used indicators of infection such as chorioamnionitis, fetal vasculitis, histological evidence, and maternal fever as indicators of placental infection. Many of these studies have shown an association between chorioamnionitis and cerebral palsy (CP) in both preterm [120] and normal birth weight infants [121]. Cerebral white matter damage, identified by ultrasound, is predictive of motor, cognitive, and perceptual disorders [122-127]. Leviton *et. al* found that indicators of maternal infection and of a fetal inflammatory response are associated with cerebral echolucency, an indicator of white matter damage [128].

Few studies have directly tested the placenta for the presence of microorganisms. One study detected infectious agents through *in situ* hybridization or reverse transcriptase-polymerase chain reaction (RT-PCR) and demonstrated an association between *in utero* infection of the placenta and neurodevelopmental abnormalities [129]. In the ELGAN study the presence of *U. urealyticum* was associated with increased risk of intraventricular hemorrhage and echolucent brain lesions in the white matter [130]. In another study of that cohort, placentas were analyzed for multiple microorganisms and infants were assessed for CP at 24 months. Microorganisms isolated from the placenta were predictive of ultrasound lesions of the brain and diparetic CP [131]. To further our understanding of the relationship between placental microorganisms and neurodevelopment, more studies are needed that are based on assays for the presence of

microorganisms as opposed to using indicators of infection. Also, assessments of the children later in life are needed to determine if the neurodevelopmental delays that have been associated with placenta microorganisms persist into later childhood and beyond.

vii. Project Approach

This research focuses microorganisms in the placenta and how they may influence neurocognitive function later in life through alterations to the placenta. **The central hypothesis of this research is that microorganisms in the placenta induce an inflammatory response via epigenetic modifications, and that this inflammatory response is associated with a decline in neurocognitive function later in life.** This project focuses on different microbial species in the placenta and their association with 10 year neurocognitive outcomes, placental CpG methylation, and mRNA expression of inflammation-related genes in the placenta. It is notable that our research is the first to examine a variety of microbial species in the placenta, as opposed to indicators of infection and analyze them in relation to (1) a broad range of neurocognitive assessments at 10-years of age, (2) genome-wide CpG methylation patterns in the placenta, and (3) placental mRNA expression of inflammation-related genes.

viii. Dissertation Organization

This dissertation is organized into three chapters. The first chapter describes the association between microbial species in the placenta and 10year neurocognitive outcomes. The second chapter begins to examine placental alterations that could explain the association identified in the first chapter by exploring placental CpG methylation patterns in the presence of microorganisms. The third chapter focuses in on the inflammation pathway and the relationship

between placental microbes and mRNA expression of inflammation-related genes. Of particular interest these studies identify mechanisms within the placenta by which microorganisms could affect later-life health outcomes.

CHAPTER 1: NEUROCOGNITIVE AND SOCIAL-COMMUNICATIVE FUNCTION OF CHILDREN BORN VERY PRETERM AT 10 YEARS OF AGE: ASSOCIATIONS WITH MICROORGANISMS RECOVERED FROM THE PLACENTA PARENCHYMA

1.1 Overview

Children born preterm, before 37 weeks, are at a higher risk for neurocognitive impairment [132]. This impairment persists into school age and manifests as poor performance in school [133, 134]. Studies have shown a higher incidence of intellectual deficit among children born preterm in both childhood [135, 136] and adolescence [137, 138]. While most studies focus on intelligence quotient (IQ), children born preterm exhibit impairment in multiple domains of neurodevelopment including motor function [134], executive function [138, 139], social cognition [140, 141], language skills [142], and mathematic ability [143]. These cognitive deficits tend to co-occur in preterm children [134, 144, 145]. As prenatal care continues to improve the survival rates of preterm children, the number of these children living with neurological deficits and disabilities is increasing [146, 147]. Thus, it is important to understand the etiology of neurocognitive impairment and the precursors of unfavorable cognitive outcomes in extremely preterm children.

The developmental origins of health and disease (DOHaD) hypothesis proposes that the prenatal environment can influence adult disease and later life outcomes [1, 6], including neurocognitive and mental health [3-5]. With relevance to DOHaD, the placenta is a critical regulator of the prenatal environment and is at the interface between the mother and developing fetus. The placenta transports nutrients from mother to fetus and produces hormones necessary to maintain pregnancy and support the fetus [148]. Once considered a sterile organ, the placenta

recently has been found to harbor microorganisms [10, 46]. The presence of bacterial species in the placenta can affect pregnancy outcomes and fetal health [7, 46]. Preterm birth has been associated with microorganisms in the placenta and intrauterine environment [7, 119, 149, 150], including *Ureplasma urealyticum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, and *Peptostreptococcus* sp. The presence of bacteria in the placenta is also associated with adverse neurological outcomes, especially in those born preterm [117-120]. Most of the studies that assessed placental bacteria and neurocognitive outcomes used indicators of infection, such as chorioamnionitis, as opposed to directly testing the placenta for the presence of microorganisms, a gap that will be addressed in this study.

1.2 Study Objectives

For this study, we set out to examine neurocognitive and social-communicative function among school-age children in relation to placental bacteria. Previously within the ELGAN study it has been observed that children whose placenta harbored two or more organisms, compared to those with no or one organism, were at heightened risk of brain abnormalities detected by ultrasound and with forms of cerebral palsy two years later [131]. Specifically, the presence of *Ureaplasma urealyticum* was associated with increased risk of intraventricular hemorrhage and brain lesions in the white matter [130]. In the present study, we assess the neurological development of the ELGAN children at 10 years of age in relation to placental microbes. The goal of these analyses is to provide insights into whether microorganisms in the placenta can alter long-term neurocognitive development and social-communicative behavior.

1.3 Materials and Methods

1.3.1 ELGAN Study Recruitment and Participation

The recruitment process of the ELGAN study has previously been described [151]. Briefly, between 2002 and 2004, women who gave birth before 28 weeks gestational age at one

of the 14 ELGAN research sites in the United States were asked to participate in the study. The Institutional Review Boards at each of the 14 study sites approved all procedures. Informed, written consent was provided within a few days before or after delivery. The mother's consent covered both her and the child's participation in the study.

A total of 1,249 mothers and 1,506 infants enrolled in the study of which 1,365 placentas were collected and analyzed for microorganisms. When children reached 2 years of adjusted age, 1102 participated in a neurodevelopmental assessment [151]. At the 10 year follow-up, 889 children were enrolled and evaluated. Of these children, 807 had their placenta parenchyma cultured and analyzed for microorganisms. These 807 participants made up the subcohort we included in this study.

1.3.2 Demographic and Pregnancy Variables

Following delivery, a trained research nurse interviewed each mother in her native language using a structured data collection form and following procedures defined in a manual. The mother's report of her own characteristics and exposures, as well as the sequence of events leading to preterm delivery were taken as truth, even when her medical record provided discrepant information.

Shortly after the mother's discharge, the research nurse reviewed the maternal chart using a second structured data collection form. The medical record was relied on for events following admission. The clinical circumstances that led to preterm delivery were operationally defined using both data from the maternal interview and data abstracted from the medical record [152]. Each mother/infant pair was assigned to the category that described the primary reason for preterm delivery.

1.3.3 Newborn Variables

The gestational age estimates were based on a hierarchy of the quality of available information. Most desirable were estimates based on the dates of embryo retrieval or intrauterine insemination or fetal ultrasound before the 14th week (62%). When these were not available, reliance was placed sequentially on a fetal ultrasound at 14 or more weeks (29%), last menstrual period (LMP) without fetal ultrasound (7%), and gestational age recorded in the log of the neonatal intensive care unit (1%).

The birth weight Z-score is the number of standard deviations the infant's birth weight is above or below the median weight of infants at the same gestational age in referent samples not delivered for preeclampsia or fetal indications [128, 153].

1.3.4 Placenta Sample Collection

Women participating in the ELGANs study were asked to provide their placentas for analysis. The placenta collection technique is as follows: delivered placentas were placed in a sterile exam basin and transported to a sampling room where they were biopsied at the midpoint of the longest distance between the cord insertion and the edge of the placental disk. Using sterile technique, the amnion was pulled back to expose the chorion. Traction was applied to the chorion and a piece of the underlying trophoblast tissue was removed. The tissue was placed into a cryo-vial and immediately immersed into liquid nitrogen. Specimens were stored until processing at minus 80°C [154].

1.3.5 Bacterial Analysis of Placenta

Study placentas were biopsied following delivery and were assessed for microorganisms as described previously [154]. Briefly, a sterile scalpel was used to remove a section of each placenta. The placental tissue was homogenized in a phosphate buffered saline solution (PBS) and serial dilutions of the homogenate were made in PBS. Aliquots of the original homogenate

as well as the dilutions were plated onto selective and nonselective media, including: pre-reduced Brucella base agar, tryptic soy agar, chocolate agar, and A-7 agar. Following the incubation period various colony types were enumerated, isolated, and identified at the Brigham and Women's Microbiology Laboratory using estimated criteria [155]. Since the constituents of the chorion parenchyma in the ELGANs study prevent the reliable detection of bacterial DNA by polymerase chain reaction techniques, this study assessed placental colonization patterns obtained only by culture techniques.

1.3.6 Procedures for the Assessments at 10-years of Age

All families who participated in the 2 year follow up were contacted by mail and then by phone to invite them to participate in the 10 year follow up. Lost to follow up families were searched for on state vaccination registries, and other openly-available websites. Facebook was also used where approved by the local institution's IRB.

Families willing to participate were scheduled for one visit during which all of the measures reported here were administered in three to four hours, including breaks. The assessments were selected to provide the most comprehensive information about neurocognitive and academic function in one testing session (**Table 1**). While the child was tested, the parent or caregiver completed questionnaires regarding the child's medical and neurological status and behavior.

Table 1. Description of neurocognitive assessment variables at 10 years of age

Measure Type	Assessment Variable	Description
Neurocognitive Measures	Latent Profile Analysis (LPA)	Includes 9 variables that assess IQ and executive function and categorize children into four neurocognitive groups: normal, low-normal, moderately impaired, and severely impaired
	Oral and Written Language Scales (OWLS) Oral Composite	Expressive and receptive language skills
	Wechsler Individual Achievement Test-III (WIAT-III) Numerical Operations	Academic function in mathematics
	WIAT-III Word Recognition	Academic function in word recognition
Social-communicative measures	Social Responsiveness Scale (SRS)	Identifies social impairment associated with autism spectrum disorder (ASD)
	Children's Communication Checklist-2 (CCC-2) Pragmatic Language	Speech, vocabulary, sentence structure, and social language skills

1.3.6.1 Neurocognitive Outcomes

1.3.6.1.1 Cognitive Function Derived from Latent Profile Analysis (LPA)

This outcome variable was derived from latent profile analysis of participants'
performances across nine measures of IQ and executive function, described in detail elsewhere
[156]. IQ was assessed with the School-Age Differential Ability Scales-II (DAS-II) Verbal and
Nonverbal Reasoning scales [157]. Executive function included two subtests from DAS-II, DAS
Recall of Digits Backward and Recall of Sequential Order, which measured verbal working
memory [157], and five subtests from the NEPSY-II (A Developmental NEuroPSYchological
Assessment-II) [158]. The NEPSY-II Auditory Attention and Response Set measured auditory
attention, set switching and inhibition, the NEPSY-II Inhibition and Inhibition Switching
measured simple inhibition and inhibition in the context of set shifting, respectively, and the

NEPSY-II Animal Sorting measured visual concept formation and set shifting. The LPA identified four subgroups of study participants with similar cognitive profiles: normal, low-normal, moderately impaired, and severely impaired.

1.3.6.1.2 Oral and Written Language Scales (OWLS) Oral Composite

Expressive and receptive language skills were evaluated with the Oral and Written Language Scales (OWLS), which assess semantic, morphological, syntactic, and pragmatic production and comprehension of elaborated sentences [159]. The OWLS yields an oral composite score that includes both listening comprehension and oral expression. To correct for small differences in age at the time of assessment and to facilitate a comparison of our findings to those reported for term children we calculated Z-scores based on distributions of values reported for the historical normative samples that are described [159].

1.3.6.1.3 Academic Function

Academic function was measured with the Wechsler Individual Achievement Test-III (WIAT-III) Word Recognition and Numerical Operations subtests [160]. For these tests, we again used Z-scores based on distributions of values reported for the historical normative samples [160].

1.3.6.2 Social-Communicative Outcomes

1.3.6.2.1 Social Responsiveness Scale (SRS)

The SRS identifies social impairment associated with autism spectrum disorder (ASD) and quantifies its severity [161]. This 65-item instrument provides a total score reflecting severity of social deficits in the autism spectrum. Raw scores are converted to T-scores to account for gender and age differences [161].

1.3.6.2.1 Children's Communication Checklist-2 (CCC-2) Pragmatic Language

The Children's Communication Checklist-2 (CCC-2) was used to assess children's pragmatic language skills [162]. The child's pragmatic language ability is assessed with four CCC-2 subscales: Initiation, Scripted Language, Context, and Nonverbal Communication. For each child, we averaged the scaled scores for these four subtests to yield a CC-2 pragmatic language composite score.

1.3.7 Data Analyses

In order to determine whether placental microorganisms are associated with neurocognitive and social-communicative function at age 10, separate logistic regression models were performed for 15 bacteria species or groups assessed including: *Lactobacillus* sp., *Prevotella bivia*, *Gardnerella vaginalis*, anaerobic *Streptococcus*, *Peptostreptococcus* sp., *Escherichia coli*, alpha-hemolytic *Streptococcus*, *Ureaplasma urealyticum*, *Mycoplasma* sp., *Staphyloccocus* sp., *Propionibacterium* sp., *Actinomyces* sp., *Corynebacterium* sp., *Streptococcus* Group B, and *Streptococcus* Group D. Each model examined whether the presence of an individual bacterial species or bacterial type was associated with increased odds of scoring one or more standard deviations below the normative mean on five different assessments: OWLS Oral Language Composite, WIAT-III Word Recognition, WIAT-III Numerical Operations, SRS, and CCC-2 pragmatic language. In the case of LPA, the models examined whether the presence of an individual bacterial species or type was associated with increased odds of having moderate or severe cognitive impairment. Confounders included in the models were infant sex, gestational age, birth weight Z-score < -1, maternal education, antenatal steroids, and mother's eligibility for government-provided medical-care insurance. These models yielded odds ratios (ORs) and 95% confidence intervals (CI) of each 10-year characteristic associated with the microbial organisms recovered from the placenta. Bacterial species were considered to be significantly associated

with a neurological function if p-value <0.05 and the OR 95% CI did not include one. An organism was considered to be associated with an increased risk of preforming poorly on an assessment when the OR and 95% CI were above one. Conversely, a microorganism was considered to have a protective effect when the OR and 95% CI were below one.

1.4 Results

1.4.1 Study Subject Characteristics

The placentas of 807 of the 889 ELGAN subjects who participated in the 10-year follow-up assessment were analyzed for microbial presence. These 807 individuals represented those for whom microbial placental data were available and make up our subcohort for this study. The subcohort is similar to the overall 10-year cohort as is demonstrated by similarities in the percentages across variables (**Table 2**). Within the subcohort there are slightly more males than females (51% versus 49%). Most of the children were born between 25 and 26 weeks (44%) while 278 (34%) were born during the 27th week and 173 (21%) were born between 24 and 25 weeks. Sixty-five percent of mothers had private insurance; 35% had public insurance. Fourteen percent of mothers had completed 12 or fewer years of formal education, 48% completed more than 12 years but less than 16 years, and 35% completed 16 or more years. Ninety percent of mothers were treated with antenatal corticosteroids. Outcome data were missing from between 5% for OWLS Oral Language Composite scores and 2% for the WIAT-III Numerical Operation score and the LPA.

Table 2. Demographics

	Overall 10-year follow up (n=889) N (%)	Subcohort of 10-year participants with placenta microbiology (n=807) N(%)
Fetal Sex		
Male	455 (51.2)	414 (51.3)
Female	434 (48.8)	393 (48.7)
Gestational Age (weeks)		
24-25	187 (21.0)	173 (21.4)
25-26	400 (45.0)	356 (44.1)
27	302 (34.0)	278 (34.4)
Birth weight (Z-score)		
< -2	53 (6.0)	48 (5.9)
≥ -2, < -1	120 (13.5)	107 (13.3)
≥ -1	716 (80.5)	652 (80.8)
SES (insurance)		
Public	307 (34.5)	271 (33.6)
Private	568 (63.9)	524 (64.9)
NS	14 (1.6)	12 (1.5)
Maternal Education, years		
≤ 12 (high school)	126 (14.2)	112 (13.9)
Some College or Associates Degree	431 (48.5)	387 (48.0)
College or Higher	306 (34.4)	284 (35.2)
NS	26 (2.9)	24 (3.0)
Antenatal corticosteroids		
Yes	788 (88.6)	723 (89.6)
No	100 (11.2)	84 (10.4)
NS	1 (0.1)	0 (0)
LPA		
Yes	874 (98.3)	792 (98.1)
No	15 (1.7)	15 (1.9)
OWLS oral composite		
Yes	849 (95.5)	771 (95.5)
No	40 (4.5)	36 (4.5)
WIAT-III word recognition		
Yes	864 (97.2)	783 (97.0)
No	25 (2.8)	24 (3.0)
WIAT-III numerical operations		
Yes	874 (98.3)	792 (98.1)
No	15 (1.7)	15 (1.9)
SRS		
Yes	866 (97.4)	787 (97.5)
No	23 (2.6)	20 (2.5)
CCC-2 Pragmatic Language		
Yes	854 (96.1)	775 (96.0)
No	35 (3.9)	32 (4.0)

NS = Not Specified

The most common bacteria present in the study placentas was *Staphylococcus* sp. which was detected in 94 (11.6%) placentas (**Table 3**). The least prevalent bacterial species detected was *G. vaginalis*, which was present in 28 (3.5%) placentas. The remaining bacterial species were found in between 36 (4.5%), in the case of *Streptococcus* Group D, and 64 (7.9%), in the case of *Corynebacterium* sp., placentas. This includes *U. urealyticum*, *Lactobacillus* sp., *E. coli*, and alpha-hemolytic *Streptococcus* which were detected in 43 (5.3%), 48 (5.9%), 49 (6.1%), and 53 (6.6%), respectively.

Table 3. Presence of microorganisms in the placenta

Bacteria	n (%)
Lactobacillus sp.	48 (5.9)
P. bivia	41 (5.1)
G. vaginalis	28 (3.5)
Anaerobic *Streptococcus*	40 (5.0)
Peptostreptococcus sp.	49 (6.1)
E. coli	49 (6.1)
Alpha *Streptococcus*	53 (6.6)
U. urealyticum	43 (5.3)
Mycoplasma sp.	42 (5.2)
Propionibacterium sp.	55 (6.8)
Actinomyces sp.	47 (5.8)
Corynebacterium sp.	64 (7.9)
Staphylococcus sp.	94 (11.6)
Streptococcus Group B	38 (4.7)
Streptococcus Group D	36 (4.5)

1.4.2 Association of Neurocognitive and Social-Communicative Function at Age 10 in ELGAN Placentas Exposed to Bacteria

Out of the four neurocognitive assessments analyzed, three (WIAT-III Numerical Operations, OWLS Oral Language Composite, and LPA of IQ and EF) were associated with statistically significant differential odds in relation to at least one type of microorganism. Neither

of the social-communicative assessments displayed differential odds in relation to any of the 15

microbial species.

1.4.2.1 WIAT-III Numerical Operation

For five of the 15 microorganisms detected, bacterial presence in the placenta was

associated with increased odds of scoring one or more standard deviations below the normative

mean on the WIAT-III Numerical Operations assessment (**Figure 3**). The strongest association

was found with *U. urealyticum* (OR, 95% CI: 2.21, 1.16 – 4.26). Other bacteria associated with a

low score on the WIAT-III Numerical Operations test were *E. coli* (OR, 95% CI: 1.94, 1.04 –

3.65), alpha-hemolytic *Streptococcus* (OR, 95% CI: 1.88, 1.03 – 3.45), *Corynebacterium* sp.

(OR, 95% CI: 1.88, 1.08 – 2.68), and *Staphylococcus* sp. (OR, 95% CI: 1.67, 1.04 – 2.68).
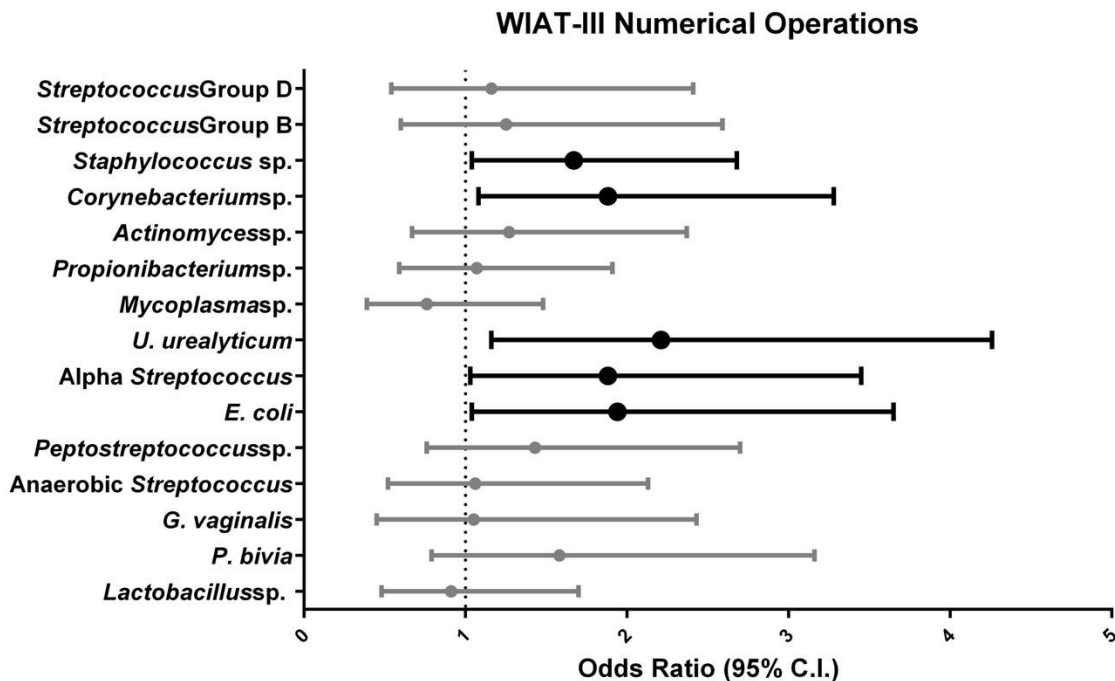
Figure 3. Odds ratios and 95% confidence intervals for WIAT-III Numerical Operations in relation to placental microorganisms. The forest plot displays ORs and 95% confidence intervals of a Z-score ≤ -1 on the WIAT-III Numerical Operations subtest at age 10 associated with the isolation of 16 bacterial species (y-axis). These odds ratios are adjusted for fetal sex, gestational age, birth weight Z-score, maternal education, public insurance, and antenatal corticosteroid use.

1.4.2.2 OWLS Oral Language Composite

For three of the 15 microorganisms in the placenta, bacterial presence was associated with increased odds of scoring one or more standard deviations below the normative mean on the OWLS Oral Language Composite assessment (**Figure 4**). The strongest association was for *G. vaginalis* (OR, 95% CI: 3.20, 1.22 – 9.99). The other two bacteria associated with a low score on the OWLS were *U. urealyticum* (OR, 95% CI: 2.38, 1.22 – 4.85) and *Staphylococcus* sp. (OR, 95% CI: 1.73, 1.07 – 2.82). The presence of *Lactobacillus* sp. was associated with decreased odds of a low score on the OWLS (OR, 95% CI: 0.5, 0.25 – 0.96).
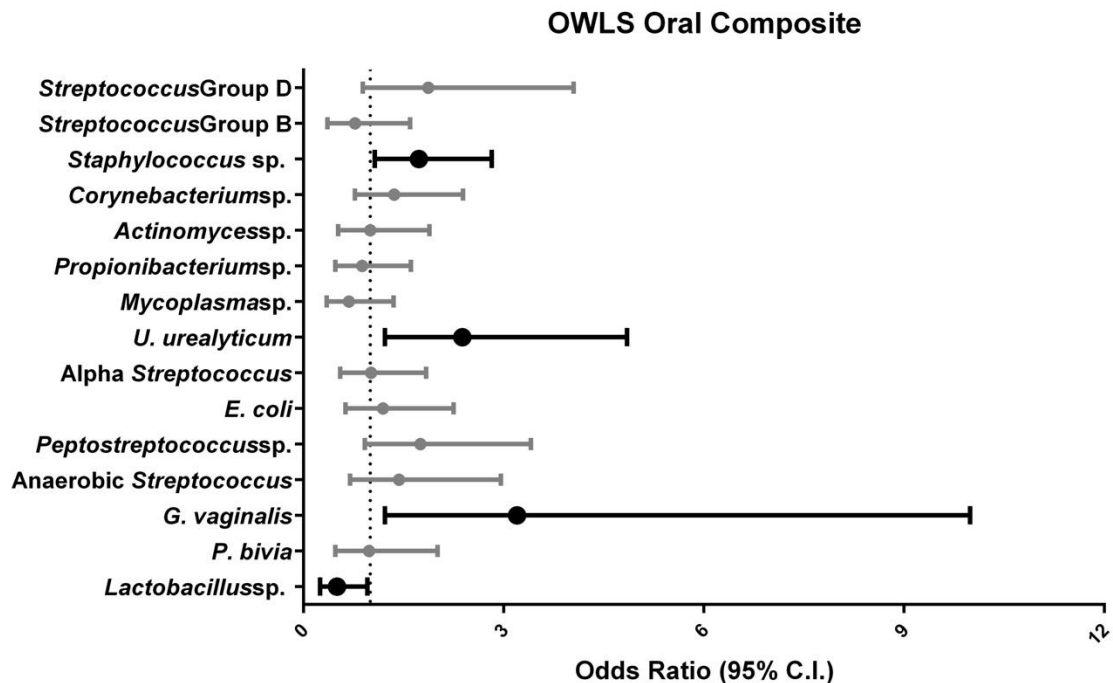
Figure 4. Odds ratios and 95% confidence intervals for OWLS Oral Composite in relation to placental microorganisms. The forest plot displays ORs and 95% confidence intervals of a Z-score ≤ -1 on the OWLS Oral Composite score at age 10 associated with the isolation of 16 bacterial species (y-axis). These odds ratios are adjusted for fetal sex, gestational age, birth weight Z-score, maternal education, public insurance, and antenatal corticosteroid use.

1.4.2.3 LPA

The presence of *Lactobacillus* sp. in the placenta was associated with decreased odds (OR, 95% CI: 0.27, 0.09 - 0.67) of having moderate or severe impairment as determined by LPA (**Figure 5**). The other 14 microorganisms were not associated with LPA outcomes.

LPA
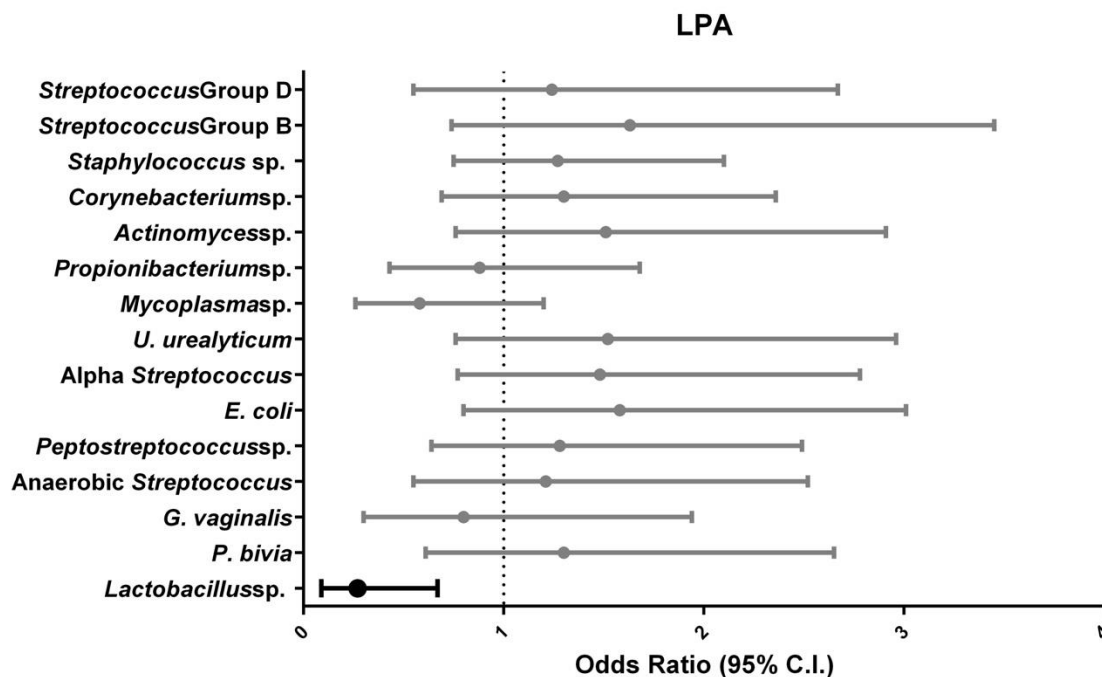
Figure 5. Odds ratios and 95% confidence intervals for LPA in relation to placental microorganisms. The forest plot displays ORs and 95% confidence intervals of a LPA subgrouping of severely or moderately impaired at age 10 associated with the isolation of 16 bacterial species (y-axis). These odds ratios are adjusted for fetal sex, gestational age, birth weight Z-score, maternal education, public insurance, and antenatal corticosteroid use.

1.4.3 *Lactobacillus* Interaction Analysis

In our analysis, *Lactobacillus* sp. was associated with lower odds of scoring low on the OWLS Oral Language Composite and lower odds of having moderate to severe cognitive impairment as characterized by LPA. Thus, we added an interaction term in the model between *Lactobacillus* sp. and the other 14 microorganisms to evaluate whether the effect of the interaction would significantly affect the ORs of our outcomes. However, we did not observe any significant effects in this analysis.

1.5 Discussion

In this study, several species of bacteria found in the placenta of children born extremely preterm were associated with neurocognitive impairments at 10 years of age. We demonstrate that bacteria associated with one or more adverse neurocognitive outcomes included *U. urealyticum,* alpha-hemolytic *Streptococcus*, *Corynebacterium* sp., *G. vaginalis* and *Staphylococcus* sp*.* In contrast*,* the presence of *Lactobacillus* sp. was associated with a lower risk of two adverse neurodevelopmental outcomes, general cognitive impairment and language deficit. Interestingly, bacterial presence in the placenta had no significant effects on social-communicative function.

Intrauterine and neonatal infection are strongly associated with negative birth outcomes, such as preterm birth [7], and also neurodevelopmental impairment [163, 164]. In the present study, infection of the intrauterine environment with *U. urealyticum* was found to be associated with deficits in language and mathematics. This same bacteria was previously associated with an inflammatory response in the chorioamnion, or chorioamnionitis [36, 165], as well as in the amniotic fluid, cord blood, and fetal tissues [166, 167]. Providing further support for the present findings, the presence of *U. urealyticum* in the amniotic fluid has been found to be predictive of neuromotor delays at age 2 in a preterm birth cohort study [168]. Within the ELGAN cohort, *U. urealyticum* in the placenta has been associated with fetal and maternal inflammation as well as white matter damage [130]. In addition to the findings with *U. urealyticum*, in the current study *E. coli* was associated with increased risk of performing poorly on WIAT-III Numerical Operations. This is interesting as in prior studies, exposure to *E. coli* increased the likelihood of white matter damage [169, 170]. White matter damage in newborns is predictive of a range of adverse neurodevelopmental outcomes including cerebral palsy, autism spectrum disorder, and psychiatric disorders [125, 171-173], but has yet to be evaluated as an antecedent of learning

limitations in children born very preterm. In terms of a mechanism linking bacterial presence to neurodevelopmental outcomes, microorganisms induce cytokine production in trophoblasts in both cell culture and in mouse models [21, 22]. Inflammation can damage the developing fetal brain [9, 16, 18, 20] and has been associated with neurological delays later in life [29, 31, 32].

In contrast to the other microorganisms, *Lactobacillus* sp. was associated with a decreased risk of oral language and general cognitive impairment, as measured by LPA. In certain contexts, *Lactobacillus* sp. has an anti-inflammatory effect; either by inhibiting NF-κB [174], a pro-inflammatory pathway, or by inducing the production of interlukin-10, an anti-inflammatory cytokine, in trophoblast cells [175]. In the ELGAN cohort, the presence of *Lactobacillus* sp. in the placenta was associated with a lower likelihood of neonatal systemic inflammation [23]. Analyses to determine to what extent the presence of *Lactobacillus* sp. counteracts the effect of the other microorganisms in the placenta did not detect an interaction, perhaps because only a small number of placentas had *Lactobacillus* sp. present along with each of the other microbial types. For example, in only 1.4% of placentas was *Lactobacillus* sp. accompanied by *Staphylococcus* sp, the species found most often in our sample of placenta. No placentas harbored *Lactobacillus* sp. and either *U. urealyticum* or *Streptococcus* Group B.

Few studies have focused on the question of whether specific microorganisms in the placenta are associated with neurocognitive and social-communicative outcomes later in life. The majority of studies on this topic have used indicators of infection, such as chorioamnionitis and fetal vasculitis, which are typically associated with genital mycoplasmas [166, 176, 177], to quantify placental infection [120, 121] instead of testing the placenta for specific microorganisms, as we did in this study. Previous ELGAN work considers specific bacterial

species in the placenta in relation to short-term neurological outcomes at age 2 [129, 131]. However, the long-term outcomes have not been previously studied.

While we have identified associations between microbes in the placenta and neurocognitive outcomes our study is not without limitations. There was a relatively low prevalence of each individual bacterium, limiting the power to detect associations. Nevertheless, 387 (48%) of the placentas in this study harbored at least one type of microorganism, which is consistent with findings reported by others [46]. Another limitation was that detection of placental microbes was based on culture techniques. Therefore, only specific bacteria were identified. Other bacterial species were likely present in the placenta samples. Future research could include a microbiome assessment to further evaluate non-culturable bacteria present in placentas and their associations with birth outcomes. Strengths of this study are the large sample size, the broad range of assessments of neurocognitive and academic achievement, and the blinding of individuals who performed the neurodevelopmental assessments to information about placental microorganisms.

In summary, there were three major findings from this study. First, the presence of several different types of microorganisms in the placenta was associated with increased risk of learning limitations at age 10 among individuals born extremely preterm. Second, with importance to identification of potential intervention-based research, *Lactobacillus* sp. was associated with a lower risk of these learning limitations. Finally, while placental microorganisms were associated with altered risk of neurocognitive outcomes they did not show an association with social-communicative function including autism. These results are relevant to the study of prenatal factors that influence neurocognitive function of children later in life.

CHAPTER 2: MICROORGANISMS IN THE HUMAN PLACENTA ARE ASSOCIATED WITH ALTERED CPG METHYLATION OF IMMUNE AND INFLAMMATION-RELATED GENES

2.1 Overview

The placenta is a critical regulator of the prenatal environment and is essential for a healthy pregnancy and fetal development. It transports nutrients from mother to fetus and produces hormones necessary to maintain pregnancy and support the fetus [148]. The placenta can also harbor bacterial communities that, depending on their composition, affect pregnancy outcomes and fetal health [7, 46]. Previously it was thought that all bacteria in the placenta originate from infections of the lower genital tract [7], however, a number of studies have found that bacteria in the placenta are derived from vaginotropic non-infectious microflora [154, 178]. In addition, bacteria derived from other tissues may contribute to the placental microbiome, such as the oral cavity [10]. For example, it has been proposed that oral bacteria can translocate to the placenta by hematogenous transmission [44].

Importantly, certain bacteria in the placenta have been associated with deleterious pregnancy outcomes including preterm birth [7]. Among these placental bacteria are *Ureplasma urealyticum*, *Mycoplasma hominis*, *Gardnerella vaginalis*, and *Peptostreptococcus* sp., which are all associated with bacterial vaginosis, a disruption in the vaginal microbiota. Other placental bacteria, Group B *Streptococcus* and *Escherichia coli*, are associated with chorioamnionitis and fetal infection [7]. Little is known about the molecular responses to microorganisms in the placenta that potentially alter pregnancy and fetal outcomes.

The placental epigenome, particularly DNA methylation, is a potential mechanism that could explain this association and has not been explored. Epigenetic changes, such as DNA methylation, have been associated with bacteria in other tissues including *Helicobacter pylori* in the gastric mucosa [70, 179, 180], uropathogenic *Escherichia coli* in uroepithelial cells [69] and *Campylobacter rectus* in murine placental tissue [24]. In human placental cell culture, the presence of bacteria has been associated with both pro- and anti- inflammatory responses mediated by cytokines [21]. With an intriguing potential for sustained inflammation, our team showed in the Extremely Low Gestation Age Newborns (ELGAN) cohort that vaginotropic placental bacteria were associated with distinct inflammatory protein profiles in newborn blood with *Lactobacillus* sp. being anti-inflammatory and bacterial vaginosis-associated bacteria being pro-inflammatory [23]. These varying inflammatory responses induced by placental bacteria might be driven by CpG methylation in the placenta which could impact both placental function and fetal well-being.

2.2 Study Objectives

To our knowledge, this is among the first studies to assess placental CpG methylation in relation to placenta bacteria. Here we investigated whether placental microbes were associated with altered placental DNA cytosine proximal to guanine (CpG) methylation patterns in the ELGAN cohort. The goal of these analyses was to provide insights into how microorganimsms in the placenta alter the placental methylome and could thereby influence pregnancy and neonatal health outcomes.

2.3 Materials and Methods

2.3.1 ELGANs Study Subject Recruitment

The recruitment process for the ELGAN study has been described in detail [151]. Briefly, between 2002 and 2004, we invited women who gave birth before 28 weeks gestational age at

one of the 14 hospitals in 5 states in the United States to participate in the study. A total of 1,249 mothers and 1,506 infants enrolled in the study of which 1,365 placentas were collected and analyzed for microorganisms. A subcohort of 84 mother/infant pairs with similar average gestational and maternal age as the overall cohort were investigated in the present study (**Table 4**).

Table 4. Demographics

	Overall with placenta microbiology (n=1365) N (%)	Subcohort (n=84) N (%)
Fetal Sex		
Male	732 (54%)	58 (69%)
Female	633 (46%)	26 (31%)
Gestational Age (weeks)	25.9 (23.0-27.9)	25.9 (23.7-27.9)
Maternal Age (years)	28.6 (13.2-47.3)	28.6 (16.0-40.6)
Maternal Race		
White	785 (58%)	38 (45%)
Non-white	562 (41%)	46 (55%)
NS	18 (1%)	0 (0%)
Delivery Method		
C-section	877 (64%)	61 (73%)
Natural	488 (36%)	23 (27%)
SES (insurance)		
Public	485 (36%)	35 (42%)
Private	741 (54%)	46 (55%)
Public & Private	46 (3%)	2 (2%)
Self-Pay Only	11 (1%)	1 (1%)
None	20 (1%)	0 (0%)
NS	62 (5%)	0 (0%)
UTI		
No	1090 (80%)	70 (83%)
Yes	203 (15%)	11 (13%)
NS	72 (5%)	3 (4%)
Vaginal Infection		
No	1120 (82%)	65 (77%)
Yes	173 (13%)	16 (19%)
NS	72 (5%)	3 (4%)
Antibiotic Use		
No	883 (65%)	61 (73%)
Yes	403 (30%)	19 (23%)
NS	79 (6%)	4 (5%)

NS = Not Specified

The Institutional Review Boards at all 14 ELGAN study sites approved all procedures. Informed, written consent was provided within a few days of delivery, either before or after. The mother's consent covered both her and the child's participation in the study.

2.3.2 Bacterial Analysis of Placenta

The placentas were biopsied as soon as possible after delivery and were assessed for microorganisms as described [154]. Briefly, a section of each placental specimen was removed using a sterile scalpel and homogenized in a phosphate buffered saline solution (PBS). Serial dilutions of the homogenate were made in PBS and aliquots of the original homogenate and the dilutions were plated onto selective and nonselective bacteriologic media, which included: prereduced Brucella base agar, tryptic soy agar, chocolate agar, and A-7 agar. Following incubation the various colony types were enumerated, isolated, and identified at the Brigham and Women's Microbiology Laboratory using established criteria [155]. Since we have determined the constituents of the chorion parenchyma in the ELGANs prevent the reliable detection of bacterial DNA by PCR techniques, this study assessed only placental colonization patterns obtained by culture techniques.

2.3.3 DNA Extraction and Assessment of DNA Methylation

DNA extraction and assessment of DNA methylation by taking a 0.2 g subsection of placental tissue was cut from the frozen biopsy of dry ice, washed with sterile 1X PBS to remove residual blood, and homogenized with B-mercaptoethanol in Buffer RLT (Qiagen, Valencia, CA). DNA and RNA sequences with 18 or more nucleotides in length were collected using the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Valencia, CA) following the manufacturer's instructions. CpG methylation was assessed using the Illumina Human Methylation450 BeadChip© array (Illumina, Inc., San Diego, CA). This technology asses the DNA methylation levels of 486,428 individual probes at single nucleotide resolution. Isolated DNA was bisulfate-

converted using the EZ DNA methylation kit (Zymo Research, Irvine, CA) and converted DNA was hybridized onto the array. The DNA methylation data was collected at Expression Analysis, Inc. (Durham, NC; www.expressionanalysis.com).

Methylation levels were calculated and expressed as β values (β = intensity of the methylated allele (M) / (intensity of the unmethylated allele (U) + intensity of the methylated allele (M) + 100) [181]. Batch effect was not a significant source of variation, as determined by principle component analysis (PCA). Based on manufacturer recommendations, probes that had high detection P-values (P>0.1) were removed from analysis (n=24,591). Following data filtration, data were normalized using the beta-mixture quantile (BMIQ) normalization method using the wateRmelon package (version 1.11.0) in R (version 3.2.3). After normalization, probes that were annotated as single nucleotide polymorphisms (SNPs) by Illumina were removed from further analysis (n=84,121), leaving a total of 377,716 probes, representing 20,418 genes. The rationale for this exclusion is that SNP variation can lead to false readings of DNA methylation signals that may be attributed to genomic variation rather alterations in actual methylation levels [182].

2.3.4 Statistical Analysis

In order to determine whether the placental microbiome is associated with differences in DNA methylation patterning of the placenta, Analysis of Covariance (ANCOVA) was performed. There were 16 bacterial species assessed including: *Lactobacillus* sp., *Prevotella bivia*, *Gardnerella vaginalis*, anaerobic *Streptococcus*, *Peptostreptococcus* sp., *Escherichia coli*, alpha-hemolytic *Streptococcus*, *Ureaplasma urealyticum*, *Mycoplasma* sp., *Staphyloccocus* sp., *Propionibacterium* sp., *Actinomyces* sp., *Corynebacterium* sp., *Staphylococcus aureus*, *Streptococcus* Group B, and *Streptococcus* Group D. Each model examined whether presence or

absence of an individual bacterial species or bacterial type was associated with differential placental methylation at any of the 377,716 probes. In the present study, variables were classified as confounding if they displayed an association with both the exposure, placental microorganisms, and the outcome, DNA methylation. Models were adjusted based on several dichotomous variables including fetal-sex, maternal race, whether the mother took an antibiotic during pregnancy, whether the mother experienced a vaginal infection during pregnancy, whether the mother experienced a urinary tract infection (UTI) during pregnancy, and whether the birth occurred via C-section. As there is no evidence that maternal age is associated with the presence of microorganisms in the placenta, it was not included as a confounding variable. Bacterial species were considered to be significantly associated with methylation if p-value <0.05 and the false discovery rate-corrected p-value <0.1 for any given probe. Each bacterial species was modeled individually and data analysis was carried out using Partek Genomic Suites 6.6.

2.3.5 Gene Set-Based Analysis

The differentially methylated probes and corresponding genes were analyzed to determine if they were enriched for specific biological functions. Four gene sets were analyzed where gene content was established using www.uniprot.org. The four gene sets that were tested were selected for their known roles in fetal development: transport-related genes (n=3,704) [183], immune-related genes (n=1,622) [183-185], inflammation-related genes (n=410) [184, 186] and growth/transcription factor-related genes (n=2030) [187-189]. A χ^2 test was used to identify enrichment of key processes. Once the enriched processes were identified a right-tailed Fisher Exact test (α=0.05) was conducted on the significant genes in the enriched pathways to

identify enriched canonical pathways and transcription factors using Ingenuity Pathway Analysis software as described in Martin *et al.*, 2015 [190].

2.4 Results

2.4.1 Study Subject Characteristics

The placentas of 84 subjects within the ELGAN cohort were analyzed for this study. The average gestational age and maternal age of the subcohort of 84 are the same as the overall cohort (n=1,365) (**Table 4**). There are some differences between the subcohort and the overall cohort, including maternal race and sex of the infant. The overall cohort is predominately white (58%) while the subcohort is 45% white and 55% non-white. The majority of the infants in both the overall cohort and subcohort are males. However, the percentage of males in the subcohort (69%) is much larger than the percentage in the overall cohort (45%). Of the 84 infants, 23 (27%) were delivered vaginally, while 61 (73%) were delivered by Cesarean section. Of these, 37 (44%) were on public insurance and 46 (55%) were on private insurance. A total of 11 (13%) of the women had a urinary tract infection, 16 (19%) had a vaginal infection, and 19 (22%) used an antibiotic during pregnancy.

The most common bacteria present in the study placentas was *Propionbacterium* sp. which was detected in 10 (11.9%) placentas (**Table 5**). This was followed closely by *Staphylococcus aureus* which was detected in seven (8.3%) placentas. *Ureaplasma urealyticum* was detected in five (6.0%) and *Actinomyces* was detected in four (4.8%). *Lactobacillus* sp. was detected in three (3.6%) of placentas and *Escherichia coli* was detected in two (2.4%) placentas. The majority of the microorganisms were present in two to three placentas and 44 (52%) of the placentas had no detectable microorganisms.

Table 5. Presence of microorganisms in the placenta and methylation of CpG probes and associated genes

Bacteria	n (%)	#	# HyperM[1] N (%)	# HypoM[2] N (%)	#	# HyperM[1] N (%)	# HypoM[2] N (%)
			CpG Probes			Genes	
Lactobacillus sp.	3 (3.6)	44	10 (23)	34 (77)	32	6 (19)	26 (81)
P. bivia	3 (3.6)	63	13 (21)	50 (79)	49	12 (24)	37 (76)
G. vaginalis	3 (3.6)	53	16 (30)	37 (70)	43	11 (26)	32 (74)
Anaerobic *Streptococcus*	4 (4.8)	0	--	--	0	--	--
Peptostreptococcus sp.	3 (3.6)	13	1 (8)	12 (92)	11	1 (9)	10 (91)
E. coli	2 (2.4)	53	24 (45)	29(55)	24	6 (25)	18 (75)
Alpha *Streptococcus*	3 (3.6)	167	35 (21)	132 (79)	105	13 (12)	92 (88)
U. urealyticum	5 (6.0)	21	11 (52)	10 (48)	11	6 (55)	5 (45)
Mycoplasma sp.	9 (10.7)	0	--	--	0	--	--
Staphylococcus sp.	7 (8.3)	40	25 (63)	15 (37)	22	8 (36)	14 (64)
Propionibacterium sp.	10 (11.9)	2	2 (100)	0 (0)	1	1 (100)	0 (0)
Actinomyces sp.	4 (4.8)	2	0 (0)	2 (100)	1	0 (0)	1 (100)
Corynebacterium sp.	3 (3.6)	24	2 (8)	22 (92)	18	1 (6)	17 (94)
Staphylococcus aureus	7 (8.3)	40	25 (63)	15 (37)	22	8 (36)	14 (64)
Streptococcus Group B	2 (2.4)	1,257	28 (2)	1,229 (98)	802	12 (1)	790 (99)
Streptococcus Group D	2 (2.4)	206	128 (62)	78 (38)	112	54 (48)	58 (52)

1 HyperM=Hypermethylated
2 HypoM=Hypomethylated

2.4.2 Identification of Differentially Methylated Probes in ELGAN Placentas Exposed to Bacteria

Placental CpG methylation differences were analyzed within the ELGAN cohort between 84 individuals with or without placental microbes for 377,716 CpG probes representing 20,418 genes. Statistical significance was adjusted using false discovery rate-corrected p-values (q<0.1). Of the 16 microbial species all but two, namely anaerobic *Streptococcus* and *Mycoplasma* sp., were associated with differentially methylated probes. A total of 1,789 probes, corresponding to 1,079 genes, were significantly differentially methylated between placentas that harbored bacteria and placentas that did not. The probes that displayed differential methylation were unique depending on which bacteria was present (**Figure 6**).
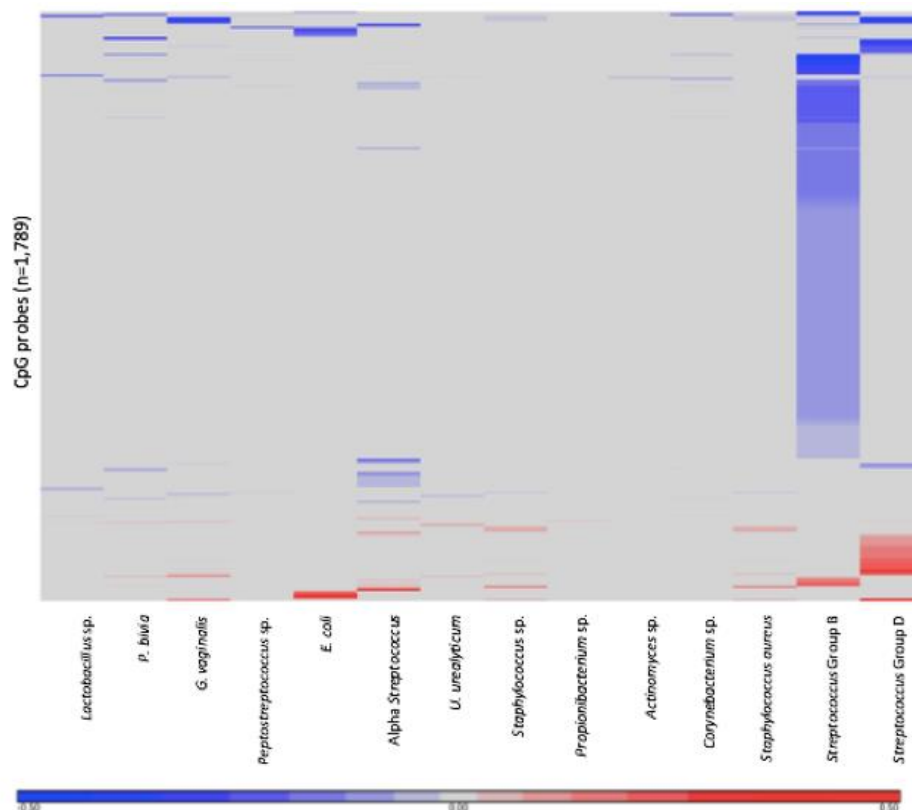
Figure 6. Heatmap of differentially methylated CpG probes corresponding to each of the microorganisms. The heatmap displays the 1,789 differentially methylated probes in relation to 17 microorganisms. Red represents increased methylation and blue represents decreased methylation.

The microbial species that was associated with the greatest number of altered CpG sites was *Streptococcus* Group B with 1,257 differentially methylated CpG probes (n=802 genes) (**Table 5**). The methylation pattern for *Streptococcus* Group B leans heavily toward hypomethylation with 98% (1,232) of the sites being hypomethylated.

Following *Streptococcus* Group B, the two microbial species that were associated with the most differentially methylated probes were *Streptococcus* Group D and alpha-hemolytic *Streptococcus* with 206 and 167 CpG probes, respectively. Most of the probes altered in relation to *Streptococcus* Group D were hypermethylated (62%) while alpha-hemolytic *Streptococcus*-associated probes were mostly hypomethylated (79%). The CpG probes correspond to 112 genes for *Streptococcus* Group D and 105 genes for alpha-hemolytic *Stretptococcus*. The presence of *E.coli* was associated with 53 CpG probes representing 43 different genes. Of those probes 55% were hypomethylated.

2.4.3 Location of Differentially Methylated Probes

The majority of the significant CpG probes for all 14 bacterial species were located in the body of the gene (**Table 6**). Both of the probes associated with *Propionibacterium* sp. were located in the gene body. *Peptostreptococcus* sp. had the next highest percentage of differentially methylated probes in the gene body at 62%, while *Actinomyces* sp. had the lowest percentage at 22%. There were also a large percentage of probes that did not correspond with a gene and therefore do not have a gene location. The remaining probes fell into the 1st exon, 3'UTR, 5'UTR, TSS1500, and TSS200 locations.

Table 6. Gene-specific location of CpG methylation

Bacteria	1st exon N (%)	3'UTR N (%)	5'UTR N (%)	Body N (%)	TSS1500 N (%)	TSS200 N (%)	N/A N (%)	# probes
Lactobacillus sp.	2 (5)	2 (5)	0 (0)	26 (59)	3 (6)	2 (5)	9 (20)	44
P. bivia	0 (0)	4 (6)	3 (5)	28 (44)	7 (11)	8 (13)	13 (21)	63
G. vaginalis	0 (0)	5 (9)	5 (9)	31 (59)	2 (4)	3 (6)	7 (13)	53
Peptostreptococcus sp.	0 (0)	0 (0)	0 (0)	8 (62)	2 (15)	1 (8)	2 (15)	13
E. coli	1 (2)	2 (4)	3 (6)	12 (22)	10 (19)	4 (7)	21 (40)	53
Alpha *Streptococcus*	8 (5)	8 (5)	10 (6)	72 (43)	17 (10)	11 (6)	41 (25)	167
U. urealyticum	0 (0)	0 (0)	5 (24)	6 (28)	0 (0)	5 (24)	5 (24)	21
Staphylococcus sp.	1 (3)	3 (7)	2 (5)	14 (35)	6 (15)	3 (7)	11 (28)	40
Propionibacterium sp.	0 (0)	0 (0)	0 (0)	2 (100)	0 (0)	0 (0)	0 (0)	2
Actinomyces sp.	0 (0)	0 (0)	0 (0)	1 (50)	0 (0)	0 (0)	1 (50)	2
Corynebacterium sp.	0 (0)	1 (4)	0 (0)	13 (54)	3 (13)	1 (4)	6 (25)	24
Staphylococcus aureus	1 (3)	3 (7)	2 (5)	14 (35)	6 (15)	3 (7)	11 (28)	40
Streptococcus Group B	33 (3)	50 (4)	107 (9)	547 (43)	148 (12)	66 (5)	306 (24)	1,257
Streptococcus Group D	9 (4)	14 (7)	22 (11)	56 (27)	33 (16)	34 (17)	38 (18)	206

2.4.4 Enrichment of Biological Functions and Pathways Among the Differentially Methylated Gene Sets

We further analyzed whether these differentially methylated probes in the placenta were enriched for four specific biological functions: inflammation, growth/transcription factors, transport, and/or immune response, selected for their known critical functions for fetal development. For this analysis, the unique genes that corresponded with differentially methylated probes were considered (n=1,080). A Yates corrected χ^2 test was used in conjunction with a right-tailed Fisher Exact test (α=0.05) to determine that immune-related proteins (n=50, p=0.000075), transcription/growth factors (n=134, p=0.00173), and inflammatory response (n=35, p=1.27E-09) were enriched amongst the CpG sites demonstrating differential methylation associated with bacterial presence (**Supplemental Table 1**).

Among the 50 immune-related genes, Nuclear Factor Kappa-light-chain-enhancer of activated B Cells (NF-κB) signaling was the top canonical pathway (p=5.98E-05)

(**Supplemental Table 1**). Five of the immune-related genes are involved in NF-κB signaling including two kinases, Bone Morphogenetic Protein Receptor Type 1A (*BMPR1A*) and Protein Kinase C Zeta (*PRKCZ*), two enzymes, TNF Alpha Induced Protein 3 (*TNFAIP3*) and Ubiquitin Conjugating Enzyme E2 N (*UBE2N*), and one transmembrane receptor, Insulin Like Growth Factor 1 Receptor (*IGF1R*). The CpG probes corresponding to these five genes were all hypomethylated. All five of the genes had at least one of their hypomethylated probes altered when *Streptococcus* Group B bacteria was present in the placenta. Alpha-hemolytic *Streptococcus* was associated with a hypomethylated probe for *BMPR1A* and *PRKCZ* while *IGF1R* had probes altered with the presence of *P. bivia*. Based on the transcription factor occupancy theory, which is a proposed mechanism of genome-wide patterning of DNA methylation [68], we identified the transcription factors of the immune-related genes that were enriched. The top five transcription factors were: Tripartite Motif Containing 24 (*TRIM24)* (p=7.27E-05), RELA Proto-Oncogene NF-κB Subunit (*RELA*) (p=2.54E-04), Tumor Protein P53 (*TP53*) (p=3.18E-04), Core-binding Factor Beta Subunit (*CBFB*) (p=6.58E-04), and POU Class 5 Homeobox 1 (*POU5F1*) (p=7.82E-04) (**Supplemental Table 2**).

2.5 Discussion

The presence of microorganisms in the placenta has been associated with inflammation and negative birth outcomes, including preterm birth [7]. Intriguing data from mouse studies and cell culture suggest that bacteria in the placenta can alter CpG methylation [21, 24]. To evaluate whether the presence of microorganisms is associated with the human placental epigenome, we integrated data for 16 different microbial species in the placenta with genome-wide CpG methylation levels. A total of 1,789 probes, representing 1,079 genes, were identified that were differentially methylated (q<0.1) in relation to placental bacteria. Interestingly, each bacteria

type corresponded with a distinct CpG methylation pattern within the placenta. The genes that corresponded to the differentially methylated probes were enriched for their roles as growth/transcription factors, immune response proteins, and inflammatory response proteins and are active in the NF-κB pathway. Interestingly, the NF-κB pathway is critical during pregnancy and for fetal development [191, 192]. Overall, our findings demonstrate that the presence of microorganisms in the placenta is associated with differences in CpG methylation. The specific genes with altered methylation could represent etiologic factors that contribute to placental function and fetal health.

The data from the study show that 14 of the bacteria species were associated with unique CpG probes, with not much overlap between the bacterial types. The three *Streptococcus* sp. displayed the most differentially methylated CpG probes. The presence of *Streptococcus* Group B was associated with the most differentially methylated probes, corresponding to 802 genes. *Streptococcus* Group D and α-*Streptococcus* followed with 206 probes (112 genes) and 167 probes (105 genes), respectively. *Propionibacterium* sp. was the most prevalent bacteria present in 10 placentas. The majority of the differentially methylated probes for each placental bacterium, were hypomethylated, meaning they displayed decreased methylation levels at a specific probe in relation to microbes. The fact that there is little overlap between the CpG probes that are differentially methylated for each bacterium shows a diverse placental epigenetic response depending on which bacterial species is present.

In the present analysis, the NF-κB pathway was enriched among the microorganism-associated differentially methylated genes. The NF-κB pathway is a pro-inflammatory signaling pathway that is activated by pathogens or stressors [193]. It has been shown that bacterial plasmid DNA activates a signaling cascade that leads to activation of the NF-κB pathway and

expression of inflammatory genes [194]. There is also accumulating evidence that NF-κB

activity increases with the onset of labour [73, 74] and is tied to children's health later in life

[18]. Of the five NF-κB genes that were differentially methylated, four are known to induce NF-

κB signaling. For example, *UBE2N* plays a role in activating the NF-κB pathway [195] and

PRKCZ is a component of the TNF/IL1β pathway that controls activation of the NF-κB pathway

[196-198] and induces contraction of myometrial tissue during late pregnancy [199]. *TNFAIP3*

is the only differentially methylated gene that inhibits NF-κB activation [200, 201]. All probes

associated with the five NF-κB genes were hypomethylated, with at least one of the probes being

altered in the presence of *Streptococcus* Group B. While there are exceptions, hypomethylation

of CpG sites often leads to the upregulation of genes [64]. From this evidence, we conclude that

the presence of microorganisms in the placenta, especially *Streptococcus* Group B, is likely

associated with activation of the NF-κB pathway. These findings are in agreement with the

positive association between anaerobic *Streptococcus* and systemic inflammation in the

ELGANs in early life, where these bacteria were associated with increased levels of five out of

16 tested inflammatory proteins that are upregulated by NF-κB activation including, IL-1β, IL-6,

TNFR1, TNFR2, and E-selectin [23].

The transcription factor occupancy theory proposes that transcription factors are drivers

of gene-specific DNA methylation patterns [68]. The transcription factor binding either prevents

or allows the DNA methylation machinery access to the DNA sequence and therefore this

binding influences gene-specific methylation. Thus, to test this theory, an analysis was carried

out to identify the enriched transcription factors of the immune-related genes. One of the

enriched transcription factors was NF-κB p65 (RelA), a transcriptional activator of the NF-κB

pathway. Six of our 50 immune-related genes are transcribed by RelA and the CpG probes that

47

are associated with these genes were all hypomethylated in the presence of *Streptococcus* Group

B bacteria. RelA is involved in the expression of IL-8, which is a chemokine that mediates an

inflammatory response. An increase in IL-8 has been associated with premature labor [202].

RelA has also been identified as a key regulator of the cytokine environment that is required for

a successful pregnancy. The suppression of RelA is critical for the shift towards Th2-type

immune responses during pregnancy [203, 204]. POU5F1 was associated with all

hypomethylated genes and is of interest because it plays a crucial role in embryonic development

and stem cell pluripotency [205]. These enriched transcription facts may influence CpG

methylation in the placenta as well as pregnancy outcomes and fetal development.

There are multiple factors that should be considered when interpreting the results from

this study. The sample size was relatively small (n=84) and bacteria prevalence was low.

Nevertheless, 52% of the placentas in the study harbored at least one type of bacteria, which is

similar to a prior study of 1,083 placentas in the ELGAN cohort that found 79% and 43% of

preterm placentas at 23 weeks and 27 weeks, respectively, carried microorganisms [154]. It is

important to note that the data on microbial presence represent live, functional bacteria detected

as colony forming units rather than simply DNA, which may be derived from dead or non-

functional microorganisms. While differential methylation of CpG sites in placentas with

microorganisms was identified, gene expression was not a part of the analysis. As a proxy for

gene expression data the placental methylome data was integrated into existing genomics

datasets to establish the functional epigenetics and biological pathways. Future research would

benefit from a larger sample size and should incorporate mRNA and protein expression data

along with the CpG methylation data.

This study is among the first to investigate the potential effect of placental bacteria on the methylome of the placenta. While outside the scope of the current study, future analysis could examine the molecular mechanism underlying microbe-CpG methylation, which could include altered DNA methyltransferase activity [69, 206, 207]. A major observation from the current study is that the CpG methylation patterning differs depending on microorganism presence in the placenta. These differences might be associated with variation in fetal development, birth outcomes and later life disease of premature babies. The genes corresponding to the differentially methylated probes in this study are associated with immune and inflammatory responses, especially the NF-κB pathway.

CHAPTER 3: INFLAMMAMATORY GENE EXPRESSION IN THE PLACENTA IN THE PRESENCE OF MICROORGANISMS AND NEUROCOGNITIVE OUTCOMES AT AGE 10 IN PRETERM CHILDREN

3.1 Overview

Children born preterm, before 37 weeks, exhibit impairments in a variety of different domains including motor and executive function, social cognition, and language and mathematic ability [132, 134, 138-143]. This impairment may persist as the child ages resulting in poor performance in school [133, 134]. The survivability of preterm children continues to increase, leading to the potential for more children to live with neurological deficits [146, 147]. However, neither the etiology nor the underlying biological mechanism of this neurocognitive impairment in preterm children is understood.

The developmental origins of health and disease (DOHaD) hypothesis proposes that prenatal exposures can affect health outcomes later in life [1, 2], including neurocognitive outcomes [3, 4]. In support of the DOHaD hypothesis, bacterial infection of the intrauterine environment (e.g. the placenta) has been associated with neurological outcomes [117-119, 164]. The presence of microorganisms in the placenta has been shown to induce the production of pro-inflammatory proteins in both cell culture and mouse model studies [21, 22, 61, 208]. In addition, within the Extremely Low Gestational Age Newborn (ELGAN) cohort, certain microbes in the placenta have been associated with epigenetic modifications, that may alter expression of genes involved in the pro-inflammatory nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) pathway [72]. However, gene expression was not assessed, a gap that this study will address.

Inflammation of the placenta and other fetal membranes due to bacterial infection has also been associated with brain injury in newborns [120] as well as neurological disorders later in life, such as cerebral palsy and autism spectrum disorder [26, 27, 107, 120]. As a potential mechanism to explain this association, there is evidence that inflammatory signals are transmitted from maternal to fetal tissues through the placenta [94] and that maternal inflammation can lead to elevated levels of pro-inflammatory proteins in the fetal circulation [9]. Once present in fetal blood, cytokines and other inflammation-related proteins cause damage to the developing fetal brain [9, 16-18, 20, 91-93].

3.2 Study Objectives

Previous research within the ELGAN cohort identified an association between the presence of culturable placental microorganisms and an increase in expression of inflammation-related proteins in newborns blood [23]. In other ELGAN studies, this increase of inflammatory-proteins in newborn blood was associated with an increased risk of neurological impairment at ages two and ten [29-32]. In the present study, we set out to test the hypothesis that microorganisms are associated with an inflammatory genomic response in the placenta and examine if placental inflammation affects later life neurocognitive outcomes. To do this, we assessed messenger RNA (mRNA) expression levels of inflammation-related genes in the placenta in relation to placental microorganisms. In addition, we determined whether the differential expression of any of the inflammatory genes is associated with neurocognitive function at 10 years of age.

3.3 Materials and Methods

3.3.1 Study Subject Recruitment and Participation

The ELGAN study recruitment process occurred between 2002 and 2004 at 14 different ELGAN research sites in the United States [151]. Briefly, women who gave birth before 28

weeks gestational age at one of the research sites were asked to participate in the study. All

procedures were approved by the Institutional Review Boards at each of the 14 study sites.

Informed, written consent was provided within a few days of delivery. The mother's consent

covered both her and her child's participation in the study.

A total of 1,506 mother/infant pairs enrolled in the study of which 1,365 placentas were

collected and assessed for microorganisms. Follow-ups were conducted when the children

reached 2 years of age and again at 10 years of age. At the 2-year follow-up 1,102 children were

enrolled and evaluated [151]. At the 10-year follow-up 889 children participated in a

neurological assessment. Of these children, 807 had their placenta parenchyma cultured and

analyzed for microorganisms and 425 of those were assessed for inflammation.

3.3.2 Demographic and Pregnancy Variables

Following delivery, each mother was interviewed by a trained research nurse interviewed

using a structured data collection form. The mother's report of her own characteristics and

exposures, as well as the sequence of events leading to preterm delivery were taken as truth, even

when her medical record provided discrepant information.

Shortly after the mother's discharge, the research nurse reviewed the maternal chart using

a second structured data collection form. The medical record was relied on for events following

admission. The clinical circumstances that led to preterm delivery were operationally defined

using both data from the maternal interview and data abstracted from the medical record [152].

Each mother/infant pair was assigned to the category that described the primary reason for

preterm delivery.

3.3.3 Placenta Sample Collection

As a part of the ELGAN study mothers were asked to provide their placentas for analysis.

Delivered placentas were placed in a sterile exam basin and transported to a sampling room.

Placentas were biopsied at the midpoint of the longest distance between the cord insertion and the edge of the placental disk. The amnion was pulled back to expose the chorion using sterile technique. Traction was applied to the chorion and a piece of the underlying trophoblast tissue was removed. The tissue sample was placed into a cryo-vial and immersed in liquid nitrogen immediately. Specimens were stored at minus 80°C for approximately 15 years until processing [154].

3.3.4 Bacterial Analysis of placenta

The placentas were assessed for microorganisms as described [154]. A portion of each placenta sample was separated using a sterile scalpel and homogenized in a phosphate buffered saline solution (PBS). Serial dilutions of the homogenate were made in PBS and aliquots of the original homogenate and the dilutions were plated onto selective and nonselective bacteriologic media, which included: pre-reduced Brucella base agar, tryptic soy agar, chocolate agar, and A-7 agar. Following incubation the various colony types were enumerated, isolated, and identified at the Brigham and Women's Microbiology Laboratory using estimated criteria [155]. Since we have determined the constituents of the chorion parenchyma in the ELGANs prevent the reliable detection of bacterial DNA by PCR techniques, this study assessed only placental colonization patterns obtained by culture techniques.

3.3.5 RNA Extraction and Assessment of Inflammation-Related Gene Expression

A small subsample of placental tissue, about 0.2 grams, was cut from the frozen sample and rinsed with 1x PBS to remove any residual blood. Samples were then homogenized in Qiagen Buffer RLT (Qiagen, Valencia CA). The extraction was completed with Qiagen's Allprep Universal DNA/RNA/miRNA kit, and was automated using Qiagen's QIAcube sample prep robotic workstation. This method captures RNA sequences that are greater than 18

nucleotides in length. Total mRNA was quantified using the NanoDrop 2000C (Thermo Fisher Scientific, Waltham, MA) and quality was assessed using TapeStation.

A total of 50 ng of mRNA extracted from each placental sample was evaluated for comparative expression of 249 inflammation-related genes using nCounter Human Inflammation Panel (NanoString Technologies, Inc., Seattle, WA). This panel was selected because our previous research indicated that inflammatory genes were differentially methylated in the presence of microorganisms and we wanted to explore whether this methylation altered gene expression [72]. This panel also includes six housekeeping genes, eight negative controls, and four positive controls. Nanostring's nCounter technology employs direct digital detection of mRNA molecules of interest which allows for a high-throughput, sensitive, and accurate measurement of gene expression from small quantities of mRNA that does not require amplification [209].

Raw expression data were extracted using the nSolver software (NanoString Technologies, Inc., Seattle, WA). Background correction was conducted to eliminate false positives using the geometric mean of the negative control probes [210]. Out of the 249 genes in the panel, 72 were not detectable above baseline, leaving 177 for analysis. The mRNA data were normalized in a two-step process as per the manufacturer's specifications [210]. First, positive control normalization was carried out to account for platform-associated sources of variation followed by housekeeping gene normalization to account for variability in mRNA input [210].

3.3.6 Procedures for the Neurocognitive Assessments at 10-years of Age

All families who participated in the 2-year follow up were contacted and invited to participate in the 10-year follow up. Families that agreed to participated were scheduled for a three to four hour visit during which all of the assessments were administered. During the child's

assessment, the parent/caregiver completed questionnaires regarding the child's medical status and neurological behavior.

3.3.6.1 Cognitive Function Derived from Latent Profile Analysis (LPA)

This outcome variable has been described elsewhere. [156]. Briefly, it was derived from latent profile analysis of data from nine intelligence and executive function variables. IQ was assessed with the School-Age Differential Ability Scales-II (DAS-II) Verbal and Nonverbal Reasoning scales [157]. Executive function included two subtests from DAS-II, DAS Recall of Digits Backward and Recall of Sequential Order, which measured verbal working memory [157], and five subtests from the NEPSY-II (A Developmental NEuroPSYchological Assessment-II) [158]. The NEPSY-II Auditory Attention and Response Set measured auditory attention, set switching and inhibition, the NEPSY-II Inhibition and Inhibition Switching measured simple inhibition and inhibition in the context of set shifting, respectively, and the NEPSY-II Animal Sorting measured visual concept formation and set shifting. The LPA identified four subgroups of study participants with similar profiles: normal, low-normal, moderately impaired, and severely impaired.

3.3.6.2 Oral and Written Language Scales (OWLS) Oral Composite

The Oral and Written Language Scales (OWLS) evaluated expressive and receptive language skills [159]. The OWLS oral composite score includes both listening comprehension and oral expression. To allow for differences in age at the times of assessment and to facilitate a comparison of our findings to those reported for children presumably born very near term we used Z-scores based on distributions of values reported for the historical normative samples that are described [159].

3.3.6.3 Academic Function

The Wechsler Individual Achievement Test-III (WIAT-III) provides standard scores in word recognition and numeric operations [160]. For these tests, we again used Z-scores based on distributions of values reported for the historical normative samples [160].

3.3.7 Data Analyses

3.3.7.1 Microorganisms in Relation to Gene Expression

In order to determine whether placental microorganisms are associated with gene expression of inflammation-related genes, Analysis of Covariance (ANCOVA) was performed. There were 15 bacterial species or groups assessed including: *Lactobacillus* sp., *Prevotella bivia*, *Gardnerella vaginalis*, anaerobic *Streptococcus*, *Peptostreptococcus* sp., *Escherichia coli*, alpha-hemolytic *Streptococcus*, *Ureaplasma urealyticum*, *Mycoplasma* sp., *Staphylococcus* sp., *Propionibacterium* sp., *Actinomyces* sp., *Corynebacterium* sp., *Streptococcus* Group B, and *Streptococcus* Group D. Each model examined whether the presence of an individual bacterial species or bacterial type was associated with differential expression of the 177 inflammation-related genes. Models were adjusted based on several dichotomous variables including fetal-sex, maternal race, whether the mother took an antibiotic during pregnancy, whether the mother experienced a vaginal infection during pregnancy, whether the mother experienced a urinary tract infection (UTI) during pregnancy, and whether the birth occurred via C-section. Bacterial species were considered to be significantly associated with gene expression if *p*-value <0.05 and the false discovery rate-corrected *p*-value <0.1 for any given gene. Data analysis was carried out using Partek Genomic Suites 6.6.

3.3.7.2 Gene Expression in Relation to 10-Year Outcomes

In order to determine whether the expression of inflammatory-related genes are associated with neurocognitive function at age 10, separate logistic regression models were

performed for the genes found to be significant in relation to placental microorganisms. Each model examined whether the expression of an inflammation-related gene was associated with increased odds of scoring one or more standard deviations below the normative mean on three different assessments: OWLS Oral Language Composite, WIAT-III Word Recognition, and WIAT-III Numerical Operations. In the case of LPA, the models examined whether the expression of inflammation-related genes was associated with increased odds of having moderate to severe cognitive impairment. Confounders included in the models were the same as in the previous analysis plus maternal education. These models yielded odds ratios (ORs) and 95% confidence intervals (CI) of each 10-year characteristic associated with inflammation-related genes. Genes were considered to be significantly associated with a neurological function if p-value <0.05 and the OR 95% CI did not include one.

3.4 Results

3.4.1 Study Subject Characteristics

The mRNA expression levels of inflammation-related genes were assessed within the placentas of 425 of the 889 ELGAN subjects that participated in the 10-year follow-up assessment. These 425 individuals make up our subcohort for this study. The subcohort is similar to the overall 10-year cohort as is demonstrated in **Table 7**. Within the subcohort there are slightly more males, 223 (52.5%), than females, 202 (47.5%). Most of the children were delivered by C-section (67%). The majority of mothers received between 12 and 16 years of education (49%) while 53 (13%) completed high school or less and 154 (36%) completed college or higher. The majority of the women did not have a vaginal infection (81%) or UTI (84%) during pregnancy and 128 (30%) took an antibiotic while they were pregnant. There are not results for each of the four assessments for every single child within our subcohort. However,

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there was a very high participation rate ranging from 97% for the OWLS oral composite scores up to 100% for the LPA.

Table 7. Demographics

	Overall 10-year Follow-up (n=889) N (%)	Subcohort (n=425) N (%)
Fetal Sex		
Male	455 (51.2)	223 (52.5)
Female	434 (48.8)	202 (47.5)
Maternal Race		
White	557 (62.7)	261 (61.4)
Non-white	319 (35.9)	159 (37.4)
NS	13 (1.5)	5 (1.2)
Maternal Education, years		
≤ 12 (high school)	126 (14.2)	53 (12.5)
Some college or Associates Degree	431(48.5)	206 (48.5)
College or higher	306 (34.4)	154 (36.2)
NS	26 (2.9)	12 (2.8)
Delivery Method		
C-section	590 (66.4)	283 (66.6)
Natural	299 (33.6)	142 (33.4)
UTI		
No	747 (84.0)	358 (84.2)
Yes	119 (13.4)	56 (13.2)
NS	23 (2.6)	11 (2.6)
Vaginal Infection		
No	740 (83.2)	345 (81.2)
Yes	126 (14.2)	69 (16.2)
NS	23 (2.6)	11 (2.6)
Antibiotic Use		
No	602 (67.7)	285 (67.1)
Yes	263 (29.6)	128 (30.1)
NS	24 (2.7)	12 (2.8)
LPA		
Yes	874 (98.3)	425 (100)
No	15 (1.7)	0 (0)
OWLS oral composite		
Yes	849 (95.5)	411 (96.7)
No	40 (4.5)	14 (3.3)
WIAT-III word recognition		
Yes	864 (97.2)	420 (98.8)
No	25 (2.8)	5 (1.2)
WIAT-III numerical operations		
Yes	874 (98.3)	424 (99.8)
No	15 (1.7)	1 (0.2)

NS = Not specified

The most common bacteria present in the study placentas was *Staphylococcus* sp. which

was detected in 55 (12.9%) placentas (**Table 8**). The least prevalent bacterial species were

anaerobic *Streptococcus* and *Streptococcus* Group B, which were present in 16 (3.8%) placentas.

The rest of the bacterial species were found in between 17 (4.0%), in the case of *G. vaginalis*,

and 34 (8.0%), in the case of *Corynebacterium* sp., placentas. This includes alpha-hemolytic

Streptococcus which was detected in 28 (6.6%).

Table 8. Presence of microorganisms in the placenta

Bacteria	n (%)
Lactobacillus sp.	32 (7.5)
P. bivia	18 (4.2)
G. vaginalis	17 (4.0)
Anaerobic *Streptococcus*	16 (3.8)
Peptostreptococcus sp.	20 (4.7)
E. coli	23 (5.4)
Alpha *Streptococcus*	28 (6.6)
U. urealyticum	21 (4.9)
Mycoplasma sp.	23 (5.4)
Propionibacterium sp.	32 (7.5)
Actinomyces sp.	24 (5.6)
Corynebacterium sp.	34 (8.0)
Staphylococcus sp.	55 (12.9)
Streptococcus Group B	16 (3.8)
Streptococcus Group D	21 (4.9)

3.4.2 Identification of Differentially Expressed Inflammation-Related Genes in Placentas Exposed to Bacteria

The differential expression of 177 inflammation-related genes in placentas were analyzed

within the ELGAN cohort between 425 individuals with or without placental microbes.

Statistical significance was adjusted using false discovery rate-corrected p-values ($q<0.1$). A

total of 31 out of the 177 genes displayed significantly differential expression between placentas

that harbored bacteria and placentas that did not (**Figure 7**). Of the 15 microbial species, three

were associated with differentially expressed genes. Alpha-hemolytic *Streptococcus* was

associated with the upregulation of two genes, Cysteinyl Leukotriene Receptor 2 (*CYSLTR2*) and

Transforming Growth Factor Beta 3 (*TGFB3*). *Staphylococcus* sp. was associated with increased

expression of three genes, Complement C4A (*C4A*), G Protein Subunit Gamma Transducin 1

(*GNGT1*), and Interleukin 4 (*IL4*). *Streptococcus* Group B was associated with the upregulation

of 28 inflammation-related genes, including *TGFB3*, *C4A*, C-C Motif Chemokine Ligand (*CCL*)
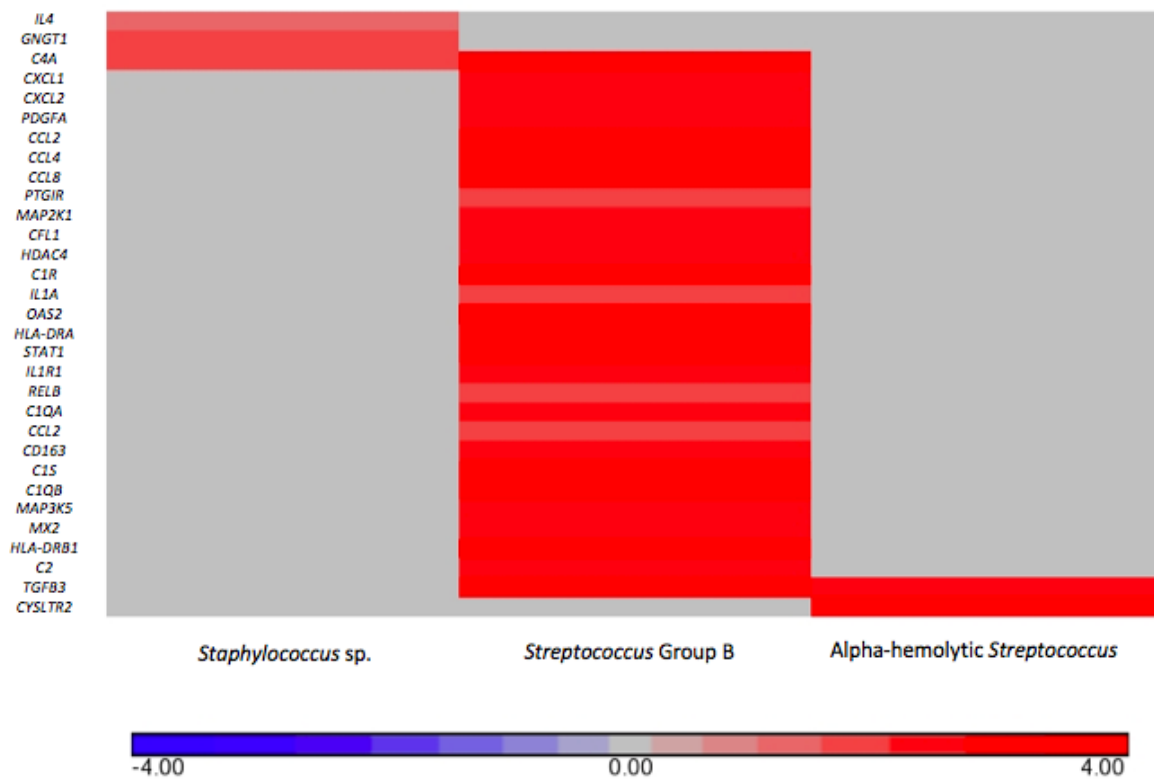
2, and *CCL4* (**Supplemental Table 3**).

Figure 7. Heatmap of differentially expressed genes corresponding to the placental microorganisms. The heatmap displays the 31 differentially expressed genes in relation to three microorganisms. Red represents increased methylation and blue represents decreased methylation

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3.4.3 Association of Neurocognitive Function at Age 10 in Relation to Inflammation-Related Gene Expression

To test whether inflammation-related genes that were differentially expressed in the presence of bacteria were associated with neurocognitive outcomes, we ran logistic regression models. Interestingly, out of the 31 genes that were differentially expressed in relation to microorganisms none of them were associated with statistically significant differential odds on any of the four neurocognitive assessments (p<0.05, data not shown).

3.5 Discussion

In this study, we set out to explore two associations. The first was whether microorganisms in the placental were associated with an inflammatory genomic response. The second was to determine whether the expression of any of the inflammatory-related genes were associated with 10-year neurocognitive outcomes. We show that the presence of alpha-hemolytic *Streptococcus*, *Staphylococcus* sp., and *Streptococcus* Group B in the placenta of children born extremely preterm were associated with increased expression of 31 inflammation-related genes in placental tissue. While these genes displayed bacterial-associated changes in expression, there was no association between placental inflammatory gene expression and neurocognitive function at 10 years of age. These data indicate that certain bacterial species in the placenta are associated with an inflammatory response but that this response in the placenta is not associated with neurocognitive function at age 10.

The presence of *Streptococcus* Group B was associated with the increased expression of 28 inflammatory genes. Our previous work showed hypomethylation of inflammation-related genes in placentas that harbored *Streptococcus* Group B [72]. While there are some exceptions, hypomethylation within gene promoters often leads to upregulation of the gene, thus in line with the findings from this study [64]. *Streptococcus* Group B is found in the lower genital tract and is

the leading cause of early onset sepsis and meningitis in newborns [211, 212]. When present in the placenta it is associated with preterm birth and histologic chorioamnionitis, inflammation of the placenta and other fetal membranes [213]. In addition, *Staphylococcus* sp. and alpha-hemolytic *Streptococcus* were only associated with increased expression of three and two genes respectively. *Staphylococcus* sp. and alpha-hemolytic *Streptococcus* are not as common of intrauterine infections as *Streptococcus* Group B, and therefore have not been studied as thoroughly. However, there is evidence of an association with both *Streptococcus* sp. and *Streptococcus* Group B and chorioamnionitis [214, 215].

In the present study, a total of 31 unique genes were upregulated in the presence of a specific microorganism. Many of these genes have been associated with pregnancy complications, such as chorioamnionitis and preeclampsia, including *TGFB3*, *C4A*, Major Histocompatibility Complex, Class II, DR Beta 1 (*HLA-DRB1*), C-X-C Motif Chemokine Ligand (*CXCL*) 1, *CXCL2*, and Cluster of Differentiation 163 (*CD163*) [190, 216-221]. In support of our results, a separate study showed that chorioamnionitis induced by Group B *Streptococcus* was associated with increased expression of *CXCL1* [222]. *CXCL1* is of interest because it is essential for neural cell development [223-225]. Providing further support for the data presented here, in a rat model, chorioamnionitis was associated with increased expression of *CXCL1* in the placenta, neonatal blood, and the developing brain, as well as brain injury [220]. *CCL2*, another gene that was upregulated in the presence of Group B *Streptococcus*, is known to have receptors in the placenta as well as the embryonic brain [226-228]. *CCL2* plays a role in the development of hypothalamic neurons that control behavior [229].

While previous research has shown an association between placental inflammation and neurological outcomes [26, 120], this is the first study to measure the mRNA expression of

inflammatory-related genes. The expression of individual inflammation-related genes in the placenta that were upregulated in the presence of bacteria were not associated with neurocognitive outcomes in 10 year olds. This could be because assays of delivered placentas may not reflect the gene expression occurring at critical points of development *in vivo* [230]. Also, these analyses focused on a targeted set (n=31) of genes. While these genes were selected for their role in inflammation in the presence of bacterial species, other genes with important relationships to children's cognition could be captured in the future using a genome-wide approach. Importantly, even though inflammation of the placental tissue was not associated with later life health outcomes, it is important to note that inflammation in newborn blood has been. For example, *CCL4*, which was upregulated in the placentas with *Streptococcus* Group B in this study, was also upregulated in the blood of newborns whose placentas harbored *Peptostreptococcus* sp. or *U. urealyticum* [23]. Increased expression of *CCL4* in newborn blood was associated with developmental delays at age 2 in another ELGAN study [28]. The data from the current study highlight the role of inflammatory processes in the placenta as responders to microbial presence.

This study is among the first to assess mRNA expression of pro-inflammatory genes in the placenta in relation to a variety of bacterial species as well as neurocognitive function at 10-years of age. Our data should be interpreted with several limitations in mind. There was a relatively low prevalence of each bacterial species, limiting the power to detect associations. Nevertheless, 209 (49%) of the placentas in this study harbored at least one type of culturable microorganism, which is consistent with previous findings [46]. While based upon a specific hypothesis focus, another limitation could be that a limited number of genes were assessed. Future research could use a genome-wide approach to identify genes outside of this set that may

be associated with placental bacterial presence and later life neurocognitive function. Strengths of this study include the large sample size, the broad range of neurocognitive assessments, and the breadth of data from the placenta at birth to the child 10 years later.

In summary, there were two major findings from this study. First, the presence of a few different microorganisms in the placenta was associated with increased expression of inflammation-related genes in individuals born extremely preterm. Second, the expression of the inflammation-related genes in the placenta was not associated with neurocognitive function at age 10. This study helps inform our understanding of prenatal factors and placental function and their influence of neurocognitive outcomes later in life.

CHAPTER 4: DISSERTATION DISCUSSION, CONCLUSIONS, AND FUTURE DIRECTIONS

Collectively, the studies presented as a part of my dissertation demonstrate that culturable microorganisms in the placenta are associated with altered placental function, through variations in DNA CpG methylation and/or gene expression in the placenta, and are associated with neurocognitive function at 10 years of age. Specifically, in Chapter 1 we demonstrated that the presence of specific bacterial species in the placenta are associated with neurocognitive function at age 10. In Chapter 2, we found that microorganisms in the placenta are associated with unique CpG methylation profiles of the placenta. In Chapter 3 we demonstrated that specific placental microorganisms are associated with the altered expression of inflammatory-associated genes. These data provide a potential mechanistic understanding for processes involved in prenatal exposure to bacteria and long term neurocognitive outcomes.

A primary goal of the work in Chapter 1 was to identify microorganisms in the placenta that were associated with neurocognitive and social-communicative function at age 10 (**Figure 8**). The hypothesis was that microorganisms in the placenta are associated with differential performance and brain function of school-age children, with a variable response depending on bacterial species. This is based on previous research that found associations between placental infection and neurodevelopmental outcomes [129] as well as brain damage in the newborns [131]. In our research, the presence of *U. urealyticum*, alpha-hemolytic *Streptococcus*, *Corynebacterium* sp., *G. vaginalis*, and *Staphylococcus* sp. were associated with increased risk of learning limitations at age 10. A proposed mechanism underlying this relationship is that

microorganisms trigger inflammatory responses in the placenta [21, 22] that may impact the development of the fetal brain [19]. This research is important as it is the first study to identify specific microbial species, as opposed to indicators of infection, in relation to later-life neurological function. In contrast to the majority of the microorganisms, *Lactobacillus* sp. presence was associated with a decreased risk of neurocognitive function. This is likely due to its anti-inflammatory effect [23, 174, 175]. In addition, we found no association between any of the placental microorganisms and social-communicative function. Taken together, this study identified specific bacterial species in the placenta that are associated with a variety of neurocognitive functions at 10 years of age.

A major goal of the work in Chapter 2 was to investigate the role of bacteria in the placenta on the placental CpG methylome (**Figure 8**). The hypothesis was that the presence of microorganisms in the placenta would be associated with altered CpG methylation of development-associated genes. This was based on studies that found differential methylation in other tissue types as well as the murine placenta in the presence of bacterial organisms [24, 69, 70]. This study identified that bacteria in the placenta are associated with differential methylation of 1,789 CpG probes. Furthermore, each bacterial type corresponded with a unique and distinct pattern of CpG methylation. The presence of *Streptococcus* Group B was associated with the largest number of differentially methylated probes. Genes that corresponded to the differentially methylated probes are enriched for growth/transcription factors, immune-related proteins, and inflammation-related proteins. Specifically, among the inflammation-related genes we found an enrichment of the NF-κB pathway. Interestingly, the NF-κB pathway has been shown to be critical pathway for pregnancy and fetal development [18, 73, 74]. Two proposed mechanisms underlying the relationship between microorganisms in the placenta and CpG methylation

include the transcription factor occupancy theory [68] and a non-gene specific process in which bacterial presence induces the production of reactive oxygen species [65] which has been shown to modulate DNA CpG methylation [66]. Taken together, data from the second study demonstrate that the presence of microorganisms in the placenta is associated with altered placental CpG methylation. Furthermore, the results provide evidence of how altered CpG methylation could contribute to placental function as well as fetal health.

The research presented in Chapter 3 examined placental mRNA expression of inflammation-related genes in relation to microbial presence in the placenta (**Figure 8**) as well as 10 year neurocognitive outcomes (**Figure 8**). The two-part hypothesis was that *first*, inflammatory-related genes will be overexpressed in placentas that contain pro-inflammatory microorganisms and *second*, children whose placentas had increased expression of inflammatory-related genes would be at a higher risk for negative neurocognitive outcomes at age 10. Given that in Chapter 2 inflammatory-related genes were differentially methylated in the presence of bacteria it seemed probable that expression of these genes would also be altered. A set of 31 inflammation-related genes were identified to be upregulated in the presence of three different microorganisms, *Streptococcus* Group B, *Staphylococcus* sp., and alpha-hemolytic *Streptococcus*. The presence of *Streptococcus* Group B was associated with 28 of the 31 differentially expressed genes, which is consistent with the findings in the Chapter 2 (e.g. *Streptococcus* Group B displayed significant association with hypomethylation of inflammation-related genes). It was surprising to find, however, that none of the differently expressed inflammation-related genes were associated with risk of neurocognitive function at age 10. This could be because gene expression in the placenta following labor may not reflect the *in vivo* gene expression that occurs earlier in pregnancy [230]. Also, it is possible that the mRNAs were

impacted by some degradation following delivery [230, 231]. Taken together, these data suggest

that inflammation-related genes are over expressed in the presence of certain bacterial species,

but, at least in the case of the placentas and the targeted genes under study here, mRNA

expression of inflammation-related genes in the placenta was not associated with later-life

neurocognitive function.
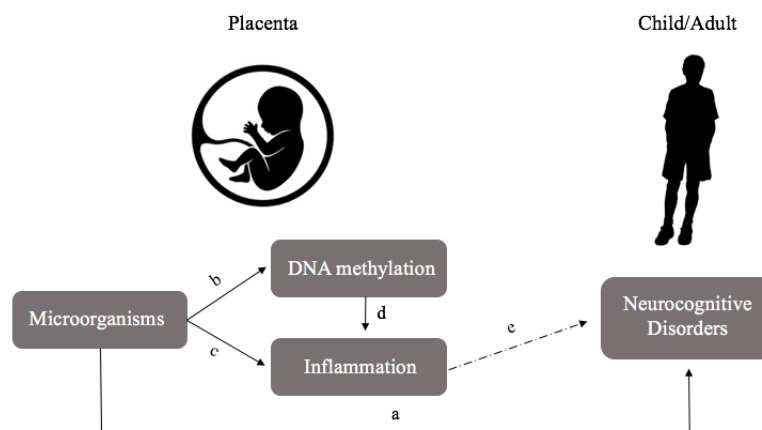
Figure 8. Integrative schematic of associations between placental microorganisms, biological functions in the placenta, and later-life neurocognitive function. The arrows indicate the associations that were analyzed within the dissertation. Solid arrows represent associations that the findings support, while dashed arrows were associations that were hypothesized but not established through this research.

In conclusion, these three studies demonstrate the associations among microorganisms in

the placenta, CpG methylation and gene expression in the placenta and their association with 10-

year neurocognitive outcomes. These studies are among the first to examine a variety of different

microbial species in the placenta and their effects on the placenta as well as later life

neurological outcomes of the child. The conclusions from these studies inform the

understandings of prenatal factors and their influence of neurocognitive function of children later

in life.

4.1 The Presence of Placental Bacteria and their Impact on Placental Function and Later in Life Neurocognitive Function

In all three chapters, the presence of 15 different bacterial types and species in the placenta were analyzed within the ELGAN placenta. The presence of the individual bacteria was fairly consistent between the placentas studied in Chapter 1 (n=807 individuals), and those studied in Chapter 3 (n=425 individuals) (**Table 9**). In both of these studies, *Staphylococcus* sp. was the most prevalent bacteria, with its presence in 94 (12%) placentas in Chapter 1 and 55 (13%) placentas in Chapter 3. This is consistent with the overall ELGAN cohort of 1,365 placentas analyzed [178]. For the placentas studied in Chapter 2 (n=84 individuals), *Propionibacterium* sp. was the most prevalent bacteria (12%) and *Mycoplasma* sp. was the second most prevalent (11%). These differences may be due to the smaller sample size that was available for placentas with CpG methylation data in Chapter 2.

Overall 51% of the ELGAN placentas analyzed were culture-positive for at least one of the 15 bacterial species [178]. This was consistent across all three of these studies. In Chapter 1 and 2, 48% of the placentas were culture-positive and in Chapter 3, 49% were culture-positive.

Table 9. Bacterial presence in the placentas across all three studies

Bacteria	Chapter 1 (n=807) N (%)	Chapter 2 (n=84) N (%)	Chapter 3 (n=425) N (%)
Lactobacillus sp.	48 (5.9)	3 (3.6)	32 (7.5)
P. bivia	41 (5.1)	3 (3.6)	18 (4.2)
G. vaginalis	28 (3.5)	3 (3.6)	17 (4.0)
Anaerobic *Streptococcus*	40 (5.0)	4 (4.8)	16 (3.8)
Peptostreptococcus sp.	49 (6.1)	3 (3.6)	20 (4.7)
E. coli	49 (6.1)	2 (2.4)	23 (5.4)
Alpha-hemolytic *Streptococcus*	53 (6.6)	3 (3.6)	28 (6.6)
U. urealyticum	43 (5.3)	5 (6.0)	21 (4.9)
Mycoplasma sp.	42 (5.2)	9 (10.7)	23 (5.4)
Propionibacterium sp.	55 (6.8)	10 (11.9)	32 (7.5)
Actinomyces sp.	47 (5.8)	4 (4.8)	24 (5.6)
Corynebacterium sp.	64 (7.9)	3 (3.6)	34 (8.0)
Staphylococcus sp.	94 (11.6)	7 (8.3)	55 (12.9)
Streptococcus Group B	38 (4.7)	2 (2.4)	16 (3.8)
Streptococcus Group D	36 (4.5)	2 (2.4)	21 (4.9)

The majority of previous research on bacteria in the intrauterine environment has focused on microorganisms of vaginal origin or organisms associated with bacterial vaginosis such as *U. urealyticum*, *Mycoplasma hominis*, and *G. vagninalis* [36-38], which were all found within the study placentas. However, there was also a presence of skin and oral bacteria in the ELGAN placentas, including *Streptococcus* sp., *Staphylococcus* sp., *Propionibacterium* sp., and *E. coli*. It is hypothesized that these bacterial species translocate to the placenta through hematogenous transmission [52].

In each of the chapters we compared the 15 microbial species analyzed in the ELGAN placentas to different placental factors or later life outcomes. In each study, the different bacterial types displayed various associations between the outcome of interest. Interestingly, two bacterial types, alpha-hemolytic *Streptococcus* and *Staphylococcus* sp., were significant across all three studies. In the first chapter, *Staphylococcus* sp. was associated with increased odds of deficits in math and language function while alpha-hemolytic *Streptococcus* was associated with increased

odds of deficits in mathematics. In the second chapter, alpha-hemolytic *Streptococcus* and

Staphylococcus sp. were associated with 167 CpG probes corresponding to 105 genes and 40

CpG probes corresponding to 22 genes, respectively. In the case of alpha-hemolytic

Streptococcus the majority of the probes were hypomethylated in the presence of the bacteria,

whereas the opposite was true for *Staphylococcus* sp. In Chapter 3, three bacterial species were

associated with differential expression of inflammation-related genes in the placenta. Alpha-

hemolytic *Streptococcus* was associated with upregulation of two inflammation-related genes

and *Staphylococcus* sp. was associated with upregulation of three genes. *Streptococcus* Group B

was associated with the most differentially methylated CpG probes, 1,257 corresponding to 802

genes, and the most differentially expressed inflammatory genes, 31 genes. However,

Streptococcus Group B was not associated with any of the neurocognitive or social-

communicative outcomes analyzed in the first chapter.

Interestingly, in the first chapter we found that the presence of *Lactobacillus* sp. was

associated with decreased odds of neurocognitive deficits as measured by LPA in the child at age

10. In all other cases the five bacteria, *U. urealyticum*, alpha-hemolytic *Streptococcus*,

Corynebacterium sp., *G. vaginalis*, and *Staphylococcus* sp., were associated with increased odds

of neurocognitive deficits. This could be explained by the fact that in other studies *Lactobacillus*

sp. has produced a net anti-inflammatory effect [174, 175] and has been associated with a lower

likelihood of systemic inflammation in the blood of newborns [23]. In Chapter 2 *Lactobacillus*

sp. was associated with differential methylation of 44 genes corresponding to 32 genes. The

majority, 77%, of them being hypomethylated. *Lactobacillus* sp. was not associated with

differential expression of any of the 177 inflammation-related genes analyzed in Chapter 3.

However, this was expected because there was no overlap between the genes analyzed in

Chapter 3 and the 32 genes differentially methylated in the presence of *Lactobacillus* sp. in Chapter 2.

Taken together, the three studies show that about 49% of the ELGAN preterm placentas harbored at least one of the 15 microorganisms. In the ELGAN study, culture techniques were used to identify specific microorganisms in the placenta. However, other microbial species that were not analyzed for could be present and having an effect on the placenta as well as the developing fetus. Future research could include a microbiome assessment of the placenta in order to account for all microbial species present using a 16S rRNA gene sequencing approach. We show that each bacterial type has a varying effect on neurocognitive outcomes at age 10, placental CpG methylation, and inflammation-related gene expression in the placenta.

4.2 Assessment of mRNA Levels and CpG Methylation in Relation to Placental Microbes

In Chapter 2 we observed an enrichment among the differentially methylated genes for roles in inflammatory processes in the cell. This influenced our interest in the subsequent analysis of the mRNA expression of inflammation-related genes in the placenta using the NanoString nCounter Human Inflammation Panel in relation to placental bacteria. This panel included 249 inflammation-related genes, 10 of which were differentially methylated in relation to placental bacteria in Chapter 2. The ten genes that overlapped are C-C Motif Chemokine Receptor 3 (*CCR3*), CD4 Molecule (*CD4*), Histone Deacetylase 4 (*HDAC4*), Interferon Regulatory Factor 7 (*IRF7*), SHC Adaptor Protein 1 (*SHC1*), SMAD Family Member 7 (*SMAD7*), Transforming Growth Factor Beta 3 (*TGFB3*), TNF Alpha Induced Protein 3 (*TNFAIP3*), Toll Interacting Protein (*TOLLIP*), and Thymic Stromal Lymphopoietin (*TSLP*). These ten genes are associated with a total of 17 differentially methylated probes that were identified to display differential CpG methylation in the presence of four different bacteria (**Figure 9**).
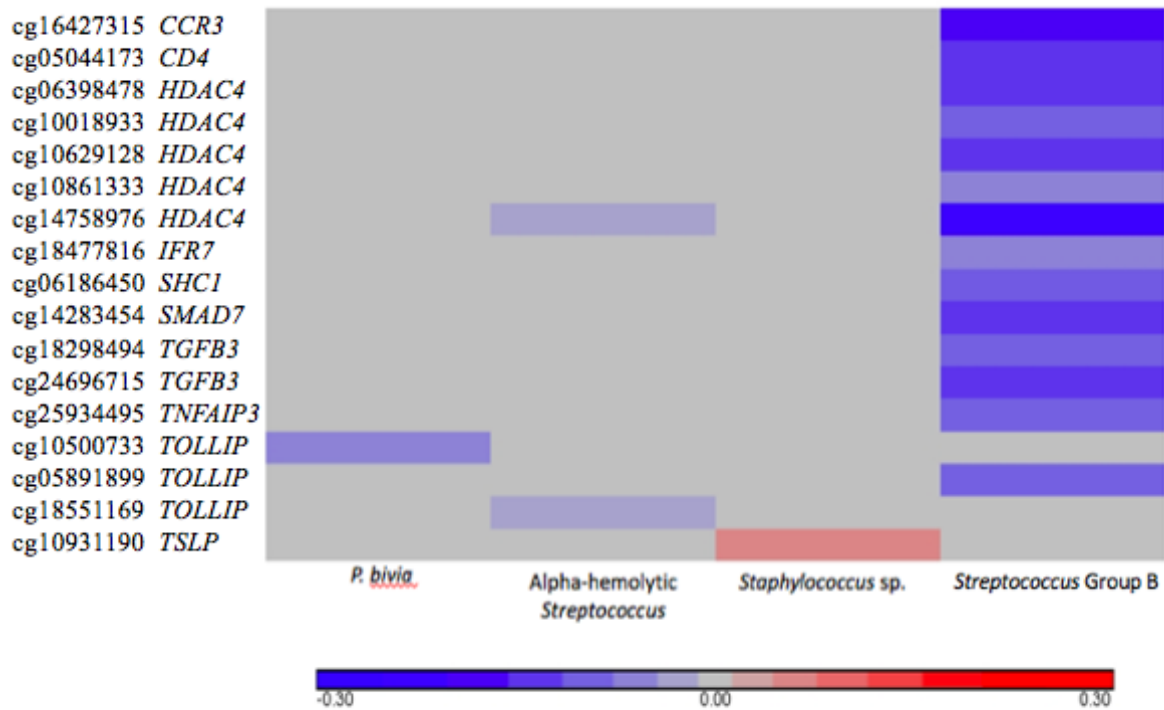
Figure 9. Heatmap of differentially methylated CpG probes of genes analyzed for mRNA expression. The heatmap displays the 17 differentially methylated probes associated with the 10 genes which were analyzed for expression in relation to placental microorganisms. The 17 probes are along the y-axis. Red represents increased methylation and blue represents decreased methylation.

Streptococcus Group B was associated with the hypomethylation of the probe associated with *CCR3*, *CD4*, *IRF7*, *SHC1*, *SMAD7*, *TNFAIP3*, and *TOLLIP*, two probes associated with *TGFB3*, and five probes associated with *HDAC4*. The presence of alpha-hemolytic *Streptococcus* is associated with hypomethylation of one of the *HDAC4* probes and a *TOLLIP* probe. A different *TOLLIP* probe is also hypomethylated in the presence of *P. bivia*. Among these ten genes, only one had a probe that was hypermethylated in the presence of bacteria, *TSLP* in the presence of *Staphylococcus* sp. These data indicate that the presence of *P. bivia*, alpha-hemolytic *Streptococcus*, and *Streptococcus* Group B in the placenta would likely be associated with an activated inflammatory-response since hypomethylation of a CpG site, in many cases, leads to upregulation of the gene [64].

Of the 10 genes that showed differential CpG methylation in Chapter 2 and were analyzed for gene expression on the inflammation panel in Chapter 3, two demonstrated differential expression in the presence of bacteria (**Figure 8**). The two were *TGFB3* and *HDAC4* in the presence of *Streptococcus* Group B. These probes were found to be hypomethylated in Chapter 2 and upregulated in Chapter 3 in the presence of *Streptococcus* Group B. Again, this is consistent with the theory that hypomethylation of CpG sites often leads to the upregulation of the genes [64]. Interestingly both *TGFB3* and *HDAC4* had multiple probes hypermethylated in the presence of *Streptococcus* Group B. While the other eight genes only had one probe differently methylated in the presence of bacteria. Since multiple CpG sites are associated with each gene it is logical that the more differentially methylated probes associated with a gene, the more likely there will be an alteration in gene expression.

In addition, of the four bacterial species that were associated with differential methylation of the inflammation-related genes analyzed for gene expression, three were associated with increased expression of inflammation-related genes, namely *Streptococcus* Group B, *Staphylococcus* sp., and alpha-hemolytic *Streptococcus*. The only bacteria that was not associated with differential expression of any of the inflammation-related genes was *P. bivia*. In the case of *Streptococcus* Group B, it was associated with differential CpG methylation of 1,080 genes, 28 of which were related with the inflammatory response, and increased expression of 31 inflammation-related genes. While *Staphylococcus* sp. and alpha-hemolytic *Streptococcus* did not have a direct overlap between differential methylation and differential expression of the same gene they still showed differential methylation and differential expression of inflammation-related genes. In this case, differential methylation of one gene could lead to downstream effects and differential expression of a different inflammation-related gene.

Out of the 84 placentas were analyzed for CpG methylation, 79 of these placentas were assessed for expression of inflammation-related genes. Linear regression was used to compare the methylation beta differences of the 17 CpG probes to the expression of the 10 inflammation-related genes that were associated with the probes in the 79 placentas. Out of the 17 CpG probes, 11 of the probes exhibited a negative correlation between methylation and expression (**Figure 10**). This finding is anticipated given that hypermethylation, or increased methylation, often leads to downregulation of a gene [64]. This was apparent in the probes associated with *CD4*, *SHC1*, *SMAD7*, *TNFAIP3*, *TSLP*, three of the five probes associated with *HDAC4*, both of the probes associated with *TGFB3*, and one of the probes associated with *TOLLIP*. The *TOLLIP* probe that exhibits this negative correlation with gene expression is the probe associated with *Streptococcus* Group B. The six other probes exhibit positive correlation when comparing methylation to gene expression.
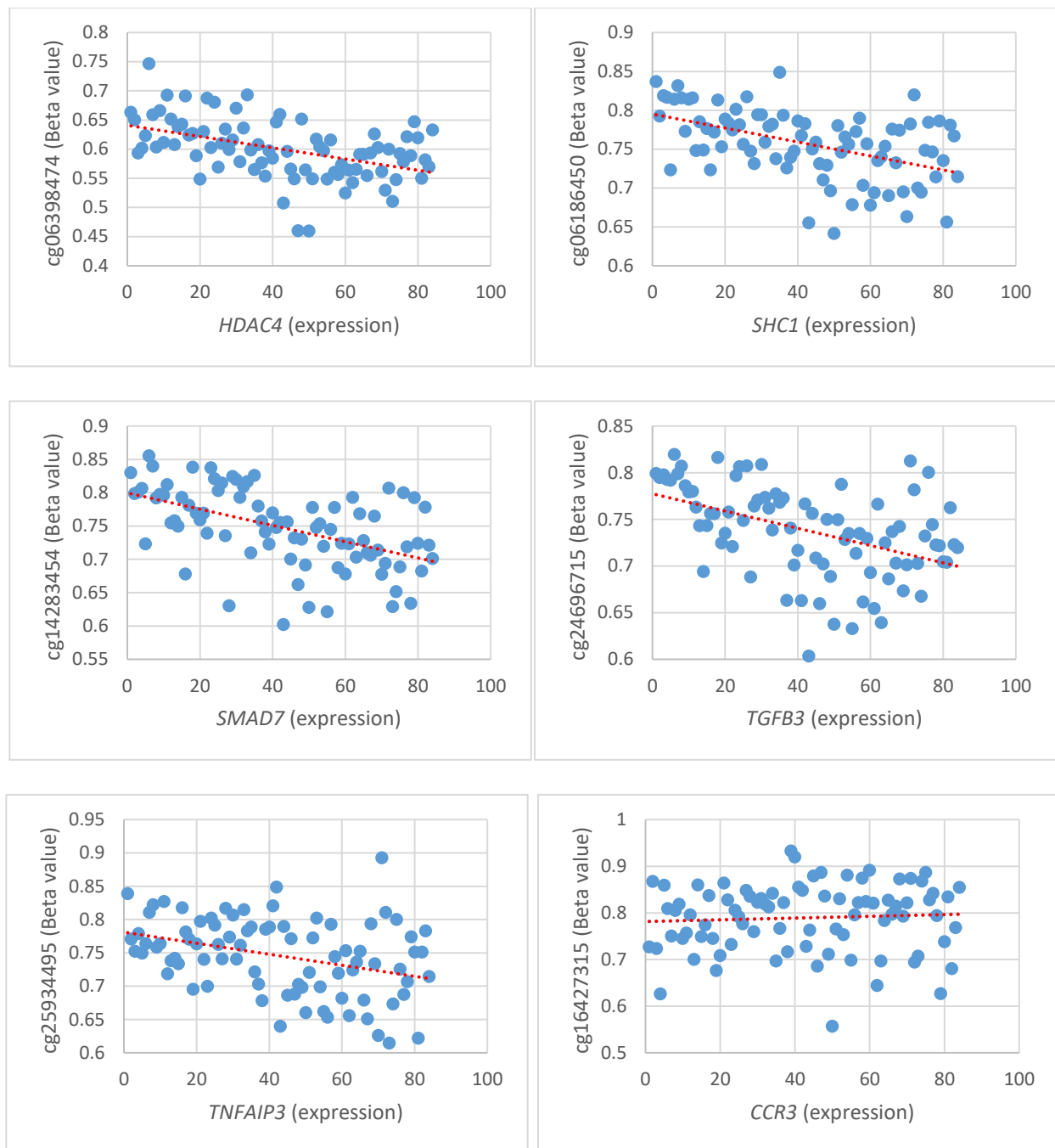
Figure 10. Linear regression of CpG methylation of probes and mRNA expression of associated genes. The six plots represent a comparison of CpG methylation and expression of corresponding genes in 79 placentas from the ELGAN cohort. Gene expression is represented on the x-axis and the beta value, indicating CpG methylation, of each probe is represented along the y-axis. Each blue dot represents one placenta. The red line is the linear regression trend line of the data.

Overall, there is indication that CpG methylation of an individual probe is associated with altered expression of the corresponding gene (**Figure 10**). However, we also see that placentas that exhibited differential CpG methylation of genes enriched for the inflammatory response in the presence of microorganisms also had increased mRNA expression of inflammation-related genes when those same microorganisms were present, even when the specific inflammation-related genes did not exhibit differential methylation. For example, when *Streptococcus* Group B was present in the placenta, Complement C2 (*C2*) was overexpressed, even though no probes associated with this gene were differentially methylated in the presence of *Streptococcus* Group B. Conversely, *CCR3* was hypomethylated in placentas that harbored *Streptococcus* Group B but this inflammation-related gene was not differentially expressed. This could be explained by the idea that differential CpG methylation of upstream regulators can lead to differential expression of genes downstream.

4.3 Public Health Relevance

Globally, preterm birth is the leading cause of death in children under the age of five [232]. In the United States alone preterm birth occurs in half a million pregnancies every year [233]. Newborns that are born prematurely are at a higher risk of morbidity, mortality, and developmental problems throughout their lives, including neurocognitive delays [234]. Multiple factors contribute to preterm birth, including bacterial infection during pregnancy [7]. Recently the placenta, which connects the mother to the developing fetus and regulates the prenatal environment, has been shown to harbor microorganisms [10, 46]. However, there has been little research on how these microorganisms impact the placenta, its function, and the development of the fetus. Our research examines the relationship between microorganisms in the placenta and three different factors: neurocognitive function at age 10, placental CpG methylation, and

expression of inflammation-related genes in the placenta. Importantly, it reveals that each microbial species has varying effects on the placenta and the fetus.

Our findings indicate that microorganisms in the placenta are associated with increased odds of decreased neurocognitive function at age 10, except when *Lactobacillus* sp. is present. *Lactobacillus* sp. has a protective effect on neurocognitive function as assessed by LPA score. While more research needs to be done, our findings indicate that *Lactobacillus* sp. could potentially be used as a way to counteract the effects of other bacterial species in mothers that have an intrauterine infection. Additionally, our findings indicate that the presence of *Streptococcus* Group B in the placenta leads to the most differentially expressed genes in the placenta as well as the strongest inflammatory response. While this inflammation was not associated with neurocognitive outcomes at 10 years of age, previous research has shown that inflammatory cytokines can damage the developing fetal brain [16-20].

Streptococcus Group B is the leading cause of sepsis and meningitis in newborns [211, 212] and its presence in the placenta leads to preterm birth and chorioamnionitis [213]. Currently, testing for *Streptococcus* Group B is part of routine prenatal care. However, this testing usually occurs between the 35th and 37th week of pregnancy. For babies that are born preterm they may not have had this testing. Also, these screenings only indicate whether *Streptococcus* Group B is present in the vagina. It does not indicate whether the bacteria have ascended into the intrauterine environment. Earlier screenings could indicate whether mothers are carriers of *Streptococcus* Group B and if they are susceptible to ascending infection. Given the impact that *Streptococcus* Group B has on the placenta, earlier treatment could be useful. Taken together these studies support the DOHaD hypothesis and provide evidence that prenatal

exposures, through epigenetic alterations in the placenta, can influence later life function of the child.

4.4 Current Research as a Platform for Future Hypotheses Testing

Our research revealed the association between bacteria in the placenta, biological signaling pathways in the placenta, and child health later in life. Even though our findings contribute to understanding the underlying biological mechanisms that link placental infection and later-life neurocognitive function there are still gaps in the knowledge. For this reason, there are clear areas to target to improve the mechanistic understandings and public health relevance collected from this study. Our findings provide the basis for hypothesis generation and future research questions, include those detailed here.

4.4.1 What Other Microorganisms are Present in Preterm Placentas and are they Associated with Neurocognitive Function at 10 Years of Age and Placental Cpg Methylation?

Results from our study suggest that the presence of different bacterial types in the placenta have varying effects on placental CpG methylation, inflammation-related gene expression, and neurocognitive function at age 10. Given that our bacterial analysis used culture techniques to identify bacteria we were only able to identify targeted bacteria. Other bacterial species were likely present in the placenta and could have been contributing to placental alterations and later life outcomes. In our analysis, we also focused on individual bacteria types. However, in many of the placentas multiple bacteria were present. Examining the bacterial community as a whole might give more insight into and better understanding of how the bacteria are affecting the placenta and the developing fetus.

One technique that would allow for this type of analysis is 16S rRNA gene sequencing A major strength of 16S rRNA gene sequencing is that it allows for the full microbiological diversity in a sample to be detected, even if species are of low-abundance [13]. This is possible

because the 16S rRNA gene is highly conserved across bacterial species while also allowing for differentiation between species [235]. One drawback of this method is that it cannot differentiate between living and dead bacteria [12]. The questions that could be tested would be the similar to those asked in Chapter 1 and 2: (1) are <u>microorganisms in the placenta associated with differential performance and brain function of school-age children, with variable response depending on bacterial species?; (2) Is</u> <u>the presence of microorganisms in the placenta associated with altered CpG methylation of development-associated genes?</u>

Analyzing the placentas for bacterial presence using 16S rRNA gene sequencing would allow for the identification of the full microbial community present in the samples. With this information, we could run the same models that we ran in Chapter 1 and Chapter 2 and determine whether any other bacteria are associated with neurocognitive or social-communicative function at age 10 or differential CpG methylation in the placenta.

4.4.2 Is Genome-Wide Gene Expression Associated with Microorganisms in the Placenta and 10-Year Neurocognitive Outcomes?

In Chapter 3 we used a targeted-gene approach to focus on inflammation-related genes and examine whether their expression was associated with placental microorganisms or neurocognitive outcomes later in life. While we found an association with microorganisms in the placenta there was no association with the specific genes we selected and neurocognitive outcomes. Future research could use a genome-wide approach to identify genes outside of the targeted set of 229 inflammation-related genes that may be associated with placental bacteria presence and later-life neurocognitive function. The question that could be tested is: <u>Is mRNA expression of any genes associated with bacterial presence in the placenta and neurocognitive function at age 10?</u>

This question could be assessed by analyzing the placentas using RNA sequencing (RNA-Seq), also known as whole transcriptome shotgun sequencing. Then, using the same statistical models used in Chapter 3, gene expression of all genes could be compared to the presence of placental bacteria and then subsequently to neurocognitive outcomes at 10-years of age. However, this would not address the limitation that mRNA may begin to degrade in the placenta following labor [230].

4.4.3 Is CpG Methylation in the Placenta a Mediator or a Moderator of Placental Bacteria and Neurocognitive Function at Age 10?

Our research demonstrated an association between microorganisms in the placenta and school-aged neurological function as well as differential placental CpG methylation. While we hypothesize that CpG methylation is an underlying biological mechanism, it is unknown whether placental bacteria directly affect CpG methylation in the placenta and whether this in turn affects neurocognitive function later in life. Support for this could be tested using a mediation analysis. The question that could be tested is: Does the methylation status of placental CpG sites act as a mediator of placental microorganisms and neurocognitive outcomes at age 10?

The mediation analysis could be done using the existing data from the ELGAN cohort on placental microorganisms, CpG methylation in the placenta, and neurocognitive function at age 10. However, analyzing more of the ELGAN placentas for methylation would help increase the statistical power. Examining whether CpG methylation is a mediator or moderator of the association between microorganisms in the placenta and neurological function later in life would allow for a better understanding of the underlying biological processes at work in the placenta that could have long-lasting effects on the fetus.

4.5 Conclusions

In summary, a major contribution of this work is the finding that microorganisms in the placenta are associated with altered signaling pathways in the placenta as well as long term neurological outcomes in the child. This is demonstrated by associations between various placental microbes and neurocognitive function at age 10, differential methylation of CpG probes in placental tissue, and placental upregulation of inflammation-related gene. Collectively, these studies increase knowledge related to the potential mechanisms by which bacteria in the placenta contributes to later-life outcomes, acting as a foundation for future research on the topic.

APPENDIX 1: SUPPLEMENTAL TABLE 1

Supplemental Table 1. Genes associated with enriched biological functions and associated canonical pathways

Immune-Related (n=50)	Transcription/Growth Factors (n=134)	Inflammatory response (n=35)
ABL1	*EGFR*	*ABL1*
ADAR	*FGFR2*	*ADAR*
ARHGEF2	*IGF1R*	*ATG5*
ATG5	*FGF2*	*BMPR1A*
BMPR1A	*ABTB1*	*CACNA1C*
C4BPA	*TNK2*	*CCL28*
CACNA1C	*ABL1*	*CD4*
CCL28	*ADD1*	*CHGA*
CD4	*AKAP13*	*CSF1R*
CHGA	*PRMT2*	*DDX60*
CLEC4C	*ADRA2C*	*DEFA6*
COL4A3BP	*AKT2*	*EIF2AK4*
CSF1R	*TFAP2A*	*GGT1*
DAB2IP	*AP2M1*	*IGF1R*
DDX60	*ANKFY1*	*IL4R*
DEFA6	*ANKRD27*	*IRF4*
EIF2AK4	*ANKS1A*	*IRF7*
GGT1	*API5*	*LAX1*
HLA-H	*ARID1B*	*LGALS3BP*
IGF1R	*ARHGEF11*	*NECTIN1*
IL4R	*ASCL1*	*NPY*
IRF4	*BAIAP2*	*ORAI1*
IRF7	*BMP7*	*PRKCZ*
LAX1	*BCAR1*	*PTK2B*
LGALS3BP	*BMP8B*	*PYCARD*
LY86	*BMPR1A*	*SLC39A4*
NLRP1	*CDKN1C*	*SPACA3*
NPY	*CELSR3*	*SRC*
ORAI1	*CHSY1*	*TNFAIP3*
PRELID1	*CREG1*	*TOLLIP*
PRKCZ	*CSNK2B*	*TRIM26*
PTK2B	*DAB2IP*	*TRIM27*
PVRL1	*DOCK1*	*TYRO3*
PYCARD	*DRG1*	*UBE2N*
		UNC5B

RNF135	*DNM2*
SFTA3	*DPF3*
SKAP2	*EIF2AK4*
SLC39A4	*ENG*
SMAD6	*EGR4*
SPACA3	*EHD1*
SRC	*EGFL8*
TNFAIP3	*EGF*
TNK2	*EPS15*
TOLLIP	*ERCC1*
TRIM26	*EPS8L1*
TRIM27	*FBN3*
TYRO3	*PTK2B*
UBE2N	*FGF14*
UNC5B	*FEZ1*
ZNF683	*FOXK1*
	GAS7
	GDF5
	GDF9
	GRHL2
	GSK3B
	GXYLT2
	HYAL1
	IGFBP7
	HTRA1
	IGFBP4
	INPP5K
	ITGB5
	IRS2
	CAMK1D
	KCNC1
	PRKCZ
	RPS6KA2
	KSR1
	LEFTY2
	TRIM71
	LRP1
	LEFTY1
	MCM7
	MAPK12

MAP2K5

SBF1

NAIF1

NCK2

SLC9A3R1

NTN1

NPHP4

SLC34A2

NUBP1

PA2G4

PCSK6

PAK4

PARD3

GIGYF1

PLXNA1

PPP1CA

PSPN

PTPRJ

RAB35

RERE

RAB11FIP2

RHBDF1

RPTOR

POLR2B

RUVBL1

SASH3

SIK1

SMAD6

SMAD7

SKI

SFRP5

SHC1

SOX10

SLC9A1

SOX5

SPSB4

SSH1

STAT5A

SPTB

SRC

STEAP3

TAOK3

TEAD4

TGFB3

PLAT

TSC2

USP4

TYRO3

USP9X

VASN

SORBS3

WNT7A

WWOX

WFS1

WWP2

HIVEP3

ZFHX3

ZIC1

EEF1G

CSF1R

Associated Canonical Pathways (P-values)

-NF-κB Signaling (5.98E-05)	-Molecular Mechanisms of Cancer (2.82E-13)	-NF-κB Signaling (1.09E-05)
-Role of Osteoblases, Osteoclasts and Chondrocytes in Rheumatoid Arthritis (1.97E-04)	-Human Embryonic Stem Cell Pluripotency (3.79E-13)	-Calcium-induced T Lymphocyte Apoptosis (1.72E-04)
-Molecular Mechanisms of Cancer (2.17E-04)	-Role of Osteoblasts, Osteoclasts and Chondrocytes in Rheumatoid Arthritis (2.23E-11)	-CCR5 Signaling in Macrophages (1.96E-04)
-Calcium-induced T Lymphocyte Apoptosis (4.84E-04)	-Axonal Guidance Signaling (7.02E-11)	-STAT3 Pathway (2.41E-04)
-CCR5 Signaling in Macrophages (5.52E-04)	-Glioma Signaling (1.10E-10)	-Macropinocytosis Signaling (3.15E-04)

Supplemental Table 2. Enriched transcription factors and associated genes from gene set

Transcription Factor	Associated Genes
Tripartite Motif Containing 24 (TRIM24)	Tripartite Motif Containing 26 (*TRIM26*) Galectin 3 Binding Protein (*LGALS3BP*) Interferon Regulatory Factor 7 (*IRF7*) DExD/H-Box Helicase 60 (*DDX60*)
RELA Proto-Oncogene, NF-κB Subunit (RELA)	Collagen Type IV Alpha 3 Binding Protein (*COL4A3BP*) Insulin Like Growth Factor 1 Receptor (*IGF1R*) Interferon Regulatory Factor 4 (*IRF4*) Interferon Regulatory Factor 7 (*IRF7*) ORAI Calcium Release-Activated Calcium Modulator 1 (*ORAI1*) TNF Alpha Induced Protein 3 (*TNFAIP3*)
Tumor Protein p53 (TP53)	Rho/Rac Guanine Nucleotide Exchange Factor 2 (*ARHGEF2*) Colony Stimulating Factor 1 Receptor (*CSF1R*) Insulin Like Growth Factor 1 Receptor (*IGF1R*) Interleukin 4 Receptor (*IL4R*) Interferon Regulatory Factor 7 (*IRF7*) PRELI Domain Containing 1 (*PRELID1*) Protein Kinase C Zeta (*PRKCZ*) PYD and CARD Domain Containing (*PYCARD*) SMAD Family Member 6 (*SMAD6*) SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase (*SRC*) Unc-5 Netrin Receptor B (*UNC5B*)
Core-Binding Factor Beta Subunit (CBFB)	CD4 Molecule (*CD4*) Colony Stimulating Factor 1 Receptor (*CSF1R*) Galectin 3 Binding Protein (*LGALS3BP*)
POU Class 5 Homeobox 1 (POU5F1)	Bone Morphogenetic Protein Receptor Type 1A (*BMPR1A*) Insulin Like Growth Factor 1 Receptor (*IGF1R*) ORAI Calcium Release-Activated Calcium Modulator 1 (*ORAI1*) PYD and CARD Domain Containing (*PYCARD*) SRC Proto-Oncogene, Non-Receptor Tyrosine Kinase (*SRC*)

Supplemental Table 3. Genes associated with placental microorganisms

Bacteria	Gene	p-value	q-value	Fold Change
Alpha-hemolytic *Streptococcus*	*CYSLTR2*	0.002	0.075	3.88
	TGFB3	0.002	0.075	2.63
Streptococcus **Group B**	*C1QA*	0.031	0.046	2.25
	C1QB	0.002	0.014	3.28
	C1R	0.001	0.014	3.70
	C1S	0.004	0.015	2.83
	C2	0.044	0.049	2.23
	C4A	0.001	0.014	2.85
	CCL2	0.026	0.043	2.00
	CCL3	0.002	0.014	2.97
	CCL4	0.014	0.032	2.71
	CCL8	0.007	0.020	2.82
	CD163	0.038	0.046	2.48
	CFL1	0.042	0.049	2.10
	CXCL1	0.003	0.015	2.12
	CXCL2	0.009	0.025	2.35
	HDAC4	0.029	0.046	2.14
	HLA-DRA	0.038	0.046	3.32
	HLA-DRB1	0.005	0.015	3.70
	IL1A	0.050	0.053	1.87
	IL1R1	0.034	0.046	2.47
	MAP2K1	0.020	0.036	2.59
	MAP3K5	0.020	0.036	2.28
	MX2	0.038	0.046	2.64
	OAS2	0.004	0.015	3.36
	PDFGA	0.016	0.034	2.23
	PTGIR	0.032	0.046	1.84
	RELB	0.019	0.036	1.92
	STAT1	0.011	0.027	3.24
	TGFB3	0.002	0.014	3.078
Staphylococcus **sp.**	*C4A*	0.050	0.096	1.67
	GNGT1	0.026	0.096	1.84
	IL4	0.032	0.096	1.42

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