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HOST-MICROBE INTERACTIONS: GUT MICROBIOTA AND ITS EFFECTS ON DEVELOPMENTAL PROGRAMMING OF THE BRAIN, PLACENTA AND TESTIS

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Stockholm 2014

Published by Karolinska Institutet.
Printed by Åtta.45 Tryckeri AB

ISBN 978-91-7549-755-6

Host-Microbe Interactions: Gut Microbiota and its Effects on Developmental Programming of the Brain, Placenta and Testis
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“All life is an experiment. The more experiments you make the better “

Ralph Waldo Emerson

إلى والديَّ الكريمين

إلى زوجي الغالي: عمر

إلى قرة عيني: دانة

وإلى كل من كافح في سبيل العلم

ABSTRACT

Reproduction of a mammalian organism involves inherited genetic programming and environmental factors that collectively shape organ development and function in the new offspring. One such factor are the indigenous microbiota and their interactions with the host. In mammals, the placenta ensures the supply of nutrition and oxygen to the fetus *in utero*. Microbes are thought to contribute to establishment of barrier functions, activation of the immune system and supply of nutrients to the host. The objectives of my thesis were to assess whether microbes can modulate barrier functions connected to the placenta, brain, and testis, as well as influence the physiological functions of these organs. All three have distinctive tissue barriers that control the passage of molecules between the blood and tissue in order to optimize function.

Paper I – **Maternal microbes influence placental development and reduce maternal metabolic stress during pregnancy.** Germ-free (GF) pregnant mice exhibited elevated glucocorticoids levels and increased gluconeogenesis and ketone body production. As a result, these dams showed marked impairment of placental development and establishment of the blood-placental barrier (BPB), with impaired capillary microstructure and reduced expression of tight junction proteins (TJPs). Metabolically, GF dams showed altered lipid and carbohydrate metabolism and drastically reduced hepatic levels of glycogen, as well as elevated levels of angiopoietin-4 (ANGPTL4), which is known to inhibit lipoprotein lipase and thus lipogenesis.

Paper II – **Maternal microbes contribute to the establishment and integrity of the blood-brain barrier (BBB).** During intra-uterine life, the BBB in GF mice was more permeable than that of specific-pathogen-free (SPF) animals, a difference that persisted into adulthood and was associated with reduced expression of TJPs. Exposure of adult GF mice to the gut microbiota of SPF animals reduced this permeability and up-regulated the expression of some TJPs. Furthermore, perfusion with Evans blue revealed that monocolonization of the intestine of adult GF mice with either *Clostridium tyrobutyricum*, a bacterial strain that produces butyrate, or *Bacteroides thetaiotaomicron*, which produces mainly acetate and propionate, was sufficient to reduce BBB permeability. Moreover, oral administration of the bacterial metabolite butyrate mimicked this effect. This effect of gut microbiota and butyrate may be mediated by an epigenetic mechanism, since administration of butyrate or monocolonization with *Clostridium tyrobutyricum* elevated levels of histone acetylation in brain lysates.

Paper III – **Gut microbes modulate the permeability of the blood-testis barrier (BTB) and regulate endocrine functions of the testis.** Establishment of the BTB, which normally occurs 16 days postpartum, was delayed in GF mice. Perfusion with Evans blue demonstrated increased BTB permeability associated with reduced expression of TJPs in these same mice during adulthood. The testis- pituitary axis was also affected by the lack of gut microbiota, since GF mice exhibited lower serum levels of gonadotropins (LH and FSH) and lower intratesticular levels of testosterone than the SPF animals. Interestingly, exposure of GF mice to *Clostridium tyrobutyricum* restored the integrity of the BTB and normalized testosterone levels.

In conclusion, the present work documents the influence of indigenous microbiota on the functions of the murine BPP, BBB and BTB, as well as their ability to support the mother during pregnancy. These findings suggest that microbes contribute to programming during critical windows of development.

LIST OF SCIENTIFIC PAPERS

I. **Al-Asmakh M**, Anuar F, Phua T, Mabel YSF, Kundu P, Zadjali F, Rafter J, Hibberd ML, Fundele R, Tan NS, Parini P, Hedin L, Pettersson S. **Commensal microbiota supports placental development and maternal metabolism.** *Manuscript*

II. Braniste V[*], **Al-Asmakh M**[*], Kowal C[*], Anuar F, Abbaspour A, Tóth M, Korecka A, Bakocevic N, Guan NL, Kundu P, Gulyás B, Halldin C, Hultenby K, Nilsson H, Hebert H, Volpe BT, Diamond B, Pettersson S. (2014) **The gut microbiota influences blood-brain barrier permeability in mice.** Science Translational Medicine. 2014; 6(263).

III. **Al-Asmakh M**[*], Stukenborg JB[*], Reda A, Anuar F, Strand ML, Hedin L, Pettersson S, Söder O. (2014) **The gut microbiota and developmental programming of the testis in mice.** PLoS One. 2014; 9(8).

[*]Shared first authorship

RELATED PUBLICATIONS

I. **Al-Asmakh M**, Anuar F, Zadjali F, Rafter J, Pettersson S. (2012). **Gut microbial communities modulating brain development and function. (**Review) Gut Microbes. 2012; 3(4):366-73.

CONTENTS

LIST OF ABBREVIATIONS

ABC	Active efflux carriers
ACTH	Adrenocorticotrophic hormone
AJ	Adherens junctions
ANGPTL4	Angiopoietin-like 4
TBC	Tubulobulbar complex
BBB	Blood-brain barrier
BTB	Blood-testis barrier
BTeta	*Bacteroides thetaiotaomicron*
CBUT	*Clostridium Tyrobutyricum*
CNS	Central nervous system
CRH	Corticotropin-releasing hormones
DOHaD	Developmental origins of health and diseases
ES	Ectoplasmic specialization
FFA	Free fatty acids
GC	Glucocorticoids
GR	Glucocorticoid receptor
GRE	Glucocorticoid response elements
GF	Germ-free
GLUT	Glucose transporter
hCG	Human chorionic gonadotrophin
11βHSD	11β-hydroxysteroid dehydrogenase
HPA	hypothalamo-pituitary-adrenal
IGF2	Insulin-like growth factor 2
ISF	Interstitial fluid
NaBu	Sodium butyrate
PPAR	Peroxisome proliferator-activated receptor
SCFAs	Short chain fatty acids
SLCs	Solute carriers
SPF	Specific-pathogen -free

SYN	Syncytiotrophoblasts
T2DM	Type 2 diabetes mellitus
TJ	Tight Junctions
VEGF	Vascular endothelial growth factor
ZO	Zonula occludens

1 INTRODUCTION

1.1 MICROBES

The vast array of intestinal bacteria (gut microbiota), which weigh 1-2 kg in an adult human is currently estimated to contain 100 trillion members of 40,000 bacterial species belonging to 1800 genera. These numbers are astonishing, meaning that there are 150-fold more bacterial than human genes in the human intestinal lumen (1, 2). The term microbiome is used to describe all constituents of these microbiota including genes, proteins and metabolites (3). During the course of evolution gut bacteria ceased acting as prey and began helping to break down otherwise undigestable molecules. This role appear to have become more diverse and complex in the more advanced compartmentalized gastrointestinal tracts of mammals than in the relatively simple tube of the ancient cyclostomatida (4, 5).

The gut microbiota of invertebrates contains small number of species than in vertebrates. Invertebrates have no adaptive immune system and it has been proposed that the expansion of microbiota in vertebrates could have occurred as a result of the ability of the adaptive immune system to recognize and remember specific microorganisms. Despite significant interindividual variation in the composition of the microbiota of different genera and strains, this composition appears to be conserved within a given species (Figure 1.1). For example, the microbiota communities of humans are dominated by the two bacterial phyla Firmicutes and Bacteroidetes with smaller contribution from Actinobacteria and Proteobacteria (4, 6). The mouse is highly similar to humans both at the taxonomic and genetic levels (99% shared genes with humans), so that findings on murine host-microbiota interactions should be applicable to humans (4).

An estimated 80% of the bacterial species in the mammalian colon have yet to be cultivated, although advances in metagenomic now allow such characterization without the need for culturing. Diet help shape the microbiota community: the diversity increases from carnivory to omnivory to herbivory (7) and changes in diet lead to shift in the composition. For instance, adding fiber to the diet of dogs increases the number of Firmicute bacteria (8).

Furthermore, obesity influences the composition of the gut microbiota. Thus, most people living in the United States possess a gut microbiota adopted to and capable of digesting a high-fat, high-protein diet; whereas those living in rural Malawi and the Amazona region of Venezuela have distinct microbial consortia optimized for the breakdown of complex carbohydrates. Interestingly, in some occasions and in response to a change in diet the gut microbiome does not only alter its composition, but also change its genetic make-up via horizontal gene transfer. For example, the gut of certain Japanese harbors the bacterium *Bacteroides plebeius*, which bears a gene transferred horizontally from the marine bacterium *Zobellia galactanivorans* that enables seaweed polysaccharides to be degraded. In addition to diet, the composition of the gut microbiota is also influenced by the genotype, social group, medical history, and advanced age of the host (5, 9).

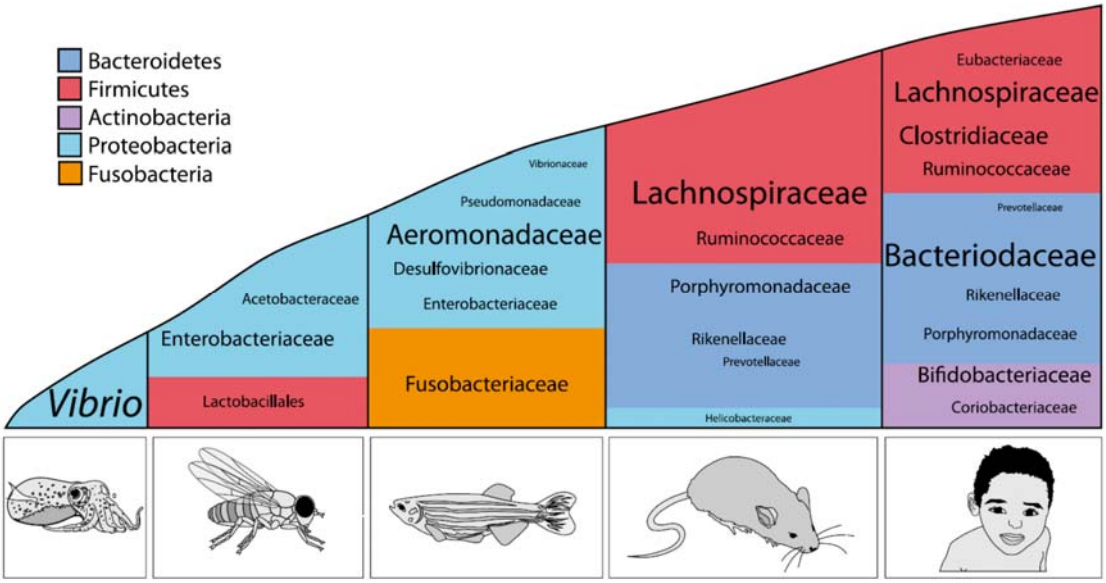

Figure 1.1 The composition of the microbiota across species. Although there can be significant interindividual variation, there are general trends within a given species, particularly at the phylum level. Different phyla are represented by different colors, and the relative abundance of the lower taxonomic levels indicated by font size (4).

1.1.1 Interactions between gut microbiota and the host

Accumulating evidence reveals that the gut microbiota plays a major role in promoting health, as a result of which it is often referred to as the 'forgotten organ'(10). The relationship between the host and microbiota is symbiotic and mutualistic, each deriving benefits from the other. These two terms are similar but mutualism is defined as 'an interaction between species that is beneficial to both of them' and symbiosis as 'the living together of two organisms in close association'(11). While the host provides the microbiota with a protected and nutrient-rich environment, the microbiota enhance, e.g., digestion, immunity and neuronal development.

This symbiotic partnership has led to the concept of holobiont, i.e., the host and all its associated microbiota. Since such mutual association not only involves sharing biological and chemical needs, but also genetic information, the concept of the hologenome defined as the sum of the host genome and associated microbiota has emerged (Figure 1.2). The genetic wealth of the microbiota is thought to contribute to the holobiont's fitness (adaptation, survival, development, growth and reproduction) (12, 13).

The hologenome theory of evolution proposed by Zilber-Rosenburg and Rosenberg (2007) states that natural selection favors not a single organism, according to traditional Darwinism, but rather the holobiont with its hologenome (12, 14). Thus, drosophila prefer to mate with other flies raised on the same diet (e.g. starch) and this mating preference is eliminated by antibiotic treatment, indicating that the fly microbiota might be involved (15, 16). Furthermore, Brucker and Bordenstein (2013) demonstrated that the microbiomes of individual wasps of closely related *Nasonia girault*i and *Nasonia longicornis* species were more similar than that of the more distantly related *Nasonia vitripennis*. Moreover, dysfunctional interactions between the microbiomes formed by crosses of *N. vitripennis* with *N. girault*i

resulted in the death the of hybrid larvae. Remarkably, when these hybrid larvae were reared on a bacteria-free diet, they survived and exhibited almost normal fitness (17).

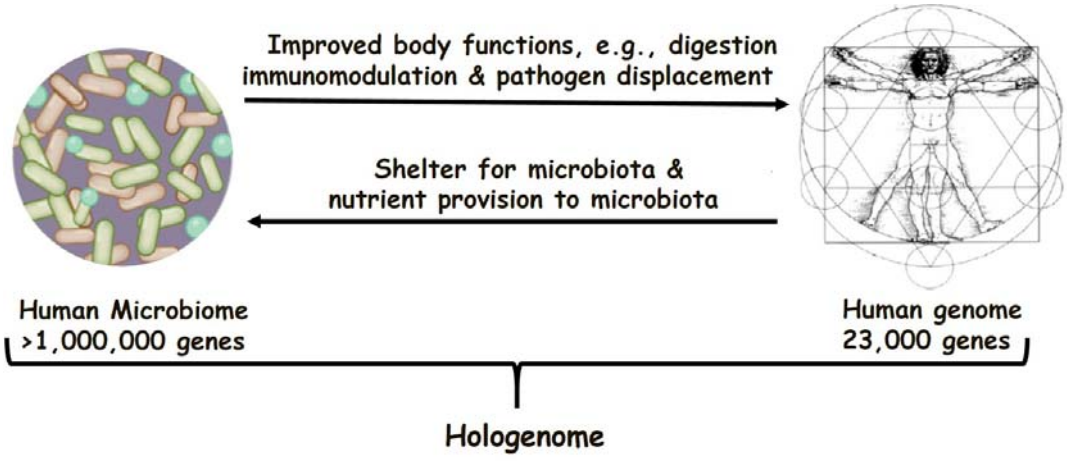

Figure 1. 2 The concept of hologenome. Modified from (13) and (14).

The term commensal, also used to describe the microbiota, means harmless and 'eating at the same table', i.e., one organism benefits without affecting the other, which is not the case with the host-microbial relationship (11). The mutualistic/symbiotic interaction between the microbiota and host requires both that the microbes colonize and persist in the host and that the host's immune system can tolerate and control them (18). Disruption of this tight relationship (dysbiosis) has been related to a spectrum of diseases, including non-alcoholic fatty liver disease, diabetes, hypertension, inflammatory bowel disease, colon cancer, allergies, asthma and, very recently, even neuropathologies (19).

1.1.2 Functions of the gut microbiota

The most extensive microbial population is present in the large intestine, where it confers many benefits on the host, including pathogen displacement, development of the immune system, barrier fortification, production of vitamin and absorption of nutrients. These microbiota are key factors in maintaining homeostasis, with functions affecting virtually every organ in the body, e.g., regulation of bone mass, brain development and behavior, hepatic function, and aspects of adipose tissues and the cardiovascular system (Figure 1.3).

Recent studies also point to the involvement of the microbiota in the development of personalized medicine and in xenobiotic metabolism. Certain environmental toxins and drugs are metabolized by the gut microbiota into less or more harmful substances. Several biological active compounds are also produced by the gut microbiota such as short chain fatty acids (SCFAs), conjugated linoleic acid, phenoles, indoles, or trimethylamine (3).

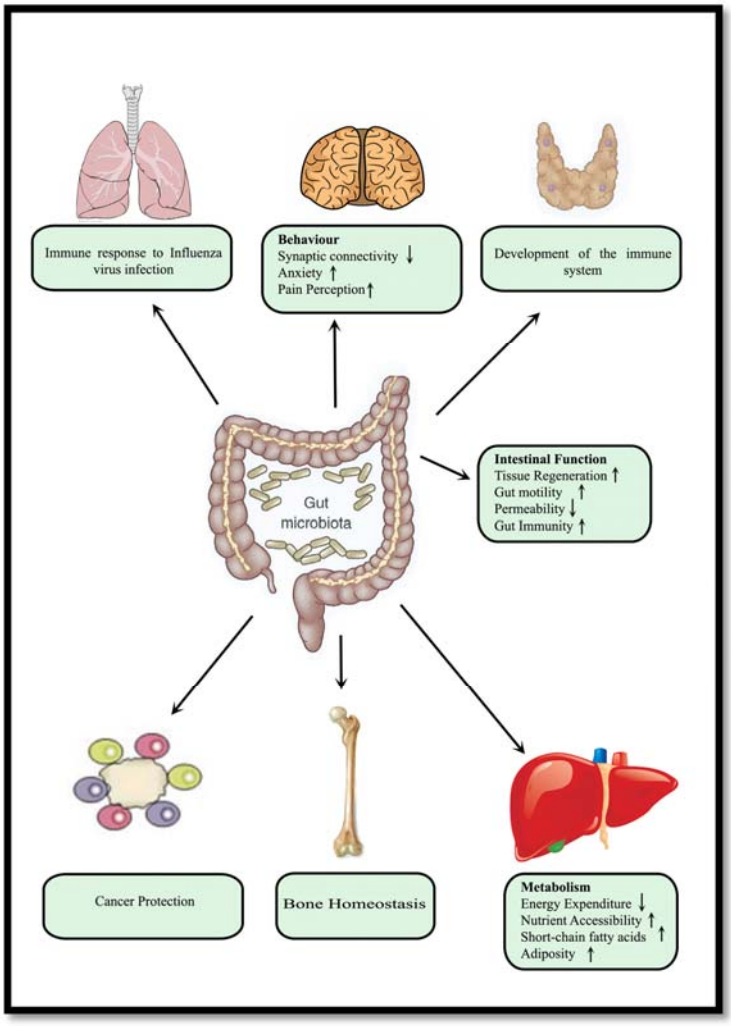

Figure 1.3. Microbial impact on host physiology. Modified from (20) and (21).

1.1.3 Short chain fatty acids (SCFAs)

Among the signaling molecules produced by the gut microbiota that could affect host physiology are the short-chain (1-6-carbons) fatty acids (SCFAs) such as butyrate, acetate and propionate that arise from the fermentation of fibers (22). Bacteria of the Bacteroidetes phylum (e.g., *Bacteroidetes thetaiotamicron*) produce large amounts of acetate and propionate; whereas bacteria of the Firmicutes phylum (e.g., *Clostridium tyrobutyricum*) produce large amounts of butyrate. In this context, the most abundant SCFA is butyrate (C4), followed by acetate (C2) and propionate (C3) (22).

Butyrate, the major fuel for colonocytes, appears to participate in the regulation of intestinal cell growth and differentiation (22); increases the expression of tight junction proteins (TJPs) *in vitro* (23); and induces angiogenesis in the small intestine *in vivo* (22). Acetate and propionate are transported to the liver and peripheral organs, where they act as substrates for gluconeogenesis and lipogenesis. In addition to providing energy, SCFAs have been implicated in regulation of the intestinal immune system (affecting the oxidative burst, degranulation and phagocytosis (24)), as well as in promoting mineral absorption, mucin production, and the expression of antimicrobial peptides (25).

SCFAs enter cells both by simple diffusion and through the action of transporters of monocarboxylates and other solutes. Butyrate and propionate, but not acetate control gene expression by inhibiting histone deacetylase (HDAC), resulting in hyperacetylation of both histones and non-histone proteins (26). All three of these SCFAs can also activate cells through G-protein-coupled receptors (GPCRs), such as GPR41 or GPR43, with differing ligand specificities and potencies. Propionate is the most potent activator of both GPR41 and GPR43; acetate has higher affinity for GPR43; whereas butyrate activates GPR41 more potently (27).

Activation of GPR41 and GPR43 by SCFAs stimulates secretion of peptide YY, which slows down gastrointestinal transit. Stimulation of GPR43 by SCFAs is crucial for the regulation of energy balance and adiposity and in adipocytes, signaling via GPR41 induces lepitn secretion and elevates adipogenesis (28). Moreover, signaling via GPR43 has anti-inflammatory effects as reflected in the observation that GPR43-kockout (*Gpr43*$^{-/-}$) murine models of colitis, arthritis and asthma display exacerbated or unresolved inflammation (29).

Exposure to metabolites produced by the gut microbiota, such as SCFA, begins already *in utero* and may contribute to developmental programming and susceptibility to disease development later in life.

1.2 MICROBES AND DEVELOPMENTAL PROGRAMMING

The prenatal period is the most important and critical phase of mammalian development, preparing the fetus for postnatal life. Exposure to environmental stimuli during this sensitive and vulnerable period could be detrimental to adult health. For instance, there is now much evidence that a nutrient poor *in utero* environment due to a poor maternal diet or placental insufficiency 'programs' the fetus in such a way as to enhance the risk of developing cardiovascular and metabolic diseases later in life. Moreover, convincing epidemiological and experimental findings support a strong association between maternal undernutrition or fetal overexposure to stress-related hormones such as the glucocorticoids and subsequent risk of developing a number of pathologies as an adult, such as diabetes, hypertension, obesity, immune dysfunction, and behavioral problems (30, 31). Such pathological conditions undoubtedly affect the quality of life and, ultimately, reduce life expectancy. Accordingly, if organ systems are indeed programmed earlier interventions designed to correct developmental defects should be more effective than later interventions.

1.2.1 The concept of developmental programming

The concept of developmental programming was first proposed by Barker as the 'fetal origin of adult disease', i.e., a correlation between poor fetal environment and later risk for disease (32). However, the realization that human development continues postnatally, influenced primarily by the composition of and nutrition in breast milk, led to a change in terminology to the 'developmental origins of health and diseases (DOHaD)' (33). The relative contribution of genes and environment to the association between early life experience and later health is still the subject of intense debate.

In 1962, the geneticist James Neel put forward the 'thrifty genotype' hypothesis in an attempt to explain the relatively high incidences of obesity and type 2 diabetes mellitus (T2DM) among certain ethnic groups (34, 35). This hypothesis postulates that evolutionary exposure of

humans to food scarcity and famine selected for "thrifty genes" designed to maximize metabolic efficiency and searching for food. In modern-day societies in which food is abundant and the life-style sedentary, these genes predispose to diseases caused by excess caloric intake, such as obesity and insulin resistance (34).

The thrifty genotype hypothesis is often cited alongside the 'thrifty phenotype' hypothesis proposed 30 years later by Hales and Barker (1992), who suggested that fetal response to energy insufficiency induces a thrifty phenotype characterized by insulin resistance and a shift in the circulation to protect the growth of vital organs such as the brain and heart at the expense of other tissues such as muscle, the liver and the endocrine pancreas (36, 37). Such adjustment and growth plasticity enhance immediate fetal survival, but elevate the subsequent risk of developing metabolic diseases caused by nutritional excess and consequent weight gain (36). This hypothesis might explain, at least in part, why the incidences of metabolic and cardiovascular diseases are very low in areas of Africa where poor foetal nutrition is followed by poor postnatal nutrition and high physical activity, while these incidences are strikingly higher among individuals in these same areas who move from rural regions to urban areas with better nutrition and less physical activities (38).

Evidence from human studies supports this thrifty phenotype hypothesis. First, men and women exposed *in utero* to food shortage (the Dutch famine) at the end of the Second World War exhibit poorer glucose tolerance than those not exposed to the famine (39). Secondly, growth-restricted (or small-for-gestational-age) babies born to mothers who smoked during pregnancy are more prone to develop obesity and T2DM (40). And thirdly, among homozygotic twins, the twin with the lowest birth weight is more likely to develop diabetes (41). Experimental data from animal studies also provide strong support for this hypothesis (42).

Concerning the thrifty genotype hypothesis certain mutations (e.g., in the gene encoding glucokinase that result in insulin resistance) have been associated with low birth weight and later development of T2DM (43). However, such mutations appear to be rare, indicating that the environment also makes a substantial contribution in this connection (38).

At first glance, the 'thrifty genotype' and 'phenotype' hypotheses may appear to be in conflict. In fact, the 'thrifty genotype' describes long-term effects of selection on a population, whereas the 'thrifty phenotype' concerns the adaptive nature of offspring, including their plasticity during early development (44). (The term 'developmental plasticity' refers to permanent influence of environmental conditions encountered during development on a trait (33)).

The 'thrifty phenotype' hypothesis is more widely accepted and several proposals concerning such adaptive developmental plasticity have emerged in recent years (reviewed in (44)). One model proposes that foetal adjustments to the prenatal environment are not designed merely to improve immediate survival, but rather a 'predictive adaptive response' meant to promote survival during reproductive life. The metaphor used in this context is the weather forecast: the developing organism responds adaptively to information gained via the placenta or during lactation to make a forecast concerning the external environment in which it will later grow and, in particular reproduce (44-46).

1.2.2 Gut microbiota and developmental programming

Since microbial colonization of mammals controls a variety of physiological functions in the host, early alterations in this colonization might influence the risk of developing diseases later in life. Initial microbes are provided *in utero* by the maternal microbiota and influenced thereafter by the mode of birth and type of infant feeding and exposure to antibiotics (47, 48). Consequently, the microbiota is heterogeneous and unstable until approximately 2–4 years of age, when it becomes more stable and begins to resemble the adult microbiota (49).

Experimental, clinical and epidemiological findings indicate that early microbial colonization during pregnancy and birth may exert a long-lasting impact on the risk of developing allergic, autoimmune and metabolic diseases in adulthood. For example, exposure of a pregnant woman to farm animals, which presumably leads to more extensive fetal contact with microbes protects the infant from immune-mediated conditions such as asthma and eczema. Such observations forms the basis for the 'hygiene hypothesis', which states that a relative lack of microbial exposure due to the hygienic conditions in developed countries hampers proper maturation of the immune system, thereby predisposing individuals to allergies and autoimmune diseases (50). This hypothesis also fits well with the concept of developmental programming discussed above.

In animals, raising the fiber content of the maternal diet during pregnancy and lactation caused specific alterations in the pup's microbiota, including populations enriched in *Bifidobacterium spp* and *Lactobacillus spp* (51). Maternal administration of antibiotics exerted enhanced permeability and systemic inflammation in the offspring (52), while interestingly supplementation of the maternal diet with the probiotic bacterium *Lactobacillus plantarum* restored intestinal permeability and stimulated the growth of the offspring's intestine (52). These later responses occurred only in rats exposed to this probiotic bacterium early between postnatal days 3 and 10 (53), emphasizing the early window of action.

The role of gut microbiota in the developmental programming of the rodent brain, influencing both brain chemistry and behavior has been documented in several recent reports (54-56) For example, germ-free (GF) mice display less anxiety (54, 55) and more motor activity (54) than animals free from specific pathogen (SPF). Notably, the behavior of GF mice could only be normalized when they were colonized prenatally (i.e., through the mothers) (54).

1.2.3 The placenta and developmental programming

The placenta evolved to support fetal growth and its role in nutrient transfer is pivotal with respect to developmental programming. Defects in placental development cause growth restriction preceded by impairment of placental nutrient transport (57). Available evidence reveals the remarkable ability of the placenta to ameliorate, rather than exacerbate, the influence of environmental cues involved in developmental programming.

Initially, the placenta adapts to changes in the maternal environments by optimizing nutrient and gas transport to promote survival of the fetus. Resorption or abortion occurs only under the most extreme conditions, i.e., when the life of the mother is threatened (58). Although maternal illness, such as diabetes and pre-eclampsia, exert profound effects on placental development, there is growing evidence that the placenta is also affected by more subtle

signals related to maternal nutrition, body composition and life-style (e.g., exercise, smoking and alcohol intake) (57, 59). Placental adaptations to such insults include alterations in vascularization, thickness of the placental barrier, the expression and activities of key transporters of nutrients and epigenetic modifications of genes (60). To date, few experimental or epidemiological studies addressing the thrifty phenotype hypothesis have considered the placental contribution to developmental programming, which must be understood if efficient interventions are to be developed in the near future.

1.3 MICROBES AND THE PLACENTA

While the present dogma is that the mammalian embryo/fetus lives in a sterile environment as a result of the placenta barrier, it has become clear that bacteria are naturally present in cord blood (61) and meconium (62). Indeed, the placenta itself appears to harbor a unique microbiome (63). Consequently, it is of the utmost importance to examine the impact of interaction between the maternal microbiome and the placenta on health.

1.3.1 The mammalian placenta

The placenta, a transiently vascularized chimeric organ composed of both maternal and fetal tissues, is used by all mammals for reproduction, with the exceptions of monotremes (egg-laying mammals) such as the duck-billed platypus and four species of echidna (also known as spiny anteaters). The word *placenta*, derived from the Latin word for 'flat cake', was introduced in the sixteenth century by the Italian anatomist Renaldus Columbus (64, 65). Throughout history this organ held a place of honor and is, indeed, considered sacred in many cultures and societies. In ancient Egypt, the placenta was believed to be one of the gods and was paraded before the Pharaoh during royal processions (Figure 1.4) (64, 65). In other cultures, this organ is viewed as the older sibling and is buried ritually by the mother. In modern culture, the placenta is usually discarded, used for research or even sold as a cosmetic product (64).

Figure 1.4. The placenta (far left) depicted in ancient Egypt as an organ with two lobes attached to the umbilical cord.

The placenta, unique in connecting two genetically distinct organisms, delivers nutrients and oxygen from the mother to the fetus and also adapts maternal metabolic, endocrine, cardiovascular and immune functions to promote fetal growth and survival. Although long considered to be merely a passive transporter of maternal resources and fetal waste, advances in imaging technology and immunological methodology have revealed that the placenta produces a wide array of signalling molecules (hormones, cytokines, molecules that influence immune function and, indeed, all other classes of signaling molecules), that act both locally and at a distance. The placenta has been referred to appropriately by the late and eminent reproductive endocrinologist Samuel Yen as the 'third brain' of pregnancy (Yen 1994) (66). The placenta has a substantial capacity to respond to the intrauterine environment, while the fetal brain is immature (64).

1.3.2 Anatomy of the human and murine placenta

Despite the huge variation in placental development among mammals, the placentas of humans and mice share a number of structural, cellular and molecular features, as described extensively in several reviews (67, 68) . In both species, the placenta is divided into three regions: 1) The region proximal to the fetus, termed the labyrinth in mice and chorionic villi in humans, is specialized for the exchange of nutrients and gases containing fetal and maternal blood vessels. The tree-like branches in this region of human placenta provide a large surface area for exchange. On the other hand, in the murine the branches are much more interconnected, generating a maze-like pattern or labyrinth (67, 69, 70).

2) The middle layer consists of densely packed trophoblast cells, referred to as cytotrophoblast cell columns and the spongiotrophoblast layer in humans and mice, respectively. The precise function of this layer is unknown, although its integrity is absolutely vital to fetal survival. It may provide support for the underlying labyrinth/villi, although in mice spongiotrophoblast cells are known to secrete several polypeptide hormones (67, 69). Also in mice, the spongiotrophoblast layer contains another type of cell known as trophoblast glycogen cells, which provide substantial energy, especially during late gestation (67, 71). Unlike the labyrinth, the spongiotrophoblast layer does not contain any fetal blood capillaries, but it is traversed by maternal blood channels (the central artery and lateral veins) (67, 72).

3) The decidua, the outer layer of the placenta bordering the maternal side consists of cells that invade maternal blood vessels and have been designated as extravillous cytotrophoblasts and trophoblast giant cells in humans and mice, respectively (67). Under the influence of steroid hormones, primarily progesterone, uterine stromal cells are transformed into large secretory decidual cells. This process, known as decidualization, is characteristic of species with hemochorial placentas (including humans and mice) and is an essential prerequisite for implantation (64). In mice, embryo implantation induces decidualization; whereas in humans signs of decidualization are present as early as day 23 of the normal menstrual cycle and if fertilization does not occur the transformed uterine tissue is shed during menstruation (64, 68). In humans, trophoblast invasion normally extends up to, but not beyond, the inner third of the myometrium (The muscular wall of the uterus), whereas in mice trophoblasts do not invade into the myometrium (67, 68). A schematic illustration of the human and murine placenta is shown in Figure 1.5.

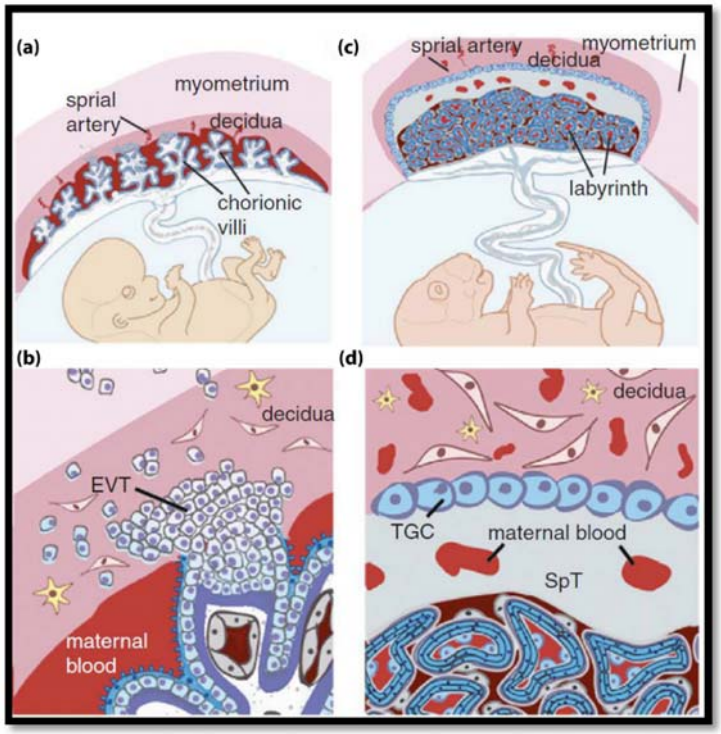

Figure 1.5. Comparative anatomy of the human and murine placentas. **(a)** The villous structure of the human placenta. **(b)** Cross-section of trophoblast region of the human uterus showing extravillous trophoblasts (EVT) invading the decidua. **(c)** The labyrinth structure of the murine placenta **(d)** The spongiotrophoblast (SpT) region and trophoblast giant cells (TGC) (analogous to EVT) in the murine placenta (73).

1.3.3 The placental barrier

The placenta forms a barrier that protects the fetus from pathogens, drugs and xenobiotics, acting as a so called "fetal armor" (74). Moreover, the placenta aids the fetus in peroids of acute maternal starvation by breaking down its own tissue (placental autophagy) in an attempt to nourish energy-demanding organs such as the fetal brain (75). Actually, Bonnin and colleagues (2011) have proposed a novel direct role for the placenta in modulating fetal brain development by converting maternal tryptophan into the neurotransmitter serotonin (76).

While the placenta promotes fetal survival by maintaining appropriate physiological condition in the uterus (67), it also functions as an immunological barrier that prevents the fetus from being rejected by the maternal immune system. Many mechanisms have been invoked to explain this fetomaternal tolerance, including the expression of non-classical MHC molecules by trophoblast cells, tryptophan catabolism by Indoleamine 2,3-dioxygenase, T-cell apoptosis, and the complement system (77). The placental barrier in rodents and humans is formed by a continuous layer of fused multinucleated fetal trophoblasts known as syncytiotrophoblasts (SYN) (67, 73, 78). This is quite different than other nutritive and protective epithelial (e.g., the intestine and testis) and endothelial (e.g., the brain and retina) barriers in mammals, which are composed of individual cells tightly linked.

A growing body of evidence indicates that this unique structure of the SYN provide extensive protection, resisting infection by diverse pathogens such as *Listeria monocytogenes* (79) and the protozoan parasite *Toxoplasma gondii* (73). In addition, in cultures of placental cells cytomegalovirus (CMV) infects cytotrophoblasts, but not SYN, and the herpes simplex virus (HSV) can only infect if the overlaying SYN is damaged (73). In humans, the SYN also secrets hormones such as estrogens and progesterone required for the maintenance of gestation and fetal well-being. In mice, however, these two hormones continue to be secreted by the corpus luteum in the ovary, so that ovariectomy results in termination of murine pregnancy (68, 80). Interestingly, towards the end of pregnancy when fetal demands are greatest, the placental barrier (SYN) layer decreases in thickness to facilitate exchange of materials between the mother and fetus (81, 82).

Although they are both hemochorial (i.e., maternal blood comes in direct contact with the trophoblasts), the human and murine placental barriers differ in structure. The human barrier involves a single layer of SYN, wherase the murine placental barrier is composed of three layers, the outer layer, which consists of mononuclear trophoblast cells (cytotrophoblasts) bathed directly by maternal blood (trophoblast Layer I), while both inner layers (trophoblast Layers II and III) are multinucleated and syncytial in nature (67, 78, 83) (Figure 1.6).

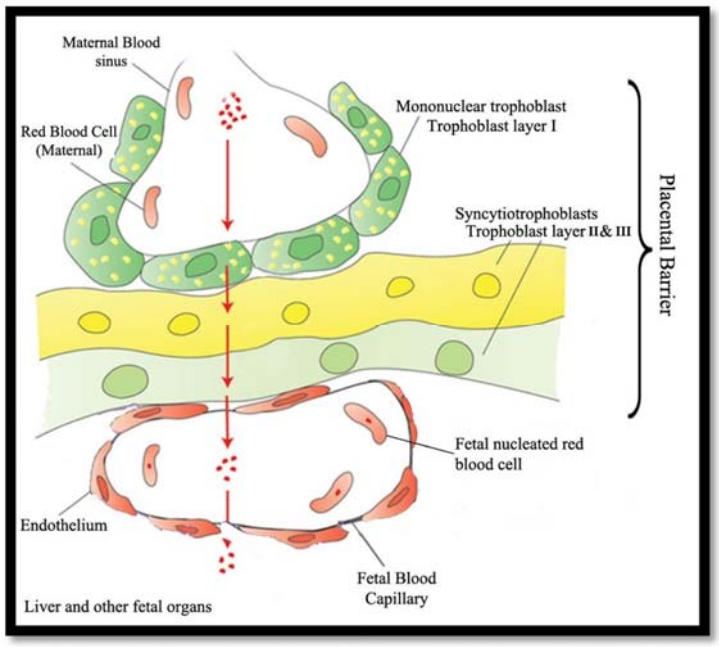

Figure 1.6. The structure of the placental barrier in mice. Modified from (84).

1.3.4 Metabolic changes during pregnancy.

Pregnancy requires exceptional amount of energy and mammals have evolved regulatory endocrine systems that respond to alteration in maternal nutritional status. Many homeostatic parameters are adjusted during gestation, e.g., the circulating levels of glucose, insulin, cortisol and leptin are all elevated; adaptive changes designed to ensure the supply of sufficient nutrition to the fetus. For example, maternal insulin resistance in the third trimester is considered to be a physiological event, favoring the transport of maternal glucose, the sole source of this vital carbohydrate to the growing fetus (64, 85).

During the early phase of pregnancy, maternal metabolism is anabolic leading to larger maternal fat depot and minor enhancement of insulin sensitivity, i.e., storage of nutrients to meet fetal and maternal demands during late gestation and lactation. In the later part of pregnancy, maternal metabolism becomes catabolic in order to support the growth spurt of the fetus with reduced insulin sensitivity (86) and enhanced maternal hepatic gluconeogenesis, lipolysis and ketogenesis (87) (referred to as controlled "accelerated starvation"). The attenuated insulin-mediated utilization of glucose promotes maternal use of lipids as an energy source, sparing other fuels such as glucose and amino acids for fetal use (87).

1.4 MICROBES AND GLUCOCORTICOIDS (GC)

Glucocorticoids (GC), steroid hormones produced predominantly by the adrenal cortex, mediate a variety of physiological processes, including regulation of energy metabolism and suppression of inflammation. The more nuanced role of GC in response to stress promotes homeostasis and is beneficial for short-term survival and recovery from challenge. On the other hand, long-term exposure to high levels of GC can lead to serious metabolic, immune and psychological dysfunctions (88).

Accordingly, the secretion of GC is under efficient feedback control by the hypothalamo-pituitary-adrenal (HPA) axis (88). Physiological and psychological stressors potently activate hypothalamic secretion of the corticotropin-releasing hormones (CRH) that induces the anterior pituitary gland to release adrenocorticotrophic hormone (ACTH), which then stimulates GC synthesis in the adrenal cortex. GC itself functions as a negative feedback signal, inhibiting the release of both CRH and ACTH (89).

The major GC in humans is cortisol, while in rodents, which lack the 17α-hydroxylase required for hydroxylation of pregnenolone to produce cortisol, corticosterone is present in highest levels. The equilibrium between biologically active and inactive forms of the GC is determined by two isoenzymes of 11β-hydroxysteroid dehydrogenase (11β-HSD): in humans, type 1 (11βHSD1) converts inert cortisone into active cortisol (that can bind and activate GC receptors), while metabolism of cortisol to inactive cortisone is catalyzed by both type 1 and type 2 (11βHSD) (90) (Figure 1.7).

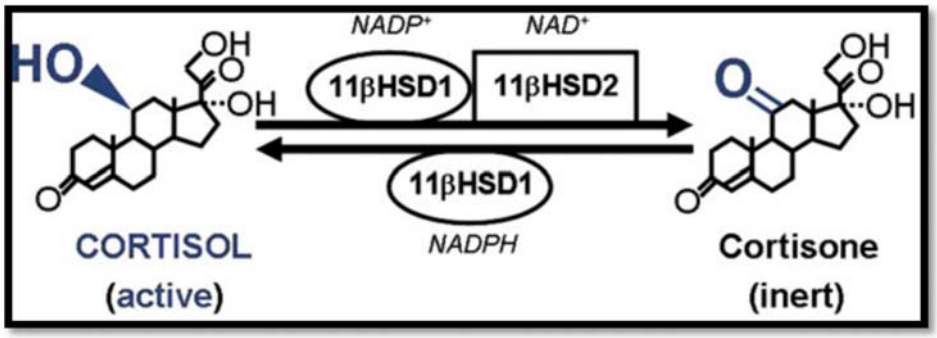

Figure 1.7. Cortisol-cortisone inter-conversion by hydroxysteroid dehydrogenase (11βHSD) enzymes type 1 and 2 (90).

The glucocorticoid receptors (GRs) belong to the superfamily of nuclear receptors and are classified into three subtypes, α, β and γ. Upon binding hormone, the GR-α dimer translocates from the cytoplasm into the nucleus, where it associates with specific glucocorticoid response elements (GREs) on DNA to trigger the transcription of target genes (90, 91). GRs are ubiquitously expressed and necessary for survival, as reflected in the finding that mice which lack GRs are not viable (91).

A decade ago Sudo and co-workers (92) showed that in the absence of microbiota the HPA axis develops abnormally, leading to exaggerated responsiveness to stress and elevated corticosterone levels. It is noteworthy that bacterial colonization of GF mice after weaning did not alter this change in the HPA axis indicating the occurrence of a critical window of time very early in life (92). In contrast, probiotic supplementation of the dams during pregnancy and lactation normalized the levels of corticosterone and CRH in neonates separated from their mothers (93). The basal level of corticosterone in the absence of stress is higher in GF mice and mice exposed to antibiotic than in animals raised conventionally (94). The mechanism by which the microbiota influences the HPA axis has yet to be determined.

1.4.1 Glucocorticoids and metabolism

The term "glucocorticoids" derives from early observations on the involvement of these compounds in regulating glucose metabolism. GC is diabetogenic, elevating blood levels of glucose by opposing the action of insulin in peripheral tissues, mainly through a reduction in glucose uptake via GLUT4 receptors and elevated hepatic gluconeogenesis. Therefore, excess GC exerts anabolic effects on the liver and catabolic effects on muscle and fat decreasing lean body and muscle mass and increasing energy expenditure. At the same time, GC lead to greater fat mass by enhancing appetite (91).

In the case of the liver, elevated levels of GC promote lipogenesis by increasing the production of low-density lipoprotein (VLDL) and triglycerides (TG) thereby triggering hepatic fat accumulation (steatosis) and high blood levels of lipid. In the case of fat, GC promote lipolysis and reduced free fatty acids (FFA) uptake in the peripheral depots, while inducing hypertrophy and differentiation of adipose cells in central depots thus leading to abdominal obesity and insulin resistance (91). Thus, insulin resistance, fatty liver, increased breakdown of skeletal muscle mass and hyperglycemia occur in patients with Cushing's syndrome, a disease characterized by hypersecretion of GC by pituitary adenomas or ectopic, ACTH-producing tumors (91). It was recently shown that the hyperglycemia, insulin resistance, and hypertension triggered in wild-type mice by GC are not observed in animals lacking the nuclear receptor peroxisome proliferator-activated receptor (PPAR)α (95). Remarkably, selective cutting of the vagus nerve attenuates the PPARα-dependent metabolic dysfunction induced by GC, improving insulin sensitivity and reducing glucose levels (96).

1.4.2 The influence of glucocorticoids on fetal and placental development.

GC are essential for normal fetal development, including maturation of the fetal liver, lungs, gut, skeletal muscle and adipose tissue in preparation for extra-uterine life. Their levels rise during late pregnancy, stimulating the lung to produce surfactant in preparation for extrauterine life. Thus, synthetic GC are effectively administered to preterm infants in whom pulmonary immaturity threatens viability, although the treatment is not without negative side-effects (88, 97, 98).

GC modulate a variety of developmental processes, from embryo implantation to subsequent growth of the fetus and placenta. For example, GC regulate uterine synthesis of prostaglandins, which play important roles in implantation and the initiation of labor. Moreover, synthetic GC (dexamethasone and triamcinolone) stimulate the secretion of chorionic gonadotrophin (hCG) by human term trophoblasts 10-fold. GC are also involved in preventing immunological rejection of the fetal semiallograft by inhibiting eosinophil infiltration. Another immunosuppressive effect of GC involves suppression of uterine natural killer (uNK) cells, which have been implicated in recurrent miscarriages (90, 98).

In summary, appropriate exposure to GC *in utero* is critical for fetal organ development, but excess levels are detrimental and may predispose to diseases such as hypertension, diabetes and stroke later in life. Such overexposure exerts adverse effects on the development of the fetal brain and enhances the post-natal activity of the HPA axis (90). For example, treatment of pregnant rats with dexamethasone altered post-natal learning and cognitive functioning in the pups, whose cholinergic neurons were also abnormally sensitive to neurotoxins (99). In addition, elevation of GC levels in pregnant rats by pharmacological blocking of the feto-placental 11βHSD2 activities, the physiological "barrier" to maternal GC, impaired post-natal behavior and expression of hypothalamic GR by the offspring (100). Likewise, the offspring of transgenic mice lacking the 11βHSD2 exhibit abnormally high anxiety (101).

Moreover, exposure of non-human primates (marmoset monkeys) to dexamethasone during early or late pregnancy impaired proliferation of dentate gyrus cells without affecting their differentiation (102). Few long term follow-up studies on preterm children who received antenatal GC, have been reported, some showing an effect on behavior (103, 104), but others not (105-107). Recently, an association between prenatal exposure to synthetic GC and mental health in children and adolescents, was found (108), but the number of participants in this study was small.

The above effects of GC on the fetus are mediated, at least in part, via effects on placental growth and function. In a number of animal species and in humans as well, exposure to elevated GC during pregnancy reduces placental weight to an extent dependent on the timing and duration of exposure as well as the dose and type of GC (98, 109). In rodents elevated exposure to GC compromises placental growth by promoting trophoblast apoptosis (110) and attenuating IGF2 expression (111), processes that are actually more sensitive to GC than fetal growth. Moreover, administration of synthetic GC to the pregnant dams restricts placental vascular development by inhibiting vascular endothelial growth factor (VEGF) and downstream activation of PPARγ (112, 113). In a similar manner the placentas of asthmatic

women taking high doses of GC display hypovascularized fetal villi with reduced placental blood flow (98).

In addition, the thickness of the placental barrier thickness and its capacity to transport glucose and amnio acids are influenced by maternal GC treatment. In mice, dexamethasone reduced the thickness of the area of diffusion in the labyrinth zone and concurrently elevated the surface area for nutrient exchange (98). In trophoblast cells, GC downregulate GLUT1 and GLUT3 expression *in vitro* (109) and injection of GC into the rat placenta, lowers the levels of both GLUT1 and GLUT3 transcripts and proteins (98).

The placenta actively protects the fetus from overexposure to GC by metabolizing these compounds via 11βHSD2, which is expressed by the syncytiotrophoblasts and converts cortisol and corticosterone to inactive metabolites (although many synthetic GC are metabolized poorly by this enzyme). As a result, maternal cortisol/corticosterone levels are as much as 10-fold higher than those in fetal blood depending, on fetal age. Indeed, inhibition of 11βHSD2 during pregnancy retards fetoplacental growth and lead to abnormalities in cardiovascular and metabolic functions in the adult offspring (97).

1.5 MICROBES AND CONTROL OF BARRIERS

In specialized compartments of the body, movements of molecules between the blood and cells in tissues are hindered by so-called gatekeepers. Such blood-tissue barriers were first described about 100 years ago in pioneer experiments showing that dyes administered to laboratory animals failed to stain the testis and the brain (114-116), leading to the concepts of the blood-brain barrier (BBB) and the blood-testis barrier (BTB). These two barriers are considered the tightest in the body, but differ in structure and function.

1.5.1 Comparison of the BBB and BTB

The BBB is formed by TJPs between endothelial cells lining the blood vessels, whereas the BTB is the result of TJPs between epithelial cells referred to as Sertoli cells (117) and consequently Russell and Peterson (1985) coined the term ‘Sertoli blood barrier’ for the BTB (118). Another fundamental difference is the organization of the TJPs: In the brain, these junctions are localized only at the apical surface of the endothelium, sealing the intercellular space with adherens junctions (AJ) immediately below the tight junction fibrils. In the BTB, on the other hand, TJPs coexist with basal ectoplasmic specializations (basal ES) and basal tubulobulbar complexes (basal TBC) (of which both are testis-specific, actin-based adherens junctions) and the desmosome-like junctions (Fig. 1.8) (117, 119).

Nevertheless, the TJPs that form these two barriers display remarkable molecular similarities, both being formed by strands of occludin, JAM, and claudin molecules linked to the cytoskeleton through the zonula occludens (ZO-1, ZO-2 and ZO-3) (119). In knock-out mice, loss of claudin-5 leads to disruption of the BBB (120) and lack of occludin is associated with calcification of the brain with no change in the permeability of the BBB (121). In the testis, loss of occludin, claudin-3 and 11, the dominant forms there, disturbs spermatogenesis and causes sterility (116, 122, 123). Moreover, under pathological conditions such as stroke (124),

multiple sclerosis (125) and Alzheimer disease (126) (where the BBB is disrupted), orchitis (127) and male infertility (116) (with disrupted BTB) the TJPs are down-regulated or redistributed. Interestingly, diabetes mellitus affects the paracellular and transcelluar permeability of the BBB and BTB negatively (128).

Both barriers physically separate their respective organs into apical and basal compartments exerting strict control over the transfer of ions and molecules. The BTB develops postnatally in rodents and during puberty in humans (119), while the BBB is formed prenatally in both rodents (129) and humans (130). Both barriers create 'immune-privileged sites', i.e. tissue transplanted into the brain (131) or the interstitial space of the testis (127) is not rejected.

This 'privilege' is due to the absence of draining lymphatic vessels and an almost complete lack of circulating immune cells, which are prevented from entering by the BTB and BBB. In the case of the BTB, mature sperm cells (spermatozoa) expressing new antigens that can be recognized as 'foreign' arise after puberty but these new autoantigens are tolerated and do not normally evoke an immune response by the testis (127). From an evolutionary perspective, immune privilege is regarded as a protection for vulnerable organs with limited capacity to regenerate. While the role of gut microbiota in fortifying the intestinal barrier is well documented, its influence on other blood-tissue barriers requires further examination.

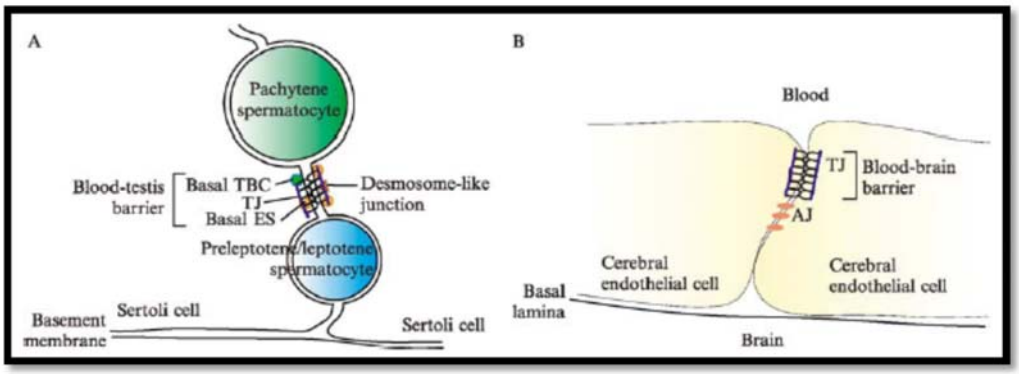

Figure 1.8. A simplified diagram illustrating the morphological differences between the blood-testis barrier (BTB) and the blood-brain barrier (BBB). (A) In the BTB, tight junctions (TJs) coexist with basal ectoplasmic specializations (ES), basal tubulobulbar complexes (TBC), and desmosome-like junctions. (B) In the BBB TJs are restricted to the apical surface of the endothelium, sealing the intercellular space, with adherens junctions (AJ) located immediately below (117).

1.5.2 The blood-brain barrier (BBB)

As mentioned above, the BBB is formed by TJPs between endothelial cells that line cerebral microvessels (Figure 1.9). Small gaseous molecules (O_2 and CO_2) and small lipophilic agents, including drugs such as ethanol, caffeine, nicotine, heroin and methadone, can diffuse freely through the lipid membranes of the BBB; whereas hydrophilic molecules such as glucose, several amino acids and neurotransmitters must be carried across by specific transporters. Two major groups of transporters are involved, i.e., the solute carriers (SLCs) and active efflux carriers (ABC transporters), both expressed on the luminal and/or adluminal surface of the BBB. Large hydrophilic molecules such as proteins can only be translocated across

membranes via endocytosis (receptor-mediated transcytosis or adsorptive-mediated) (132), which is however, uncommon in brain endothelium. The high metabolic demands placed on cerebral endothelial cells by active transport are reflected in higher abundance of mitochondria than in systemic endothelial cells (133).

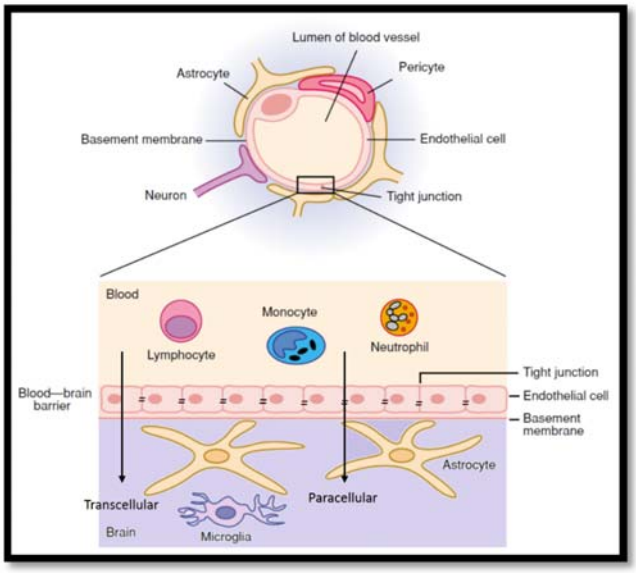

Figure 1.9. The blood–brain barrier (BBB). Molecules cross the BBB either transcellulary or paracellularly between the cells through the junctions. Modified from (134).

1.5.2.1 Development of the BBB

During embryonic angiogenesis, neural progenitors induce endothelial cells to express BBB-specific proteins such as TJPs and nutrient transporters. At E13, pericytes then strengthen the barrier properties by sealing the interendothelial TJPs, limiting the rate of transcytosis, down-regulating the expression of leukocyte adhesion molecules and inducing the expression of efflux transporters. A functional BBB that excludes tracers administered intravenously from the CNS parenchyma is present at E16. Normally, astrocytes appear postnatally to provide additional support to the functional BBB during adulthood, as well as in connection with injury and disease (Figure 1.10) (129, 135).

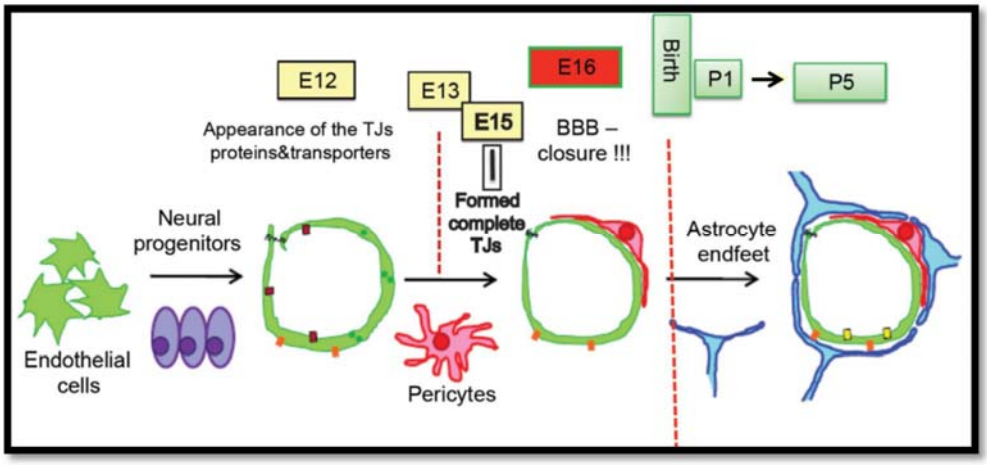

Figure 1.10. Schematic illustration for the time-course of BBB development**.** Modified from (129).

1.5.2.2 Functions of the BBB

The many vital roles played by the BBB include supplying the brain with important nutrients, mediating the efflux of numerous waste and toxic substances and regulating ion trafficking between the blood and brain via specific ion transporters and channels to produce a brain interstitial fluid (ISF) of optimal composition for neuronal function. This composition is similar to that of blood plasma except that the protein content is much lower, the K^+ and Ca^{2+} concentrations are also lower and the level of Mg^{2+} is higher. Importantly, the BBB protects the brain from fluctuations in ionic composition that can occur following exercise or after a meal. Furthermore, since immune surveillance in the CNS is limited, the BBB acts as a shield against infection and foreign materials (132, 136).

1.5.2.3 Cell types associated with the BBB

The brain endothelial cells that form the BBB are surrounded by or closely associated with several types of cells, including the perivascular end-feet of astrocytic glia, pericytes, microglia and neurons (132, 136) (Figure 1.9). The close association between such cells and brain capillaries suggests that they are involved in specific features of the BBB and, indeed, transplantation studies have demonstrated that formation of the BBB is induced by interactions between endothelial cells and the neural cells (137). There is now strong evidence, particularly from cell cultures, that astrocytes can up-regulate many features of the BBB involved in creating effective tight junctions (138). In addition, pericytes are required for the integrity of this barrier both during embryogenesis (129) and in adulthood (139). Thus, the BBB of adult mice lacking pericytes is leaky to water and a range of low and high-molecular-weight tracers. During development, pericyte–endothelial cell interactions are crucial for the formation of TJPs in the BBB, as well as for vesicle trafficking by CNS endothelial cells.

The possible influence of other cell types on the BBB is less well characterized. Some investigations suggest an inductive role for microglia, macrophages derived from blood monocytes and resident in the CNS. Accordingly, co-culture of brain endothelial cells with blood macrophages enhanced barrier tightness (140). Some indirect evidence indicates that smooth muscle cells may also influence BBB functions (141).

1.5.3 The blood-testis barrier (BTB)

The BTB is formed by TJPs between two adjacent Sertoli cells at the seminiferous tubules, with the peritubular layer of myoid cells that encircle the seminiferous tubules and the testis endothelial cells in the interstitium also making a significant contribution (142, 143) (Figure 1.11). The primary functions of this barrier are to segregate the haploid male germ cells from the immune system, create polarity and help to create a unique environment for germ cell differentiation. At the same time, the BTB poses an obstacle to the development of non-hormonal male contraceptives by sequestering drugs (e.g., adjudin) in the apical compartment (144).

The BTB forms during puberty in human (at ~ 12-14 years of age), while in mice a functional BTB is established ~ 15-16 days after birth (119, 145), which coincides with the time-point at which the testis cords are transformed into seminiferous tubules with a lumen (146, 147), as well as when the Sertoli cells cease to divide and become terminally differentiated (148).

Thus, in adult mammals the number of Sertoli cells is thought to remain relatively constant (119, 148), although there are reports that these cells can proliferate and divide in adult rodents under experimental conditions (149) and even under physiological conditions in humans (150). The number of Sertoli cells determines the number of germ cells that can be supported during spermatogenesis and thus sperm production and the sperm count in adulthood (147, 148).

Sertoli cells are highly dynamic, changing their three-dimensional structure during the course of spermatogenesis and spermiogenesis (151, 152). This causes the BTB to change as well: the TJPs undergo remodeling (opening and closing) to allow the passage of preleptotene spermatocytes from the basal to the adluminal compartment, where they undergo meiosis (151). This structural disassembly and reassembly of the TJPs occurs at stage VIII in the rat and is tightly regulated by testosterone and cytokines (153-155).

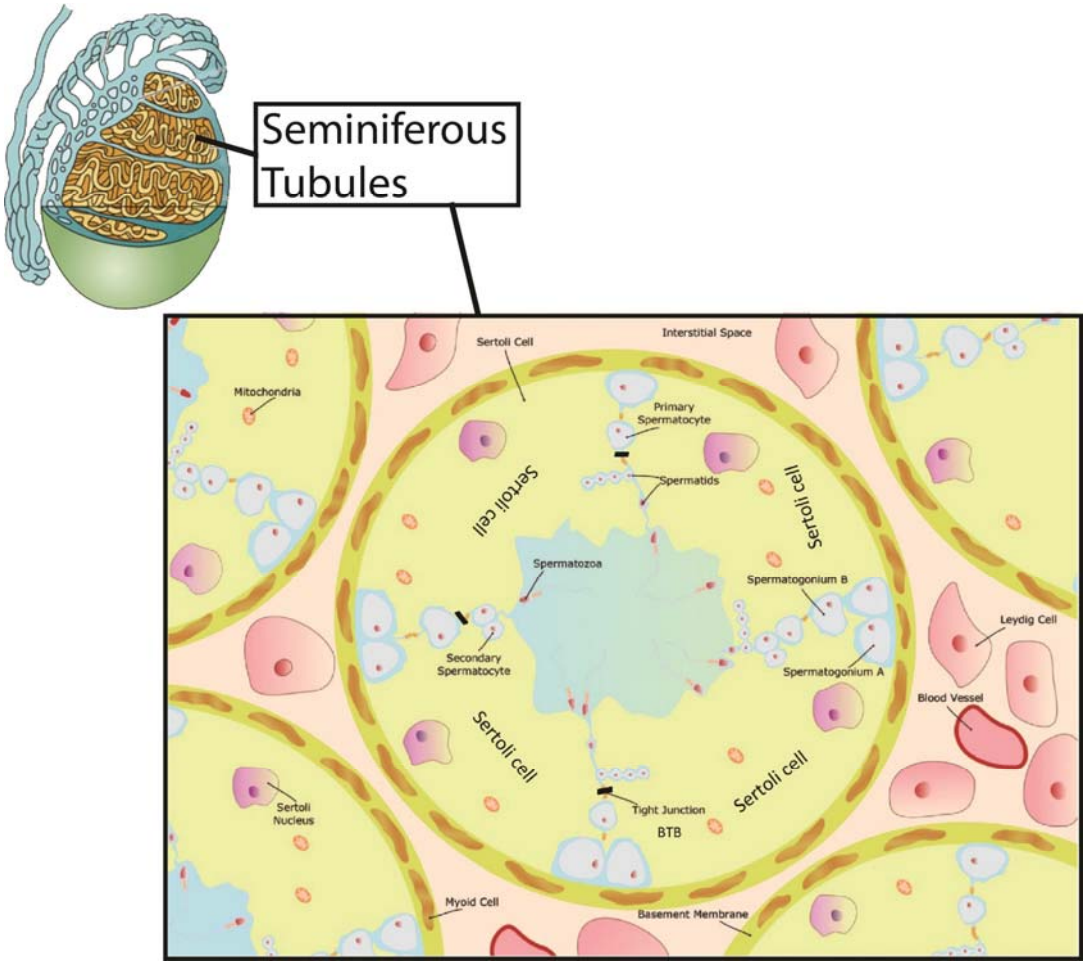

Figure 1.11. Schematic illustration of the seminiferous tubule and the blood-testis barrier (BTB). The BTB is formed by tight junctions between Sertoli cells. Blood vessels and the Leydig cells, which produce testosterone, are located in the interstitial space. Adjacent to the basement membrane are several layers of modified myofibroblastic cells, termed peritubular cells (156).

1.6 MICROBES AND TESTOSTERONE

Testosterone produced by the Leydig cells, is essential for male sexual differentiation, spermatogenesis, and expression of male secondary sex characteristics. Its biosynthesis is primarily under the control of the pituitary derived luteinizing hormone (LH), which upon binding to its receptor on the plasma membrane of Leydig cells, stimulates formation of cAMP from ATP and cAMP, in turn, activates protein kinase A, which is required for the transport of cholesterol from the cytoplasmic pool into mitochondria. Cholesterol transfer from the outer to the inner mitochondrial membrane is facilitated by the steroidogenic acute regulatory protein (StAR) and peripheral benzodiazepine receptor (PBR) (89, 157).

The P450 side-chain cleavage (P450scc or CYP11a) enzyme, which resides on the matrix side of the mitochondrial inner membrane, converts cholesterol into pregnenolone, which then diffuses to the smooth endoplasmic reticulum (SER) for conversion to progesterone by 3β-hydroxysteroid dehydrogenase Δ5-Δ4-isomerase (3β-HSD or Hsd3b). Progesterone is then converted (158) in two steps involving 17α-hydrosylase (17α-OH-lase) and C17-20 lyase to androstenedione, which is finally converted to testosterone by 17β-hydroxysteroid dehydrogenase type III (17 HSD3) (Figure 1.12). Subsequently, testosterone can be converted to estradiol by the P450 aromatase (Cyp19). Earlier studies revealed that the gut microbiota influences testosterone production , but it remains unclear whether this involves regulation of the circulating level of pituitary LH and/or of Leydig cell steroidogenesis (157).

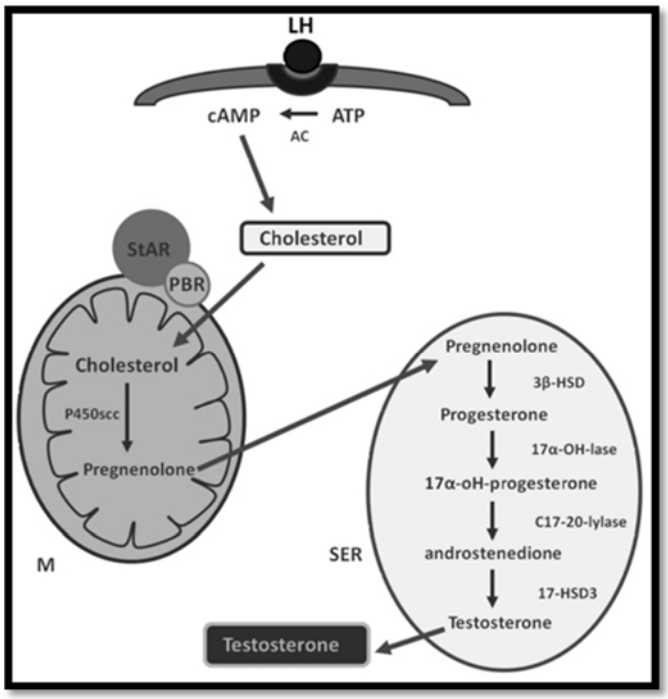

Figure (1.12). Schematic illustration of Leydig cell steroidogenesis. Adenyl cyclase (AC), steroidogenic acute regulatory protein protein (StAR), peripheral benzodiazepine receptor (PBR), the mitochondrion (M), cytochrome P450 side-chain cleavage (P450 scc), smooth endoplasmatic reticulum (SER), 3β-hydroxysteroid dehydrogenase Δ5-Δ4-isomerase (3β-HSD), 17α-hydrosxylase (17α-OH-lase), and C17-20 lyase, 17β-hydroxysteroid dehydrogenase type III (17 HSD3) (157).

2 AIMS

The overall aim of the present thesis was to characterize host-microbe interactions concerning three immune-privileged organs i.e., the placenta, brain and testis. In particular, we evaluated the influence of the normal gut microbiota on the developmental programming of these organs. The specific aims were therefore as follows:

I. To determine the role played by the gut microbiota in maternal metabolism during pregnancy (Paper I).

II. To investigate the influence of the maternal microbiome on placental development (Paper I).

III. To evaluate the involvement of the gut microbiota in establishing the integrity of the blood-placenta, blood-brain and blood-testis barriers (Papers I, II and III).

IV. To characterize the cross-talk between the gut microbiota and its metabolites and the brain (Paper II).

V. To assess the influence of the gut microbiota on testosterone production and male reproduction (Paper III).

3 METHODOLOGICAL HIGHLIGHTS

Detailed descriptions of the methods employed in this thesis are presented in the individual papers. Therefore, this section will describe the germ-free mouse model utilized in all three publications. The advantages and disadvantages of the procedures applied to assess the permeability of the three barriers (BPB, BBB, BTB) will also be discussed.

3.1 GERM-FREE AND GNOTOBIOTIC MICE

GF animals provide an invaluable experimental tool for examining interactions between a host and its microbiota. The term **germ-free** (axenic) refers to an animal demonstrably free from microbes including bacteria, viruses, fungi, protozoa and parasites, throughout its lifetime (159, 160). GF animals selectively colonized with one or more bacterial species are referred to as **gnotobiotic** (161, 162) (a term sometimes used synonymously with GF). This term is derived from the Greek 'gnotos', meaning "known", and 'bios' or a life with a fully defined flora.

3.1.1 Historical aspects of GF experimentation

The concept of a germ-free animal was recognized more than a century ago by Louis Pasteur (1885), although he concluded that bacteria-free existence is impossible. Ten years later in 1895, Nuttle and Thierfelder at Berlin University produced the first GF animals (guinea pigs), which survived for as long as 13 days. However, due to the lack of knowledge concerning nutrition, it took 50 more years until the first GF rat colonies were established in the late 1940s. Subsequently, the first GF mice were successfully developed by Pleasants in 1959 (159, 160, 163).

The GF animal facility at the Karolinska Institutet, one of the oldest in the world established in the 1950s by Professor Bengt Erik Gustafsson, a pioneer in the design of equipment and procedures for producing GF rats. Figure 3.1 depicts a stainless steel isolator designed by Gustafsson (1959) (164) and located at our previous GF facility. After moving to a new building in the beginning of 2013, we now keep all GF mice in plastic isolators (Figure 3.2).

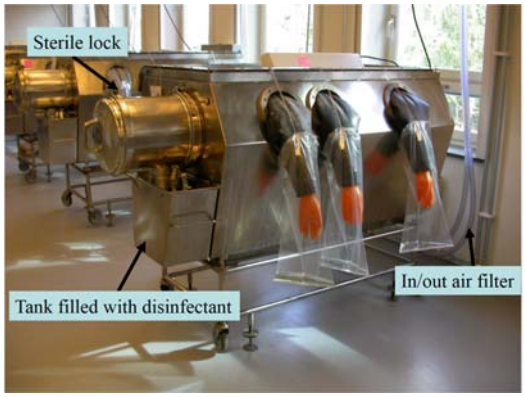

Figure 3.1. Gustafsson steel isolator at the GF facility at the Karolinska Institutet.

3.1.2 Isolator technology

Isolators provide physical barriers that allow creation of a sterile environment. These devices have an air supply, air inlet and outlet, transfer port and arm-length gloves, as well as a special tank filled with disinfectant and used for the transfer of mice in and out (Figure 3.3). Maintaining an isolator is very laborious work and requires special training. All manipulation of mice and supplies occurs inside the isolator through gloves and sleeves attached to the isolator walls. In terms of potential contamination, the gloves are most vulnerable and the most common cause of contaminations were due to holes in the gloves.

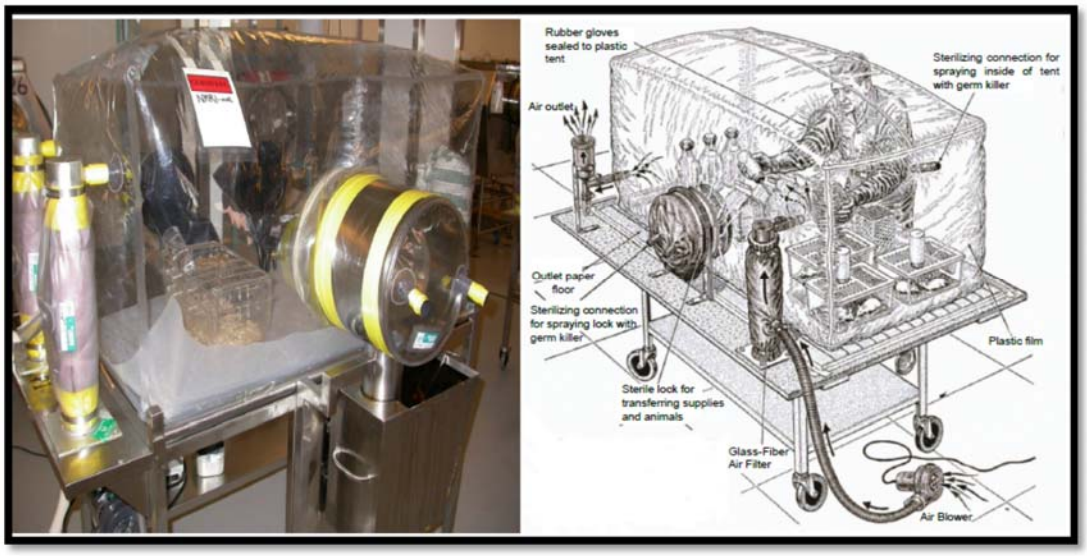

Figure 3.2 A Plastic isolator at the GF facility at the Karolinska Institutet.

Bedding, food, water, and equipment, including cages, must first be sterilized (autoclaved) and are then put into the isolator through the so-called the sterile lock. Sterilization of entire steel isolators is accomplished by autoclaving the whole isolator, as well as with portable vacuum and steam equipment. In the case of plastic isolators, which cannot tolerate the heat of steam sterilization, sterilization is accomplished with germicidal vapour (2% peracetic acid and chlorine dioxide). Air is sterilized upon entry and exhaust by mechanical filtration under positive pressure.

Figure 3.3 Transfer of mice from inside the isolator. The mouse is placed in an autoclaved glass jar and transferred through a sterilized lock into the tank filled with disinfectant.

3.1.3 Establishment of GF mice

Establishment of new strains of GF mice requires that the fetus remain sterile in the uterus. The pups are most commonly delivered by sterile Caesarean section and then transferred while still in the uterine sac to a GF foster mother (Figure 3.4). Thereafter, it is relatively straightforward to maintain and breed colonies of GF mice in isolators with free access to autoclaved food and water (162, 165). It is not advisable to use the first generation of GF mice for experiments, since their mother was not GF and virus, bacteria and bacterial metabolites can be transmitted transplacentally from the mother to the fetus. At our facility, the GF status of the mice is confirmed weekly by in-house quality assurance involving collection of fecal samples to be cultured for aerobic and anaerobic bacteria and fungi. For bacteria that cannot be cultured, 16S PCR testing is occasionally performed.

Figure 3.4. Establishment of GF mice by Caesarian section. (A) The uterine sack is removed and clamped together at the top of each horn and at the base close to the cervix. (B) The uterine sac is placed in a glass jar containing desinfectant. (C) The uterine sack is transfered into the isolator, where it is opened and the pups removed cleaned and stimulated to breath. (D) The pups are introduced to the GF foster mother.

3.1.4 Establishment of the control group for GF mice

GF and gnotobiotic mice are compared to the specific pathogen-free (SPF) animals free from known pathogens that causes clinical or subclinical infections that can bias research findings (162). Although SPF mice are usually housed in special rooms (including the ones at our facility), for reliable comparison they should be housed in the same environment as the GF mice (i.e., also in isolators), but this is seldom done because isolators are too expensive.

Our SPF mice are screened and tested for pathogens 3 or 4 times a year, as recommended by the Federation of Laboratory Animal Science Associations (166). In this connection, one SPF mouse from each rack is sent to the National Veterinary Institute (Uppsala, Sweden), along

with GF mice, which are always negative for pathogens. It is important to note that SPF animals are normally colonized with commensal bacteria, but the diversity and type of colonization is rarely known with any accuracy. To achieve balanced and identified colonization, commercial breeders and animal facilities tend to expose SPF mice to the modified Schaedler flora, containing 8 species of bacteria, 5 belonging to the genera *Clostridium, Eubacterium*, and *Bacteroides*; one a spirochete from the *Flexistipes* group *(Mucispirillum schaederli)*; and two *Lactobacillus* species (162).

3.1.5 Anatomical and physiological characteristics of GF mice

If their diet is supplemented with vitamins, including K and B, GF mice are viable and healthy However, these animals show a number of important developmental and physiological differences in comparison to SPF animals. For example, the cecum is enlarged by 4-8-fold due to the accumulation of mucus and undigested fibers. This is in contrast to other GF animals, including dogs, pigs, sheep, goats and chickens that due to the anatomy of the junction between their small and large intestine show little or no such enlargement. When body weight is corrected for cecal weight adult GF rodents weigh less than their SPF counterparts.

Moreover, the small intestine of GF rodents is less developed, with a considerably smaller surface area, slower peristalsis, irregular villi and reduced renewal of epithelial cells. Consequently, the ability of GF animals to utilize nutrients is compromised. Interestingly, GF rats live longer and develop spontaneous cancers less frequently than SPF rats (159). GF animals are also more prone to infections and have altered immune systems. Additional differences between SPF and GF mice are presented in Table 3.1.

3.1.6 The advantages and disadvantages for GF mice as experimental models

GF and gnotobiotic mice are valuable experimental tools for examining host-microbe interactions. GF mice can be selectively colonized with a single bacteria, as we monocolonized them by oral gavage with *B.thetaiotaomicron (Bteta)* (Paper II) and with *Clostridium tyrobutyricum (CBUT)* (Papers II and III). Furthermore, genetically modified mice can be made germ-free in order to study interactions between any particular gene and the microbiome.

The major questions concerning host-microbe interactions include how colonies of microbiota are established and maintained, how these affect their host, how the host shapes the populations of microbiota and how the microbiota influence the development of diseases. However, information obtained by comparing GF and SPF mice cannot be directly applied to humans and it often remains uncertain whether a disruption in the microbiota associated with a disease in humans is a cause, contributing factor, or merely a consequence of the disease state. Although such comparisons provide hints concerning the pathogenesis of diseases such as cancer, cardiovascular disease, diabetes and multiple sclerosis, the underlying mechanisms remain unknown and as a result, GF findings can seldom be readily translated into treatments and/or prevention.

Several factors could contribute to this failure. One caveat is that several bacterial species that colonize the murine gut are not found in humans. Secondly, the immune responses of mice differ from those of humans. Furthermore, the distinct physiology and anatomy (including skin, fur, orapharyneal structures and compartmentalization of the GIT) and behavior (e.g., coprophagia) of mice will undoubtedly influence microbial communities (4, 9, 20, 161, 167).

Despite these pitfalls, the GF mouse remains the most powerful model system for studying host-microbe interactions.

3.2 PERMEABILITY TESTS

Various experimental procedures are utilized to assess the integrity of blood-tissue barriers. Here we employed three methods to evaluate the permeability of the BPB, BBB and BTB. **1) Evans blue perfusion** in Papers II and III. This fairly new approach involves perfusing the anesthetized mouse intracardially with the tracer (the Evans blue dye, MW 961 Da) and the standard fixative solution of formaldehyde (168). Evans blue (EB) emits red fluoresce when bound to proteins, predominately serum albumin (MW 65 kDa) (169) and leakage of EB-albumin through the barrier can be observed under the fluorescence microscope. This method is inexpensive and, unlike more quantitative optical densitometry, enables localization of the areas of the barriers that are disrupted and visualization of the structures affected. Another advantage is that perfusion with EB is more rapid than traditional intravenous or intraperitoneal injection of the tracer which must be allowed to distribute in the body (168). However, to obtain reliable results the perfusion rate must be kept low enough as not to damage capillaries and/or disrupt the barriers.

2) Positron emission tomography (PET), utilized for the BBB in Paper II, involves intravenous injection of a tracer, the extravasation of which can then be visualized in real time. This method is a non-invasive, with no need to sacrifice the mice, and highly sensitive and also allows quantitation of tissue radioactivity and calculation of kinetic parameters of permeability. Its main drawbacks are its very high cost and the fact that the animal is exposed to radiation (170).

3) Tracer injections, utilized for the BPB in Paper I and BBB in Paper II, involves intravenous injection of a tracer; then allowing time for it to distribute (30 min-2 hr), sacrificing the mouse; and finally examining extravasation of the tracer usually by fluorescence microscopy. This is the most common approach and a broad spectrum of tracers with different molecular sizes can be used to obtain detailed information about the extent, nature, and dynamics of barrier impairment (139). However, such tracer extravasation might not reveal minor alternations in barrier function. Furthermore, in comparison to EB perfusion, this approach resulted in less intense staining of disrupted areas of the BBB (168).

Table 3.1. Anatomical and physiological features of germ-free mice that differ from those of specific-pathogen-free and wild-type mice (162).

Characteristic	Difference
Nutrition	Requirement for vitamins K and B in diet Decreased percentage body fat Normal or increased food intake
Fluid balance	Increased intake of water
Metabolism	Decreased basal metabolic rate Increased secretion of free amino acids and urea and little excretion of acetic acid More urea and little ammonia in intestinal contents More nitrogen in the cecal contents and feces Elevated oxidation-reduction potential of the cecal contents Altered response to anesthetics
Circulation	Reduced total volume of blood Decreased cardiac output Decreased blood flow to skin, liver, lungs and digestive tract Increased cholesterol level, numbers of red blood cells and hematocrit in blood
Liver	Reduced size Increased levels of ferritin and cholesterol
Lungs	Thinner alveolar and capsular walls Fewer reticuloendothelial elements
Intestinal morphology	Reduction in total intestinal mass Decrease in the total surface area of the small intestine Slender and uniform villi of the small intestine Shorter ileal villi and longer duodenal villi Shorter crypts of the small intestine Lamina propria of the small intestine thinner, with fewer cells and slower cell renewal Larger cecum with a thinner wall
Intestinal motility	Increased muscle tissue, with elongated and hypertrophied muscle cells in the cecum Longer transit time
Intestinal physiology	Reduced osmolarity in the small intestine Elevated oxygen tension and electropotential in the small intestine
Intestinal function	Enhanced absorption of vitamins and minerals, alterations in the absorption of other ingested materials Altered enzyme content, elevated levels of typsin, chymotrypsin and invertase in the feces High levels of mucin (mucoproteins and mucopolysaccharides) in the feces Less fatty acids and no cyclic or branched-chain fatty acids in the intestinal content, excretion of primarily unsaturated fatty acids
Endocrine function	Less uptake of iodine by the thyroid Decreased motor activity and hyperresponsiveness to epinephrine, norephinephrine and vasopressin
Electrolyte status	More alkaline cecal contents High levels of calcium and citrate and little phosphate in the urine Somewhat less sodium and low levels of chloride in the intestinal content

4 RESULTS AND DISCUSSION

4.1 PAPER I: COMMENSAL MICROBIOTA SUPPORTS PLACENTAL DEVELOPMENT AND MATERNAL METABOLISM

Animal who lay eggs will deposit a fixed amount of energy in the yolk sac. In contrast, mammals have evolved a more complex and flexible system to provide energy to the growing offspring. As gestation progresses, the metabolic demands on the mother increases to ensure sufficient supply of nutrient to the growing fetus. Accumulating evidence indicate that gut microbiota and their metabolites act as critical regulators of metabolism in adults (171). However, much less is known about such metabolic-microbial interaction during pregnancy.

In Paper I, I assessed the maternal microbiome and its potential influence on pregnancy and placental development. We demonstrated that the serum levels of glucocorticoids in GF dams are elevated even in the absence of pregnancy, which is indicative of metabolic stress. Indeed, this metabolic stress may explain the observation that GF mice must spend approximately 30% more time eating in order to maintain their body weight (172). Moreover, corticosterone levels in serum are normally elevated during pregnancy (173) in order to secure glucose and the additional elevation when GF female mice become pregnant indicates that the maternal microbiome may contribute to metabolic support to the female during pregnancy.

During pregnancy, the predominant source of energy for the offspring are carbohydrates (174) and ketone bodies (175) provided by the mother. However, in a situation of nutritional constrain, placental development may be impaired and placental structures altered to optimise nutrient transfer and secure sufficient energy for the offspring (176). In early pregnancy, following embryo implantation and decidualization, formation of the placental labyrinth requires a considerable amount of energy in order to develop correctly. We found that the morphology of the GF placenta is impaired, with reduced labyrinth size, disrupted vascularization and reduced development of barrier functions in late gestation.

Our analysis designed to correlate such morphological differences to metabolic parameters revealed that hepatic gluconeogenesis, lipolysis and ketogenesis were enhanced to meet the increased nutritional demands by the growing fetus. During late pregnancy, ANGPTL4 was specifically activated in order to block lipoprotein lipase in GF, but not in SPF dams further underscoring the severe metabolic stress experienced by the former. These finding indicates that in rodents, at least, the maternal microbiome plays an important role in optimizing metabolic functions during pregnancy, modulating maternal lipid and carbohydrate metabolism and regulating placental development.

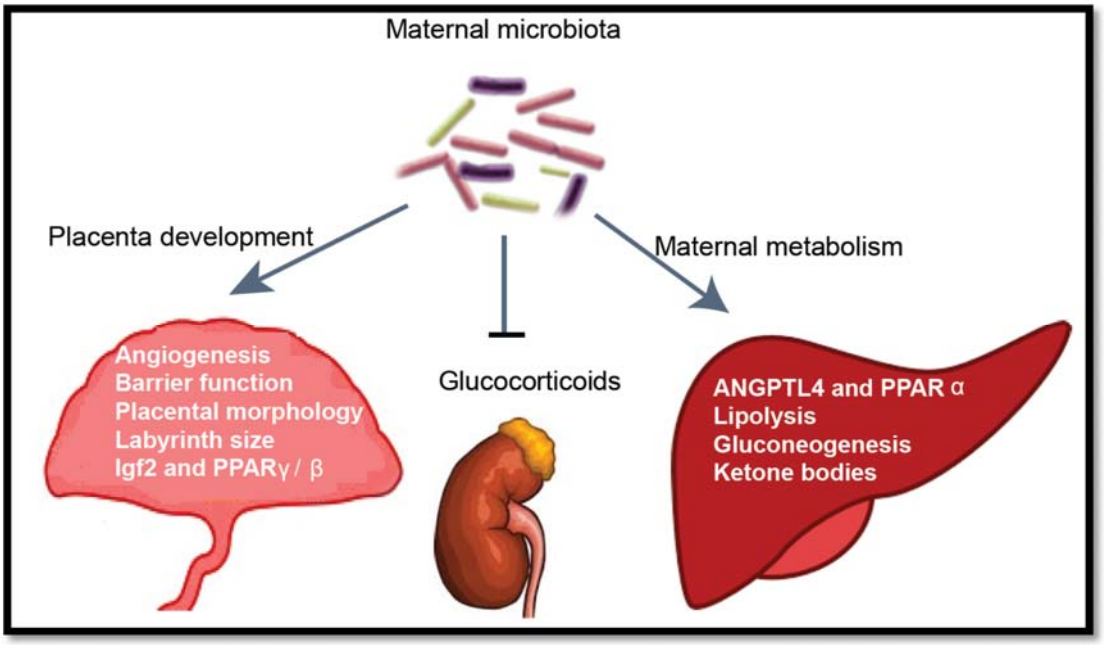

Figure 4.1 Graphic summary of Paper I

4.2 PAPER II: THE GUT MICROBIOTA INFLUENCES BLOOD-BRAIN BARRIER PERMEABILITY IN MICE

As described above, the gut microbiota influence several key processes in the brain, including synaptogenesis and production of neurotransmitters and neurotrophic factors, thus apparently contributing to normal brain development and function (177). It is also well known that the development and function of the brain require a functional BBB to ensure an optimal microenvironment (177).

In Paper II, we assessed the potential impact of the gut microbiota on BBB integrity and thus permeability by comparing specific-pathogen-free (SPF) mice, germ-free (GF) mice and GF-mice colonized with a complete SPF flora.

Injection of an antibody carrying a moiety that absorbs infrared light into pregnant mice and subsequent imaging revealed that at around E17 this antibody penetrated the brain parenchyma of GF but not SPF foetuses. Complementary studies in adults using several independent techniques (i.e. Evans blue (EB) perfusion, [11C]Raclopride PET imaging and i.v. injection of an antibody) demonstrated that this BBB "leakiness" is observed in the adult GF mice as well. *In vivo* imaging using TRITC-Dextran and staining for pericytes showed no major quantitative differences in the structure of larger brain vessels in GF and SPF brains. Although we cannot totally exclude differences in microcapillary structures. We did observe decreases in the levels of the tight junction proteins (TJPs) occludin and claudin 5 in the GF brain, which could be partially reversed by faecal transfer of microbiota to adult GF mice.

Perfusion with Evans blue revealed that monocolonization of the intestine of adult GF mice with either *Clostridium tyrobutyricum* (CBUT), a bacterial strain that produces butyrate, or oral administration of the bacterial metabolite butyrate, was sufficient to reduce BBB permeability (Figure 4.2). This effect of gut microbiota and butyrate may be mediated by an epigenetic

mechanism, since administration of butyrate or monocolonization with CBUT elevated levels of histone acetylation in brain lysates.

The results in Paper II, underscore our previous findings that the gut microbes can contribute to brain development and function (177). Our findings indicate that the gut microbiota may be one of several environmental cues that contribute to the BBB integrity required for correct spatial and temporal programming of brain development and maturation. TJPs, the target of microbiota, control endothelial polarity and impart the high transendothelial electrical resistance that restrict permeability and result in immune quiescence. Moreover, our present observations may have implications for understanding the development of neurodegenerative diseases known to involve altered BBB permeability.

4.3 PAPER III: THE GUT MICROBIOTA AND DEVELOPMENTAL PROGRAMMING OF THE TESTIS IN MICE.

In this study we found that the lack of gut microbiota can lower sperm count, levels of testosterone, expression of TJPs and increase permeability of the BTB in adult GF mice. Moreover, development of the BTB at postnatal day 16 was also affected with significantly fewer open tubules in the testes of GF than SPF males, a difference that can be reversed by colonization of the GF animals with CBUT. Perfusion with Evans blue demonstrated restored BTB permiability when GF mice were either colonized with SPF microbiota (CV), monocolonized with CBUT or treated with butyrate (NaBu) (Figure 4.2).

In addition to underscoring the importance of the gut microbiota for the establishment of barriers to protect reproductive organs, these findings indicate the essential role of the microbiota in regulating testosterone levels and sperm count. It is tempting to speculate that probiotic supplementation might improve sperm count in men suffering from oligospermia and azoospermia.

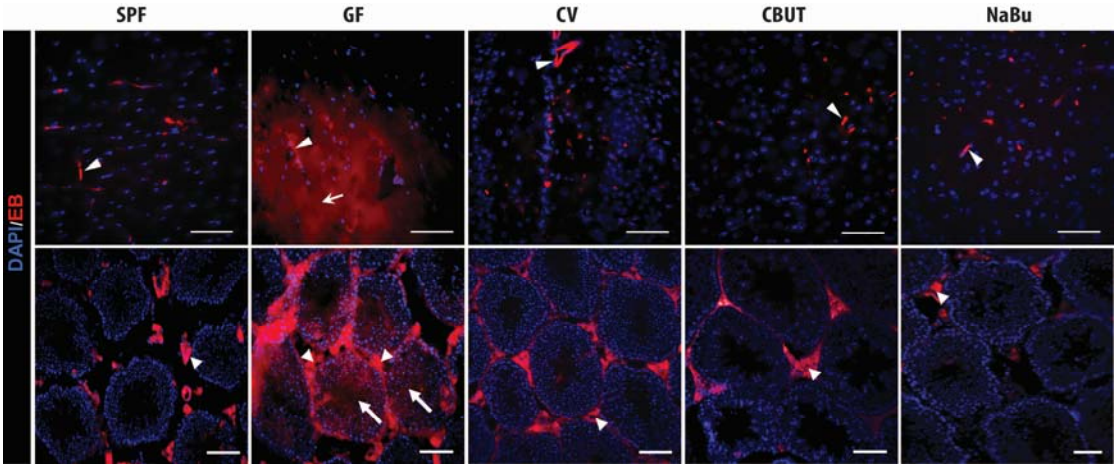

Figure 4.2. Evans blue (EB – red) and nuclear staining (DAPI – blue) of brain frontal cortex (upper panel) and seminiferous tubules (lower panel) of adult SPF, GF, CV, CBUT and NaBu mice. Arrowheads: brain blood vessels and interstitial cells of the testis. Arrows: EB extravasation into the brain parenchyma and the lumen of seminiferous tubules.

5 CONCLUSIONS AND FUTURE PERSPECTIVE

This thesis support that our microbes are important regulators of host barriers protecting immune-privileged organs such as the placenta, brain and testis. Why should microbes be involved in regulation of barrier integrity and functions? Perhaps as a way to improve the host innate immunity as these physical barriers represent the first line of defense against foreign molecules and pathogens. Such protection will also favor the growth and survival of the microbiota. Moreover, results from this thesis further support the model that maternal microbes are important regulators of metabolism during pregnancy. To what extent these findings can explain some of the GF phenotypes previously reported in adults remains an open question.

Future studies should focus on unraveling the signaling pathways and the identification of the metabolites involved in the establishment of barrier functions. Such research may improve our understanding of host-microbe crosstalk and perhaps pave the way for novel microbiota-based interventions. Detailed characterization of bacterial metabolites (e.g., SCFA) and target genes could prove fruitful in this context?

However, many questions regarding maternal host-microbe interaction during pregnancy remain un-answered. How does the microbiota communicate with the fetus through the placenta? How does it control maternal metabolism? Here, we focused on one metabolic maternal organ, the liver, and maternal adipose tissue and muscle should also be examined. Is the supply of probiotics to mothers during pregnancy and perhaps during lactation something to consider? Future studies should also address the potential involvement of microbes in pregnancy-associated diseases such as pre-eclampsia and gestational diabetes mellitus.

In addition, our data imply that microbes can modulate testosterone production. It is therefore of great interest to consider evaluating whether host microbes can influence prostate function, a testosterone-dependent organ. In addition, is there an association between certain microbes and prostate hyperplasia and/or prostate cancer? It is still puzzling that even though male GF mice have an altered BTB and very low levels of testosterone, they are nonetheless fertile. How do these animals compensate and reproduce? Moreover, I focused here on the influence of microbiota on the primary male reproductive organ, the testis, and it will now be of considerable importance to look at the female gonads, the ovaries, as well as the potential effects of microbiota on levels of female hormones (estrogen and progesterone).

6 ACKNOWLEDGMENT

Coming from a conservative country with a different lifestyle, I found it challenging to immigrate to Sweden alone with my two-year-old daughter. The first year was extremely tough, but with time I began to make new friends and meet amazing people. I also started to get used to and learn to cope with the freezing weather during the very long winter in Stockholm. During the 6 years of my PhD studies, I have matured and learned how to deal with tough situations, unfulfilled expectations and set-backs. This dream would not have become true without the continuous support, guidance and kind patience of my PhD supervisors, friends and family, whom I would now like to thank.

I would like to start by thanking my main supervisor, **Sven Pettersson**, for introducing me to the world of gut microbiota and giving me the opportunity to work in his laboratory. Thanks for all the fruitful brain-storming sessions and for teaching me to think outside the box and look at my results from a different angel. Despite all the frustration and tough times, I must say that you molded me into an independent scientist. Thank you, Sven, for your unending scientific ideas and challenges that made me a better scientist.

Lars Hedin, my external mentor and supervisor, you are the reason I came to Sweden. I didn't even know Karolinska Institutet existed before meeting you. Despite the geographical distance, you have been a constant presence in my studies. Thanks for all your Skype and Viber calls! I always feel invigorated and energized after our talks. Thanks for your continuous support and encouragement. I am sure we will continue to collaborate in Qatar, be it on gut microbiota or something else.

I would like to thank my co-supervisor**, Olle Söder,** for his care and support, for helping me to publish the blood-testis barrier paper and for your kindness, good advice and scientific discussions.

I would like to express my sincere gratitude to **Jan-Bernd Stukenborg,** my co-author, and in a way, also my German supervisor. It was great fun working with you. Thanks for believing in me and in my blood-testis barrier project, despite all the obstacles. We got it published in the end! Thanks for making this happen. I am extremely grateful for your patience, guidance and mentorship from the beginning of this project until the end. Thanks for teaching me all about the male reproductive system and for making me attend the pediatric endocrinology course, from which I have benefited immensely. Hopefully we will continue to collaborate.

Farhana Anuar, a Singaporean post-doc, mentor and my co-author, I must say I was totally lost in the lab until you came. Thanks for teaching me all the necessary techniques and for showing me how to plan experiments and interpret and analyze my results. I greatly appreciate your daily guidance and scientific discussions during the first two years of my PhD studies. You actually shaped my whole project by noticing the leakiness in the brain and testis barriers and, later, in the placenta. Thank you from my heart.

I also wish to thank **Velmurugesan Arulampalam**, I can't imagine our research group without you. You are always available if someone needs help and although we worked on different projects, you were always there to discuss my results and exchange ideas during group meetings. I always come to you when I am down and stressed and you always manage to cheer me up even before you became the student director. Thanks for your kindness, support and advice. In addition to the scientific environment, thanks for all the wonderful dinners and group gatherings at your place. You are a great cook!

Fahad Al-Zadjali, my Omani external mentor, colleague and co-author, Thanks for your endless support during the last part of my PhD journey. Despite your tight schedule as an associate dean at Sultan Qaboos University, you always reply to my emails and WhatsApp messages. I remember when you were with me step-by-step on WhatsApp when I was running the hepatic glycogen assay. I always turn to you when I face any problem with my experiments and, amazingly, you always manage to solve it. Thanks for helping me with all the figures and cartoons for my papers and for designing the cover of my thesis. Thanks for your fruitful input and advices about my PhD. I am really eager to explore your SOCS2 knockout mice once I have finished my PhD, so please be prepared for that.

I would like to also thank all present and past members of Sven Pettersson's group. **Kerstin, Annika, Bogdan, Delina, Gedas, Gosia, Ing-Marie, Kripitch, Linda, Markus, Nima, Parag, Rossana, Sasha, Sammy, Sebastian, Shawon, Terri, Viorica, Ying-Hui, Check Mai, Song Hui** and Prof. **Joseph Rafter.** Special thanks go to: **Agata,** for company along the PhD journey we started together and, finally, we both made it. Thanks for all your technical help at the lab and all the wonderful scientific, non-scientific, philosophical and religious discussions at the coffee table. **Terri,** for your amazing daily company at lunch during the last year of my PhD studies. Thanks for making me eat a decent lunch every day. I really enjoyed your company, laughs and discussions. **Parag**, my old neighbor and the zymogram expert, thanks for all the scientific discussions about the MMPs and social events full of delicious Indian cuisine. **Viorica**, for getting the BBB paper published, for exposing us to Moldovan culture and for organizing most of the group's social events. **Afrouz**, our young Persian scientist and the immunofluorescence expert, it was pleasure working with you on the BBB paper when you first joined the lab. I wish you the best of luck in your PhD journey. **Shawon,** our muscle expert, you brought a new twist to the lab. Good luck in your post-doctoral training. **Song Hui**, thank you so much for helping me with a major animal experiment at MTC and for all the laughs and discussions about science and the Singaporean culture. **Annika,** our lab manger and research technician, thanks for your tremendous help throughout my PhD studies especially concerning ordering things and work with animals. Thank you also so much for providing the amazing photos of the GF isolators, used in this thesis. **Kerstin**, thanks for your administrative help when I started my PhD and for gossip at the coffee table.

The staff at the Germ-free animal facility deserve huge thanks: **Frida Tjärnberg**, **Johanna Aspsäter**, **Pia Andersson**, **Evelina Edén**, and **Therese Fröling**, thank you all for your help throughout the years with breeding, gavaging and handling the GF mice. I really appreciate this.

Thanks to all my friends and colleagues in the lab corridors at MTC, especially: **Sunitha**, thanks for your care and for inviting Dana and me several times to your place. Thanks for

teaching Dana all about Indian culture. **Young-Keun,** for performing all the TEER assays; even though the results were not included in the BBB paper, I really appreciate your tremendous help. **Sadia**, for all the wonderful chats in the corridor and all the fun we share when you try to communicate with me in Arabic. **Pradeepa,** thanks for our fun chats in the corridor. Many thanks to **Benedict,** our neighbor across the corridor, for your endless sarcasm and jokes that really brought life to the working environment. **Carina,** for all our technical talks about the BBB. Prof. **Martin Rottenberg,** thanks for the scientific talks about the BBB and for allowing me to use the ELISA reader in your lab several times. The members of your group are really very helpful.

I would like to especially thank Prof. **Marie Arsenian-Henriksson**, the head of MTC, for her care and support. Thanks for the fruitful meeting we had about my PhD. I will always remember your words: *Never look back.* I would also like to thank Prof. **Anders Gustafsson,** the Dean of Doctoral Education, for your support and for discussing my PhD progress. Thanks for your fruitful suggestions and for discussing the alternative options. A special thank also to Prof. **Gunnar Norstedt,** thanks for opening your door when all other doors were shut. My friend Amira is really lucky to have you as her supervisor.

I also thank my colleagues outside MTC: **Maarja Mäe** from MBB, for your help with the tracer experiments, and **Ljubica Perisic** from CMM, for your help and guidance.

Now, I would like to acknowledge all the amazing friends and other people who played such a huge part in the journey to my PhD degree. My two wonderful friends from Oman: **Manal**, thanks for the amazing trips we made together (Brussels, Venice, Paris and skiing at Engelberg) and for organizing the latest kayaking trip. Thanks for taking care of Dana when I had to attend a conference in France. Thanks for your concern, care and kindness. **Amira**, thanks for being there for me when I needed most and for putting up with my bad temper at the end of this long battle. Sorry I exploded at you once, but you always understand me. I already miss you and miss your company in Stockholm. Thanks for printing and nailing up my thesis. I can always depend on you. My two amazing medical doctors from Qatar: **Mariam Al-Mahmood** and **Safa Najar**: my life in Stockholm was dull and boring until you guys came. Thanks for all the wonderful weekends we spent together. **Mariam**, my fashion advisor and trip organizer: thanks for organizing all the skiing trips (Åre and Engelberg) and for organizing our latest trip with your mother to Italy (Milan, Genoa and Portofino). I really enjoyed her company. God bless her. **Safa,** I always respect and enjoy your religious ideas and fatwas. Thanks for your care, love and all the amazing trips with Manal. A special thanks also to **Nada** from Sudan and my Egyptian neighbor **Sally** for all the good times. **Marja and John**, such an amazing Swedish family. Thanks for your love and care towards Dana. She still remembers all the good times she spent with you and Manal. **Ahemd and Marwa**, a wonderful Egyptian couple thanks, for sharing the good times and I really pray for Marwa to recover and get better. **Amani,** my good friend. Thanks for all the great weekends we spent together when I first arrived to Stockholm.

I wouldn't have achieved this PhD without my country's support through the higher education institute and scholarship office at Qatar university. Thanks for paying my bench fees and for your continuous follow-up. **Cesar Wazen,** the director of the scholarship office, thanks for your support and your quick reply within minutes to my emails. **Dr.Asma Al-Thani**, head of the health department at Qatar University, thanks for your tremendous support and

understanding. I am eager to come back and work under your wings. **Dr. Eman Mustafawi,** the Dean and President **Prof. Sheikha Abdulla Al-Misnad,** I am so grateful for your continuous support and kindness. I aim to come back and bring knew knowledge and expertise to Qatar University. Of most, I wish to thank my brother-in-law, **Dr.Talal Al-Emadi,** for his guidance throughout this journey. Thanks for all your fruitful advices and help about my scholarship and career at Qatar University.

Last but not least, I am truly and deeply indebted and blessed to have the support of my family. **My mother**, your continuous prayers days and night made this dream come true. You always encouraged me to complete my postgraduate education and here I am, I finally did it, and I hope you are proud of me. **My father**, thanks for looking after me and for building my new house while I was away in Stockholm. **My grandmother**, I know you never liked the idea of me being away and living alone in Stockholm, but I had to follow my dreams. Thanks for your tremendous love and for your prayers. May Allah keep you in good health. **My sisters**, **Laila, Reem, Sara, Mona and Huda,** thank you all for your prayers, encouragement and support. I can't imagine my life without my 5 sisters. I really missed our Thursday gatherings while I was in Stockholm and I am glad to be back with all of you. **My brothers**, **Hassan, Mohammed and Khalid,** thanks for caring and asking about me all the time. **My in-laws**, I am blessed to be part of your family. Thanks for your love and support. My precious daughter and little princess, **Dana** you are a gift from God. I have enjoyed our life together in the small apartment in Stockholm. You actually became so Swedish and Westernized. Now it is time to come back and fit into the new Qatari society. Finally, I wish to thank my **husband** and soul mate, thanks for your inspiration and protection. You always make me feel safe in this crazy world of science and believe in me when I am in serious doubt. Despite our imposed long-distance relationship for the sake of this PhD, your spirit has always surrounded me. I am so happy we are re-united and I look forward to the better future ahead of us.

الْحَمْدُ لِلَّهِ الَّذِي بِنِعْمَتِهِ تَتِمُّ الصَّالِحَات

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