Gut microbial metabolomics to understand allergies in early life

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Chapter 1

General Introduction and Scope

Food allergies

In the past decades the prevalence of food allergies, especially in Western countries, has been on the rise.[1,2] The term "food allergy" encompasses various clinical conditions, sharing a common underlying mechanism: a loss of clinical and immunological tolerance to ingested food proteins.[3] Consequently, individuals with food allergies experience an exaggerated immune response to otherwise harmless food proteins.[4] Allergic reactions are mainly classified as immunoglobulin E (IgE)-mediated, non-IgE mediated, and mixed based on clinical symptoms and immunological mechanism.[5] IgE-mediated food allergies are characterized by IgE antibody production and immediate onset of symptoms following allergen ingestion.[5] In comparison, non-IgE mediated food allergies are marked by a delayed response to the allergen and involve immunological mechanisms that are not yet well-defined.[5] Symptoms of food allergy range from mild skin, respiratory, gastrointestinal symptoms to life-threatening anaphylaxis.[3]

Often allergic disease follows a temporal progression from atopic dermatitis and food allergy in infancy to allergic asthma and allergic rhinitis in childhood, also known as “atopic march”.[6,7] Notably, early life atopic dermatitis is also a well-established risk factor for food allergies later in life.[3] Immune tolerance to the food allergen is often, but not always, acquired with time leading to outgrowth of the food allergy.[5] For instance, hen egg and cow’s milk allergies are often outgrown in childhood, whereas nut allergies can persist into adulthood.[5]

Despite the increasing knowledge on food allergies treatment and prevention, allergen avoidance remains the most common management strategy.[3] Considering the reduction of quality of life of allergic individuals and the associated financial burden,[8] the development of prevention and treatment strategies is urgently needed.

Multiple hypotheses have been suggested to explain the increase in allergies in Western countries with a leading hypothesis proposing that reduced microbial exposure in the developed world has disturbed the once beneficial commensal human-microbe relationship leading to a loss of certain immunoregulatory pathways.[9] In other words, this theory suggests that reduced bacterial exposure results in an ill-trained immune system, which is incapable of recognizing a friend from a foe, resulting in food sensitization and thus allergies.

The gut microbiome and early life

Our guts harbor to the greatest number of humoral immune cells in our bodies[10] and are home to densely populated microbial communities which have coevolved with us in a symbiotic relationship.[11] We, as hosts, provide the gut microbiome (GM) with a hospitable habitat and nutrients and in turn the GM aids nutrient absorption, produces vitamins, protects against pathogenic microorganisms and more.[11] The colonization of infants’ gut starts at birth and is very rapid in the first 3 years of life, especially in the first year[12,13] (**Figure**

1). Prior the introduction of solid food, the GM of healthy breastfed infants is dominated by *Bifidobacterium* species, including *B. breve*, *B. longum*, *B. bifidum*.[14] Their prevalence is a result of their excellent capability to (co-)ferment human milk oligosaccharides (HMOs) abundant in breastmilk.[13] Following solid food introduction, the microbiome starts diversifying and eventually closely resembles that of an adult at 3 years of age[12,13] (**Figure 1**).

The microbiome composition in early life is highly dependent on multiple factors, including birth mode, feeding mode, solid food introduction, and antibiotic usage[13,15–17] (**Figure 1**). For instance, at birth the GM of vaginally delivered infants resembles that of mother's vagina, whereas that of the infants delivered via C-section – the environment and mother's skin.[18] Meanwhile, the consumption of infant formula promotes the colonization of microbial taxa commonly found in adults.[19]

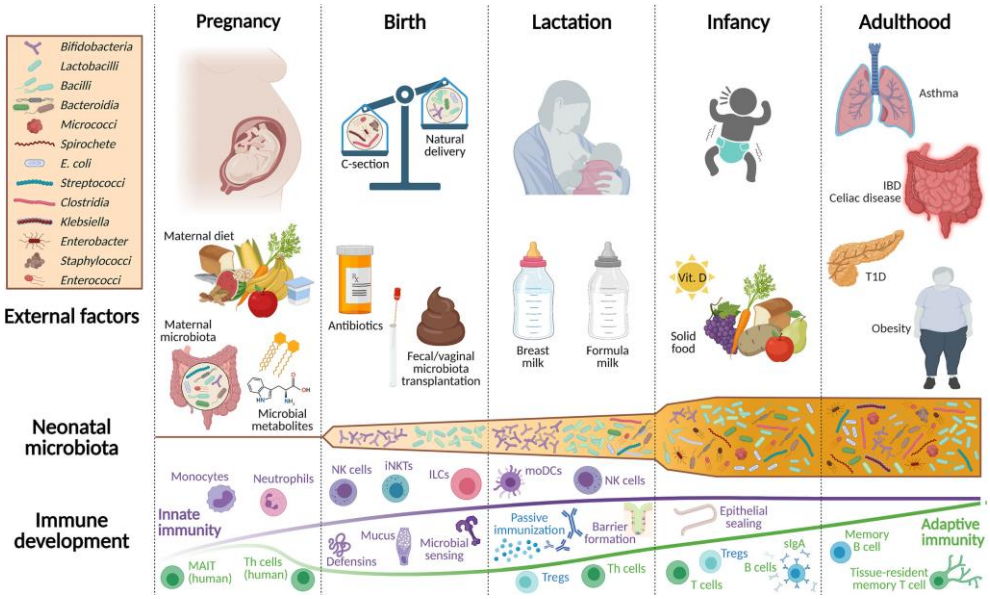

Figure 1. *Overview of factors shaping the development of the GM and mucosal immune system through pregnancy, birth, lactation, infancy, and adulthood. Abbreviations: IBD, inflammatory bowel disease; sIgA, secretory immunoglobulin A; ILCs, innate lymphoid cells; iNKTs, invariant natural killer T cells; MAIT, mucosal-associated invariant T cells; moDCs, monocyte-derived dendritic cells; NK, natural killer cells; Th cells, T helper cells; Treg, regulatory T cells; T1D, type 1 diabetes. Image derived from[15]*

The development of the immune system starts prior to birth and is influenced by the maternal diet[15] as well as by gut microbial-derived metabolites which reach the fetus via the placental barrier.[20] At birth, simultaneous to the rapid temporal development and maturation of the infants GM, the immune system of the infant also develops substantially[21] (**Figure 1**). Considering their simultaneous development and high density in the gut, it is not surprising that the host's immune system and GM have coevolved to influence each other.

Consequently, the proper development and maturation of the GM in early life is essential for optimal immune system development.[15,22,23] Among others, bifidobacterial species *B. breve*, *B. longum*, *B. bifidum* have well-recognized beneficial effects for the immune system development in early life.[24] Meanwhile, disturbances in the GM composition in early life, also known as dysbiosis, and reduction in bifidobacteria in particular, have been linked to multitude immune-mediated diseases, including food allergies.[25,26] Because of that, the first three years of life are often viewed as a critical period during which GM disturbances can lead to adverse health outcome and disease.[27–29] Alternatively, the period can be viewed as a window of opportunity in which the dynamic nature of the GM and its ability to adapt to changes can be advantageous and be used to treat and prevent diseases, including allergies.[27–29]

Infant diet and bifidogenic interventions

Human milk is considered the golden standard for infant nutrition.[30] In addition to the macromolecules (carbohydrates, protein, fat), human milk also contains minerals, vitamins, hormones, immune cells, cytokines, and other bioactive components such as the above-mentioned HMOs.[31] Exclusive breastfeeding is highly recommended for the first six months of life, followed by breastfeeding alongside solid food consumption until age of two or beyond.[30] The alternative feeding mode in the cases when infants cannot receive breastmilk is formula-feeding. Infant formulas commonly use cow's milk or soymilk as a base and are often supplemented with minerals, vitamins, nucleotides, and fatty acids in the attempt to resemble human breastmilk as much as possible and meet all nutritional needs of the infant.[31] When infants cannot tolerate standard formula, specialized formulas are prescribed instead. In those formulas the milk proteins are either partially or extensively hydrolyzed to reduce their allergenicity.[32] When the infant cannot tolerate the partly and extensively hydrolyzed formula, an amino acid-based formula is assigned instead.[31]

In contrast to breastmilk, cow's milk and soymilk used in infant formula do not contain HMOs, which are essential for the growth and proliferation of bifidobacteria in infants' guts.[31] Due to the importance of bifidobacteria for the immune system development and maturation, infant formulas are sometimes supplemented with *Bifidobacterium* probiotic or bifidogenic (bifidobacteria-enhancing) prebiotics and synbiotics.[33,34] Probiotics are microorganisms with known beneficial effect on health; prebiotics are non-digestible food ingredients that promote the growth of beneficial microorganisms; while synbiotics are a blend of probiotics and prebiotics.[31] *Bifidobacterium* probiotics include but are not limited to *B. breve*, *B. longum*, *B. bifidum*, whereas prebiotics include non-digestible carbohydrates such as galactooligosaccharides (GOS), fructooligosaccharides (FOS), and inulin.[33,34] The latter two are linear fructose chains ending in a glucose unit that vary in chain length: 3–5 units in short-chain FOS, 6–10 in long-chain, and up to 200 in inulin.[35]

Microbiome analysis

To study the link between the GM and disease, the microbiome composition and/or function could be analyzed in fecal samples. In the past, microbiome research was limited by traditional culturing, which was biased toward aerobic microorganisms, limiting their ability to capture the predominantly anaerobic gut microbiota.[36] Fluorescence *in situ* hybridization (FISH) is a culture-independent molecular technique that enables quantification of bacterial taxa such as *Bifidobacterium* spp.[37] This technique targets the 16S rRNA gene, which is ubiquitously found in bacteria.[36] This gene consists of constant regions shared by all bacteria, and hypervariable regions which facilitate phylogenetic differentiation at the genus or species level.[36] Next-generation sequencing technologies revolutionized GM research by offering high-resolution large-scale analysis of microbial communities.[38] 16S rRNA gene sequencing, which as the name suggests also utilizes the 16S rRNA gene, has been considered the golden standard in compositional microbiome analysis for many years.[13] The technique provides relative abundance data, typically at genus level.[38] Alternatively, a metagenomic approach, also known as shotgun metagenomics, allows for the examination of the entire microbial genome.[13,39] This methodology offers much richer data, providing insights into the genetic functional potential of the GM.[13,39] Although shotgun metagenomics enables taxonomic resolution down to the species and strain level, its higher cost and computational demands make 16S rRNA sequencing the more widely used approach.[38]

Metabolomics

Functional insights into host–GM interactions can be derived through metabolomic analysis.[40] Metabolomics is the scientific field that focused on the comprehensive analysis of metabolites - small molecules (≤ 1500 Da) involved in metabolic processes within biological systems.[41] By producing a wide range of metabolites, the GM impacts gut physiology in both beneficial and harmful ways.[40] Studying these microbial metabolites provides direct insight into host-microbiota interactions.[40] Different biological matrixes (feces, urine, plasma) could be subjected to metabolomic analysis in the field of GM research depending on the research question. Plasma is used to study the circulating metabolome, capturing microbial metabolites that have been absorbed from the gut into the blood and may affect the immune system.[42] However, plasma is an invasive matrix, difficult to obtain from infants. Fecal samples are widely used in microbiome and metabolomic research, particularly in infant studies, due to their non-invasive nature, and their ability to reflect gut microbial composition and activity.[43] Fecal metabolomics provides detailed insights into the metabolic interactions between the host, diet, and gut microbiota.[44]

Metabolomics measurements are typically acquired using either nuclear magnetic resonance (NMR) or mass spectrometry (MS).[40] Even though superior to MS in terms of absolute quantification capability, NMR's low sensitivity limits its application only to the

most abundant analytes.[40] The high sensitivity, broad dynamic range, and high throughput make MS ideal for GM research.[40] Typically, MS is coupled to a chromatographic separation technique to decrease the sample complexity and enhance the detectability and identification capabilities of MS. Gas chromatography (GC) is suitable for the analysis of volatiles and of non-volatiles after suitable derivatization.[40] Meanwhile, liquid chromatography (LC) is suitable for the analysis of a wide range of polar (Hydrophilic Interaction LC - HILIC) and apolar metabolites (reverse phase LC).[40,45]

Translational gut-on-a-chip

In vitro gut-on-a-chip models are an alternative approach to study host–microbiome interactions mechanistically in a controlled manner, with some platforms enabling high-throughput experimentation.[46] In recent years, there has been a significant shift from traditional 2D in vitro models, where microbes are cultured under static conditions, to advanced 3D models like gut-on-a-chip, which more accurately replicate human gut physiology.[46,47] Even though no model can capture the full complexity of the human gut, a diverse range of gut-on-a-chip models have been reported reflecting different aspects of gut physiology.[46,47] The selection of models is driven not only by technological progress but also by the specific research question, as some can be adequately addressed with simple models.[46,47] Design aspects worth mentioning are the choice of epithelial cell lines, e.g., Caco-2 monolayer; presence and choice of immune and vascular cells; the addition of mucus; choice of microbiota, e.g., pathogens, probiotic species; the inclusion of flow to mimic in vivo fluid flow and shear stress; and oxygen gradient.[46,47] The latter is necessary when coculturing aerobic epithelial cells and anaerobic gut microbiome taxa.[46,47] The flexibility in the choice of model components has enables studies on disease mechanisms and therapeutics, including probiotic interventions.[47,48]

Outline and scope of the thesis

The early life gut microbiome (GM) is a dynamic and rapidly evolving ecosystem that plays a crucial role in immune system development and has been associated with allergic disease. Despite increasing recognition of importance of the GM, the complex interplay between the GM, environmental and dietary factors, and the allergic disease remains poorly understood. To unravel the intricate host–microbiome interactions, there is a growing shift toward metabolomic studies, which offer functional insights beyond taxonomic profiling. The research is based on the hypothesis that the GM in early life influences the development and resolution of allergies via the production of metabolites and that metabolomics can be used to study the function of the GM. The aim of this research is to study the links between allergy and intestinal health, the GM, and external factors by exploring the metabolome in longitudinal clinical studies and in vitro gut-on-a-chip models.

Chapter 2 aims to provide a systematic review on the role of the GM in the most common food allergy in early life: IgE-mediated cow's milk allergy (CMA). To offer a comprehensive overview of the current knowledge in the field, this review focusses on the microbiome,

transcriptome, proteome, metabolome, and immune response data from studies in children (≤12 years of age) and from animal models. Case-control and intervention studies are included to detail both disease-associated alterations and the impact of microbiome-based interventions. As most studies focus on microbiome compositional analysis, functional insights into the host-microbiome interplay remain scarce. The limited coverage of the available metabolomics studies hampers mechanistic understanding, highlighting the need for more comprehensive metabolomics and integration with other omics analyses.

Addressing the limited metabolomic scope in the existing literature (**Chapter 2**), in **Chapter 3** and **Chapter 4** the aim is to study the role of a broad range of host and microbial metabolites, including aromatic amino acid metabolites, bile acids, and short chain fatty acids in early-life allergy. In **Chapter 3** infants, exclusively breastfed for at least 16 weeks and at risk of developing allergies are followed during the first year of their lives, the period when "atopic march" typically starts, and microbial colonization is most rapid. This study aims to (1) explore the fecal metabolome and microbiome association with allergy development in the first year of life, and (2) evaluate the impact of age, delivery mode, and feeding practices, i.e. breastfeeding, formula feeding, and complementary feeding on the fecal metabolome and microbiome during this critical period.

IgE-mediated CMA is typically managed through elimination diets, including the use of amino acid-based formulas (AAF) in formula-fed infants. Given the growing evidence of the beneficial role of *Bifidobacterium* spp. for the immune development and their association with allergy, as shown in **Chapter 2** and **Chapter 3**, bifidogenic supplementation of AAF has emerged as a promising strategy in the management of IgE-mediated CMA. **Chapter 4** aims to study the links between IgE-mediated CMA, the GM, and bifidogenic synbiotic supplementation. For this, samples of the PRESTO clinical trial are analyzed. This trial follows infants diagnosed with IgE-mediated CMA who receive either standard AAF or AAF supplemented with synbiotic blend of probiotic *Bifidobacterium breve* M-16 V and prebiotic inulin and oligofructose. This study aims to (1) assess the changes in the fecal metabolome associated with the acquisition of tolerance to cow's milk protein, and (2) to evaluate the impact of bifidogenic synbiotic supplementation on the fecal metabolome.

To investigate how allergy onset, tolerance acquisition and synbiotic supplementation modulate the immune responses and intestinal barrier function, physiologically relevant in vitro experimental models are essential. Gut-on-a-chip systems provide a promising platform to replicate key aspects of intestinal physiology. However, to ensure robust and reproducible results, optimization of experimental conditions, particularly the selection of appropriate cell culture media, is crucial. The goal of **Chapter 5** is to examine how exposure to proinflammatory cytokines impacts intestinal barrier integrity and the secretion of signaling lipids under serum-containing and serum-free medium conditions. Using Caco-2 tubules in a membrane-free microfluidic organ-on-a-chip platform, barrier integrity is examined simultaneously using transepithelial electrical resistance (TEER), DRAQ7 staining, and actin cytoskeletal analysis. Meanwhile, lipid mediators are profiled using targeted LC -

tandem MS method across apical and basolateral compartments of the tubule to study the impact of the culture medium on the inflammatory responses and lipid mediator profiles in response to proinflammatory cytokine exposure.

Finally, **Chapter 6** offers a general conclusion of the studies described in this thesis. Perspectives and recommendations on further research are also discussed.

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