Gut microbial metabolomics to understand allergies in early life

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Chapter 6

Conclusion and perspectives

The global rise in allergy incidence is a growing concern, as allergies can significantly diminish quality of life and, in severe cases, be life-threatening. The first 1000 days of life are critical for immune system development and gut microbial colonization. Considering the high immune cell and microbial density in the gut, the microbiome and immune system closely influence each other, making early-life microbiome colonization essential for proper immune development. Disruptions in gut microbiome (GM) composition in early life, particularly reduction in bifidobacteria, have been linked to immune-mediated diseases, including allergies. However, there is a considerable knowledge gap in understanding the link between the GM and allergies, and how microbial-based interventions can be used for allergy management. As the GM impacts host physiology through the production of diverse metabolites, examining these small molecules offers direct mechanistic insights into host–microbiome interactions. The research described in this thesis aimed to study the links between allergy and intestinal health, the GM, and external factors by exploring the metabolome in longitudinal clinical studies and gut barrier functioning in vitro gut-on-a-chip models.

Chapter 2 gave a comprehensive overview of current knowledge on the role of the GM in IgE-mediated CMA and the effect of microbiome-based interventions on the GM in children and animal models. The review showed that IgE-mediated CMA is consistently associated with a reduction in *Bifidobacterium* spp., and that probiotic *Bifidobacterium* interventions effectively induce bifidobacterial growth in the gut. Importantly, the chapter showed that most of the research on the topic is primarily focused on microbiome compositional analysis and that shotgun metagenomics, (meta-)transcriptomics, and (meta-)proteomics research is lacking. What is more, metabolomics studies were found to be greatly limited by the narrow range of metabolites studied, mainly focused on short-chain fatty acids (SFCAs), amino acids, and organic acids. The review highlighted the need for metabolomics studies examining a broader range of gut microbial metabolites to study the interplay between the microbiome and the host. It also greatly emphasized the need for multi-omics studies to get a mechanistic understanding of the link between the GM and CMA in early life.

As stated in the introduction, the studies presented in **Chapter 3** and **Chapter 4** addressed the limited metabolomic scope reported in the literature by covering a wide range of host- and microbiota-derived metabolites, including microbial amino acid metabolites, bile acids, and short-chain fatty acids. In **Chapter 3**, infants at risk of developing allergies, exclusively breastfed for at least 16 weeks, were followed during the first year of their lives, a period of rapid microbial colonization and potential onset of the atopic march. Alteration in the fecal metabolome and key microbiome members were examined in relation to age, diet, delivery mode, and allergy development during this period. The findings revealed significant age-related metabolomic shifts likely driven by concurrent alterations of the host metabolism, feeding practices, and microbiome composition. These included increases in amino acid derivatives, bile acids (BAs), B vitamins, nucleosides, SCFAs, and phenolic acids, along with decreases in long-chain fatty acids and acylcarnitines. We showed that C-section

was significantly associated with fecal metabolome alterations up to six months of age. Owing to the prospective nature of the study, it was possible to identify that infants who developed allergies within the study period had lower levels of *Bifidobacterium* spp. and significantly elevated levels of long-chain fatty acids prior to onset of clinical manifestations. Even though the findings of this research require validation in a larger cohort, the study advanced our understanding of the fecal metabolome development in early life and factors that shape it during this critical period of immune system and microbiome development. Future research should examine larger cohorts and stratify the IgE-mediated and non-IgE-mediated cases, as well as differentiate among various allergy types (skin, food, or respiratory). Differentiation of the allergy subtypes acknowledges the difference in immune mechanisms, which is essential for identifying disease-specific alterations with greater clinical relevance. To elucidate the potential link between elevated long-chain fatty acid levels and allergy development, integrated analyses of the fecal microbiome, plasma metabolome, and maternal breastmilk lipid composition, e.g. free fatty acids, are essential.

Chapter 4 explored the link between IgE-mediated CMA, the GM, and bifidogenic-synbiotic supplementation by means of fecal metabolomics. The longitudinal data analysis revealed minor metabolome alterations associated with tolerance acquisition to cow's milk protein, including alterations to the branched-chain SCFAs, BAs, and amino acid levels. Notably, infants who developed tolerance exhibited significantly elevated citrulline levels, suggesting reduced gut permeability, as well as an insignificantly lower serotonin and 5-hydroxytryptophan, which are involved in inflammation. One of the study's key findings is that the impact of synbiotic supplementation on the fecal metabolome was most pronounced after six months of intervention, with changes largely diminishing by 12 months, suggesting that early intervention is required to maximize the effect of synbiotics. Specifically, synbiotic supplementation led to increased levels of aromatic lactic acids, purine metabolites, long-chain fatty acids, and BAs, reflecting changes in GM activity. Among these, indolelactic acid and 4-hydroxyphenyllactic acid, aromatic amino acid metabolites of infant-type *Bifidobacterium*, were significantly elevated and positively correlated with the abundance of the *Bifidobacterium* genus. These findings complement the microbiome and (meta-)proteomics findings from the same cohort,[1] further supporting the efficacy of the synbiotic intervention in promoting *Bifidobacterium* growth and activity in the gut. While the synbiotic had no statistically significant effect on tolerance acquisition,[2] the observed increase in anti-inflammatory indolelactic acid suggests that synbiotic supplementation may still confer immunological benefits. Larger cohort studies are required to verify these findings and their clinical implication. Additionally, in vitro gut-on-a chip models may be utilized to understand how the tolerance-associated and synbiotic-driven metabolome associations relate to immune response.

Using Caco-2 tubules in a membrane-free microfluidic organ-on-chips platform (OrganoPlate), **Chapter 5** demonstrated how cytokine exposure impacts intestinal barrier integrity and the secretion of signaling lipids under serum-containing and serum-free

medium conditions. Pro-inflammatory cytokine exposure significantly increased intestinal permeability, cellular permeability, and induced actin remodeling under both conditions. While the intestinal permeability and cellular permeability alterations were comparable between media conditions, actin remodeling was significantly lower in serum-free compared to serum conditions. The impaired gut barrier was associated with elevated prostaglandin levels in the apical, but not in the basolateral compartment, with this effect being more pronounced under serum-free conditions. The developed integrated model offers a valuable framework for exploring the interplay between inflammation, barrier integrity, and lipid metabolism in intestinal pathophysiology which is also relevant for allergy research. Future work should refine model systems by co-culturing epithelial cells with immune or mucus-producing cells to better reflect gut physiology.

Perspectives

Research is increasingly revealing the crucial role of the GM in shaping immune function and influencing susceptibility to immune-mediated diseases. Despite that, still the link between immune health, the GM, and nutrition is only slowly being understood. There remains a pressing need for mechanistic studies to clarify how microbial communities influence host's immune response and how nutritional interventions can be utilized as prevention and treatment strategies for immune-mediated diseases, such as allergies. The perspectives presented in this chapter are structured around four key topics: clinical study design considerations, technical considerations, multi-omics data integration, and in vitro modeling.

Clinical study design considerations

To better understand the host-microbiome interaction in the context of allergy in addition to the fecal samples analyzed in this thesis it is also crucial to examine the circulating metabolome. Profiling the plasma metabolome gives insights into which microbial metabolites enter the bloodstream and may influence the host's immune response. The standard sampling of blood is, however, invasive and non-ethical in infants. Thanks to recent developments, blood micro-sampling is emerging as a highly promising, minimally invasive technique, already used for neonates.[3] Since many gut microbial metabolites (e.g., indoxyl sulfate and hippuric acid) get absorbed into the bloodstream and subsequently excreted in urine, profiling the urine metabolome is also relevant. Urine sampling is particularly advantageous in infants, as it is non-invasive and can be achieved using cotton balls as adsorption material.

Immune markers in blood, feces, and saliva are of interest for understanding the relation between host's immune response and the GM. Integrating these markers with metabolomic profiling can enhance the understanding of host-microbiome interactions in allergy as well as following microbiome-targeted interventions. Gastrointestinal motility and transit time, though often overlooked, are important factors affecting the gut microbiome and metabolome[4] and are associated with allergy.[5] Future studies investigating allergy

development and resolution in infants should consider whether observed microbiome and metabolome differences are mediated by variations in intestinal motility. Throughout early life, fecal frequency and consistency may serve as proxies for transit time, with the sweet corn test providing a more direct measure of transit time once solid foods are introduced. Given that certain metabolites are associated with both intestinal immunity and motility, metabolomic data should be interpreted alongside gastrointestinal motility indicators and (fecal) biomarkers of intestinal immune function and barrier integrity.

Considering the dynamic nature of the early-life microbiome and metabolome, future clinical studies in this field should adapt longitudinal designs, as in this thesis. The study design can be further improved by enrolling participants of a narrow age range and doing more frequent sampling throughout the study period. To reduce the variation due to dietary differences, it is recommended that infants receive an age-appropriate standardized diet prior to sampling when feasible. Alternatively, detailed dietary records, particularly after the introduction of complementary foods, can be collected. Such study design would enhance the ability to isolate effects of interest, such as allergy development, and to detect subtle yet potentially important metabolome alterations.

In studies involving breastfed infants, information on the mother's nutrition through dietary records and breastmilk compositional analysis (e.g. HMO, lipid, and metabolomic profiling) would also be valuable in revealing possible associations between maternal diet, breastmilk composition, and infant microbiome and metabolome in the context of allergy development. Such research may guide the design of nutritional interventions for mothers during gestation and breastfeeding periods. Future clinical trials should also examine alternative allergy prevention and treatment strategies for formula-fed infants. Human milk oligosaccharides (HMOs) are structurally diverse components of human breast milk that play a crucial prebiotic role in supporting *Bifidobacterium* growth. The HMOs complexity is not reflected by the galactooligosaccharides and fructooligosaccharides commonly used as prebiotics in infant formula and only a few HMOs have been shown to be both safe and well-tolerated for supplementation in infant formula.[6] Future studies should focus on examining the effect of prebiotic HMOs supplementation, preferably within a synbiotic blend, in the context of allergies.

As highlighted in the introduction of this thesis, *Bifidobacterium* spp. are undoubtedly beneficial for infants' immune system development and maturation. In addition to *B. breve*, used as a probiotic in **Chapter 4** of this thesis, other bifidobacterial species i.e. *B. longum* and *B. bifidum* are also key members of infants' gut and play crucial immunoregulatory roles.[6] Combining those beneficial bifidobacteria as a probiotic blend may thus better reflect the gut community and improve clinical outcomes. Given the variations in HMO degradation capabilities and cross-feeding between bifidobacteria,[8] such probiotic mixtures may be particularly valuable and necessary when combined with prebiotic HMOs blend. Other than bifidobacteria, *Lactobacillus* species are also key infant GM members and have beneficial effects on the immune system[9] and have been used for allergy treatment

(**Chapter 2**). Their probiotic potential as a blend with bifidobacteria species should also be explored in formula-fed infants.

Technical considerations

In this thesis, reverse-phase liquid chromatography coupled with high-resolution mass spectrometry (LC-HRMS) was the primary analytical technique used for fecal metabolomics. Data were acquired in untargeted full-scan mode (MS1 data), while preprocessing was performed in a targeted manner, focusing on known host- and microbially-derived metabolites. Several aspects of data acquisition and processing can be improved to strengthen future exploratory studies. Analyzing the data in an untargeted manner would allow for the detection of both known and novel microbial metabolites potentially involved in the interplay between allergies, the GM, and external factors. However, for untargeted metabolomics, accurate mass and chromatographic retention time information is insufficient for confident metabolite identification. To enhance compound identification and improve biological interpretation, future studies should acquire data using tandem mass spectrometry (MS/MS) using data dependent acquisition or data independent acquisition such as Sequential Windowed Acquisition of All Theoretical Fragment Ion Mass Spectra (SWATH). Additionally, the application of electron activated dissociation (EAD)[10] alongside the commonly used collision induced dissociation (CID) holds promise for enhancing metabolite annotation, as the two techniques are complementary - a potential that remains largely underexplored. Following untargeted metabolomics, targeted quantitative analysis should be performed to validate and confirm key findings.

Future studies should expand analyte profiling to include bioactive lipids like prostanoids and sphingolipids, known for their key role in inflammation and allergic responses.[11] Alterations in the bioactive lipids derived from enzymatic oxidation of polyunsaturated fatty acid such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are expected considering the altered LCFA profiles in infants prior allergy onset observed in **Chapter 3**. Given the role of BAs in regulating immune response[12] and their association with tolerance acquisition observed in **Chapter 4**, future research should also explore the role of recently discovered "microbially conjugated BAs"[13] in immune response and allergy.

Multi-omics data integration

The host–microbiome relationship is inherently complex and multifactorial. While individual omics approaches, such as metabolomics, (meta-)genomics, (meta-) transcriptomics, and (meta-)proteomics, offer valuable insights, none alone can fully capture the complexity of this dynamic interplay. To unravel the mechanistic links between allergies, the GM, and environmental and dietary factors, integrated multi-omics strategies are essential. Ideally, combining all omics levels would provide the most comprehensive picture, however, such an approach is often constrained by high costs, time-limitations and complex data integration. To gain mechanistic insights into the host–microbiome interplay, I envision the integration of metabolomics with whole metagenome sequencing as a

powerful approach, capturing functional microbiome activity along with high-resolution taxonomic profiles and genetic potential. For exploratory research, combining metabolomics with 16S rRNA sequencing still offers valuable, insights into both microbial composition and function. Integrating microbiome and metabolome data remains a significant challenge, necessitating broader metabolite coverage, advanced metabolite annotation, and improved data integration strategies to deepen our understanding of the host–microbiome interplay.

In vitro modeling

By combining epithelial-immune cell co-culture with an anaerobic microbial compartment, gut-on-a-chip models offer powerful platforms for studying intestinal inflammation and host-microbiome interactions.[14] Moving forward, such models should be leveraged to gain mechanistic insights into the link between the GM, intestinal barrier, and host's immune system in the context of allergies.[15] To achieve that, ideally, the microbiome compartment of the gut-on-a-chip model would mimic the complexity of the infant GM. An emerging approach is to culture bacterial isolates from fresh infant fecal samples under anaerobic conditions.[16] A co-culture reflecting key members of the infant GM could be a preferred alternative. Both approaches come with their technical challenges including but not limited to the maintenance of oxygen gradient that supports the anaerobic microbiome and the aerobic human cells. This could be studied in the gut-on-chip platform used in **Chapter 5** using an intestinal epithelium tubule with apical (lumen) channel perfused with an anaerobic bacterial co-culture and a basolateral channel perfused with aerobic medium. To ensure allergen sensitization instead of tolerance acquisition, such models could be developed by firstly skewing the immune compartment toward the T helper cell 2 (Th2) phenotype, followed by exposing the epithelial compartment to the allergen of interest. Such gut-on-a-chip models can be applied following exploratory clinical research, such as that presented in this thesis. Those models have the potential to shed light on how metabolomic alterations associated with allergy development and tolerance acquisition affect the immune response and intestinal barrier and help uncover the underlying immunological mechanisms. Additionally, they can facilitate the evaluation of how intervention-driven metabolomic changes influence the host immune system. The insights gained from the models would be invaluable for designing follow-up clinical trials and guiding prevention and treatment strategies. Gut-on-a-chip models also hold promise in preclinical research, where they can be used to evaluate the effects of novel probiotics and synbiotics on the immune response and intestinal barrier.

Through fecal metabolomics and in vitro modelling, this thesis has contributed to advancing our understanding of the complex interactions between allergy and intestinal health, the GM, and external factors. Future research should prioritize well-designed longitudinal clinical studies, comprehensive metabolomic profiling, immune response assessment, multi-omics integration, and the use of physiologically relevant in vitro models such as gut-on-a-chip systems. In parallel, alternative allergy prevention and treatment strategies that

mimic healthy breastfed infant gut environment should be explored as potential modulators of infant immune development. Achieving these will require multidisciplinary collaboration, ultimately paving the way for microbiome-based nutritional strategies to prevent and treat immune-mediated diseases such as allergies in early life.

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