Exploring the gut-thyroid axis

The role of the microbiome in thyroid autoimmunity

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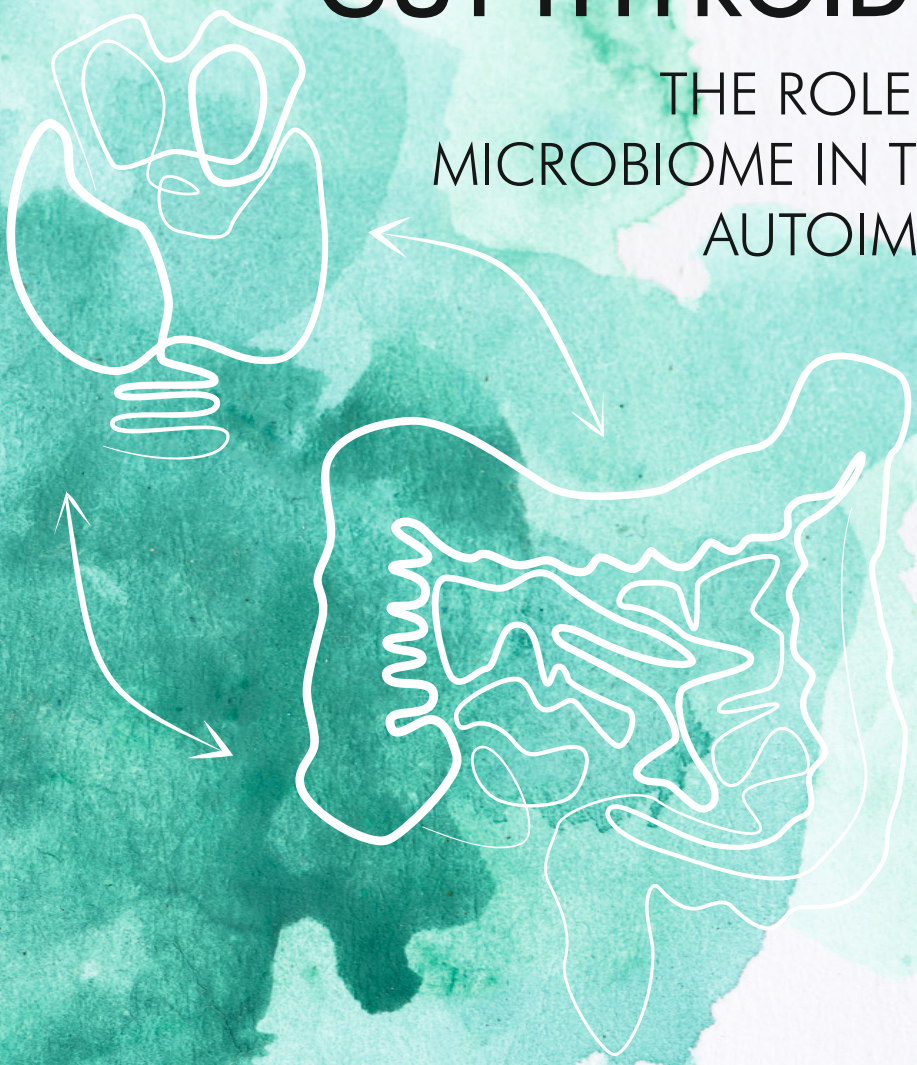

ALINE CAROLIEN FENNEMAN

Exploring the Gut-Thyroid Axis: The Role of the Microbiome in Thyroid Autoimmunity

Aline Carolien Fenneman

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Exploring the Gut-Thyroid Axis: The Role of the Microbiome in Thyroid Autoimmunity

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TABLE OF CONTENTS

1

GENERAL INTRODUCTION AND OUTLINE OF THIS THESIS

GENERAL INTRODUCTION

Thyroid hormone production

Thyroid hormones (TH) play a crucial role in the development of the brain and body during fetal life and infancy, as well as in regulating metabolic activity in adults, impacting the functioning of almost every organ system. These hormones are synthesized by the thyroid gland and released into the bloodstream, where their concentration is precisely controlled by the hypothalamus-pituitary-thyroid (HPT) axis[1,2]. The HPT axis functions through an endocrine negative feedback loop (**Fig. 1**), which enables the brain to continually monitor the circulating TH levels and adjust the production of TH via the hypothalamus and the pituitary.

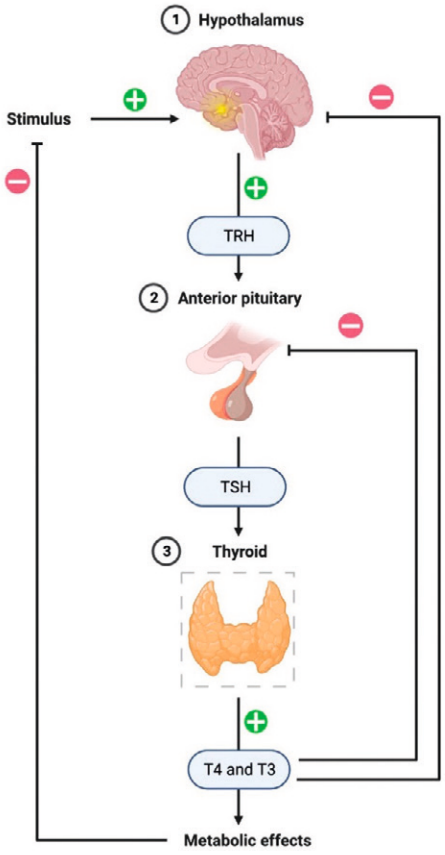

Figure 1. Schematic overview of the hypothalamus-pituitary-thyroid (HPT)-axis.

TRH; thyrotropin-releasing hormone, TSH; thyroid stimulating hormone, T4; thyroxine, T3; triiodothyronine.
Adapted from "Hypothalamic-Pituitary-Organ Axis", by BioRender.com (2023). Retrieved from https://app.biorender.com/biorender-templates

Within the paraventricular nucleus (PVN) of the hypothalamus, thyrotropin-releasing hormone (TRH) is produced by neurons projecting to the median eminence, from where it is released into blood vessels and transported to the anterior pituitary gland, where it stimulates the secretion of thyroid-stimulating hormone (TSH). TSH, in turn, stimulates the thyroid gland to synthesize TH, mainly in the form of the prohormone thyroxine (T4)(80%) and, to a lesser extent, the biologically active hormone triiodothyronine (T3)(20%)[3]. Conversion of T4 to T3 occurs at the cellular and tissue level with the aid of deiodination enzymes (DIO), enabling the local regulation of TH bioavailability[4].

Autoimmune dysregulation of the thyroid gland

The thyroid gland is susceptible to autoimmune dysregulation, whereby autoantibodies and infiltrating autoreactive lymphocytes target and destroy the gland's hormone-producing cells. This was first reported in 1912 by the Japanese physician Hakaru Hashimoto and is presently known as Hashimoto's thyroiditis (HT) disease[5]. HT is regarded as a T cell-mediated autoimmune disease where thyroid antigen-specific T cells infiltrate the thyroid gland and destroy thyroid hormone-producing follicular cells, eventually resulting in low serum fT4 and fT3 and elevated TSH levels. This hypothyroid state is characterized by reduced metabolism, causing symptoms such as fatigue, cold intolerance, constipation, brain fog, and weight gain. On the other side of the spectrum, Graves' disease derives from stimulation of the TSH receptor by autoantibodies, resulting in elevated fT4 and fT3 and suppressed TSH serum levels. This hyperthyroid state may manifest as anxiety, weight loss, diarrhea, increased heart rate, and in some cases, atrial fibrillation or thyroid eye disease (Graves' orbitopathy).

Thyroidology research has made significant progress since the early 20th century. Just two years after Hashimoto's discovery, Edward C. Kendall isolated thyroxine in pure form from extracts of hog thyroid glands in 1914 – an achievement he was awarded a Nobel Prize in 1950[6]. Chemical synthesis of this hormone in 1927 by British chemists Charles Robert Harington and George Barger[7] paved the way for substitution treatment of hypothyroid patients. Since then, levothyroxine has been the standard treatment for hypothyroidism. In 1942, Edwin Bennett Astwood discovered chemical compounds that could inhibit TH synthesis and were successfully used to treat hyperthyroid patients two years later[8,9]. These antithyroid drugs, called thionamides (methimazole, carbimazole, and propylthiouracil), are now cornerstones in the daily management of Graves' hyperthyroidism[10]. Around the same time, in 1946, the physician Saul Hertz conceived the idea of using radioactive iodine therapy (RAI) to block TH synthesis[11]. RAI ablation with I-^{131} of the thyroid gland is nowadays commonly used to treat Graves' disease and thyroid cancer. It was in the late 1980s when the effect of the gut microbiome on TH metabolism was first established as gut

microbiota were shown to promote the intestinal reabsorption of free iodothyronines into the enterohepatic circulation[12–15].

Despite all these findings and efforts made, autoimmune thyroid disease (AITD) remains challenging to treat with increased morbidity and no preventative or curative treatment for HT and Graves' disease. A significant number of levothyroxine treated and euthyroid HT patients (5-15%) continue to experience various persistent symptoms[16], highlighting the need for continued efforts to better understand the underlying pathophysiology and the development of more effective treatment options. Therefore, further investigation is necessary, including identifying environmental risk factors contributing to AITD, such as the potential role of the gut microbiome.

Gut microbiome

The intestines harbor trillions of bacteria (10^{13} to 10^{14}), collectively called the gut microbiota. Their genetic content, including viruses, fungi, microbial metabolites, and structural elements are referred to as the microbiome, although these terms after often used interchangeably[17]. Environmental factors, such as diet, xenobiotics, lifestyle, and BMI, primarily influence the composition and function of these microbes[18,19], while host genetics and ethnicity only account for a small percentage of the explained variance of the inter-person microbiome variability (1.9 – 8.1 and 6%)[20,21]. The contributory role of the gut microbiota and its metabolites in the host's health and disease has been increasingly studied over the past decade. It has been linked to a variety of conditions ranging from infectious diseases (*Clostridioides difficile* diarrhea), cardiometabolic diseases (diabetes mellitus type 2, metabolic syndrome, and hypertension), and psychiatric disorders (depression and autism) to systemic autoimmune diseases, including multiple sclerosis, diabetes mellitus type 1 (T1DM), ulcerative colitis, and Crohn's disease. However, the relationship between the gut microbiome and the thyroid gland remains largely unexplored.

One potential explanation for the onset of endocrine autoimmune diseases, including those affecting the thyroid gland, is the occurrence of molecular mimicry. Epitopes from bacterial strains share sequence similarities to auto-antigens, which may lead to a T and B cell-mediated autoimmune reaction against the endocrine gland tissue[22,23]. Several bacteria, including *Borrelia, Yersinia Enterocolitica, Bifidobacteria*, and *Lactobacilli,* have been proposed to be involved in this phenomenon in autoimmune thyroid disease but have been showing conflicting results[22,24,25]. This connection represents one pathway within the intricate network of the so-called gut-thyroid axis.

Gut-thyroid axis

The gut-thyroid axis involves a complex and bidirectional communication between the gut and the thyroid gland, influencing TH metabolism, immune regulation, and, potentially, disease onset and progression.

The gut is a target organ of TH as they are essential in regulating gut health and function and maintaining intestinal homeostasis. Previous studies showed that the gut was the most affected organ in mice lacking TH receptors, with reduced mucosa thickness and diminished intestinal function[26–28]. Similarly, humans with a TH receptor defect, in particular the TH receptor alpha isoform, also experience a dysfunctional gastrointestinal tract[29]. These findings align with the observation that both hypothyroid and hyperthyroid patients can experience gut problems, consisting of decreased intestinal mobility, constipation, dyspepsia, and heartburn in HT patients, while diarrhea is frequently present on the other end of the spectrum in patients with hyperthyroidism.

Conversely, gut microbiota influences TH metabolism, as initially demonstrated by experimental studies. In short, rodents treated with antibiotics to disrupt their gut microbiota composition showed higher fecal excretion of TH compared to untreated rats, suggesting diminished reabsorption of TH from the gut back into the systemic circulation[13–15]. Recent studies revealed important aspects of this symbiosis in humans, including significant differences in the diversity and composition of microbial strains between patients with AITD and healthy controls[30]. Several microbial genera correlated with parameters of thyroid status, as discussed in more detail in Chapters 2 and 5. However, a number of key questions remain to be answered. It needs to be determined which specific thyroid patient groups have a dysbiotic gut microbiota composition, and whether the dysbiosis is related to thyroid status, to thyroid hormone substitution or antithyroid drugs treatment, or to thyroid autoimmunity *per se*. Additionally, the long-term implications of gut-thyroid axis interactions remain uncertain. What will happen over time? What will happen with the thyroid reserve capacity after restoring a disrupted microbiome with fecal microbiota transplantations? Therefore, conducting adequately powered human intervention studies is imperative before suggesting a causal relationship between gut microbiota and AITD disease pathogenesis.

THESIS OUTLINE

This thesis comprises three parts that explore different aspects of the gut-thyroid axis and its clinical implication.

Part I. Gut-Thyroid Axis

Part I focuses on the role of the gut in TH metabolism and the impact of thyroid medication on gut health. **Chapter 2** provides a comprehensive overview of TH metabolism in the gut and its clinical implications. **Chapter 3** investigates the association between levothyroxine use and the risk of colorectal cancer, and **Chapter 4** examines the effect of sodium iodide (NaI) supplementation on the gut microbiota composition in NOD.H-2^{h4} mice, a mouse strain widely used to study the spontaneous development of autoimmune thyroiditis.

Part II. Gut Microbiota in Hashimoto's Thyroiditis

Part II explores the relationship between the gut microbiota and Hashimoto's thyroiditis, a common autoimmune disease that affects the thyroid gland. **Chapter 5** examines the role of the gut microbiota and its metabolites in the pathogenesis of the endocrine diseases HT and T1DM. **Chapter 6** investigates whether a disrupted gut microbiota composition exists even before clinical disease onset in participants susceptible to HT using a multi-ethnic euthyroid population with thyroid autoimmunity. **Chapter 7** presents a protocol for a randomized clinical trial to assess the effect of fecal microbiota transplantations on thyroid reserve in patients with subclinical autoimmune hypothyroidism. **Chapter 8** discusses the challenges and costs of donor screening for fecal microbiota transplantations.

Part III. Gut-Thyroid Axis and Beyond

Part III extends the investigation of the gut-thyroid axis to other areas of endocrinology and gastroenterology. **Chapter 9** explores the association between intestinal permeability and Graves' orbitopathy, a manifestation of Graves' disease that affects the eyes. **Chapter 10** investigates the role of antibiotics in the pathogenesis of diabetes and inflammatory diseases of the gastrointestinal tract. Finally, **Chapter 11** summarizes and addresses several topics relevant to translational thyroid research and its clinical impact.

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PART I

GUT–THYROID AXIS

2

A COMPREHENSIVE REVIEW OF THYROID HORMONE METABOLISM IN THE GUT AND ITS CLINICAL IMPLICATIONS

Aline C. Fenneman
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ABSTRACT

Background

The gut is a target organ of thyroid hormone that exerts its action via the nuclear thyroid hormone receptor α1 (TRα1) expressed in intestinal epithelial cells. Thyroid hormones are partially metabolized via hepatic sulfation and glucuronidation resulting in the production of conjugated iodothyronines. Gut microbiota play an important role in peripheral thyroid hormone metabolism as they produce and secrete enzymes with deconjugation activity (β-glucuronidase and sulfatase), via which TH can re-enter the enterohepatic circulation.

Summary

Intestinal epithelium homeostasis (the finely tuned balance between cell proliferation and differentiation) is controlled by the crosstalk between T3 and TRα1 and the presence of specific TH transporters and TH-activating and inactivating enzymes. Patients and experimental murine models with a dominant-negative mutation in the TRα exhibit gross abnormalities in the morphology of the intestinal epithelium and suffer from severe symptoms of a dysfunctional gastrointestinal tract.

Over the past decade, gut microbiota has been identified as an essential factor in health and disease, depending on its compositional and functional profile. This has led to a renewed interest in the so-called gut-thyroid axis. Disruption of gut microbial homeostasis (dysbiosis) is associated with autoimmune thyroid disease (AITD), including Hashimoto's thyroiditis (HT), Graves' disease (GD), and Graves' orbitopathy (GO). These studies reviewed here provide new insights into the gut microbiota roles in thyroid disease pathogenesis and may be an initial step toward microbiota-based therapies in AITD. However, it should be noted that cause-effect mechanisms remain to be proven, for which prospective cohort studies, randomized clinical trials, and experimental studies are needed.

Conclusion

This review aims to provide a comprehensive insight into the interplay between thyroid hormone metabolism and gut homeostasis.

INTRODUCTION

It has now been established that there is essential crosstalk between thyroid hormones (THs) and the gut. TH controls the intestinal epithelium homeostasis via the interaction between T3 and TRα1, the dominant TR isoform expressed in intestinal epithelial cells, the presence of specific TH transporters, and TH-activating or inactivating enzymes. In turn, the gut microbiota, consisting of 10^{13} to 10^{14} bacterial cells, has been identified as an important regulator of health and disease, including autoimmune thyroid diseases. This review addresses the different aspects of this so-called gut-thyroid axis: The role of THs in intestinal epithelium homeostasis, the important function of the gut microbiota in peripheral thyroid hormone metabolism, and the interaction between these gut microbiota and thyroid autoimmunity.

Intracellular Thyroid Hormone Metabolism

Thyroid hormones (THs) are tyrosine-based molecules that contain iodine atoms at three or four positions of the aromatic rings. The two principal thyroid hormones are thyroxine (also known as T4 or L-3,5,3',5'-tetraiodothyronine) and triiodothyronine (T3 or L-3,5,3'-triiodothyronine). THs are produced and secreted by the thyroid gland under strict control of the hypothalamus-pituitary-thyroid (HPT)-axis via a negative feedback loop [1,2]. The thyroid mainly produces T4, the prohormone, and to a lesser extent T3, the active hormone. T4 requires conversion into T3 in the peripheral tissues to become biologically active as T3 is the only form that binds the thyroid hormone receptor (TR). The conversion of T4 occurs at the cellular level by specific enzymes, the so-called deiodinases, that can remove an iodine atom from the inner or outer ring of TH. Before TH can be metabolized, it must be transported into the cell by active TH transporters. Several TH transporter families have been described; monocarboxylate transporters (MCT8 and MCT10), the organic anion transporter polypeptides (OATP), large neutral amino acid transporters (LAT), and recently, SLC17A4 of the solute carrier family. The expression of the different TH transporters is cell-specific, and the affinity of these transporters for other TH metabolites also differs; e.g., MCT8 prefers T4 whilst MCT10 prefers T3 [2–5].

Three types of deiodinases can be distinguished; type 1 deiodinase (DIO1), type 2 deiodinase (DIO2), and type 3 deiodinase (DIO3) **(Fig 1.)**. DIO1 is capable of both inner and outer ring deiodination and is highly expressed in the liver, where it is thought to be the main source of local T3 and is important for the clearance of rT3 [6]. DIO2 selectively removes an iodine atom of the outer ring of TH resulting in the production of T3 out of T4. DIO2 is important in generating local T3 in specific tissues and cells and plays a significant role in negative feedback regulation as it is expressed in the hypothalamus and pituitary [7]. DIO3 removes an iodine atom of the inner ring, thereby converting T4 and T3 to their respective inactive metabolites rT3 and T2 [5,8]. Deiodinases are differentially expressed between various cell and tissue types [5].

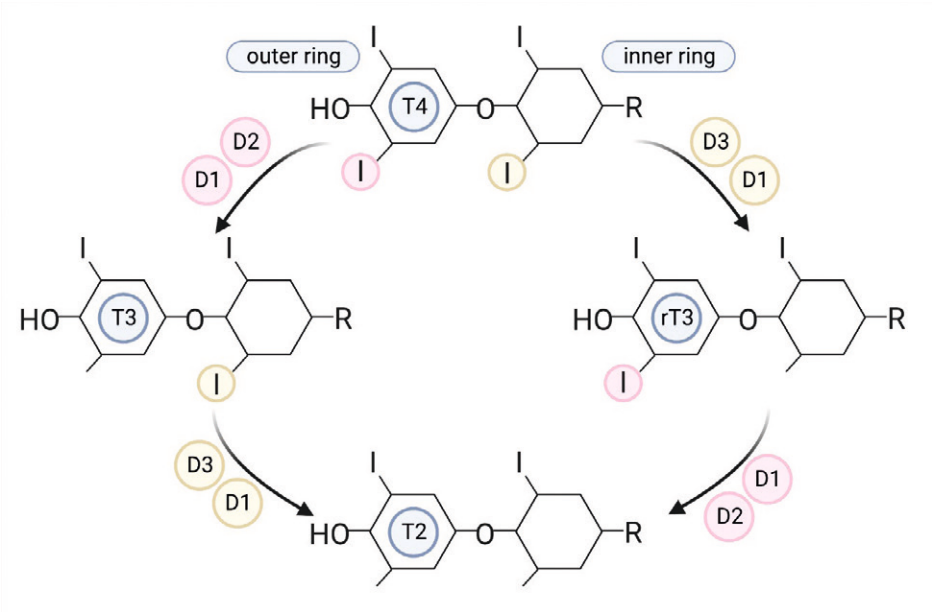

Figure 1. Different types of deiodinase enzymes.

The interplay between the transporters and deiodinases determines the local TH availability in cells and tissues.

T3, the active hormone, exerts its action via binding to the thyroid hormone receptor (TR), a nuclear receptor expressed in a wide variety of cell types. Several TR isoforms have been described, but only three isoforms can bind T3; TRα1, which is widely expressed in cardiac and skeletal muscle, the central nervous system, hematopoietic cells, bone, and intestine; TRβ1, which is mainly present in the brain, liver, and kidney; and TRβ2 which is expressed in the retina, inner ear, hypothalamus and pituitary and thereby involved in negative feedback regulation [1]. These TRs isoforms are encoded by the THRA and THRB genes, respectively.

TRs act as transcription factors and are primarily necessary to establish the actual effect of T3 either with or without (in)direct DNA binding (canonical vs. non-canonical pathways) and can be classified into four types of TH signaling pathways [9]. In the canonical pathway, activated TRs behave as ligand-dependent transcription factors. They directly bind to specific DNA sequences named thyroid hormone response elements (TRE), after which they promote the expression of the targeted genes.

In the non-canonical pathways, TRs can bind indirectly to DNA (tethered to DNA by other proteins) or participate in signaling pathways without the requirement of DNA binding and rapidly mediate second messenger signaling (by participating in

the PI3K pathway, among others). Lastly, TH can exert its effect independently of TR through binding to integrin αvβ3. Integrin αvβ3 is a transmembrane receptor for T4 and to a lesser extent T3 and has been shown to result in rapid non-genomic signaling pathways, including extracellular signal-regulated kinases (ERK1/2), with direct cellular responses [10,11].

Other Thyroid Hormone Metabolizing Pathways

Two major classical pathways of thyroid hormone metabolism are sulfation and glucuronidation of iodothyronines. About 20% of daily T4 production appears in feces, predominantly via biliary excretion of conjugated iodothyronines [12]. Conjugation of iodothyronines occurs predominantly in the liver, leading to increased water solubility of the substrates, thus enhancing their biliary and urinary clearance. Sulfation of iodothyronines is performed by sulfotransferases (SULTs) which catalyze the sulfate conjugation of the phenolic hydroxyl group, resulting in the sulfated iodothyronines substrates T4S, T3S, rT3S, and 3,3'T2S. This sulfation is a primary step towards rapid and irreversible inactivation of thyroid hormones (T4 and T3), as these sulfated iodothyronines are highly efficient substrates of DIO1 (and are not processed by DIO2 and DIO3)[13,14]. The rapid clearance of sulfated iodothyronines by DIO1 explains the low serum, bile, and urine levels of sulfated TH in adults. However, under conditions with low DIO1 activity (*i.e.,* hypothyroidism, selenium deficiency, and non-thyroidal illness), the inactivation of TH by sulfation is reversible due to the expression of sulfatases in different tissues [15,16] and by gut microbiota [17], which converts T3S back to T3.

Hepatic UDP-glucuronyltransferases (UGT) are responsible for catalyzing the glucuronidation of the phenolic hydroxyl group of iodothyronines, as glucuronic acid is the first step in the enterohepatic cycle, resulting in the production of T4G, T3G, and T2G [2,18]. In contrast to the sulfates, glucuronidated iodothyronines are rapidly eliminated via biliary excretion in the intestine. After the biliary excretion of the glucuronidated iodothyronines (mainly T4G in humans) in the intestine, gut microbiota, specifically obligatory anaerobes, can hydrolyze T3G and T4G back to T3 and T4 by using the bacterial enzyme β-glucuronidase [2,18]. This process promotes the intestinal reabsorption of free iodothyronines into the enterohepatic circulation, where they are again available to the liver. These findings suggest that glucuronidated iodothyronine may serve as an intestinal thyroid hormone reservoir, thereby preventing fluctuation of serum TH levels.

A small fraction of TH is metabolized by oxidative deamination or decarboxylation of the alanine side chain of TH. Deamination of T4 and T3 produces 3,3',5,5' -tetraiodothyroacetic acid (tetrac, TA4) and 3,3',5-triiodothyroacetic acid (triac, TA3) respectively. Decarboxylation followed by deiodination results in the formation of thyronamines, including 3-T1AM[2]. Interestingly, the entire molecular machinery required for this T1AM biosynthesis is expressed in the (murine) intestinal tissue[19].

Another minor TH metabolizing pathway (less than 5% of TH disposal) is ether-link cleavage, resulting in the formation of diiodotyrosine (DIT) [20]. For more information on these forms of thyroid hormone metabolism, the reader is referred to a review by Wu and colleagues, discussing these alternate pathways of thyroid hormone metabolism [20].

The Role of Thyroid Hormone in the Gut

The interplay of TH transporters, deiodinase enzymes, and thyroid hormone receptors (TR) is of great importance for the bioavailability of TH in cells and tissues and, therefore, for the effect of TH. The integrated action of these TH signaling cascade components is cell type-specific, meaning that circulating TH concentrations do not necessarily reflect intracellular TH bioavailability [21]. The solute carrier family (SLC17A4) transporters are the predominant transporters in small intestinal and colonic epithelial cells, followed by LAT- and OATP-transporters [3]. All deiodinase enzymes are present in the gastrointestinal tract, with expression rates depending on different stages of embryonic and adult life [22–24]. Both TRα and TRβ1 isoforms are present in the gastrointestinal tract, although TRα1 is the predominant subtype in the intestinal tissue to which T3 can bind [25].

TH is involved in several processes in the gut. The intestinal epithelium consists of rapid and continuous cell renewal and is a well-established TH target [26]. Epithelial lineages derived from intestinal stem cells consist of rapidly proliferating progenitor cells in the crypts (invaginations of the intestinal wall) in both the small intestine and colon. The small intestine consists of differentiated cells in the villi (extensions of the intestinal wall), followed by apoptosis in the apex of the villi, whereas the colon consists of a flat surface with highly differentiated cell types in the upper part **(Fig 2)**. Several studies have shown that the intestinal epithelium's developmental and physiological functions are controlled by TH signaling [26–28]. The first evidence that supported this hypothesis derived from the observation that intestinal epithelium remodeling in amphibians completely depends on TH [29]. The observation that Dio3 is highly expressed in the intestinal epithelium of the human fetus fits within the idea that the regulation of intracellular TH concentrations is highly relevant during embryogenesis [22]. Interestingly, Dio3 is re-expressed in intestinal epithelium in adult life upon neoplastic transformation (intestinal adenomas and carcinomas) compared to healthy intestinal tissue [24,30], indicating an essential role for TH in the homeostasis of intestinal cell proliferation and differentiation. It must be noted that TH and TRα1 are also coordinated and integrated with other signaling pathways in both mammalian and amphibian intestine [31,32]. As such, TH directly affects the Wnt/β-catenin pathway and Notch signaling, regulators of self-renewal and differentiation of stem/progenitor cells [26,33].

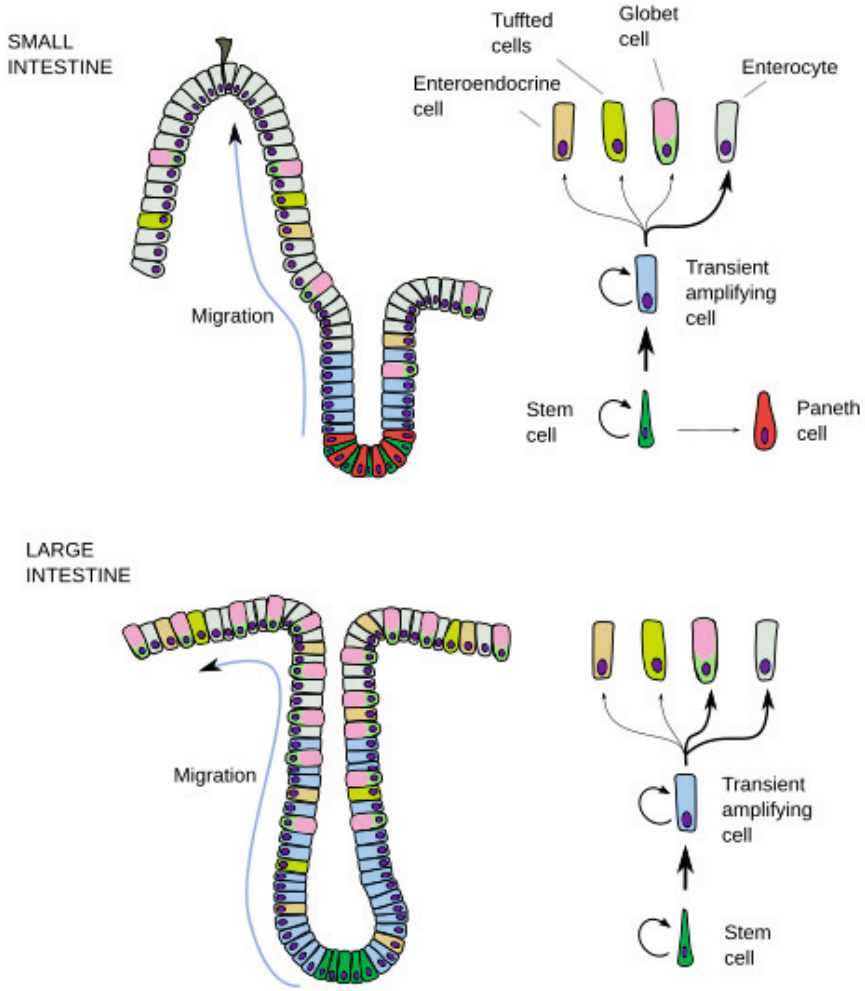

Figure 2. Drawing of the main cell lineages of the small intestine (upper part) and large intestine (bottom part). Thick arrows indicate a larger population. (Reproduced with permission from https://mmegias.webs.uvigo.es/02-english/8-tipos-celulares/enterocito.php)

Intestinal alkaline phosphatase (IAP) is a brush border enzyme secreted by intestinal epithelial cells, of which the highest expression is located in the duodenum [34,35]. The primary function of IAP consists of a gut mucosal defense factor. It dephosphorylates the proinflammatory bacterial endotoxin lipopolysaccharide (LPS), thereby preventing the translocation of LPS into the systemic circulation and subsequent TLR4 responses of host cells [36]. The IAP gene is a T3-responsive gene, as shown by an increased IAP gene transcription in enterocytes in response to T3 [37–40]. Diminished

IAP levels were seen in hypothyroid rats [41] displaying marked hypoplasia of crypts and villi. Interestingly, fecal samples of IAP-knock out (C56BL/6) mice showed a significantly reduced diversity of gut microbiota composition compared to their wild-type counterparts [39], which was restored after oral supplementation of IAP. IAP-deficient zebrafish were highly susceptible to LPS toxicity, resulting in a steep influx of intestinal neutrophils, whereas the intestines of germ-free zebrafish lacked neutrophils [42]. Overall, these studies indicate that THs are not only an important regulator of intestinal development but also promote mucosal tolerance to the commensal gut bacteria and may preserve gut microbiota composition via their action on IAP [42].

In this regard, the availability of transgenic mouse models made it possible to study the role of TH in the gut in more detail. TRα$^{-/-}$ mice (in which the TRα isoforms are abolished but the TRΔα isoforms remain) showed postnatal growth arrest with delayed maturation of the small intestine and bones. The intestine was the most affected organ in TRα$^{0/0}$ mice (lacking TRα1, TRα2, and the shorter TRΔα transcripts) [43]. These mice showed a reduced mucosal thickness compared to wild-type mice and a reduction of villus height, as well as decreased levels of digestive enzymes (lactase, sucrose, aminopeptidase) and gut transcription factors (Cdx-1 and Cdx-2) in the small intestine, indicating a diminished intestinal function. In contrast, no intestinal development retardation was seen in mice lacking the TRβ isoform (TRβ$^{-/-}$ mice), demonstrating that the proliferation of intestinal cells (crypts) is enhanced by T3 in a TRα1-dependent manner [43]. Moreover, TRα$^{0/0}$ mice have an altered TH metabolism, indicated by a lower serum rT3/T4 ratio compared to their wild-type counterparts (2.63 ±0.18 vs. 5.09 ±0.22, $p < 0.001$, respectively) [44]. These findings align with results from *ex vivo* experiments with 3D intestinal epithelium organoids, which showed a reduced development and stem cell activity in organoids prepared from crypt cultures of TRα$^{0/0}$ mice compared to crypt cultures from either wild-type or TRβ$^{-/-}$ mice. Additionally, accelerated stem cell proliferation and unbalanced differentiation were seen upon T3 treatment in wild-type 3D intestinal organoids compared to non-treated control organoids [45]. Thus, T3 and TRα control gut development and homeostasis through the modulation of intestinal crypt cell proliferation and stem cell activity [45]. Specifically, they regulate the rate of cell renewal in normal conditions and of apoptosis and cell renewal during the process of epithelial regeneration in response to DNA damage [26,33].

The dominant role of TRα in intestinal epithelium homeostasis was also seen in the *Thra1*$^{PV/+}$ mouse model (transgenic mice with a dominant-negative mutation in TRα that prevents binding of T3 to the TR, thereby repressing T3 positive receptor function), in which severe constipation was observed [46]. The murine intestine showed severe defects, consisting of shorter villi, increased differentiated cells in the crypts,

and reduced stem-cell proliferation in the intestine compared to age-matched wild-type littermates [46].

Patients with a dominant-negative mutation in the TRα (so-called resistance to thyroid hormone alpha (RTHα) were first described a decade ago[47]. Since then, over 40 patients with RTHα have been described with approximately 25 variants in the THRA gene [48]. The observed mutations, comparable to the situation in the *Thra1*$^{PV/+}$ mouse, result in a dominant-negative effect on the wild-type TRα, in which it fails to bind intracellular T3 and thereby actively represses T3 positive target gene transcription [49]. RTHα patients have only mildly affected circulating TH and TSH levels. Of interest is that up to 84% of RTHα cases show symptoms of a dysfunctional gastrointestinal tract, mostly suffering from various degrees of constipation due to an increased intestinal transit time concomitant with bowel dilation [48]. Constipation is also one of the main characteristics in patients with primary hypothyroidism, defined by insufficient TH levels in the circulation. Whereas constipation usually ameliorates after initiating levothyroxine (LT4) treatment in hypothyroid patients, constipation in RTHα patients barely respond to levothyroxine supplementation, which might be caused by weakened smooth muscle contractility of the rectum in the latter patients [50].

To date, no data have been reported on the clinical features of human RTHα intestines. Animal studies observed abnormal morphology of the intestinal epithelium of RTHα mice characterized by shortened villi, increased differentiation in crypt cells, and decreased stem cell proliferation [48]. This is phenotypically different from RTHβ mice, in which the gastrointestinal tract is not affected. This can be explained by the fact that the TRβ1 receptor is restricted to the differentiated epithelial cells of the villi, and no overt function for this protein has been described in the intestine [51].

In summary, intestinal epithelium homeostasis is controlled by T3. This process is regulated by the crosstalk between T3 and TRα1 and the presence of specific TH transporters and TH-activating and inactivating deiodinase enzymes. However, it must be noted that these findings were primarily obtained from studies with genetically modified mice or experimental *in vitro* models using colorectal cell lines. It may therefore be difficult to translate directly to humans, although phenotypes observed in human RTHα patients do support a crucial role for TRα mediated T3 signaling in intestinal function.

Gut Microbiome and Thyroid Hormone Metabolism

The human gut microbiome (the collective genomic content of microorganisms) consists of 10^{13} to 10^{14} bacterial cells (microbiota) and has been identified as an important factor in various processes that impact host health and the occurrence and progression of disease [36,52,53]. The gut microbiota produce several gut-derived microbial metabolites, which act as signaling molecules allowing the gut microbes

to exert their effect within the host. These microbial metabolites derive directly from bacteria or from the transformation (fermentation) of indigestible dietary components by the gut microbiota and include short-chain fatty acids (SCFAs) such as butyrate, acetate, and propionate, trimethylamine N-oxide) and branched-chained amino acids of which valine, isoleucine, and leucine are the most abundant. These metabolites regulate immune responses [54] and maintain (intestinal) homeostasis. Changes in the abundance of these compounds are associated with several metabolic disorders, including metabolic syndrome, obesity, and type 2 diabetes [55–58]. Other essential functions of the gut microbiota consist of the production and secretion of several vitamins (Vit K, folic acid, Vit B2, B3, B5, B6, B7, and B12) and the inducing of gut hormones secretion (leptin, ghrelin, GLP1)[59,60]

It has been known for many years that the gut microbiota affects thyroid hormone metabolism in the gut, which was first demonstrated by experimental rodent studies [13,17,18,61]. Several experimental studies have been performed with different administration routes of radiolabeled ([^{125}I])-T3, -T3S, and -T3G in conventionally raised rats and rats without intestinal bacteria (using fecal suspensions from germ-free as well as from orally decontaminated rats). It was shown that conventionally raised rats administrated [^{125}I]-T3 intravenously excreted less radioactivity with feces and urine compared to decontaminated rats (feces 15.8% vs. 25.1% and urine 17.5% vs. 23.6%)[18], suggesting decreased enterohepatic reabsorption in the latter. The form of T3 and T3 conjugates excreted in the feces also differed between the two groups. Fecal samples of conventionally raised rats contain more T3 than samples from decontaminated rats (52.5% vs. 29.6%, respectively). Moreover, no conjugates of T3 or 3-3'-T2 were detected in feces of conventionally raised rats, whereas only a small proportion of the glucuronidated iodothyronines were hydrolyzed in fecal samples of decontaminated rats, resulting in the excretion of substantial amounts of conjugated T3 (11.5% T3G and 10.9% T3S, respectively) in the feces of decontaminated rats **(Fig 3.)**[18].

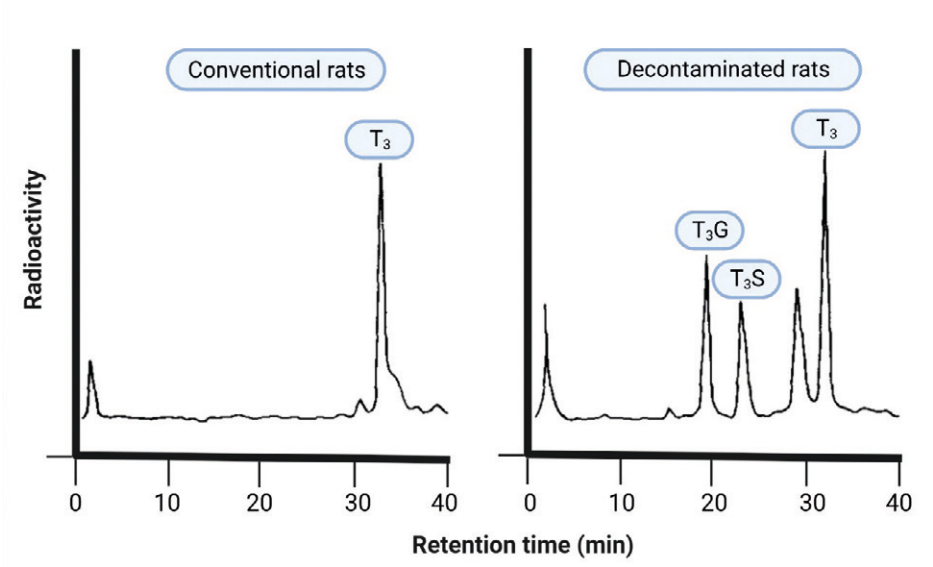

Figure 3. HPLC analysis of radioactivity from feces from conventional and decontaminated rats collected up to 38 hours after intravenous injection of [^{125}I]T_3. Adapted from "*On the enterohepatic cycle of triiodothyronine in rats; importance of the intestinal microflora*" by W.W. de Herder, 1989, *Life Sciences, 45,* p 852. Reprinted with permissio

In line with these findings, HPLC analysis of fecal samples of decontaminated rats receiving intragastric [^{125}I]-T3G showed that radioactivity still existed in the form of T3G, whereas this was not the case in their conventionally raised counterparts [18]. Oral administration of [^{125}I]-T3S or [^{125}I]-T3G led to 3 to 5 times lower resorption of T3 in decontaminated rats compared to CV rats. Incubation of sulfated iodothyronines with feces from decontaminated rats resulted in no hydrolyzation of any of the iodothyronine sulfates [17]. In contrast, complete hydrolyzation of the various sulfate conjugates and T3G was observed when using fecal samples from conventionally raised rats, indicating that (anaerobic) gut microbiota possess sulfatase activity [17].

In conclusion, these studies demonstrate that hydrolysis of conjugated iodothyronines in the intestine is prevented by disturbing the gut microbiota composition, probably due to the lack of bacterial enzymes with deconjugation activity, β-glucuronidase and sulfatase. Of note, although β-glucuronidase-producing bacteria have been isolated from human feces[61,62], most of these mechanistic findings are derived from animal models or *in-vitro* experiments and warrant validation in controlled human clinical trials. It remains unknown whether disruption of the gut microbiota composition, known as dysbiosis, affects the metabolic pathway of conjugated iodothyronines in humans.

Gut Microbiome and Autoimmune Thyroid Disease

A potential effect of the gut microbiota composition on the thyroid gland was first hypothesized in the 1970s and was investigated using experimental rodent studies. The thyroid glands of rats exposed to antibiotics showed a significantly decreased uptake of radioactive iodine, indicating reduced thyroid functioning [63]. Studies with germ-free mice (lacking gut microbiota) revealed an increased TSH secretion of 25% compared to their conventionally raised counterparts [64]. Interestingly, specific pathogen-free (SPF) rats were less susceptible to autoimmune thyroiditis than conventionally raised rats, suggesting that some gut microbiota might constitute a protective effect on the thyroid gland [65].

Further evidence to support this metabolic symbiosis between host and gut microbiota has only been demonstrated over the past decade. As most gut microbiota taxa are mainly obligate or facultative anaerobes, traditional culture techniques could not identify specific microbial species [66]. With the development of recent novel culture techniques combined with microbial gene sequencing (16s rRNA gene analysis, whole-genome shotgun sequencing, and metagenomics), a renewed interest has emerged in the human-microbiota interaction in health and disease [36,67]. As such, various studies have recently been published on the association between gut microbiota composition and autoimmune thyroid disease (AITD), such as Graves's disease, Graves' orbitopathy, and Hashimoto's thyroiditis (**Table 1** and **Table 2**) and have been recently reviewed by Virili and colleagues [68]. By comparing AITD patients' fecal samples to healthy controls, these studies have shown significant differences in the diversity and composition of microbial strains between these two groups. Several of these studies [69–72] reported significant correlations between the relative abundance of the gut microbiota and diagnostic parameters of thyroid status (serum levels of TSH and FT4) and thyroid antibodies (TPOAb, TgAb, and TRAb), indicating the potential clinical significance of dysbiosis in these patients: The genera *Bacteroides, Dorea, Faecalibacterium,* and *Coprococcus* showed a significant inverse association with TPOAb or TRAb, whereas *Blautia, Lactobacillus, Alistipes, Ruminococcaceae*, and *Enterobacteriaceae* were positively correlated with the presence of TPOAb, which may be due to molecular mimicry from autoepitopes from these bacterial strains drive a T and B cell-mediated autoimmune reaction against thyroid gland tissue [73].

The productive function of the intestinal microbiota in the development of thyroid disease has also been studied [70,74–77] using a random forest analysis to find discriminative microorganisms that could distinguish between patients and the control group. As such, hyperthyroid patients (AUC-values are ranging between 0.76 and 0.98, **Table 2**) as well as hypothyroid patients (AUC-value of 0.92 [74], **Table 1**), could be identified with high accuracy based on their microbial composition. The identified microbiota **(Table 1 and Table 2)** are of particular interest, as these might be useful for finding new biomarkers.

Table 1. Characteristics of studies that investigated the association between gut microbiota and Hashimoto's thyroiditis disease.

Author	Date/Location	N of fecal samples per category		Use of LT4?	Key Findings *abundance is disease vs healthy control
		Hashimoto's thyroiditis	Healthy controls		
Ishaq [84]	2017 / China	29	12	No	• Discordant results of richness and diversity indices • ↑ *Bacteroides, Escherchia-Sighella* and *Parasutterella* • ↓ *Bifidobacterium, Lactobacillus, Prevotella_9* and *Dialister*
Zhao [85]	2018 / China	28 (explorative cohort) 22 (validation cohort)	16 (explorative cohort) 11 (validation cohort)	Yes	• Bacterial richness and diversity were not significantly different (p = 0.11) • ↑ *Blautia, Roseburia, Ruminococcus_torques, Romboutsia, Dorea, Fusicatenibacter,* and *Eubacterium Halli* ↓ *Faecalibacterium, Bacteroides, Prevotella_9,* and *Lachnoclostridium*
Cornejo-Pareja [71]	2020 / Spain	9	11	Yes	• Significantly increased bacterial richness • ↓ *Faecalibacterium* • ↑ *Victivallaceae* • *Alistipes, Faecalibacterium, Ruminococcaceae unclasificated,* and *Enterobacteriaceae* were significantly correlated with TPOAb.
Su [74]	2020 / China	52	40	No	• Significantly increased bacterial richness • Significantly decreased bacterial diversity and F/B ratio • ↑ *Phascolarctobacterium, Ruminococcus, Neisseria*, and *Streptococcus* • ↓ *Sutterella, Prevotella, Lactobacillus, Veillonella, Dorea, Butyricimonas, Roseburia, Lachnospira, Fusobacteriu Clostridium, Bacteroides, Paraprevotella* • Random forest analysis showed that *Veillonella, Paraprevotella, Neisseria,* and *Rheinheimera* could distinguish between untreated HT and healthy controls [AUC 0.92]
Liu [86]	2020 / China	45 (euthyroid) 18 (hypothyroid)	34	Mixed	• Significantly decreased bacterial richness and diversity • ↑ *Phascolarctobacterium* • ↓ *Lachnospiracaeae_incertae_sedis, Lactonifactor, Alistipes,* and *Subdoligranulum*

Table 1. Continued

Author	Date/Location	N of fecal samples per category		Use of LT4?	Key Findings *abundance is disease vs healthy control
		Hashimoto's thyroiditis	Healthy controls		
Cayres [88]	2021 / Brazil	40	53	Mixed	• Bacterial richness and diversity are not reported • ↑ *Bacteroides* • ↓ *Bifidobacterium* • Difference in composition between patients with and without use of LT4: ↑ *Lactobacillus* without LT4
El-Zawawy [87]	2021 / Egypt	7	30	Mixed	• Bacterial richness and diversity were not significantly different • ↑ *Bacteroidetes, Prevotella* • ↓ *Firmicutes,* F/B ratio • *Bacteroidetes, F Prausnitzii, Firmicutes,* and *Prevotella* were significantly correlated to TPOAb
Gong [69]	2021 Meta-Analysis	73 Including [71,84,85,87]	80	Mixed	• Significantly decreased bacterial richness: SMD 0.68 [0.16 – 1.20] • Overall conclusion indicates an association between AITD and dysbiosis at family, genus, and species levels.

LT4, levothyroxine; AITD, autoimmune thyroid disease; AUC, area under the curve; SMD, standardized mean differences;↑, increased; ↓, decreased.

The above mentioned studies suggest the presence of a so-called gut-thyroid axis, representing a bidirectional signaling axis that regulates thyroid homeostasis [78,79]. However, the cross-talk pathways remain to be unraveled as these studies have yielded inconsistent and conflicting results in terms of diversity and specific microbiota identified. For example, one study [80] found a significantly higher diversity in GD patients, whereas others [81–83] showed a reduced diversity among these patients. Similar conflicting results have also been found in studies with hypothyroid patients [71,74,84–87]. This might be due to the fact that the studies have been conducted with relatively small heterogeneous patient groups (treated vs. untreated thyroid patients0 (**Tables 1 and 2**) with the inclusion of anti-TPO-negative patients [74,86], different ethnicities [88] and using different techniques for microbial assessment (RT-PCR, high throughput sequencing of 16s rRNA and/or PCR-DGGE). Moreover, most of these studies lacked a functional assessment of the gut microbiota and did not report on the participants' dietary intake. Lastly, these studies were mainly conducted in China **(Tables 1 and 2**), which might impact the generalizability of the results to other regions of the world, as ethnicity and geography are significant factors in the variation of the gut microbiota composition [89,90].

Altogether, these data show an association between gut microbiota composition and (autoimmune) thyroid diseases but do not imply causality. Proving a causal relationship between dysbiosis and disease onset remains challenging, as the composition of the gut microbiota is influenced by many factors, including host delivery mode (vaginal delivery vs. C-section), use of medication (especially antibiotics), lifestyle and behavioral characteristics, genetics, and nutrition [67].

Table 2. Characteristics of studies that investigated the association between gut microbiota and Graves' disease and Graves' orbitopathy

Author	Date/Location	N of fecal samples per category			Use of antithyroid medication?	Key Findings *abundance is disease vs healthy control
		GD	GO	Healthy controls		
Zhou [80]	2014 / China, Dadlian	14	-	7	Not reported	• Significantly increased bacterial diversity • ↑ *Enterococcus, Clostridium* • ↓ *Bifidobacterium, Lactobacillus*
Ishaq [83]	2018 / Shaanxi	27		11	Untreated	• Significantly decreased bacterial richness and diversity • ↑ *Prevotella_9, Haemophilus,* and *H. Parainfluenza* • ↓ *Alistipes, Faecalibacterium, Bifidobacterium* and *Lactobacillus*
Yang [81]	2019 / China, South-West	15		15	Untreated	• Bacterial richness and diversity not significantly different • ↑ *Oribacterium, Mogibacterium, Lactobacillus,* and *Aggregatibacter* and F/B ratio
Shi [93]	2019 / China, Bejing	-	33	32	Thyrozol	• Significantly decreased bacterial diversity, bacterial richness was not significantly different • ↑ *Bacteroidetes* (phylum), *Prevotellaceae* (genus), *Prevotella_copri* (species) • ↓ *Firmicutes* (phylum), *Blautia, Fusicatenibacter, Butyricoccus, Anaerostipes* and *Collinsella* (genus), *acteroides_massiliensis, Ruminococcus, Alistpes_shahii, Eubacterium_hallii, Eubacterium_ventriosum,* and *Marseillibacter_massiliensis* (species) • *Succinivibrionaceae* and *Subdoligranulum* were positively correlated to TRAb • *Parabacteroidetes_distanosis* was inversely correlated to TRAb
Yan [82]	2020 / China, Bejing	39	-	17	Untreated	• Significantly decreased bacterial diversity • ↑ *Bacilli, Lactobacillales, Prevotella, Megamonas*, and *Veillonella* • ↓ *Ruminococcus, Rikenellaceae,* and *Alistipes*

Table 2. Continued

Author	Date/Location	N of fecal samples per category			Use of antithyroid medication?	Key Findings *abundance is disease vs healthy control
		GD	GO	Healthy controls		
Su [75]	2020 / China, Shandong	58	-	63	Untreated	• Significantly decreased bacterial diversity and richness • ↑ *Bacteroidetes* (phylum) and 7 bacterial genera • ↓ *Firmicutes* (phylum) and 33 bacterial genera, F/Br ratio • Random forest analysis showed that *Bacteroides, Alistipes,* and *Prevotella* could distinguish best between GD patients and healthy controls [AUC 0.85] • Significantly increased serum LPS levels in GD patients ($p < 0.0001$) • Transplanting gut microbiota of GD patients increased GD incidence in SPF BALB/c mice
Chen [72]	2021 / China, Jiangsu	15	-	14	Methimazole	• Significantly decreased bacterial richness and diversity • Before thyroid treatment: o ↑ *Lactobacillus, Veillonella,* and *Streptococcus* • After 3-5 months of methimazole treatment: o ↑ *Phascolarctobacterium* o ↓ *Blautia, Corynebacter, Ruminococcus,* and *Streptococcus* • *Lactobacillus* and *Ruminoccocus* were significantly correlated with TRAb (indicating novel biomarkers) • *Synergistetes* and *Phascolarctobacterium* were significantly inversed correlated with TRAb (protecting the thyroid?)
Cornejo-Pareja [71]	2020 / Spain	9	-	11	Neotomizol	• Significantly decreased bacterial evenness • ↑ *Fusobacteriaceae, Fusobacterium* and *Sutterella* • ↓ *Faecalibacterium* • *Lactobacillaceae, Lactobacillus, Faecalibacterium,* and *Pasteurellaceae* were significantly correlated with TRAb (TSIAb)
Shi [76]	2021 / China, Bejing	30	33	32	Thyrozol	• Significantly decreased bacterial diversity (GD and GO vs healthy) but not for bacterial richness • Significant differences in multiple taxa at phylum, genus and species level between control vs GO, control vs GD, and GD vs GO. • Random forest analysis showed that *Deinococcus-Thermus, Cyanobacteria* and *Chlorofexi* could best distinguish between GD vs GO vs healthy [AUC 0.77] and GD vs GO [AUC 0.82]

Table 2. Continued

Author	Date/Location	N of fecal samples per category			Use of antithyroid medication?	Key Findings *abundance is disease vs healthy control
		GD	GO	Healthy controls		
Zhu [77]	2021/ China, Haikout	36 (mild) 64 (severe)		62	Not reported	• Significantly decrease in bacterial richness and diversity of severe GD versus mild GD and controls (p <0.001)* • ↑ *Eggerthella lenta, Streptococcus parasanguinis, Veillonella parvula, Fusobacterium mortiferum,* and *Streptococcus salivarius** • ↓ *Faecalibacterium prausnitzii, Butyricimonas faecalis, Bifidobacterium adolescentis,* and *Akkermansia muciniphilia** • Random forest analysis showed that microbial species could distinguish between severe GD [AUC 0.98], mild GD [AUC 0.78], and all three combined [AUC 0.88]
Jiang [70]	2021 / China, Shangia	45	-	59	Untreated	• Significantly decreased bacterial richness • ↑ *Bacteroides* and *Lactobacillus* • ↓ *Blautia, Eubacterium_hallii, Anaerostipes, Collinsella, Dorea, Peptostreptococaceae,* and *Ruminococcus_torques* • Random forest analysis showed that these nine species could distinguish between GD and controls [AUC 0.81]
El-Zawawy[87]	2021 / Egypt	13 (incl GO)		30	Mixed	• Bacterial richness and diversity not significantly different • F/B ratio was significantly decreased • ↑ *Bacteroidetes, Prevotella* • ↓ *Firmicutes* • *Bacteroidetes* and *Firmicutes* were significantly correlated to TRAb
Gong [69]	2021 Meta-Analysis	123 Including [70–72,80,83,87]		132	Mixed	• Significantly decreased bacterial richness: SMD -0.87 [-1.46 – -0.28] • Overall conclusion indicates an association between AITD and dysbiosis at family, genus, and species level.

GD; Graves' disease; GO, Graves' orbitopathy; TRAb, thyrotropic receptor autoantibodies, AITD, autoimmune thyroid disease; AUC, area under the curve; SMD, standardized mean differences;↑, increased; ↓, decreased.

Fecal Microbiota Transplants in Autoimmune Thyroid Disease

Humanized gnotobiotic mouse models are needed to unravel the underlying molecular mechanism and to better dissect the impact of healthy or dysbiotic microbiota on the progression or onset of AITD. Recent studies have investigated the effect of mice treated with fecal microbiota transplants (FMTs) with feces from healthy human individuals compared to FMTs from AITD patients (HT [74], GD [75], and GO [91], respectively). FMTs from AITD patients led to an increase in disease incidence and severity [74,75,91], concomitant with an increase in serum LPS level [74,75] and intestinal permeability [74] and a decrease in fecal SCFA concentrations [74,75]. Interestingly, mice treated with vancomycin showed a reduction in GO and GD incidence and severity accompanied by a lower microbiota diversity, whereas mice treated with FMT from GO patients inherited their GO donor's microbiota, leading to an increase in GO incidence [91]. This demonstrates a significant variation in gut microbiota composition in these murine models, correlating with GD heterogeneity [91].

However, various concerns should be addressed when translating gut microbiota research results from experimental murine models into humans, as there are notable differences in anatomy, genetics, and physiology [90,92]. Therefore, future research should include prospective studies assessing gut microbiota composition and functionality together with thyroid function, as well as randomized clinical trials that determine the effects of altering the gut microbiota composition on disease progression in AITD patients.

CONCLUSION

This review provides a comprehensive insight into the interplay between thyroid hormone metabolism and gut homeostasis, the so-called gut-thyroid hormone axis. The gut microbiota has been identified as an essential factor in health and disease, depending on its compositional and functional profile. It produces several gut-derived microbial metabolites, which act as signaling molecules allowing the gut microbes to exert their effect within the host.

Thyroid hormone is effectively metabolized via sulfation and glucuronidation (conjugation) of T3 and T4. Glucuronidated iodothyronines (T3G and T4G) are rapidly eliminated via biliary excretion into the intestine. Once excreted in the intestine, T3G and T4G can be hydrolyzed back to T3 and T4 by the gut microbiota, which can be reabsorbed into the enterohepatic circulation. This suggests that T3G and T4G serve as an intestinal thyroid hormone reservoir.

TH is involved in several processes in the gut. The homeostasis of the intestinal epithelium is controlled by T3 through its interactions with TRα1, the dominant TR isoform in the intestine. This homeostasis depends on tight regulation of local T3

concentrations, regulated by specific TH transporters and deiodination enzymes in the intestine. Patients and experimental murine models with a dominant-negative mutation in the TRα exhibit gross abnormalities in the morphology of the intestinal epithelium and suffer from severe symptoms of a dysfunctional gastrointestinal tract supporting the crucial role of TRα-mediated T3 signaling in intestinal function.

Disruption of gut microbial homeostasis (dysbiosis) is associated with autoimmune thyroid disease. However, a causal role of dysbiosis in autoimmune thyroid disease is yet to be established. Recent studies using experimental murine models suggest that fecal microbiota transplants can be a promising tool to treat AITD patients in the future. However, more research is needed to better understand the effects of altered gut microbiota composition on disease progression in AITD patients.

Author Contributions

Aline Fenneman and Anita Boelen researched data for the article, made substantial contributions to the discussion of the content, wrote the article, and reviewed/edited the manuscript before submission. Anne van der Spek made substantial contributions to the content discussion and edited the manuscript before submission. Max Nieuwdorp and Eveline Bruinstroop reviewed the manuscript before submission.

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Competing Financial Interests Statement

Max Nieuwdorp is on the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands. None of these are directly relevant to the current paper. There are no patents, products in development, or marketed products to declare. The other authors declare no competing financial interests.

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3

LEVOTHYROXINE USE AND THE RISK OF COLORECTAL CANCER: A LARGE POPULATION–BASED CASE–CONTROL STUDY

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ABSTRACT

Objective
Whether an association between oral levothyroxine use, leading to supraphysiological exposure of the colon to thyroid hormones, and risk of colorectal cancer exists in humans is unclear. We therefore aimed to assess whether the use of levothyroxine is associated with a reduced risk of colorectal cancer in a linked cohort of pharmacy and cancer data.

Design
Population-based matched case-control study.

Methods
A total of 28,121 patients diagnosed with colorectal cancer between 1998-2014 were matched to 106,086 controls. Multivariable logistic regression was used to estimate the association between levothyroxine use and occurrence of colorectal cancer, adjusted for potential confounders. Results were stratified by gender, age, tumour subtype and staging as well as treatment duration and dosing.

Results
A total of 1,066 colorectal cancer patients (4%) and 4,024 (4%) controls had used levothyroxine at any point before index date (adjusted odds ratio 0.95 [0.88-1.01]). Long-term use of levothyroxine was seen in 323 (30%) colorectal cancer patients and 1,111 (28%) controls (adjusted odds ratio 1.00 [0.88-1.13]). Stratification by tumour subsite showed a borderline significant risk reduction of rectal cancer, while this was not seen for proximal colon cancer or distal colon cancer. There was no relationship with treatment duration or with levothyroxine dose.

Conclusions
In this study, no reduced risk of colorectal cancer was seen in levothyroxine users. When stratifying by tumour subsite, a borderline significant risk reduction of rectal cancer was found and may warrant further research.

INTRODUCTION

Primary hypothyroidism is a common condition with a rapidly rising global prevalence. In the Netherlands, the prevalence of overt hypothyroidism has increased from 0.4 to 2.9% over the past 15 years[1] (www.nivel. nl/nl/nivel-zorgregistraties-eerste-lijn/jaarcijfers-aandoeningen-incidenties-en-prevalenties). Treatment of primary hypothyroidism consists of hormone substitution therapy with daily oral administration of the synthetic thyroid hormone levothyroxine. The majority of levothyroxine is absorbed in the upper gastrointestinal tract, ranging from 40 to 80%[2]. Consequently, 20 to 60% of this synthetic drug is excreted in the stool, resulting in a supraphysiological exposure of the colonic epithelium to thyroid hormone.

Thyroid hormones (TH) play an important role in cellular growth, proliferation, and differentiation[3]. Alterations in TH bioavailability have been implicated in the development of several forms of cancer, including basal cell carcinomas[4], lung cancer[5,6], and colorectal cancer (CRC)[7]. CRC is one of the world's most common cancers, with an estimated global number of 1.8 million diagnoses each year[8]. Interestingly, it has been shown that in CRC, intracellular TH concentrations are reduced due to upregulation of the TH-inactivating enzyme deiodinase type III (Dio3)[7], whereas increased intracellular levels of TH led to a potent reduction in the growth rate of human colorectal tumour cell lines[9]. This raises the question whether levothyroxine users are protected against CRC development due to supraphysiological intestinal concentrations of TH.

Previous studies have explored the association between the use of levothyroxine and the risk of CRC but have shown conflicting results. Some studies found evidence for a protective effect of levothyroxine, while others found no association[10-13]. Furthermore, some of these studies are limited by including a relatively small number of patients, relying on self-reported information, and lacking details on the dose and duration of levothyroxine use as well as tumour location and staging.

Therefore, in this study, we investigated whether the use of levothyroxine is associated with a reduced risk of CRC and whether such effect is associated with dose and duration of levothyroxine use. In addition, differences in tumour location were assessed as well since clinical features of CRC may vary depending on the anatomical site.

MATERIALS AND METHODS

Data sources

Data from the Netherlands Cancer Registry (NCR) were linked on a patient-level to the Out-patient Pharmacy Database of the PHARMO Database Network.

The NCR is a population-based registry that is maintained by the Comprehensive Cancer Centre the Netherlands and comprises information on newly diagnosed cancer patients in the Netherlands. The NCR is notified of new patients with cancer by pathology departments, general hospitals, and radiotherapy institutes. On a daily basis, trained data managers register data from hospital records within all Dutch hospitals using the NCR's registration and coding manual.

The Out-patient Pharmacy Database of the PHARMO Database Network comprises general practitioner (GP) or specialist prescribed healthcare products dispensed by the outpatient pharmacy. The dispensation records include information on the type of product, date, strength, dosage regimen, quantity, route of administration, prescriber specialty, and costs. Drug dispensations are coded according to the WHO Anatomical Therapeutic Chemical (ATC) Classification System (https://www.whocc.no/atc_ ddd_index/).

The cohort resulting from this linkage covers a catchment area of the PHARMO Database Network representing approximately 4.2 million residents (~25% of the Dutch population) that all have complete information available on GP or specialist prescribed healthcare products dispensed by the outpatient pharmacy. Detailed information on the methodology of the used record linkage method can be found elsewhere[14-16].

Study population

All subjects who were diagnosed in the period between 1998 and 2014 with primary CRC (ICD 10-CM code C18-C20) were identified. The first date of CRC diagnosis was defined as the index date. To reduce the likelihood of including patients with heritable CRC syndromes, patients younger than 40 years of age at diagnosis were excluded.

Each CRC case was randomly matched to between one and four controls based on sex, birth year (with a variation of 2 years), zip code, and start year of enrolment in the Out-patient Pharmacy Database (to ensure equal time windows to measure exposure). Matched controls received the same index date as their matched CRC case. Cases and controls were not allowed to have a diagnosis of cancer before the index date. Furthermore, controls had to be alive and known in the Out-patient Pharmacy Database at index date and could not be matched more than once.

Exposure definition

Of all CRC cases and their matched non-cancer controls, all levothyroxine dispensations (ATC code H03AA01) prior to the index date were extracted from the Out-patient Pharmacy Database. Use of levothyroxine was defined as having at least one dispensation of levothyroxine at any time point prior to the index date. Treatment episodes of uninterrupted levothyroxine were constructed based on quantity dispensed and prescribing information to assess the cumulative days of exposure. In case of an interruption between two dispensations, use of levothyroxine was considered uninterrupted if the duration of this gap was less than half the period of the given dispensation, according to the method of Catalan and LeLorier[17]. Timing of initiation was defined as the first dispensation of levothyroxine prior to the index date.

Furthermore, the total cumulative dose of levothyroxine was calculated as the sum of all dispensed doses during the levothyroxine episodes prior to the index date and expressed in milligram.

Statistical methods

Characteristics of CRC cases and their matched non-cancer controls were reported descriptively. Differences in characteristics were assessed using chi-square tests for categorical variables and ANOVA tests for continuous variables.

Multivariable logistic regression was used to estimate the odds ratio (OR) and two-sided 95% CI for CRC with the use of levothyroxine, adjusted for drugs that potentially decrease the risk of CRC including use of aspirin, nonsteroidal anti-inflammatory drugs (NSAIDs), statins, antidiabetics (both oral antidiabetics and insulin), hormone replacement therapy, and oral contraceptives. Non-users of levothyroxine served as the reference group for all analyses. Analyses were conducted stratified by timing of initiation, cumulative dose, and cumulative duration of use. Furthermore, the association between CRC and use of levothyroxine was examined stratified by age, gender, tumour stage, and tumour subsite. To enable stratification by tumour stage and subsite, all non-cancer controls were provided with artificial tumour information similar to their matched CRC case. Proximal colon cancers included malignant neoplasms of the cecum, appendix, ascending colon, hepatic flexure, and transverse colon. Distal colon cancers included malignant neoplasms of the splenic flexure, descending colon, and sigmoid colon. Rectal cancer included malignant neoplasm of the rectum.

All data were analysed using SAS programs organized within SAS Enterprise Guide version 7.1 (SAS Institute Inc., Cary, NC, USA) and conducted under Windows using SAS version 9.4.

RESULTS

A total of 28,121 patients with CRC could be matched to 106,086 non-cancer controls. Baseline characteristics of study participants are provided in **Table 1**. As a result of matching on birth year with a variation of 2 years, patients with CRC were statistically significantly younger compared to non-cancer controls (69.6 years vs 71.1 years, *P* < 0.0001); however, this is not a clinically relevant difference. The mean available observation period prior to the index date was 7.3 years for CRC cases and 7.4 years for non-cancer controls (*P* < 0.01).

Table 1. Characteristics of colorectal cancer cases and matched non-cancer controls

	Colorectal cancer cases	**Non-cancer controls**	***p-value***
	N = 28,121	**N = 106,086**	
Age (years), n (%)			
<75	17,638 (63)	60,942 (57)	*<.0001*
≥75	10,483 (37)	45,144 (43)	*<.0001*
mean (±SD)	69.6 ± 11.5	71.1 ± 11.5	*<.0001*
Gender, male, n (%)	15,892 (57)	59,625 (56)	*0.35*
Available observation time period before index date			
mean (±SD)	7.3 ± 4.1	7.4 ± 4.1	<.01
Tumour stage, n (%)			
I	4,962 (18)	NA	*NA*
II	8,041 (29)	NA	*NA*
III	8,009 (29)	NA	*NA*
IV	5,958 (21)	NA	*NA*
Unknown	1,151 (4)	NA	*NA*
Tumour subtype, n (%)			
Colon	18,754 (67)	NA	*NA*
Proximal	9,304 (33)	NA	*NA*
Distal	8,900 (32)	NA	*NA*
Unspecified	550 (2)	NA	*NA*
Rectum	8,147 (29)	NA	*NA*
Rectosigmoid	1,220 (4)	NA	*NA*
Comedication*, n (%)			
Oral contraceptives	1,200 (4)	3,508 (3)	*<.0001*
Hormone replacement therapy	2,126 (8)	7,531 (7)	*<.01*
Aspirin	4,847 (17)	17,420 (16)	*<.01*

Table 1. Continued

	Colorectal cancer cases	Non-cancer controls	*p-value*
	N = 28,121	N = 106,086	
NSAIDs	15,965 (57)	53,531 (51)	<.0001
Statins	7,945 (28)	27,092 (26)	<.0001
Antidiabetics	3,683 (13)	11,693 (11)	<.0001

SD = standard deviation; NSAID = Non-Steroidal Anti-Inflammatory Drug; NA = Not Applicable; *defined as having a dispensation in the period before index date (i.e., ever use)

At the time of initial diagnosis, the majority of all tumours had grown into the outermost layers of the colon or rectum but had not spread to distant sites (stage II and stage III disease both account for 29% of all tumours). A primary tumour located in the proximal colon was seen in 33% of the CRC patients, 32% had a primary tumour in the distal colon, 29% in the rectum, and 4% in the rectosigmoid junction. Furthermore, the use of medication prior to index date that is potentially associated with a reduced risk of CRC was lower among non-cancer controls compared to CRC cases ($P < 0.001$). **Table 2** provides the overall OR for levothyroxine use and the risk of CRC. All results were adjusted for the use of comedications (including aspirin, NSAIDs, statins, antidiabetics, hormone replacement therapy, and oral contraceptives) that are potentially associated with a reduced risk of CRC. A total of 1066 CRC cases (4%) had used levothyroxine at any point before the index date compared to 4024 (4%) non-cancer controls. A modestly increased risk of CRC was seen among those initiating levothyroxine in the 2 years before the index date, albeit not significant (OR 1.12 (95% CI 0.97–1.290). Among levothyroxine users, 342 CRC cases (32%) and 1338 non-cancer controls (33%) had used levothyroxine for less than 2 years (adjusted OR 0.94 (95% CI 0.83–1.06)). Use of levothyroxine for more than 6 years was seen among 323 (30%) CRC cases compared to 1111 (28%) non-cancer controls, yielding an adjusted OR of 1.00 (95% CI 0.88–1.13) for CRC associated with long-term use of levothyroxine.

Table 2. Use of levothyroxine and the risk of colorectal cancer, overall and by amount, and duration, and intensity of use

	Colorectal cancer cases N = 28,121	Non-cancer controls N = 106,086	OR matched (95% CI)	OR adjusted (95% CI)†
Never use of levothyroxine	27,055 (96)	102,062 (96)		
Use of levothyroxine at any point*	1,066 (4)	4,024 (4)	1.00 (0.93-1.07)	0.95 (0.88-1.01)
Timing of initiation (years)				
0-≤2	249 (23) ‡	830 (21) ‡	1.13 (0.98-1.30)	1.12 (0.97-1.29)
>2-≤4	241 (23) ‡	857 (21) ‡	1.06 (0.92-1.22)	1.02 (0.88-1.18)
>4-≤6	178 (17) ‡	787 (20) ‡	0.85 (0.72-1.00)	0.81 (0.69-0.95)
>6-≤8	155 (15) ‡	614 (15) ‡	0.95 (0.80-1.14)	0.88 (0.74-1.05)
>8	134 (13) ‡	496 (12) ‡	0.98 (0.85-1.13)	0.89 (0.77-1.02)
Cumulative duration (years)				
>0-≤2	342 (32) ‡	1,338 (33) ‡	0.96 (0.86-1.09)	0.94 (0.83-1.06)
>2-≤4	247 (23) ‡	889 (22) ‡	1.05 (0.91-1.21)	1.00 (0.87-1.15)
>4-≤6	154 (14) ‡	686 (17) ‡	0.85 (0.71-1.01)	0.80 (0.67-0.96)
>6	323 (30) ‡	1,111 (28) ‡	1.10 (0.97-1.24)	1.00 (0.88-1.13)
Cumulative dose (mg)				
Unknown	3 (<0.5) ‡	14 (<0.5) ‡	-	-
<100	493 (46) ‡	1,964 (49) ‡	0.95 (0.86-1.05)	0.91 (0.83-1.01)
100-<150	137 (13) ‡	510 (13) ‡	1.01 (0.84-1.22)	0.97 (0.80-1.17)
≥150	433 (41) ‡	1,536 (38) ‡	1.06 (0.96-1.18)	0.98 (0.88-1.09)

OR = odds ratio; †Adjusted for use of statins, antidiabetic, oral contraceptives, hormone replacement therapy, NSAIDs and aspirin (ever use); *defined as at least one dispensation of levothyroxine before index date (i.e., ever use) ‡Percentage relative to the number of levothyroxine users

Analysing the use of levothyroxine by cumulative dose also yielded ORs close to one. No changes in CRC risk were found when stratifying by gender, age, and tumour stage (**Fig. 1**). Stratification based on CRC location revealed a modest borderline significant decreased risk in rectal cancer (OR 0.86 (95% CI 0.75–1.00)) in levothyroxine users, whereas no variations in OR for CRC in proximal and distal colon were observed. Of note, malignant neoplasms of overlapping sites of colon, unspecified sites of colon, and the rectosigmoid junction were not presented in the stratification by tumour subsite due to the low sample size of patients exposed to levothyroxine in these groups. There was a clear difference in the proportion using levothyroxine between men and women. Of all women with CRC, 7% (798 of 12,229) had used levothyroxine,

compared to 2% (268 of 15,892) of all men with CRC. This difference was similar for non-cancer controls: 7% of all women (2999 of 46,461) used levothyroxine compared to 2% (1025 of 59,625) of all men. However, no difference in the risk of CRC was seen between men and women. Finally, limiting the results to long-term users of levothyroxine (use for more than 6 years) did not change the results (data not shown).

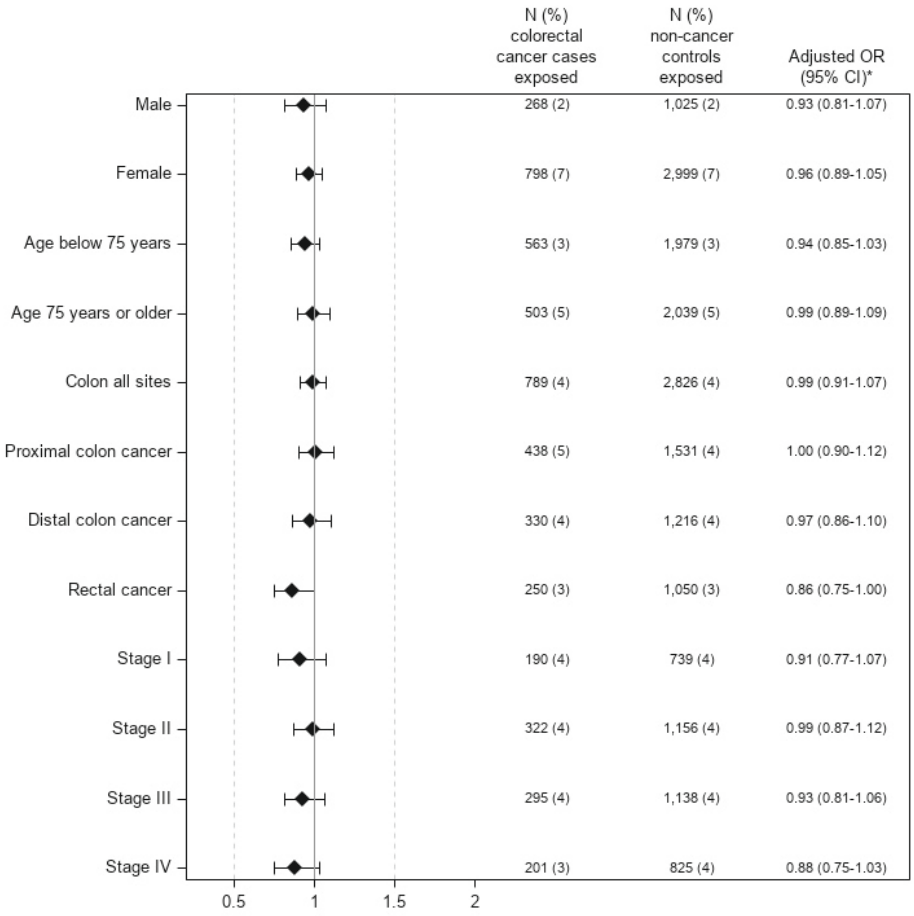

Figure 1. Use of levothyroxine and colorectal cancer risk by patient subgroups

*Adjusted for use of statins, antidiabetic, oral contraceptives, hormone replacement therapy, NSIDs and aspirin (ever use)

DISCUSSION

Our study shows that there is no evidence of an association between the use of levothyroxine and the risk of CRC, even when taking into account treatment duration

or dose– response relationships. These results were consistent between subgroup analyses across different age and sex groups and tumour stages. Likewise, no statistically significant risk variation was seen in the different tumour subsites. The observed differences in levothyroxine use between men and women reflect the well-known gender differences in hypothyroidism, as Hashimoto's thyroiditis is four to eight times more common in women than in men[18]. Contrary to our expectation, CRC patients more often used comedication such as oral contraceptives, hormone replace therapy, aspirin, NSAIDs, statins, and antidiabetics. Those drugs potentially decrease the risk of CRC; however, this might be a reflection of cancer patients having a poorer physical health status, resulting in more comorbidities. Adjustment for these comedications only marginally changed the results.

To date, limited data on the association between levothyroxine use and CRC risk are available and have yielded conflicting results. In contrast to our study, a previous study by Rennert *et al.* found a statistically significant and inverse association between long-term levothyroxine use and CRC for women but not in men[13]. However, this study used self-reported information on levothyroxine use which is subject to recall bias, especially when obtaining information going several years back. Incomplete information on levothyroxine use among CRC patients can create an artificial appearance of drug benefit. In a later study, Friedman *et al.* assessed the association with levothyroxine use for both colon cancer and rectal cancer separately and found a reduction in the risk of rectal cancer but not for colon cancer[11]. In their gender-stratified analysis, this negative association between rectal cancer and levothyroxine use was statistically significant in men but not in women. In line, we also found a modestly decreased risk of rectal cancer among levothyroxine users, although this did not reach statistical significance. Another study by Boursi *et al.* found that long-term TH replacement was associated with a decreased risk of CRC[10]. This protective association became stronger with cumulative duration of levothyroxine supplementation, with the highest protection given by more than 10 years of use. However, the study did not report on cumulative dosage, tumour location, or staging. In this regard, a previous study by L'Heureux *et al.* found that both hypothyroidism and hyperthyroidism were inversely associated with the risk of being diagnosed with CRC within an East Asian population cohort[12]. However, after stratification for tumour location, the statistically significant association for hypothyroidism was only found for rectal cancer (adjusted OR 0.55 (0.40–0.76)) but not for colon cancer (adjusted OR 0.92 (0.74–1.16))[12]. Interestingly, in a subgroup analysis for use of levothyroxine by patients with hypothyroidism, the association between hypothyroidism and a lower risk of CRC was no longer observed (adjusted OR 0.93 (0.66–1.30))[12]. In general, evidence linking CRC onset and levothyroxine intake remains controversial as distinct studies find varying results of the use of levothyroxine and the risk of CRC.

T4 is the prohormone of the biological active triiodothyronine (T3). These pleiotropic THs play an important role in cellular metabolism, differentiation, and growth[3]. The use of levothyroxine, which is a synthetic form of T4, lowers the serum (free) T3:(free)T4 ratio[19]. However, circulating TH concentrations do not necessarily reflect intracellular TH bioavailability. Intracellular TH concentrations are subject to cell-specific regulation by TH transporters and the deiodinase enzymes which can activate or inactivate cellular TH, whereas type I and type II deiodinase (Dio1 and Dio2) initiate TH action by conversion of T4 into T3. Dio3 is the inactivating enzyme and mediates the local attenuation of TH by converting T4 and T3 into the inactive metabolites rT3 and T2[3]. Importantly, Dentice *et al.* disclosed that colon adenomas and carcinomas express elevated Dio3 levels compared to surrounding normal intestinal mucosa and identified Dio3 as a transcriptional target of the Wnt/β-catenin pathway, which is aberrantly hyperactivated in almost all CRCs[7]. Indeed, knockdown of β-catenin reduced Dio3 expression levels and concomitantly induced expression of Dio2, leading to a net increase of intracellular T3. These findings indicate that increased TH may reduce cell growth and enhance cell differentiation in intestinal cells. Furthermore, increased intracellular TH levels, through Dio3 depletion, significantly reduced the tumorigenic potential of colorectal stem cells[9]. However, these results were obtained from rodent models and in experimental *in vitro* models using human and murine cell lines employing silencing of genes important for TH metabolism or CRC cell growth, which cannot be mimicked by extracellular TH supplementation as in the case of levothyroxine therapy[7,20,21]. There is currently no data available on deiodinase activity in colorectal tumour biopsies from human patients. Hence, whether this protective effect also exists in humans is less clear.

Besides the ability of thyroid hormone levels to modify activity of the Wnt/β-catenin catenin pathway, T4 has also been suggested to affect carcinogenesis via an alternate signalling pathway involving the integrin αvβ3. This integrin αvβ3 acts as a cell surface receptor for T4 and to a lesser extent T3. Binding of these hormones has been shown to result in activation of rapid non-genomic signalling pathways including ERK1/2, which has stimulated cancer cell proliferation[22]. This would suggest that high extracellular T4 levels are in fact carcinogenic. However, in our current data set, we find no effect of LT4 treatment on the incidence of colorectal cancer.

Our study has several important strengths. First, this study is a large population-based case–control study with 28,121 CRC cases matched to a large number of non-cancer controls. This large sample size led to a high statistical power and hence allowed us to conduct subgroup analyses on gender and age. Furthermore, by linking this database to the cancer registry, detailed information on characteristics of the colorectal tumour, including stage and subtype, could be obtained. This enabled us to study potential risk variations by tumour subsite as differences exists between the proximal and distal colon in terms of cellular origin and molecular and genetic

characteristics[23]. Lastly, information on levothyroxine use was obtained from the Out-patient Pharmacy Database of the PHARMO Database Network which contains computerized dispensation records with detailed information on dosing and duration. This ensured complete and high-quality assessment of drug use.

Nonetheless, this study also had some limitations. In our study, information on biochemical markers of thyroid function (serum levels of TSH and free T4, specifically) was not available. However, it is unlikely that a large proportion of our study population had untreated overt thyroid disease during the available observation period of 7.3 years. A recent cohort study from the Netherlands showed that only 0.44% of their study population had untreated hypothyroidism, as defined by an increased serum TSH level concomitant with a low serum free T4 level[24]. Although it is known that a substantial proportion of levothyroxine users appear to be undertreated (as shown by serum TSH >4.0 mE/L)[24], it is unlikely that this would skew our current findings. In fact, despite their low serum levels of free T4, undertreated levothyroxine users are still exposing their intestinal epithelium to a supraphysiological level of T4.

In an observational study, there is a potential risk of confounding bias. Although we accounted for comedication, we lacked information on other risk factors, such as smoking history, BMI, family history of cancer diagnosis at a young age, and comorbidities such as diabetes and Crohn's disease, that may influence TH levels or CRC risk. Furthermore, looking at the timing of the initiation of levothyroxine use, a modestly increased risk of CRC was seen among those starting the use of levothyroxine within 2 years before the index date. However, these findings are most likely the result of protopathic bias (e.g. early symptoms of undiagnosed cancer for which levothyroxine is prescribed) or might be explained by surveillance bias, which occurs when patients are followed up more closely than their matched controls. Finally, we have investigated the effect of levothyroxine use on CRC, which by definition also investigated the effect of Hashimoto's thyroiditis on CRC, as the majority of levothyroxine users are diagnosed with this autoimmune disease[18]. To our knowledge, there is no relation between Hashimoto's thyroiditis and CRC. Finally, although being a large population study, only a small proportion (<0.1%) of the patients in the present cohort used liothyronine and could not be analysed. Since liothyronine is just recently reimbursed, we expect the number of liothyronine users to increase in the future. However, liothyronine is almost completely (95%) absorbed in the small intestines, preventing a supraphysiological exposure of T3 to the colonic epithelium[25]. Therefore, it is unlikely that liothyronine usage would affect CRC development.

In summary, we did not find an association between the use of levothyroxine and risk of CRC. However, a borderline significant risk reduction of rectal cancer was found, which is consistent with previous studies and may warrant further research. Additional

evidence from both *in vivo* and prospective studies on the association between TH and CRC are needed in order to assess the implications for clinical practice.

Declaration of interest

Josephina G Kuiper and Ron M C Herings are employees of the PHARMO Institute for Drug Outcomes Research. This independent research institute performs financially supported studies for government and related healthcare authorities and several pharmaceutical companies. The other authors declare that they have no conflict of interest.

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Authorship contribution statement

Josephina G Kuiper and Aline C Fenneman designed the research study, collected the data, analysed the data, contributed to the interpretation of the study results and wrote the paper. Anne H van der Spek, Elena Rampanelli, Max Nieuwdorp, Myrthe P P van Herk-Sukel, Valery E P P Lemmens, Ernst J Kuipers and Eric Fliers designed the research study, contributed to the interpretation of the study results, and critically reviewed and revised the article.

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4

IODINE SUPPLEMENTATION SIGNIFICANTLY IMPACTS MURINE GUT MICROBIOME COMPOSITION

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Under preparation

ABSTRACT

Hashimoto's thyroiditis (HT) is a common autoimmune disease that has been increasing in incidence in recent decades. The gut microbiome plays an important role in host health and disease, and there is a need for experimental models for the investigation of the causal relationship of the gut microbiota in HT pathogenesis. To address this issue, in non-obese diabetic mice that are genetically susceptible to HT (NOD.H-2^{h4}), we examined the effect of sodium iodide (NaI) exposure on the gut microbiome. We found that NaI exposure caused significant alterations in microbial community composition and structure, which persisted over time in a sex-specific manner. These findings indicate that it is essential to note the effects of supraphysiological iodide concentrations on the gut microbiome composition and the need to house males and females separately when studying gut microbiome composition. These findings also suggest that iodine supplementation may have effects on the gut microbiome in other hosts.

INTRODUCTION

Over the past decades, the prevalence of Hashimoto's thyroiditis (HT) has rapidly increased, from 0.3% in 1988-1994[1] to 6.9% in 2007-2012[2] in the United States. HT is characterized by the production of antibodies against thyrocyte-derived autoantigens, such as enzyme thyroid peroxidase (TPO) and thyroglobulin (Tg), infiltration of pathogenic T cells and immunity-mediated destruction of hormone-producing thyroid follicular cells[3–5]. The sequelae of these events eventually culminate in the clinical onset of HT with a drastic decline in serum thyroxine (T4) and triiodothyronine (T3) levels. Current treatment consists of life-long continuous hormone replacement with levothyroxine, which does not affect disease progression. Novel preventative and therapeutic opportunities are greatly needed as 5-15% of the patients treated with levothyroxine experience persistent symptoms, with fatigue most common[6].

Recent evidence indicates that the gut microbiota are an important factor in host health and disease, including susceptibility to autoimmunity and the production of microbial-derived immunomodulatory metabolites[7,8]. The gut microbiome consists of trillions of bacteria, viruses, fungi, and other microorganisms that reside in our intestines. An altered gut microbiome composition with low diversity indices and increased abundance of pathogenic bacterial strains is often referred to as "dysbiosis', which may lead to reduced integrity of intercellular tight junctions in the conic wall. This 'leaky gut' may translocate microbial antigens into the intestinal tissues and subsequent systemic circulation, promoting immune system activation and triggering autoimmunity. This may occur by mimicry of microbial peptides with autoantigens due to sequence similarity, leading to cross-reactivity[9].

The microbiota structure in HT patients may be altered compared to healthy controls[10–16]. Changes in the abundance of specific microbial strains have correlated with the circulating autoantibody levels[12,17]. Antibiotic exposure also was shown to modify the susceptibility of mice to experimental autoimmune thyroiditis[18]. Such findings suggest a link between dysbiosis and HT onset and progression but studies assessing causality are still missing. Mouse models can be used to dissect the underlying molecular mechanisms and to study the impact of a healthy or dysbiotic microbiome on the progression or onset of autoimmune thyroid disease.

The NOD.H-2^{h4} mouse strain is a widely used model to study the spontaneous development of autoimmune thyroiditis[19–21]. Since this process ordinarily requires several months, NOD.H-2^{h4} mice are exposed to excess dietary sodium iodide (NaI) through drinking water to accelerate and amplify the process. After 3-4 weeks of NaI supplementation, thyroid lesions begin to develop. However, the impact of this supraphysiological iodine exposure on gut microbiome composition in NOD.H-2^{h4} mice

is currently unknown. Understanding this relationship is crucial for interpreting future intervention studies in such a mouse model to elucidate the role of gut commensals in autoimmune thyroid diseases. This study is the first to investigate the potential impact of excessive iodine supplementation on gut microbiota composition in NOD.H-2h4 mice and examines potential sex-dependent variations.

MATERIALS AND METHODS

Study Design

Male and female adult (six to seven-week-old) NOD.H-2^{h4} mice were randomized into two groups: control and NaI, separated by sex, and maintained, treated, and studied as described in Methods. In total, 37 mice were included in the study (**Figure 1**):

- Group A: females (N = 7) receiving standard drinking water;
- Group B: males (N = 8) receiving standard drinking water.
- Group C: females (N = 10) receiving NaI-supplemented water;
- Group D: males (N = 12) receiving NaI-supplemented water;

Materials

Reagent type or resource	Designation	Source of reference	Identifiers
Strain (Mus musculus)	NOD.H-2^{h4} mice	Jackson Laboratory (Bar Harbor ME)	#004447[22]
Biological sample	Mouse microbiota samples: fecal, cecal contents, ileal contents	This paper	
Chemicals	Sodium iodide	Sigma-Aldrich	Cat#217638-100G
Commercial assay or kit	DNeasy 96 PowerSoil Pro Kit (384)	Qiagen	Cat#47017
Commercial assay	QIAquick PCR Purification Kit	Qiagen	Cat#28106
Commercial assay	Maxima Hot Start PCR Master Mix	ThermoScientific	Cat#EP0601
Oligonucleotides	Bacterial Universal 16S primer pair 515F-806R	ThermoScientific	N/A
Commercial assay	Quant-iT PicoGreen dsDNA Assay Kit	Invitrogen	Cat#P11496
Commercial assay	QuBit 2.0 Fluorometer	ThermoScientific	Cat#Q32851
Software, algorithm	QIIME2 (pipeline)	doi:10.1186/s40168-018-0470-z	https://qiime2.org
Software, algorithm	SILVA v. 138 reference database	The SILVA rRNA database project	https://arb-silva.de
Software, algorithm	R Statiscal Computing Software	The R Foundation	https://www.r-project.org
Software, algorithm	MaAsLin2 package in R (package v.1.8.0)	The R Foundation	https://huttenhower.sph.harvard.edu/maaslin/

Experimental model and subject details

Animals and iodine treatments

Two week-old NOD.H-2^{h4} mice were purchased from Jackson Laboratory (Bar Harbor, ME)[22] and bred in a Specific Pathogen-Free vivarium at Rutgers University in the School of Public Health facility. Mice were maintained in humidity and temperature-controlled rooms, on a 12-hour light-dark cycle, and fed a standard diet (PicoLab Mouse Diet - Purina 5058). All animal procedures were approved by Rutgers Institutional Animal Care and Use Committee (IACUC protocol no. 201900017). At postnatal (P) day 23, the offspring pups were weaned and housed to separate males and females. All mice received reverse osmosis (RO)-treated drinking water supplied by the facility's automated water system. When six to seven weeks old, mice were randomly assigned to either the RO water or supplemented with NaI. Throughout the experiment, all mice were housed in pairs of the same sex (**Figure 1** and **Table 1**). During the first week of the experiment (D0 – D7), all mice received the RO water. After that, mice from groups C and D were given RO water supplemented with 0.05% NaI for the following two weeks (D7 – D21). The water was refreshed once weekly. At D21 – D28, a one-week wash-out period was followed, with all mice receiving the RO water again.

Group	Treatment	N
A Females	0.05% NaI	10
B Males	0.05% NaI	12
C Females	Regular water (reversed osmosis)	7
D Males	Regular water (reversed osmosis)	8

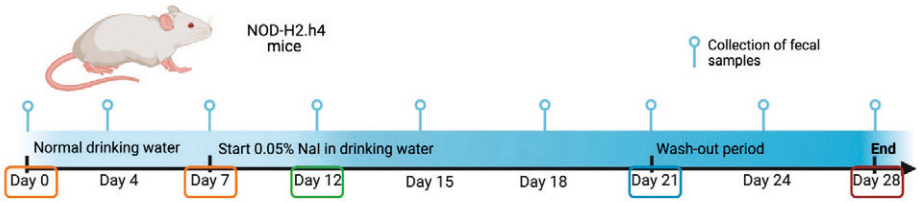


Figure 1. Study design. Male and female adult (six to seven-week-old) NOD.H-2^{h4} mice were randomized into two groups: control and NaI, separated by sex, and maintained, treated, and studied as described in Methods.

Collection of fecal and tissue samples

Fresh fecal pellets were collected from each mouse every three to four days, as described previously[23]. In short, each mouse was placed in an empty, clean beaker for 2-5 minutes to allow them to defecate normally to obtain 3-4 pellets. At the end of study (D28), mice were euthanized by carbon dioxide inhalation, after which ileum, cecal, and colon tissues were collected with their contents. All samples were directly

frozen at -80°C until further processing for DNA extraction and 16S rRNA analysis. The fecal samples that were analyzed for this study were two baseline samples (from D0 and D7) and from five (D12) and 14 days (D21) after starting 0.05% NaI supplementation and after a week of wash-out (D28).

Blood samples were collected from cardiac puncture, and serum samples were prepared and frozen at -80°C for thyroid measurements.

16S rRNA assessments of microbiota and community analysis and quantitation

Microbiome assessment was performed as described[23,24]. Briefly, microbiota DNA was extracted from fecal samples (one pellet) and ileal and cecal samples using the DNeasy 96 PowerSoil Pro Kit (Qiagen, Hilden, Germany). The V4 region of the bacterial 16S rRNA genes was amplified in triplicate reactions using barcoded fusion primers 515F/806R, which amplifies both bacterial and archaeal 16S genes[25,26]. The DNA concentration of the V4 amplicons for each sample was measured using the Quant-iT PicoGreen dsDNA assay kit (Life Technologies, Eugene OR, USA), and samples were pooled in equal quantities. These pools were treated with the Qiaquick PCR purification kit (Qiagen) to remove primers, quantified using the high-sensitivity dsDNA assay kit (Life Technologies) and the Qubit 2.0 Fluorometer (Life Technologies Corporation, Carlsbad CA, USA) and then combined at equal concentrations to constitute the sequencing library. The ~254 bp V4 region was sequenced using the Illumina MiSeq 2 × 150 bp platform (Azenta, South Plainfield NJ, USA) at the Rutgers Center for Advanced Biotechnology and Medicine.

Statistical analyses

Microbiome data were processed and analyzed using Quantitative Insights into Microbial Ecology (QIIME2, version 2022.02, https://qiime2.org/). Sequences were filtered for quality trimmed, de-noised, merged, and then the chimeric sequences were removed using the DADA2 plugin to generate the feature table and aligned using MAFF, as described[24]. Taxonomy was assigned using Silva 138 (released December 2019). All samples were rarefied to 9,180 sequences per sample for Alpha and beta diversity analyses. To assess alpha diversity, the Shannon index and observed features were calculated at the ASV level. Significant differences between experimental groups were determined using the Kruskal-Wallis method. To assess beta diversity analysis, the Jaccard index, Bray-Curtis dissimilarity, and unweighted UniFrac distances (by principal coordinates analyses) were calculated. Beta diversity was tested with Permutational multivariate analysis of variance (PERMANOVA) (permutations = 999). Differential taxa of gut microbiota were analyzed using Microbiome Multivariable Associations with Linear Models in R (MaAsLin2, package v.1.8.0; https://huttenhower.sph.harvard.edu/maaslin/), with default parameters. P-values were corrected for multiple comparisons using the Benjamini-Hochberg (FDR) method.

Significant taxa (FDR-corrected q-value <0.25) were generated heatmaps using the ggplot2, reshape2, and Complex Heatmap packages in R, based on the coefficient value as inferred by the Maaslin2 package.

In the final analysis, 836 different taxa were used, including 109 at family level, 231 at genus level, and 398 were ASVs.

RESULTS

This present study aimed to investigate the effect of NaI supplementation on gut microbiome composition in a mouse strain commonly used as a model for autoimmune thyroiditis. Individual mice were weighed weekly during the entire experiment to determine whether NaI supplementation impacts the body weight of NOD.H-2^{h4} mice. *As expected*, male mice weighed significantly more than female mice. NaI-treated mice showed no apparent weight change during the treatment period compared to their control counterparts (**Figure S1**).

Diversity Analysis

NaI supplementation significantly altered alpha diversity and persisted over time, with sex-specific effects.

To determine the effect of NaI exposure on the intestinal microbiota, 16S rRNA genes were examined. From the 181 fecal samples, a total of 4.714.085 clean sequences were analyzed for a depth of 9,180 sequences per sample as well as for the 37 ileal and 36 cecal samples.

Before the treatment, the female NaI mice had slightly higher species richness (i.e., observed features) than the control mice, but their diversity (as measured by the Shannon index) was similar. After being exposed to NaI-supplemented water, the female mice had a significant increase in fecal alpha diversity compared to their control counterparts. This increase was observed from the first time point after NaI supplementation and persisted until the end of the study (black asterisks, **Figure 2A, C**). In contrast, NaI-treated male mice showed similar gut microbial diversity prior to the intervention but had a significantly lower alpha diversity in fecal samples at the end of the study compared to their control counterparts (black asterisk, **Figure 2B, D**). The alpha diversity of the ileal samples was similar between all male and female groups, whereas cecal samples had lower evenness and Shannon index ($p < 0.05$, respectively) in NaI-treated male mice compared to their controls (**Figure S2**).

Within each treatment group, the impact of NaI supplementation on alpha diversity also was sex-specific. NaI-treated female mice showed a significant increase in alpha diversity over time (purple asterisks, **Figure 2A, C**), while NaI-treated male mice

showed a decrease in alpha diversity after a one-week washout (purple asterisks, **Figure 2B, D**). Notably, a significant increase in observed features was observed at the end of the study compared to baseline samples in both control male and female mice (green asterisk, **Figure 2C, D**).

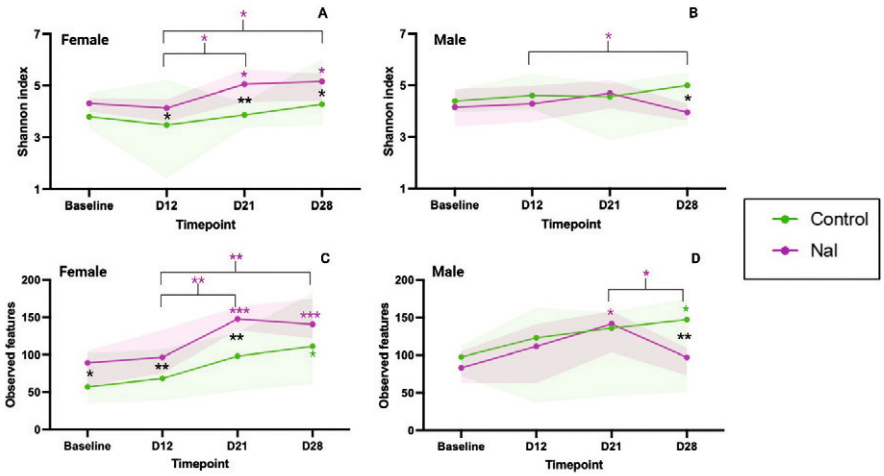


Figure 2. Alpha diversity in fecal samples over time. Results are for males and females separately, as determined by Shannon index (A and B) and Observed features (C and D). Baseline consists of samples from days D0 and D7; D12 is the first sample after NaI treatment was started; D21 is at the end of the NaI treatment; D28 is after a one-week washout period. Sequence depth: 9,180. Significance of the comparison of time points and between treatments was tested by pairwise Kruskal-Wallis test (* p<0.005, ** p<0.01, ***p<0.001). Plots show median values with 95% confidence interval. Asteriks: *, significant difference between the groups at that specific time point; *, significant difference within the control group over time, compared to baseline or to other time points; *; significant difference within the NaI-treated group over time, compared to baseline or to other time points.

The community structure was affected by NaI supplementation, with sex-specific effects. Throughout the study period, there was substantial overlap in community structure (β diversity) between the control and NaI fecal samples across both sexes (**Figure 3**). However, there were notable changes in both intra- and intergroup diversities in fecal samples over time.

After excluding all baseline samples (to emphasize the effect of NaI since all baseline samples were similar), significant differences were observed in the community structure of fecal samples between NaI-treated and control groups using the Jaccard matrix method (p = 0.016) but not using the Bray-Curtis dissimilarity index (males and females combined) (**Figure 3A, B**). Interestingly, a gender disparity was observed

in the community structure, with significant differences in Bray-Curtis and Jaccard indices between female and male NOD.H-2^{h4} mice, irrespective of the treatment group (**Figure 3C, D**).

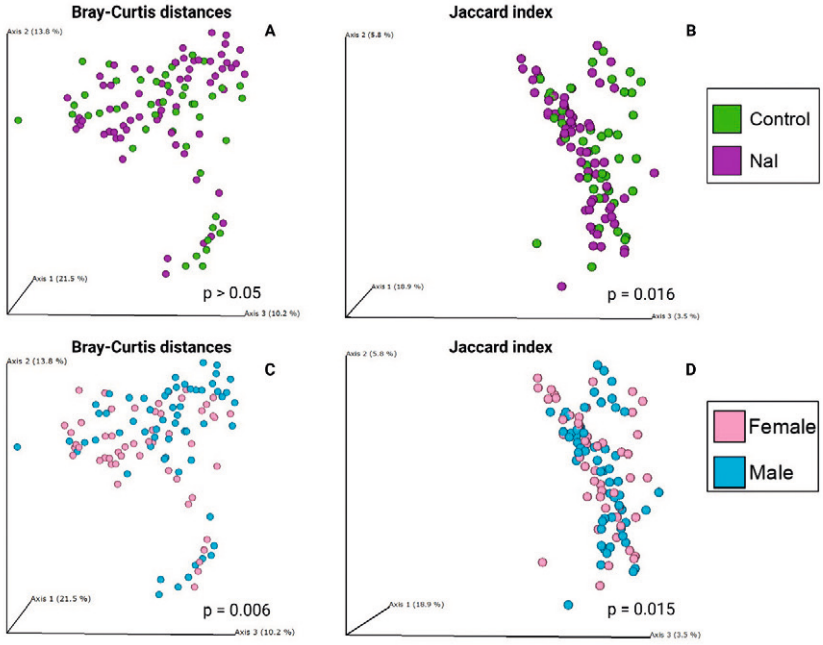
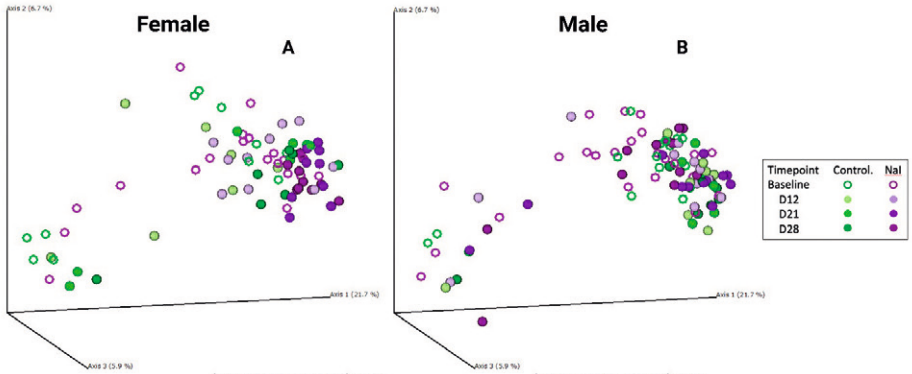


Figure 3. Principal coordinate analysis (PCoA) of Beta diversity of fecal samples from mice treated with NaI or not (control). Panels show Bray-Curtis (A,C) and Jaccard (B,D) analysis of 110 samples (baseline samples were excluded to emphasize the effect of NaI). Top panels: treatment effects; bottom panels: sex. Statistical significance determined with pairwise PERMANOVA. Depth: 9,180.

The community structure of each group's baseline samples was compared to every subsequent time point (**Figure 4**) in intragroup comparisons. Fecal samples from control mice (both females and males) and male mice treated with NaI had consistent and stable community structures throughout the study, while the community structure of female NaI mice changed from baseline (**Figure 4**). Specifically, Beta diversity (unweighted UniFrac) of NaI-treated female mice was significantly decreased after two weeks of NaI supplementation (D21), persisted after a week of wash-out (D28), and differed significantly from their control counterparts at all time points after baseline (intergroup comparison). These results imply that the administration of NaI may have been a selective pressure that favored certain microbial taxa over others. These effects were sex-specific, as no differences in Beta diversity were observed in male mice.



Intragroup comparisons

Group	Control F	Control M	NaI F	NaI M
Base vs D12	>0.05	>0.05	>0.05	>0.05
Base vs D21	>0.05	>0.05	0.001**	>0.05
Base vs D28	>0.05	>0.05	0.002**	>0.05
D12 vs D21	>0.05	>0.05	>0.05	>0.05
D12 vs D28	>0.05	>0.05	0.003**	>0.05
D21 vs D28	>0.05	>0.05	>0.05	>0.05

Intergroup comparisons

	Control vs. NaI	
Timepoint	Female	Male
Baseline	>0.05	>0.05
D12	0.024*	>0.05
D21	0.006**	>0.05
D28	0.029*	>0.05

Figure 4. Principal coordinate analysis (PCoA) of Beta diversity over the experimental course. Unweighted UniFrac analysis involved 181 samples from baseline to sacrifice, and significance determined by pairwise PERMANOVA test, followed by Bonferroni's correction for multiple testing. Depth: 9,180.

The Beta diversity of male mice receiving NaI treatment remained similar to controls, but a significant alteration was observed longitudinally, with fecal samples obtained on D21 and D28 showing a marked difference compared to their respective baseline samples (**Figure 5**)($p<0.05$), similar to NaI females ($p<0.01$) but not for controls. Lastly, significant differences in ileal community structures were observed in NaI females compared to their controls ($p=0.024$), while cecal diversity remained similar (**Figure 6**). No differences were observed in the ileal and cecal samples between NaI-treated and control male mice.

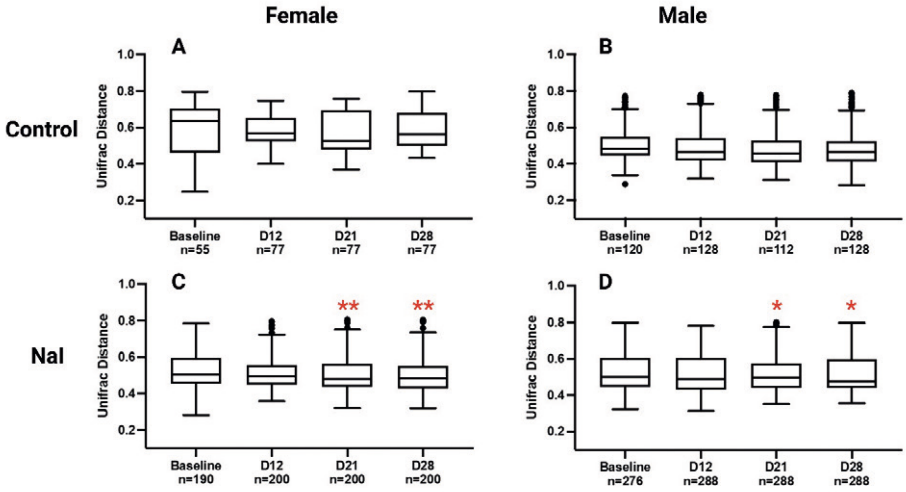
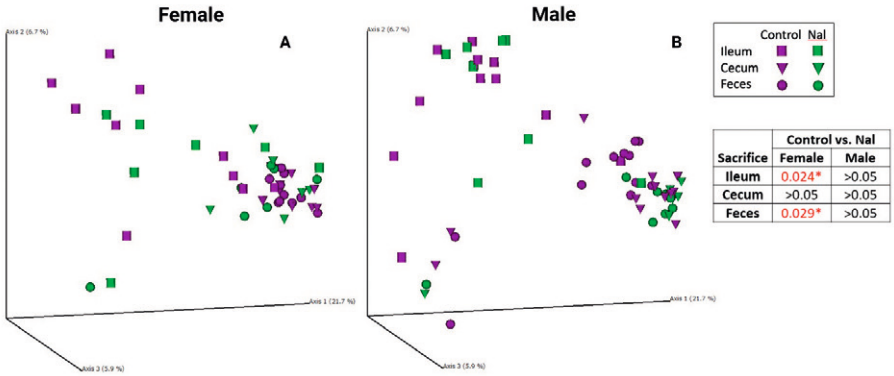


Figure 5. Longitudinal changes of fecal Beta diversity (unweighted UniFrac). Comparisons are between baseline vs. three other time points. Statistical significance determined with pairwise PERMANOVA, followed by Bonferroni's correction for multiple testing (* p<0.005, ** p<0.01). Depth: 9,180.



	Control vs. Nal	
Sacrifice	Female	Male
Ileum	0.024*	>0.05
Cecum	>0.05	>0.05
Feces	0.029*	>0.05

Figure 6. Principal coordinate analysis (PCoA) of Beta diversity of fecal, cecal, and ileal samples from mice treated with NaI or not (control). Unweighted UniFrac analysis involved 37 fecal, 36 cecal, and 37 ileal samples. Left panel: female mice. Right panel: male mice. Statistical significance determined with pairwise PERMANOVA. Depth: 9,180.

Differential taxa of gut microbiota in NaI and control mice

The impact of NaI treatment on bacterial taxa abundance was assessed using the MaAsLin2 tool, revealing sex-specific differences in the NaI-treated mice (**Figure 7**). In NaI males, 35 taxa displayed significant changes over time compared to their baseline samples, while NaI females had significant variations in the relative abundance of 44 taxa. Of these, twelve overlapping taxa were found with shifts in the same direction. These taxa included *Family_XIII_AD3011, Enterorhabdus, Gordonibacter, Lachnospiraceae_UCG.001*, *Lachnospiraceae_UCG.004, Lachnospiraceae_FSC020, Lachnospiraceae_UCG.006, Muribaculum, Parasutterella, Rhodospirillales_uncultured,* and *Ruminococcaceae_uncultered.* These changes were specific to NaI supplementation and were not observed in the control group. Noteworthy differences between the sexes were observed in the abundance of *Eubacterium coprostanoligenes*, *Lachnospiraceae_ASF356*, and *Marvinbryantia*; those taxa were depleted in NaI males but over-represented in NaI females. These changes were specific to NaI supplementation and were not observed in control mice.

The control groups also displayed changes over time in a small number of taxa. In particular, three taxa in control males showed significant alterations, two of which displayed a similar shift as in NaI-treated males. Interestingly, there was an opposite shift in the relative abundance of the *Lactobacillus* genus, with a decrease at D12 in control males (and a decrease at D21 and D28 in NaI females) compared to baseline samples, whereas an increase of *Lactobacillus* was seen in NaI males at D28 compared to baseline. In control females, seven taxa were significantly altered, and six of these showed a similar trend as their NaI-treated counterparts, although with variations in the timing.

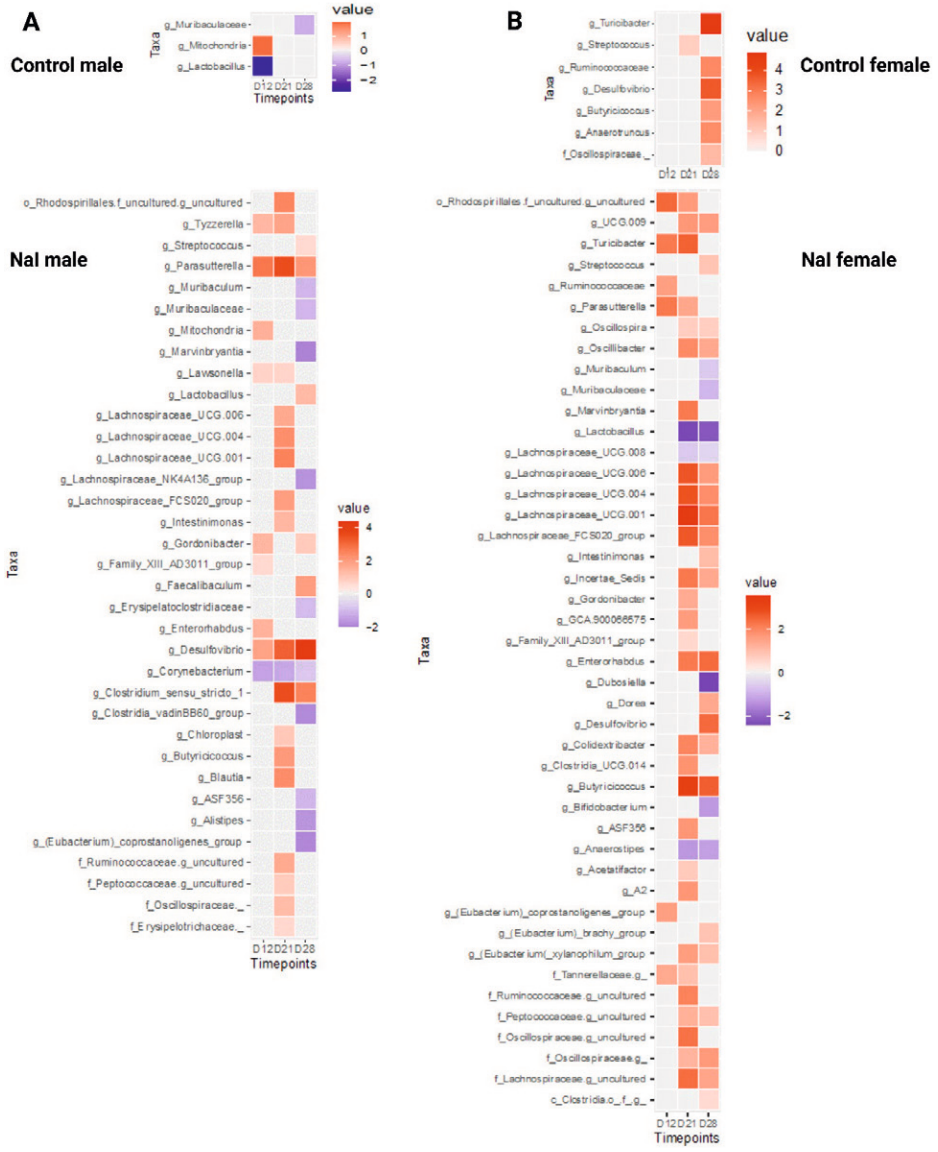


Figure 7. Heatmap representing fecal taxa (at genus level) that significantly differ between baseline vs. three other time points in the control and NaI-treated male (A) and female (B) mice, as assessed by MaAsLin2 analysis. Each row represents a different taxon, and each column is a different timepoint; plot shows taxa differences with q<0.25. Cells that denote significant associations (coefficient values) are colored. Red indicates over-representation, and blue indicates under-representation.

DISCUSSION

This study shows that a short period of continuous exposure to a high dose of sodium iodide (NaI) supplementation via drinking water induced distinct changes in the gut microbiome compared with regular drinking water, as demonstrated by the consistent changes within each group in alpha and beta diversity and the taxonomic profile in NOD.H-2^{h4} mice. These differences were maintained even one week following withdrawal of the exposure. Moreover, we found that NaI supplementation has a greater impact on the microbial community in female mice, while the gut microbiota profile of males responds in a different manner to the same exposure. These findings may have important bearing when this murine model is used to study microbial-immune interactions within the gut-thyroid axis.

NOD.h2h4 mice

Non-obese diabetic (NOD).H-2^{h4} mice are completely protected from developing diabetes due to the expression of differing MHC haplotypes (H-$2K^k$ and I-A^k, respectively) on the NOD genetic background[19,22]. This results in the development of spontaneous autoimmune thyroiditis (SAT) and production of IgG autoantibodies when mice receive 0.05% NaI in drinking water, with an essentially universal incidence after 6-8 weeks post-treatment or by four months of age in both sexes. Without treatment, thyroiditis is delayed and incomplete, with ~60-70% incidence in mice that are 7-10 month-old[22]. Since NOD.H-2^{h4} mice do not develop clinical signs of hypothyroidism (*e.g.,* T4 serum levels remain within the normal range), they are an optimal murine model for investigating new potential (microbiota) therapeutic interventions over long periods[27].

Iodine is essential for the formation of thyroid hormones.

The thyroid gland produces two principal iodine-containing thyroid hormones. Iodine (I) is incorporated into the thyroid gland in the ionized form [iodide (I-)] by active transport via the sodium-iodide symporter (NIS)[28]. Within the thyroid gland, iodide is concentrated, undergoes oxidation by the enzyme thyroid peroxidase, and is incorporated into thyroglobulin to produce monoiodotyrosine (MIT), diiodotyrosine (DIT), which ultimately combine to form the thyroid hormones triiodothyronine (T3) and thyroxine (T4), a process called organification[29–31]. These intrinsic regulatory mechanisms maintain thyroid homeostasis and are able to compensate for acute periods (~24h) of excess iodine intake (known as the Wolff-Chaikoff effect)[32,33]. However, chronic periods of excessive iodine ingestion can lead to either iodine-induced hypo- or hyperthyroidism[34]. The prevalence of thyroid autoantibodies was increased by 150% in the Danish population following the iodine fortification of salt[35]. In clinical practice, supraphysiological levels of iodine are administered to hyperthyroid patients who are undergoing thyroid storm to achieve an acute decrease in the release of thyroid hormones (**Box 1**)[32,36]. Conversely, excessive iodine intake

also can cause hyperthyroidism and thyrotoxicosis in individuals using high iodine-containing drugs, such as amiodarone and potassium-iodine pills used in radiation emergencies[33].

Iodine physiology in the gut.

The absorption of dietary iodide constitutes the first step of iodide (I-) metabolism. The uptake occurs in the small intestine and is mainly facilitated by the expression of NIS proteins on the apical surface of enterocytes. The expression of NIS in the small intestinal enterocytes is regulated by intracellular I- concentrations[37,38]. Rats given 0.05% potassium iodide (KI) in their drinking water showed a significantly reduced NIS-mediated I-uptake of 55% after 24 hours and 83% after 48 hours[38]. These results suggest that the 0.05% NaI supplementation used in our study leads to a supraphysiological colonic concentration of iodide, which might explain the changes we observed in gut microbiota composition. Interestingly, gut microbiota may also play a role in the absorption of iodide, as shown by a study in which rats exposed to the antibiotic kanamycin had a significantly reduced radioiodine uptake compared to untreated rats[18].

Box 1: How excessive is 0.05% NaI supplementation?

- An adult mouse drinks 4 ml of water and consumes 3 to 5 grams of food daily.
- At a concentration of 0.05% NaI, the daily iodine intake for mice is 2000 µg daily via drinking water.
- The estimated iodine requirement for a mouse is 150 µg/kg of diet, equivalent to 0.75 µg daily[57.]
- Thus, the mice in our study have a greater than 3-$\log_{10}$ (2666-fold) increase in daily iodine intake.

- The recommended daily iodine intake for human adults is 150 µg daily, with a tolerable upper intake level of 1,100 µg daily[58].
- At a concentration of 0.05% NaI, the daily iodine intake for humans would be 1 gram per 2L of water intake.
- To provide context, a 200mg tablet of amiodarone contains 75mg of iodine, and approximately 9 mg of iodine is released during the daily metabolism of a 300mg dosage per day[33].
- Supraphysiological administration of iodine can be used to treat hyperthyroid patients experiencing thyroid storm or as a pre-operative measure. This treatment involves using a saturated solution of potassium iodide containing 1,000mg of iodine daily (to block the thyroid hormone release, combined with propylthiouracil to block thyroid hormone synthesis).[32,36]
- In a nuclear accident involving I^{-131}, individuals >12 years old may be recommended to take a single dose of 130mg KI, containing 100mg iodine, which is approximately 333 times higher than the recommended daily iodine intake[59].

The reduced iodide uptake may result from decreased lipopolysaccharide (LPS) binding from Gram-negative bacteria to toll-like receptor 4 (TLR4) present on thyrocytes. LPS enhances TSH-induced iodide uptake and NIS protein expression by activating the NF-kB signaling pathway in thyrocytes, indicating a bacterial interaction that interferes with thyroid homeostasis[39–42].

How does 0.05% NaI supplementation alter the gut microbiota composition?
One explanation for the mechanism by which NaI supplementation affects the microbiota is related to the fact that, like chloride, iodide is a halogen and is known for its antimicrobial effects, such as used in povidone-iodine solutions[43]. Exposure to high doses of iodide may therefore cause gut microbiota toxicity by disrupting the outer bacterial membrane via binding iodide to amino acids Tyr and His and by oxidation of cytoplasmic and nuclear components[44,45]. To date, only few studies have evaluated the effect of iodine on intestinal microbiota composition. Perineal disinfection of the mother by povidone-iodine during vaginal delivery led to a steep decline in alpha diversity and *Lactobacillus* abundance, while this was the dominant bacteria in the non-disinfected control group[46]. In another study, iodine-enriched cows' milk had a lower relative abundance of the genus *Pseudomonas* (73.2% to 32.9%), whereas the genus *Lactococcus* was increased (22.1% to 66.4%) compared to regular cow's milk[47].

A study of non-obese ICR mice administered KIO_3 (18 µg/kg/day) via gavage showed no significant differences in gut microbiota composition compared to saline[48]. However, when administered $NaIO_3$ (18 µg/kg/day) via gavage once daily, both male and female mice had a significant increase in alpha diversity compared to the control[49]. Furthermore, PCoA visualization of weighted UniFrac analysis revealed significant separation between male and female mice, rather than a separation based on treatment group[49], in line with the gender disparity observed in our study.

Our study revealed that even after a one-week washout period, the microbiota of NaI-exposed mice remained distinct from that of the control group, indicating that the effects of NaI supplementation have a more lasting impact on the microbiota. Four microbes from the Lachnospiraceae family (*Lachnospiraceae UCG.004, UCG.006, UCG.001,* and *FCS020*) were enriched in fecal samples of both NaI-treated males and females. *Lachnospiraceae* abundances have differed from one another in prior studies. While one study reported a significant decrease in members of the Lachnospiraceae family in NaI-treated mice[49], other studies have shown that the relative abundance of Lachnospiraceae was significantly higher in patients with autoimmune thyroid disease[16] and in participants with detectable serum thyroid autoantibodies[50].

The relative abundances of *Lactobacillus* were not uniform in our study, as it was enriched after iodine treatment in females and control males but depleted in NaI-treated males. These findings highlight the potential gender-specific effects of NaI supplementation on gut microbiota composition. Interestingly, in a separate study, a probiotic mixture containing *Lactobacillus* and *Bifidobacterium* in hypothyroid patients led to a slight decrease in levothyroxine dose ($p = 0.007$)[51], suggesting that these two taxa may have an impact on thyroid hormone metabolism. This is consistent with our results, showing a significantly reduced *Bifidobacterium* abundance in NaI-treated females at the end of the study but not in NaI males.

Sexual dimorphism in NOD.H-2^{h4} mice.
Our study observed a remarkable gender difference in the gut microbiome of NOD.H-2^{h4} mice, irrespective of the treatment group, with significant differences in both Bray-Curtis and Jaccard indices between females and males. These findings align with the well-established sex difference in the prevalence of HT in humans, with an 8:1 female-to-male ratio[52]. The increased incidence of HT in post-partum and postmenopausal women suggests hormone-dependent regulation of autoimmune hypothyroidism[52,53]. Recent research has shown that such hormonal changes coincide with a shift in microbiota profiles in mice. In a prior study, transferring commensal microbiota from adult males to immature females altered sex hormone levels and protected against type 1 diabetes development in NOD mice predisposed to T1D [53].

This is the first study that directly examines the effect of iodide on gut microbiome composition. Our study has several notable strengths. To identify potential sex-related differences in response to NaI regarding changes in gut microbiome composition, mice were housed in pairs of the same sex. This approach also minimizes the confounding effect of their coprophagic behavior, which can significantly affect the composition of ileal and cecal microbiota[54]. The washout period of one week also enabled us to establish the long-term disruptive effects of iodide on the gut microbiota profile. As the microbial community does not return to the baseline composition after normalization of the environmental conditions (e.g., re-administration of regular drinking water), it suggests the possibility of chronic and potentially detrimental consequences for the host.

Several limitations in this study also should be considered. Although our study found a correlation between high iodide intake and alteration in taxa and metagenomics, it does not infer a causal link between gut microbiota dysbiosis and the onset of HT. Future research could investigate this potential link by performing fecal microbiota transplantations (FMTs) from NaI-treated mice to control mice on a regular water diet and comparing the onset of thyroiditis between FMT-treated and non-FMT-treated control mice, both of which would be consuming normal drinking water. Such research could contribute to a better understanding of the role of the gut microbiota in HT development.

Secondly, it remains challenging to translate our results directly to human (gut) physiology, as the gut microbiome composition from mice is distinct from humans[55]. Some human taxa do not colonize rodents, while others do colonize but are present in very different communities or phenotypes[56]. It would therefore be interesting to analyze fecal samples of patients before and during use of oral amiodarone (consisting of 37% iodine by weight) to provide more insight into the effect of iodide in human gut microbiota due to its rich iodine content. Lastly, by conducting functional profiling of the microbiome through whole-genome sequencing, it may be possible to

gain valuable insights regarding whether there is a selection for strains that exhibit resistance to NaI or, conversely, selection for strains that can effectively use NaI and could therefore either be beneficial or detrimental for host health.

CONCLUSION

In conclusion, our study has shown that the gut microbiome composition is significantly altered in NOD.H-2^{h4} mice after administering 0.05% NaI in drinking water, a method commonly used in this murine model. Secondly, sex-specific differences exist in the gut microbiota profile, independent of the drinking water method used. Further studies would be needed to determine the specific mechanisms driving these observed differences.

The insights gained from our study are essential for designing studies with this specific murine model that aim to provide causal evidence linking the gut microbiome to HT pathogenesis. Specifically, when transplanting fecal microbial communities from individuals with and without autoimmune thyroid disease into the NOD.H-2^{h4} mouse model, it is essential to consider the effects of supraphysiological iodide concentrations on the gut microbiome composition. Moreover, our findings emphasize the importance of housing males and females separately when studying gut microbiome composition in murine models of autoimmunity to control for sex-related differences.

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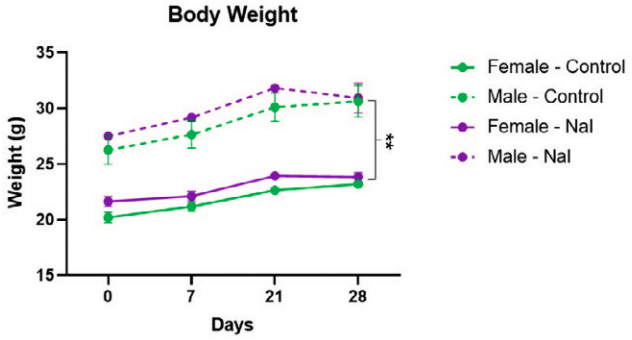
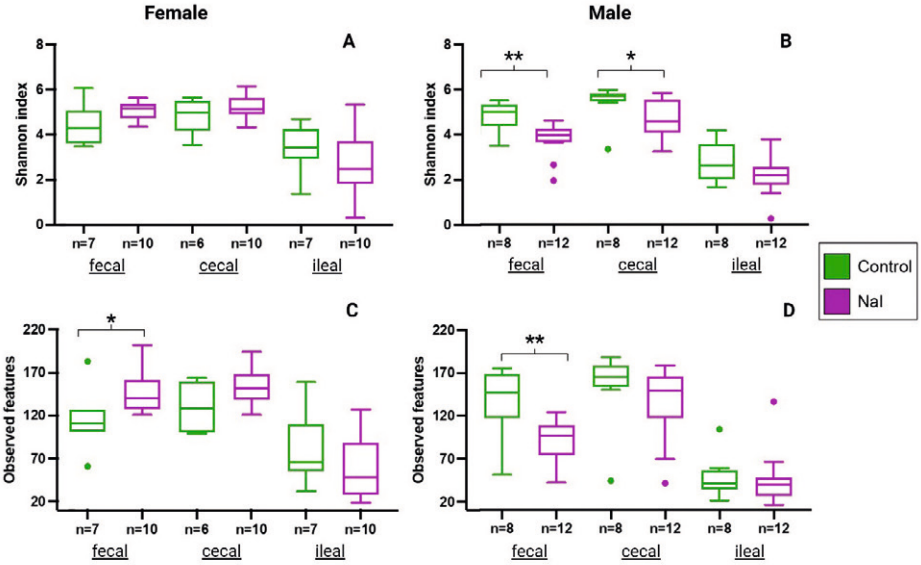
SUPPLEMENTARY MATERIAL



Supplementary Figure 1. Body weight changes during the study period. Each point shows weight mean ±SEM (*n* = 7-12 per group). Significance between NaI and control mice was tested separately by gender and for each time point by Two-way ANOVA; and for all females vs. all males at the final time point (** p<0.01).



Supplementary Figure 2. Alpha diversity of fecal, cecal, and ileal samples. Results are for males and females separately, as determined by Shannon index (A and B) and Observed features (C and D). All samples collected during the sacrifice (D28). Sequence depth: 9,180. Significance of the comparison of time points and between treatments was tested by pairwise Kruskal-Wallis test (* p<0.005, ** p<0.01). Plots show median values with 95% confidence interval.

PART II

GUT MICROBIOTA IN HASHIMOTO'S THYROIDITIS

5

GUT MICROBIOTA AND METABOLITES IN THE PATHOGENESIS OF ENDOCRINE DISEASE

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ABSTRACT

Type 1 diabetes (T1D) and Hashimoto's thyroiditis (HT) are the two most common autoimmune endocrine diseases that have rising global incidence. These diseases are caused by the immune-mediated destruction of hormone-producing endocrine cells, pancreatic beta cells and thyroid follicular cells, respectively. Both genetic predisposition and environmental factors govern the onset of T1D and HT. Recent evidence strongly suggests that the intestinal microbiota plays a role in accelerating or preventing disease progression depending on the compositional and functional profile of the gut bacterial communities. Accumulating evidence points towards the interplay between the disruption of gut microbial homeostasis (dysbiosis) and the breakdown of host immune tolerance at the onset of both diseases. In this review, we will summarize the major recent findings about the microbiome alterations associated with T1D and HT, and the connection of these changes to disease states. Furthermore, we will discuss the potential mechanisms by which gut microbial dysbiosis modulates the course of the disease, including disruption of intestinal barrier integrity and microbial production of immunomodulatory metabolites. The aim of this review is to provide broad insight into the role of gut microbiome in the pathophysiology of these diseases.

INTRODUCTION

Over the past few decades, the incidence of autoimmune thyroid diseases (AITD) and that of type 1 diabetes (T1D) have each increased dramatically[1,2]. Recent research suggests a possible link with these trends, in that immune disorders, including autoimmunity, are intimately connected to imbalances in bacterial gut communities (dysbiosis). This review will provide broad insights into the role of the gut microbiome in the pathophysiology of T1D and Hashimoto's thyroiditis (HT, the most common AITD). Both diseases are governed by cellular autoimmune responses rather than humoral autoimmunity as in Graves' AITD. Although these diseases affect different glands, they share common pathogenetic pathways dictated by the interplay between genetic susceptibility, altered gut microbiome, and loss of self-immunotolerance (**Figure 1**). Insights into this nexus may offer novel therapeutic opportunities, which are greatly needed considering the unavailability of effective therapies other than continuous hormone treatment and the risk of co-morbidities.

Pathogenesis

T1D and HT are, respectively, characterized by progressive destruction of insulin-producing beta cells and thyroid hormone-producing thyrocytes leading to an absolute hormone deficiency, for which the only current treatment is life-long hormone supplementation. The overriding feature of T1D and HT consist in breakdown of immunotolerance to autoantigens derived from pancreatic beta cells and follicular thyroid cells, respectively, resulting in circulating autoantibodies, lymphocyte infiltration in the targeted glands and ultimately T cell-mediated destruction of hormone-producing endocrine cells. The latter events culminate in the clinical manifestation of the diseases owing to decline and, at late stages, deficiency in circulating insulin in T1D or thyroxine (T4) and triiodothyronine (T3) in HT cases. During the pancreatic- or thyroid–homing of leukocytes (normally referred as insulitis or thyroiditis), activated cytotoxic CD8T cells account for the direct destruction of beta or follicular cells, whereas other immune cells (macrophages, effector CD4T cells and B lymphocytes) endorse tissue damage and inflammation by secreting chemokines, inflammatory cytokines and sustaining CD8T cell immunity. Importantly, self-tolerance is normally ensured by regulatory lymphocytes (Treg and Breg) which suppress the function of effector CD4T helper cell[3-5]. Dysfunction of Treg cells or aberrant Th responses can cause inflammation to 'go haywire' with consequent breakdown of immune tolerance. As T cells display high plasticity in lineage differentiation and cytokine profile, shifts towards other Th phenotypes are regarded as prominent features in these autoimmune disorders[3-5].

Both T1D and HT are characterized by production of antibodies against autoantigens derived from beta cells, such as glutamic acid decarboxylase 65 (GAD 65), islet cell, insulin (IAA, IA-2A), and Zinc transporter 8 (ZnT8), or from thyrocytes, such as

enzyme thyroid peroxidase (TPO) and thyroglobulin (Tg) in the case of HT. Notably, the production of autoantibodies precedes the clinical manifestation of disease and may be used as prognostic markers[3,6-8].

Genetic susceptibility and environmental components play an important role in the etiology of both diseases. Genetic susceptibility is mainly accounted by the carriage of high-risk class II human leukocyte antigen (HLA) haplotypes as well as polymorphisms in genes encoding cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and protein tyrosine phosphatase, non-receptor type 22 (PTPN22), both regulators of T cell activation. However, the steep increase in the incidence of both T1D and HT in the Western world cannot be explained solely by genetic variants as shown by the discordant rate in the lifetime risk of monozygotic twins[9-11]. A plethora of environmental triggers have been identified as risk factors for the development of autoimmune disease[12-14]. Recent evidence suggests the importance of the gut microbiome as an environmental risk factor[15-18]. **Table 1** shows an overview of the common hallmarks of both endocrine diseases. Of note, many environmental risk factors are known determinants of microbiota composition: diet, drugs, infections (**Figure 1**).

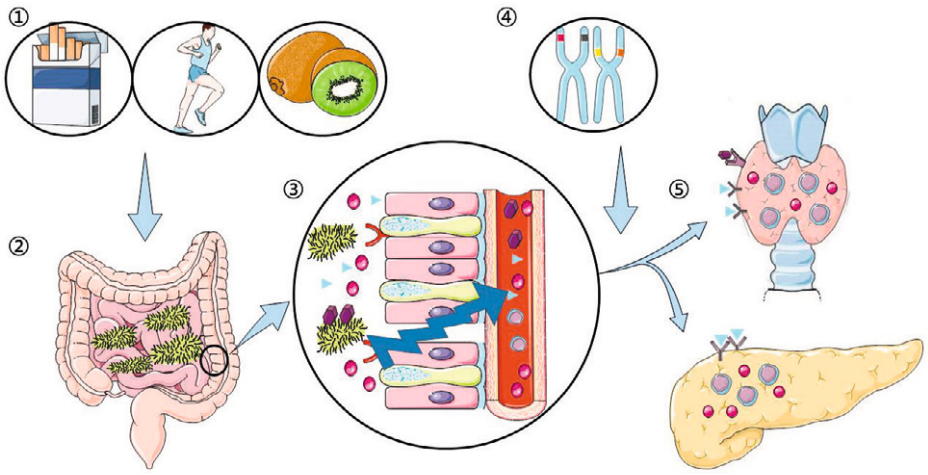

Figure 1. Simplified overview of the role of gut microbiome in endocrine diseases. An ever growing body of evidence have illuminated the intertwined relationship between indigenous bacteria and host immunity. In this complex interplay, environmental factors (1) are known determinants of microbiota composition (2). There are several potential mechanisms by which gut microbial dysbiosis modulate the course of the disease, including disruption of the intestinal barrier leading to a 'leaky gut' (3). Together with genetic susceptibility (4), this plays an important role in the etiology of both type 1 diabetes and Hashimoto's thyroiditis (5).

Table 1. Common hallmarks of type 1 diabetes (T1D) and Hashimoto's thyroiditis (HT): autoimmunity involves both genetic and environmental factors

Disease	Incidence	Age onset	Genetic association	Environmental factors	Auto-antibodies
T1D	*Per 100.000 person-years; in children 0 – 19 years.* The Netherlands: 11.1 - 21.4 [19] Finland: 64.3 [20] United States: 13.1 - 25.9 [21] Global estimate: 4.9 [22]	Early in life	HLA-DR3 [23] HLA-DR4 [23] PTPN22 [24] CTLA-4 [25] IFIH1 [26] INS [23] Probandwise concordance rate in MZ: 6% [9] - 42.9% [10]*	*Infections* Coxsackie A [27] Coxsackie B [27] Cytomegalovirus [28] Rotavirus [29] Enterovirus [30,31] *Dietary components* Vitamin D deficiency [32] Early exposure to Cow's milk [33]	anti-GAD [34] anti-IA-2 [35] IAA [35] anti-ZnT8A [8] ICA [35]
HT	*Per 1000 persons;* The Netherlands [36] F: 2.1 per 1000 M: 0.4 per 1000 United Kingdom [37] F: 4.75 per 1000 M: 1.09 per 1000 United states [38] F: 3.5 per 1000 M: 0.8 per 1000	45-65 years	HLA-DR3 [39] HLA-DR5 [39] CD40 [39] CTLA-4 [39,40] PTPN22 [41] FOXP3 [39] CD25 [39] Probandwise concordance rate in MZ: 55% [11]	*Infections:* Hepatitis C [42,43] Helicobacter pylori [44] Yersinia enterolitica [45] Borrelia burgdorferi [45] *Dietary components:* Mineral deficiency: iodide, selenium, iron, zinc deficiency [46] Vitamin D deficiency [47] *Medication:* Amiodaron [48] Lithium [49] IFN-α [43]	anti-TPO [6] anti-Tg [6]

HLA, human leukocyte antigens, PTPN22, encodes the lymphoid protein tyrosine phosphatase important for regulating T-cell receptor signaling; CTLA-4, cytotoxic T-lymphocyte-associated molecule-4; IFIH1, interferon-induced helicase; INS, insulin; MZ, monozygotic twins; anti-GAD, antibodies to glutamic acid decarboxylase; anti-IA-2A, anti-tyrosine phosphatase-like insulinoma antigen 2; IAA, antibodies to insulin; anti-ZnT8, antibodies against Zinc transporter 8; ICA, islet-cell antibodies; FoxP3, forkhead box P3; CD40, cluster of differentiation 40; CD25, cluster of differentiation 25; anti-TPO, antibodies to thyroid peroxidase; anti-Tg, antibodies to thyroglobulin; F, female; M, male. *Dependent on age of diagnosis.

The Gut Microbiome

The human is the host of hundreds of trillions of microorganisms, consisting of commensal bacteria, archaea, viruses, fungi and yeasts, all living in a symbiotic state[50]. Prior studies often reported that in the human body bacteria outnumber the human cells by an estimated 10-fold. More recently, however, this has been lowered to a more equal ratio of 3 : 1 or 1 : 1[51].

The recent introduction of new molecular techniques including high-throughput technology to sequence the bacterial 16S rRNA genes has allowed new insights into bacterial communities in health and disease. Moreover, the advent of metagenomics has allowed to address open questions on functional and strain-specific differences in healthy and 'disease' microbiomes. Depicting the microbiome composition revealed that, despite the interpersonal variation, only a limited number of phyla are dominant in the intestinal microbial community: the Gram-negative phyla *Bacteroidetes* and Proteobacteriae and the Gram-positive phyla *Firmicutes, Actinobacteria* and *Verrucomicrobia*[52].

Colonization of the microbiome essentially begins at birth with mode of delivery (caesarian section versus vaginal birth) and diet during infancy (formula feeding or breast milk) as major colonization pattern determinants[53-54]. During life a variety of factors can regulate the composition: (prior) use of medication (especially antibiotics), smoking, diet, gender and even ethnicity, geographical regions and cultural differences play an important role[55-57], which challenges the reproducibility of the results of studies reporting a link between the gut microbiome and disease. These patients' characteristics as well as differences in the study methodologies and data analysis could explain the differences found in in the microbiome composition across studies.

'All disease begins in the gut.'—Hippocrates, 400 years BC

Although a universal characterization of 'healthy microbiota' has not yet been defined, a key accepted feature of healthy microbiota is microbial diversity, a high richness of different taxa, renders the microbiome resistant to environmental perturbations. When the gut microbiota composition is disrupted and the microbial ecosystem becomes imbalanced, that can be defined as the occurrence of dysbiosis. Across all metabolic and inflammatory disorder (obesity, diabetes, inflammatory bowel disease, autoimmune diseases) currently linked to altered gut microbiota composition, dysbiosis is commonly characterized by the loss of diversity (reduction in alpha-diversity) with concomitant reduction in (beneficial) commensals and an overgrowth of pathogenic bacterial strains[58]. This results in reduced resistance against microbial and inflammatory imbalance, and failure to maintain of immune homeostasis, a form of resilience.

A growing body of evidence has illuminated the complex interplay of environmental factors and intestinal characteristics, including immune status and host genotype, together modulate the composition of commensal communities[59-61]. Particular microbial lineages provide beneficial tolerogenic signaling, while others induce and/or amplify inflammation. In addition, the microbiota may control host negative regulatory mechanisms that reduce the antimicrobial responses and could contribute to dietary, commensal and self-antigen immunotolerance ('balanced signal hypothesis')[62]. Therefore, dysbiosis may increase susceptibility to autoimmunity or alter the trajectory of an established disease and may interfere with both the process of innate immune receptor activation and the production of microbial-derived immunomodulatory metabolites (such as short chain fatty acids and tryptophan derivatives).

However, only associations and correlations between gut microbiota and disease pathogenesis have been shown for most dysbiosis-related diseases; causality has not been demonstrated. The dysbiosis may be driving the illness, may result from the illness, or may reflect medications used to treat the illness; in fact these are not exclusive categories, which makes analysis more difficult.

Alterations in gut microbial composition in T1D and HT

In the following paragraphs, we will explore current knowledge about the microbiome alterations associated with T1D/HT and the potential mechanisms linking dysbiosis to disease onset and progression. **Table 2** shows the concordant and discordant changes found in the gut microbiome signatures in T1D and HT reported by different human studies performed in different geographic regions.

Table 2. Overview of taxonomic gut microbiota signatures in T1D and HT

Taxonomic level	Organism	T1D	HT*	Possible functional effects
Phylum	Actinobacteria	Discordant results (63-65)	Increased [NS] (66)	
Phylum	Bacteroidetes	Increased (64,67-69)	Decreased (66,70)	
Phylum	Firmicutes	Decreased (63-65,71)	Discordant results (66,70)	Comprise conversion to secondary BA (72)
Phylum	Fusobacteria	Discordant results (67,73)	Increased [NS] (66)	
Phylum	Proteobacteria	Discordant results (64,65)	Increased [NS] (66)	
Phylum	Verrucomicrobia	Decreased (73)	Decreased [NS] (66)	
Family	*Bacteroidaceae*	Increased (64,68)	Discordant results (66,70)	
Family	*Enterococcaeae*	Decreased (53,67)	Increased [NS] (66)	
Family	*Lachnospiraceae*	Decreased (35,64,65,73-75)	Increased (66,70)	
Family	*Peptostreptococcaceae*	Decreased (75)	Increased (70)	

Table 4. Continued

Taxonomic level	Organism	T1D	HT*	Possible functional effects
Family	*Prevotellaceae*	Discordant results [53,73]	Decreased [66,70]	
Family	*Ruminococcaceae*	Decreased [53,64,67]	Decreased [NS] [66]	
Family	*Streptococcaceae*	Not assessed	Increased [70]	
Family	*Veillonellaceae*	Decreased [64,67]	Decreased [NS] [66]	
Genus	*Akkermansia*	Discordant results [53,73,75]	Not assessed	Inducing Treg [15]
Genus	*Alistipes*	Discordant results [76,77]	Increased [NS] [66]	Promote mucus production [78]
Genus	*Bacteroides*	Increased [63,65,67,68,75,77,79]	Discordant results [66,70]	Inducing Treg [15] SCFA producer [70]
Genus	*Bifidobacterium*	Discordant results [63,65,73,75,77]	Decreased [66,70]	Regulating translocation of intestinal bacteria [80] Possible antigenic in HT [81]
Genus	*Blautia*	Increased [35,65,74,75]	Increased [70]	
Genus	*Clostridium*	Discordant results [63,71,82]	Not assessed	Potent driver of Treg expansion and differentiation [83]
Genus	*Dialister*	Discordant results [35,53,67,71,73,84]	Decreased [NS] [66]	
Genus	*Dorea*	Decreased [75]	Increased [70]	
Genus	*Escherichia-Shigella*	Increased [71,73]	Increased [NS] [66]	
Genus	*Faecalibacterium*	Decreased [75]	Decreased [70]	
Genus	*Fusicatenibacter*	Increased [NS] [53]	Increased [70]	
Genus	*Lachnoclostridium*	Increased [NS] [53]	Decreased [70]	
Genus	*Lactobacillus*	Decreased [63,73,76]	Decreased [66]	Inducing Treg [15] Possible antigenic in HT [81]
Genus	*Prevotella*	Discordant results [63-65,73,77,79]	Decreased [66,70]	
Genus	*Romboutsia*	Decreased [NS] [53]	Increased [70]	
Genus	*Roseburia*	Discordant results [65,73,75,85]	Increased [70]	
Genus	*Ruminococcus*	Discordant results [53,65,74,75]	Discordant results [66,70]	
Genus	*Streptococcus*	Discordant results [53,65,73,74]	Not assessed	Inducing Treg [15]
Genus	*Subdoligranulum*	Decreased [73]	Increased [NS]	
Species	*Alistipes shahii*	Increased [85]	Not assessed	
Species	*Bacteroides clarus*	Increased [86]	Increased [NS] [66]	
Species	*Bacteroides dorei*	Discordant results [69,82,86]	Increased [NS] [66]	
Species	*Bacteroides fragilis*	Decreased [64]	Increased [NS] [66]	Induce IL-10 secretion [87] Enhance bacterial translocation [68]

Table 4. Continued

Taxonomic level	Organism	T1D	HT*	Possible functional effects
Species	*Bacteroides vulgatus*	Discordant results [69,86]	Increased [NS] [66]	
Species	*Bifidobacterium adolescentis*	Decreased [68,82]	Not assessed	SCFA producer (acetate and lactate) [68] Induce Th17 cell response [15]
Species	*Bifidobacterium longum*	Increased [82,84,86]	Decreased [NS] [66]	
Species	*Bifidobacterium pseudocatenulatum*	Discordant results [68,85]	Not assessed	
Species	*Dialister invisus*	Discordant results [74,84]	Not assessed	
Species	*Escherichia coli*	Increased [64]	Increased [NS] [66]	Induce Th17 cell response [15]
Species	*Eubacterium hallii*	Decreased [NS] [68]	Increased [70]	
Species	*Faecalibacterium prausnitzii*	Discordant results [64,68,82,86]	Not assessed	Regulating Th17 cell [70]
Species	*Lactobacillus gasseri*	Not assessed	Decreased [66]	
Species	*Lactobacillus lactis*	Decreased [85]	Not assessed	
Species	*Olsenella sp. SK9K4*	Not assessed	Decreased [66]	
Species	*Roseburia faecis*	Decreased [68]	Not assessed	
Species	*Roseburia hominis*	Increased [85]	Not assessed	
Species	*Ruminococcus flavefaciens*	Not assessed	Increased [66]	
Species	*Ruminococcus gnavus*	Decreased [64,74]	Not assessed	Inducer of proinflammatory polysaccharide [88]
Species	*Streptococcus mitis/ oralis/pneumonia*	Increased [85]	Not assessed	
Species	*Streptococcus thermophilus*	Decreased [85]	Not assessed	
General characteristics				
α-diversity		Decreased [64,74]	Discordant results [66,70]	
Bacterial richness		Decreased [35]	Discordant results [66,70]	
F/B ratio		Decreased [63,74]	Increased	

T1D, type 1 diabetes; HT, Hashimoto's thyroiditis; [NS], FDR adjusted P-value (Q-value) was not significant; α-diversity is the variety of microorganisms within a single sample; bacterial richness is the total number of different species; F/B ratio is the Firmicutes to Bacteroidetes ratio.
* To date, only two studies have analyzed the gut microbiome composition in HT patients. Discordant results observed in these two studies may be due to the different thyroid functional status of the patients involved (euthyroid HT patients in study of Zhao et al. and hypothyroid HT patients in the study of Ishaq et al.)

Type 1 diabetes

The relationship between gut microbiome dysbiosis and T1D has been extensively studied in past decades and these studies have been summarized elsewhere[61,90,91]. The role of the gut microbiome as a regulator of T1D progression is strongly supported by evidence from murine studies with non-obese diabetic (NOD) mice, a polygenic model for spontaneous autoimmune diabetes, in which T1D incidence strongly depends on environmental/microbial exposure[92-95]. In support of the 'balanced signal hypothesis', Burrows et al.[94] elegantly showed that T1D development depends on microbiota-induced signaling through TLRs and the adaptor signaling molecule MyD88. Specifically, deletion of MyD88 protected mice from T1D development under specific pathogen-free (SPF) conditions, but not in germ-free (GF) vivaria. Such data provide evidence that host interactions with the commensal bacteria occurring through MyD88 produces pathogenic signals. Colonization of GF NOD.MyD88−/− mice with a probiotic mix containing *Lactobacillaceae* suppressed insulitis[94]. This can be interpreted as showing that the probiotics have disease-reducing interactions that do not require the MyD88 pathway related to TLR signaling. Analysis of SPF/GF NOD mice lacking TLRs or the downstream adaptor TRIF indicated that TLR4 and TRIF signaling act as microbiota-induced tolerizing pathways, whereas TLR2 mediates (indirectly) microbial pro-diabetic signals[94]. The protective phenotype of MyD88 knockout mice was associated with an increased intestinal abundance of Lactobacillaceae (*Firmicutes*), Rikenellaceae and Porphyromonadaceae (both *Bacteroidetes*). The protective effect was successfully transmitted to GF NOD mice that were exposed to microbiota from SPF NOD.MyD88−/− mice, as assessed by reduced insulitis and increased proportion of intact islets compared with the uncolonized GF NOD mice[92]. Thus, the unperturbed microbiota has a tolerizing effect on the disease phenotype. Overall, these studies define important roles of microbial-immune cross-talks on the progression of T1D.

While comparing taxonomic changes of gut microbiome in T1D patients with those in healthy controls has revealed some concordant microbial signatures (**Table 2**), other studies have observed contradictory trends. Common features to the diabetogenic human gut microbiome are lower *Firmicutes* to *Bacteroidetes* (F/B) ratio[63,74], as well as decreased diversity[64,74] and richness[35]. Importantly, the existence of diabetogenic microbiota appears to precede disease onset. In an American study, Alkanani et al.[76] reported that the microbiota composition of seropositive and seronegative first-degree relatives was similar but distinct from that of unrelated healthy controls or new-onset T1D subjects. Strikingly, the authors found an increase in the relative abundance of *Bacteroide*s and a decreased abundance of *Prevotella* in seropositive subjects with multiple versus one autoantibody. In a European longitudinal study examining the microbiota of children from birth until 3 years of age, a marked drop in alpha-diversity was found after seroconversion in patients that progressed to T1D[74]. The reduced microbiota diversity was accompanied by the outgrowth of *Blautia*, the

different pre-clinical and clinical stages[53,85]. Applying metagenomics sequencing analysis of stool samples from 783 children (seroconverter and/or confirmed T1D cases with their time-matched controls) collected from 3 months of age until clinical end-point (seroconversion or T1D), Vatanen et al. showed that healthy controls contained higher levels of *Lactobacillus rhamnosus* and *Bifidobacterium dentium*, whereas children with autoimmunity had higher abundance of *Streptococcus group mitis/oralis/pneumoniae* species. Notably, the progressors to T1D contained higher levels of *Bifidobacterium pseudocatenulatum, Roseburia hominis* and *Alistipes shahii* species, and non-progressors had more *Streptococcus thermophilus* and *Lactococcus lactis* species instead[85]. *S. thermophilus* may be a marker for probiotic exposure.

Due to the complexity of microbial communities, it remains difficult to identify specific beneficial or detrimental lineages and to determine whether the altered microbiota contributes to or results from compromised immune function. In this regard, *Akkermansia muciniphila* was identified as a protective symbiont against T1D onset in NOD mice as it was abundant in T1D low-incidence colonies (NOD/MrkTac) and absent from high-incidence (NOD/Jax) colonies. In addition, oral transfer of *Akkermansia* to NOD/Jax mice delayed diabetes onset, reduced insulitis severity and promoted intestinal barrier function. Similarly, it induced remote protective effects in pancreatic islet with an increased Foxp3+ Treg cell count, elevated expression of the anti-inflammatory cytokines IL-10 and TGF-β, reduced infiltration by mononuclear leukocytes, diminished TLR2 and TLR4 levels, and in total, delayed T1D onset[96]. Since *A. mucinophila* is regarded as an organism that increases in abundance opportunistically, it is a marker of compromised microbiota; its association with protection is consistent with the maxim with NOD mice that 'dirty protects'. Notably, two separate studies found *A. muciniphila* levels to be associated with diminished risk of developing T1D-associated autoantibodies in children at risk[73,97] indicating consistency with the studies in NOD mice[73,97] . Several studies have reported correlations between significantly different fecal taxa and clinical parameters of glycemic control, mainly reported by HbA1c levels[35,67,75]. As such, T1D patients with reduced abundance of beneficial microbes *Bacteroidetes* and *Lactobacillales* tend to have higher levels of HbA1c[77]. Furthermore, *Subdoligranum* was associated with poorer metabolic control[98], the abundance of *Blautia* positively correlated with HbA1c and T1D-associated antibodies[35], and the F/B ratio inversely correlated with the plasma glucose level[63]. It should be noted that, similar to the discordant results of taxonomic signatures shown in **Table 2**, conflicting results of possible functional effects of bacteria have been reported. For instance, while Salamon et al.[75] found a negative correlation between the abundance of the family *Erysipelotrichaceae* and HbA1C levels, other studies have suggested a highly immunogenic role and positive association with T1D onset for this bacterial family[53,99].

A role for the microbiota in protecting from or inducing T1D has been indicated in multiple studies in which antibiotics have been used in NOD mice[100-104]. Studies have shown both enhanced induction of T1D by certain antibiotics (notably broad spectrum combinations, macrolides, and vancomycin) and protection by others. This point to multiple immunological mechanisms. Loss of small intestinal Treg cell populations and alterations in other T helper cells is one attractive mechanism for the altered diathesis[100,102,103]. Studies of intestinal epithelial gene expression show that the antibiotic-perturbed microbiota differentially induces important innate immune pathways, including those related to TLRs, SAA and NO, as well as adaptive immune effects as well (e.g. Th17, and Tregs)[100,101]. Importantly, timing of the antibiotic exposure is important with effects seen prenatally[102], or in early life[100,101]. Such results are consistent with the hypothesis that the early life microbiota affects immunological development, and the nature of the immunological tone, which affects the development of autoimmunity in the NOD mouse. Transfer of the antibiotic-altered microbiota resulted in similar phenotypes[100,102], indicating that it is the antibiotic-altered microbiota per se that is having the effect. Such studies move the question of microbiome effects on T1D development in human children to the earliest months of life, in many cases years before the development of T1D.

Hashimoto's thyroiditis

Although fewer studies have addressed the link between microbiome and HT, the topic is receiving growing attention[46,60]. An association between the gut microbiome and thyroid function was already postulated in murine models by the 1970s. Modifying the microbiome in rats by exposing them to antibiotics led to reduced thyroid gland function, measured by the uptake of radioactive iodine[105]. Another study showed a 25% increase in TSH levels in GF mice compared with conventionally raised mice with normal intestinal microbiota[106]. Similarly to the NOD model, early-life environmental exposures influence the susceptibility to the disease. Using thymectomy and irradiation to induce experimental autoimmune thyroiditis (EAT), Penhale et al.[107] discovered that maintenance of female PVG/c rats under SPF conditions until weaning conferred resistance to AITD, whereas antibiotic treatment and microbiota transfer from conventionally reared rats into newly weaned SPF rats increased the autoimmune susceptibility of the latter. On the contrary, daily administration of isolated 'probiotic' strains *Lactobacillus rhamnosus HN001*(HN001) and *Bifidobacterium lactis HN019* (HN019) had no impact on autoimmune responses, assessed by autoantibody levels, spleen weight and lymphocyte infiltration into thyroid glands, after inducing EAT in CBA/CaH (H-2k) mice by consecutive injections of mouse thyroglobulin[108].

More recently, two Chinese studies have compared the fecal microbiota of HT patients with matched healthy controls, and described a HT-associated dysbiosis[66,70]. Ishaq et al.[66] included hypothyroid HT patients, whereas Zhao et al.[70] studied the fecal samples

of euthyroid HT patients[70]. This difference in thyroid functional status, and treatments are major confounding factors possibly affecting the gut microbiome composition and may explain some of the discordant observations in fecal microbiome characteristics shown in **Table 2**. Notably, independent of the thyroid functional state, both reports identified a reduction in abundance of the species *Prevotella_9* in subjects with HT. The Zhao study found no significant increase in microbiota species richness and diversity in HT patients, but taxon-dependent analysis showed an overall different population structure, with 27 genera found significantly altered between the HT and healthy microbiomes, and the effects markedly associated with clinical parameters of thyroid disease (anti-TPO, anti-Tg, fT4 and TSH)[70]. The genera enriched in HT patients that were positively correlated with the thyroid antibodies anti-TPO and anti-Tg mostly belong to the phylum *Firmicutes*, whereas the genera depleted in HT patients that were inversely correlated with these antibodies mostly were in the phylum *Bacteroides*. The genus *Alloprevetolla* was positively correlated with FT4 levels, whereas an inverse correlation was observed between the genera *Fusicatenibacte*r and *Romboutsia* and FT4 and TSH levels. Prediction modeling selected 10 of the 27 species as biomarkers of HT-associated microbiome in both the exploratory cohort and in a second validation cohort of HT patients and healthy controls[70]. Interestingly, as reported in the diabetogenic microbiome, the microbiota of HT patients was enriched in *Blautia* genera, belonging to the order *Clostridiales*, which are important mediators of intestinal homeostasis and tolerogenic immunity[83,109].

Overall these data indicate that the characteristics of the gut microbiota may be associated with disease progression; however, further investigations in EAT models are needed to uncover the possible mechanisms linking dysbiosis and the growth of specific bacteria to the development of HT.

Mechanisms linking the gut microbiome to autoimmune T1D and HT

Microbiota influence on immune system: from development to function

In the evolutionary process of animal life on earth, the gut microbiome and the immune system have co-evolved profoundly leading to a reciprocal microbiome-immune system interplay. Previous studies with GF mice[110,111] have shown extensive deficits in the development of gut-associated lymphoid tissue (GALT), a secondary lymphoid organ present throughout the gastro-intestinal tract. These deficits include fewer and smaller mesenteric lymph nodes with lower numbers of CD4+CD25+Foxp3T-lymphocytes[112] and IgA-secreting plasma cells[113]. Notably, these immunologic deficiencies reversed within a few weeks after colonization of GF mice, and the normal development and maturation of the GALT was followed[111,112]. Commensal bacteria are important for both immune system development and function. Previous studies have demonstrated that microbiota composition influences the balance between two major effector T cell populations, IL-17+ Th17 and CD25+ Foxp3+ Treg. Ivanov

et al.[114] reported that intestinal colonization with a single commensal microbe-segmented filamentous bacterium (*Savagella*) was sufficient to induce Th17 cells in the intestinal lamina propria. Intestinal colonization by other mucosal-associated bacteria (*Escherichia coli, Bifidobacterium adolescentis, Staphylococcus aureus*) also have been shown to induce Th17 cell responses, although with distinct cytokine profiles[15].

Administration of *Bacteroides fragil*is-derived polysaccharide A (PSA) restored immunologic deficits in GF mice by inducing CD4+ T cell expansion systemically and by correcting the Th1/Th2 imbalance[115]. *B. fragilis*-PSA exerts systemic anti-inflammatory activities by enhancing activated T cell-induced IL-10 production and by promoting the frequency and function of IL-10+Foxp3+ Treg cells[89,115,116]. A mixture of *Clostridia* strain, isolated from human microbiota, was identified as potent driver of Treg expansion and differentiation in GF mice[83]. Other microbiota members capable of inducing Treg include *Escherichia, Akkermansia, Bacteroides, Lactobacillus,* and *Streptococcus* strains, as well as the altered Schaedler flora, a defined commensal community[15].

In light of these previous discoveries, it is reasonable to assume that alterations in the relative abundance of specific symbiotic strains in T1D- and HT-associated microbiomes, such as *Akkermansia* and *Bacteroides*[76,96], may impact the plasticity of effector T cell differentiation and hence the course of disease. Both of these studies show the remarkable impact that colonization by a single bacterium can have with associated alterations in specific immune cell-types affecting autoimmune diabetes development.

The indigenous microbiota also indirectly regulate gut barrier integrity and the expression of innate immunity. In the extreme example, GF mice exhibit an altered mucosal layer, an impaired development of GALTs[117], and a lower expression of intestinal bacterial pattern recognition receptors (PRRs). The expression of functional PRRs, such as Toll- and NOD-like receptors, by intestinal epithelial cells (IECs) and GALT-resident myeloid cells is essential for the establishment of host-microbial symbiosis and for host control of microbiota composition. Upon bacterial recognition, PRRs drive inflammatory responses and initiate processes involved in mucus production, regeneration of IECs, and antimicrobial peptide production[59,118]. Dysbiosis has been reported in several mouse models of innate immune deficiency (e.g. MyD88−/− and NOD2−/−), and activation of these innate receptors has been shown to influence the incidence and severity of T1D in NOD mice. Therefore, the microbiome can be viewed as a changeable component of both innate and adaptive immunity, influencing their function, while living symbiotically inside the gastrointestinal tract.

Intestinal permeability: the 'leaky gut'

Increased intestinal permeability occurs in the setting of disruption of gut homeostasis, allowing food-derived antigens, intestinal toxins, and microbial factors to breach the endothelial barrier.

Aberrant functional integrity of the gut has been reported in both human and animal studies of T1D[68,78,119]. As such, down-regulated intestinal expression of tight junction encoding genes and up-regulation of serum levels of zonulin, a marker of gut permeability, were documented in human T1D subjects accompanied by changes in microbiome composition[65,120]. Moreover, increased intestinal permeability, as measured by the lactulose/mannitol test, was detectable prior to clinical onset[121], strengthening the hypothesis of a causative role of 'leaky gut' in T1D development. In support of this concept, Costa et al.[122] observed bacterial translocation to pancreatic lymph nodes in streptozotocin-injected wild-type mice and further demonstrated that this translocation mediates inflammation and hyperglycemia by activating the NOD2 innate receptor in pancreas. In a more recent study, using BDC2.5XNOD mice, which carry a beta cell-specific T cell receptor but do not develop spontaneous T1D, Sorini et al. showed that disruption of intestinal integrity activates the isletspecific T cells within the gut mucosa in a microbiota-dependent manner. These T cells, expressing a gut homing marker, can subsequently appear or be tracked to the pancreas. Hence, this study provides evidence that diabetogenic T cells are activated by intestinal microbiota under 'leaky gut' conditions and later can migrate to the pancreas to induce diabetes[123].

Similarly, altered intestinal permeability evaluated by the lactulose/mannitol test, was reported in HT patients together with morphological changes in duodenal enterocytes[124]. In HT patients, the decreased metabolism and longer gastrointestinal transit time, typical of hypothyroidism, have been found to impair microvilli function and affect the intestinal homeostasis. This intestinal motor dysfunction may lead to small intestinal bacterial overgrowth (SIBO), which has been reported in HT patients[59,66,125]. Eventually, bacterial overgrowth may prompt bacterial translocation, inducing systemic inflammatory complications such as autoimmune thyroiditis[59]. Since these changes may be a consequence rather than a cause of HT, further investigations are needed to address this thyroid-GI motility-microbiota nexus.

In both diseases, a 'leaky gut' may be triggered by an imbalance between microbiota taxa that enhance the mucous barrier and mucolytic strains when dysbiosis occurs, prior to manifestation of the disease (**Figure 1**). In a T1D cross-sectional study, metaproteomic analysis of microbial proteins in human stool samples (integrated with microbiota taxonomic profiling data) revealed alterations in mucin degradation in seroconverters and depletion of microbial taxa associated with particular host proteins involved in maintaining the mucous barrier and exocrine pancreas function in

new-onset T1D subjects[78]. Likewise, alterations in the composition of mucin degrading bacteria was associated with early development of anti-islet cell autoimmunity in children[97]. Since T1D per se may alter the microbiota through several functional changes, the studies that show abnormalities prior to full T1D development have the greatest significance.

Gut microbial-derived metabolites and pathways

The gut microbiome participates in multiple crucial physiological processes that impact host immunity and energy metabolism, including synthesis of microbiota-specific metabolites and vitamins (B and K), production and secretion of (intestinal) hormones (leptin, ghrelin and glucagon-like peptide 1), synthesis of secondary bile acids, and absorption of mineral nutrients by competing with the host (e.g. iodide, iron, and zinc important for thyroid function)[46]. We next discuss the roles of a few microbial metabolites linked to inflammation and the pathophysiology of both T1D and HT[46].

Short-chain fatty acids

The saccharolytic fermentation of non-digestible complex carbohydrates leads to the production of short-chain fatty acids (SCFAs), predominantly butyrate, acetate and propionate (in a general ratio of 60 : 20 : 20) primarily by phylum Bacteroidetes organisms. After production, most (~95%) SCFAs are absorbed by colonocytes through diffusion or co-transport[126]. SCFAs have been intensively researched due to their roles in metabolic responses and their anti-inflammatory properties. SCFAs are histone deacetylase (HDAC) inhibitors and also ligands for G-protein coupled receptors (GPCR) 43, 41, and 109a, which are expressed by multiple cell-types including immune cells and IECs. Through GPCR signaling and epigenetic modulation, SCFAs, particularly butyrate, restrain the NFκB-elicited production of proinflammatory cytokines in myeloid cells, and promote generation of regulatory T cells and increase their suppressive activity via inhibition of proinflammatory HDAC[15,127-131]. Furthermore, butyrate is a major energy source for enterocytes and plays an important role in maintaining gut integrity by inducing mucus production and promoting tight junction expression in IECs[129]. Thus, SCFAs are vital for immune homeostasis by both counteracting proinflammatory responses and fortifying the IEC barrier[129].

In longstanding T1D patients, there was no significant difference in fecal SCFA levels, which constitute ~5% of net SCFA production; however, a lower abundance of SCFA-producing microbes was observed, accompanied by lower levels of plasma SCFA and decreased fecal levels of the butyryl-CoA:acetate-CoA transferase gene[100]. Another study in human children genetically susceptible to diabetes hinted to a protective effect of butyrate in T1D development, since lower abundances of microbial genes for the butyrate production were associated with early autoantibody development[97].

These results were consistent with a previous murine study which found that blood and fecal concentration of the SCFAs acetate and butyrate were decreased in NOD mice with higher T1D incidence. Moreover, a 5-week acetate- and butyrate-yielding diet provided long-term protection against T1D development with a decline in autoreactive T cells, concomitant expansion of Treg, and improved gut barrier integrity[18,130]. In NOD mice, SCFAs promoted a tolerogenic pancreatic immune environment by controlling the beta cell-mediated production of the antimicrobial peptide CRAMP, which elicited a protective effect against diabetes onset[132].

Nevertheless, supplementation of butyrate alone to longstanding T1D patients did not impact immune responses or metabolic markers of disease[133]. Whether oral SCFAs can slow down autoimmunity-induced beta cell destruction in new-onset human T1D has yet to be studied.

Importantly, a multicenter prospective study revealed that T1D-associated microbiome alterations are taxonomically diverse but functionally more coherent: pathways related to bacterial fermentation and SCFA synthesis were the most significantly differential functional profiles of control and T1D microbiomes, but these changes were not consistently specific for microbial taxa across geographically diverse centers[85]. This suggests that functional rather than phylogenetic characterization of the microbiome might better serve for disease monitoring and biomarker discovery.

Small-scale human studies comparing HT and healthy individuals have observed a decreased abundance of SCFA-producing bacteria in HT[66,70]. However, fecal and plasma levels of SCFA in HT have yet to be studied. We speculate that SCFAs elicit beneficial immune functions in AITD, similar to those shown in T1D.

Secondary bile acids

The gut microbiome has an essential role in bile acid metabolism. The production of cholesterol-derived primary bile acids (BAs) is regulated by the nuclear farnesoid X receptor (FXR) and the G protein-coupled receptor TGR5[134]. The colonic microbiome, of which primarily the genus Clostridium, carry out the conversion to secondary BAs through the bacterial 7α-dehydroxylation reaction[72]. Murine models indicate that one or more feedback loops between the host and the microbiota may characterize BA metabolism: the gut microbiota regulates host primary BA synthesis by reducing levels of a FXR antagonist[135], and FXR-regulated pathways can in turn alter bile acid composition, regulating microbiota composition[136,137].

BAs are recognized as signaling molecules that are involved in both lipid metabolism and energy expenditure by activating TGR5, and taurine-conjugated secondary BAs are the most potent TGR5 ligands. Activation of TGR5 stimulates type 2 iodothyronine deiodinase (D2) in brown adipocyte tissue, increasing local production of the bioactive

thyroid hormone T3[138,139]. In another feedback loop, thyroid hormone regulates BA metabolism by increasing the expression of CYP7A1 in the liver[140]. Additionally, deoxycholic acid (DCA) is proposed to have a strong and selective antimicrobial effect by inducing membrane damage and therefore reducing bacterial overgrowth[141]. Metabolic profiling showed that DCA is the dominant bile acid in HT patients[142,143], which may reflect that small intestinal bacterial overgrowth (SIBO) is common in patients with HT[125]. Whether or not DCA contributes to HT pathogenesis or merely reflects its presence is not known. BAs also act on hepatic glucose metabolism through the FXR and TGR5 receptors[144,145]. In both mice and humans, complete cessation of insulin production alters bile acid pool size, composition, and homeostasis, perhaps suggesting a feedback loop between glucose and BA metabolism[146-148]. In addition, plasma levels of DCA were significantly higher in children with T1D, even when well-controlled[147]. However, it is uncertain whether these changes are causal factors in the onset and progression of T1D.

In conclusion, accumulating evidence implicates the microbiome in the modulation of (extra)intestinal immunopathologies. However, the exact nature of the molecular and cellular signals interconnecting the two remains an active area of research.

FUTURE PERSPECTIVES

- *Highlight importance of the field*: In the Western world there is rapidly rising incidence of auto-immune type 1 diabetes and Hashimoto's thyroiditis. Current treatment consists of providing hormone replacement treatment, rather than intervening in the pathophysiology. In recent years, numerous studies have suggested a potential causal role of the gut microbiome in the pathogenesis of disease.
- *Provide a summary of current thinking:* Both common and discordant changes in the gut microbiome signature in T1D and HT have been found: the overall structure of the T1D/ HT-associated dysbiosis is characterized by loss of diversity, reduction in protective bacteria, such as SCFA producing strains, and overgrowth of potentially pathogenic strains, which provide proinflammatory signals and/or encompass mimic peptides of disease relevant auto-antigens. These changes precede the clinical manifestation of the disease and appear to play a causal important role in the onset of disease.
- *Comment on future directions:* The gut microbiota constitutes a new putative target of intervention in early stage of autoimmune disease. Restoration of healthy microbiota has the potential to re-establish intestinal and immune homeostasis and can be accomplished by fecal microbiota transplantation. The latter represents a safe promising therapeutic approach to counteract immune disorders and also an opportunity to expand our understandings of microbial-immune interactions for specific human diseases. This might well lead to new

therapeutic options for dysbiosis-driven diseases: novel probiotics with multiple specific beneficial strains identified from the fecal infuses leading to personalized patient care.

Competing Interests

A.C.F., E.R., and E.F. declare not conflict of interest. M.N. is on the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands; M.N. is on the Scientific Advisory Board of Kaleido, USA; M.J.B. is on Scientific Advisory Boards for Dupont, Procter & Gamble, Elysium, and Seed, Inc. None of these are directly relevant to the current paper. There are no patents, products in development or marketed products to declare. The other authors have no conflicts of interest. Funding The writing of this review was supported by Le Ducq consortium grant 17CVD01 and a Novo Nordisk Foundation GUT-MMM 2016 grant. M.N. is supported by ZONMW-VIDI 2013 grant (016.146.327) and Dutch Heart Foundation CVON IN CONTROL -II. Supported in part by U01-AI22285 from the National Institute of Allergy and Infectious Diseases, and the Sergai Zlinkoff Fund.

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All authors contributed to the writing and reviewing of the manuscript and all authors read and agreed to the final version.

Abbreviations

(f)T4, (free) thyroxine; AITD, autoimmune thyroid disease; AP-1, activator protein 1; BA, bile acids; Breg, B regulatory lymphocytes; CRAMP, cathelin-related antimicrobial peptide; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; D2, deiodinase 2; DCA, deoxycholic acid; EAT, Experimental autoimmune thyroiditis, a mice model for Hashimoto's thyroiditis; F/B ratio, Firmicutes to Bacteroidetes ratio; FXR, nuclear farnesoid X receptor; GAD 65, glutamic acid decarboxylase 65; GALT, gut-associated lymphoid tissue; GCPR, G-protein coupled receptors; GF, germ-free mice; HDAC, histone deacetylase; HLA, human leukocyte antigen; HMP, Human Microbiome Project; HT, Hashimoto's thyroiditis; IA-2A, tyrosine phosphatase like protein, autoantigen in T1D; IAA, antibodies to insulin; IECs, intestinal epithelial cells; IFIH1 gene, interferon-induced helicase; IGRP, islet-specific glucose-6-phosphatase catalytic subunit–related protein; IRF3, Interferon regulatory factor 3; LPS, lipopolysaccharide; Mgt, bacterial magnesium transporter; MHC-1, major histocompatibility complex I; MyD88, Myeloid Differentiation Primary Response 88; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NIH, national institute of health; NO, nitric oxide; NOD mice, non-obese diabetic mice, a polygenic model of spontaneous autoimmune diabetes; NOD2 receptors, nucleotide-binding oligomerization domain containing 2 innate receptor; PBMCs, peripheral blood mononuclear cells; PRR, pattern recognition receptors; PSA, polysaccharide A; PTPN22, protein tyrosine phosphatase, non-receptor type 22; SAA, serum amyloid A; SCFA, short chain fatty acids; SFB,

segmented filamentous bacterium, a commensal microbe; SIBO, small intestinal bacterial overgrowth; SPF mice, specific pathogen-free mice; T1D, type 1 diabetes; T3, triiodothyronine; Tg, thyroglobuline; TGF-β, Transforming growth factor beta 1; TGR, G-protein-coupled bile acid receptor or Gpbar1; Th1/2/17, T helper lymphocytes; TLR, toll-like receptor; TPO, thyroid peroxidase; Treg, T regulatory lymphocytes; TRIF, TIR-domain-containing adapter-inducing interferon-β; TSH, thyroid stimulating hormone; Znt8, against Zinc transporter 8.

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6

A CHARACTERIZATION OF THE GUT MICROBIOME COMPOSITION IN A MULTI–ETHNIC EUTHYROID POPULATION WITH THYROID AUTOIMMUNITY

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ABSTRACT

Background

Previous studies have reported gut microbiome alterations in Hashimoto's autoimmune thyroiditis (HT) patients. However, it is unknown whether an aberrant microbiome is present before clinical disease onset in participants susceptible to HT or whether it reflects the effects of the disease itself. We report the first study to examine taxonomic and functional profiles of the intestinal microbiota in euthyroid participants with anti-TPO antibodies (TPOAb)(N=159) in relation to seronegative healthy controls (N=1,309).

Methods

A prospective cohort study including European Dutch, Moroccan, and Turkish subjects from the multi-ethnic HEalthy Life In an Urban Setting (HELIUS) cross-sectional study. Fecal microbiota composition was profiled using 16S rRNA sequencing. The data was analyzed based on overall composition (alpha and beta diversity), as well as differential abundance of microbial taxa and functional pathways using multiple tools.

Results

We found no ethnicity-specific differences in thyroid markers nor overall shift in the microbial signature of seropositive individuals. Nonetheless, association analysis of the gut microbiome community revealed that TPOAb-status is nominally significantly linked with 138 taxa, of which thirteen taxa were consistently found nominally significant with four separate difference abundance methods, and several functional pathways.

Conclusion

There is weak evidence to suggest that perturbations of the gut microbiota might precede clinical HT onset, which could be pivotal in pathogenesis. Future studies are needed to evaluate whether the microbiome composition differences are related to HT clinical course.

INTRODUCTION

Hashimoto's autoimmune thyroiditis (HT), characterized by the progressive destruction of thyroid hormone-producing thyrocytes, is the most common autoimmune endocrine disorder, affecting ~5% of the population in industrialized Europe.[1] The prevalence of HT has increased worldwide, including in The Netherlands, which has risen from 2.3% in 2011 to 2.9% in 2020.[2] The tissue destruction of the thyroid may ultimately lead to deficiency of triiodothyronine (T3) and thyroxine (T4), reflecting reduced thyroid function, which is defined as overt or clinical hypothyroidism. Since thyroid hormones affect many physiological processes, thyroid gland dysfunction has multiple clinical manifestations, including growth retardation, constipation, and an increased risk for metabolic and cardiovascular disease.[3,4] The clinical onset of HT is preceded by high serum concentrations of autoantibodies targeting thyroid peroxidase (TPOAb),[3,5] which have been linked to an increased risk of developing thyroiditis6.

The loss of immune self-tolerance to thyrocyte antigens is driven by a combination of genetic susceptibility and environmental exposures. However, HT penetrance in monozygotic twins[7] is concordant in only 55% of cases, underscoring the importance of environmental factors in HT development, and genetics alone cannot explain the global increase in HT prevalence. Given its high prevalence and the knowledge gaps about specific environmental factors involved in HT onset, better understanding of HT pathogenesis is needed. Since the intestinal microbiota plays an important role in the induction, training, and functioning of innate and adaptive immunity [8–11], alterations in the crosstalk between the gut microbiome and host immunity could be pivotal in autoimmune disease pathogenesis.[8,12–14]

Prior cross-sectional studies have reported perturbations in the microbiota composition of HT patients, characterized by reduced bacterial diversity[15–19], with correlations with clinical thyroid function parameters.[18,20] However, because those patients already had clinically significant hypothyroidism, it was unclear whether the microbial abnormality preceded the disease or vice versa. Thus, the nature of the microbiome structure prior to disease onset has remained unstudied. Moreover, the prior studies were conducted in small numbers of HT patients, included levothyroxine-treated patients, and usually did not account for patient ethnicity and geography, which significantly affect gut microbiome composition, as shown in the multi-ethnic HEalthy Life In an Urban Setting (HELIUS) cohort.[21] However, serum thyroid antibody concentrations differ between ethnic groups[22] and persons of Western descent are more susceptible to autoimmune diseases than other ethnicities.[23,24]

To address these issues, we now examine the relationship between gut microbiota composition and autoimmune thyroiditis in 1,468 participants of European Dutch,

Moroccan, and Turkish descent who were clinically euthyroid from the multi-ethnic HELIUS cohort.[25] These subjects differed in whether or not they had anti-thyroid peroxidase autoantibodies with concomitant differences in the likelihood of developing clinical hypothyroidism.

METHODS

Detailed descriptions of the methodology are listed in the Supplemental Methods section.
The cross-sectional data used were obtained during baseline visits of the prospective multi-ethnic HELIUS cohort study.[25] Based on the availability of fecal 16S rRNA sequencing data, a total of 1,468 European Dutch, Moroccan, and Turkish participants ≥35 years old were included in this study. Excluding criteria are shown in **Fig. S1**.

Blood samples were collected during morning study visits under fasting conditions. Reference values ranged from 0.5-5.0 mU/L for TSH and 12-22 pmol/L for fT4; TPOAb serum levels <30 kU/L were considered negative5, whereas TPOAb serum levels ≥60 mU/L were considered as positive.

Fecal bacterial compositions were profiled by sequencing the V4 region of the 16S rRNA gene on an Illumina MiSeq platform (Illumina RTA v1.17.28; MCS v2.5, San Diego, CA, USA). Statistical analyses were performed in the R statistical framework (v. 4.0.3, R Foundation for Statistical Computing, Vienna, Austria). Microbial data were summarized to phylum level. The four most abundant phyla were analyzed using MaAsLin2 (Microbiome Multivariable Associations with Linear Models) in R (package v. 1.8.0)[26], with age, sex, BMI, smoking, and ethnicity as covariates. LOG transformation, no normalization, the LM analysis method, and no filter for abundance or prevalence were used as settings for MaAsLin2.

RESULTS

Sex-specific, but comparable ethnic differences in thyroid markers distribution.

The majority of all 1,468 participants, in whom thyroid markers were analysed, were of European Dutch descent (57.2%), followed by individuals of Moroccan (26.1%) and Turkish descent (16.8%). There were no differences in the distribution of concentrations of the serum thyroid markers TSH, fT4, and TPOAb between these three ethnic groups (**Fig. 1**). Serum TSH and TPOAb levels were significantly positively correlated with each other in European Dutch and Moroccan but not in Turkish participants (**Fig 2A**); in contrast, serum TSH and fT4 levels showed inverse correlations in all three ethnic groups (**Fig. 2B**).

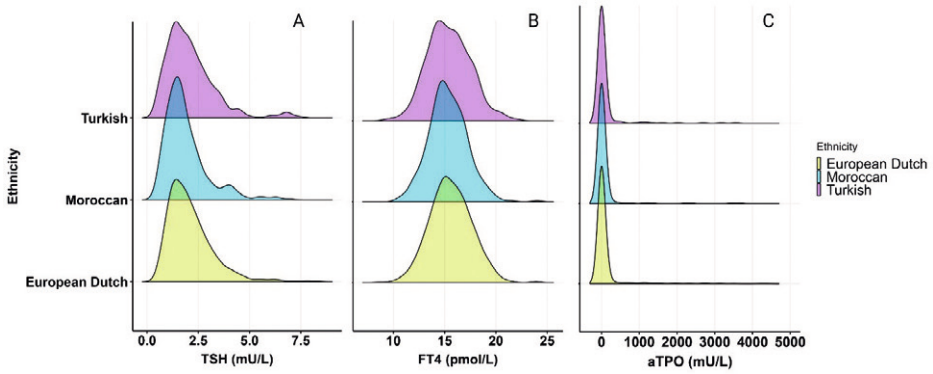

Figure 1. Thyroid markers are equally distributed among the 246 Turkish, 383 Moroccan, and 839 European Dutch participants.

(A). in TSH in mU/L
(B). in FT4 in pmol/L;
(C). in TPOAb in mU/L.
European Dutch participants (N=839) are represented in purple; Moroccan participants (N = 383) are represented in green, and Turkish participants (N = 246) are represented in blue.

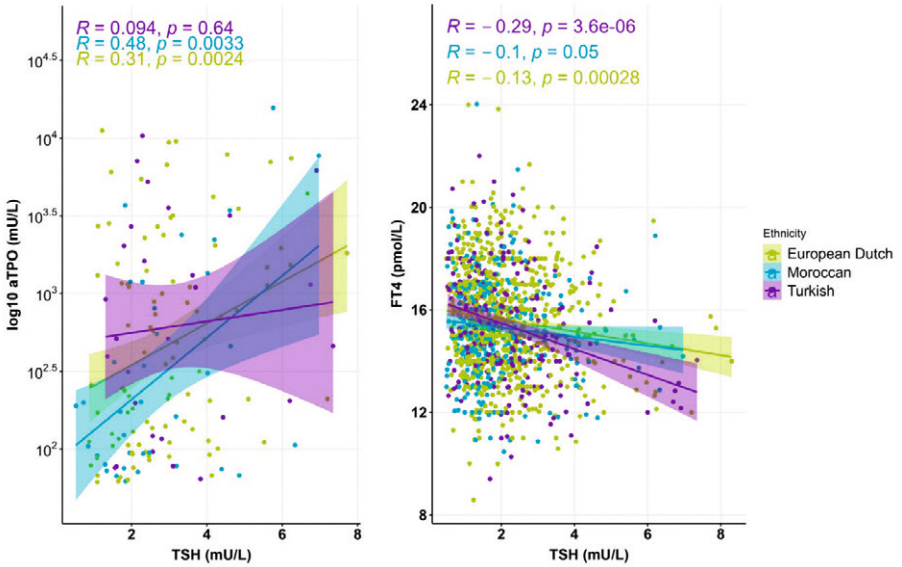

Figure 2. Significant correlation between the concentration of serum thyroid makers among the three different ethnicities.

(A). There is a significant correlation between TSH (mU/L) and TPOAb;
(B). There is a significant correlation between TSH (mU/L) and FT4 (pmol/L)
European Dutch participants (N=839) are represented in purple; Moroccan participants (N = 383) are represented in green, and Turkish participants (N = 246) are represented in blue.

Of the 1,468 participants, 1,309 had no detectable serum levels of TPOAb (**Table 1**). As expected[27,28], the prevalence of high TPOAb levels was greater in women than men (13.9% vs. 8.2%, $p < 0.001$). TPOAb-positive participants had significantly higher serum TSH (median 2.56 vs. 1.71 mU/L respectively, $p = < 0.001$) and lower serum fT4 (mean 15.1 vs. 15.5 pmol/L respectively, $p = 0.010$) levels compared to TPOAb-negative individuals. No significant differences were found in anthropometric parameters, medication use, and metabolic profile between TPOAb-negative and TPOAb-positive participants. Similarly, dietary intake, including total calories (kcal/day), carbohydrates (g/day), fiber (g/day), protein (g/day), and fat (g/day), was similar between the two groups.

TPOAb-seropositivity is not a differentiating factor of gut microbiota composition and diversity, but ethnicity is.

There were no differences in alpha diversity of the fecal microbiota between the two groups in analyses with all six metrics used (**Fig. 3A**). Moreover, the microbiome population structure of seropositive and seronegative participants was overlapping; for example, analysis of Aitchison distance, a beta-diversity metric, showed no significant difference between the two groups (**Fig. 3B)**, indicating that TPOAb presence per se is not a major contributing factor to gut microbial composition (**Table S1**). Other indices of beta-diversity (Bray-Curtis index, Jaccard index, weighted and unweighted UniFrac distances) corroborated no significant difference between the seropositive and seronegative groups (**Fig S2**). In addition, none of the beta-diversity indices were significantly different for dispersion (data not shown), indicating homogenous variation in the gut microbiome composition within each group. Thus, the overall ecological parameters of the two groups were indistinguishable in both composition and dispersion. Nevertheless, alpha and beta diversity analyses demonstrated that ethnicity is one of the most significant differentiating factors of gut microbial composition (2.3%, $R^2 = 0.02$, **Table S1**).

Table 1. Patient characteristics of 1,468 HELIUS participants

Characteristics	TPOAb-negative	TPOAb-positive	p-value
N	1,309	159	
Female	574 (43.9)	93 (58.5)	**0.001**
Age (years)	52.00 [45.00, 60.00]	51.00 [47.00, 59.00]	0.611
Ethnicity			0.577
- European Dutch	744 (56.8)	95 (59.7)	
- Turkish	218 (16.7)	28 (17.6)	
- Moroccan	347 (26.5)	36 (22.6)	
Smoking (yes)	269 (20.6)	27 (16.8)	0.306
BMI (kg/m^2)	27.15 (4.70)	27.26 (4.56)	0.794
Sys BP (mmHg)	127.90 (17.47)	127.29 (17.61)	0.678
Dia BP (mmHg)	80.30 (10.36)	79.44 (10.72)	0.326
Serum thyroid hormone markers			
TPOAb (kU/L)	0.00 [0.00, 0.00]	313.60 [106.10, 1375.50]	**<0.001**
TSH (mU/L)	1.71 [1.28, 2.40]	2.56 [1.87, 3.76]	**<0.001**
fT4 (pmol/L)	15.54 (2.00)	15.11 (1.96)	**0.010**
Metabolic profile			
Total cholesterol (mmol/L)	5.19 (0.97)	5.20 (1.03)	0.861
HDL (mmol/L)	1.42 (0.45)	1.49 (0.46)	0.106
LDL (mmol/L)	3.22 (0.87)	3.17 (0.92)	0.443
Triglycerides (mmol/L)	1.21 (0.81)	1.22 (0.90)	0.846
Diabetes (N)	118 (9.0)	13 (8.2)	0.839
Waist (cm)	95.58 (12.64)	95.58 (12.64)	0.345
Hip (cm)	102.86 (8.39)	103.27 (7.42)	0.526
WHR (waist-hip-ratio)	0.93 (0.09)	0.91 (0.09)	0.063
Antidiabetic drugs (N)	74 (5.7)	10 (6.3)	0.884
Lipid-lowering drugs (N)	166 (12.7)	17 (10.7)	0.555
Antidepressants (N)	62 (4.7)	6 (3.8)	0.728
Dietary intake			
Total caloric intake (kcal/day)	2289.29 (850.15)	2233.90 (826.28)	0.695
Carbohydrate intake (g/day)	242.65 (106.28)	242.65 (106.28)	0.818
Fibre intake (g/day)	26.28 (11.13)	26.77 (11.49)	0.791
Protein intake (g/day)	91.21 (34.52)	92.26 (35.72)	0.855
Fat intake (g/day)	85.88 (36.33)	83.02 (33.39)	0.635

For normally distributed parameters, data are presented as mean ± SD, and p values were calculated using Student's t-test. For non-normally distributed parameters, data are presented as median [IQR], and the p-value was calculated using the Mann-Whitney U test. Nominal variables are presented as n (%).

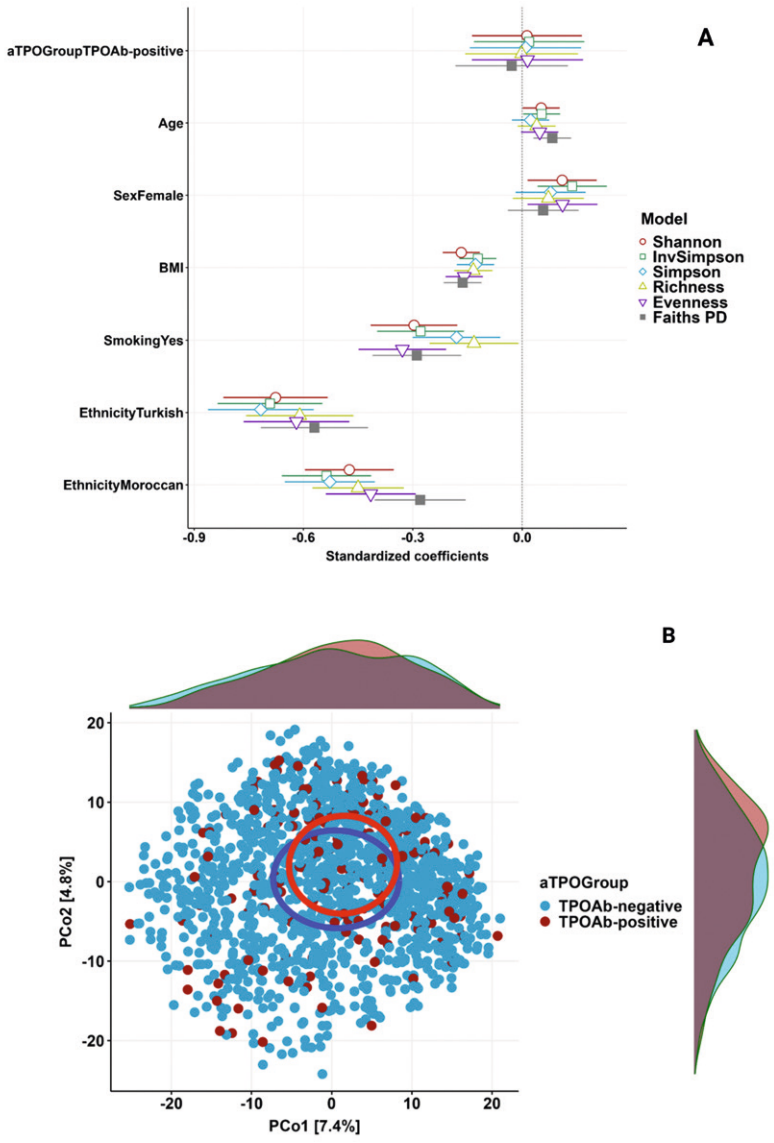

Figure 3. TPOAb presence is not a major contributing factor in gut microbiome diversity

(A). Multivariable linear regression analysis of six alpha diversity indices (Shannon index, Inverse Simpson index, Simpson index, Richness, Evenness, Faith's Phylogenetic Diversity) (N=1,468), including covariates age, gender, BMI, smoking, and ethnicity.

(B). Microbial compositional differences between TPOAb-negative and TPOAb-positive participants (beta-diversity) are visualized using principal coordinate analysis (PCoA) of Aitchison distance. The first two principal coordinates (PCo1 and PCo2) are plotted. The variance explained by the PCoAs is indicated in the parentheses on the axes. (N=1,468). Ellipses show the confidence interval of a multivariate t-distribution at a 30% confidence level. P-value = 0.39.

Specific gut microbial taxa and functional pathways are significantly associated with TPOAb status.

Applying the MaAsLin2 (Microbiome Multivariable Associations with Linear Models26, software version 2) algorithm with confounder adjustment (age, sex, BMI, ethnicity, and smoking), we found 138 nominally significant taxa associated with TPOAb status **(Table S2, visualized at family level Fig. 4).** A total of seven families were consistently and positively associated with the presence of TPOAb at the nominal p-value level of significance. The taxa with the highest effect-size coefficients belong to the *Desulfovibrionaceae* family, followed by *Acidaminococcaceae, Mollicutes RF39, Flavobacteriales, Corynebacteriaceae, Defluviitaleaceae,* and *Coriobacteriaceae.* In contrast, five taxa from three families had consistently negative associations with TPOAb-presence (*Erysipelotrichaceae* and *Clostridiales vadin BB60 group*). The family with the highest negative coefficient was the *Clostridiales vadinBB60 group.* Three taxa of the *Erysipelotrichaceae* had a nominally significant association with TPOAb absence. The families *Prevotellaceae*, *Christensenellaceae, Ruminococcaceae, Lachnospiraceae, Family XIII*, and *Veillonellaceae* consisted of multiple nominally significant taxa with abundances pointing in opposing directions. No taxa were significantly associated with TPOAb status after correcting for multiple testing using Benjamini-Hochberg q-values (adjusted p-value > 0.05**, Table S2**).

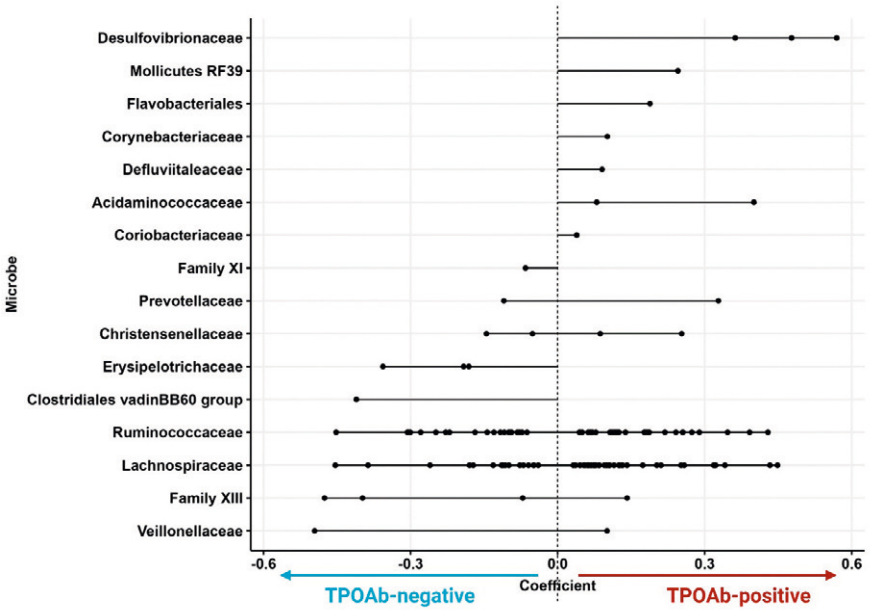

Figure 4. **Microbial taxa associated with TPOAb-status**

Lollipop chart showing the discriminating families (or when family was not annotated; orders) between TPOAb-positive versus TPOAb-negative participants. Each dot represents a differentially significant abundant taxon (N=138 individual taxa) at a certain phylogenetic level (ASV, genus, family), which are then grouped by the family level (each row). The direction of the line indicates the phenotype association (positive or negatively associated with TPOAb-presence). The line length indicates the effect size (coefficient) with corresponding SE. Two significant lineages, *Mollicutes RF39* and *Flavobacteriales,* are not annotated on the family level, and are shown on the order level. Exact p-values are stated in Table S2.

To substantiate the findings of MaAsLin2, we applied three additional differential abundance (DA) tools (ALDEx2, CLR transformed Wilcoxon test and ANCOM-BC). All methods showed similar results (**Table S3**); thirteen taxa were nominally significant in all four DA methods (**Fig. 5A and B**), but no significant changes in microbial abundances between the two groups were observed after adjusting for multiple testing.

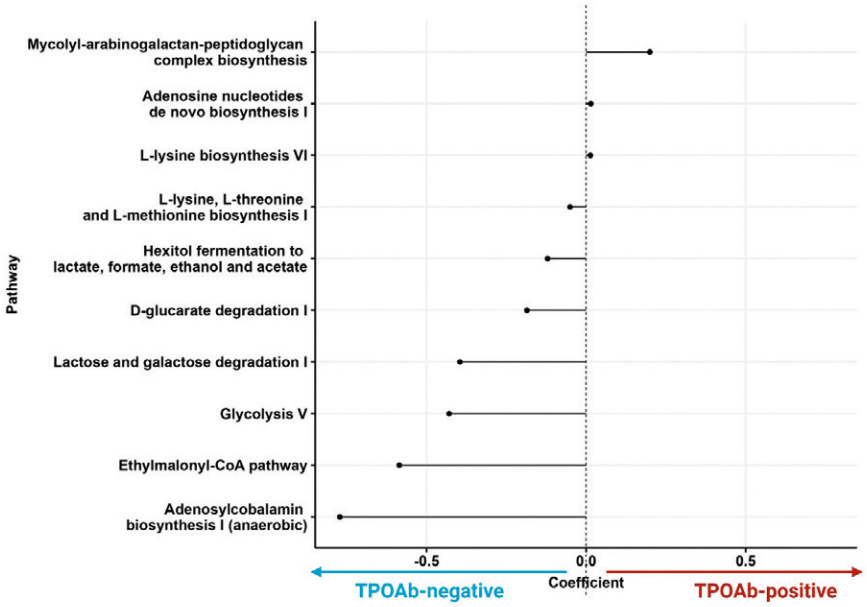

Figure 5. Microbial pathways associated with TPOAb-status

Lollipop chart showing the discriminating microbially expressed pathways as imputed by PICRUSt2 between TPOAb-positive versus TPOAb-negative participants. Each dot represents one of the 10 differentially significant abundant pathways. The direction of the line indicates the phenotype association (3 positive and 7 negatively associated with TPOAb-presence). The line length indicates the effect size (coefficient). P-values for each pathway are in Table S3.

We also investigated the differential expression of predicted microbial pathways, as predicted by PICRUSt2, in participants with or without TPOAb. At the nominal significance level, we found multiple pathways to be differentially abundant between the TPOAb groups (**Fig. 6 and Table S4**). Several pathways such as D-glucarate degradation (glucardeg PWY), lactose and galactose degradation (lactosecat PWY), glycol metabolism and degradation (glycol glyoxdeg PWY), glycolysis V, ethymalonyl-CoA, and adenosylcobalamin biosynthesis I pathways were all decreased in TPOAb-positive participants. Three pathways were increased in TPOAb-positive participants, of which the mycolyl-arabinogalactan-peptidoglycan complex biosynthesis pathway (PWY 6397) had the highest effect size. No microbial pathway was significantly associated with TPOAb status after correcting for multiple testing using Benjamini-Hochberg q-values (adjusted p-value > 0.05).

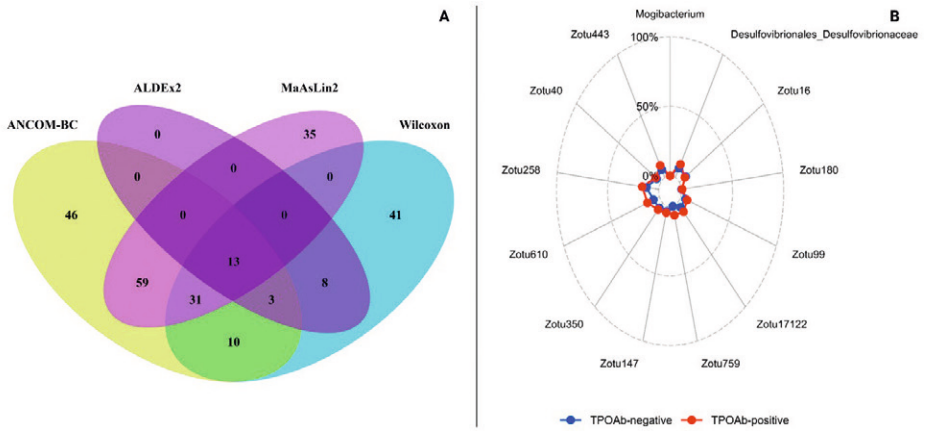

Figure 6. Comparing MaAsLin2 results with other DA tools identified thirteen nominally significant overlapping taxa.

(A). The differential abundances (DA) of the MaAsLin2 were compared to those of ALDEx2, CLR transformed Wilcoxon test, and ANCOM-BC DA metrics to verify the DA findings of our primary MaAsLin2 tool. In total, thirteen taxa were nominally significant in all four metrics used.
(B). Radar chart showing the abundance of the thirteen overlapping taxa that were nominally significant in all four DA metrics (ZOTU: Zero-radius operational taxonomic unit).

DISCUSSION

This study aimed to determine whether structural and/or functional gut microbiota perturbations occur in the early stages (seroconversion) of autoimmune thyroiditis. Given the lack of studies investigating the microbial pattern prior to disease onset, our study constitutes a step forward to better understanding the potential contribution of the gut microbiome in the pathogenesis of autoimmune thyroiditis. The main results from our large multi-ethnic population-based cohort study suggest that euthyroid persons with TPOAb (prior to the clinical onset of autoimmune thyroid disease) exhibit modest gut microbial variation at the taxonomic level compared to those without these antibodies, although no significant differences in global alpha and beta diversity were found. Based on our discovery analysis, 138 gut microbiota taxa were nominally associated with TPOAb status, independent of age, sex, BMI, ethnicity, and smoking. Of these, none passed multiple testing correction, though 13 taxa were found consistently across four separate DA methods. These findings underscore the need for a better understanding of the roles of altered gut microbiota composition in the pathophysiology of autoimmune diseases, including hypothyroidism. Future research is needed to effectively evaluate whether new gut microbiome-based diagnostics, preventives, or therapeutics for autoimmune hypothyroidism would reduce the need for the current replacement with daily oral administration of synthetic thyroid hormone (levothyroxine) after the thyroid gland has sustained substantial damage.

Similar to prior cohort studies in adults[5,27], 10.8% of the 1,468 euthyroid participants in the HELIUS cohort had TPOAb serum antibodies. The observed difference in the proportion of TPO-positivity between men and women reflects the well-known sex difference, as HT is four to eight times more common in women than in men.[27,28] Variance in biomarker values has been noted between different ethnic groups[29], highlighting the importance of ethnicity-specific reference intervals for biomarkers. Our study did not find any differences in serum thyroid markers or thyroid autoimmunity between these three ethnic groups living in an iodine-replete area. However, consistent with prior analyses of multi-ethnic population studies[21], we show that ethnicity is an important correlate with microbiome diversity.

A growing body of evidence has shown that gut microbiota dysbiosis may be a marker of several autoimmune diseases, including type 1 diabetes (T1D), inflammatory bowel disease, and rheumatoid arthritis.[9,30–32] However, studies on the association between autoimmune thyroiditis and gut microbiome composition have been limited and have yielded conflicting results.[14,16–20,33–35] This may reflect small sample sizes with heterogeneous groups and varying definitions of autoimmune thyroiditis.[35] For example, two studies investigated the differences in intestinal microbiota in hypothyroid participants without detectable TPOAb serum levels,[16,33] thus including non-autoimmune causes of hypothyroidism. The conflicting outcomes observed in prior studies may reflect thyroid status differences of the subjects. Whereas some studies included only euthyroid HT patients using levothyroxine (LT4)[19,20], others included both euthyroid and hypothyroid HT patients[16–18], or solely included hypothyroid (medication-naïve) patients with autoimmune thyroiditis.[33,34] Such methodological differences make comparison of results difficult; for example, the impact of LT4 use on gut microbiota composition is currently unknown. Many of these studies were conducted in China [16,20,33,34] and might not generalize to other populations as ethnicity and patient geography are important covariates of the gut microbiome composition.[21] In conclusion, it remains difficult, if not impossible, to validate our results with previously published cohorts.

The fecal taxa most predictive of TPOAb-positive status belonged to the family *Desulfovibrionaceae* and was found nominally significant in analyses with all four DA tools. While prior studies on the gut microbiome composition of patients with HT did not report on the abundance of the *Desulfovibrionaceae* family, higher abundances of *Desulfovibrio,* a genus within this family, have been inversely associated with other autoimmune diseases such as T1D[36]; *Desulfovibrio piger* presence in duodenal biopsies predicted preserved beta-cell function in T1D patients, consistent with a protective effect.[36]

Several taxa within the same family (*e.g.,* the families *Prevotellaceae, Christensenellaceae,* Family *XIII, Ruminococceae,* and *Lachnospiraceae)* had bidirectional associations with TPOAb-presence. These findings reflect the

heterogeneity of these taxonomic families, which substantially vary in genetic content and, thus function. Considering the large effect size and high average abundance of two taxa belonging to the same family (e.g., two *Veillonellaceae* taxa*)*, those might represent two separate species having opposing effects on HT pathogenesis. Our imputation of pathways using PICRUSt2 did not permit discrimination between the genetic functions of these microbes. Accordingly, differences within major taxa might also explain the discordances found in prior studies[14,16–20,33,34] but do not rule out a role in HT pathogenesis.

In all four DA tools, seven of the thirteen taxa that were found nominally significant belonged to the *Lachnospiraceae* family, while four belonged to the Ruminococcaceae family. *Lachnospiraceae* are obligate anaerobe core members of the gut microbiota that colonize the host's intestinal tract from birth and increase in abundance throughout life. They are characterized by their production of short-chain fatty acids and other metabolites, such as vitamin B12 and alcohol, and are therefore considered 'beneficial' bacteria with anti-inflammatory, immunostimulatory, and homeostasis-maintaining effects.[37,38] A recent meta-analysis showed that the percentage of *Lachnospiraceae* was higher in autoimmune thyroid disease patients (24.3 [95%CI -0.026 to 0.512]) compared to healthy controls (17.8% [95%CI 0.040 to 0.316]).[14] This finding had a significant effect size (Z=2.89, p=0.004), and the abundance ratio between HT (30.3%) and controls (21.6%) was 1.40. Our results were comparable to those of previous studies, as all nominally significant *Lachnospiraceae* members had higher abundance in TPOAb-positive participants except for *Agathobacter* [Zotu16], a high butyric acid producer.[37] Further research is warranted to explore if and how changes in the abundance of *Lachnospiraceae* could contribute to HT's pathogenesis.

Functional profiling of gut microbial pathways showed a decrease in the D-glucarate degradation pathway in the TPOAb-positive participants. D-Glucaric acid is the end-product of D-glucarate degradation, serving as an energy source for certain bacteria[39], and is a potent β-glucuronidase inhibitor.[40] β-glucuronidase promotes the reabsorption of free thyroid hormones into the enterohepatic circulation by hydrolyzing conjugated iodothyronines.[41,42] It is unknown whether this observed decrease in the D-glucurate degradation pathway affects (conjugated) iodothyronine concentrations. Another bacterial pathway significantly reduced in the seropositive participants contributes to the synthesis of adenosylcobalamin, an active form of vitamin B12. One study[43] found vitamin B12 levels negatively correlated with anti-TPO autoantibodies, and that vitamin B12 deficiency was associated with autoimmune hypothyroidism, although this could not be verified.[44,45] In conclusion, the differently abundant families and functional pathways identified in this study do not indicate a priori immune-regulatory function, thus their significance is uncertain.

Strengths and limitations of the study

This study has several strengths, including its large sample size, inclusion of three ethnicities, and use of the same analytical tests and study instruments across the population. All study participants were euthyroid and did not use levothyroxine at the time of fecal sampling, leading to a highly homogenous cohort without the confounding effect of thyroid treatment. This is the first study to assess the gut microbiome composition in euthyroid TPOAb-positive patients who did not have overt clinical autoimmune thyroid disease. Another notable strength is that we addressed potential confounders since both intrinsic and extrinsic factors modulate gut microbiota composition. Study participants with recent antibiotic use were excluded, and we adjusted our analyses for other relevant confounding factors. Lastly, at the compositional level (alpha- and beta-diversity) and for the DA results, we applied multiple indices, dissimilarity metrics, and methods to substantiate our findings.

One study limitation is its cross-sectional design. To understand the drivers of the gut microbiome profile, it is necessary to follow participants prospectively. Although approximately 10% of the general population is positive for TPOAb, fortunately, not all will develop overt hypothyroidism.[46] The natural course of autoimmune hypothyroidism is marked by slow development over years, evolving from positive serum levels TPOAb with serum thyroid hormone levels remaining within the reference range (euthyroidism), evolving to subclinical and eventually overt hypothyroidism. The risk of developing overt hypothyroidism over 13 years for euthyroid TPOAb-positive women was 55.2% (37.1–73.3%).[47] Longitudinal data are thus needed to investigate whether the differences in microbiome composition are indeed related to progression to overt hypothyroidism. Such longitudinal cohorts also will be better suited for studying the effect of LT4 supplementation on microbiome composition.

As this is a hypothesis-generating study, we report results at the nominal significance level; however, this means that the results may be false positive findings, and all associations require validation in independent cohorts. Currently, multiple DA tools are available, and they are frequently used interchangeably, resulting in inconsistent findings that may hinder comparability and reproducibility across studies.[29] To address this issue, a consensus approach or standardized guideline is necessary for analyzing complex microbiome data. Hence, we employed multiple DA methods and focused on significant features identified by most tools to increase our results' robustness.

Furthermore, the microbial functional pathways were imputed using PICRUSt2, which is based on reference genomes for the 16S gene region sequenced; this is less accurate than shotgun metagenomic sequencing, which provides the actual genetic profile of the fecal microbiome.[48] Finally, the distribution of TPOAb is skewed towards zero, as

1,309 participants showed no detectable anti-TPO antibodies. Despite reporting the largest cohort of HT participants, this study still has low statistical power due to the high heterogeneity of the human gut microbiome and the relatively low incidence of TPOAb in the population.

CONCLUSIONS

Neither by global ecological patterns nor by microbial taxa or functional pathway level did we find robust evidence of gut microbiome differences in relation to TPOAb status. However, we report several nominally significant taxa associated with TPOAb presence, thirteen concordant across four DA methods. Future clinical prospective studies and translational animal investigations are warranted to elucidate the potential contribution of the identified taxa in relation to susceptibility to HT onset and/or accelerating disease progression.

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AUTHOR CONTRIBUTIONS

Conceptualization, supervision, project administration, M.N. Methodology, software, formal analysis, visualization, writing—original draft, A.C. and U.B. Resources, B.-J.v.d.B. and H.G. Data curation, U.B and H.G. Writing—review and editing, A.C., U.B., D.C., A.v.d.S, E.R, B.-J.v.d.B., H.G., A.H.Z., E.F. M.J.B. and M.N. Funding acquisition, B.-J.v.d.B and M.N.

DECLARATION OF INTERESTS

M.N. is on the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands. None of these are directly relevant to the current paper. There are no patents, products in development, or marketed products to declare. The other authors declare no competing financial interests.

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STAR METHODS

Materials

REAGENT OR RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Human participants from the HELIUS cohort	HELIUS executive board	http://www.heliusstudy.nl/en/over-helius
Deposited data		
16S rRNA gene sequences have been deposited in the European Genome-Phenome archive	N/A	EGAD00001004106; https://ega-archive.org/
Software and algorithms		
R Statistical Computing Software	The R Foundation	https://www.r-project.org
SILVA v. 132 reference database	[53,54]	https://www.arb-silva.de/
PICRUSt2 v. 2.3.0-b	[55]	https://huttenhower.sph.harvard.edu/picrust/
USEARCH (v11.0.667_i86linux64)	[56]	https://www.drive5.com/usearch/
MaAsLin2 package in R (package v. 1.8.0)	[28]	https://www.r-project.org
Other		
Cobas C8000 analyzer	Roche Diagnostics, Basel, Switzerland	https://diagnostics.roche.com/nl/en/products/systems/cobas-8000-analyzer-series-sys-128.html
Kryptor Compact Plus analyzer	BRAHMS Thermo Scientific, Henningsdorf, Germany	https://www.brahms.de/en-gb/products/kryptor-analyzers.html

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact: Max Nieuwdorp, MD, PhD, Department of Internal and Vascular Medicine, Amsterdam University Medical Centres, location AMC, 1105 AZ Amsterdam, The Netherlands. E-mail: m.nieuwdorp@amsterdamumc.nl.

Materials availability

Thyroid markers TSH, FT4, and anti-TPO antibodies were measured using fasted serum samples from participants of the HELIUS cohort. This study did not generate new materials.

Data and code availability

As earlier reported the 16S rRNA gene sequences have been deposited in the European Genome- phenome Archive (accession number EGAD00001004106). The HELIUS data are owned by the AMC in Amsterdam, the Netherlands. Any researcher can request the data used in this study by submitting a proposal to the HELIUS Executive Board as outlined at http://www.heliusstudy.nl/en/researchers/collaboration/.Collaboration and data that does not include confidential patient information will be provided upon reasonable request. In addition to this, this paper analyzes existing publicly available data, which are listed in the key resources table.

METHOD DETAILS

Data collection

The cross-sectional data used were obtained during baseline visits of the prospective multi-ethnic Healthy Life in an Urban Setting (HELIUS) cohort study [25]. Data collection took place between 2011 and 2015. Participants in the age range of 18-70 years were randomly sampled, stratified by ethnic origin (*i.e.* European Dutch, Surinamese, Ghanaian, Moroccan, or Turkish origin), through the municipality register of Amsterdam. A total of 24,788 participants were included. All participants provided written informed consent and the study was approved by the medical ethical review board of the Amsterdam University Medical Center)(Amsterdam UMC), location AMC, and followed the principles of the Declaration of Helsinki (revisions 6 and 7). All participants completed extensive questionnaires regarding sociodemographic information and dietary intake (FFQs, in a subsample of n=5084) amongst others, and during the examinations, anthropometric characteristics, fasting blood samples, and stool samples (in a subsample of n=6048) were gathered [25]. Based on the availability of fecal 16S rRNA sequencing data, a total of 2,110 European Dutch, Moroccan and Turkish participants ≥35 years old were included in this study. After excluding participants who used antibiotics within 3 months before stool sample collection (or with unknown antibiotic use), and participants with subclinical (defined as serum TSH < 0.5 mU/L) or overt hypothyroidism (defined as serum TSH > 5.0 mU/L and fT4 < 12.0 pmol/L) or a borderline increased serum level of TPOAb (between 30 and 60 kU/L), or current use of levothyroxine, leaving a total of 1,496 participants. Since 28 participants had missing values in one or more of the covariates, the sample size for the definitive analyses was 1,468 **(Fig. S1).**

Thyroid markers.

Blood samples were collected during morning study visits under fasting conditions. Serum levels of thyroid stimulating hormone (TSH) and free thyroxine (fT4) were determined by electrochemiluminescence ECLIA using the *Cobas C8000* analyzer (Roche Diagnostics, Basel, Switzerland). Serum levels of TPOAb were determined on TRACE technology with a *Kryptor Compact Plus* analyzer (BRAHMS Thermo Scientific,

Henningsdorf, Germany). Reference values ranged from 0.5-5.0 mU/L for TSH, from 12-22 pmol/L for fT4; TPOAb serum levels <30 kU/L were considered negative[5], whereas TPOAb serum levels ≥60 mU/L were considered as positive.

Profiling of fecal microbiota composition

In stool samples, collected as described [25], DNA was extracted and purified from a 150 mg aliquot of fecal samples using a repeated bead-beating protocol [54]. Bacterial compositions were profiled by sequencing the V4 region of the 16S rRNA gene on an Illumina MiSeq platform (Illumina RTA v1.17.28; MCS v2.5, San Diego, CA, USA) (2 × 250 bp paired-end reads). PCR was performed in duplicate reactions, using a reaction mixture containing 1x Five Prime Hot Master Mix (5PRIME GmbH), a total of 400 nM of reverse and forward primers, 0.4 mg/mL bovine serum albumin (BSA), 5% dimethylsulfoxide, and 20 ng of genomic DNA (total volume of 25 µL).

Pre-processing of the raw sequencing data was performed as described [21], using USEARCH (v11.0.667_i86linux64, [53]) for the raw sequence reads. Amplicon Sequence Variants (ASVs) were obtained after merging paired-end reads, quality filtering, dereplication of contigs, and denoising of unique sequences (using UNOISE3). The function assignTaxonomy from the dada2 R package (v. 1.12.1) to allocate taxonomy using the SILVA (v. 132) reference database.[50,51] The ASV table was rarefied to 14,932 counts per sample and sequenced samples with <5,000 counts were excluded. The final dataset contained 6,032 samples and 22,532 ASVs, which was subset as described above in the section Data collection.

Characteristics of gut microbiota composition

Statistical analyses were performed in the R statistical framework (v. 4.0.3, R Foundation for Statistical Computing, Vienna, Austria).

Alpha and beta diversity

For all analyses of microbial alpha and beta diversity, age, sex, ethnicity, BMI, and smoking status (“Yes currently” or “No”) were included as covariates, based on the methodology previously specified[25]. To assess alpha diversity, we calculated the richness (‘specnumber’ vegan v.2.5.7 R package’), Shannon index, Simpson index, inverse Simpson index (‘diversity’ vegan v.2.5-7 R package), evenness (Shannon index/log(richness)) and Faith’s Phylogenetic Distance (‘pd’ picante v.1.8.2 R package) at the ASV level.

Multivariable linear regression of alpha diversity indices was tested using ‘lm’ in R with the above-mentioned covariates, with TPOAb presence as outcome. For beta-diversity, the Jaccard index, Bray-Curtis dissimilarity (function ‘vegdist’ vegan v. 2.5-7 R package), Aitchison distance (based on rarefied count data with a pseudocount of 1, with ‘clr’ from the chemometrics v.1.4.2 R package, followed

by vegdist(method='euclidean')), and weighted and unweighted UniFrac distances (function 'UniFrac' phyloseq v 1.30.0 R package; [55]) were calculated. Beta diversity was tested with a Permutational multivariate analysis of variance (PERMANOVA) (function 'adonis2' vegan v. 2.5-7 R Package, Distance ~ Age + Sex + BMI + Smoking + Ethnicity + Outcome (TPOAb presence), permutations = 1000) as well as with an anova of the betadisper (function 'betadisper' vegan v. 2.5-7 R Package).

Phyla abundance
The microbial data were summarized to phylum level, and the four most abundant phyla were analyzed using MaAsLin2 (Microbiome Multivariable Associations with Linear Models) in R (package v. 1.8.0)[26], with age, sex, BMI, smoking, and ethnicity as covariates. We used LOG transformation, no normalisation, the LM analysis method, and no filter for abundance or prevalence as settings for MaAsLin2.

Next, we analyzed differences at different taxa levels. The microbial data were summarized to family, genus, and ASV levels. All of these taxa were filtered using the nearZeroVar function from the caret package in R (v. 6.0-92), using the parameters uniqueCut = 5 and freqCut =150/5. Finally, highly correlated taxa were removed using the findCorrelation function from the caret package in R, using a Spearman correlation cutoff of 0.9. The taxa were transformed to relative abundance, for each taxonomic level. In the final analysis, 2,665 different taxa were used, including 134 at genus level, 38 at family level, and 2,493 were ASVs. We used the LM analysis method from MaAsLin2 (multivariable linear regression) on each taxon with TPOAb status as outcome, using CLR normalization and no transformation. Results were visualized by depicting the MaAsLin2 calculated effect size of each taxon, summarized at family level. Additionally, the taxa were tested using a Wilcoxon test of the CLR (with 1 pseudocount of the raw count data) transformed abundances. Finally, the ANCOM-BC method was applied on the raw counts, with the same confounders as described previously. The raw counts were input to aldex2, with aldex.clr function used with the parameter mc.samples=100, otherwise default parameters. The function aldex.kw was used with default parameters to test the differential abundance. Multiple testing correction was calculated in R using p.adjust with BH method, for all taxa (2,665) for each method.

Functional profiling
To generate functional profiling of gut microbial pathways, the 16S sequence reads were used as input for Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt2, v. 2.3.0-b; [52]) with default settings. The resulting functional pathways were mapped using the MetaCyc ontology database. Based on this, nearZeroVar with uniqueCut=5 was used to filter pathways, resulting in 351 pathways, before they were converted to relative abundance. The same MaAsLin2 tool was used to predict which pathways were differentially abundant between

participants with present or absent TPOAb. The following settings were used for MaAslin2; no normalization, log transformation, and LM analysis method.

SUPPLEMENTAL INFORMATION

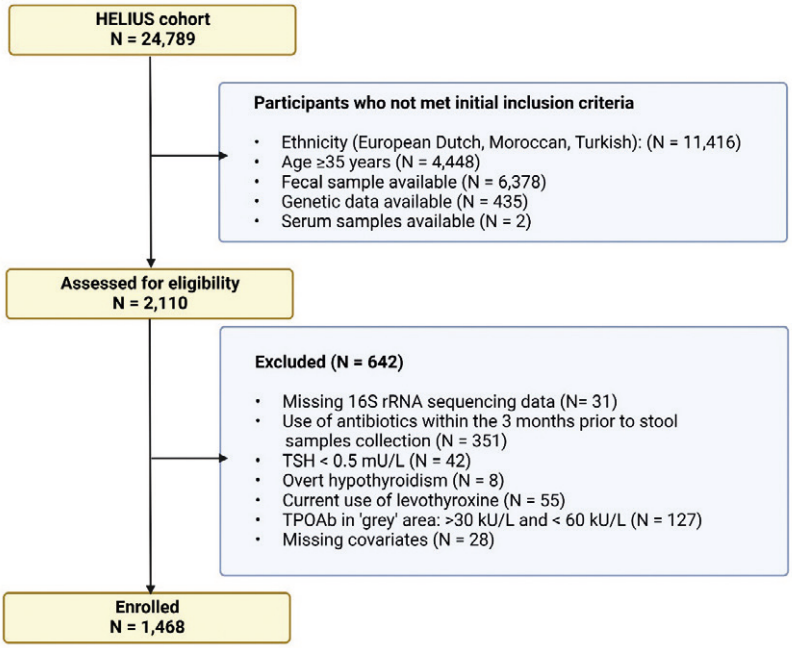

Figure S1. Flowchart of HELIUS participants assessed for eligibility, who were included in the study and analyzed.

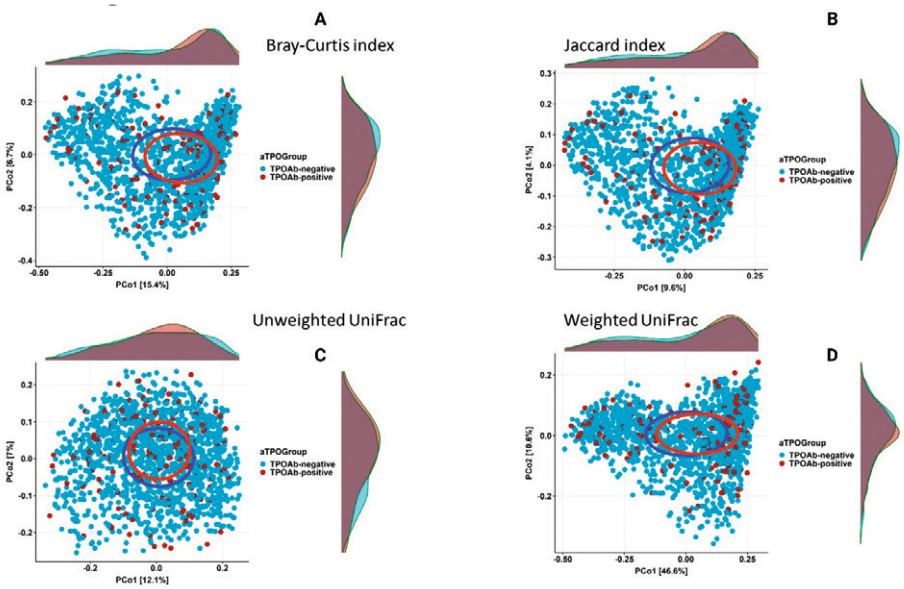

Figure S2. Principal coordinate analyses of the beta-diversity indices Bray-Curtis dissimilarity index (A), Jaccard (B), unweighted (C), and weighted (D) UniFrac. The first two principal coordinates (PCo1 and PCo2) of each index are plotted. The variance explained by the PCOs is indicated in the parentheses on the axes.

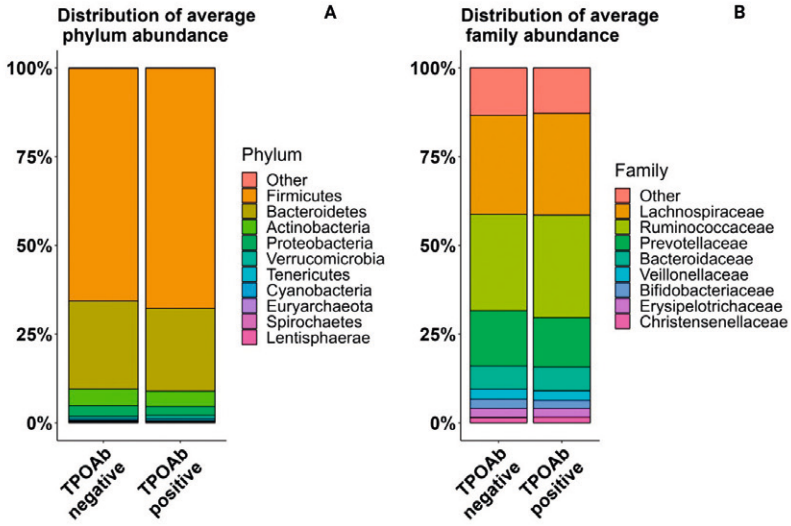

Figure S3. No taxonomical differences in the gut microbiota composition between TPOAb-negative and -positive participants. The taxonomical differences in the gut microbiota composition between TPOAb-negative and TPOAb-positive participants are shown by the relative abundance of phyla (A) and family (B).

Table S1. TPOAb presence is not a major contributing factor to gut microbial composition, but ethnicity is.
Multivariable PERMANOVA analysis to identify variation (R^2) in microbial beta-diversity (Aitchison distance) explained by study characteristics. Explained variance (%) is calculated by R^2 multiplied by 100.

Table S2. Microbial taxa that are nominally significantly different between TPOAb-positive and TPOAb-negative participants.
Multivariable MaAsLin2 modeling of the association between the 138 nominally significant microbial taxa and the presence of TPOAb. Beta-coefficients (β) represent the effect size for the difference in abundance between TPOAb-positive vs. TPOAb-negative participants with model-based standard errors of the mean (SEM) and nominal p-values. Q-values are presented as corrections for multiple testing (via MaAsLin "default").

Table S3. Differences in the functional pathway of the microbial taxa between TPOAb-positive and TPOAb-negative participants.
The functional pathways of the microbial taxa that discriminated between the 159 TPOAb-positive and 1,309 TPOAb-negative participants were predicted using the MetaCyc ontology database. Beta-coefficients (β) represent the effect size for the difference in abundance between TPOAb-positive vs. TPOAb-negative participants with model-based standard errors of the mean (SEM) and nominal p-values. Q-values are presented as corrections for multiple testing (via MaAsLin "default").

Table S4. Comparing MaAsLin2 results with other DA tools identified thirteen nominally significant overlapping taxa.
The differential abundances (DA) of the MaAsLin2 were compared to those of ALDEx2, CLR transformed Wilcoxon test, and ANCOM-BC DA metrics. Beta-coefficients (β) represent the effect size for the difference in abundance between TPOAb-positive vs. TPOAb-negative participants with model-based standard errors of the mean (SEM) or standard error (SE) and nominal p-values. Q-values are presented as corrections for multiple testing. Additionally, the Kruskal-Wallis tests are shown for ALDEx2, and the statistic W is shown for ANCOM-BC and Wilcoxin (CLR) DA metrics.

7

PROTOCOL FOR A RANDOMIZED, DOUBLE–BLINDED, PLACEBO–CONTROLLED TRIAL TO ASSESS THE EFFECT OF FECAL MICROBIOTA TRANSPLANTATIONS ON THYROID RESERVE IN PATIENTS WITH SUBCLINICAL AUTOIMMUNE HYPOTHYROIDISM: THE IMITHOT TRIAL

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ABSTRACT

Background

Hashimoto's thyroiditis (HT) is a common endocrine autoimmune disease affecting roughly 5% of the general population and involves life-long treatment with levothyroxine, as no curative treatment yet exists. Over the past decade, the crosstalk between gut microbiota and the host immune system has been well-recognized, identifying the gut microbiome as an important factor in host health and disease, including susceptibility to autoimmune diseases. Previous observational studies yielded a link between disruption of the gut microbiome composition and HT. This is the first study that investigates the potential of restoring a disrupted gut microbiome with fecal microbiota transplantations (FMTs) to halt disease progression and dampen autoimmunity.

Methods and analysis

The IMITHOT trial is a randomized, double-blinded, placebo-controlled study evaluating either autologous or allogenic FMTs in medication-naïve patients with subclinical autoimmune hypothyroidism. In total, 34 patients will be enrolled to receive either three allogenic or autologous FMTs. FMT will be made of fresh stool and directly administered into the duodenum. Patients will be evaluated at baseline before the first FMT is administered and at 6-, 12-, and 24-months post-intervention to assess efficacy and adverse events. The primary outcome measure will be the net incremental increase (iAUC) on thyrotropin-stimulated fT4 and fT3 release at 6 and 12 months compared with baseline.
Results will be disseminated via peer-reviewed journals and international conferences. The recruitment of the first patient and donor occurred on 18 Dec 2019.

Ethics and dissemination

Ethics approval was obtained from the hospital Ethics Committee (METC) at Amsterdam University Medical Center (Amsterdam UMC). The results of this trial will provide high-quality evidence to assess the potential of future clinical application of this new therapy.

Trial registration number: NL7931

ARTICLE SUMMARY

Strengths and limitations of this study

- This is the first interventional study that aims to preserve thyroid function by restoring gut microbiota composition in medication-naïve patients with subclinical autoimmune hypothyroidism;
- Each intervention group will undergo three FMTs using either the same donor or participants' own stool to enhance the successful engraftment of the gut microbiota.
- Participants will be followed for two years after the first FMT to study the sustainability of the fecal microbiota engraftment.
- Patients reported outcomes will be measured with the validated Thyroid-related quality of life (ThyPRO) questionnaire.
- This study protocol lacks a control group without FMT intervention (*e.g.,* a group receiving a saline solution instead of fecal suspension).

INTRODUCTION

Hashimoto's thyroiditis (HT), characterized by the progressive destruction of thyroid hormone-producing thyrocytes, is an autoimmune endocrine disorder resulting from genetic susceptibility accompanied by particular environmental factors. HT is the most common form of hypothyroidism in iodine-sufficient areas, affecting roughly 5% of the general population with a female predominance (8:1 female to male ratio)[1].

The natural course of HT is a slow development over years, evolving from positive serum levels of antibodies to thyroid peroxidase (TPOAb) with serum thyroid hormone levels still within reference range (euthyroidism) to subclinical (elevated TSH with normal serum fT4 levels) and eventually overt hypothyroidism (elevated TSH with decreased serum fT4 levels). Approximately 10% of the general population have positive TPOAb serum levels[2,3], but not all of these people will develop overt hypothyroidism. Previous studies have shown that the likelihood of developing overt hypothyroidism significantly rises in subjects with elevated TSH and positive TPOAb serum levels: a twenty-year follow-up study revealed an odds ratio of 28 [95% CI 22-65] and 173 [95% CI 81-370] in this specific group of women and men, respectively [4]. A different study showed that 66.6% of participants (aged 55 years or older) with a TSH level of ≥10 mIU/L progressed to overt hypothyroidism after an average of 18.3 months[5]. Moreover, another study demonstrated a significantly increased relative risk of 15.6 of developing overt hypothyroidism in patients with autoimmune subclinical hypothyroidism with a TSH level of ≥12 mIU/L, with an annual progression rate of 11.4 ±3.0%[6]. Thyroid ultrasound examination may provide additional information in patients with thyroid autoimmunity, as a hypoechoic and inhomogeneous ultrasound pattern with nodules is a risk factor for the development of HT[7,8].

Current treatment of HT consists of life-long hormone substitution therapy with daily oral administration of the synthetic thyroid hormone levothyroxine (LT4). In 2021, over 500.000 people in the Netherlands were using LT4; making it one of the most prescribed medications in patients between 65 and 74 years (3.8%) in the Netherlands[9]. However, approximately 5-15% of euthyroid HT patients receiving LT4 treatment still experience various persistent symptoms, with fatigue being the most significant[10,11]. The interpretation and optimal management of these symptoms are still being determined at present[12]. No curative treatment is available to restore normal thyroid function, as the underlying autoimmune etiology is not fully understood.

The hallmark of HT is the drastic loss of thyroid hormone-producing follicular cells by T cell-mediated autoimmune responses. The loss of self-tolerance against the main autoantigen thyroid peroxidase (TPO) results in infiltration of the thyroid gland by mainly autoreactive T and B cells and is postulated to be driven by an overt activation

of T helper (Th) type 1 (T1) and Th17 cells at the expense of the immunosuppressive activity of regulatory T cells (Treg)[13–16]. This extensive stimulation is followed by a predominantly lymphocytic infiltration of several B cell phenotypes in the thyroid gland with well-defined germinal centers. In contrast, no distinct lymphocytic infiltration was found in the thyroid tissue of healthy controls[13]. Interestingly, while untreated hypothyroid patients had a similar proportion of IL-10+ regulatory B cells (Breg) as healthy individuals[17], euthyroid HT patients who received thyroxine treatment showed an increased proportion of functional Breg cells[19]. Therefore, our research aims to investigate the proportion of T and B cells, including B regulatory cells, in both peripheral blood and thyroid gland tissue obtained through ultrasound-guided fine needle aspiration, to gain a comprehensive understanding of the immune cell dynamics associated with Hashimoto's thyroiditis.

The human gut microbiome (the collective genomic content of microorganisms) consists of 10^{13} to 10^{14} bacterial cells (microbiota). As the gut constitutes the largest immune component in humans (residing up to 70-80% of all immune cells), the gut microbiota, epithelial layer, and mucosal immune system are closely connected[18,20]. The gut microbiome has now been identified as an important factor in the regulation of host health and disease, including the susceptibility to autoimmunity and the production of microbial-derived immunomodulatory metabolites[21–24].

Previous studies[25–31], including a recent systematic review[32], have linked an altered intestinal microbiota composition (dysbiosis) to HT pathophysiology. However, these studies were primarily cross-sectional and observational in nature[33], and causality has yet to be demonstrated. Two recent studies observed a significant positive (but minimal clinical) effect of synbiotic supplementation on serum thyroid markers in patients with hypothyroidism using LT4 compared with their placebo-treated counterparts. Unfortunately, both studies lacked information on gut microbiota composition and functionality before and after the intervention[34,35]. The present IMITHOT study is the first to test the effect of restoring intestinal homeostasis with healthy microbiota using fecal microbiota transplantations (FMTs) on thyroid function in medication-naïve patients with subclinical autoimmune hypothyroidism. Recently, our group has shown that this therapeutic approach has been shown to be safe and effective in preserving endocrine function in autoimmune diabetes and halting disease progression[36]. Potential mechanisms underlying a beneficial effect of FMT include changes in the production of immunoregulatory metabolites by commensal gut microbes and the acquisition of an immunoregulatory phenotype of T cells trafficking through the intestinal lymphoid tissue[37,38]. Therefore, we hypothesize that changing the gut microbiota composition with multiple FMTs might dampen the autoimmunity of these T cells and may halt the destruction of the thyroid gland, thus delaying or even preventing the need for exogenous thyroid hormone supplementation with LT4 in patients at high risk of developing overt hypothyroidism.

The ultimate aim of this study is to develop the first known treatment to prevent the development of autoimmune hypothyroidism by targeting microbial-immune interactions within the gut-thyroid axis.

Primary objective

The study aims to investigate whether compositional changes of the gut microbiome induced by multiple fecal microbiota transplantations (FMTs) from either allogenic (healthy) or autologous (own) donor stool, administrated via a nasoduodenal tube, have beneficial effects on residual thyroid functions (fT4 and fT3 excretion upon rTSH (Thyrogen) injection) in patients recently diagnosed with subclinical autoimmune hypothyroidism.

Key secondary objectives

- Study the effect of FMTs on changes in immune-phenotypes and cell activation in circulating PBMC and immune cells infiltrating the thyroid gland (retrieved by fine needle aspiration)
- Study the effect of FMT on oral and fecal microbiota composition as well as (microbiota-derived) fasted plasma metabolites measured at baseline and at 6, 12, and 24 months follow-up.
- Study the effect of FMT on intestinal transit time, measured by the amount of ingested radiopaque markers (Transit-Pellets) seen on an abdominal X-ray.
- Study the effect of FMT on quality of life using the validated thyroid-specific patient-reported outcome (ThyPRO) questionnaire.
- Assess dietary intake via a food frequency questionnaire (via mijn.voedingscentrum.nl/nl/eetmeter)

METHODS AND ANALYSES

Study design

The IMITHOT trial is a double-blinded, randomized-controlled, exploratory, single-center trial. Each patient will receive three FMTs, with two months between each FMT. Patients will be randomized to one of the two following treatment arms (Fig. 1 and 2):

1. Three allogenic (healthy donor) fecal infusions at baseline, 8 and 16 weeks.
2. Three autologous (patients' own) fecal infusions at baseline, 8 and 16 weeks.

Eligible patients will be followed up for two years after the first intervention to monitor residual thyroid function and ensure a high FMT engraftment success rate. This study protocol is reported as per the Standard Protocol Items: Recommendations for Interventional Trials (SPIRIT) guidelines[39].

Study setting

The participants are recruited through general practitioners affiliated with the Amsterdam University Medical Center (Amsterdam UMC), advertisements via posters, and the patient's association *Schildklier Organisatie Nederland*. All study interventions will be performed at a single center, Amsterdam UMC, location AMC, the Netherlands. This is an academic center with over ten years of experience in the administration of FMTs[36,40–42].

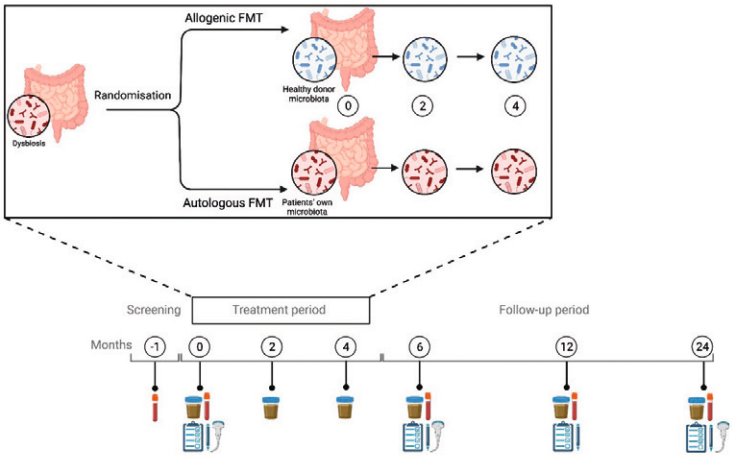

Figure 1. Schematic overview of the study design.

	Enrolment	Allocation	During treatment		Follow-up		
TIMEPOINT	***T-4***	**T0**	***T2***	***T4***	***T6***	***T12***	***T24***
	-4 weeks	***Baseline***	***2 months***	***4 months***	***6 months***	***12 months***	***24 months***
	Screening patient	**1st FMT**	**2nd FMT**	**3rd FMT**			
ENROLMENT:							
Eligibility screen	X						
Informed consent	X						
Allocation		X					
INTERVENTIONS:							
Allogenic FMT		←	—	→			
Autologous FMT		←	—	→			
ASSESSMENTS:							
rTSH test (residual thyroid function)		X			X	X	X
Ultrasound-guided FNA thyroid gland		X			X		X
Oral and stool samples		X	X	X	X	X	X
Fasted blood samples		X	X	X	X	X	X
ThyPRO questionnaire		X	X	X	X	X	X
Transit-Pellets radiopaque markers		X			X	X	X

Figure 2. Schematic overview of the study design according to the SPIRIT guidelines.

Eligibility criteria

Inclusion criteria patients

The inclusion criteria for subclinical autoimmune hypothyroid patients are as follows:

- Males and females between 18 – 70 years of age at the time of inclusion
- Non-obese BMI (18 – 30 kg/m^2)
- Confirmed subclinical autoimmune hypothyroidism:
  - TSH ≥ 10 mU/L
  - FT4 within normal references values (0.5 – 5.0 pmol/L)
  - Anti-TPO positive (> 60 kU/L)
  - History of at least three consecutive abnormal blood results, with the second test performed at least three months after the first test.
- Ability to give informed consent
- Residing in the Netherlands

Exclusion criteria patients

- Diagnosis or symptoms of other autoimmune diseases (*e.g.,* type 1 diabetes mellitus (T1D), coeliac disease, autoimmune gastritis, rheumatoid arthritis, or inflammatory bowel diseases (IBD) such as Crohn's disease and ulcerative colitis);
- Following specific diets, including vegan, keto, and paleo diets;
- Use of any medication, including proton pump inhibitors, antibiotics, and pro-/prebiotics in the past three months or during the study period;
- History of chronic diarrhea (≥ 3 defecations/day for >4 weeks), chronic constipation (<2 defections/week for >3 months), or irritable bowel syndrome (IBS) according to Rome IV criteria;
- Smoking or illicit drugs use (MDMA, amphetamine, cocaine, heroin, GHB) in the past three months or during the study period;
- Use of >5 alcoholic units on an average daily basis in the past three months or during the study period;
- History of cholecystectomy;
- Prolonged compromised immunity (due to recent cytotoxic chemotherapy or human immunodeficiency viruses (HIV) infection with a CD4 < 240/mm^3).

Donors

Potential healthy stool donor candidates were recruited among non-healthcare workers of the Amsterdam UMC and preclinical medical students. Informed consent was obtained after oral and written information about the screening and donation process. Financial compensation was offered for qualified donors (€50,- per donation). The screening process, in-, and exclusion criteria are in accordance with the European consensus of FMT in clinical practice[43]. A comprehensive report on the specific donor screening process has been recently published[44] and can be found in the online supplemental material (Tables S1 and S2).

Inclusion criteria donors

- Males and females of ≥ 18 years of age at the time of inclusion
- Normal BMI (18 – 25 kg/m^2)
- Regular morning stool pattern
- Ability to give informed consent
- Residing in the Netherlands

Exclusion criteria donors

Exclusion criteria are presented in the online supplementary Table S3.

Interventions

A schematic overview of all study activities can be found in Figures 1 and 2.

Fecal Microbiota Transplantation (FMT)

A fresh morning stool sample (100-200 grams) will be collected by both the recipient and the healthy donor and processed directly (<2 hours) in the laboratory. An independent lab technician will randomize and blind the two collected samples. Either the autologous or allogenic feces will be mixed with saline in a 1:1 ratio, homogenized and filtered. Meanwhile, a nasoduodenal tube is inserted with a CORTRAK* Enteral Access System, after which bowel lavage with 2-3 liters of Kleanprep (via the nasoduodenal tube) will be performed to ensure complete bowel lavage (3-4 hours). Finally, the FMT will be infused in the duodenum through the positioned tube (within 6 hours of the stool sample donation).

Residual thyroid function test

The residual thyroid function will be measured via a dynamic thyroid function stimulation test. At baseline, 6, 12, and 24 months after the first FMT, a single intramuscular injection of 0.9mg recombinant TSH (Thyrogen) will be administrated, as previously described[45]. Blood samples will be drawn over the following 5 hours. The incremental area under the curve (iAUC) will be used to determine the net increase of the area under the concentration vs. time between 0 and 300 minutes of serum fT4 and fT3 levels after subtracting the baseline value to account for the between-subject variation in fasting serum TH levels. The iAUC values will be derived according to the trapezoidal rule.

Ultrasound-guided fine needle aspiration thyroid gland

An ultrasound-guided fine needle aspiration (FNA) of the thyroid gland will be performed at baseline, 6, and 24 months by an experienced radiologist, who will also measure thyroid volume and echogenicity. Nodules (if present) will be scored using the ACR TI-RADS classification[46]. The FNA biopsy will be done from normal thyroid tissue.

Gut microbiota composition
Morning oral and stool samples will be collected during each study visit to assess the effect of FMT on gut microbiota composition. Both an oral swab of the upper front teeth rim, as well as saliva in overnight fasted patients who refrained from tooth brushing that morning, are used to study the oral microbiota composition. All samples will be immediately stored at -80°C and analyzed at the end of the study by sequencing the V3-V4 region of 16S rRNA genes with the Illumina MiSeq sequencer. Established protocols will be followed for DNA extraction to ensure the reliability and accuracy of the results, as described previously[47,48].

Intestinal transit time
Radiopaque Transit-Pellets™ markers will be used at baseline, 6, 12, and 24 months to measure colonic transit time, given that patients with (subclinical) hypothyroidism often experience constipation[49,50]. In short, the patient's intestinal transit time is measured by swallowing one capsule containing ten markers each morning for six days prior to the study visit. An additional capsule is taken on the evening before the visit, 12 hours before the abdominal X-ray. This ensures a 144-hour interval between the first marker and the X-ray. The radiologist then counts the visible capsules on the X-ray image.
To calculate the colonic transit time, we determine the mean oro-anal transit time (OATT) for the markers swallowed daily. With a daily dose of ten markers, the transit time in days is obtained by dividing the number of markers counted from the X-ray film (M) by 10. This calculation is facilitated using the provided Medifactia tool[50].

Fasted blood drawn
Overnighted fasted blood is drawn at baseline, 6, 12, and 24 months to assess biochemistry, endocrinology, metabolomics, and for PBMCs isolation. Samples will be centrifuged at 3000 RPM, at 4°C for 15 min, and stored at -80°C.

Outcomes

Primary outcome
The primary effect parameter is the preservation of (Thyrogen stimulated) fT4 and fT3 release at 6, 12, and 24 months compared with baseline (0 months). This dynamic thyroid functional endpoint is chosen because Thyrogen acts as an amplifier, which can magnify any underlying abnormality in thyroid hormone secretion by the thyroid gland, making it possible to detect subtle changes in thyroid functioning. Moreover, static thyroid serum markers (*e.g.*, a single, fasted measurement) could be affected by external factors, such as seasonal variation, the timing of blood draw, exercise, diet and lifestyle, and BMI[51–54]. In a dynamic function test measuring the net incremental increase in the AUC, these factors may have less influence on the results.

Secondary outcomes

- Gut microbiota composition by sequencing the V3-V4 region of 16S rRNA genes with the Illumina MiSeq sequencer (overall composition by alpha- and beta-diversity indices, relative abundances of families, phyla, and ASVs, and principal component analysis of the taxonomic profiling).
- Profiling of immune cell subsets in PBMCs and thyroid tissue, using a single-cell high-dimensional profiling assay of Maxpar Direct Immune Profiling Assay.
- Fasting plasma targeted (microbial-derived) metabolomics will be measured by Metabolon (Durham, NC), using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UPLC, as previously described[36]). Raw data will be normalized to account for interday differences. The levels of each metabolite will be rescaled to set the median equal to 1 across all samples. Missing values, generally due to the sample measurement falling below the detection limit, will be imputed with the minimum observed value for the respective metabolites.
- Intestinal transit time will be measured by the amount of ingested radiopaque markers (Transit-Pellets) seen on an abdominal X-ray.
- The thyroid-specific patient-reported outcome (ThyPRO) questionnaire will assess the patient-reported quality of life.
- Total caloric intake, macronutrients (carbohydrates, proteins, fats, and fibers), and micronutrients (selenium and iodine, among others) will be reported by food frequency questionnaires (via mijn.voedingscentrum.nl/nl/eetmeter).

Participant timeline

Potential patient participants will be screened at the first visit (V1) (Fig 1 and 2, Table 1). Baseline data will be collected on the first FMT (V2) day. The second (V3) and third (V4) FMT are scheduled for two months between each visit. Follow-up will be conducted for two years after the first FMT, during which the participants will visit thrice (V5-V7). The last visit (V7) will be completed two years after the first FMT. In accordance with the recruitment of the study protocol, the participants should not change their original eating habits and are not allowed to ingest pre-, pro-, or synbiotic supplements during the trial period.

Table 1. Specification of patient screening

Anthropometric measurements	
Demographics	Lifestyle (exercise, diet, alcohol intake)
Physical examination	
Serum screening	
Hematology	
Alanine aminotransferase (ALAT)	Gamma-glutamyl transferase (GGT)
Alkaline phosphatase (AF)	Glucose (fasted)
Aspartate aminotransferase (ASAT)	Hemoglobin
Bilirubin	Kreatinin
Complete Bound Count (CBC)	Lipid spectrum: total cholesterol, HDL, LDL, Lp(a)
C-reactive protein (CRP)	Ureum
Estimated Glomerular Filtration Rate (eGFR)	
Endocrinology	
Free triiodothyronine (fT3)	Thyroid peroxidase antibodies (TPOAb)
Free thyroxine (fT4)	Thyroid stimulating hormone (TSH)
Viruses *(CLIA or PCR)*	
Cytomegalovirus (CMV): IgG and IgM	Epstein-Barr Virus (EBV): VCA IgG and EBNA IgG

Patient allocation and blinding

Patients are allocated to either allogenic or autologous FMTs through computer-generated block randomization (block size = 4) in a 1:1 ratio to ensure equal sample sizes and avoid selection bias. After all patients have completed the study and the data have been locked, the investigator will unblind the materials.

Data collection and management

In the IMITHOT trial, data are collected during seven study visits, as defined in Figures 1 and 2. Data collection will be performed by trained local research staff and data entry in the Clinical EDC, CASTOR database. The Clinical Research Unit (CRU) of the Amsterdam UMC will perform and monitor data entry and looks after timely CRF (case report forms) delivery. Any other parameters necessary to evaluate the study endpoints and reason for end-of-protocol treatment are also documented. All subject data will be pseudonymized with a study code. The subjection identification log, which links subjects to the code, is kept in a trial file only accessible to study personnel. All research data will be stored for fifteen years. An independent data monitoring committee will perform an interim analysis when the first 20 patients have finished the trial.

Statistical methods

Sample size calculation
As this is a phase III trial, a reliable sample size calculation is not feasible but is based on previous research[36] . A sample of 17 patients in each group (34 patients in total) is needed to provide 80% power to detect a 10% difference in the Thyrogen-stimulated fT4 and fT3 incremental area under the curve (iAUC) between treatment groups at 6, 12, and 24 months, with a two-sided test at α= 0.05 and assuming a 10% dropout. All power calculations were performed with an online power calculator (www.biomath.info/power/)

- Autologous arm: decline of Thyrogen-stimulated fT4 and fT4 $iAUC_{(0\text{-}300\ min)}$ from 150% to 100% at 12 and 24 months
- Allogenic arm: decline of Thyrogen-stimulated fT4 and fT4 $iAUC_{(0\text{-}300\ min)}$ from 150% to 120% at 12 and 24 months

Statistical analysis
All statistical tests will be conducted as a two-sided test with a p-value of less than 0.05 considered statistically significant. Unpaired Student's t-test or the Mann-Whitney U test will be used for baseline differences between the two groups, dependent on the distribution of the data. Data will be expressed as mean ± the standard deviation or the median with the interquartile range. The incremental AUC for the 5-hour residual thyroid function test (fT4 and fT3 after Thyrogen injection) will be calculated using the trapezoidal method. Depending on the data distribution, either the Pearson correlation or Spearman's Rank test will be used for correlation analyses. A linear mixed model (LMM) will be used to compare the primary end-point, in which 'allocation' and 'time point' will be fixed effects and 'study ID' a random effect. The p-value for the interaction between 'allocation' and 'time point' will be reported. Additionally, parameters will be compared between groups at various time points using the Mann-Whitney U test with multiplicity correction.

XGBoost machine learning classification algorithm will be applied to determine which microbial strains and/or metabolites predict the response to FMT treatment, immune profiling changes, and residual thyroid function.

Ethics and dissemination

Ethics approval was obtained in the Netherlands from the Medical Ethics Committee (METC) of the Amsterdam UMC, in accordance with the Declaration of Helsinki (updated version Oct 2013, Fortaleza Brasil) and in accordance with the Medical Research Involving Human Subjects Act (WMO). The trial is registered with the Netherlands Trial Register NL7931. A manuscript with the results of the primary study

outcomes will be published in a peer-reviewed journal. All participants will provide written informed consent.

Adverse events and safety
Patients will be submitted to multiple intramuscular injections of 0.9mg Thyrogen, three ultrasound-guided fine needle aspirations (FNA) of the thyroid gland (performed by an experienced radiologist), three insertions of a nasoduodenal tube, and several vena punctions. Prior studies have shown that FMT is a safe therapy, and strict conditions apply for donor screening (Table S1-S3). The possible complications are cited in the written patient information.
Adverse events (AEs) are defined as any undesirable experience occurring to a patient during the clinical trial, whether or not related to the trial, and will be reported by the investigator. A severe adverse event (SAE) is any medical occurrence or effect that at any dose results in;
- Death;
- Life-threatening situation (at the time of the event);
- Hospitalization or prolongation of existing inpatients' hospitalization;
- Persistent or significant disability;
- A congenital anomaly or birth defect;
- An SAE, but this was prevented due to timely intervention.

DISCUSSION

Hashimoto's thyroiditis is a chronic condition requiring life-long hormone supplementation. Previous studies have documented significant differences in the gut microbiome composition of patients with Hashimoto's thyroiditis compared to healthy controls, but causal evidence linking the gut microbiome to disease development is lacking. To our knowledge, the IMITHOT trial is the first clinical study that investigates the effect of restoring the gut microbiome composition on thyroid function in subclinical autoimmune hypothyroid patients at high risk of developing overt hypothyroidism. This study aims to halt disease progression.

This study will enable us to determine if gut microbiota plays an essential role in disease progression and, secondly, provides an indication of which microbiota components might predict a positive response to FMT in the pathophysiology of HT. A strength of this study protocol is that patients will receive multiple FMTs administered via a nasoduodenal tube to obtain a high bacterial strain engraftment success rate. Subsequently, the long follow-up period of two years allows us to assess the long-term impact and sustainably of FMT engraftment.

There is no established standard for determining the FMT treatment period. In our study, we have chosen to administer three FMTs every two months based on the

treatment protocol employed in a recent clinical trial conducted by our group[36]. This trial investigated the efficacy of a similar study design in patients with new-onset type 1 diabetes. Given the focus of our current study on another autoimmune endocrine disease, targeting the thyroid gland, we have opted for the same treatment protocol[55,56].

Next, previous research by our group has shown that the beneficial effects of FMT are transient. We observed only a short-term improvement in insulin sensitivity, whereas no long-term effects were seen at 18 weeks after FMT[57]. This finding suggests that performing FMT more frequently over an extended period may be beneficial. In line with this, a study by Li et al.[58] indicated that the engraftment of donor bacterial strains could be transient, and individuals possess their own unique fecal core microbiome. Consequently, a single FMT may not lead to sustained changes in the intestinal microbiota composition. Another study demonstrated that three FMTs significantly improved long-term microbiota engraftment ($p < 0.05$)[59]. Thus, our decision to conduct three FMTs every two months in our study is based on the goal of achieving a higher engraftment rate and promoting more sustained changes in the intestinal microbiota. We acknowledge that further research is required to determine the optimal treatment period for FMT, and we will thoroughly evaluate the results of our study to contribute to the understanding of this question.

Lastly, only medication-naïve patients with relatively high TSH serum levels and positive TPOAb serum levels will be included in this study as these patients are less likely to normalize to a euthyroid state spontaneously[8], and LT4 treatment might affect gut microbiota composition. If patient's serum fT4 level decreases during the study period or if they develop severe symptoms of hypothyroidism, we prioritize their well-being and refer them back to their own family doctor who can prescribe levothyroxine as appropriate. These patients are then excluded from our study.

A limitation of this protocol is the relatively small sample size, which allows us only to encounter major effects of the FMTs. Secondly, patients are randomized to either autologous or allogenic FMTs, but a control group without FMT intervention (e.g., an observational control group) is lacking due to ethical reasons.

In conclusion, disentangling specific signatures of the gut microbiota that might be involved in improved thyroid function may pave the way to a microbial-targeted therapy in patients prone to develop overt HT.

AUTHORS' CONTRIBUTIONS

ACF contributes to patient selection and will contribute to data analysis and manuscript writing. ER is contributing to laboratory procedures and will be contributing to data analysis and writing the manuscript. AvdS will be contributing to data analysis and writing the manuscript. MN and EF have written the protocol.

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This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflicts of interests
MN is co-founder and member of the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands. None of these are directly relevant to the current paper. There are no patents, products in development, or marketed products to declare. The other authors declare no competing financial interests.

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Table S1. Time interval of donor rescreening

Screening	Every 6 months	Prior every individual FMT	60-days
Short rescreening questionnaire		X	
Extensive screening questionnaire	X		
Feces			
Parasites	X		
Bacteria	X		
Viruses	X		
Calprotectine	X		
SARS-CoV-2	X		X
Multidrugs resistant bacteria	X		X
Serum			
Haematology	X		
Endocrinology	X		
Bacteria (ELISA)	X		
Viral loads (CLIA or PCR)	X		
Parasites (ELISA)	X		

Table S2. Specification of donor screening

Anthropometric measurements	
Demographics	Lifestyle (exercise, diet, alcohol intake)
Physical examination	
Feces screening	
Calprotectin (ELISA)	
Bacteria (PCR or stool antigen detection)	
Clostridium difficile (GDH and toxine)	Salmonella spp.
Helicobacter pylori	Shiga toxin-producing Escherichia coli 1 and 2 (STEC)
Pathogenic Campylobacter spp.	Shigella spp (EIEC)
Pleisiomonas shigelloides	Yersinia enterocolitica
Multidrug-resistant organisms (culture)	
Carbapenem-resistant Enterobacteriaceae (CRE)	Methicillin-resistant Staphylococcus aureus (MSRA)
ESBL-producing Enterbactereacceae	Vancomycin-resistant Enterococcus
Multi-drugs resistant Gram-negatives (MRGN) 3	MGNR 4
Viruses (RNA or DNA PCR)	
Adenovirus non-40/41	Norovirus Type I and II
Adenovirus type 40/41	Parechovirus
Astrovirus	Rotavirus
Enterovirus	Sapovirus
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)	Hepatitis E virus
Parasites (PCR and/or microscopic evaluation)	
Blastocystis spp.	Entamoebe moshkovski
Cryptosporidium spp.	Entamoeba pelecki
Cyclospora	Giardia lamblia
Dientamoeba fragilis	Iodamoeba bütschlii
Endolimax nana	Isospora spp.
Entamoeba coli	Larvae
Entamoebe dispar	Microsporidium spp.
Entamoebe gingivalis	Parasitic worm eggs (Ridley)
Entamoebe hartmann	Protozoan Cysts and Oocysts (Ridley)
Entamoebe histolytica 1	

Table S2. Continued

Anthropometric measurements	
Serum screening	
Hematology	
Alanine aminotransferase (ALAT)	Complete Bound Count (CBC)
Alkaline phosphatase (AF)	C-reactive protein (CRP)
Aspartate aminotransferase (ASAT)	Estimated Glomerular Filtration Rate (eGFR)
Bilirubin	Kreatinin
Gamma-glutamyl transferase (GGT)	Lipid spectrum
Hemoglobin A1c (HbA1c)	Ureum
Endocrinology	
Free thyroxine (FT4)	
Thyroid peroxidase antibodies (TPOAb)	
Thyroid stimulating hormone (TSH)	
Bacteria (ELISA)	
Treponema pallidum	
Viruses (CLIA or PCR)	
Cytomegalovirus (CMV): IgG and IgM	Hepatitis C virus: HCV IgG total
Epstein-Barr Virus (EBV): VCA IgG and EBNA IgG	Human immunodeficiency virus (HIV): Ag and Ab
Hepatitis A virus: Ig total and IgM	Human T-lymphocytic virus Type I and II (HTLV)
Hepatitis B virus: HBsAg, HBcore IgG total, and anti HbsAg	
Parasites (antibodies)	
Strongyloides stercoralis	

Table S3. Inclusion and exclusion criteria for healthy donors

Healthy donors
Inclusion criteria
Males and females of ≥ 18 years of age at the time of inclusion;
Normal BMI (18 – 25 kg/m^2);
Regular morning stool pattern;
Ability to give informed consent.
Exclusion criteria
Use of any medication, including proton pump inhibitors, antibiotics, and pro-/prebiotics in the past three months or during the study period;
History of, or known exposure to HIV, hepatitis B (HBV) or C (HCV) virus, syphilis, human T-lymphotropic virus (HTLV) I and II, malaria, trypanosomiasis, tuberculosis
Known systemic infection not controlled at the time of donation;
Smoking or illicit drugs use (MDMA, amphetamine, cocaine, heroin, GHB) in the past three months or during the study visit;
Use of >5 alcoholic units on an average daily basis in the past three months or during the study period;
History of cholecystectomy;
Risky sexual behavior, including anonymous sexual contacts; contacts with prostitutes, drug addicts, individuals with HIV, viral hepatitis, or syphilis; work as a prostitute; history of a sexually transmittable disease;
Previous reception of tissue/organ transplant;
Previous (<12 months) reception of blood products;
Recent (<6 moths) body tattoo, piercing, earring, or acupuncture;
Recent (<6 months) needles tick accident;
Recent (<6 months) medical treatment in poor hygienic conditions;
Risk of transmission of diseases caused by prions;
Recent parasitosis or infection from rotavirus, Giardia lamblia, and other microbes with gastrointestinal (GI) involvement;
Recent travel in tropical countries, countries at high risk of communicable diseases, or traveler's diarrhea (period based on recommendations Sanquin for blood donors)
Recent (<6 months) history of vaccination with a live attenuated virus, if there is a possible risk of transmission;
Healthcare providers having frequent patient contact (to exclude the risk of transmission of multidrug-resistant organisms);
Individuals working with animals (to exclude the risk of transmission of zoonotic infections);
History of IBS (according to Rome IV criteria), IBD, functional chronic constipation, coeliac disease, and other chronic GI disorders;
History of chronic, systemic autoimmune disorders with GI involvement;
History of, or high risk for, GI cancer or polyposis;
Recent (< 6 months) appearance of diarrhea (≥3 stools/day) or hematochezia;

Table S3. Continued

Healthy donors
History of neurological/neurodegenerative disorders;
History of psychiatric conditions;
Presence of chronic low-grade inflammation or metabolic syndrome (NCEP criteria)
Presence of T1D, T2D, or hypertension;
History of cholecystectomy;
Positive serologic test for HIV 1/2, hepatitis A virus (HAV), HBV, HCV, hepatitis E virus (HEV), active cytomegalovirus (CMV) or active Epstein–Barr virus (EBV), Strongyloides or lues;
Presence of faecal bacterial pathogens Salmonella spp., Shigella spp., Campylobacter spp., Yersinia spp., C. difficile, H. pylori, shigatoxigenic Escherichia coli (STEC), Aeromonas spp. or Pleisiomonas Shigelloides in faeces;
Positive Dual Faeces Test (DFT) for Giardia Lamblia, Dientamoebe fragilis, Entamoeba histolytica, Microsporidium spp., Cryptosporidium spp., Cyclospora, Isospora or Blastocystis Hominis. Positive microscopic exam for eggs, cysts, and larvae (*e.g.*, helminth eggs)
Presence of extended-spectrum beta-lactamase (ESBL) producers, Carbapenem-resistant Enterobacteriaceae (CRE), vancomycin-resistant enterococci (VRE), or methicillin-resistant Staphylococcus aureus (MRSA) in faeces;
Presence of Rotavirus, Norovirus I/II, Enterovirus, Parechovirus, Astrovirus, Sapovirus, or Adenovirus in faeces;
Presence of SARS-Cov2 in faeces;
Abnormal liver or renal function (creatinine >110 µmol/l, ureum >8,2 mmol/l, ASAT >40 U/L, ALAT >45 U/L, AF >120 U/L, GGT >60 U/L, bilirubin >17µmol/L) or impaired immunity (CRP >5 mg/L, haemoglobin <8,5 mmol/L, MCV 80-100 fL, leukocytes 4,0-10,5 x10^9/L, thrombocytes 150-400 x10^9/L).
Abnormal thyroid function (TSH < 0.5 or > 5.0 mU/L; FT4 < 12.0 or > 22.0 pmol/L; anti-TPO antibodies >60 kU/L).

8

CHALLENGES AND COSTS OF DONOR SCREENING FOR FECAL MICROBIOTA TRANSPLANTATIONS

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ABSTRACT

Background

The increasing interest to perform and investigate the efficacy of fecal microbiota transplantation (FMT) has generated an urge for feasible donor screening. We report our experience with stool donor recruitment, screening, follow-up, and associated costs in the context of clinical FMT trials.

Methods

Potential stool donors, aged between 18−65 years, underwent a stepwise screening process

starting with an extensive questionnaire followed by feces and blood investigations. When eligible, donors were rescreened for MDROs and SARS-CoV-2 every 60-days, and full rescreening every 4−6 months. The costs to find and retain a stool donor were calculated.

Results

From January 2018 to August 2021, 393 potential donors underwent prescreening, of which 202 (51.4%) did not proceed primarily due to loss to follow-up, medication use, or logistic reasons (e.g. COVID-19 measures). 191 potential donors filled in the questionnaire, of which 43 (22.5%) were excluded. The remaining 148 candidates underwent parasitology screening: 91 (61.5%) were excluded, mostly due to *Dientamoeba* fragilis and/or high amounts of *Blastocystis* spp. After additional feces investigations 18/57 (31.6%) potential donors were excluded (mainly for presence of Helicobacter Pylori and ESBL-producing organisms). One donor failed serum testing. Overall, 38 out of 393 (10%) potential donors were enrolled. The median participation time of active stool donors was 13 months. To recruit 38 stool donors, €64.112 was spent.

Conclusion

Recruitment of stool donors for FMT is challenging. In our Dutch cohort, failed eligibility of potential donors was often caused by the presence of the protozoa *Dientamoeba* Fragilis and *Blastocystis* spp.. The exclusion of potential donors that carry these protozoa, especially *Blastocystis* spp., is questionable and deserves reconsideration. High-quality donor screening is associated with substantial costs.

INTRODUCTION

Fecal microbiota transplantation (FMT) is defined as the infusion of feces from healthy individuals into diseased recipients. FMT is thought to be effective because it has the potential to restore a recipient's distorted microbiota, by introducing a new and diverse microbiome associated with a healthy state to normalize microbiota composition and function. In daily practice, FMT is a widely accepted and highly effective treatment for recurrent *Clostridioides difficile* infection (CDI)[1,2]. Over the past couple of years, evidence is growing for the application of FMT as a treatment for other diseases, such as inflammatory bowel disease (IBD)[3], irritable bowel syndrome (IBS)[4], obesity and related metabolic diseases[5], acute graft-versus-host disease[6], and autism spectrum disorder[7]. The interest in FMT increased tremendously recently, with more than 357 registered ongoing clinical trials worldwide at the time of writing[8-10].

This increasing interest in FMT has generated an urge for feasible donor screening programs to secure an ongoing supply of healthy stool donors. Enrolled donors need to fulfill strict safety criteria, which are continuously adjusted to new insights[11]. For example, due to the current COVID-19 pandemic, additional screening procedures to assess COVID-19 symptoms before donation and regular testing for SARS-CoV-2 RNA are needed[12,13]. In addition, measures to reduce the risk of transmitting multi-drug resistant organisms (MDROs) via FMT were advised earlier by the United States Food and Drug Administration (FDA) after two immunocompromised adults developed invasive infections with extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli*[11]. Although international recommendations on donor screenings exist[14], stool donor selection processes in practice are highly heterogeneous, and standardized procedures are lacking[9]. Experience from clinical practice indicates that finding a safe, eligible stool donor is complicated. Previous studies performed in the USA, Canada, Hong Kong, and Denmark have shown variable donor acceptance rates ranging between 0.8 - 31%[15-21]. Challenges in donor screening comprise initial donor recruitment and prolonged donor eligibility. A major disadvantage of the extensive screening procedures is the high associated costs[15], leading to an economic burden for patient care and research departments. Therefore, more insights into donor screening programs and accompanying costs are warranted to optimize and further standardize donor screening procedures.

At present, limited data is published on FMT donor screening and associated costs within the context of clinical trials. In recent years, one of the largest University hospitals in the Netherlands −the Amsterdam UMC− has conducted four randomized controlled clinical FMT trials: the FAIS[22], IMITHOT and PIMMS trials have evaluated the efficacy of multiple donor FMTs using fresh fecal material in respectively IBS (in adolescents), subclinical autoimmune hypothyroidism and metabolic syndrome, whereas the TURN2-trial is evaluating the efficacy of frozen fecal suspensions in

active ulcerative colitis. To perform these trials, a pool of healthy stool donors who were able to provide regular stool donations was established. The donor screening was performed according to a predefined standardized screening protocol. With the current study, we aim to describe the process of recruiting and screening stool donors, evaluate the follow-up of eligible donors, and report the associated costs in the context of clinical FMT trials in a Dutch tertiary University hospital.

METHODS

Donor recruitment

In this retrospective observational cohort – study, potential healthy fecal donors were recruited through advertisements via posters, announcements in the hospital magazine and intranet network (employee website), and word-of-mouth advertising among staff at the Amsterdam UMC (location AMC). The Amsterdam UMC, location AMC, is a University hospital with over 7000 employees and 2300 healthcare student placements. Potential donors were invited to participate in the FAIS, IMITHOT, PIMMS, and/or TURN2-trial and oral and written information about the study aims, donation process, and screening requirements were provided. Clinical trials registration numbers are NCT03074227, NL7931, NL8289, and NL7770, respectively. All trials were approved by the Medical Ethics Research Committee of the Amsterdam UMC, the Netherlands. Written, signed and dated informed consent forms were obtained separately for each study as participation in multiple trials was optional. Financial compensation was offered, with reimbursements ranging between €10 – 50 per donation plus additional travel expenses, depending on the trial.

Population and screening procedure

The study population consisted of non-smoking adults, aged 18 − 65 years (except for the TURN2 trial, in which the age ranged between 18 – 54 years), and with a body mass index between 18 − 25 kg/m.^{2} No specific diet restrictions were required. After informed consent was signed, potential donors were thoroughly screened based on the screenings protocol of the Netherlands Donor Feces Bank (NDFB)[23], a Dutch stool bank that supplies FMT for the treatment of CDI in the Netherlands since 2016. Before accepting a donor, a rigorous screening was performed as shown in **Table 1**. The screening started with an extensive questionnaire regarding risk factors for infectious diseases and factors potentially perturbing the intestinal microbiota. When potential donors passed the screening questionnaire, they subsequently underwent elaborate fecal and blood laboratory testing in a stepwise approach (**Table 2**). First, stool samples -collected in a plastic stool container- were screened for parasites presence by a combination of PCR and direct microscopy (Dual Feces Test). Next, feces samples were tested for pathogenic bacteria and viruses, multi-drug resistant organisms and calprotectin.

Table 1. Exclusion criteria donor recruitment

Risk of infectious agent
Active hepatitis A, B-, C- or E-virus infection or known exposure within recent 12 months
Acute infection with *Cytomegalovirus* (CMV) or *Epstein-Barr virus* (EBV)
An extensive travel behaviour
Higher risk of colonization with multidrug resistant organisms including: o *Health care workers with direct patient contact* o *Persons who have recently been hospitalized or discharged from long term care facilities* o *Persons who regularly attend outpatient medical or surgical clinics* o *Persons who have recently engaged in medical tourism*
History or current use of (IV) drugs
Individual working with animals[a]
Positive blood tests for the presence of: HIV, HTLV, *Treponema pallidum*, *Strongyloides stercoralis*
Positive fecal test for MDROs, pathogenic bacteria, viruses and parasites as listed in Table 2
Previous reception of blood products (<12 months) or recent needle-stick accident (<6 months)[a]
Tattoo or body piercing placement within last 6 months
Unsafe sex practice (assessed with standardized questionnaire)
Gastrointestinal comorbidities
A positive history/clinical evidence (e.g. elevated fecal calprotectin) for inflammatory bowel disease, including Crohn's disease or ulcerative colitis
A positive history/clinical evidence for other gastrointestinal diseases, including chronic diarrhea or chronic constipation
Abnormal bowel motions, abdominal complaints or symptoms indicative of irritable bowel syndrome
Factors affecting intestinal microbiota composition
Antibiotic treatment in the past 12 weeks[b]
History of or present known malignant disease and/or patients who are receiving systemic anti-neoplastic agents
History of cholecystectomy
History of treatment with growth factors
Patients receiving immunosuppressive medications and/or a positive history/clinical evidence for autoimmune disease including: o *Type 1 Diabetes Mellitus* o *Hashimoto's hypothyroidism* o *Graves' hyperthyroidism* o *Rheumatoid arthritis* o *Celiac disease*
Recent (gastrointestinal) infection within last 6 months[c]
Smoking
Use of any medication including PPI, except contraceptives and over the counter medication
Use of pre- and probiotics in the past 12 weeks[a]

Table 1. Continued

Risk of infectious agent
Other conditions
Abnormal liver function[d]: ASAT >40 U/L, ALAT >45 U/L, AF >120 U/L, GGT >60 U/L, bilirubin >17µmol/L
Abnormal renal function[d]: creatinine >110 µmol/l, urea >8,2 mmol/l
Alcohol abuse (>3 units/day)
Chronic pain syndromes (e.g., fibromyalgia)[c]
Impaired immunity[d]: CRP >5 mg/L, haemoglobin <8,5 mmol/L, MCV 80-100 fL, leukocytes 4,0-10,5 x10^9/L, thrombocytes 150-400 x10^9/L
Known chronic neurological/neurodegenerative disease (e.g., Parkinson's disease, multiple sclerosis)
Known psychiatric disease (i.e., depression, schizophrenia, autism, Asperger's syndrome)
Known risk of Creutzfeldt Jacob's disease
Major relevant allergies (e.g., food allergy, multiple allergies)
Presence of diabetes mellitus type 1 and 2 or hypertension[d]
Presence of chronic low-grade inflammation or metabolic syndrome (NCEP criteria)[e]

[a] Not included in screening protocol of FAIS and TURN2-trial; [b] For the TURN2-trial the exclusion criteria included antibiotic treatment in the past 4 weeks; [c] Additional exclusion criteria FAIS trial; [d] Not included in screening protocol of TURN2-trial; [e] Additional exclusion criteria PIMMS trial
Abbreviations: AF, alkaline phosphatase; ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; GGT, gamma-glutamyl transferase; CRP, c-reactive protein; HIV, human immunodeficiency virus; HTLV, human T-lymphotropic virus; MCV, mean corpuscular volume; MDROs, multidrug-resistant organisms, NCEP, National Cholesterol Education Programs; PPI, proton pump inhibitors.

Table 2. Specification of donor screening and associated costs

Feces screening		**€**
Calprotectin[a] (ELISA)		***20,-***
Bacteria *(PCR or stool antigen detection[b])*		***150,-***
Clostridium difficile	*Salmonella spp.*	
Helicobacter pylori	Shiga toxin-producing *Escherichia coli* (STEC)	
Pathogenic *Campylobacter spp*.	*Shigella spp*.	
Plesiomonas shigelloides	*Yersinia enterocolitica*	
Multidrug resistant organisms *(culture)*		***150,-***
Carbapenem-resistant *Enterobacteriaceae* (CRE)	Multidrug-resistant Gram-negatives (MRGN) 3	

Table 2. Continued

Feces screening		
ESBL-producing *Enterobactereacceae*	MRGN 4	
Methicillin-resistant *Staphylococcus aureus* (MRSA)	Vancomycin-resistant *Enterococcus* (VRE)	
Viruses *(PCR)*		***125,-***
Adenovirus non-41/41	Norovirus Type I and II	45
Adenovirus type 40/41	Parechovirus	
Astrovirus	Rotavirus	
Enterovirus	Sapovirus	
Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)		45
Hepatitis E virus		35
Parasites *(PCR and/or microscopic evaluation)*		***212,-***
Blastocystis spp.[c]	*Entamoeba moshkovskii*[d]	
Cryptosporidium spp.	*Entamoeba polecki*[d]	
Cyclospora	*Giardia lamblia*	
Dientamoeba fragilis	*Iodamoeba bütschlii*[d]	
Endolimax nana[d]	*Isospora spp.*	
Entamoeba coli[d]	Larvae[c]	
Entamoeba dispar[d]	*Microsporidium spp.*	
Entamoeba gingivalis[d]	Parasitic worm eggs[c]	
Entamoeba hartmanni[d]	Protozoan Cysts and Oocysts[c]	
Entamoeba histolytica		
Serum screening		
Hematology[a]		***44,-***
Alanine aminotransferase (ALAT)	Complete Blood Count (CBC)	
Alkaline phosphatase (AF)	C-reactive protein (CRP)	
Aspartate aminotransferase (ASAT)	Estimated Glomerular Filtration Rate (EGFR)	
Bilirubine	Kreatinine	
Gamma-glutamyl transferase (GGT)	Ureum	
Bacteria *(ELISA)*		***8,-***
Treponema pallidum		

Table 2. Continued

Serum screening		
Viruses *(CLIA or PCR)*	***Serology: 119,-***	***PCR: 293,-***
Cytomegalovirus (CMV)	36,-	35,-
Epstein-Barr Virus (EBV)	25,-	
Hepatitis A virus[a]	15,-	
Hepatitis B virus	10,-	67,-
Hepatitis C virus	11,-	77,-
Human immunodeficiency viruses (HIV)	11,-	63,-
Human T-lymphotropic virus Type I and II (HTLV)	11,-	
Parasites *(ELISA)*	***18,-***	
Strongyloides stercalis	18	

[a] Not included in screening protocol of TURN2-trial
[b] All bacteria were detected with the use of PCR, with exception of Helicobacter pylori were ELISA was used
[c] Microscopic evaluation, exclusion of donor only if high amounts *Blastocystis* spp. are seen, defined as 'moderate' or 'many'[38]
[d] Presence of only one non-pathogenic parasite is acceptable
Abbreviations: ELISA, quantitative enzyme-linked immunosorbent assay; CLIA, chemiluminescence immunoassay.

Subsequently, routine biochemical analysis of blood was performed, followed by serological testing for pathogenic viruses, bacteria, and parasites. Once qualified as fecal donors, rescreening of active fecal donors was performed regularly to reduce the risk of transmission of infectious diseases as much as possible. In line with FDA recommendations[11], screening for MDROs (fecal culture) and molecular stool testing on SARS-CoV-2 was performed every 60 days. Frozen FMT material (TURN2 trial) remained quarantined until successful complete rescreening, performed every four months. Complete rescreening was executed every six months when fresh FMT was used (other trials). During the trials, the study staff were in regular contact with the active stool donors, especially before each donation. If there were any concerns about symptoms or risk factor exposure of the fecal donor, donation was suspended and an additional rescreening was performed. In addition, since the outbreak of coronavirus pandemic in 2019 (COVID-19) questions to assess the risk on SARS-CoV-2 infection were asked, including the presence of fever, cough, sore throat, dyspnea, anosmia or ageusia, or close contact to subjects with suspected or proven infection. Independent of SARS-CoV-2 vaccination status, in case of any suspicion on COVID-19 infection, nasopharyngeal swab and reverse transcription polymerase chain reaction (RT-PCR) were performed and the potential donor was temporarily excluded. During

the screening and rescreening process, all positive laboratory tests were discussed with the (potential) donor and counselling was provided accordingly. Qualified fecal donors were matched to patients based on gender (with exception of the TURN2-trial) and cytomegalovirus (CMV)/ Epstein–Barr virus (EBV) status. Donors of the TURN2-trial were additionally selected on a putatively favorable microbiota profile based on results from a previous TURN1 trial, including high alpha-diversity and high predicted butyrate production[24,25].

Data and statistics

Data were collected from January 2018 to August 2021. To date, donor recruitment is still carried out for the IMITHOT and TURN2-trial. Data were collected in the Electronic Data Capture system Castor EDC. Descriptive statistics were used to summarize variables. Normally distributed continuous data are expressed as mean (SD). Not normally distributed continuous data are presented as median (IQR). Categorical data are displayed as frequencies (percentages). Data were analyzed using IBM SPSS Statistics for Windows, Version 26.0 (Armonk, NY: IBM Corp).

RESULTS

Initial donor screening

From January 2018 to August 2021, a total of 393 potential donors underwent prescreening. A flowchart of donor screening is presented in **Fig 1.** The main causes for failing prescreening were lost to follow-up (N=97), logistics problems (N=35, *e.g.,* working from home as a result of national COVID-19 measures), occupation as a health care worker with direct patient contact (N=23), and the use of medication, including pre- and probiotics (N=19). Eventually, only half of the initial respondents signed informed consent and continued the screening procedure (N=195). After consenting, four individuals did not respond to further communication and were lost to follow-up. All other potential donors filled in the online screening questionnaire (N=191). Based on 191 completed questionnaires, 43 individuals (23%) were excluded for various reasons (**Fig 1**). Hereafter, 148 potential donors remained and sent in fecal samples for parasitology screening. This screening step resulted in the largest relative loss of potential donors, with positive test results in 91 out of 148 samples (61%). Potential donors tested most frequently positive for *Dientamoeba fragilis* (N=26, 29%), microscopic quantification of 'moderate' or 'many' *Blastocystis spp. (*N=25, 27%), or a combination of both (N=34, 37%). Asymptomatic infestation with *Giardia Lamblia* and *Cryptosporidium* resulted in the exclusion of two additional donors. One donor was dismissed from further screening steps because remarkably high amounts of yeasts were noticed during microscopy evaluation of the stool. Next, 57 potential donors continued screening and delivered stool samples for biochemical, bacterial, and viral analysis.

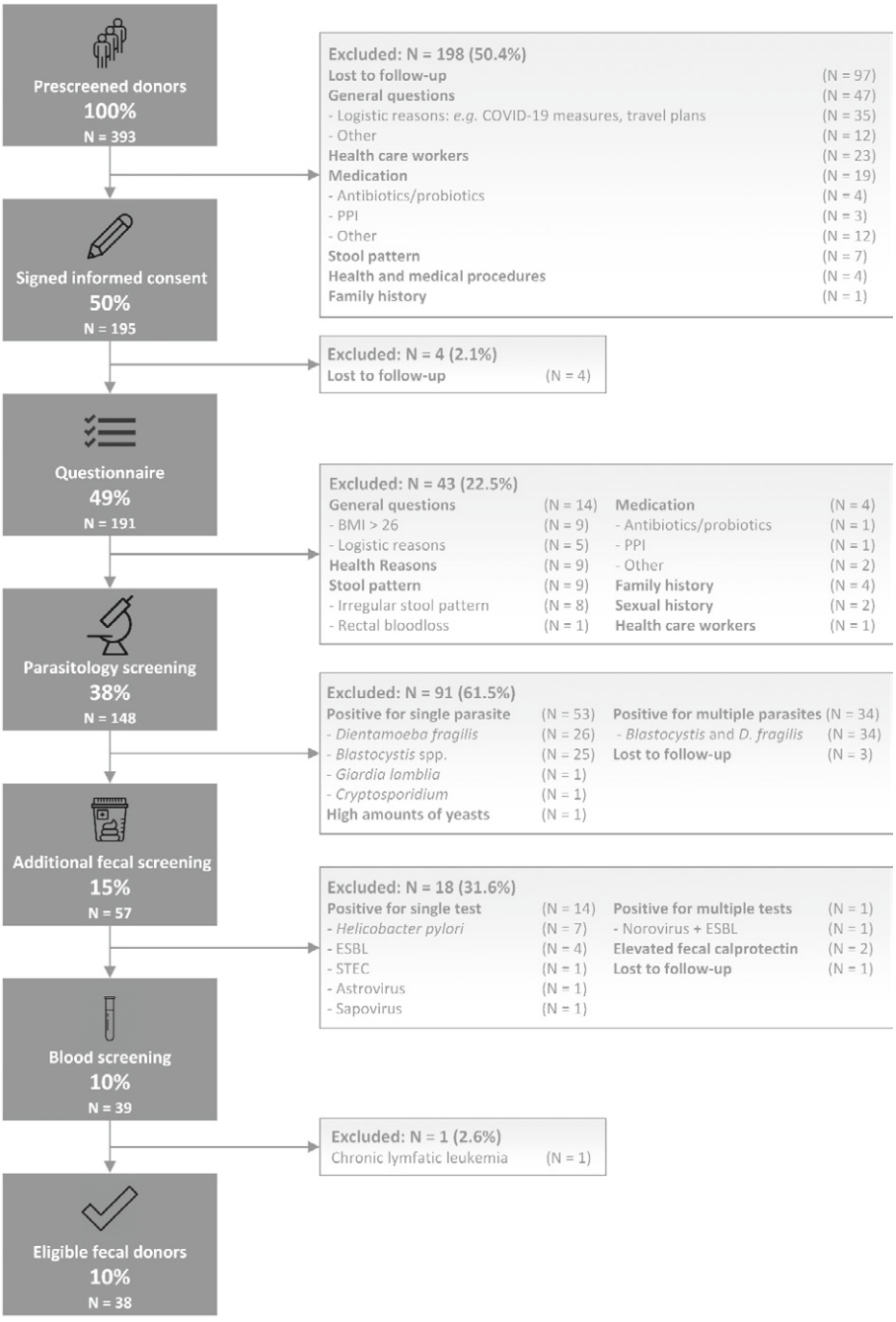

Figure 1. Flow diagram of donor screening outcomes.

Eighteen out of 57 individuals (32%) failed these stool tests: 7 had Helicobacter pylori, 4 an ESBL-producing strain of E. coli, 1 individual had a Shiga toxin-producing *E.coli* (STEC), 2 potential donors tested positive for a pathogenic virus (astrovirus, sapovirus) and one individual tested positive for multiple tests (norovirus plus an ESBL). Two additional potential donors were excluded due to elevated fecal calprotectin levels (79 and 87 ug/g). The penultimate screening step consisted of blood analysis and resulted in the exclusion of only one individual who had remarkably high levels of lymphocytes and was later diagnosed with chronic lymphatic leukemia. Serum screening for the presence of Hepatitis B and C, HIV, recent infection of CMV and EBV, *Strongyloides,* and *Treponema pallidum* didn't result in any positive tests. In the end, only 38 of the initial 393 individuals (10%) could be enrolled as fecal donors.

Eligible fecal donors

A flowchart of the follow-up of eligible donors is presented in **Fig 2.** The median age of the 38 eligible fecal donors was 28 years (IQR: 25 – 31.5 years), and 14 donors (36.8%) were male. Eligible donors had a healthy weight with a median BMI of 22.5 kg/m^2 (IQR: 20.3 – 24.0 kg/m^2). Twenty-four of the 38 eligible fecal donors (63.2%) donated at least one time, further referred to as 'active donors'. The other 14 'non-active' donors could not be matched to a patient due to their microbiota profile (TURN2-trial), gender and/or CMV/EBV status, and therefore did not donate (demographic and referral reasons are listed in **S1 Table**). The number of donations per active donor ranged from 2 to 48 with a median of twelve donations (IQR: 5.3-18.8). Seven donors donated for and participated in multiple studies. The active donors (N=24) had a median participation time of 13 months (IQR: 8 – 16 months). Additional screenings due to symptoms or exposure to risk factors were performed in 11 donors with a total of 34 tests, of which 11 (32.6%) returned positive. Five donors had transient positive tests that didn't lead to definite exclusion, most frequently a transient infection with enterovirus. Reasons for definite exclusion of active donors varied; six donors were excluded due to recurrent positive stool testing of which the majority tested positive for *Dientamoeba fragilis* and/or microscopic quantification of 'moderate' or 'many' *Blastocystis spp.* (N=4). Demographic characteristics of the active donors, details on (re)screenings, and reasons for later exclusion are listed in **S2 Table**. At time of writing (August 2021), only five out of the 38 eligible donors (13%) were still qualified and active donators. The median time of their participation up till August 2021 was 9 months (IQR: 4 – 21.5 months).

initial screening, one full rescreening (every six months), four times an additional 60-day screening, and average costs of additional screenings per active donor (€197,-). In the TURN2-trial, in which frozen feces is used, the total screening costs per year are even higher; €5,388 a year per active donor, including full initial screening, two complete re-screenings (every four months) with PCR assays, three additional 60-day screening, and average costs of additional screenings per active donor (€197,-).

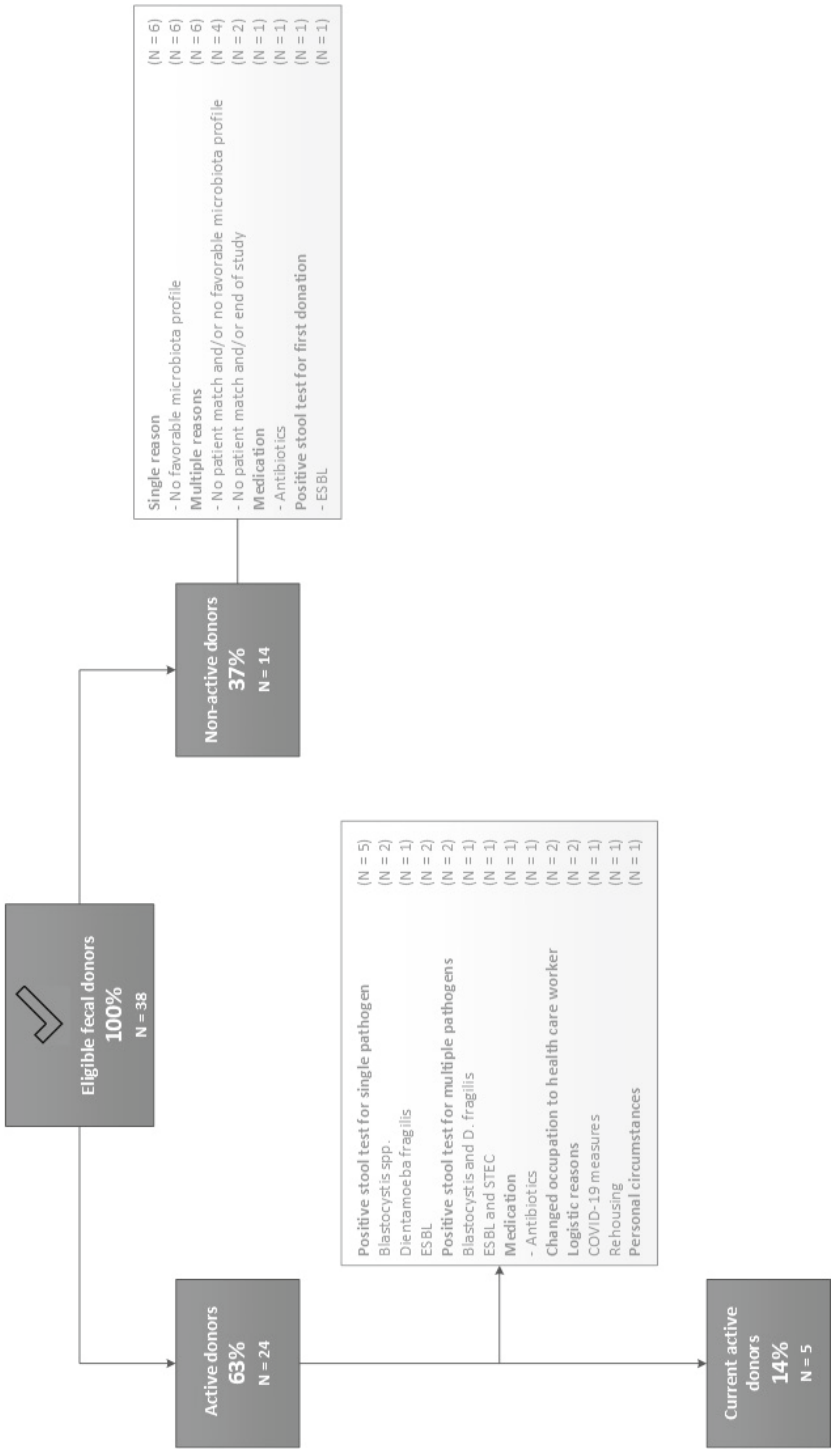

Figure 2. Flow diagram of follow-up of eligible donors.

Screening costs

An initial safety screening at our center amounted to €846 for all fecal and blood tests only, not including microbiota profiling (TURN2), costs for location, travel allowance and compensation for donors, and wage of study coordinators (**Table 3**). The total cost of all performed biochemical tests was €64,112 to find 38 eligible fecal donors. Total screening costs per active donor were estimated at €2,774 a year, including full

Table 3. Total costs donor screening procedure

Screening	€
Full screening	**846**
Feces screening	*657*
Serum screening	*189*
60 - day screening	**195**
Multidrug resistant organisms	*150*
SARS-CoV-2	*45*
Full rescreening 4 months (*TURN2*)	**940**
Feces screening	*612*
Serum screening[a]	*328*
Full rescreening 6 months (*FAIS, IMITHOT, PIMMS)*	**846**
Feces screening	*657*
Serum screening	*189*
Additional rescreening[b]	**Variable**
Feces bacteria	*150*
Feces MDRO	*150*
Feces viral gastroenteritis	*45*
Feces SARS-CoV-2	*45*
Feces parasites	*212*
Serum haematology	*44*

[a] Full rescreening in TURN2-trial included PCR assays of HIV, CMV, HBV and HCV instead of serology;
[b] In case of concerns donation was suspended and an additional rescreening was performed depending on symptoms and/or exposed risk factor of the fecal donor.

DISCUSSION

The expanding use of FMT in daily practice and clinical trials is accompanied by a need for more long-term available fecal donors and feasible donor screening programs. In this study, we reported our experience with stool donor recruitment, screening, follow-up, and associated costs in the context of clinical FMT trials. Our study showed that only 10% of potential donors passed all screening steps and could be enrolled as stool donors. Adding to the current literature, we reported the follow-up of eligible donors. In our experience, once qualified, active donors were eligible to donate for about a year before exclusion. Recruiting eligible donors is not only challenging, but also costly; we spent over €64,000 on biochemical tests only to detect 38 suitable

fecal donors. This study highlights the obstacles in donor screening and provides practical insights for FMT researchers.

Previous research on donor screening showed variable success rates between 0.8 - 31%[15-21]. Our 10% eligibility rate is similar to smaller studies performed by Craven et al.[15] and Paramsothy et al.[19]. A higher success rate compared to our data was reported in a Danish study, and may be explained by the fact that in this study potential donors were recruited among an existing cohort of eligible blood donors, in which the risk of transmittable infectious diseases by blood transfusion is already assessed[21]. Lower success rates were published by Openbiome, the first public stool bank based in the USA, in which over 15.000 candidates were (pre-)screened and only 3% eventually qualified as fecal donors. The majority of candidates (66%) failed prescreening mainly due to social history reasons and body-mass index higher than 30 kg/m^2 [27]. In our cohort approximately half of all potential donors failed prescreening (N=198) of which half was lost to follow-up after initial contact (N=97). Especially during the COVID-19 pandemic, when in periods employees were requested to work from home in accordance to national measures, we experienced high rates of exclusion due to logistics of stool donations. It could be assumed that the COVID-19 pandemic also impacted our high rates of lost to follow-up during prescreening. More insight into motivation and preferences around stool donation is needed to improve initial donor recruitment and to reduce drop-out rates. Limited data on this subject is available[28]. Based on a multinational questionnaire study, McSweeney and colleagues identified that a male gender and being a blood donor is associated with a high willingness to stool donation, whereas a lack of knowledge on FMT and logistic burdens around screening and stool donation were reported as deterrents[28]. These variables should be taken into consideration. In general, the process of screening and donating should be as easy and convenient for donors as possible.

The global distribution of donor exclusion reasons varies not only as a result of different screening criteria between FMT centers and stool banks[9] but also on diagnostic approach and geographic location. For example, in the Hong Kong study stool tests were failed by the majority (86%) due to the carriage of ESBL-producing *Enterobacteriaceae*[16]. High prevalence of ESBL in this area is the result of several factors, including a high population density and diet habits. High carrier ship of ESBL-producing organisms seems less of an issue for donor selection in the USA and the Netherlands, where stool bank Openbiome tested only 3 of 571 (0.5%) stool donors positive for MDROs[27], and in our experience, ESBL positive stool tests accounted for the exclusion of five (8.8%) Dutch individuals at initial screening. Moreover, the US FDA has warranted screening for enteropathogenic E. coli (EPEC) by stool nucleic acid amplification testing (NAAT) in addition to Shiga toxin-producing E. coli (STEC)[29]. In our cohort one individual failed stool testing due to the presence of STEC. Currently, EPEC is not included in our screening, because data on pathogenicity of EPEC is

inconclusive[30]. Including EPEC in our screening protocol could result in even higher rates of donor exclusion.

In our cohort, we found positive parasite testing as the most common exclusion reason during the laboratory screening stage (91 out of 148 stool samples, 61.5%), in specific the presence of *Dientamoeba fragilis* or high amounts of *Blastocystis* spp. This is why, at least in certain cohorts, parasitology testing should follow as first step of the laboratory testing phase after (pre-)screening questionnaires. *D.fragilis* and *Blastocystis* spp. were also leading reasons for exclusion in the Canadian study by Craven et al.[15] and the Australian study by Paramsothy et al.[19], but not in others[16,17]. Despite the recommendation of an international guideline to screen and exclude for these protozoa, heterogeneity between screening procedures in practice exists. According to a systematic review evaluating 168 FMT studies, only 15.7% and 14.5% of studies specifically report screening for *D. fragilis* and *Blastocystis* spp., respectively[9]. Moreover, many studies do not state the methods to screen for these organisms, even though the specific diagnostics used has a considerable influence on the detection rate. To illustrate this, the introduction of a *Blastocystis spp.* polymerase chain reaction (PCR) test by the NDFB in 2018 resulted in the discovery that feces from previously by-microscopy-regarded *Blastocystis* spp.-negative donors did actually contain DNA of *Blastocystis subtypes 1* or *3* and that these *Blastocystis* spp. were transferred to 31 patients via FMT[31]. Importantly, this did not have a negative effect on the efficacy of treatment for CDI nor resulted in gastrointestinal symptoms. The potential risk of harming recipients by transferring *Blastocystis* spp., might be overestimated. In fact, patients that received *Blastocystis* spp.-positive donor stool evaluated their defecation pattern in the long-term as more improved than those receiving *Blastocystis spp.*-negative donor stool[31].

Current consensus recommendations for screening stool donors are based on safety criteria, drawn up by FMT experts in the field, and aim to minimize the risk of inadvertently transmitting a communicable disease to an FMT recipient. Once a potential pathogen is added to the screening norms it can be difficult to defer it later. However, since the field of FMT research is still relatively new, these criteria are not always supported by solid data and should therefore be adjusted to risk-benefit analysis and progressive insights. For example, whether the exclusion of *D. fragilis*- and *Blastocystis* spp.-positive donors is justified could be questioned, especially for *Blastocystis* spp. of which the pathogenicity is still under debate[32,33]. Both *Blastocystis* spp. and D. *fragilis* appear more commonly in asymptomatic individuals than in patients with gastrointestinal symptoms or disorders, suggesting that these protozoa can have a commensal relationship with human hosts[34-36]. Interestingly, recent literature shows a link between the presence of the above-mentioned single-cell eukaryotes, especially *Blastocystis* spp., and gut microbiota features[37]. For example, stool containing *Blastocystis* spp. has been associated to higher bacterial diversity and

distinct microbial profiles (*e.g.* enterotype Bacteroides[38] and co-occurrence with the beneficial bacteria *Akkermansia*[39]), and their presence may reflect a healthier state of the gut microbiota[38-43]. The application of the current consensus screening protocol that suggests the exclusion of *Blastocystis* spp. positive donors[14] could therefore result in the elimination of stool donors that have a favorable bacterial community. This led us to adjust our initial screening protocols where we now accept donors with microscopic quantification of 'rare' or 'few' *Blastocystis* spp. and only exclude individuals with 'moderate' of 'many'*Blastocystis* spp.[26]. Due to the double-blinded nature of the described ongoing clinical studies, it is not yet established if *Blastocystis* spp. positive FMT products have been transferred to our study patients. To prevent unnecessary elimination of valuable stool donors, future research should look into the influence of co-transplantation of common protozoa (and their subtypes) on the microbiota structure and efficacy of FMT.

Since there is limited understanding of what constitutes a successful stool donor for different conditions, most current screening protocols do not comprise potential predictors for FMT efficacy. Nevertheless, it is clear that FMT can improve disease outcome in some recipients (responders), but not in all (non-responders). Hence, the current 'one stool fits all' approach may not be the way to go[44-46]. A more personalized donor-recipient matching strategy where donors are screened for taxa associated with metabolic pathways, or directly for metabolites[47], that are disturbed in a particular disease phenotype, might enhance FMT efficacy. Conversely, the more tailor-made matching strategies will become, the harder the search for suitable donors will be. Evidently, future larger-scale studies in the FMT field are needed to further explore donor-dependent predictors of treatment success.

In the current trials, 14 eligible donors could not be matched to recipients based on gender and/or CMV/EBV status. These mismatches led to expiration of costly screening results and non-activity of valuable stool donors. This waste of screening costs is partly explained by the fact that the current trials started with establishing a pool of healthy stool donors, whereas at that time no patients were included and stool donation was not yet required. Donor-recipient incompatibilities could be prevented by a more synchronous approach of execution of donor screening programs and patient recruitment. Alternatively, especially in trials using fresh fecal material for FMT, another approach could be applied where patients are first recruited and serologically profiled and subsequently a suitable donor is being sought. The stepwise approach for donor screening could then start with serological testing for pathogenic viruses. Only in case of gender and/or CMV/EBV match, the potential donor could continue full screening program. However, postponing the search of stool donors until a study patient has been screened, might result in an unnecessary delay in the start of study treatment.

Direct costs of an initial safety screening at our center was €846 (891 USD) per donor. These costs did not include overhead, administration costs and personnel. Limited data is available on associated donor screening costs in other centers. In accordance with our study, Kazerouni et al.[48] evaluated screening costs for Openbiome to be 885 USD per donor, including clinical assessment, stool and serum screening. The Canadian study by Craven et al.[15] reported that the costs for a full donor screening work-up (including history, examination, blood, stool, and urine screening, and administration) were approximately 440 USD per donor. Differences in costs can be explained by lower costs of biochemical tests in Canada. As discussed previously, minor differences in screening protocols occur since no current consensus on the perfect screening program exists. It should be considered that stricter regulations can lead to increased rates of (temporarily) donor disqualification and even higher associated donor screening costs. Examples of stricter regulations compared to our donor protocol are more regular rescreening of active fecal donors, screening for more enteric pathogens (e.g. EPEC implemented by OpenBiome[49]), broader assessment of conditions (e.g. anti-nuclear antibody test for autoimmune diseases[50]), and mandatory donation of feces in a supervised bathroom. By reporting the average costs associated with our donor screening program we provide an estimate for clinicians thinking of establishing a pool of healthy stool donors for FMT research. Collaboration with other FMT researchers or national stool banks, in order to share screening costs and eligible donors, will presumably be more cost-effective. Furthermore it lowers the chance of discarding valuable FMT products when a suitable patient match cannot be found within a relatively small study cohort.

Nowadays, FMT is a widely accepted treatment for recurrent *Clostridioides difficile* infection[1,2]. The application of FMT as a treatment for other conditions associated with alterations in the gut microbiome, is limited to the context of clinical trials[8-10]. This barrier has driven some patients to seek for alternative options, including Do-It-Yourself-FMT procedures with self-administration of (mostly) unscreened donor feces[51]. The high rates of donor exclusions in seemingly healthy individuals reported by our study and other FMT programs[15-21] illustrates that Do-It-Yourself-FMT procedures can be accompanied by several risks, most importantly the risk of inadvertent transmission of an infectious disease to an FMT recipient. Ekekezie et al. studied factors that influenced willingness to pursue DIY-FMT. Results showed that majority of respondents would have preferred to have FMT performed in a clinical setting[51]. However, lack of access drives these patients to try FMT at home. Regulated stool banks could partially attenuate this problem by enabling compassionate use of FMT in carefully defined clinical cases. A major advantage of regulated (national) stool banks is to ensure safety of FMT products by following strict safety criteria for screening stool donors. Nevertheless, health care professionals must acknowledge the fact that DIY-FMT is an actual phenomenon and therefore clinicians should discuss concerns regarding safety and potential harms with patients considering such a

procedure. On the other hand, commercial developers argue that the development of synthetic microbial community products seem to be a safe and sustainable alternative to conventional FMT[52]. However, most colonic bacteria are yet unculturable not and current synthetic microbial products contain limited strains and therefore poorly represent the gut microbiome. Data on clinical efficacy of these products as well as their longterm safety is yet unavailable. Also, data on transmission of uncovered harmful species (i.e. potentially procarcinogenic or pathogenic) can only be derived retrospectively from performed conventional FMT studies[53]. Using synthetic microbial products in FMT trials would rule out the possibilities for these ancillary findings.

This study has several strengths. Firstly, our study included data regarding recruitment and selection procedures of healthy fecal donors from four different clinical FMT trials, creating a large cohort. Secondly, by presenting follow-up data we provided information on the time frame in which donors were qualified to donate feces after successful screening. Furthermore, this study included an estimation of donor screening costs. By presenting discussed data, this study provides insights in the challenges for creating a sustainable feces donor pool and is accordingly relevant for researchers setting up clinical FMT trials.

Nonetheless, this study also has some limitations. First, the FMT trials required donors to deliver fresh fecal samples to the hospital for rapid procurement. Therefore, only donors living within a short travel distance were included, comprising mostly urban areas. This potentially influenced the presence of pathogenic microorganisms as mentioned above and limits the generalizability of our results to other regions and countries. Secondly, due to our stepwise screening approach not all fecal and blood laboratory tests were executed on every potential donor. Therefore, presented data on donor deferral reasons per step should be interpreted with caution. Lastly, as discussed, minor differences in the screening protocols of the four included clinical trials were present. Pre-screening approaches through advertising and short telephonic interviews to discuss in- and exclusion criteria were not standardized. As a consequence, possible exclusions of potential donors and multiple donor deferral reasons could have been missed. Nevertheless, the most relevant in- and exclusion criteria were similar and our approach is in line with current available screenings protocols[14,23]. Therefore, we believe that the effect of the minor (pre-) screening differences is limited.

CONCLUSION

In conclusion, this study shows that a thorough screening protocol for stool donors in the context of clinical FMT trials results in only 10% being eligible donors and is associated with substantial costs. The majority of healthy asymptomatic donors failed stool testing, predominately due to positive parasite testing. The need to exclude

donors that carry certain protozoa, especially *Blastocystis* spp., is questionable. The high rates of donor exclusions in seemingly healthy individuals reported by our study illustrates that Do-It-Yourself-FMT procedures can be accompanied by several risks, Further research into the centralization of stool donor screening and procurement of FMT products is warranted.

SUPPORTING INFORMATION

S1 Table. Demographics and reasons of exclusion of non-active donors. [a] based on genderand/or CMV/EBV status; [b] PIMMS or FAIS study; [c] Donors of the TURN2-trial were additionally selected on a putatively favorable microbiota profile based on results from a previous TURN1 trial. Abbreviations: ESBL, extended spectrum beta-lactamase.

S2 Table. Demographics, specifications of screening, and reasons of exclusion of active donors. [a] Determined microscopically by an experienced laboratory analyst[26]; [b] PIMMS or FAIS study; [c] based on gender or CMV/EBV status. Abbreviations: CBC, complete blood count; CRP, c-reactive protein; DFT, dual feces test; ESBL, extended spectrum beta-lactamase; NA, not applicable; STEC, shiga toxin-producing Escherichia coli; MDROs, multidrug resistant organisms; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

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SUPPLEMENTARY MATERIAL

Table S1. Demographics and reasons of exclusion of non-active donors.

Donor	Included study/ studies	Age	BMI	Sexe	Reason of exclusion
1	PIMMS	41	24,7	M	Antibiotic use
2	IMITHOT	24	21,3	F	ESBL-strain *Escherichia coli*
3	FAIS / TURN2	25	23,8	F	No patient match [a], end of study [b]; no favorable microbiota profile [c]
4	PIMMS	28	24,8	M	No patient match [a], end of study [b]
5	FAIS / TURN2	23	23,4	F	No patient match [a], end of study [b]; no favorable microbiota profile [c]
6	FAIS	28	22,8	M	No patient match [a], end of study [b]
7	TURN2	29	19,0	F	No favorable microbiota profile [c]
8	TURN2	43	24,9	F	No favorable microbiota profile [c]
9	TURN2	33	23,9	F	No favorable microbiota profile [c]
10	TURN2	31	23,5	F	No favorable microbiota profile [c]
11	FAIS / TURN2	26	20,1	F	No patient match [a], end of study [b]; no favorable microbiota profile [c]
12	IMITHOT/ TURN2	27	22,4	M	No patient match [a], end of study [b]; no favorable microbiota profile [c]
13	TURN2	29	23,9	F	No favorable microbiota profile [c]
14	TURN2	30	19,7	F	No favorable microbiota profile [c]

[a] based on gender and/or CMV/EBV status; [b] PIMMS or FAIS study; [c] Donors of the TURN2-trial were additionally selected on a putatively favorable microbiota profile based on results from a previous TURN1 trial. Abbreviations: ESBL, extended spectrum beta-lactamase.

Table S2. Demographics, specifications of screening, and reasons of exclusion of active donors.

Donor	Included study/ studies	Age	BMI	Sexe	Donations	Full screenings	60 days screenings	Additional screenings	Transient positive tests	Transient pathogens	Reason of exclusion
1	IMITHOT	34	19.6	F	6	2	3	0	0	NA	Antibiotic use
2	FAIS	27	21.8	F	12	5	3	5 (DFTs)	2	*Blastocystis* spp. ('rare' or 'few') [a]	*Blastocystis* spp. ('moderate' or 'many') [a]
3	FAIS / TURN2	27	20.4	F	5	1	0	1 (serum CBC, CRP)	0	NA	*Blastocystis* spp.
4	TURN2	28	20.2	F	21	4	3	0	0	NA	Changed occupation to health care worker
5	TURN2	29	25.0	M	19	3	3	5 (feces viral)	2	Enterovirus	Changed occupation to health care worker
6	IMITHOT / FAIS	25	20.2	M	12	3	5	0	0	NA	COVID-19 measures
7	TURN2	22	24.7	F	18	4	2	6 (DFTs)	0	NA	*Dientamoeba fragilis*
8	FAIS	29	23.9	M	2	2	0	0	0	NA	*Dientamoeba fragilis* + *Blastocystis* spp.
9	PIMMS	29	23.7	M	9	2	2	0	0	NA	End of study [b]
10	PIMMS	33	20.6	M	3	2	1	1 (feces bacterial)	1	Yersinia enterocolitica	End of study [b]
11	IMITHOT / FAIS	38	24.4	F	2	2	1	0	0	NA	End of study [b]
12	TURN2	51	22.6	F	7	2	3	3 (MDROs)	0	NA	ESBL-strain *Escherichia coli*
13	PIMMS / TURN2	45	21.1	M	18	4	3	0	0	NA	NA (active donor)

Table S2. Continued

Donor	Included study/ studies	Age	BMI	Sexe	Donations	Full screenings	60 days screenings	Additional screenings	Transient positive tests	Transient pathogens	Reason of exclusion
14	IIMTHOT / FAIS	23	23	M	13	3	4	1 (SARS-CoV-2)	0	NA	NA (active donor)
15	TURN2 / IMITHOT / FAIS	25	24.0	F	28	3	5	5 (feces viral)	5	Noro-, Sapo-, Enterovirus	NA (active donor)
16	TURN2	21	24.2	M	16	2	0	0	0	NA	NA (active donor)
17	IMITHOT	23	21.7	F	6	1	1	0	0	NA	NA (active donor)
18	TURN2 / IMITHOT / FAIS	27	19.0	F	28	4	3	1 (SARS-CoV-2)	0	0	No patient match [c]
19	FAIS	31	21.0	F	2	1	0	0	0	NA	No patient match [c]
20	IMITHOT	22	21.6	M	3	1	3	3	0	NA	No patient match [c]
21	TURN2	47	25.4	M	48	5	5	0	0	NA	Personal circumstances
22	IMITHOT / PIMMS FAIS	28	25.3	F	27	1	1	0	0	NA	Rehousing
23	TURN2	26	18.9	F	14	3	4	3 (feces viral, STEC, MDROs)	1	Enterovirus	STEC + ESBL-strain *Escherichia coli*
24	FAIS / PIMMS	25	20.7	F	6	2	1	0	0	NA	Travel

[a] Determined microscopically by an experienced laboratory analyst.[38]; [b] PIMMS or FAIS study; [c] based on gender or CMV/EBV status. Abbreviations: CBC, complete blood count; CRP, c-reactive protein; DFT, dual feces test; ESBL, extended spectrum beta-lactamase; NA, not applicable; STEC, shiga toxin-producing Escherichia coli; MDROs, multidrug resistant organisms; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2.

PART III

... AND BEYOND

9

INTESTINAL PERMEABILITY IS ASSOCIATED WITH AGGRAVATED INFLAMMATION AND MYOFIBROBLAST ACCUMULATION IN GRAVES' ORBITOPATHY: THE MICROGO STUDY

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ABSTRACT

Background

Graves' disease (GD) and Graves' orbitopathy (GO) result from ongoing stimulation of the TSH receptor due to autoantibodies acting as persistent agonists. Orbital pre-adipocytes and fibroblasts also express the TSH receptor, resulting in expanded retro-orbital tissue and causing exophthalmos and limited eye movement. Recent studies have shown that GD/GO patients have a disturbed gut microbiome composition, which has been associated with increased intestinal permeability. This study hypothesizes that enhanced intestinal permeability may aggravate orbital inflammation and, thus, increase myofibroblast differentiation and the degree of fibrosis.

Methods

Two distinct cohorts of GO patients were studied, one of which was a unique cohort consisting of blood, fecal, and retro-orbital tissue samples. Intestinal permeability was assessed by measuring serum lipopolysaccharide-binding protein (LBP), zonulin, TLR5, and TLR9 ligands. The influx of macrophages and accumulation of T-cells and myofibroblast were quantified in orbital connective tissue. The NanoString immune-oncology RNA targets panel was used to determine the transcriptional profile of active fibrotic areas within orbital sections.

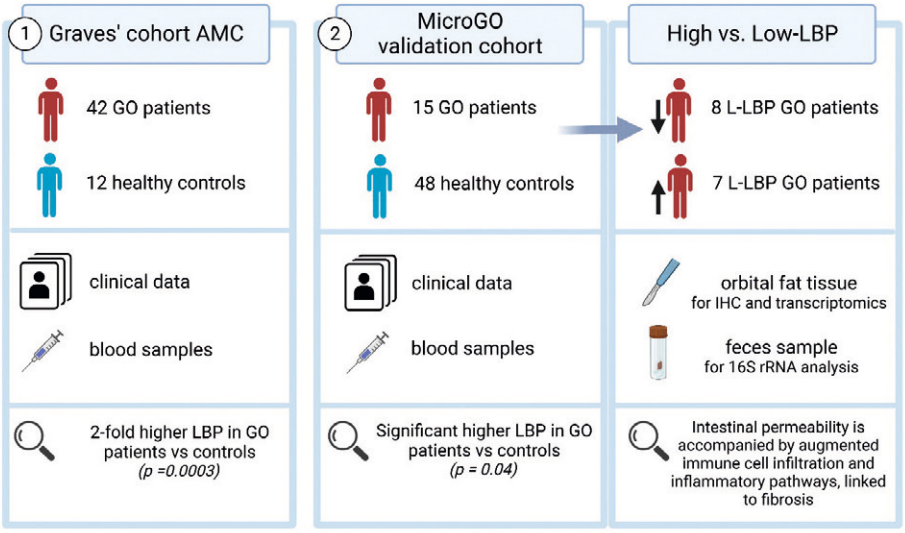
Results

GO patients displayed significantly higher LBP serum concentrations than healthy controls. Within the MicroGO cohort, patients with high serum LBP levels also showed higher levels of zonulin and TLR5 and TLR9 ligands in their circulation. The increased intestinal permeability was accompanied by augmented expression of genes marking immune cell infiltration and encoding key proteins for immune cell adhesion, antigen presentation, and cytokine signaling in the orbital tissue. Macrophage influx was positively linked to the extent of T cell influx and fibroblast activation within GO-affected orbital tissues. Moreover, serum LBP levels significantly correlated with the abundance of specific Gram-negative gut bacteria, linking the gut to local orbital inflammation.

Conclusion

These results indicate that GO patients have enhanced intestinal permeability. The subsequent translocation of bacterial compounds to the systemic circulation may aggravate inflammatory processes within the orbital tissue and, as a consequence, augment the proportion of activated myofibroblasts, which actively secrete extracellular matrix leading to retro-orbital tissue expansion. These findings warrant further exploration to assess the correlation between specific inflammatory pathways in the orbital tissue and the gut microbiota composition and may pave the way for new microbiota-targeting therapies.

Graphical abstract



INTRODUCTION

Graves' disease (GD), characterized by TSH-receptor stimulating antibodies and increased thyroid hormone serum levels, is an autoimmune disease affecting roughly 3% of the general population[1]. GD is the most common form of hyperthyroidism [1]and up to 40% [CI 0.32 – 0.48] of GD patients have clinically apparent abnormalities of orbit soft tissue, known as Graves' orbitopathy (GO) or thyroid eye disease (TED)[2].

Manifestations of GD/GO result from a B cell-mediated autoimmune response against the thyrotropin receptor (TSHR), resulting in the plasma cell production of autoantibodies targeting the thyrotropin receptor (TRAb). These autoantibodies bind and stimulate the TSH receptor resulting in excess secretion of thyroid hormones, namely triiodothyronine (T3) and thyroxine (T4), thus causing the clinical manifestations[3,4]. TSHR is also expressed in extra-thyroidal tissue, including the orbital fat tissue and extra-ocular muscles; therefore TRAbs can lead to GO development. The latter is characterized by profound orbital tissue remodeling, with inflammation and extracellular matrix deposition being the drivers of the clinical GO manifestations, including periorbital edema, exophthalmos, limited ocular movement, and in severe cases, optic nerve compression and blindness. Mechanistically, GO is induced by both autoantibodies and immune cell influx within the retro-orbital tissue. Orbital pre-adipocytes and fibroblasts express the TSH receptor, which, once activated by activating autoantibodies, creates a cross-talk with the insulin-like growth factor 1 receptor (IGF1R), leading to the induction of adipogenesis and differentiation of fibroblasts into myofibroblasts producing extracellular matrix[5]. The net result is an expansion of the retro-orbital tissue causing eye protrusion and limited movement. In addition, orbital fibroblasts can engage with autoreactive T cells through CD40 expression, resulting in the production of cytokines and more immune cell influx[3,4].

Both GO and GD are driven by a combination of genetic susceptibility (accounting for 79%) and environmental exposures (accounting for 21%)[6,7]. However, in recent years, gut microbiome composition and functionality have been implicated as another driver of host's health and disease, including various autoimmune diseases[8,9]. Interestingly, the gut microbiome is predominantly influenced by environmental factors, with only 2-8% of the variation explained by the host's genetics[10,11].

In addition to the well-recognized cross-talk between gut commensals and the host immune system[12], there seems to be an essential bidirectional signaling axis between the gut microbiome and the local thyroid gland, regulating thyroid homeostasis by iodine uptake, degradation, and enterohepatic circulation[13]. It has been shown that perturbations of the gut microbiome composition are present in human GD and GO fecal samples[14–18] and could therefore be pivotal in the pathophysiology of the disease.

Deviations in the gut microbiome have often been associated with impaired intestinal integrity and increased intestinal permeability[19,20]. This phenomenon, in popular terminology called a "leaky" gut, results in the translocation of bacterial components into the circulation, such as lipopolysaccharide (LPS), an endotoxin located in the outer membrane of many Gram-negative bacteria[21–23]. The leakage of bacterial compounds may promote systemic inflammation as they are recognized by innate receptors ubiquitously expressed by immune and parenchymal cells[20]. LPS-binding protein (LBP), a soluble glycoprotein that enhances the host's immune response to endotoxins, is used as a serum biomarker of intestinal permeability[23]. LBP has been previously reported to be increased in the circulation of GD patients[24].

In this observational study, we hypothesize that enhanced intestinal permeability, as inferred by measuring circulating levels of LBP, zonulin, TLR5 and TLR9 ligands, may aggravate orbital inflammation and, thus, increase myofibroblast differentiation and the degree of fibrosis. For this, we use two distinct cohorts of GO patients, one of which is a unique cohort with available fecal and blood samples as well as retroorbital adipose tissue biopsies.

METHODS

Study population

The cross-sectional data were obtained during study visits between 2013 and 2017. All participants provided written informed consent. The study was approved by the medical ethical review board of the Amsterdam University Medical Center (Amsterdam UMC), location AMC, and followed the principles of the Declaration of Helsinki (revisions 6 and 7).

Two distinct cohorts were used in this study. The first cohort is the "*Graves' cohort AMC"* and comprises a cross-sectional cohort including 42 Graves' patients with GO (N=21 with inactive moderate-to-severe GO and N=21 with active moderate-to-severe disease) and 12 healthy controls, of which clinical data and blood samples were collected. All Graves' patients in this cohort were currently using antithyroid drugs and levothyroxine supplementation therapy (**Table 1**).

The second cohort is our validation cohort, called the "*Graves' orbitopathy (MicroGO) cohort"*. This cohort comprises a smaller group of 15 GO patients and 48 healthy controls. It includes clinical data and blood samples of both groups, and fecal specimens and biopsies of orbital fat tissues of the Graves' patients.

All MicroGO cohort patients underwent orbital decompression surgery based on established clinical protocols from the Graves Orbitopathy outpatient clinic and were on stable antithyroid treatment for over three months (**Table 2**). During this

procedure, orbital connective tissue was removed to reduce the degree of proptosis. One part of the excised orbital tissue was snap-frozen in liquid nitrogen and stored at -80^0 until analysis; the other was held in 4% formalin for histological analysis. One day before the surgery, anthropometric characteristics, fasting blood samples, and stool samples were collected. Serum samples of healthy controls were collected similarly via the Amsterdam UMC Liquid Biopsy Centre and were matched to the GO patients based on gender, age, and BMI. All healthy controls were non-smokers and did not use any medication. Unfortunately, fecal samples and orbital tissue of healthy controls were not available. Individuals (GO patients and healthy controls) who underwent treatments with not eligible antibiotics, prednisone, or proton pump inhibitors within three months prior to the scheduled operation date, were not included to avoid confounding effects on the gut microbiota composition. A total of 15 GO patients and 48 healthy controls between 18-70 years old were included in the study.

Table 1. Patient characteristics of MicroGO cohort subjects.

Characteristic	GO patients (N = 15)	Healthy controls (N = 48)	p-value
Male sex (%)	5 (33.3)	22 (45.8)	0.579
Age (yr)	46.47 ±11.90	41.96 ±12.72	0.229
Current smoker (%)	5 (33.3)	0 (0.0)	**<0.001**
BMI (kg/m^2)	25.43 ±4.17	23.60 ±2.79	0.055
TSH (mU/L)	1.25 [0.46 – 3.57]	1.60 [1.08 – 2.60]	0.496
FT4 (pmol/L)	18.74 ±3.77	15.85 ±2.13	**0.001**
FT3 (pmol/L)	4.28 ±0.59	4.96 ±0.66	**0.001**
TBII (U/L)	3.42 [0.00 – 9.10]	0.00 [0.00 – 0.00]	**<0.001**
LBP (µg/m)	11.13 [9.89 – 13.95]	9.06 [6.87 – 11.39]	**0.044**

For normally distributed parameters, data are presented as mean ± SD, and p values were calculated using a Student's t-test. For non-normally distributed parameters, data are presented as median [IQR], and the p-value was calculated using the Mann-Whitney U test. Nominal variables are presented as n (%).

The 7-point Clinical Activity Score (CAS)[25] for assessing disease activity (**Table S1**) and EUGOGO classification (mild, moderate-to-severe, or sight-threatening) (**Table S2**)[26] for assessing disease severity were determined during an outpatient visit before the surgery by either an endocrinologist or an ophthalmologist.

Table 2. Patient characteristics of the MicroGO patients, separated by LBP serum level

Characteristic	Low LBP group (N = 8)	High LBP group (N = 7)	p-value
Male sex (%)	2 (25.0)	3 (42.9)	0.855
Age (yr)	42.12 ±12.76	51.43 ±9.31	0.136
Current smoker (%)	2 (25.0)	3 (42.9)	0.855
BMI (kg/m^2)	24.20 ±2.77	26.83 ±5.23	0.237
TSH (mU/L)	0.72 [0.26 - 3.54]	1.60 [1.25 - 3.35]	0.482
FT4 (pmol/L)	20.59 ±3.65	16.63 ±2.79	**0.037**
FT3 (pmol/L)	4.39 ±0.35	4.16 ±0.80	0.471
TBII (U/L)	1.71 [0.00 - 6.12]	5.17 [1.68 - 13.41]	0.473
LBP (µg/m)	9.89 [8.15, 10.40]	14.33 [13.18, 20.81]	**0.001**
CAS score	3.0 [1.5 - 4.0]	2.0 [0.75 – 2.25]	0.195
Hertel OS (mm)	25.0 ±2.8	22.9 ±1.6	0.089
Hertel OD (mm)	21.2 ±2.9	23.4 ±2.3	0.211
Thyroid medication (%)			0.117
- Block-and-replace therapy	8 (100.0%)	4 (57.1)	
- Levothyroxine only	0 (0.0%)	1 (14.3)	
- No (thyroid) medication	0 (0.0%)	2 (28.6)	
Severity			
- Mild	0 (0.0%)	0 (0.0%)	1.000
- Moderate to severe	8 (100%)	8 (100%)	
- Sight-threatening	0 (0.0%)	0 (0.0%)	

For normally distributed parameters, data are presented as mean ± SD, and p values were calculated using a Student's t-test. For non-normally distributed parameters, data are presented as median [IQR], and the p-value was calculated using the Mann-Whitney U test. Nominal variables are presented as n (%).

Thyroid markers

Serum levels of thyroid-stimulating hormone (TSH) and free thyroxine (fT4) were determined by electrochemiluminescence assay (ECLIA) using the *Cobas C8000* analyzer (Roche Diagnostics, Basel, Switzerland). Free triiodothyronine (fT3) was determined by Chemiluminescent Microparticle Immunoassay (CMIA) using the *Alinity I system* (Abbott Laboratories, Lake Bluff, Illinois, USA). Autoimmune hyperthyroidism was diagnosed by measuring serum thyroid-binding inhibitory immunoglobulins (TBII), which exploit the antibodies' ability to inhibit labelled-TSH binding to the TSHR[27]. TBII levels were determined on TRACE technology with a *Kryptor Compact Plus* analyzer (BRAHMS Thermo Scientific, Henningsdorf, Germany). Reference values ranged from 0.5-5.0 mU/L for TSH, from 12-22 pmol/L for fT4; from 2.5-5.1 pmol/L for fT3; TBII serum levels ≤1.0 U/L were considered negative, whereas TBII serum levels ≥1.8 U/L were considered as positive.

Intestinal permeability markers

Concentrations of LBP in serum (in ug/ml) were assessed by ELISA (Human LBP ELISA, Hycult Biotech, Leiden, The Netherlands) accordingly to the manufacturer's instructions. Participants were divided into either high- (H-LBP) or low-LBP (L-LBP) serum levels based on the median value of serum LBP. Zonulin serum concentrations (in ng/ml) were measured by ELISA (Human Haptoglobin DuoSet ELISA, R&D systems, Minnesota, USA) following the manufacturer's instructions. Presence of active ligands for TLR5 and TLR9 was tested using the HEK-Blue human TLR5/TLR9 reporter cell lines (InvivoGen). For this, 20 uL serum samples were used per well (in a 96-well plate) and mixed with 180 uL of 1.4x1E5 cells/mL in HEK-Blue SEAP (secreted embryonic alkaline phosphatase) detection media (InvivoGen, San Diego, USA), which allowed the detection of SEAP after 5-hour exposure as the reporter protein is secreted by HEK-Blue cells upon TLR5/TLR9 signaling activation.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) sections of orbital biopsies were utilized for immunohistochemical staining. Slides were deparaffinized in 100% xylene and rehydrated in ethanol (100%, 96%, and 70%) and H2O, followed by blocking endogenous peroxidase in 3% H2O2 methanol for 20 minutes and heat-induced epitope retrieval (HIER) in citrate buffer pH 6.0 at 98°C for 10 minutes. FFPE sections were then incubated with primary antibodies anti-CD68 (KP1; Cell Signaling Technology, Danvers, USA), anti-CD3 (D7A6E; Cell Signaling Technology, Danvers, USA), anti-smooth muscle actin alpha (1A4 clone, DAKO,), following by incubation with the secondary Poly-HRP-conjugated antibodies (BrightVision, Gothenburg, Sweden) for 30 minutes at room temperature. Staining was visualized with a 3,3'Diaminobenzidine (DAB) kit (Sigma Aldrich, St Louis, USA).

Image quantification: After immunohistochemistry, images of distinct areas of orbital tissue sections were taken in a blinded–manner. Staining for CD68, α-SMA (ASMA), and CD3 were quantified with the Image J software and are presented as a percentage of positive areas.

GeoMx Digital Spatial Transcriptome profiling

Orbital FFPE sections (8µm thick) were used to determine the transcriptional profile of specific fibrotic areas within the retro-orbital biopsies from GO patients. This assay is developed by Nanostring (NanoString Technologies Inc, Seattle, Washington, USA) and employs oligo-labeled probes (complementary sequences) that specifically align to targeted mRNA transcripts. Here we used the NanoString immune-oncology RNA targets panel, including six negative probes and five probes targeting housekeeping RNA transcripts. To compare the gene expression across multiple samples, the raw gene expression data were first normalized to the signal from negative probes and afterward to the housekeeping genes.

Several regions of interest per orbital section were selected based on immunofluorescence staining of morphology markers: DNA (nuclear staining), CD45, ASMA, and FABP4. This allowed us to navigate the slide and determine which areas were active fibrotic areas and which were occupied by mature adipocytes. As fibroblast activation and fibrosis are at the core of GO pathogenesis, we selected the region of interest within the ASMA-positive area. These selected regions were used by the GeoMx instrument for gene expression quantification.

Gut microbiota analyses

DNA was extracted according to the in-house 16S rRNA gene-based PCR amplification protocols performed at the University of Helsinki, using primers detecting the V3 regions of the 16S rRNA genes[28]. Samples were sequenced by Illumina HiSeq (Illumina, San Diego, USA). Sequences were truncated to 150 nucleotides. This read length gives accurate quality scores, which start dropping after 160-180 nucleotides, as reported previously[28]. Only forward reads were processed as these are most reliable and provided an accurate prediction of taxa when tested with the mock community (see Korpela et al. [28] and the MARE package manual in R, version 1.0, https://github.com/katrikorpela/mare). The minimum read abundance (sequences that occur less frequently than the threshold were discarded to avoid sequencing errors) was set to 10^{-05}. Consequently, sequences that appear fewer than 6 times were removed from preprocessing. The low threshold is set this way because of the exploratory nature of the sample type. Both databases "silva_v3v4_Gut.udb (confidence level 0)" and "silva_v3v4.fasta" were used for the annotation of OTUs. Three different non-template controls were preprocessed with the samples.

Data availability 16S rRNA gene sequencing data are deposited in the European Nucleotide Archive. The gene expression data obtained with Nanostring DSP GeoMx technology in formalin-fixed paraffin-embedded orbital tissues will be available upon request.

Statistical analysis

Mann-Whitney U or unpaired Student's t-tests were used to analyze differences between the two groups. Multiple comparisons (e.g., gene expression data analysis) were done by one-way ANOVA tests, followed by the Kruskal-Wallis test. Spearman nonparametric rank correlation was used to study relationships between variables. Statistically significant differences are shown by * for p-values equal or below 0,05, ** for p-values equal or below 0,01, *** for p-values equals or below 0,001.

RESULTS

GO is accompanied by increased intestinal permeability.
To investigate whether GO is accompanied by increased intestinal permeability, we measured the LBP concentrations, a common marker of intestinal permeability, in serum samples from the *Graves' cohort AMC*. We found that serum LBP levels are significantly increased (approximately 2-fold) in GO patients with inactive moderate-to-severe GO (N=21) and active moderate-to-severe (N=21) GO compared to healthy controls (N=12) (**Fig. 1A, Table S3**). However, the lack of a significant difference between the two patient groups may indicate that intestinal permeability is an early event that occurs equally at the beginning of GO pathogenesis and throughout its clinical manifestations.

To determine whether increased intestinal permeability influences the inflammatory milieu in the orbital tissue, we used a different unique cohort of 15 GO patients (*MicroGO* cohort) in which biopsies of orbital tissues were taken during surgery. Measurement of LBP in serum samples from this cohort validated the increased LBP levels in amount and significance found in GO patients as compared to healthy matched controls (**Fig. 1B**).

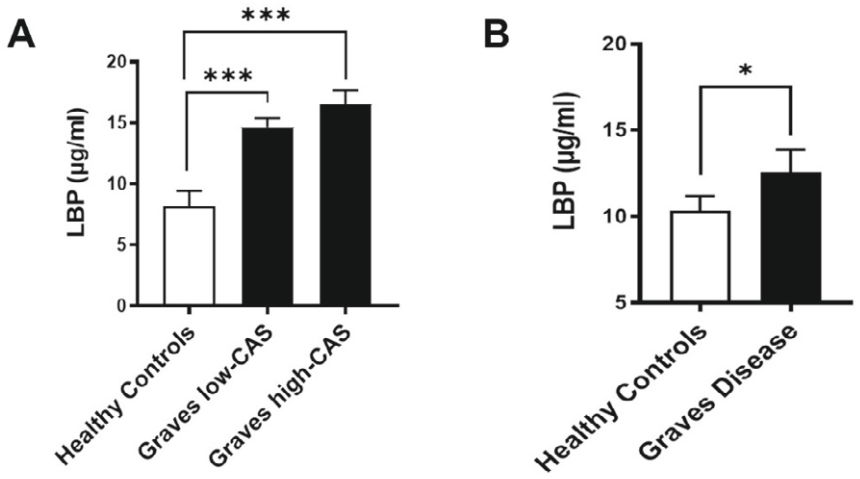


Figure 1. Serum LBP levels (ug/ml) in two distinct cohorts.

A. Graves' cohort AMC: Patients with inactive (CAS<3) moderate-to-severe (N=21) and active (CAS ≥4) moderate-to-severe (N=21) Graves' Orbitopathy (GO) compared to healthy controls (N=12). p=0,0003 between healthy and inactive GO, p=0,0001 between healthy and active GO groups.
B. Graves' orbitopathy (MicroGO) cohort (validation cohort): GO patients (N=15) compared to healthy matched controls (N=48); p=0.04.
A,B. Data shown as mean +/- SEM (standard error of the mean). Statistical significance determined with Mann-Whitney U test.

The baseline characteristics of the MicroGO cohort study (validation cohort) are provided in **Table 1**. Briefly, the majority of all 63 participants were female (57.1%), with an average age of 43.0 years. On average, the 15 GO patients were 46.5 years of age; 66.6% were female. GO patients had significantly different thyroid serum levels compared to healthy controls, with higher FT4 serum levels and lower FT3 and TSH serum levels. As expected, GO patients had significantly higher serum levels of TBII and LBP.

The GO group was then divided into high- (H-LBP) versus low-LBP (L-LBP) patients, based on the median serum levels LBP (11.13 ug/ml) (**Table 2**), to investigate whether differences in inflammatory and fibrotic markers within the orbital tissues occur as a result of different degrees of intestinal permeability. This resulted in eight patients with low serum levels of LBP (median 9.89 ug/ml) and seven patients with high LBP serum levels (median 14.33 ug/ml)(**Fig. 2A**). No significant differences were found in TBII serum levels, Hertel measurements, EUGOGO severity classification, anthropometric parameters, and medication use between the patients with low and high LBP. Interestingly, patients of the H-LBP group showed higher levels of another gut permeability marker, serum zonulin (p=0.054), than that of the L-LBP group (**Fig. 2B**). In line with higher permeability and higher translocation of bacterial components from the gut lumen to the circulation, the serum samples from the H-LBP group had a greater capacity, albeit not significant (p=0.1), to induce the activation of TLR5 and TLR9 signaling when compared to patients from the L-LBP group (**Fig. 2C,D**). Importantly, no significant differences in autoantibodies titers nor in clinical features were found between patients of the two groups (**Fig. 2E, Table 2**), indicating that any differences found in inflammatory markers in the orbital tissues are not attributable to the discrepancy in the levels of TSHR autoantibodies or different GO phenotypes, but rather due to the loss of intestinal barrier integrity. Similarly, the CAS score was not significantly different between H-LBP and L-LBP groups, suggesting that "intestinal leakage" occurs both in mild and severe GO and may be an early event in GO disease (**Fig. 2F**).

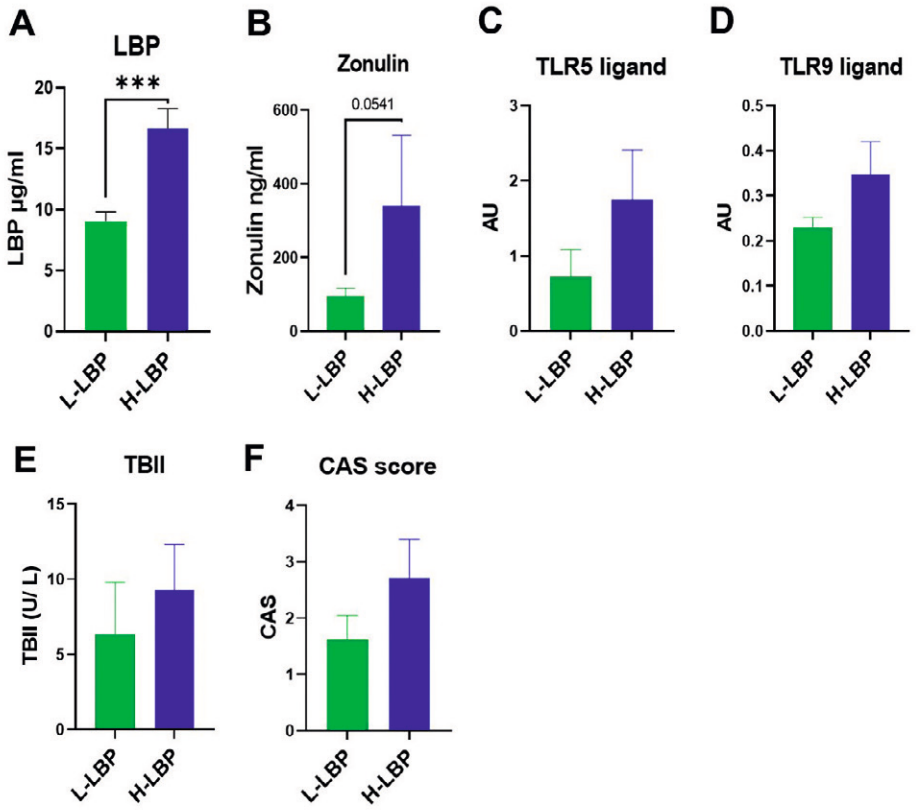


Figure 2. GO patients of the MicroGO cohort were divided into low- (L-LBP) versus high- (H-LBP) lipopolysaccharide-binding protein (LBP) serum levels.

A. Serum LBP levels in ug/ml; p=0,0003 B. serum zonulin concentrations in ng/ml; p=0,054 C,D. HEK-Blues reporter activity as a proxy of circulating levels of active bacterial ligands of (C) TLR5 (flagellin) and (D) active bacterial ligands of TLR9 (unmethylated CpG motifs of bacterial DNA); E. serum levels of TSH-binding inhibitor immunoglobulin (TBII) in U/L; F. Clinical Activity Score (CAS). Data is shown as mean +/- SEM (standard error of the mean). Statistical significance determined with Mann-Whitney U test.

Serum LBP levels are linked to specific gut commensal bacteria.

Using fecal DNA isolated from stool samples of the GO patients within our validation Graves' orbitopathy cohort, we performed 16S rRNA amplicon sequencing to determine the taxonomic profile of the fecal microbiota. This allowed us to determine that the relative abundance of two Gram-negative species, *Bacteroides spp.* and *Dialister spp.*, were positively correlated with the concentration of serum LBP (**Fig. 3A).** Moreover, we found that the relative level of *Lactobacillus* spp. was very strongly (over 10-fold) increased in the H-LBP group **(Fig. 3B)**. Notably, *Lactobacillus* abundance in

stool samples was shown to be associated with the severity of GO and specifically with orbital adipogenesis[29].

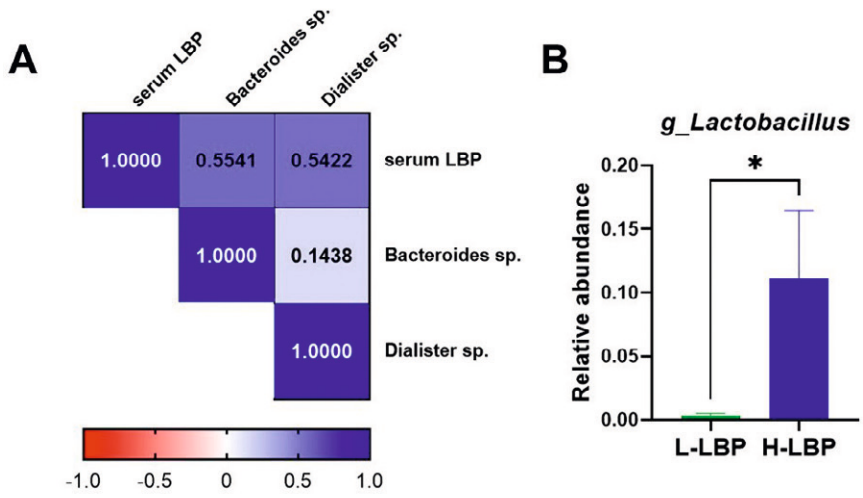


Figure 3. Relationship between gut microbiota and serum LBP levels in GO patients.

A. Heatmap showing Spearman's correlation rho coefficients of serum LBP and the abundance of two Gram-negative species, *Bacteroides sp.*(p=0,042) and *Dialister sp.* (p=0,048) (respective annotations: Bacteroidetes_Bacteroidia_Bacteroidales_Bacteroidaceae_Bacteroides_unculturedorganismHQ761051.1.1439 and Firmicutes_Negativicutes_Selenomonadales_Veillonellaceae_Dialister_unculturedorganism);B. Relative abundance of genus *Lactobacillus* (relative to total genera found in fecal microbiota) in H-LBP GO patients (N = 7) and L-LBP (N=8) p=0,033. Data displayed as mean +/- SEM (standard error of the mean).

Higher intestinal permeability is accompanied by augmented immune cell infiltration and inflammatory pathways in orbital tissues.

Immunohistochemistry of formalin-fixed paraffin-embedded orbital sections was employed to quantify the influx of CD68-positive macrophages. We found a significant positive correlation between the influx of macrophages (CD68+) in the orbital tissue and serum LBP level (r = 0.4, p = 0.043; **Fig 4A**), linking a "leaky" gut with orbital inflammation. However, the increased influx of macrophages in the H-LBP group was not statistically significant (**Fig. 4B**).

In line, the influx of macrophages was also significantly correlated with active myofibroblasts, assessed by immunostaining for α-smooth muscle actin (ASMA) (r = 0.5, p = 0.011), whereas ASMA was significantly correlated (r = 0.4, p = 0.043) with CD3 T cells as well (**Fig. 4A and B**).

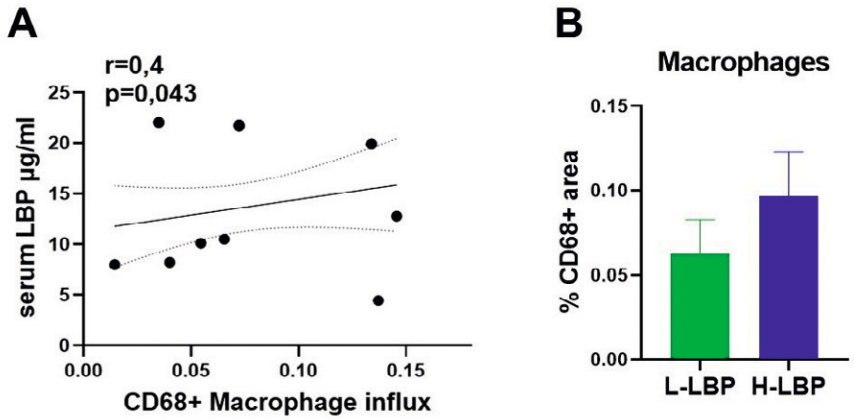


Figure 4. Augmented infiltration of CD68+ macrophages in GO patients is correlated to serum LBP levels.
A. Spearman correlation between the influx of CD68+ macrophages (shown as percentage of CD68-positive areas) in the orbital tissue and serum LBP levels of GO patients (N=15), p = 0.043; B. Influx of CD68+ macrophages in L-LBP (N=8) vs H-LBP (N=7) GO patients.

Next, to quantify the relationship of serum LBP levels with the inflammatory profile of GO patients, the GeoMx Digital Spatial profiler technology was used to profile the gene expression specifically within the active fibrotic area (ASMA-positive) of the orbital tissue, which was enriched with CD45-positive immune cells. This enabled the simultaneous quantitation of multiple inflammatory genes in a specific area of interest. **(Fig. 5 and Fig. S1**). Similarly to the macrophage influx, expression of immune cell markers *LY6E*, *CD20*, *CD3*, *CD4,* and *CD8* showed that increased intestinal permeability is accompanied by enhanced recruitment in the orbital tissue of granulocytes, B cells, and T cells (specifically CD4+ T cells) as the H-LBP group displays a marked increase in the expression of these phenotypic immune cell markers (**Fig. 5A,B**). In line, the expression of genes involved in antigen presentation (both via MHC class I and II, *B2M* and *CD74,* respectively) and immune cell adhesion (*PECAM1*) were significantly upregulated in patients of the H-LBP group compared to those of the L-LBP group (**Fig. 5C,D**). In addition, patients of the H-LBP group displayed an enhanced expression of genes involved in the type 1 interferon pathway, such as interferon-alpha/beta receptor and signaling molecule STAT1, and the expression rate of interferon-alpha and -beta receptor subunit 1 (IFNAR1) positively correlates with the serum concentration of LBP (**Fig. 5E,F**). Notably, this pathway is important in autoimmunity as it boosts antigen presentation.

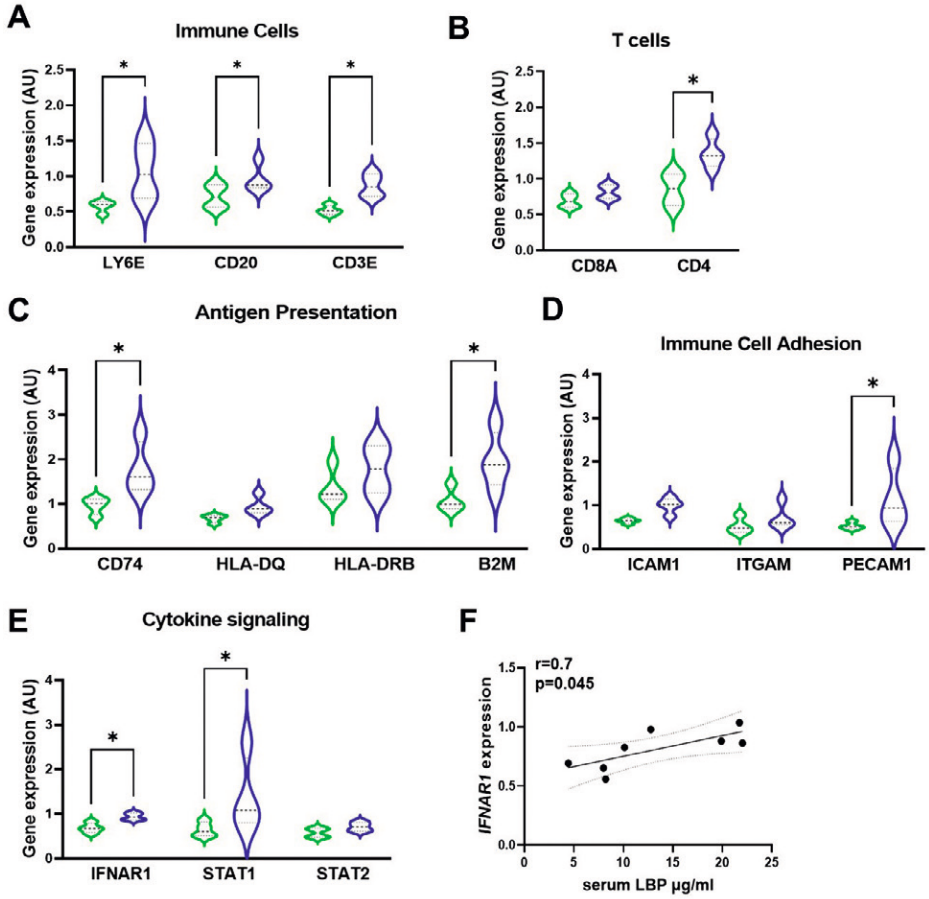


Figure 5. Gene expression profile within the active fibrotic ASMA-positive area of orbital tissue of L-LBP (green, N=8) versus H-LBP (blue, N=7) GO patients.

A. Gene expression of immune cells markers LY6E (neutrophil granulocytes), CD20 (B lymphocytes), and CD3E (T lymphocytes); B. Gene expression of T-cells markers CD8a and CD4; C. Gene expression of antigen presentation cells markers CD74, HLA-DQ, HLA-DRB, and B2M; D. Gene expression of Immune Cell Adhesion and Migration markers ICAM1, ITGAM, and PECAM1 as markers; E. Gene expression of cytokine signaling markers IFNAR1, STAT1, and STAT2; A-E. Data shown as mean =/- SEM; gene expression assessed by GeoMx digital spatial profiler technology, raw data normalized for negative probes and housekeeping gene; p<0,05. F. Significant Spearman correlation between serum LBP levels and IFNAR1 gene expression in GO patients (N = 15), p = 0.045, r=0.7.
Statistical significance determined by one-way ANOVA tests, followed by the Kruskal-Wallis test.

Orbital inflammation is linked to fibroblast activation.

Lastly, we found that the rate of macrophage influx (assessed by immunohistochemistry for CD68) is positively associated with higher expression of genes encoding for protein pivotal in cytokine signaling, cell adhesion, apoptosis, as well as with the

percentage of active α-smooth muscle actin-positive myofibroblasts (detected by immunohistochemistry) (**Fig. 6A**). The latter are active secretors of collagen and contribute to the expansion of the retro-orbital tissue. The number of myofibroblasts within the orbital tissue was significantly and positively associated with the influx of macrophages (r = 0.5, p = 0.011) and T cells (**Fig. 6B,C**).

As persistent inflammation is known to be linked to fibrosis development[30], here we reveal that increased gut permeability is associated with the immune cell infiltration of orbital tissue in GO, the degree of local inflammation, and the differentiation of fibroblasts in ASMA-positive myofibroblasts, which actively secrete extracellular matrix.

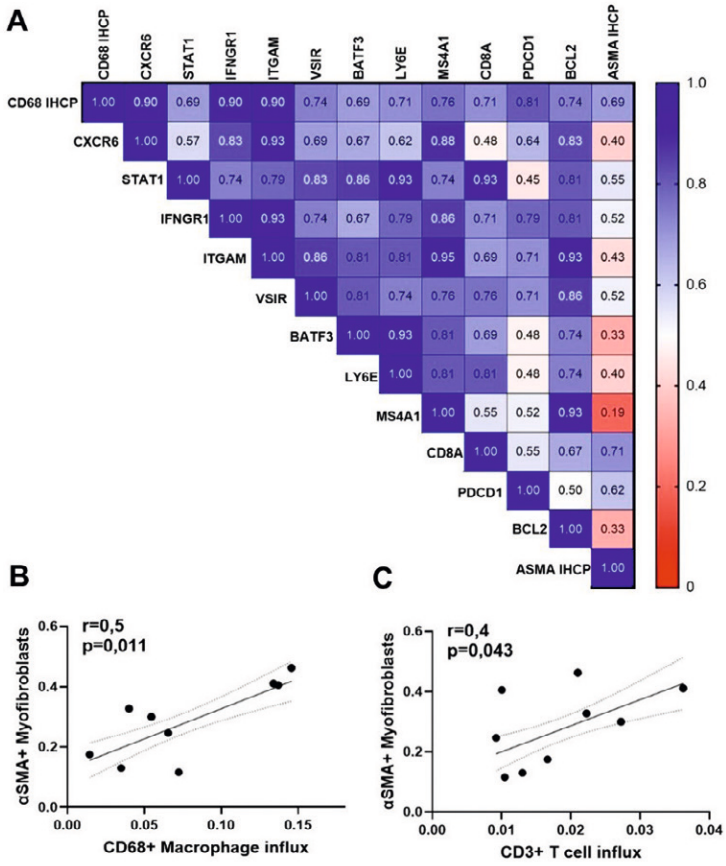


Figure 6. Orbital inflammation is linked to fibroblast activation in GO patients (N=15). A. Heatmap of Spearman's rho rank correlation coefficients between gene expression rates, influx of macrophages, and accumulation of myofibroblasts; B. Spearman correlation between the number of orbital macrophages and CD3 T lymphocytes; C. Spearman correlation between CD68+ macrophages (p=0,011, r=0,6) and ASMA-positive myofibroblasts (p=0,043, r=0,4).

DISCUSSION

In this pilot study, we have demonstrated a positive link between increased intestinal permeability and local inflammation and fibrosis within the orbital tissue of GO patients.

The occurrence of a "leaky gut" during Graves' orbitopathy is supported by a previous study that found significantly elevated serum levels of LPS, I-FABP, zonulin, and D-lactate in patients with initial GD compared to healthy controls[24].

The zonulin family peptide is a potent regulator of intercellular tight junctions of the intestinal epithelium. Mucosal defects can lead to increased serum zonulin levels and can be used as a leaky intestinal barrier, dysbiosis, and inflammation biomarker[31,32]. GO patients with high serum LBP levels also had elevated zonulin levels. In line with this finding, bacterial ligands of Toll-like receptors 5 and 9, namely flagellin and unmethylated CpG motifs of bacterial DNA, appeared to be increased in the circulation of obese patients supporting the hypothesis that intestinal barrier dysfunction leads to the translocation of bacterial components into the bloodstream[33].

The thyroid biomarkers TSH, FT4, and FT3 were all within the normal reference range in these patients, indicating that the intestinal leakage persists after achieving a euthyroid state. We did not compare our subjects based on the serum levels of thyroid biomarkers or CAS score since these are values to identify the GO phase, while orbitopathy may remain despite remission of Graves' disease, revealed by the TBII-negative serum levels in some patients.

Immunohistochemistry and Multiplexed RNA in-situ hybridization via NanoString digital spatial profiling technology enabled the assessment of the degree of immune cell recruitment and inflammatory processes activated in GO tissues. Moreover, we could link orbital inflammatory markers with the rate of intestinal permeability as well as the degree of myofibroblast expansion, which causes the clinical GO manifestations. In GO, fibroblasts are activated by TSHR-binding autoantibodies and can differentiate into adipocytes or myofibroblasts, with consequent extracellular matrix deposition and retro-orbital tissue expansion[3]. For this reason, when investigating the degree of inflammation and the differences in gene expression between patients in the low- and high-LPB groups, we selected the ASMA-positive regions, which are active fibrotic regions and showed enrichment in CD45-positive leukocytes as compared to the area occupied by mature adipocytes only.

We demonstrated a positive association between serum LBP levels and macrophage influx in GO orbital tissues, establishing a link between "leaky gut" and macrophage influx. Interestingly, a recent paper demonstrated distinct macrophage

immunophenotypes in GO orbital tissues, with M1-like proinflammatory macrophages being predominant in active GO and M2-like anti-inflammatory macrophages dominating in stable GO[34]. The expression of IL-6 and transforming growth factor-ß was found respectively higher in M1 and M2 macrophages. However, in our study, these markers were not significantly different between high- and low-LBP groups possibly indicating a similar macrophage composition in GO tissues, although this would require future targeted investigations. In addition to macrophages, the expression of granulocyte, B cell, and T cell markers was significantly elevated with higher intestinal permeability. Particularly CD3+ T cell infiltration was found to be linked to the accumulation of myofibroblasts and postulated to be a central player in GO tissue remodeling[35]. Indeed, T cells have been shown to infiltrate the retro-orbital tissue at an early stage of GO in humans[36] and analysis of antigen receptor variable region repertoires has shown that the autoreactive T cells infiltrating the thyroid gland are found in retroorbital tissues[37]. We may speculate that the systemic inflammation caused by intestinal permeability aids the infiltration of TSHR-reactive T cells into secondary organs. Various studies have recently reported significant associations between the relative abundances of the gut microbiota and diagnostic parameters of thyroid status and thyroid antibodies in patients with Graves' disease[38–45] and/or Graves' orbitopathy[17,46,47] compared to healthy controls. Interestingly, similar to our results, Shi and colleagues showed an increased abundance of *Bacteroides sp.* and Lactobacillus in GO patients[17]. In a recent study, *Bacteroides spp.* was identified as one of the top bacterial biomarkers for predicting the severity of GO and was significantly correlated to TSH and FT4 levels, however the whole Bacteroides phylum was decreased in the GD group[18]. Given the observational study design, conclusive evidence of a causal relationship or its direction cannot be drawn from our study. Specifically, it remains unclear whether the bacterial species *Bacteroides spp.* and *Dialister spp.*, identified to correlate with LBP levels, play an active role in triggering gut barrier permeability.

Two recent studies have investigated the effect of transplanting gut microbiota of GD/GO patients in a GD/GO mouse model[42,48]. The gut microbiota composition of medication-naïve GD patients differed significantly from healthy controls, which led to a higher disease incidence in mice after fecal microbiota transplantations (FMTs) from these GD patients compared to mice treated with FMT from healthy human controls (73.3% vs. 28.6%, respectively, $p = 0.03$)[42]. A second study showed significant variation in gut microbiota composition in murine models of GD/GO correlating with GO heterogeneity, including enlarged volume of orbital brown adipose tissue[48]. Analysis of fecal microbiota profiles revealed an increased Bacteroides to Firmicutes ratio in severe GO patients versus healthy controls. Assuming that the GO patients in this study also had elevated LBP levels, this would confirm our present finding that the abundance of *Bacteroides sp.* was correlated with high LBP levels. The fecal samples of the GO patients were transferred via FMT into mice immunized with human

thyrotropin receptor (as a GD model), resulting in a hyperplastic thyroid and increased fat area in the middle orbital tissue. Here, an inverse correlation between the relative abundance of *Lactobacillus* spp. and TRAb was observed in the FMT-treated mice. In line, another study showed significant enrichment of *Lactobacillus* spp. in GO mice ($p = 0.018$), positively correlated with orbital adipogenesis and serum fT4[29]. These findings are in line with our results showing that the H-LBP patients displayed a higher relative *Lactobacillus* spp. abundance and simultaneously a higher rate of activated orbital fibroblasts. However, the mice results should be interpreted with caution as the *Lactobacillus* levels in mice are much higher than those in humans[49].

A notable strength of our study is the combination of biopsies from the orbital tissue with serum and fecal samples, which allowed us to link intestinal permeability with a distant organ. The Digital Spatial Profiling technology enabled us to quantify the inflammatory gene expression in the selected regions of active fibrosis within orbital biopsies. Of note, active fibrosis is a pivotal process in the pathogenesis of Graves' orbitopathy, yet it cannot be included in the clinical activity score and is not associated with the CAS severity. Indeed, GO disease may appear clinically quiescent and, yet, exhibit certain features of the disease, such as those seen in EUGOGO[26]. In our study, the degree of fibrosis could not be accurately compared with other clinical indicators, such as serum thyroid hormone levels, as nearly all patients were taking thyroid medication on the day of surgery (as shown in Table 2).

Previous studies revealed that GO phenotype features were linked to thyroid autoantibody serum levels[50,51]. Both TSAb and TBII levels were significantly associated with a higher CAS score and more severe proptosis. However, our study did not find any correlation between LBP and these clinical features of GO, suggesting that variations in inflammatory markers in the orbital tissues are not attributed to differences in levels of thyroid autoantibodies or GO phenotypes and underscoring a potential role of a "leaky" gut in orbital inflammation.

As orbital decompression surgery is a last resort in treating GO[25], it remained difficult to retrieve a large cohort. Moreover, orbital surgery is only performed after extensive initial medical treatment with anti-thyroid medications and steroids, which most likely influences the gut microbiota composition. It is yet not known whether our finding of enhanced intestinal permeability in GO patients is associated with local orbitopathy or just an accompanying sign of autoimmune hyperthyroidism. Fecal samples from GD patients without eye disease, as well as mild medication-naïve inactive GO patients, might be an interesting source to investigate whether gut microbiota are a key factor in the pathogenesis of GO/GD.

This explorative study shows a positive association between gut permeability and orbital inflammation, which in turn is linked to fibrosis. Nonetheless, the causative

relationship of this phenomenon has to be further studied in further intervention trials. Indeed, a human randomized clinical trial employing fecal microbiota transplantation from healthy donors in GD patients versus placebo would help uncover whether the gut microbiota are a causative factor in the onset of GD/GO pathogenesis or whether it merely reflects the effects of the disease itself. These and other interventions could be an alternative way to show that compromised barrier function is a causative factor in GD/GO pathogenesis. Such studies not only would address the possibility that a perturbed microbiome is causing GD on its own but also may aggravate or accelerate disease progression by influencing systemic immune responses.

CONCLUSION

In conclusion, this study shows that GO is associated with enhanced intestinal permeability and the degree of fibrosis positively correlates with higher inflammatory tone within the orbital tissue of GO patients. Particularly, patients with high LBP serum levels presented an increased expression of genes involved in antigen presentation, immune adhesion, IFN-α signaling, and immune cell markers of macrophages, B, and T cells. This suggests that in patients with enhanced intestinal permeability, the subsequently increased translocation of bacterial compounds to the systemic circulation triggers a local inflammatory immune response in the orbital tissue. These initial findings warrant further exploration in larger GD/GO cohorts to assess how (and why) specific inflammatory pathways (e.*g.,* type I interferon and antigen presentation) in the orbital tissue correlate with gut microbiota composition and provide a basis for developing microbiota-targeting therapeutic interventions.

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Author contributions
Aline Fenneman and Elena Rampanelli contributed to patient selection, data analysis, and laboratory procedures and wrote the manuscript. Stephan Havik contributed to the performance of immunohistochemistry on orbital tissues. Willem de Vos provided the microbiota analysis by r16S-sequencing. Anne van der Spek contributed substantially to the content discussion and edited the manuscript before submission. Eric Fliers and Max Nieuwdorp reviewed the manuscript before submission. All authors read and approved the manuscript.

Competing Financial Interests Statement

Max Nieuwdorp and Willem de Vos are co-founders and members of the Scientific Advisory Board of Caelus Pharmaceuticals, the Netherlands. None of these are directly relevant to the current paper. There are no patents, products in development, or marketed products to declare. The other authors declare no competing financial interests.

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SUPPLEMENTARY MATERIAL

Table S1. Components of the Clinical Activity Score (CAS)

Components of the Clinical Activity Score (CAS)
Spontaneous retrobulbar pain
Pain with eye movement
Redness of the eyelids
Redness of the conjuctiva
Swelling of the eyelids
Swelling of the caruncle
Conjuctival edema (chemosis)

The CAS score is calculated according to the presence or absence of the characteristics listed. One point is given for each presence of the characteristics listed; with 0 – 3 scored as low CAS score and 4 to 7 as a high CAS score.

Table S2. Classification of severity of Graves' orbitopathy (GO), according to the guidelines of the 2021 European Group on Grave's orbitopathy (EUGOGO), as reported by Bartalena and colleagues[26].

Classification	Features
Mild GO	Patients whose featuer of GO have only a minor impact on daily life that have insufficient impact to justify immunomodulation or surgical treatment. They usually have one or more of the following: - Minor lid retraction (<2mm); - Mild soft-tissue involvement; - Exophthalmos <3mm above normal for race and gender; - No or intermittent diplopia and corneal exposure responsive to lubricant.
Moderate-to-severe GO	Patients without sight-threathening GO whose eye disease has suffient impact on daily life to justify the risks of immunouppresion (if active) or sugical intervention (if inactive). They usually have two or more of the following: - Lid retraction ≥2mm - Moderate or severe soft-tissue involvement - Exophthalmos ≥3mm above normal for race and gender; - Inconstant or constant diplopia.
Sight-threathening (very severe) GO	Patients with dysthyroid optic neuropathy and/or corneal breakdown

Table S3. Patient characteristics of moderate-to-severe Graves' disease patients of the Graves' cohort AMC, divided by the clinical activity.

Characteristic	Inactive GO (N = 21)	Active GO (N = 21)	p-value
Age (years)	61±11	61±11	0.97
BMI (kg/m^2)	25.3±4.8	25.9±5.3	0.62
CAS score	0.8±0.65	4.9±0.87	**<0.0001**
Hertel OS (mm)	20±4	23±5	**0.001**
Hertel OD (mm) (mm)rightright(mm)	20±4	23±4	**0.001**
TSH (mE/L)	2.58±2.66	3.26±7.22	0.58
FT4 (pmol/l)	17.4±9.4	16.6±6.7	0.66
T4 (nmol/l)	116±51	120±35	0.68
T3 (nmol/l)	2.0±0.7	2.1±0.7	0.80
T3 uptake	1.06±0.16	1.01±0.14	0.12
FT4 index	124±75	122±43	0.91
AntiTPO (kU/L)	593±906	876±1170	0.23
TBII (E/L)	9.4±16.6	25.7±38.9	**0.02**
Glucose (mmol/l)	5.6±0.8	5.9±1.3	0.26
Alkalic Phosphatase (U/L)	92±49	84±28	0.36
Gamma-GT (U/L)	31±25	32±23	0.94
LBP (µg/mL)	15.70±5.83	16.26±5.20	0.66

For normally distributed parameters, data are presented as mean ± SD, and p values were calculated using a Student's t-test. Inactive GO was considered as a CAS score < 3. Active GO was considered as a CAS score ≥4. All patients were classified as moderate-to-severe GO.

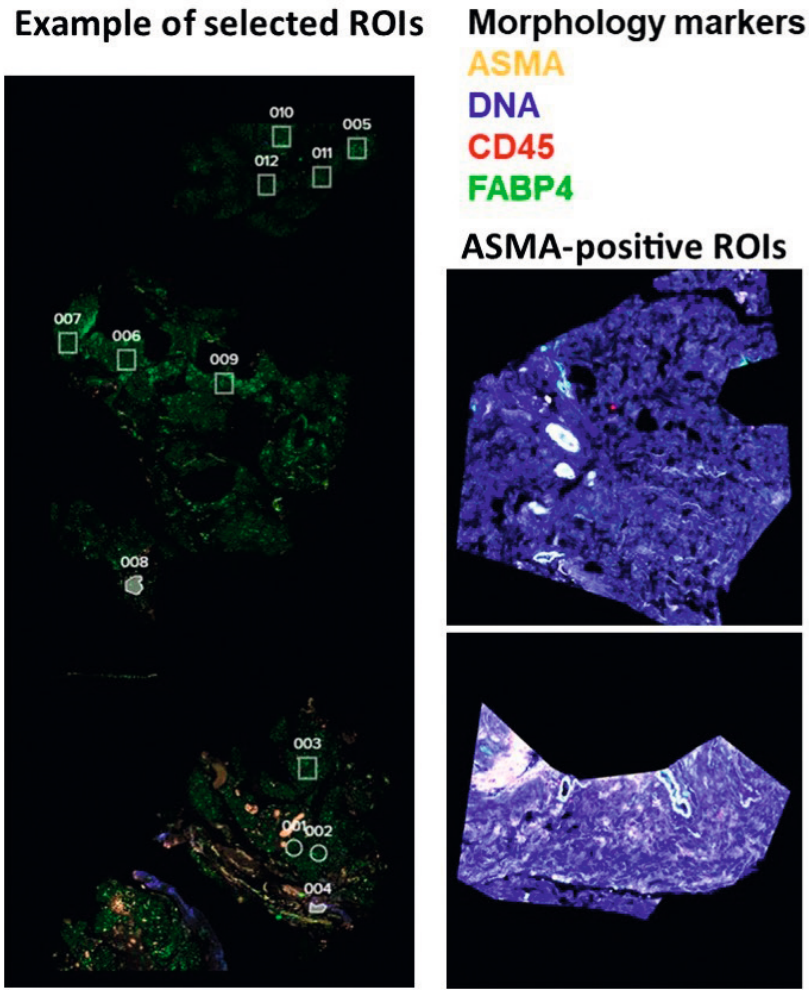

Figure S1. Regions Of Interest (ROIs) of orbital tissue of GO patients, using Multiplexed RNA in-situ hybridization via NanoString digital spatial profiling technology.

10

ANTIBIOTICS IN THE PATHOGENESIS OF DIABETES AND INFLAMMATORY DISEASES OF THE GASTROINTESTINAL TRACT

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ABSTRACT

Antibiotic use is increasing worldwide. However, the use of antibiotics is clearly associated with changes in gut microbiome composition and function, and perturbations have been identified as potential environmental risk factors for chronic inflammatory disorders of the gastrointestinal tract. In this Review, we examine the association between the use of antibiotics and the onset and development of both type 1 and type 2 diabetes, inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, as well as coeliac disease and eosinophilic oesophagitis. We discuss the key findings of epidemiological studies, provide mechanistic insights into the pathways by which the gut microbiota might contribute to these diseases, and assess clinical trials investigating the effects of antibiotics. Such studies indicate that antibiotic exposures, varying in type, timing and dosage, could explain differences in disease risk. There seems to be a critical window early in life in which perturbation of the microbiome has a substantial effect on disease development. Identifying the antibiotic-perturbed gut microbiota as a factor that contributes to the pathophysiology of these inflammatory disorders might stimulate new approaches to prevention, diagnosis and treatment.

Key points

- The widespread use of antibiotics worldwide is consistent with a rise of chronic inflammatory diseases of the gastrointestinal tract, including inflammatory bowel disease, coeliac disease, eosinophilic oesophagitis, and type 1 and type 2 diabetes.
- Exposure to antibiotics leads to profound effects on both the composition and the functionality of the gut microbiota, leading to potential pathogenic mechanisms for disease onset.
- Experimental studies have shown that antibiotic-induced perturbations of the microbiota are transferable and affect disease development.
- Differential levels of antibiotic exposures, and their types and timing - particularly early-childhood exposure - could explain differences in disease risk.
- A growing body of evidence indicates that an antibiotic-perturbed microbiota is associated with disease development, although current knowledge is limited by microbiota complexity. Future research including prospective epidemiological studies, clinical trials and experimental studies is required.
- Novel therapies aiming to remediate the perturbation of the gut microbiome are being researched, including prebiotics, probiotics, synbiotics or fecal microbiota transplantation. However, the strong application of antibiotic stewardship is most warranted to prevent perturbing the microbiome.

INTRODUCTION

The gastrointestinal tract is subject to important chronic inflammatory diseases. These include diseases that affect the wall of the gastrointestinal tract, such as inflammatory bowel disease (IBD), including ulcerative colitis and Crohn's disease, as well as coeliac disease and eosinophilic oesophagitis (EoE) (**Box 1**). In addition, the pancreas is subject to inflammatory processes that can lead to either type 1 or type 2 diabetes. In this Review, we consider the relationship between these diseases and the gut microbiome, especially with respect to how antibiotic treatment for other indications can perturb the microbiome and affect the risk and course of these illnesses.

Humans, like other mammals, develop in a uterus that is routinely sterile or only occasionally visited by adventitious microbial pathogens[1,2]. The major exposure of the baby to the world of microorganisms occurs when after rupture of membranes and its descends through the birth canal and is exposed to maternal vaginal and fecal microorganisms[1,2]. This is followed by successional colonization and blooms of taxa in the intestine that are highly conserved across all healthy infants[3–5] . The most dynamic period for the human microbiome is the first three years of life[6], which is also the period in which immunity, metabolism and cognition become well-established. Studies in animal models show that perturbing the early-life microbiome, even transiently, can have long-term effects on these crucial developmental steps[7–12]. This conserved biology and the effects of experimental perturbation have led to the theory that an altered microbiota underlies a number of the diseases that are currently epidemic globally[13,14], including the inflammatory conditions affecting the gastrointestinal tract.

Studies have shown that visceral, inflammatory and neuropathic pain can be influenced by the gut microbiome, which is particularly relevant in conditions in which pain can be a prominent symptom, including IBD and coeliac disease[15,16]. The inflammatory foot pad pain induced by carrageenan, lipopolysaccharide (LPS), tumour necrosis factor (TNF), IL-1β, and by the chemokine CXCL1 in conventional mice was reduced in germ-free mice.[17] Similar experiments demonstrate that mice develop visceral hypersensitivity following dextran sulfate sodium (DSS)-induced colitis, even after the intestinal inflammation is resolved[18]. This hypersensitivity was transferable by transplantation of a post-inflammatory microbiome to mice that had never been exposed to DSS but not by transplanting a control microbiome[18]. These findings provide evidence that the gut microbiota in mice contributes to the development of inflammatory hypernociception[18,19]. Specific data on the effect of the gut microbiome on pain in patients with IBD and coeliac disease are preliminary. In a pilot study of 21 children with coeliac disease, statistically significant differences in relative abundance

of specific bacterial taxa were associated with symptoms including abdominal pain[20], but further studies are warranted.

Antibiotics entered the general practice of medicine in the late 1940s and have since become pillars of modern medicine. Consequently, their use has steadily grown, and health practitioners increasingly rely on them. Estimated use exceeds one course per year for every person worldwide[21], and the numbers are growing. There is extensive variation in antibiotic use within localities, regions and countries, reflecting important differences in the culture of medicine and personal characteristics of both patients and practitioners[22]. Antibiotics also vary considerably in their antimicrobial spectrum of activity[23]. Antibiotics were developed to treat infections caused by bacterial pathogens, which has been the major thrust of both their development and their use. However, when an antibiotic is taken, it also has collateral effects on the resident microbiota: inhibiting some, and thus reciprocally selecting for others. In addition to selecting for potential pathogens within the microbiome, including *Staphylococcus aureus* and *Clostridioides difficile*, antibiotics considerably perturb the human gut microbiome, with effects lasting for months or longer[24–26]. In the past, it was widely assumed that after a course of antibiotics the 'normal flora' would bounce back to its pre-treatment state. Unfortunately, using molecular tools that have greater precision than culture-based studies, it has become clear that the microbiome is perturbed for months, and might never resume to its pre-treatment state[24–26]. This finding is especially important for young children, in which the microbial succession is highly choreographed and perturbations, even if transient, can affect both microbiome and host development[3–5,8,25].

In mixed microbial populations, such as the gut microbiome, fungal numbers usually increase after exposure to antibiotics[27]. Many gut commensal fungi, including *Candida* species, interact with host epithelial and immune cells[28,29]. The host adaptive immune response is targeted to hyphal cells[29], and thus any shift in the balance between yeasts and hyphae will affect the immunological milieu. Such phenomena could contribute to antibiotic-induced exacerbations of both disease predisposition and the disease itself.

As such, there is growing interest in the hypothesis that owing to their effects on the gut microbiome, antibiotic use might have unintended collateral clinical consequences, especially when given to young children, whose developing microbiome is both plastic and interlinks with host development[30].. In this Review, we consider this hypothesis in the context of several chronic inflammatory diseases that affect the human gastrointestinal tract and that have increased in incidence during the antibiotic era (**Fig. 1**).

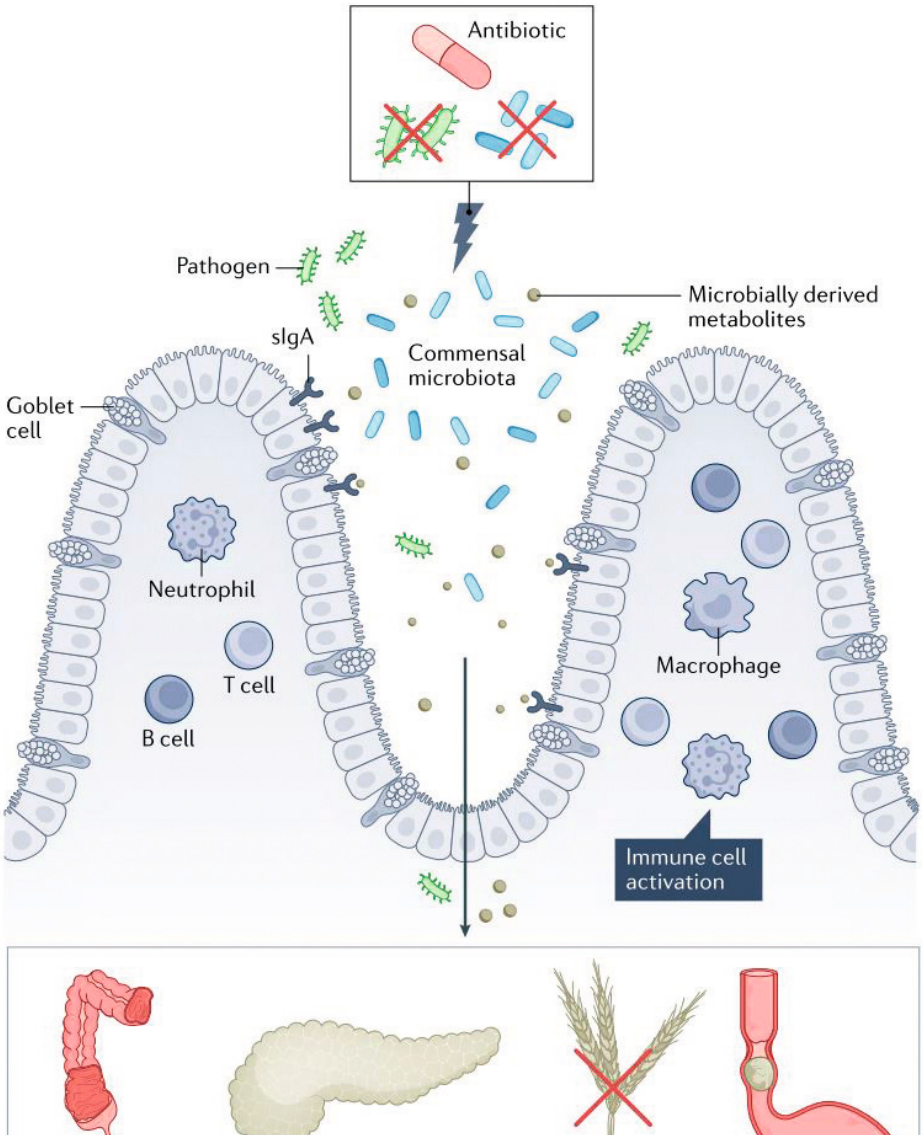

Figure 1. The gut microbiota and antibiotics in the pathogenesis of inflammatory diseases of the gastrointestinal tract. Schematic overview of the role of the microbiota in the pathogenesis of inflammatory diseases injuring organs in the gastrointestinal tract, leading to the onset and development of both type 1 diabetes mellitus (T1DM) and type 2 diabetes mellitus (T2DM), inflammatory bowel disease (IBD; including ulcerative colitis (UC) and Crohn's disease (CD)), coeliac disease and eosinophilic oesophagitis (EoE). Exposure to antibiotics leads to profound effects on both the composition and functionality of the gut microbiome, leading to decreased diversity. These changes might then lead to secondary effects involving the intestinal wall including altered epithelial cell signalling to adaptive immune effectors, and/or increased intestinal permeability, leading to translocation of microbial constituents and products into the systemic circulation, among other mechanisms. sIgA, secretory IgA.

Diabetes

Approximately 537 million people worldwide have either type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus (T2DM)[31]. Epidemiological studies of the incidence and prevalence of T1DM and T2DM in 212 countries/regions have been extensively reviewed by the International Diabetes Federation and published in the Diabetes Atlas[31]. Although hyperglycaemia is the common element in T1DM and T2DM, the diseases differ extensively in epidemiology and pathogenesis, and will therefore be considered separately.

TYPE 1 DIABETES

Epidemiology

An important factor in T1DM is the genetic predisposition provided by specific HLA haplotypes, mainly DR3-DQ2 and DR4-DQ8[32]. However, as the age of onset is swiftly decreasing, these predisposing genes cannot solely explain the rapidly rising incidence of T1DM worldwide[32–34]. Altered gut microbiome composition (referred to as dysbiosis) has been identified as a potential environmental risk factor[35]. The gut microbiota of patients with T1DM harbour a lower ratio of Firmicutes to Bacteroidetes, have decreased *Bifidobacterium* spp. abundance, reduced bacterial richness and diversity, and lower production of short-chain fatty acids (SCFAs) compared with healthy individuals[36–39]. Although such changes are seen largely in children already affected by the disease, some were already present before clinical onset.

Changing epidemiology of T1DM in the antibiotic era

In the past few decades, the worldwide incidence of T1DM has risen dramatically, particularly in children under 14 years old[31,40]. The estimated global number of newly-diagnosed children annually rose by approximately 50% from 65,000 in 2003[41] to 98,300 in 2021[31] , a 3% annual increase. In 2021, >1.2 million children and adolescents worldwide had T1DM[31]. However, there is striking geographic variation in the reported incidence of T1DM, with the highest annual incidence reported in children in Europe (~31,000 cases (5.26% of all children in Europe)) and the lowest in children in the Western Pacific (~11,600 cases (1.88% of all children in the Western Pacific))[31]. Discrepancies between regions must be interpreted with caution, as data sources on T1DM incidence in low-income regions are scarce.

Epidemiological linkages with the disease

The exact causes of the steep increases in incidence of T1DM are not yet known[42]. Rapid changes within a short span of time are more likely a result of changes in environmental risk factors than changes in genetic risk[32]. Intriguingly, the rising incidence of T1DM in children began in many countries/regions in the middle of the 20th century, coinciding with the start of the antibiotic era[43]. For example, the rise in Finland preceded the widespread introduction of antibiotics, which is consistent with

changes in sanitation, such as the use of chlorinated drinking water. These improved hygiene conditions led to reduced exposure to infectious agents in early childhood. This 'hygiene hypothesis' is supported by the negative correlation between hygiene conditions and T1DM incidence[44].

Two important changes in medical practice in the second half of the 20th century were antibiotics and the increased frequency of caesarian section. Antibiotic use early in life clearly leads to changes in the intestinal microbiome[3,45]. Similarly, children born by caesarian section begin life with an altered microbiome[3,5,46,47], and the changes, including reduced *Bacteroides* species, and altered community composition can persist throughout the first year of life[3,46–48]. Birth by caesarian section is usually a compounded insult to normal microbiome development, involving loss of the natural passage through the birth canal, and the administration of high doses of antibiotics to the mother in the peripartum period[49]. Children born by caesarian section also seem to be more likely to receive antibiotics in early life **(Table 1)**[50,51]. Antibiotics are widely administered to children on the basis of the clinical premise of important benefit and minimal risk; however, antibiotic overuse is well-documented in children as well as in older people[52,53], and prescribing rates vary widely with differences between countries/regions (both high and low-middle income countries) as well as regional differences, even among children with similar clinical presentations[22]. Parallel statements can be made about caesarian section[49]. Currently, only limited data on the association between the risk of T1DM and the use of antibiotics are available, mostly provided by Scandinavian nationwide cohort studies[50,51,54,55] **(Table 1)**, where most children are exposed to antibiotics early in life.

Two longitudinal cohort studies from Sweden and Denmark found an increased risk of T1DM after antibiotic exposure early in life[50,51]. However, mode of delivery is a strong confounder, as a larger effect was observed in children delivered by caesarian section compared with vaginal delivery. Although similar in the magnitude of the increased risk ratio, two other cohort studies from Denmark and Norway found no significant association between the use of antibiotics and T1DM onset, irrespective of antimicrobial spectrum or use in an age-specific period[54,55] **(Table 1)**. These results might reflect the differences between countries/regions, types of antibiotics used, and exposure to probiotics, among other factors. Such variation might lead to non-significant associations. More homogeneous cohorts, with varying ethnicities and geographical regions, will better assess whether perturbation of gut microbiome composition as a result of caesarian section and/or early-life antibiotic exposure influences the onset of T1DM.

Table 1. Clinical studies evaluating antibiotic exposure and risk of diabetes and childhood obesity

Study	Antibiotic exposure timing	Date (location)	Study design	Antibiotic data source	Disease diagnosis	Key findings[a]
Type 1 diabetes mellitus						
Wernroth et al.[50]	Prenatal to 12 months	2020 (Sweden)	Cohort	Prescriber registry (ATC code)	Database diagnostic code for T1DM (ICD-10: E10)	Increased risk of T1DM before age of 10 years: ≤1 year: aHR 1.19 (1.05–1.36): 44.3/100,000 person-years among children exposed versus 39.0/100,000 person-years among non-exposed children. ≤ 6 mo: aHR 1.26 (1.04 – 1.5) Modified by mode of delivery
Clausen et al.[51]	Birth to 24 months	2016 (Denmark)	Cohort	Prescriber registry (ATC code)	Database diagnostic code for T1DM	Increased risk after exposure to broad-spectrum antibiotics HR 1.13 (1.02–1.25) Modified by mode of delivery
Hviid et al.[54]	12 months – year 2005	2009 (Denmark)	Cohort	Prescriber registry (ATC code)	Database diagnostic code for T1DM (ICD-10: E10)	Differences not significant RR 1.16 (0.91– 1.50)
Tapia et al.[55]	Prenatal to 18 months	2018 (Norway)	Cohort	Repeated questionnaires	Database diagnostic code for T1DM (ICD-10: E10)	Differences not significant Prenatal: aHR 1.09 (0.85 – 1.35) Early in life: aHR 1.11 (0.81 -1.50)
Childhood obesity						
Trasande et al.[90]	< 6 mo 6 - 14 months 14 - 23 months	2013 (UK)	Longitudinal birth cohort	Repeated questionnaires	Measured during five study visits	Increased body mass at 10 to 38 months, after antibiotics exposure during first 6 months of life aOR 1.22 (P = 0.029)
Bailey et al.[91]	0 - 23 months 24 - 59 months	2014 (Philadelphia, USA)	Cohort	Outpatient prescriptions and patient-reported medications	Measured during recurring study visits	Cumulative exposure to antibiotics was associated with development of obesity. ≥4 courses: RR 1.11 (1.02-1.21) Stronger effect for broad-spectrum antibiotics: RR 1.16 (1.03 – 1.19)

Table 1. Continued

Study	Antibiotic exposure timing	Date (location)	Study design	Antibiotic data source	Disease diagnosis	Key findings[a]
Azad et al.[92]	0 – 5 years	2014 (Canada)	Longitudinal birth cohort	Prescription records	Measured at age 9 and 12 years	Increased risk of overweight and central adiposity in preadolescent boys, but not girls. Age 9: aOR 2.19 (1.06 -4.54) Age 12: aOR 5.35 (1.94 – 14.72)
Murphy et al.[93]	0 – 12 months	2014 (18 countries/ regions)	Cross-sectional	Repeated questionnaires	Self-reported or measured	With antibiotic exposure, increased childhood BMI in boys aged 5-8 years, but not girls. BMI + 0.107 kg/ m^2 ($P < 0.0001$)
Aversa et al.[94]	0 – 6 months 6 – 12 months 12 – 24 months	2021 (Minnesota, USA)	Population-based cohort	Medical-records linkage system	Medical-records linkage system	Increased risk for overweight and obesity, depending on number, type, and timing of antibiotic exposure. - Girls with overweight: HR 1.19 (1.09-1.30) - Girls with obesity: HR 1.13 (0.99 – 1.29) - Boys with overweight: HR 1.22 (1.12-1.34) - Boys with obesity:: HR 1.12 (1.08-1.39)
Mueller et al.[48]	Prenatal	2014 (New York, USA)	Cohort	Questionnaire	Measured at age 7 years	Exposure to antibiotics in the second or third trimester associated with higher risk of childhood obesity. aRR = 1.77 (1.25 – 2.51)

Table 1. Continued

Study	Antibiotic exposure timing	Date (location)	Study design	Antibiotic data source	Disease diagnosis	Key findings[a]
Mbakwa et al.[95]	Birth to 10 years	2016 (The Netherlands)	Longitudinal cohort	Repeated questionnaires	Self-reported over 7 time points	Increased height and weight in children exposed to: - One course during first 6 months of life; adjusted ß 0.24 and 0.23 - Two or more courses during second year of life; adjusted ß 0.34 and 0.29
Type 2 diabetes mellitus in adults						
Mikkelsen et al.[87]	Adults	2015 (Denmark)	Case-control	Prescriber registry (ATC code)	First-ever prescription of a noninsulin glucose-lowering agent (ATC A10B)	Dose-dependent relationship with number of antibiotic courses: For 2-4, OR 1.21 (1.19-1.24) For ≥5, OR 1.53 (1.50-1.55)
Davis et al.[88]	Adults	2019 (USA)	Retrospective cohort	Outpatient antibiotic prescriptions >6 months prior to diabetes diagnosis	≥2 ICD-9 codes for diabetes or ≥2 prescriptions of diabetes medications, other than metformin	Increased risk after exposure to >1 prescription of antibiotics HR = 1.13 (1.01 – 1.25)
Boursi et al.[89]	Adults	2015 (UK)	Nested case-control	Antibiotic prescriptions >1 year before diabetes diagnosis	At least one Read code (general practitioners)	No significant difference after a single antibiotic course Dose-dependent relationship for number of antibiotic courses (≥2), with OR depending on antibiotic type.

aHR, adjusted hazard ratio; aOR, adjusted odds ratio; aRR, adjusted relative risk; ATC, Anatomical Therapeutic Chemical classification system; ICD, International Classification of Disease; RR, rate ratio; T1DM, type 1 diabetes mellitus. [a] Value ranges in parentheses are 95% confidence intervals; adjusted β are adjusted generalized estimating equation estimates in relation to z-scores.

Experimental studies

The composition of the intestinal microbiota early in life has a large effect on immunological development in both intestinal and systemic sites[10,11,56–59]. Therefore, perturbations of microbiome composition during this critical window might have a key role in T1DM onset, which is shown by studies using non-obese diabetic mice (NOD), an experimental model resembling T1DM in humans[60]. The variation in T1DM incidence in these NOD mice is dependent on the microbial composition to which the mice are exposed[61–63]. A general rule of thumb is that 'dirty protects'; NOD mice reared in ultra-clean facilities develop T1DM at higher rates than those in more standard facilities[64]. As such, germ-free mice are more prone to develop T1DM compared with NOD mice exposed to a single bacterium[62,63]. NOD mice with deficient innate immunity owing to a null mutation of the Toll-like receptor (TLR) adapter signalling molecule MYD88 are protected from T1DM development under specific-pathogen-free (SPF) conditions, but not in germ-free conditions[65], indicating that the microbiota signal is transduced through MYD88. A particular taxon, the genus *Candidatus Savagella* (formerly known as segmented filamentous bacteria (SFB)), protected NOD mice against T1DM by inducing small intestinal T helper 17 (Th17) cell populations[63]. These results indicate an important and complex interplay between the microbiota and immunologic effectors in T1DM (**Fig. 2**).

Timing and nature of antibiotic exposure

As specific types of antibiotics exert differential effects on gut microbiota composition, exposure to particular antibiotic classes can differentially affect T1DM development[66–68]. Timing of antibiotic exposure is also a potential factor[66,69]. In NOD/Caj mice, a substrain of NOD mice used to understand the role of B cells as antigen-presenting cells, maternal (prenatal) exposure to neomycin or vancomycin both induced long-term changes in the gut microbiome composition of the offspring compared with the offspring of untreated control mice[66]. However, only vancomycin (which targets mainly Gram-positive bacteria and anaerobes) strongly accelerated T1DM development[66]. In mice prenatally treated with vancomycin, T cells from the spleen, pancreatic lymph nodes, and Peyer's patches showed statistically significant decreases in naïve T cell markers ($CD44^{-}CD62L^{+}$) and increased numbers of $CD4^{+}$ memory T cells ($CD44^{+}CD62L^{-}$). Consistent with this more-activated T cell repertoire, these T cells expressed higher levels of the pro-inflammatory cytokines interleukin-17 (IL-17), interferon-γ (IFNγ) and TNF-α. In contrast, prenatal treatment with neomycin (which targets mainly aerobic microorganisms) significantly protected the progeny from T1DM compared with untreated counterparts, and was associated with increased Bacteroidetes abundance[66]. The protection was also associated with induced immunotolerogenic responses of antigen-presenting cells in both spleen and mesenteric lymph nodes[66].

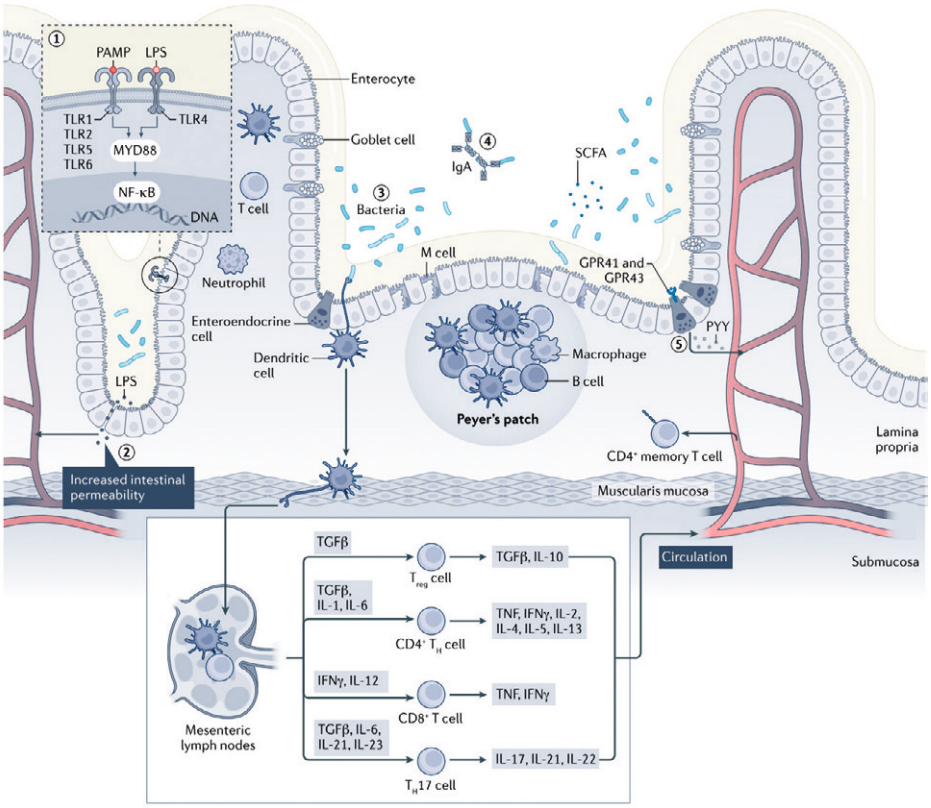

Figure 2. Complex interplay between the gut microbiota and the immune system in diabetes and IBD. Microbially derived peptides, such as pathogen-associated molecular patterns (PAMPs) and lipopolysaccharide (LPS), bind to Toll-like receptors (TLRs) on the cell membrane of enterocytes (1). Activation of these TLR/MYD88-dependent signalling pathways leads to translocation of nuclear factor-κB (NF-κB) into the nucleus, promoting transcription of numerous cytokines[63,65,239]. Dysbiosis of the gut microbiota can lead to intestinal barrier dysfunction and increased intestinal permeability (2). This facilitates the translocation of PAMPs and LPS into the systemic circulation, leading to a persistent, low-grade inflammatory state of liver, muscles, and visceral and subcutaneous adipose tissue as observed in both diabetes (both type 1 and type 2 diabetes mellitus) and inflammatory bowel disease (IBD)[109–114]. Across the intestinal epithelium, antigen-presenting cells (APCs), including macrophages and dendritic cells, detect pathogenic bacteria and promote the antigens on the cell surface (3). Thereafter, the APCs migrate to mesenteric lymph nodes, mediating an alteration of T lymphocyte subsets[66,71]. Secretory IgA (sIgA) serves as the first line of defence in protecting the intestinal epithelium from enteric toxins and pathogenic microorganisms (4). Antibiotic exposure leads to lower levels of sIgA, potentially leading to an increased inflammatory environment[9]. Gut microbiota ferment diet-derived carbohydrates into short-chain fatty acids (SCFAs) (5). SCFAs are ligands of the G protein- coupled receptors GPR41 and GPR43, which are expressed by intestinal enteroendocrine cells and enhance production of peptide YY (PYY), a hormone that affects insulin utilization by increasing the intestinal transit time, and increases satiety and energy harvest from the diet[105,106]. TH cell, T helper cell; Treg cell, regulatory T cell. The original version of this figure was created with BioRender.com

In another study, lifelong treatment of NOD mice with either vancomycin or neomycin, started prenatally until the onset of diabetes, accelerated T1DM onset and altered effector T cell populations, with increased IFNγ $CD4^+$ T cells and, in contrast to the previously discussed study[66], reduced IL-17^+CD4^+ T cells[70]. In male but not female NOD mice treated with a broad-spectrum high-dose antibiotic cocktail (streptomycin, colistin and ampicillin) or vancomycin only, T1DM incidence was significantly increased compared with untreated control mice[68] ($P<0.0001$ for the antibiotic cocktail and $P<0.0004$ for vancomycin) and showed a significant decrease in IL-17A gene expression or IL-17-producing cells in colon, Peyer's patches and mesenteric lymph nodes[68]. However, another study showed that T1DM was attenuated in NOD mice that received vancomycin from birth through weaning at age 4 weeks[69]. These conflicting results might reflect the nature of the microbiota in the mouse colony being studied, but nevertheless they provide experimental evidence that antibiotic perturbation of the microbiome affects T1DM development.

Most studies investigating the effects of antibiotics in murine models have used a continuous antibiotic regimen, often at super-therapeutic levels[67,68]. However, such interventions do not mimic paediatric antibiotic use, which consists of discrete courses that are modelled better by therapeutic-dose pulsed antibiotic treatment (PAT). A study in which NOD mice received PAT (with a macrolide) early in life showed accelerated development of T1DM and insulitis compared with mice continuously treated with subtherapeutic antibiotic treatment (STAT) and controls[71]. Male mice exposed to PAT showed reduced α-diversity and β-diversity in their microbial population structure and decreased proportions of small intestinal lamina propria Th17 and regulatory T (Treg) cells before T1DM onset. These immunological changes were accompanied by altered ileal gene expression (concomitant with upregulated cholesterol biosynthesis) and altered metabolomic profiles in the caecum, liver and serum[71]. Transfer of the antibiotic-altered microbiota to adult germ-free mice showed similar changes in intestinal T cell populations, confirming that the perturbed microbiome was responsible for the altered immunological signal. However, transfer of antibiotic-perturbed microbiota from 6-week-old mice to pregnant germ-free mice showed an unexpected protection of the offspring from T1DM[71]. One potential explanation for this observation is that the 6-week time point (P42) microbiota were highly selected for opportunistic microorganisms, and their transfer to the newborn mice led to tolerance, consistent with the adage in NOD mice that 'dirty protects'. This complexity, shown in a single series of experiments by the same group[9,72], illustrates that the relationship between the microbiota and host phenotypes depends on antibiotic type, dosage and timing. In independent experiments, even a single 5-day macrolide PAT course early in life was sufficient to accelerate and enhance T1DM onset in male mice, leading to profound changes in expression of genes encoding immunological effectors in the ileum[72]. A study published in 2021 explored whether the phenotype of mice that had been exposed to antibiotics that perturbed the

microbiome, changed immunological phenotypes and accelerated and enhanced T1DM, can be rescued by attempting to restore their microbiota[73]. To investigate this, during a period between 3-7 days after antibiotic treatment ended, mouse pups were gavaged with caecal microbiota of mothers obtained on the day of their pup's birth. This treatment largely restored the baseline T1DM phenotype, partially restored the intestinal microbiome composition, metagenome and metabolome, and restored ileal RNA and microRNA expression. These studies demonstrate the importance of the effect of antibiotic perturbation of the microbiome on T1DM development, and point toward the role of post-exposure restorative approaches. They also provide a path to discovery of relevant microorganisms, microbial genes, metabolites, and host genes that influence the propensity for T1DM[73].

Clinical trials

An intervention study that used the antimicrobial fusidic acid in 28 patients with newly diagnosed T1DM showed no statistically significant differences in beta-cell function, C-peptide values, or quantitative insulin requirements compared with a control group who received placebo[74]. In addition, a clinical trial with an oral dose of the SCFA butyrate showed no effect on either innate or adaptive immunity markers in 30 patients with long-standing T1DM[75,74]. In contrast, in a pilot trial including 20 patients with recent T1DM onset, use of fecal microbiota transplantation (FMT) led to an increased abundance of both *Desulfovibrio* strains and microbiota-derived plasma metabolites of tryptophan origin, which were associated with the stabilization of residual beta cell function[76]. This result provides a proof-of-principle that even after T1DM commencement, interventions affecting gut microbiome composition and activity can have salutary effects, extending the findings observed in mice. Taken together, such findings warrant added caution in the use of antibiotics in pregnant women and newborns, and minimizing the practice of caesarian sections.

TYPE 2 DIABETES AND CHILDHOOD OBESITY

Epidemiology

Changing epidemiology of the disease in the antibiotic era

In the United States, the prevalence of T2DM has increased from 0.93% in 1958 to 7.40% in 2015[77]. This steep rise coincides with an increased prevalence of obesity, one of the hallmarks of T2DM, as well as with the cumulative and increasing use of antibiotics[52,53,78]. The worldwide prevalence of T2DM is estimated to increase to 12.2% in 2045[31].

Metagenomic analysis of the gut microbiome of patients with T2DM revealed distinct perturbations in composition and function, characterized by a decreased abundance of butyrate-producing bacteria and an enrichment of opportunistic pathogens, often

mucin-degrading (*Akkermansia muciniphila*) and sulphate-reducing *(Desulfovibrio* sp.)[79]. Although some studies showed an increased Firmicutes:Bacteroidetes ratio in patients with T2DM[80,81], this should not be considered a T2DM hallmark as the relative abundance of these phyla is highly variable between individuals with T2DM[82,83]. These compositional changes are partly explained by differences in ethnicity; lower α-diversity was observed in the gut microbiomes of populations of South-Asian and African origin compared with those of European origin living in the same city[84,85]. Such differences correspond to higher risk of T2DM in these ethnic minority populations[86].

Epidemiological linkages with the disease

As T2DM mostly affects adults, studies examining the association between antibiotic use and T2DM diagnosis have only been performed in adult cohorts[87–89] **(Table 1)**. However, multiple longitudinal cohort studies have shown that exposure to antibiotics in the first three years of life is associated with an increased risk of childhood obesity and central adiposity, which are well-known risk factors for T2DM[48,90–95] **(Table 1)**. Strikingly, these studies show that most (>70%) children in high-income countries/regions are exposed to antibiotic therapy at least once by the age of 2 years[90], whereas the average incidence of antibiotic use in low and middle-income countries/regions is even higher (4.9 courses per child per year)[96]. This enormous use is of a scale consistent with the extent of disease incidence; antibiotic use in developing countries/regions started later, but cumulatively they are catching up quite rapidly[97].

Antibiotic use during pregnancy also has effects on the development of the infant microbiome[98]. Exposure to antibiotics in the second or third trimester has been associated with a 84% (95% CI 33-154) higher risk of childhood obesity, including higher waist circumference (3.13 cm (95% CI 0.68-5.59)) and increased body fat percentage (1.86% (95% CI 0.33-3.39))[48]. Although no direct information on T2DM was provided in these childhood studies, these findings provide consistent evidence that exposure to antibiotics during the critical window early in life, even if brief, could lead to long-term effects that increase the risk of developing T2DM.

Adult cohort studies consistently show that antibiotic exposure at least 6 months before the date of diabetes diagnosis is associated with increased risk of T2DM **(Table 1)**. The risk increases with more frequent antibiotic exposure[87–89] and varies depending on antibiotic type[88,89]. However, these studies vary in their methodological approaches. For example, not all studies adjusted the results for variables including obesity, dyslipidaemia, hypertension and other cardiovascular comorbidities[88], conditions that are part of the metabolic syndrome and are associated with T2DM as well as gut dysbiosis[99,100]. That the gut microbiome is influenced by ethnicity[84], as well as environmental factors such as diet, physical activity, smoking and other medications[101], limits the interpretation of epidemiological studies. As such,

the causal link between antibiotic exposure and its subsequent perturbation of the gut microbiome, with T2DM development, has not been established by the epidemiological studies.

Experimental studies

Murine models and fecal transplant experiments have provided mechanistic insights into how the gut microbiota might contribute to T2DM development. In particular, they have shown that the intestinal microbiota influences both host metabolism and immunological interactions. Early-in-life STAT exposure in C57BL/6J mice, either continuously or for a short time span only, led to statically significantly increased fat mass compared with control mice[7,8,102,103], with increased incretin secretion and glucose intolerance compared with controls[7,8,103]. Antibiotic treatment also altered expression of hepatic and ileal genes involved in fatty acid metabolism and triglyceride uptake, as well as hepatic steatosis[9,102,103]. The gut microbiota of STAT mice showed a shift in taxonomic composition, with higher levels of Firmicutes and lower levels of Bacteroidetes[7,8,102], similar to that observed in ob/ob (leptin deficient, obese) mice[99,104]. Importantly, the onset of adiposity occurred after the alterations in microbiome composition and remained later in life (32 weeks)[7,8], even after the perturbation of the microbiome recovered following STAT withdrawal[8,102]. Transferring caecal contents from STAT mice into germ-free mice by oral gavage replicated the obesity phenotype[8]. These data are consistent with the hypothesis that there is a critical early-life period in which later-in-life metabolic development is set, and that alterations to the gut microbiota in that period have long-term consequences.

Caecal contents of STAT mice showed substantially higher levels of SCFAs (acetate, butyrate and propionate), which indicates an increased capacity to harvest energy by bacterial fermentation of complex dietary carbohydrates[7]. SCFAs are ligands of the G-protein-coupled receptors (GPCRs) GPR41 and GPR43, which are expressed by intestinal enteroendocrine cells that produce peptide YY[105], a hormone that affects insulin utilization by increasing intestinal transit time and increases satiety and energy harvest from the diet. However, the role of GPR41 and GPR43 in obesity is inconsistent between studies. A study with GPR41-knockout mice showed that they had reduced body weight, less fat accumulation, and less insulin resistance than their wild-type counterparts[106]. Another study showed that weight gain was suppressed in mice that overexpressed GPR43 in white adipose tissue and that were fed a high-fat diet, whereas GPR43-knockout mice become obese, even on a normal diet[107]. These seemingly contradictory results might reflect factors such as differences in the disease models used, the inbred mouse strains used and their microbiota, or non-specific effects of the knockouts themselves[108]. Epithelial cell expression of GPR41 and GPR43 in human physiology needs more study. Nevertheless, these studies provide evidence that SCFAs, and therefore the gut microbiota, regulate host energy

expenditure. As such, disruption of gut microbiome composition and functionality by antibiotic exposure affects host energy balance, at least in part.

High circulating levels of inflammatory effector molecules, including TNF, IL-6, IFNγ, and bacterial lipopolysaccharide (LPS), have been consistently associated with both obesity and T2DM, providing a potential mechanism for the persistent, low-grade inflammatory state of liver, muscle and adipose tissue frequently observed in both obesity and T2DM[109–114]. These changes might reflect increased intestinal permeability, leading to translocation of microbial constituents and products into the systemic circulation[110]. Conventionally raised mice receiving continuous LPS infusions showed metabolic responses, including increased hepatic insulin resistance, similar to those in mice receiving a high-fat diet[109]. In 6-week-old ob/ob mice characterized by high LPS levels, antibiotic treatment lowered plasma LPS levels and inflammatory markers in adipose tissue[110]. These changes occurred concomitantly with improved metabolic parameters (such as improved glucose tolerance, less weight gain and lower fat mass). Notably, similar beneficial effects on glucose metabolism, including improved glucose tolerance and reduced fasting glucose levels, were seen in lean, healthy male mice after different 4-week antibiotic regimens[115]. These improved metabolic processes were accompanied by changes in hepatic and ileal gene expression involving glucose regulation and bile metabolism. Thus, after perturbations that increase translocation of intestinal contents, antibiotic treatments reduce secondary effects, and are therefore useful tools for future experiments.

However, early-life exposure to antibiotics also directly affects host immune phenotypes, as illustrated by altered $CD4^+$ T cell subsets and reduced intestinal secretory IgA levels in mice exposed to antibiotics compared with conventionally raised mice[9]. Germ-free mice exposed to antibiotics did not exhibit any substantial immunological changes, indicating that the metabolic and immunological effects were not a direct result of the antibiotics but rather are consequences of the antibiotic-induced gut microbiome alterations[9] (**Fig. 2**). Thus, perhaps paradoxically, although early-life antibiotic exposures can drive the altered pathophysiology, once the damage has been done (from whichever cause), antibiotics have the potential to improve outcomes.

Dietary intake is one of the key modifiable extrinsic factors that influences gut microbiome composition and contributes to the onset of both T2DM and obesity. Antibiotic exposure early in life aggravates the negative effects (which include dyslipidaemia, insulin resistance, and increased visceral fat mass) that accompany a high-fat diet (HFD) in female BALB/c mice compared with mice similarly exposed to antibiotics but fed a normal diet, even when the dysbiosis progressively recovers[116]. In another study, mice fed an obesogenic diet showed increased weight and fat mass when receiving lifelong STAT compared with their unexposed HFD-fed counterparts[103].

Such an obesogenic diet also affects antibiotic susceptibility. HFD-fed mice had impaired efficacy of bactericidal antibiotics compared with normally fed mice, a difference that was not observed in microbiota-depleted animals[117]. These findings suggest that antibiotic exposure worsens the diet-induced adiposity phenotype, leading to increased T2DM risk, and that obesity also reduces antimicrobial susceptibility.

Clinical trials

Owing to concerns about antibiotic resistance, there are few clinical trials that have investigated the effects of antibiotic exposure on weight gain in children. The trials that have been performed show conflicting results, with early-in-life exposure to antibiotics either having pronounced effects on weight gain[118] or no effect[119]. A meta-analysis of 10 randomized controlled trials including 4,316 children showed that undernourished children (<12 years old) from low and middle-income countries/regions treated with antibiotics were significantly taller (0.04 cm/month (95% CI 0.00-0.07)) and had increased weight gain (23.8 g/month (95% CI 4.3–43.3)) compared with their placebo (9 studies) or untreated (1 study) control groups[117]. However, there was significant geographic variation in weight gain, with children from trials conducted in Africa gaining 35.6 g/month (95% CI 12.8-58.3) of body weight more than children from other regions, which possibly is a reflection of more-severe malnutrition[118]. A United States trial showed that 302 children (<6 years) using long-term oral trimethoprim-sulfamethoxazole prophylaxis did not gain substantially more weight than the 305 control individuals, although approximately 25% of children in both groups had overweight or obesity[119].

Studies of antibiotic interventions to alter T2DM progression have been limited in number. A broad-spectrum antibiotic mixture in healthy lean men led to a drastic reduction in the abundance of gut microbiota (in colony forming units (CFU)), but without significant changes in fasting or postprandial glucose, insulin secretion, and plasma lipid levels[120]. Similar effects on gut microbiota composition were seen after oral vancomycin therapy[121,122]. Bacterial diversity was significantly ($P < 0.001$) reduced, with lower abundance of Gram-positive bacteria and increased abundance of Gram-negative bacteria, concomitant with increased levels of circulating LPS[121,122]. These perturbations affected glucose metabolism by decreasing peripheral insulin sensitivity in both lean individuals and patients with metabolic syndrome. Another study showed that 48 patients with bacterial endocarditis treated with intravenous vancomycin and gentamicin substantially increased BMI (+2.3 kg/m^2 (± 0.9)), which persisted one year after treatment[123]. However, as the therapy improved clinical status, weight gain could have been a reflection of the overall health improvement.

Novel therapies that aim to restore the dysbiotic gut microbiota in individuals with obesity and T2DM have been investigated in humans using different prebiotic[124],

probiotic[125] or synbiotic[126] regimens, but beneficial effects have been elusive. FMT has been studied as a means of counteracting the dysbiotic gut microbiome to improve insulin sensitivity in patients with metabolic syndrome or T2DM. Transfer of feces from healthy donors to patients with metabolic syndrome improved insulin sensitivity in some, but not in all, patients[127,128]. This dichotomy might reflect baseline intestinal bacterial differences, with responders having lower diversity before FMT[128], or differences in donor FMT composition and its administration.[129]

INFLAMMATORY BOWEL DISEASE

Definitions and epidemiology of IBD

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the intestines, with two main subtypes: ulcerative colitis and Crohn's disease. Although the precise aetiology of IBD remains unknown, increasing data suggest that alterations in the intestinal microbiota are a major contributor to IBD risk. As such, antibiotic exposure might have a role in IBD development in the current era.

One feature of IBD that might help in understanding its underlying pathophysiology is its temporal variation. Not only in disease activity, extent or behaviour within a patient, but also in epidemiological features that have evolved globally over time. Although the disease initially seemed to mostly be limited to Northern and Western Europe, the United States and Canada, data from 1960-2017 show that IBD has become increasingly prevalent worldwide, including in Central and South America, Africa, and the Caribbean and Asia-Pacific regions[130–132]. Furthermore, incidence in newly identified hotspots might outpace that of regions with traditionally high prevalence[130,131], which is consistent with the rising cumulative exposure to antibiotics in those areas.

The demographics of those with IBD have also evolved. Ulcerative colitis is the predominant IBD subtype in regions with newly-incident IBD, and Crohn's disease incidence then increases over time[130]. Countries/regions with traditionally high prevalence tend to have similar distributions of Crohn's disease and ulcerative colitis[130]. Among the 34 countries/regions in the Organization for Economic Co-operation and Development (OECD), IBD hospitalizations (a proxy for disease severity) were highest in traditionally high-prevalence regions (North America, Europe, and Oceania) and lowest, but also most rapidly increasing, in new-incidence regions (Asia, Latin America, and the Caribbean)[133]. In the Asia-Pacific Crohn's and Colitis Epidemiology Study (ACCESS) inception cohort of 413 patients (181 with Crohn's disease, 22 with ulcerative colitis, and 10 with unclassified IBD), ~20% of patients with Crohn's disease who initially presented with a non-fistulizing, non-stricturing phenotype developed these complications after a median follow-up of 18 months[134], which is similar to rates in Western populations[135]. However, patients

with ulcerative colitis in the ACCESS cohort were less likely to need advanced medical therapies and also less likely to have colectomies compared with their counterparts with Crohn's disease, as well as in comparison to patients with ulcerative colitis in Western populations[134,136].

Although the time of IBD onset can span from infancy to older age, diagnoses occur most commonly around the third decade of life[132,137]. However, there has been an increase in paediatric-onset IBD incidence in the past few decades, particularly in children <10 years[137–139], in countries/regions with a high prevalence of IBD. For example, a population cohort study in Ontario, Canada, reported an annual increase of 9.7% from 1999 to 2008, although this might reflect improved early diagnosis[137]. However, IBD incidence increases following immigration from a low to a high-prevalence country/region, with stronger risk associated with younger age at time of immigration (similar to T1DM) and among subsequent generations born in the high-prevalence countries/regions[140–142]. Such studies provide direct evidence of the importance of environmental factors in IBD risk. Although genome-wide association studies[143,144] and twin studies[145,146] have clearly identified genetic contributors to IBD risk, the changes in global IBD patterns and the time frame in which they have occurred emphasize the critical role of environmental triggers. Notably, many of the environmental factors identified to be associated with IBD risk or protection (for example, migration, diet, breastfeeding)[145,146] are related to changes in the intestinal microbiome[147–149].

Antibiotic exposure and IBD risk in humans

Several large cohort studies have implicated the use of antibiotics in risk of IBD. Two large national database studies, one from the UK[150] (a registry of 1,072,426 children <18 years with 6.6 million person-years of follow-up from 1994-2009) and another from Denmark[151] (a registry of 577,627 children with 3,173,117 person-years of follow-up from 1995-2003), both found that antibiotic exposure in childhood is associated with IBD risk in a dose-dependent manner. This relationship has been confirmed in both paediatric-onset and adult-onset IBD (with approximate odds ratios ranging from 1.3-3.4), although the risk is higher with childhood exposure, with greatest quantity of evidence and strength of effect in children exposed to antibiotics before the age of 1 year[150–157]. Although some studies have found associations between antibiotic treatment and subsequent development of both Crohn's disease and ulcerative colitis[152,154], in other studies the relationship was either stronger for Crohn's disease (that is, statistically significant findings at lower exposure levels)[150] or for Crohn's disease only[151,155,158]. However, many of these studies were conducted in children, and the distribution of Crohn's disease compared with ulcerative colitis, as well as the primary drivers of disease aetiology, may differ from adults[158]. For example, although the increasing incidence of very-early-onset IBD in Canada (that is, onset before age 6 years)[159] suggests that its aetiology contains an environmental component, it is more

commonly associated with monogenic mutations than cases of IBD that start later in childhood or in adulthood[160]. A potentially important confounding factor is the nature and severity of the illnesses for which antibiotics are prescribed. One hypothesis is that antibiotic exposure is merely a proxy for the underlying acute infections, which themselves might be the primary factor for increased risk of IBD, or alternatively be a protective factor against IBD (the 'hygiene hypothesis'). Unsurprisingly, such issues are difficult to tease out from studies of human children[161], which is why models in experimentally exposed animals, in the absence of initiating infections, are useful.

Animal models of antibiotics in IBD

Given the lag between antibiotic exposure and IBD onset, as well as potential clinical confounders associated with antibiotic use, animal studies have been particularly helpful in establishing causal relationships between antibiotic treatment and changes in intestinal inflammation in IBD models. IL-10-deficient[162] and SAMP1/YitFc[163] mice spontaneously develop bowel inflammation, and disease activity is responsive to microbiome manipulations[164,165]. Studies in both models have shown that antibiotic treatment improves intestinal inflammation when used in either preventive or treatment strategies[165,166]. Studies in the IL-10-deficient model demonstrated that particular antibiotic classes improved colitis in different intestinal regions, suggesting that specific microbiome populations have roles in modulating intestinal inflammation[166]. In experiments that model how vertical transmission of the maternal human microbiome to infants might affect IBD development**,** Schulfer et al. gavaged gnotobiotic IL-10-deficient mouse dams with fecal microbiota from wild-type mice that had either been perturbed by antibiotics administered at weaning in their drinking water or had not (control microbiota)[167]. In the absence of any other intervention, pups born to dams gavaged with the antibiotic-perturbed microbiota developed substantially more-severe colitis than those born to dams given the control microbiota. In this experiment, the fact that neither the pups nor their mothers were actually exposed to any antibiotic established that the antibiotic-perturbed microbiota passed down from the mothers was sufficient for the enhanced disease. Parenthetically, this experiment also provides evidence that in diseases with familial tendencies, the risk factors might not only be the inherited host genes, but also the intergenerational transfer of microorganisms. Similar experiments were conducted by Miyoshi et al. with similar results[168]. Taken together, these studies provide an important link between cumulative antibiotic use in populations and the increasing IBD disease risks observed over the past few decades. They are consistent with the notion that antibiotic effects are cumulative across generations, as previously postulated[169]. Human studies investigating the effect of inheriting a perturbed microbiome on IBD risk are ongoing[170].

Another important experimental model of IBD is dextran sulfate sodium (DSS)-induced colitis[171,172]. Ozkul and colleagues[173] investigated whether previous exposure

to antibiotics worsened the course of DSS colitis. They observed that mice with early-life exposure to a macrolide developed more-severe colitis than mice without the exposure. That the antibiotic exposure ended more than two weeks before the DSS challenge indicates potential latency in antibiotic effects, which is consistent with observations in human children[153]. In a subsequent experiment, the investigators transferred antibiotic-perturbed microbiota obtained 30 days after the exposure ended to germ-free mice, who were subsequently challenged with DSS; the recipients of the antibiotic-perturbed microbiota developed more-severe colitis than those given the normal microbiota. Taken together, these studies suggest that antibiotic perturbation of the microbiota, in the absence of any infection, worsens experimental models of colitis. In other experiments in which the antibiotic challenge preceded exposure to a colonic pathogen of mice (*Citrobacter rodentium*) by as much as 80 days, there was worsened inflammation compared with mice unexposed to antibiotics[174]; the antibiotic-perturbed microbiota also transmitted more-severe disease to germ-free mice that had been conventionalized, indicating that the perturbed microbiota per se had pathogenic properties[174].

Antibiotics in the treatment of IBD

A separate question is whether antibiotics can modulate disease activity in established IBD, as with diabetes. There has been a long-established, but understudied, clinical practice of using antibiotics in treating complications of IBD, such as fistulae, abscesses and pouchitis[175–178]. A related question is whether antibiotics can be used more routinely to alter the natural history of IBD. However, randomized controlled trials (and related meta-analyses) have yielded conflicting results[179,180]. **(Table 2).** The literature is difficult to interpret given the diversity of antibiotics studied as well as the differing indications and timing of treatment. The endpoints for some of these trials were based on clinical symptoms, and were therefore subject to potential confounding by treatment of irritable bowel syndrome, a condition that frequently co-exists with IBD[181] and for which antibiotic treatment can have some efficacy[182]. Nonetheless, one of the clearest examples of the utility of antibiotics in the management of IBD is the use of nitroimidazoles, specifically metronidazole and ornidazole, to prevent post-operative recurrences of Crohn's disease. Compared with placebo, both medications reduced (by 25-30%) the proportion of patients with endoscopic recurrence 3 months after surgery[183,184]. In the setting of defined clinical interventions, such as surgery, antibiotics can clearly improve outcomes.

Table 2. Clinical studies evaluating antibiotic exposure and inflammatory bowel disease

Study	Antibiotic exposure timing	Date (location)	Study design	Antibiotic data source	Disease diagnosis	Key findings[a]
Kronman et al.[150]	Childhood	2012 (UK)	Retrospective cohort	Health records registry	Diagnosis (Read) codes	HR IBD 5.51 (1.66–18.28) – antibiotic exposure before 1 yo; HR 2.62 (1.61–4.25) – by 5 yo; HR 1.57 (1.35–1.84) -by 15 yo. Dose dependency.
Hviid et al.[151]	Childhood	2011 (Denmark)	Retrospective cohort	National health registry	Diagnosis (ICD) codes	IBD RR 1.84 (1.08-3.15) Dose dependency.
Nguyen et al.[152]	All	2020 (Sweden)	Case-control	National health registry	Diagnosis (ICD) codes	aOR IBD 1.94 (1.85-2.03) Dose dependency.
Shaw et al. [153]	Infants < 1 years	2010 (Manitoba, Canada)	Nested case-cohort	Provincial health registry	Diagnosis (ICD) codes	OR IBD 2.9 (1.2-7.0) Dose dependency.
Shaw et al. [154]	2-5 years before diagnosis	2011 (Manitoba, Canada)	Case-control	Provincial health registry	Diagnosis (ICD) codes	aOR IBD 1.27 (1.20-1.35) Dose dependency
Card et al. [157]	2-5 years before diagnosis	2004 (UK)	Case-control	Research database	Diagnosis (Oxmis, Read) codes	aOR IBD (1.05-1.65) No dose dependency
Virta et al. [158]	Childhood	2012 (Finland)	Case-control	National health registry	Diagnosis (ICD) codes	aOR CD 1.87 (1.37-2.56) aOR UC 1.18 (0.92-1.52)
Ungaro et al.[155]	Variable	2014 (Europe, Canada, New Zealand)	Meta-analysis	Mostly health registries, some surveys	Varies	Pooled OR IBD 1.57 (1.27-1.94)
Zou et al. [156]	Childhood	2020 (North America, Eurasia, Oceania)	Meta-analysis	Varies	Varies	Pooled OR IBD 1.5 (1.22-1.85)

aOR, adjusted odds ratio; CD, Crohn's disease; IBD, inflammatory bowel disease; ICD, International Classification of Disease; RR, rate ratio; UC, ulcerative colitis. [a]Value ranges in parentheses are 95% confidence intervals.

Beyond the direct effect of antibiotic-perturbed microbiota on IBD risk and activity, antibiotics might also influence disease by altering the metabolism of IBD medications by the gut microbiota[185,186] (**Fig. 2, Fig. 3**). Sulfasalazine, which was among the earliest recognized IBD treatments, is comprised of an anti-inflammatory 5-aminosalicylate (such as mesalamine) joined with the antimicrobial sulfapyridine via a diazo-bond. The cleavage of this bond, and the subsequent release of the active moiety, is mediated by diazo-reductases, which are produced by many gut bacterial taxa but in the largest quantity by Clostridia[186,187]. Notably, antibiotic-treated germ-free rats do not excrete cleaved sulfasalazine[186,187]. The gut microbiota also has roles in metabolism of other IBD medications including glucocorticoids[188], methotrexate[189] and thioguanine[190]. Thus, antibiotic-induced microbiome manipulation might affect established IBD in addition to having effects on disease development.

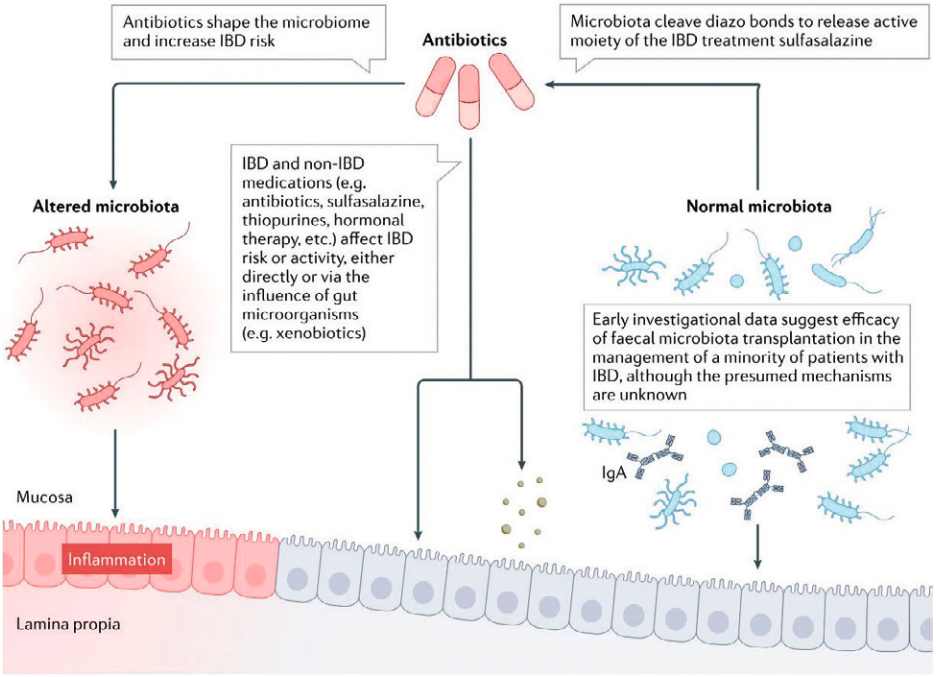

Figure 3. Influence of the gut microbiota on inflammatory bowel disease activity. Antibiotics shape the microbiota and increase the risk of inflammatory bowel disease (IBD)[150–158,161]. Normal microbiota constituents cleave sulfa–diazo bonds to release the active moiety of the IBD treatment sulfasalazine[186,187]. IBD and non-IBD medications (for example, antibiotics, sulfasalazine, thiopurines, hormonal therapy, etc.) affect IBD risk or activity, either directly or with the influence of gut microorganisms (for example, xenobiotics)[150–158,161,175–180,183,184,186–190,240–242]. Early investigational data suggest efficacy for crude faecal microbiota transplantation in IBD management, although the precise mechanism is unknown[191,193,243]. The original version of this figure was created with BioRender.com

FMT as an approach for treating IBD

The growing interest in FMT, coupled with high-throughput sequencing, permits new understanding of the interactions between the intestinal microbiota and IBD activity. Despite differing delivery routes and treatment regimens, three of the four randomized clinical FMT trials for treatment of mild to moderately active ulcerative colitis yielded remission rates of ~25-30% compared with 5-10% in controls[191–194]. In a pilot, randomized, controlled study of 12 patients with mild-to-moderate ulcerative colitis published in 2021, remission was numerically more common in the treatment group but was not statistically significant (2/6 versus 0/6, respectively)[195]. Another negative study, published in 2015, evaluated 50 patients who were randomized to either two donor FMTs over 3 weeks by nasoduodenal tube or placebo (autologous FMT). 37 patients completed the primary endpoint evaluation at 12 weeks. In both the per-protocol and intention-to-treat analyses, there were no significant differences between groups, although some have attributed the negative result to the less-intensive dosing interval compared with the positive FMT trials[192]. Across trials, consistent taxa or mechanisms underpinning the successful FMTs have not been identified. Nevertheless, the proof of principle underlying these trials is promising, and has spurred development of defined microbial consortia instead of stool. Although there have been a few open-label studies and case reports of FMT for Crohn's disease that report positive results[196], the only two randomized clinical trials to date did not show significant effects[197,198]. However, data interpretation is limited by heterogeneity in disease location, behaviour and extent, which makes Crohn's disease difficult to study.

COELIAC DISEASE

Definition and epidemiology

Coeliac disease is a chronic immune-mediated inflammatory disease that affects the small intestine and is triggered by gluten exposure in a genetically susceptible host. Coeliac disease affects about 1% of the population worldwide and is associated with increased morbidity and mortality[199–202]. A meta-analysis published in 2019 revealed that the incidence of coeliac disease has been increasing by 8.4% (95% CI 6.0-10.8) annually over the past few decades (since the 1990s), with female predominance[203]. Coeliac disease can occur at any age, and studies demonstrate that a loss of gluten tolerance can occur during adulthood[204]. Although increased incidence and prevalence might reflect improved detection, disease development in adulthood and the dramatic increases observed suggests that environmental factors are contributing to risk of coeliac disease.

Environmental factors

Coeliac disease has been strongly linked to HLA variants within the DQ2 and DQ8 heterodimers[205]. Although the presence of risk alleles is generally necessary for coeliac

disease development, it is not sufficient, indicating the importance of environmental factors[205]. Earlier studies evaluating the microbial populations associated with coeliac disease found intestinal dysbiosis in patients, with an increased abundance of *E. coli* and *Bacteroides* and a decreased abundance of *Bifidobacterium*[206–211]. Some differences in relative abundance resolve with a gluten-free diet, whereas others persist [206–211]. In 2015, Galipeau et al. showed that in mice with genetic susceptibility (that is, expressing the human HLA-DQ8 gene) for celiac disease, antibiotic exposure influenced gluten-induced immunopathogenicity dependent on the specific bacterial taxa expanded by the antibiotic[212]. These findings are consistent with cohort studies that show an increased risk of celiac disease is associated with such microbiome perturbations as caesarean delivery and proton pump inhibitor exposure[213,214], but studies now suggest particular taxa potentially implicated in pathogenesis.

Additional evidence is emerging that gut microbiota composition and function contribute to the development of coeliac disease in genetically susceptible hosts[212,215–217]. Opportunistic pathogens can induce immune activation of gluten-specific T-cells relevant for celiac disease, either through bacterial elastase modification (increasing immunogenicity and mucosal translocation)[215,216] or via molecular mimicry[217] in animal and preclinical studies. Caminero et al. colonized germ-free mice with opportunistic pathogens derived from small intestinal biopsy samples of patients with coeliac disease, including *Pseudomonas aeruginosa*, or with *Lactobacillus* spp. from healthy control individuals[216]. *P. aeruginosa* showed enhanced mucosal translocation in the mouse intestine[216], and *P. aeruginosa* modified gluten peptides which were then recognized by activated gluten-specific T-cells from patients with coeliac disease, leading to increased immune recognition. By contrast, *Lactobacillus* spp. from healthy individuals degraded gluten peptides, resulting in decreased immunogenicity[216]. Taken together, multiple factors modulated by specific opportunistic pathogens associated with coeliac disease could reduce tolerance towards gluten in genetically susceptible individuals.

Clinical studies

Coeliac disease and antibiotic exposure

To date, there have been 10 studies evaluating antibiotic exposure and risk of developing coeliac disease (**Table 3**). In addition, systematic reviews and meta-analyses of the literature from 2018 to 2020 have demonstrated increased risk of coeliac disease and antibiotic exposure[218–220]. Jiang et al. reported increased risk of coeliac disease after antibiotic exposure (pooled OR 1.2 (95% CI 1.04–1.39)), and specifically for antibiotic exposure during childhood (OR 1.15 (95% CI 1.02–1.29))[218]. Kamphorst et al. also concluded that antibiotic exposure in the first 2 years of life is associated with coeliac disease risk[219]. Although the studies evaluated in this meta-analysis were of high quality, there were only four, and the odds ratios were modest,

ranging from 1.13 to 1.4[221–223]. However, the clear dose-response relationship in three of the studies provides further evidence of a relationship[221–223] **(Table 3)**. In another population-based birth cohort study of >14,000 children born in Olmsted County, Minnesota between January 2003 and December 2011, antibiotic exposure in the first 2 years of life was studied as a possible risk factor for development of 10 conditions with childhood onset, including coeliac disease. In the cohort, which included 45 children who were subsequently diagnosed with coeliac disease, there was a significant antibiotic dose-dependent relationship, which was stronger in girls than in boys.

Timing of antibiotic exposure

Five studies showed an increased risk of coeliac disease associated with antibiotic exposure. In four of the studies, the exposure was within the first 2 years of life[94,221–223], whereas fifth study examined exposures at all ages in childhood[224] (**Table 3**). It is biologically plausible to interpret these findings as supporting a causal relationship given that the gut microbiota becomes well-established by 3 years of age[6]. Both studies that evaluated maternal (prenatal) antibiotic exposure did not show a statistically significant difference between prenatal exposure to antibiotics compared with no exposure and risk of coeliac disease in the offspring (**Table 3**)[225,226]. Taken together, these findings suggest that timing of exposure might be a significant factor for the risk of developing coeliac disease[94,221–223,225,226]. In addition, two large cohort studies evaluated the relationships between coeliac disease and antibiotic exposures in specific at-risk populations, specifically children with T1DM with coeliac disease-permissive HLA alleles (**Table 3**)[227,228]. These studies followed children from birth, based on parental reporting of antibiotic exposure, monitoring for development of positive coeliac serologies, including tissue transglutaminase IgA, and in one study for development of histologically proven coeliac disease[227]. In these restricted populations, neither study showed an association between antibiotic exposure and either development of coeliac disease or positive serologies.

Table 3. Clinical studies evaluating antibiotic exposure and coeliac disease risk

Author	Antibiotic exposure timing	Date (location)	Study design	Antibiotic data source	Coeliac disease diagnosis	Key findings[a]
Marild et al.[225]	Prenatal	2014 (Sweden)	Cohort	Prospective questionnaire	Histology (Marsh 3) and either positive coeliac serologies or symptoms consistent with coeliac which resolved on GFD[b]	No significant difference HR 1.33 (0.69-2.56)
Marild et al. [226]	Prenatal	2017 (Norway)	Cohort	Prospective questionnaire	Questionnaire or database diagnostic codes for coeliac disease[c]	No significant difference aOR 1.16 (0.94-1.43)
Myleus et al.[232]	Birth to 6 months	2012 (Sweden)	Case-control	Parental questionnaire	3 consecutive duodenal biopsy samples (Marsh 3)	No significant difference between coeliac disease and controls OR 1.2 (0.87-1.6)
Canova et al.[221]	Birth to 12 months	2014 (Italy)	Cohort	Prescriber registry	Database diagnostic codes for coeliac disease	OR 1.3 (1.10-1.56) Dose response relationship with ≥5 antibiotic courses: OR 2.66 (1.79-3.95)
Sander et al.[222]	Birth to 12 months	2019 (Denmark and Norway)	Observational cohort	Prescriber registry	Database diagnostic codes for coeliac disease	OR 1.26 (1.16-1.36) Dose-dependent relationship for each additional antibiotic: OR 1.08 (1.05–1.11)
Kemppainen et al.[228]	Birth to 48 months	2017 (Finland, Germany, Sweden and USA (TEDDY))	Cohort with T1DM and permissive HLA for CD	Prospective questionnaire	Risk of coeliac disease defined as 2 consecutive positive serum TTG IgA at least 3 months apart[d]	No increased risk of positive TTG IgA and antibiotic exposure HR 1.00 (0.98-1.02)

Table 3. Continued

Author	Antibiotic exposure timing	Date (location)	Study design	Antibiotic data source	Coeliac disease diagnosis	Key findings[a]
Bittker et al.[223]	Birth to 48 months	2019 (USA)	Case-control Internet-based survey	Parental report	Diagnosis from medical professional	aOR 1.13 (1.04-1.24) Dose-dependent relationship for number of antibiotic courses: For 4-7, OR 1.62 (1.03-2.55) For >8, OR 2.48 (1.29-4.75)
Aversa et al.[94]	Birth to 24 months	2021 (USA)	Cohort	Prescriber registry	Database diagnostic codes for coeliac disease	Dose-dependent relationship Gender specific for girls: For one or two antibiotic prescriptions, HR 8.12 (1.03-64.10) For more than five antibiotic prescriptions, HR 12.32 (1.56-97.32)
Simre et al.[227]	Birth to 60 months	2016 (Estonia and Finland (DIABIMMUNE))	Cohort with T1DM and permissive HLA for CD	Parental report of antibiotic exposure	Positive celiac serology and duodenal biopsy sample (Marsh 3)	No significant difference (number of antibiotic courses 1.1 versus 1.0 Finland)
Marild et al.[224]	All ages	2013 (Sweden)	Case-control	Prescriber registry	Histology Database (Marsh 3)	OR 1.40 (1.27-1.53) Also found increased risk with inflammation (Marsh 1 and 2) and those with normal histology (Marsh 0) but positive coeliac serologies

aOR, adjusted odds ratio; CD, coeliac disease; GFD, gluten-free diet; OR, odds ratio; T1DM, type 1 diabetes mellitus; TTG, tissue transglutaminase. [a]Value ranges in parentheses are 95% confidence intervals. [b]Data obtained from prior study published in 2004. [c]Validation study performed by authors. [d]Primary outcome was risk of coeliac disease.

Pathogenesis

Several high-quality studies have shown associations between the development of coeliac disease and early-in-life antibiotic exposures with dose-dependent relationships[94,221–223]; however, we know little about the specific mechanisms, other than the evidence of major changes in immunological development seen in other studies[10,68,94,166]. There seems to be a critical window, perhaps within the first 2 years of life, in which antibiotic exposure, by perturbing the gut microbiome and consequently altering immunological maturation, affects coeliac disease development. However, other windows might exist later in life, given that celiac disease can develop in adulthood. Certain HLA haplotypes affect disease onset. For example, having two copies of HLADQB1*02 has been associated with earlier disease onset, classical clinical presentation and more-severe histologic damage[229]. Future studies to help better understand the interplay between genetic susceptibility and environmental contributions, such as that from a perturbed microbiota, should also include HLA genotyping. Our understanding of the pathogenetic steps has been limited by the lack of a proper animal model. The development of a mouse model in 2020 that approximates coeliac disease through overexpression of IL-15 and expression of the predisposing HLA-DQ8 molecule, leading to development of villous atrophy after ingestion of gluten, has great promise for developing greater mechanistic understanding[230].

DISCUSSION AND CONCLUSIONS

In this Review, we consider several distinct diseases. Yet, all are centered on the development of abnormal patterns of inflammation of the gastrointestinal tract, an organ system that hosts an enormous, complex and varied microbiota. Although our discussion of the aetiology of these diseases centers on perturbation of the hindgut microbiome, we also consider another disease that is increasing in incidence, eosinophilic oesophagitis (EoE) (**Table 4**), for which foregut microbiota perturbation might be important (**Box 1**). In reality, the principles being considered are parallel for the foregut and hindgut.

Table 4. Clinical studies evaluating antibiotic exposure and eosinophilic oesophagitis risk

Study	Timing of antibiotic rxposure	Age of EoE onset	Date (location)	Study design	Antibiotic data source	EoE diagnosis	Key findings[a]
Witmer et al.[233]	Birth to 6 months	Paediatric	2018 (USA)	Case-control	Pharmaceutical coding records	National military database	aOR 1.31 (1.10-1.56)
Jensen et al.[234]	Birth to 12 months	Paediatric	2013 (North Carolina, USA)	Case-control	Retrospective survey	Database[b]	OR 6 (1.7-20.8)
Radano et al.[235]	Birth to 12 months	Paediatric	2014 (Boston, MA, USA)	Case-control	Retrospective questionnaire	Database[b]	OR 3.61 (1.11–11.7)
Jensen et al.[236]	Birth to 12 months	Paediatric	2018 (Cincinnati, OH, USA)	Case-control	Retrospective questionnaire	Database[b]	aOR 2.30 (1.21-4.38)
Dellon et al.[237]	Birth to 12 months	Adult	2021 (North Carolina, USA)	Nested case-control[c]	Retrospective questionnaire	Database[b]	OR 4.64 (1.63-13.2)
Slae et al.[238]	Birth to 12 months[d]	Paediatric	2015 (Canada)	Case-control	Retrospective questionnaire	Database[b]	No significant difference OR 1.00

aOR, adjusted odds ratio; EoE, eosinophilic oesophagitis; OR, odds ratio. [a]Value ranges in parentheses are 95% confidence intervals. [b]Database included histopathological data to confirm EoE diagnosis based on accepted definitions. [c]Case–control study nested within a previously conducted prospective cohort study of adults undergoing outpatient oesophagogastroduodenoscopy for evaluation of gastrointestinal symptoms. [d]This study included questions regarding recent antibiotic exposure, which was defined as ‘less than once a year’, ‘once a year’, ‘two to three times per year’ and ‘four or more times per year’.

Box 1. Eosinophilic oesophagitis

Eosinophilic esophagitis (EOE) is a chronic immune-mediated disease that is defined by the presence of symptoms consistent with oesophageal dysfunction, with an oesophageal biopsy sample showing ≥15 eosinophils per high power field, while excluding other causes of oesophageal eosinophilia[244,245].
The immune response in EOE is mainly mediated by T helper 2 (Th2) interleukins[244,246]; overexpression of IL-13 selectively induces eotaxin 3 (also known as CCL26) expression in oesophageal epithelial cells, leading to eosinophilic infiltration and activation within oesophageal tissue[246–249].

Epidemiology

As with other atopic diseases, the prevalence of EOE has increased in the past few decades[250–254]. EOE now accounts for approximately one-quarter of histologically proven oesophageal disease in children undergoing oesophagogastroduodenoscopy (EGD)[250,251]. EOE affects all age groups, is male-predominant, and is global, although it seems to be more common in temperate climates and in those of European descent[244]. Improved detection alone does not account for the increase in prevalence[250,252,253,255,256]. Although genetic variants might relate to EOE risk, a retrospective cross-sectional study utilizing two cohorts, including an international registry of EOE twin probands, showed that there was a stronger environmental contribution than genetic contribution to EOE[255]. In the twin cohort, genetic heritability was 14.5 ± 4%, and common family environment contributed 81 ± 4% to risk[255].

Inverse association with *Helicobacter pylori*

Helicobacter pylori has been inversely associated with atopic diseases such as asthma[257] and oesophageal diseases including gastroesophageal reflux disease (GERD)[258–261], Barrett oesophagus[261] and oesophageal adenocarcinoma[262]. Six studies have shown an inverse relationship between the presence of *Helicobacter pylori* and either oesophageal eosinophilia and/or EOE[263-268]. Humans have been colonized by *H. pylori* for at least 100,000 years, and likely longer[118]. Loss of key microbial species, including *H. pylori,* as a result of antibiotic exposures could plausibly contribute to EOE risk[269].

Clinical studies

In six clinical studies evaluating the relationship between antibiotic exposure and EOE risk, the odds ratios ranged from 1.3 to 6 (**Table 4**)[233-238]. A 2021 meta-analysis of five of these studies showed significant associations between antibiotic exposure and EOE risk in four studies[219,233–236]. In one study, there were similar rates of early-life exposure to antibiotics between EOE and GERD cohorts (81% and 73%, respectively), which were higher than the exposure rate (42%) for a control cohort of asymptomatic children[234]. Although these findings provide evidence that antibiotic exposure early in life might increase the risk of developing EoE, they are limited by the small numbers of individuals studied.

Timing of EOE onset and antibiotic exposure

A case-control study evaluated patients with EOE who did not develop symptoms until ≥18 years of age, nested within a prospective cohort study of adults undergoing EGD for evaluation of gastrointestinal symptoms[237]. Both the individuals their mothers provided information about early-life antibiotic exposures; antibiotic exposure in the first year of life was associated with adult-onset EOE (OR 4.6)[237] (**Table 4**).

EOE disease severity and antibiotic exposure

In a retrospective review of Italian children and adults with EOE, antibiotic exposure in relation to EOE disease activity was studied[270]. The researchers defined refractory EOE as symptom 'flare-ups' and responsive EOE as asymptomatic, but histological data to confirm EOE disease activity was not reported. Antibiotic exposure was defined as repeated courses (≥3 courses per year) in the first 3 years of life. Adults with refractory (symptomatic) EOE were significantly more likely to have repeated antibiotic exposure (as defined by the authors): 70% versus 33.3% in asymptomatic EOE (P=0.03)[270]. Further studies are needed to assess the relationship between antibiotic exposure and EOE disease severity.

For each of these diseases, there is a growing body of evidence that a perturbed microbiota is associated with onset and pathogenesis. There is a complex interplay between host genetics and environmental factors that influence gut microbiota composition and functionality, with multiple confounding factors. Epidemiological studies suggest that antibiotic treatments, especially early in life, are associated with increased risk of these diseases by altering the microbiota. However, as they are designed to test associations rather than causal roles, they can never be conclusive. However, experimental murine studies of several antibiotic treatments have shown substantial early-life perturbations of the gut microbiota, with downstream metabolic and immunological effects. From such experiments, it is possible to reach conclusions about causality (in mice), which can be juxtaposed with the human epidemiological and clinical data to reach broader conclusions. Experiments further indicate that variation in antibiotic types, dosages and timing could explain differences in disease risk.

If this notion is correct, then it can help us to understand the pathogenesis of each of these illnesses in new ways, beginning with how microbial populations in the lumen are signalling to host tissues in beneficial or pathogenic ways. Understanding the pathogenetic mechanisms might lead to new approaches to diagnosis and treatment of these diseases. In addition, such investigations should lead us to a new appreciation of the biological costs of antibiotic treatments. Although antibiotic treatment can be life-saving, most patients receive antibiotics for treatment of mild infections, often without strong indications of utility[22]. Such wide use reflects a general sense by both practitioners and the public that antibiotics are very safe, and that benefits outweigh any risks. But if antibiotic exposure is indeed playing a part in any or all of these illnesses, then consideration of their risks must grow, and their use must be tempered by a more transparent risk-benefit assessment.

Antibiotics have been pillars of medicine for the past 75 years, and have so much benefit and so little immediate cost that they are widely used even for the most marginal indications[22]. However, increasing evidence of long-term costs propels us to find alternative approaches to control bacterial infections. One important avenue is the development of narrow-spectrum agents, whether they be antibiotics, peptides, bacteriophages or other approaches, to reduce the unintended collateral consequences of broad-spectrum agents. Other approaches are ecological: to select for, or introduce, competitors of the pathogens; this could be done with single agents or mixtures of beneficial organisms, using probiotics, prebiotics or FMT. Retreating from antibiotic ‘carpet bombing’ of the intestinal microbiota will likely prevent much future disease.

However, as discussed throughout this Review, the data are incomplete, and limited by the complexity of testing hypotheses that involve exposures months, years or even

decades before the development of a disease. Going forwards, these issues should be considered in light of the nine criteria for understanding causal relationships developed in 1965 by Sir Austin Bradford Hill[231]. These criteria, which include the strength of the association (effect size), consistency (reproducibility), specificity, temporality, biological gradient (dose-response relationship), plausibility, coherence, experiment, and analogy, are highly relevant to the questions raised here. For each of the illnesses, there is a partial match with the criteria, but the data are incomplete to fully assess the link and its magnitude. As such, data interpretation in humans is challenging, and many pathways remain to be discovered (**Box 2**). Further knowledge will require more prospective epidemiologic studies, clinical trials, and disease models in experimental animals that mimic the conditions of interest. Nevertheless, this interface involving some of the most common medicines used in the world represents an important frontier of medical science for diseases of global significance.

Box 2: Open research questions

- What is the relationship of the timing of antibiotic exposure to disease risk?
- Do all antibiotics have similar metabolic and immunological effects mediated by microbiota disturbances?
- Are there particularly bad combinations of antibiotic exposures that magnify risk?
- What is the relationship between antibiotics and other exposures that perturb the early-life microbiome (for example, cesarean birth, formula feeding)?
- What are the mechanisms by which a perturbed microbiota aberrantly signals to host tissues?
- After a damaging exposure, can there by restoration to baseline?
  - o What is the relevant time window?
  - o Is there a point of no return?
  - o What are the optimal ways to accomplish this (for example, prebiotic, probiotic or synbiotic supplementation, or fecal microbiota transplantation)?

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Author contributions

A.C.F. and M.J.B. researched data for the article, made substantial contributions to discussion of content, wrote the article, and reviewed/edited the manuscript before submission. M.W., and L.A.C. researched data for the article, wrote the article, and reviewed/edited the manuscript before submission. M.N. researched data for the article and reviewed/edited the manuscript before submission.

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The authors declare no competing interests.

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GLOSSARY

Dysbiosis Perturbation of the homeostasis of gut microbiota composition, potentially leading to changes in both functional and metabolic activities.

Germ-free mice Mice born and raised in sterile conditions and thus free of bacteria and fungi.

Lipopolysaccharide (LPS). A microbiota-derived endotoxin found in the outer membranes of Gram-negative bacteria. Bacterial LPS has a key role as an elicitor of innate immune responses through binding to CD44, LBP and TLR4.

Non-obese diabetic mice (NOD mice) A mouse strain developing an autoimmune illness resembling type 1 diabetes mellitus in humans. The substrain NOD/Caj have membrane-bound immunoglobulins and are not secreting antibodies, so as to understand the role of B cells as antigen-presenting cells rather than production of auto-antibodies only.

Prebiotic Dietary components, mostly consisting of non-digestible fibres, that might have a potential beneficial effect on gut microbial composition and function.

Probiotics Viable microorganisms that reach the intestine in an active state and might elicit a favourable effect on host metabolism or re-establishment of gut microbial composition.

Short-chain fatty acid (SCFA) Predominantly butyrate, propionate and acetate. Metabolic products of complex carbohydrate fermentation, chiefly from anaerobic bacteria, that are both energy sources for hosts as well as signalling molecules to host tissues.

Synbiotic Nutritional supplements that consist of a synergistic combination of both prebiotics and prebiotics.

11

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

This thesis provides novel insights into the well-established concept of the gut-thyroid axis. An extensive body of literature is currently available describing the impact of thyroid hormones (TH) on intestinal homeostasis and, more recently, the reciprocal effect of the gut (specifically its microbiota) on TH metabolism.

Chapter 2 provided a comprehensive overview of the bidirectional communication between the gut and the thyroid and its clinical consequences in the two autoimmune thyroid diseases: Hashimoto's thyroiditis (HT) and Graves' disease. Despite the recent increase in research interest on this topic, many questions remain unanswered, making it difficult to establish whether the gut-thyroid axis has a potential causal role in the pathogenesis of autoimmune thyroid disease. In other words, is it the chicken or the egg? Addressing these and other questions is needed before microbiota-based therapy can be considered in clinical practice as a treatment modality for autoimmune thyroid diseases. To achieve this, we first delved deeper into the role of TH in the gut.

THE ROLE OF THYROID HORMONES ON THE GUT

The impact of TH on maintaining gut homeostasis has been demonstrated through various studies, including studies on colorectal cancer (CRC). Disruption of this homeostasis precedes the development of tumorous cells, characterized by abnormal cell differentiation and proliferation. Experimental and ex vivo studies showed that CRC cells have lower intracellular TH bioavailability while increasing TH levels reduced tumor growth in vitro. This raised the question of whether high intestinal exposure to TH could reduce the risk of CRC. One population that can provide insights into this question is levothyroxine (LT4) users. LT4 is a synthetic form of TH that is incompletely absorbed in the gastrointestinal (GI) tract. As a result, the colonic epithelium is exposed to supraphysiological levels of TH. Prior cohort studies tried to investigate this question but suffered from several methodological shortcomings, including small sample sizes or failing to adjust for LT4 treatment duration or for the use of other medication. To this end, in **Chapter 3** we conducted a large population-based case-control study on the effect of LT4 use on CRC risk. We concluded that there is no reduced risk of CRC in levothyroxine users but did find a borderline significant reduction in rectal cancer risk. However, while this provides valuable information on the effect of high intraluminal TH levels, it does not provide data on the intracellular mechanism.

In unpublished data, we have observed a reduction in T4 and an increase in reverse T3 in human colon carcinoma samples compared to healthy intestinal samples (**Fig. 1**). These findings suggest an upregulation of DIO3 activity, consistent with high DIO3 expression in human CRC cell lines[1]. Increased DIO3 activity causes degradation of (L)T4, which may explain why our study did not show a protective effect. To increase TH levels, DIO2 activity needs to be enhanced instead of DIO3.

Interestingly, we detected the presence of 3,3',5-triiodothyronine (3,5'T2) in both our carcinoma and healthy intestinal samples, which is intriguing considering that this particular form of T2 is not expected following an increase in DIO3 activity (as this would lead to the production of 3,3'T2). However, we could not detect the presence of 3,3',5'-triiodothyronine (3,3'T2) in either sample type, which adds complexity to the interpretation of our results. Therefore, quantifying DIO3 and DIO2 activity could provide valuable insights into intracellular TH activity in enterocytes.

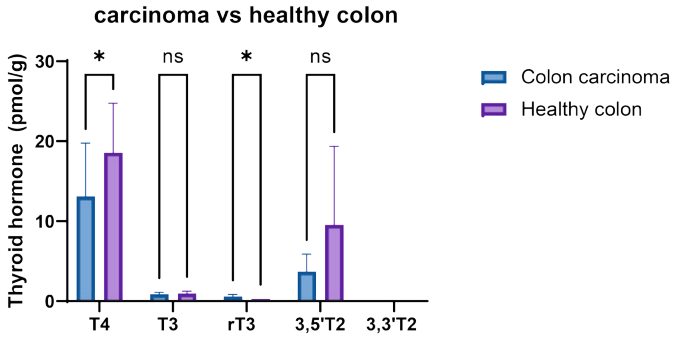

Figure 1. Thyroid hormone levels in colon carcinoma and healthy colon tissue obtained from the same patient (N=6), measured using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Statistical significance was determined using paired t-tests, * means $P < 0.05$

Chapter 4 demonstrated that iodide affects the gut microbiota composition in mice with a remarkable sex-specific effect, suggesting that high-iodine-containing drugs (such as amiodarone) may have unexpected consequences in other hosts. However, despite being extensively used in scientific research, the use of such gnotobiotic murine models has been controversial. Physiological differences between rodents and humans challenge the translation of findings to human health. For instance, some human bacterial taxa do not colonize rodents, and other taxa result in different phenotypes or ecological outcomes[2].

The novel *gut-on-a-chip* models are an innovative tool for studying the interactions between TH and enterocytes[3,4]. These artificial guts are microfluidics-based cell culture devices designed with two channels separated by a porous membrane to enable intercellular crosstalk. The upper channel replicates the host's intestinal lumen, while the lower channel represents the mucosal side, including endothelium and immune cells. It can be used to study the impact of various physiological intestinal functions, such as drug absorption (e.g., levothyroxine), nutrient digestion (e.g., iodine and selenium), intestinal barrier formation, and, interestingly, host-microbiome interactions. Additionally, such a model could reduce the dependence on animal models and enhance the translation of murine findings to human health. When

combined with other organ-on-a-chip models, these novel gut-on-a-chip approaches can also explore the interconnection with extra-intestinal organs. Although a *thyroid-on-a-chip* has been developed to replicate thyroid functionality[5], a gut-microbiome-on-a-chip connected to a thyroid-on-a-chip model has yet to be investigated.

THE ROLE OF THE GUT MICROBIOTA ON THYROID HORMONES

The second part of this thesis aimed to explore the role of gut microbiota on TH and its potential impact on HT. **Chapter 5** elaborated on the current knowledge in this field and identified potential mechanisms by which gut microbial dysbiosis affects the disease. Our findings showed common patterns of compositional and functional shifts in both type 1 diabetes and HT, characterized by loss of diversity, reduction in protective commensals, and overgrowth of potentially pathogenic strains. But even among HT studies, significant discrepancies in the relative abundance of bacterial taxa (either enriched or depleted) have been reported in literature. These discordant changes in the gut microbiome signature lead to inconclusive results and highlights several challenges in microbiome research, including establishing a clear definition of a healthy gut microbiome. Furthermore, the definition of HT, as with many other diseases, is not unequivocal and may or may not include the presence of anti-TPO antibodies (TPOAb), hypothyroidism, or goiter[6].

To address these challenges, we investigated whether a disrupted gut microbiota composition exists before clinical disease onset in participants susceptible to HT. In **Chapter 6**, we compared the taxonomic and functional gut microbiota profile of euthyroid participants with positive serum levels of TPOAb to seronegative healthy controls. We studied three ethnic groups (European Dutch, Turkish, and Moroccan) in a multi-ethnic cross-sectional cohort. Results of four separate differential abundance (DA) tools showed thirteen taxa consistently found nominally significant (enriched in the seropositive/-negative groups, however, none passed adjustment for multiple corrections. These results should be interpreted cautiously, as no standardized approach for analyzing complex microbiome data exists. Multiple DA analysis methods are often used interchangeably, resulting in inconsistent results that impede the comparability and reproducibility of findings across different studies[7–9]. Such inconsistencies in analytical methods can arise, e.g., due to disagreements regarding correcting for differing read depth across samples. For example, rarefaction is a frequently applied method for library size normalization of amplicon sequencing data and normalizes samples of varying sample sizes by subsampling each to a shared threshold (typically between 0 and 1). Some researchers advocate using rarefied data for accounting for this variation as it draws a biologically meaningful diversity analysis, while others argue against it as this might increase sparsity and, thereby, the zero count amplifies the issues around zero substation due to the omission of valid data. As advancements in computational methods continue to evolve rapidly,

establishing fixed standardized guidelines or approaches may be challenging. Therefore, it is essential to justify the computational techniques employed in each published study thoroughly.

Due to this study's cross-sectional design, long-term implications of gut-thyroid axis interactions remain uncertain, leaving the question of what will happen over time unanswered. Extensive longitudinal studies with frequent fecal and blood sampling to monitor gut microbiota and thyroid function would provide valuable insight into the role of gut microbiome composition in the disease progression of thyroid autoimmunity., Thus, a follow-up study on the 159 euthyroid TPOAb-positive participants to investigate their thyroid function over the next five years would be very informative. Additionally, the effect of levothyroxine treatment on the gut microbiota composition and functionality could also be explored in this follow-up study. This follow-up study's results might help answer important questions, such as who is prone to develop HT, as not everyone with TPOAb will eventually develop hypothyroidism. Can we potentially predict who needs to be more intensively monitored?

Chapter 7 outlined the most ambitious project of this thesis, the IMITHOT study. This randomized clinical trial investigates whether multiple fecal microbiota transplantations (FMT) from a healthy donor prevent the decline of thyroid reserve capacity in medication-naïve patients with subclinical autoimmune hypothyroidism compared to autologous (patients' own) FMT. FMT is a treatment approach that involves replenishing the gut microbiota to restore its function and alleviate disease progression. Based on previous studies in other pathologies, we hypothesized that this treatment modality might dampen the T cell-mediated autoimmune attack against thyrocytes, thereby halting the destruction of the thyroid gland. In turn, this might delay or even prevent the need for exogenous thyroid hormone supplementation with LT4 in patients at high risk of developing overt hypothyroidism.

At the time of writing this thesis, the IMITHOT study is still ongoing, and the last study visit is scheduled for September 2024. We have encountered various hurdles and challenges in conducting an FMT study, as outlined in **Chapter 8**. Out of 393 potential donors, only 10% had successfully passed the complete screening and were eligible to serve as stool donors. The screening costs were substantial, with €64.112 being spent on screening for the participation of only one year. With the results of the IMITHOT trial still pending, we can only speculate about the potential for personalized treatment of autoimmune thyroid disease in the future.

Graves' orbitopathy (GO), or thyroid eye disease, is a frequent manifestation of Graves' disease and is characterized by orbital fat expansion and fibrosis. Its pathogenesis is incompletely understood, and treatment can be very challenging. In **Chapter**

9, we presented evidence linking the pathogenesis of Graves' orbitopathy (GO) pathophysiology to the gut microbiome through impaired intestinal permeability. In two distinct cohorts, we found that the intestinal barrier was compromised in Graves' patients compared to healthy individuals[10]. GO patients showed significantly higher circulating levels of lipopolysaccharide-binding protein (LBP) compared to controls, a marker of intestinal permeability. The serum LBP level correlated with the degree of inflammation within the orbital adipose fat tissue, which underlies myofibroblast activation and, thus, fibrogenesis. When disrupted, bacterial compounds, including their DNA or microbial components of the cell wall or flagellum, might translocate to the systemic circulation[10], leading to systemic inflammation and potentially aggravating the inflammatory processes within the orbital tissue. Future intervention trials - preferably a human randomized clinical trial – should be conducted to gain further insight into these correlations. A potential study might involve administering FMT from healthy donors in GO/GD patients, compared to a placebo, along with collecting (small) intestinal biopsies.

FUTURE PERSPECTIVES FOR (PERSONALIZED) MICROBIOME-BASED THERAPY IN AUTOIMMUNE THYROID DISEASE

Diet-induced modulation of the gut microbiota is an area of research that holds great promise. In recent years, profit-driven companies have claimed they can provide tailored recommendations and even personalized recipes for improving health based on an individual's gut microbiota composition. With this growing interest and the potential for personalized medicine to modulate gut microbiota, there is a need to explore whether this approach can be adopted for autoimmune thyroid diseases.

While some small cohort studies suggest that a paleolithic, autoimmune protocol or gluten-free diet may benefit autoimmune thyroid diseases[11,12], randomized clinical trials are still lacking. Similarly, studies on the effects of pro-, pre-, or synbiotic supplementation on thyroid function have shown only marginally beneficial effects[13,14].

The concept of restoring gut health by manipulating intestinal microorganisms has a long history, with early Chinese physicians prescribing *yellow soup* as a remedy for digestive problems as early as the 4th Century BC[15]. In recent years, FMT as a clinical treatment approach has gained renewed attention since the pioneering trial in 2013 by van Nood and colleagues on the infusion of healthy donor feces as a treatment modality in recurrent *Clostridioides difficile* infection (rCDI)[16].

Since then, the potentially beneficial effects of FMT have been extensively studied. It significantly improved the response rate in acute graft-versus-host-disease patients post-hematopoietic stem cell transplantation compared to steroids alone (82.4% vs.

25-40%, respectively)[17–19]. FMT has also shown promise in stabilizing residual beta cell function in patients with type 1 diabetes[20]. Furthermore, FMT led to an increase in the proportion of patients achieving clinical and endoscopic remission of ulcerative colitis, although the maintenance of remission remains uncertain[21]. Unfortunately, the effect was less pronounced for Crohn's disease. An impressive number of 449 studies are currently registered to investigate the effect of FMT in many different conditions (Search of: FMT - List Results - ClinicalTrials.gov).

However, the demonstrated variability in individual responses to fecal microbiota transplantation (FMT) in previous studies suggests that a '*one-size-fits-all*' approach may not effectively treat autoimmune thyroid disorders, and the combination of microbiota donors and recipient characteristics may dictate the therapeutic outcome[22]. Predicting which patients will be responders or non-responders remains challenging[18]. Therefore, individualized assessment and management are necessary, as what works for one patient may not be appropriate for another.

One possible explanation for the inconsistent effects of FMT treatment is that it comprises not only the transplantation of the host's bacteria but also other microorganisms that reside in feces, including fungi, viruses, bacteriophages, and fecal-excreted hormone metabolites[23,24]. It has been shown that these other microorganisms and compounds may play a significant role in the efficacy of FMT, as demonstrated in studies on the impact of *Candida albicans* on FMT effectiveness in rCDI[25]. It is unlikely that a single bacterium or bacterial community contributes to the colonization or resistance of the microbiota engraftment to prevent or halt the progression of autoimmune thyroid diseases by FMT. Instead, the impact of FMT results from transplanting an utterly functional ecosystem, consisting of a complex interplay of multiple microbiota communities and networks contributing to producing metabolites and immune modulation together to promote resilience and protection.

Recent advancements in FMT techniques and strategies have enabled the development of lyophilized FMT capsules using freeze-dried feces, which hold promising potential for addressing the challenges faced with nasoduodenal insertion of fresh feces FMT. This method has demonstrated preserved viability of gut microbiota and comparable engraftment with other FMT preparation and administration methods[26,27]. One advantage of this approach is the ability to accurately analyze the donor microbiome before treatment, prepare the capsules in advance, and reduce the burden of the FMT recipient. Additionally, the capsules can be supplemented with beneficial bacterial strains and metabolites such as tryptophan and short-chain fatty acids (SCFA) to enhance therapeutic efficacy.

Lastly, as the age-old adage goes, "prevention is better than cure," a maxim inherent in a balanced gut microbiome. In the final chapter of this thesis (**Chapter 10**), we

elaborated on the critical window in early life in which microbiome perturbations substantially affect disease development. Hence, it is imperative to exercise caution with excessive and irrational use of antibiotic drugs in the young, not only due to the concerns regarding antimicrobial resistance but also because of the potentially harmful effects of disrupting the gut microbiota, which may manifest only later in life.

CONCLUSION

In this thesis, we have explored the intriguing connection between the thyroid gland and the gut with its residing microbiota through a comprehensive review of the current literature and several original data studies. We have established that LT4 does not appear to have a protective effect on colorectal cancer. Next, the marginally significant link between dysbiosis and thyroid autoimmunity in subjects who have not yet developed hypothyroidism suggests that dysbiosis may precede disease onset, but the correlation is weak. We also designed the first human interventional study to determine whether restoring a disrupted microbiome will halt disease progression.

Further research and interdisciplinary collaborations are necessary to address the remaining challenges and future perspectives on the role of gut microbiota in thyroid health. Microbiome research requires the combined efforts of clinical physicians (including immunologists, endocrinologists, and radiologists), fundamental researchers, laboratory technicians, and bioinformaticians. As research in this field expands, joining forces may provide novel microbiota-based approaches for preventing and treating thyroid disorders, thereby improving patient outcomes and quality of life for individuals with thyroid disease.

To conclude, a perturbed microbiome may be one of the multiple precipitating factors leading to the disruption of thyroid homeostasis. However, the directionality of whether a perturbed microbiome leads to increased AITD susceptibility or vice versa or merely reflects its presence remains unknown. While restoring dysbiosis might help alleviate symptoms by breaking a vicious circle, it is unlikely to eliminate the underlying pathogenesis completely.

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Appendices

SUMMARY

The research in this thesis was aimed to shed new light on the bidirectional relationship between the gut and the thyroid gland and its clinical consequences in autoimmune thyroid diseases. It comprises a comprehensive review of the current literature, an epidemiological study, a randomized clinical trial, and basic research.

A short introduction to the regulation of the thyroid gland and microbiome is given in the first chapter, followed by an outline of the studies presented in this thesis. Three sections can be discerned: The first part (**Chapters 2 - 4**) focuses on the so-called gut-thyroid axis. The second part (**Chapters 5 – 8**) discusses the role of gut microbiota in Hashimoto's thyroiditis. Finally, the third part (**Chapters 9 – 10**) investigates various topics related to gut microbiota and their clinical impact.

Together, these studies can be seen as the beginning of an ongoing exploration of the emerging field of gut microbiota research in relation to thyroid health.

Key findings of this thesis:

- The gut-thyroid axis is a well-established entity, as discussed in **Chapter 2.** It consists of an essential interplay between TH metabolism and gut homeostasis, including the involvement of gut microbiota in peripheral TH metabolism and a finely tuned balance of intestinal epithelium proliferation and differentiation controlled by TH signaling.
- The oral administration of levothyroxine results in a supraphysiological exposure of TH to colon cells. While experimental studies showed that elevated levels of TH within colon cells potentially reduce the growth rate of human colorectal tumor cell lines, our population-based matched case-control study did not reveal a reduced risk of colorectal cancer among levothyroxine users, except for a borderline significant reduction in rectal cancer risk (**Chapter 3**).
- Experimental studies involving murine models are needed to establish a causal link between perturbed microbiota and the onset or progression of disease. However, the significant impact of sodium iodide should be considered when using the widely used NOD.H-2^{h4} mouse model to establish gut microbiota's role in autoimmune hypothyroidism. In **Chapter 4**, we showed that sodium iodide supplementation significantly altered both alpha- and beta diversity and the taxonomic profile of the gut microbiota, and these differences persisted even after one week of withdrawal. Interestingly, we observed that these effects were sex-specific.
- Much can be learned from previous research conducted on the gut microbiome in various disease entities. By examining the common pathogenic pathways of the interplay between gut microbiota and type 1 diabetes and Hashimoto's thyroiditis, **Chapter 5** provides valuable insights into the mechanisms of gut

microbial dysbiosis in two common autoimmune endocrine diseases affecting different glands.

- In **Chapter 6**, we found that the influence of the gut microbiome on thyroid autoimmunity appears to be relatively modest in the preclinical stage. In comparison to intestinal diseases, such as *Clostridioides difficile* infection, ulcerative colitis and Crohn's disease, its impact appeared to be less pronounced in HT. A follow-up study on the 159 euthyroid TPOAb-positive participants to investigate their thyroid function over the next five years would provide valuable insight into the role of gut microbiome composition in disease progression of thyroid autoimmunity.
- As per guidelines outlined in **Chapter 7,** the administration of FMT treatment poses significant challenges for patients and researchers. The process involves several critical steps, such as identifying a compatible donor, preparing fresh feces for FMT, and inserting a nasoduodenal tube with laxatives to ensure proper engraftment. Additionally, as highlighted in **Chapter 8**, the associated costs of this treatment can be substantial.
- Graves' orbitopathy (GO) patients exhibit a significantly higher serum concentration of lipopolysaccharide-binding protein, a marker of LPS (a major component of Gram-negative outer membrane) in the bloodstream, than healthy controls, indicating enhanced intestinal permeability (**Chapter 9**) in these patients. This may contribute to the aggravation of orbital inflammation and trigger myofibroblast activation, causing the retro-orbital tissue expansion that is a hallmark of GO. However, whether the gut microbiota play a causal role in this process or merely reflect the effects of the disease itself remains to be further studied in future intervention trials, preferably a randomized clinical trial employing FMT from healthy donors in GD patients versus placebo.
- There is a critical window in early life in which microbiome perturbations substantially affect disease development (**Chapter 10**). Hence, it is imperative to exercise caution with excessive and irrational use of antibiotic drugs in the young, not only due to the concerns regarding antimicrobial resistance but also because of the potentially harmful effects of disrupting the gut microbiota, which may manifest only later in life.

NEDERLANDSE SAMENVATTING

De onderzoeken in dit proefschrift hadden als doel om nieuw licht te werpen op de bidirectionele relatie tussen de darm en de schildklier en de klinische gevolgen daarvan bij auto-immune schildklierziekten. Het omvat een uitgebreid overzicht van de huidige literatuur, een epidemiologische studie, een gerandomiseerde klinische studie en fundamenteel onderzoek.

In het eerste hoofdstuk wordt een korte introductie gegeven over de regulatie van de schildklier en het microbioom, gevolgd door een overzicht van de studies die in dit proefschrift welke drie delen bevat. Het eerste deel (**hoofdstukken 2 - 4**) richt zich op de zogenoemde darm-schildklier-as, met de focus op de rol van schildklierhormonen op de homeostase van de darmen. Het tweede deel (**hoofdstukken 5 - 8**) bespreekt de rol van de darmmicrobioom bij Hashimoto's thyroïditis. Ten slotte onderzoekt het derde deel (**hoofdstukken 9 en 10**) verschillende onderwerpen met betrekking tot het microbioom van de darmen en hun klinische impact. Samen kunnen deze studies gezien worden als het begin van een voortdurende verkenning van het opkomende onderzoeksveld van de darmmicrobioom in relatie tot de schildklier.

De belangrijkste bevindingen van dit proefschrift zijn:

- De darm-schildklier-as is een gevestigde entiteit, zoals besproken in hoofdstuk 2. Het bestaat uit een essentiële wisselwerking tussen schildklierhormoon metabolisme en darm homeostase, inclusief de betrokkenheid van darmmicrobioom in perifere schildklierhormoon metabolisme en een fijn afgestemd evenwicht van intestinale epithelium proliferatie en differentiatie, gecontroleerd door schildklierhormoon signalering.
- De orale toediening van levothyroxine resulteert in een suprafysiologische blootstelling van schildklierhormoon aan darm epitheelcellen. Hoewel experimentele studies aantoonden dat verhoogde levels van schildklierhormoon in darmcellen mogelijk de groeisnelheid van menselijke colorectale tumorcellijnen verminderen, toonde ons retrospectief cohortonderzoek geen verminderd risico op colorectale kanker bij levothyroxinegebruikers, behalve een marginale vermindering van het risico op rectale kanker (hoofdstuk 3).
- Experimentele studies met muismodellen zijn nodig om een causaal verband vast te stellen tussen een verstoorde darmmicrobioom en het ontstaan of de progressie van ziekten. Echter, bij het gebruik van het veelgebruikte NOD.H-2^{h4} muismodel om de rol van de darmmicrobioom bij auto-immuunhypothyreoïdie vast te stellen, moet echter rekening worden gehouden met het significante effect van het toedienen van natriumjodide. In hoofdstuk 4 toonden wij aan dat natriumjodidesuppletie de alfa- en bètadiversiteit en het taxonomisch profiel van het darmmicrobioom aanzienlijk veranderde, en dat deze verschillen zelfs na een

week van na het staken bleven bestaan. Interessant genoeg zagen we dat deze effecten geslacht specifiek waren.

- Er kan veel geleerd worden uit eerder onderzoek naar het darmmicrobioom bij verschillende ziekte-entiteiten. Door de gemeenschappelijke pathogene paden te onderzoeken van de wisselwerking tussen het darmmicrobioom en type 1 diabetes en Hashimoto's thryreoïditis, biedt hoofdstuk 5 waardevolle inzichten in de mechanismen van darmmicrobiële dysbiose bij deze twee veel voorkomende auto-immuun endocriene ziekten.
- In hoofdstuk 6 vonden we dat de invloed van het darmmicrobioom op schildklier auto-immuniteit relatief bescheiden lijkt te zijn in het preklinische stadium. In vergelijking met darmaandoeningen, zoals *Clostridioides difficile* infectie, colitis ulcerosa en de ziekte van Crohn, lijkt de invloed ervan bij Hashimoto's thryreoïditis minder uitgesproken. Een vervolgonderzoek bij de 159 euthyroïde TPOAb-positieve deelnemers om hun schildklierfunctie gedurende de komende vijf jaar te onderzoeken, zou een waardvol inzicht verschaffen in de rol van de samenstelling van het darmmicrobioom bij de ziekteprogressie van schildklierauto-immuniteit.
- Zoals beschreven in het protocol van de IMITHOT-studie in hoofdstuk 7, brengt de toediening van een FMT aanzienlijke uitdagingen met zich mee, voor zowel patiënten en onderzoekers. Het proces omvat verschillende kritieke stappen, zoals het identificeren van een geschikte donor, het voorbereiden van verse feces voor FMT, en het inbrengen van een nasoduodenale sonde met laxeermiddelen om een goede engraftment te waarborgen. Bovendien kunnen, zoals in hoofdstuk 8 wordt uiteengezet, de kosten van deze behandeling aanzienlijk zijn.
- Patiënten met Graves' orbitopathie (GO) hebben een aanzienlijk hogere serumconcentratie van lipopolysaccharide bindend eiwit, een marker van Gram-negatieve bacteriële activiteit, dan gezonde controles, hetgeen wijst op een verhoogde darmpermeabiliteit bij deze patiënten (hoofdstuk 9). Dit kan bijdragen tot de toename van de orbitale ontsteking en myofibroblast-activering, wat leidt tot retro-orbitale weefselexpansie, een kenmerk van GO. Of de darmmicrobiota een causale rol spelen in dit proces of slechts de effecten van de ziekte zelf weerspiegelen, moet echter nog verder worden onderzocht in toekomstige interventiestudies, bij voorkeur middels een gerandomiseerde klinische studie met FMT van gezonde donoren bij GD-patiënten versus placebo.
- Er is een kritieke periode in het vroege leven waarin verstoringen van het darmmicrobioom de ontwikkeling van de ziekte aanzienlijk beïnvloeden (hoofdstuk 10). Het is daarom van groot belang om beducht te zijn op het overmatig en irrationeel gebruik van antibiotica bij jonge kinderen. Niet alleen vanwege de zorgen over antimicrobiële resistentie, maar ook vanwege de potentieel schadelijke effecten van het verstoren van de darmmicrobioom, welke zich mogelijk pas later in het leven zullen manifesteren.

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LIST OF PUBLICATIONS

IN THIS THESIS

Fenneman AC, Rampanelli E, Yin YS, Ames J, Blaser MJ, Fliers E, Nieuwdorp M; Gut microbiota and metabolites in the pathogenesis of endocrine disease. *Biochem Soc Trans* 30 (2020); 48 (3): 915–931. doi: 10.1042/BST20190686

Kuiper JG*, **Fenneman AC***, van der Spek AH, Rampanelli E, Nieuwdorp M, van Herk-Sukel MPP, Lemmens VEPP, Kuipers EJ, Herings RMC, Fliers E. (2022). Levothyroxine use and the risk of colorectal cancer: a large population-based case–control study, *Endocrine Connections,* 11(1) e210463. doi: 10.1530/EC-21-0463

Bénard MV*, de Bruijn CMA*, **Fenneman AC**, Wortelboer K, Zeevenhoven J, et al. (2022) Challenges and costs of donor screening for fecal microbiota transplantations. *PLOS ONE* 17(10): e0276323. doi 10.1371/journal.pone.0276323

Fenneman, AC, Weidner M, Chen LA, Nieuwdorp M, Blaser MJ. Antibiotics in the pathogenesis of diabetes and inflammatory diseases of the gastrointestinal tract. *Nat Rev Gastroenterol Hepatol* 20, 81–100 (2023). doi: 10.1038/s41575-022-00685-9

Fenneman AC, Bruinstroop E, Nieuwdorp M, van der Spek AH, Boelen A. A Comprehensive Review of Thyroid Hormone Metabolism in the Gut and Its Clinical Implications. *Thyroid*. (2023) 32-44. doi: 10.1089/thy.2022.0491

Fenneman AC*, Boulund U*, Collard D, Galenkamp H, Zwinderman AH, van den Born BJH, Rampanelli E, van der Spek, AH, Fliers E, Blaser MJ, Nieuwdorp M. A characterization of the gut microbiome composition in a multiethnic euthyroid population with thyroid autoimmunity. Previous version available at SSRN: https://ssrn.com/abstract=4309030 (2023) doi: 10.2139/ssrn.4309030

Fenneman AC, van der Spek AH, Hartstra A, Havik S, Salonen A, de Vos WM, Soeters M, Saeed P, Nieuwdorp M, Rampanelli E. Intestinal permeability is associated with aggravated inflammation and myofibroblast accumulation in Graves' orbitopathy: the MicroGO study, Front. Endocrinol. (2023): 10.3389/fendo.2023.1173481

Fenneman AC, Rampanelli E, van der Spek AH, Fliers E, Nieuwdorp M. Protocol for a randomized, double-blinded, placebo-controlled trial to assess the effect of fecal microbiota transplantations on thyroid reserve in patients with subclinical autoimmune hypothyroidism: The IMITHOT trial. Accepted in BMJ-Open (2023).

***shared first authorship**

PHD PORTFOLIO

Name PhD student:	Aline Carolien Fenneman
PhD period:	August 2019 – May 2023
PhD supervisors:	Prof. dr. M. Nieuwdorp and Prof. dr. E. Fliers
PhD co-supervisor:	dr. E. Rampanelli and dr. A.H. van der Spek

1. PhD training

	Year	ECTS
Courses		
Clinical Epidemiology: Randomized Clinical Trials	2019	0.6
Pratical biostatistics	2019	1.4
The Amsterdam UMC World of Science	2019	0.7
Computing in R	2020	0.4
E-BROK ('Basiscursus Regelgeving Klinische Onderzoek')	2020	1.5
Good Clinical Practice	2020	0.2
Project management	2020	0.7
Scientic Writing in English	2020	1.5
Didactical Skills	2021	0.4
Statistics and data visualisation	2022	0.4
Basis Kwalificatie Onderwijs (BKO)	2021 – 23	5.8
Seminars, workshops and master classes		
Biweekly Diabetes Research Seminars	2019 – 23	2.8
Weekly Department Research Seminars – Vascular Medicine	2019 – 23	5.6
Weekly Department Research Seminars – Endocrinology	2019 – 23	5.6
Weekly Department Clinical Seminars	2019 – 23	2.8
Weekly Department Research Seminars – dr. Blaser lab	2021 – 22	1.2
Masterclasses by TLC (culturele diversiteit in Onderwijs, Zorg ICT - Wat kun je ermee	2021	0.1
Workshops by TLC (hoe begeleid ik een TBL sessie, welke activerende werkvorm past bij mij)	2022	0.1
Presentations		
Regioavond Endocrinologie Amsterdam	2019	0.5
Leducq Consortium, Amsterdam	2019	0.5
Nederlandse Donor Feces Bank, online	2020	0.5
Annual Dutch Thyroid Day, online	2021	0.5
Leducq Consortium, Berlin	2022	0.5
Leducq Consortium, New Jersey	2022	0.5
(Inter)national conferences		
21th Gut Day, Amsterdam	2019	0.25
Leducq Consortium Amsterdam	2019	0.5
1st Dutch Diabetes Academy	2020	0.25
Dutch Diabetes Meeting, Utrecht	2020	0.25
World of Microbiome, Digestive & Metabolic Health	2020	0.5
Vasdia Symposium	2020	0.5
Annual Symposium of the Dutch Thyroid Research Foundation	2021	0.25
Leducq Consortium meetings, online	2021	0.5
Annual Symposium of the Dutch Thyroid Research Foundation	2022	0.25
Leducq Consortium Berlin	2022	0.5
Leducq Consortium New Jersey	2022	0.5
Other		
AGEM PhD student retreat, online	2021	0.2
AGEM symposium "Owning Obesity & Negating NASH", online	2021	0.25
AGEM symposium "treating NASH", Amsterdam, the Netherlands	2022	0.25
AGEM PhD student retreat, Garderen, the Netherlands	2023	1.0

2. Teaching

	Year	ECTS
Lecturing		
Lectures on 'Professionele Ontwikkeling' for 2nd year bachelor students of Medicine	2020 – 21 2020 – 22	
Lectures on 'leerlijn 'Opmaat naar de Praktijk' for 3rd year bachelor students of Medicine	2021 – 22	
Lectures on 'Just in Time Learning, Internal Medicine' for 1st year master student of Medicine		
Tutoring, Mentoring		
Mentor for 2nd year bachelor student of Medicine	2020 – 21	
Supervising		
Research proposal of 1st year master students of Biomedical Sciences: *Riham Ghalaiyini and Alessandra Boggian*	2019	
Research report of sophomore student of Biomedical Sciences: *Haril Shah*	2021	

3. Parameters of Esteem

	Year
Funding and (travel) grants	
Amsterdam Young Talent Award	2020
Prins Bernhard Cultuurfonds - Cultuurfondsbeurs	2021
Stichting de Drie Lichten	2021
Stichting Atheros	2021
Amsterdam University Fund - Spinoza Fonds travel grant	2021

4. Media

	Year
Dokters van Morgen - episode 'Ontstekingen'	2020

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“Alone we can do so little; together we can do so much.” - Helen Keller.

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ochtend wakker werd met een plakkerige navel van de belly-shots. Is het al tijd voor koffie (met havermelk)??

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Poepgroep vanaf het eerste uur: **Koen, Klaartje en Melanie.** Het uitvoeren van een FMT-studie uitvoeren (in Covid tijd) gaat niet zonder uitdagingen, holy shit! We hebben onze krachten gebundeld en zo een donorpool opgezet, elkaar geholpen bij het inbrengen van de Cortrak-sonde, en daarbij de nieuwste tips en trucs uitgewisseld. En het resultaat mag er zijn: een prachtig gepubliceerd paper!

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Papa en mama, jullie warme hart en onvoorwaardelijke steun hebben mij gemaakt tot wie ik nu ben. Samen met **Annet** en **Nico** vormen we een prachtige en liefdevolle modern family. Ik ben ontzettend dankbaar dat jullie allemaal in mijn leven zijn.

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CURRICULUM VITAE

Aline Carolien Fenneman was born on July 31, 1993, in Doetinchem, the Netherlands. She spent her childhood in a small village in the Achterhoek, together with her parents and the twin brothers, Jesse and Achiel. After graduating from St.-Ludgerscollege te Doetinchem in 2011, Aline moved to Amsterdam to start Medical School at the Vrije Universiteit.

It was during her scientific internship at the lab of Dr. Wulf at the Beth Israel Deaconess Medical Center in Boston, USA, that Aline's interest in science and research was ignited. After obtaining her medical degree, Aline worked at the Internal Medicine ward at Tergooi Hospital before embarking on her Ph.D. trajectory in August 2019. Under the supervision of Prof. dr. Max Nieuwdorp and Prof. dr. Eric Fliers, Aline investigated the role of the gut microbiome in thyroid autoimmunity and aimed to shed new light on the gut-thyroid axis.

In the midst of the pandemic, Aline moved to Piscataway, New Jersey, for five months to conduct several experimental studies in the lab of Prof. dr. Blaser.

Aline currently lives in Amsterdam with her partner, Sam, but will soon be moving to a suburb called Heemstede. She recently started her residency in Internal Medicine at the Amstelland Hospital, but whenever she's not working, she enjoys working out, whether it's running, road cycling, or CrossFit.