Microbiome and Graves' Orbitopathy

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Abstract

Background: Studies from animal models of autoimmunity have highlighted the potential importance of microorganisms and their metabolic products in shaping the immune system. **Summary:** This review provides an introduction to the current state-of-the-art in microbiome research both from the perspective of “what is known” and of methodologies for its investigation. It then summarises the evidence for a role for the microbiome in the pathogenesis of Graves' disease and Graves' orbitopathy with reference to animal models and studies in human cohorts, from both published and ongoing sources. **Key Message:** Microbiome research is in its infancy but has already provided novel insights into disease pathogenesis across the spectrum from cancer to mental health and autoimmunity.

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Introduction

As already described in the other excellent contributions to this collection, Graves' disease (GD) is an autoimmune condition in which thyroid-stimulating antibodies

(TSAb) target the thyroid-stimulating hormone receptor (TSHR), causing hyperthyroidism. About half of GD patients also have Graves' orbitopathy (GO), in which cross-reacting autoimmune responses to thyroid/orbit shared antigens (notably the TSHR) lead to expansion of the orbital contents. The overproduction of extracellular matrix and increased adipogenesis within the bony confines of the orbit produce proptosis, diplopia, and even blindness [1].

In other autoimmune conditions, animal models have been invaluable in dissecting the humoral and T-cell-mediated responses underpinning pathogenesis. Twenty years ago, a TSHR-induced model of GD/GO was established by genetic immunisation of female BALB/c mice with the human TSHR. A substantial proportion of the mice developed TSAb, thyroiditis, and GO-like symptoms. Furthermore, it was possible to transfer disease using primed T cells from TSHR-immunised mice to naïve recipients [2]. However, the model could not be reproduced in Cardiff, where the induced disease was more “humoral” (i.e., many mice had TSAb) but there was no lymphocytic infiltration in the thyroid or orbital tissues [3], in contrast to TSHR-immunised mice in Brussels. At that time, insufficient notice was paid to the microbial environment and although both animal units were “clean,” neither was specific pathogen-free.

This led the authors to suggest a role for microorganisms in priming the immune repertoire making it suscep-

tible, or not, to tolerance being broken by immunisation with autoantigenic preparations. Similar effects had been observed in other autoimmune conditions, notably in experimental autoimmune encephalitis, a murine model of multiple sclerosis, mice obtained from one supplier which consistently developed multiple sclerosis-like symptoms whilst those from another supplier were largely resistant. When the gut microbiota composition was compared in mice from the two suppliers, many significant differences were apparent. Painstaking analysis revealed that segmented filamentous bacteria (SFB) were required for experimental autoimmune encephalitis susceptibility; SFB were lacking in resistant mice but when administered led to induction of multiple sclerosis signs and symptoms. This was achieved by the ability of SFB, or more likely their metabolites, to increase the generation of Th17 cells [4].

This work is just one of many studies which have demonstrated that gut microorganisms are able to influence the development of immature T cells present in the gut-associated lymphoid tissue. At any one time up to 50% of circulating lymphocytes reside in the gut-associated lymphoid tissue, the balance between anti-inflammatory Treg and proinflammatory Th17 (found in many autoimmune lesions) is affected by species in the gut. For example, short-chain fatty acids (SCFAs), such as butyrate, favour Treg; *Intestinimonas* and *Roseburia* both secrete butyrate, whilst SFB promote Th17, most likely in a toll-like receptor-dependent manner [4]. In a healthy gut the microbiota species should be able to maintain a balance between Treg and Th17. However, as will be discussed later, many environmental factors can perturb the gut microbiota and tip the balance in favour of autoimmune and inflammatory responses (Fig. 1).

Before moving on it would be helpful to provide a few definitions and some general background on the microbiome [5]: (1) Microbiota: bacteria, viruses, protozoa, fungi that inhabit a particular niche, e.g., the gut. (2) Niche: other niches in the human body include the skin, vagina, sinuses, and eye etc. (3) Microbiome: the genomes of all the microbiota in a niche. (4) Metabolome: metabolic products, e.g., SCFAs that impact on the host. (5) Metabonome: collection of metabolomes, e.g., gut plus nasal sinus etc.

Microbiome analyses most often involve metataxonomics, a culture-independent approach based on the high-throughput sequencing of variable regions of the bacterial 16S rRNA gene, to obtain information about taxonomic diversity, i.e., identity and relative abundance of species present [6]. Polymerase chain reaction (PCR) primers used for the amplicon sequencing are often based

on the highly conserved regions of the 16S rRNA gene, while variable regions (e.g., V1–V2 or V3–V4), unique to a single microorganism, are used for phylogenetic analyses [7]. Alternatively, metagenomics or “shotgun sequencing” can be applied; this involves sequencing of the whole microbial genome (i.e., bacterial, but also *Archaea* and fungal), but it remains expensive and requires computational effort and infrastructure. In either case, the high-throughput sequencing reads are processed by bioinformatics pipelines to filter out poor-quality bases [8–10] and to align the passing filter reads to one of the 16S gene reference databases now available (e.g., SILVA [11], the Ribosomal Database Project [12], or GreenGenes [13]). Aligned tags which cluster together at a certain cut-off (usually 97%) are considered identical and referred to as an operational taxonomic unit (OTU), which approximates to a single bacterial species. OTUs can be binned into phylogenetic levels from phylum to genus. The human microbiome contains more than a million genes and dwarfs the approximately 20,000 residing in our genomes [14].

We inherit our microbiome from our mothers during delivery, hence children born naturally derive their microbiome from the vagina, whereas babies born by caesarean section have a microbiome more closely resembling maternal skin; differences also result depending on whether the child is breast- or bottle-fed [15]. The importance of the early-life microbiota composition to future disease disposition is elegantly demonstrated by studies of type 1 diabetes (T1D), a condition whose incidence has greatly increased in recent years. A study of the gut microbiome in infants in Finland (high incidence of T1D) and Russia (less susceptible to autoimmunity and allergy) from birth to 3 years of age revealed higher levels of *Bacteroides* at birth in Finland. As a consequence, lipopolysaccharide (LPS) was more likely to be *Bacteroides* derived, whereas Russian infants showed a higher prevalence of *Escherichia coli*-derived LPS. *E. coli* (phylum *Proteobacteria*) LPS is a strong stimulator of innate and adaptive immune responses and was subsequently shown to confer resistance to T1D in non-obese diabetic mice. In contrast, *Bacteroides* LPS was immunoinhibitory and had no effect on T1D in non-obese diabetic mice, leading the authors to suggest that it reduces early immune education [16].

The gut microbiome remains relatively stable and unchanged from early to mid-adulthood [17], but it is influenced by a range of factors. One of the most important is diet and “westernisation”: individuals who consume large amounts of animal protein, fat, sugar, and starch but scant fibre possess a different and less diverse gut micro-

Fig. 1. The gut microbiota and its effects on the immune system. The interaction between gut epithelium, antigen-presenting cells such as dendritic cells, and the “healthy” gut microbiota produces cytokines and other metabolites which preserve the balance between regulatory T cells (Treg) and proinflammatory Th17 and Th1 cells. When the microbiota is suboptimal, the cytokines produced favour generation of proinflammatory Th17 cells, which may lead to loss of tolerance. In an individual with a particular genetic predisposition the result of this so-called “dysbiosis” or “dysbacteriosis” could lead to GD and, in extreme cases, GO. Transforming growth factor beta (TGFβ) is secreted by gut dendritic cells and is a differentiation factor for both Th17 and Treg cells, the latter characterised by expression of the FoxP3 forkhead

transcription factor. Another product of dendritic cells, retinoic acid, enhances Treg development whilst inhibiting Th17 cells, which in turn are maintained by IL-23. IL-10 and IL-2 are secreted by gut macrophages; the latter inhibits Th17 development while the former inhibits secretion of Th17 cytokines, e.g., the IL-17 family (including IL-25 or IL-17E) which recruit neutrophils, a crucial component of innate immunity. Treg cells inhibit the development of proinflammatory Th17 and Th1 cells, the latter secrete interferon gamma (IFN-γ), which exacerbates autoimmune responses by upregulating major histocompatibility complex II expression. *B. fragilis*, *Bacteroides fragilis*; *S. typhimurium*, *Salmonella typhimurium*; SFB, segmented filamentous bacteria.

biome than those in rural communities whose diet is low in animal protein and fat but high in fibre [18]. It is also possible that seasonal variation in foods consumed may modify the gut microbiome and contribute to the clustering of disease onset at certain times of the year [19], reported for some autoimmune conditions, although this has been attributed to vitamin D intake. Major perturbations of the gut microbiota were reported after treatment with antibiotics, although its composition is thought to normalise within weeks of cessation of short-term exposure [20]; more profound and persistent changes result, however, from repeated or long-term antibiotic consumption, which may even predispose to autoimmunity [21]. Smoking is a major risk factor for GO and there are reports of its effects in reducing gut microbiota diversity, including a significant increase in *Proteobacteria* and *Bacteroidetes* phyla, whereas *Actinobacteria* and *Firmicutes* phyla were decreased [22]; similar findings were

reported in the middle meatus of the nose [23]. Stress has long been recognised as a possible trigger of GD and may even promote recurrence [24]; stress can also have major effects on the gut microbiota, gut permeability, and the cytokines released by gut-resident immune cells [25]. Of potential relevance, Zhang et al. [26] conducted proteomic profiling in European GD and GO individuals, finding significantly increased levels of zonulin in patients compared with unaffected controls. Zonulin regulates tight junctions between epithelial cells and is implicated in gut permeability.

Studies on the Role for the Microbiome in GD and GO

Following the early frustrations with animal models, a robust in vivo model of GD/GO has been established by Paul Banga and Sajad Moshkelgosha, using genetic

immunisation with the TSHR-A subunit accompanied by electroporation to enhance autoantigen expression [27–29]. This model allowed us, in collaboration with Anja Eckstein, Utta Berchner-Pfannschmidt, and Hedda Kohling/Verhasselt, to investigate the effect of the gut microbiota on disease using the same BALB/c strain but housed in two different locations [30] or of using two mouse strains of differing H2 haplotype (BALB/c and C57BL/6) but housed in the same unit [31]. We compared the gut microbiota by sequencing the bacterial 16S rRNA gene and performing standard microbiology on faecal or intestine (gut) samples derived from TSHR-immunised mice in Essen and London, both units being specific pathogen-free. The London murine gut microbial community demonstrated significantly reduced richness but not diversity when compared with the Essen mice. Consequently, the gut microbiota composition in the two centres showed good separation using appropriate statistical methods. Seven phyla were identified, with *Bacteroidetes* and *Firmicutes* being the most abundant and not differing between centres. In contrast, we observed significant differences in the abundance of 18 distinct genera, with *Lactobacillaceae*, *Ruminococcaceae*, and *Porphyromonadaceae* families more abundant but *Bifidobacteria* essentially absent in Essen mice. Results were confirmed by the traditional culture methods, which also indicated higher yeast counts in mice housed in London. The gut microbiota was also compared in Essen untreated, TSHR, or β -galactosidase control immunised mice; although no significant difference was observed between immunisation groups in alpha diversity, non-metric dimensional scaling of the weighted UniFrac distance matrix showed good separation of the three groups, which was confirmed by the permutation test and revealed that the β -galactosidase group more closely resembled the untreated group. OTUs of *Bacteroidetes* and *Firmicutes* phyla were again the most abundant, but varied by immunisation with TSHR-immunised mouse bacterial communities, demonstrating a reduction in *Bacteroidetes* (mostly gram-negative) and increase in *Firmicutes* (mainly gram-positive) phyla. Furthermore, significant differences in microbiota composition were also seen at the genus level. Longitudinal analyses of stool samples collected at various time points revealed a significant increase in the richness and diversity of the gut microbiota over the 18 weeks of the experiment, which was less apparent in the TSHR-immunised mice. We observed a significant difference in the *Firmicutes*:*Bacteroidetes* ratio (used as an indicator of bacterial shifts) following TSHR immunisation. We also

observed cage effects, which were not apparent in analyses of the gut samples collected at the end of the experiment. We then investigated disease-associated taxonomies in the gut to seek explanations for disease heterogeneity such as hyperthyroidism in mice housed in London but not Essen. In both centres, OTUs for *Firmicutes* and *Bacteroidetes* were negatively correlated with each other. In the genera with differential abundance between the two centres we found a strong negative correlation of the *Firmicutes* genus *Intestinimonas* and the levels of blocking TSHR autoantibodies (TRAb) in London but not in Essen, where this butyrate-producing genus was more abundant; no correlation was found between *Intestinimonas* and TSAb or thyroxine levels in either centre. In Essen TSHR-immunised mice, orbital adipogenesis correlated positively with *Firmicutes* OTUs and negatively with *Bacteroidetes* phyla, and this was recapitulated at the genus level. The traditional microbial culture data revealed further correlations in the TSHR-immunised mice as well as a strong positive correlation between orbital muscular atrophy and *Lactobacilli*, *Enterococci*, *Bifidobacteria*, and *Coliforms*, whilst free thyroxine positively correlated with *Lactobacilli* and *Staphylococci* spp. [30].

In the second study, even though C57BL/6 mice had TRAb, none of them had increased thyroxine levels or any orbital pathology, in contrast to the BALB/c animals. Furthermore, splenic T cells from C57BL/6 mice proliferated poorly in response to TSHR and secreted mainly anti-inflammatory cytokines such as IL-10. Significant differences in beta diversity were noted between the two strains along with differential abundance of six genera, although no significant differences were reported in the viable bacterial counts obtained through standard microbiology. A different pattern of correlations between microbiota genera and disease (both endocrinological and immunological) features was reported between the two mouse strains, e.g., enriched *Limibacter* spp. correlated negatively with TSAb in C57BL/6J mice. Interestingly, species from only one genus (*Erysipelotrichaceae*) correlated positively with the same cytokines in both strains, despite a different genetic background [31].

The results of these two studies suggested a role for the gut microbiota in shaping the immune response and thus the phenotype of TSHR-induced thyroid and eye diseases. In our most recent study we modified the gut microbiota composition prior to TSHR immunisation, using antibiotic, Lab4 probiotic (supplied by Sue Plummer at Cultech Ltd., UK), and transfer of human faecal material from patients with severe GO. Our findings strongly sup-

port our hypothesis (manuscript undergoing modification following peer review).

To date, relatively little has been reported on the gut microbiota composition of patients with autoimmune thyroid disease (AITD), despite the bowel discomfort that is often reported in these conditions, ranging from constipation in Hashimoto's thyroiditis (i.e., hypothyroidism) to diarrhoea in hyperthyroid GD [reviewed in 32]. A recent study reported altered composition of the gut microbiota in Hashimoto's thyroiditis patients compared to matched healthy controls: seven genera including *Blautia*, *Roseburia*, *Dorea*, and *Fusicatenibacter* were increased in Hashimoto's thyroiditis, while *Faecalibacterium*, *Bacteroides*, *Prevotella*, and *Lachnoclostridium* spp. were decreased and correlated with disease features [33].

At present, few studies have examined the human gut microbiome in GD and its progression to GO. One study conducted quantitative PCR analysis of selected genera and species and also 16S rRNA sequencing in 27 GD patients in whom disease was of >18 months' duration but who had not received antithyroid drug (ATD) therapy for at least 6 months and 11 healthy controls. The quantitative PCR revealed significantly reduced *Bifidobacteria* and *Lactobacilli* in patients. High-throughput sequencing identified reduced diversity in the patients, accompanied by a reduced *Bacteroidetes* and increased *Firmicutes* phyla, although not reaching significance. Significant differences were observed between patients and controls at the family, genus, and species levels, but whether these correlated with disease phenotype or severity was not investigated [34]. A similar cross-sectional study compared 33 patients with severe active GO, who were mainly hyperthyroid and receiving ATDs, with 32 healthy controls. Their finding of significantly reduced diversity in patients agreed with that of Ishaq et al. [34], but Shi et al. [35] observed significantly increased *Bacteroidetes* and decreased *Firmicutes* in their GO patients' gut microbiota. Whether the relative abundance of *Bacteroidetes* and *Firmicutes* phyla reported reflects real differences between the gut microbiota composition of GD and GO patients or the impact of ATD therapy remains to be elucidated. Subsequently, Shi et al. [36] applied network-driven analysis to investigate links between the gut microbiota and GO-related traits in 31 hyperthyroid patients with severe active GO. They found that OTUs from the *Prevotellaceae* family associated with TRAB; OTUs from the *Bacteroides* genus associated with clinical activity score, whilst OTUs from the *Bacteroides stercoris* species associated with thyroglobulin autoanti-

bodies. The same authors expanded their studies to compare their original GO cohort with 30 GD patients free of eye disease and receiving ATD if hyperthyroid. Random forest, an algorithm to predict whether an individual belongs to a particular group based on their gut microbiome, was able to identify controls, GD patients, and GO patients with 70–80% accuracy and identified bacterial phyla whose abundance was able to distinguish GD and GO patients, e.g., *Cyanobacteria* and *Actinobacteria*. The authors also reported differences at the genus level, e.g., *Blautia* and *Dorea*, which were more abundant in GD. The effect of thyroid hormone levels were not investigated since they were similar in the GD and GO patient groups, who were also matched for age and sex, but smoking status was not mentioned [37].

The INDIGO study has conducted a large-scale analysis of the gut microbiome in GD and GO patients compared with healthy controls in four European countries. In total, 211 patients and 46 controls were initially enrolled, and of those 171 and 42 provided at least one faecal sample. After removal of non-eligible patients, 121 patients and 42 controls were included. Faecal samples were obtained from untreated patients or within 6 weeks of commencing treatment at recruitment; GD with no or minimal eye signs; GO, mild or moderate-severe (as defined by EUGOGO); and healthy controls. Total DNA was extracted for microbiota analysis, using V1–V2 region primers of the 16S rRNA gene, to generate 10,000 paired-ends reads per sample (Miseq Illumina). Data were processed using the QIIME bioinformatics pipeline for analysing microbial communities. A subset was also evaluated using traditional microbiology methodology. Quality control applied rarefaction curves to confirm adequacy of sequencing depth and that sufficient numbers of samples had been included; in both cases the curves plateaued as anticipated.

A cross-sectional preliminary evaluation at baseline of the microbiota composition in GD patients ($n = 65$), GO patients ($n = 56$) and healthy controls ($n = 42$) revealed no significant differences in alpha and beta diversity indices. At the phylum level, *Bacteroidetes* were significantly more abundant in controls (38.5%) than in GD (24.2%) and GO (27.3%) patients, *Firmicutes* were more abundant in GD (59%) and GO patients (60.5%) than controls (53.2%), and the *Firmicutes*:*Bacteroidetes* ratio was significantly higher in GD patients than controls, but similar in GD and GO patients. In 2 GD patients who developed GO during the trial period there was a further decrease in the genus *Bacteroides*, confirmed using traditional microbiology. *Enterococcus gallinarum* counts, a

pathobiont reportedly involved in triggering autoimmunity, though low were significantly higher in GD and GO patients than controls. We also found that disease-associated taxonomies correlated with both endocrine (thyroxine level) and immunological (TSAb/TRAb titre) features. We adjusted for sex and smoking status in patients and controls – with interesting results. We were able to undertake a longitudinal study on 50 of the patients who were enrolled (1) at baseline, when 100% were TRAb-positive, (2) at the point where euthyroidism was achieved, when 86% were TRAb-positive, and (3) at the end of treatment, when 50% were TRAb-positive. Further analyses are ongoing.

Our results agree with some, but not all of the findings relating to GD/GO and the gut microbiome reported by others, predominantly in Chinese populations. There is emerging evidence that microbiota disease associations may vary with ethnicity [38] – just as genetic predisposition to developing AITD is driven by different HLA haplotypes [39] in patient cohorts collected around the globe.

In another aspect of the INDIGO study, Salvi et al. (manuscript in preparation) conducted a trial of the Lab4 probiotic. Probiotics are defined by the World Health Organization as “live microorganisms which when administered in adequate amounts confer a health benefit on the host” [40]. In common with many probiotics, Lab4 comprises species of *Lactobacillus* and *Bifidobacteria* and has been shown to have benefit in several conditions [41–44]. Probiotics are distinct from prebiotics, which are described as “a substrate that is selectively utilised by host microorganisms conferring a health benefit”; dietary fibre is a good example.

In the trial, patients with GD ($n = 31$) were treated with ATDs and titration thyroxine replacement and were randomised to receive Lab4 (two capsules b.i.d., 25 billion colony-forming units/capsule) or placebo for 6 months. Patients on probiotics were significantly less likely to have hyperthyroid relapse and had lower levels of circulating IgG and IgA, hinting at a systemic effect. Microbiota composition was more stable in patients receiving probiotics, who also displayed a significant reduction in counts of the *Firmicutes* phylum compared to patients receiving placebo (unpublished data).

Studies on AITD are further complicated by the possible contribution of thyroid hormones to alterations in the gut microbiota; these could mask or be mistaken for changes resulting in or the consequence of perturbation of immune balance. Interestingly, a recent meta-analysis of 2,700 individuals from the TwinsUK cohort suggested

a correlation between the gut microbiota of hyper- and hypothyroid patients (not necessarily with the autoimmune form) [45]. In addition, iodination, sulphation, and glucuronidation of thyroid hormones, which are necessary for releasing the more active triiodothyronine hormone, may be performed in certain conditions by intestinal bacteria [46, 47], and SCFAs may promote such enzymatic reactions [as reviewed in 48].

Conclusion

Small steps have been taken in trying to understand how the gut microbiota – or indeed microorganisms in other niches such as the nasal sinus – may contribute to shaping an immune system that, in genetically predisposed individuals, leads to AITD. Considerable work remains to be done, not least in identifying environmental factors that could be modified to maintain a more favourable gut microbiome. Smoking cessation is already known to decrease GO severity; it will be interesting to see whether nicotine from e-cigarettes is as harmful as traditional tobacco products and to observe the impact of both on the microbiome. Diet is an obvious candidate, and Danila Covelli investigated food sensitivity, which was found to be more prevalent in GD patients and accompanied by food antigen-specific genera enrichment (unpublished data). We are stuck with the genes we were born with – but maybe we can optimise the much bigger gene pool lurking in our gut.

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Conflict of Interest Statement

G. Masetti is involved in collaborative projects with Cultech Ltd. M. Ludgate has no conflicts of interest to declare.

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Author Contributions

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