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1 “The thyroid, the eyes and the gut: a possible connection”

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1 ABSTRACT

2 **Introduction** Graves' disease (GD) is an autoimmune disorder responsible for 60-90% of thyrotoxicosis, with an
3 incidence of 1 to 2 cases per 1,000 population per year in England. Graves' orbitopathy (GO) is the most frequent
4 extrathyroidal manifestation, not provoked directly by abnormal thyroid hormone levels, but the consequence of the
5 underlying autoimmune process. The aetiology of autoimmune disorders is due to an interplay between susceptibility
6 genes and environmental factors, such as infections, stress etc. What triggers the autoimmune reaction to a specific site
7 of the body is not yet clearly understood. The lack of knowledge in GD and GO pathogenesis implicates therapies that
8 only limit damage but do not prevent disease onset. **Material and methods** We performed on PubMed and the Cochrane
9 Library a literature search for the articles published until July 2016 by using the search terms 'graves disease' and
10 'microbiome', 'orbitopathy', 'autoimmune pathogenesis'. Reference lists of relevant studies were hand-searched for
11 additional studies **Conclusion** In this scenario, a Marie Sklodowska-Curie funded project INDIGO ([http://www.indigo-](http://www.indigo-iapp.eu/)
12 [iapp.eu/](http://www.indigo-iapp.eu/)) is investigating the role of the gut bacteria in GD and GO pathogenesis. The gut is the first and the widest area
13 of bacteria access, with the highest concentration of T cells in the human body and trained to react to microorganisms.
14 Interestingly, all the environmental factors involved in GD and GO pathogenesis can alter the balance within the
15 microorganisms located in the gut, and influence the immune system, in particular the proportions of regulatory Treg and
16 inflammatory TH17 cells. It is hoped that investigating GD and GO pathogenesis from this novel aspect will identify new
17 targets for prevention and treatment.

❖ INTRODUCTION

Graves's disease (GD) is one of the most common organ specific autoimmune disorders. Characterized by thyrotoxicosis, diffuse goitre and the presence of thyroid stimulating antibodies (TSAB), GD represents 60-90% of all causes of thyrotoxicosis, in areas where populations are exposed to adequate iodine intake. It typically affects people between 30 and 60 years old, with an incidence of 1 to 2 cases per 1,000 population per year in England and roughly eight times greater in women than in men [1].

Graves' orbitopathy (GO) is the most frequent extrathyroidal manifestation [2]. The inflammation of intraorbital tissues, increased adipogenesis and accumulation of glycosaminoglycans within the extra-ocular muscles induce expansion and remodeling of the orbital contents. A recent study shows that, the orbital fat volume is associated with the duration, while the eye muscle volume is related to the severity of the disease [3]. As reported in some recent epidemiological studies, 20.1% of GD patients present at least one of the typical manifestation of GO (e.g. periorbital oedema, eyelid retraction, proptosis, conjunctival redness, and strabismus) [4]. Fortunately, the incidence of moderate-to-severe form is approximately 5% and only 2% of GO patients develop the sight threatening ocular disease due to dysthyroid optic neuropathy (DON) [5]. The age-adjusted annual incidence of clinically relevant GO is 16 per 100,000 population in women and 2.9 in men [6]. Even though spontaneous improvement or stabilization may occur in mild GO, many patients need treatment that impact on quality of life.

As in all autoimmune diseases, when self-tolerance is broken, T cells recognize self-antigens and B cells produce antibodies targeting host cells; this amplifies when they recruit and activate other immune cells. Concerning GO, it is widely accepted that the underlying autoimmune process and not abnormal thyroid hormone levels, is responsible. [7].

The aetiology of autoimmune disorders is due to an interplay between susceptibility genes and environmental factors, such as infections, stress, drugs, radiation and others [8]. What triggers the autoimmune reaction against the thyroid instead of other sites of the body is not yet clearly understood but single-nucleotide polymorphisms in the TSH-receptor (TSHR) (please see below) might be implicated. The lack of knowledge in GD and GO pathogenesis means that available therapies treat signs and symptoms, without changing the natural course of the disease or preventing disease onset.

We will review what is known about GD and GO pathogenesis and then focus on a possible link between thyroid-eyes and gut.

❖ WHAT IS THE TARGET OF THE AUTOIMMUNITY?

Patients with GD have cell-mediated immune reactivity and antibodies against the TSHR (TRAb) [9] whose central importance is supported by the development of animal models [10,11]. Most GD patients also have thyroid peroxidase (TPO) and less frequently thyroglobulin antibodies, whilst up to 25% of active GD show low-level titers of antibodies to DNA and to liver mitochondria.

1 Moreover, thyreopathies are not uncommon in subjects affected by other “organ specific” autoimmune diseases including
2 chronic gastritis, ACTH deficiency, Addison’s disease, chronic hepatitis, celiac disease, diabetes mellitus type 1,
3 myasthenia gravis, premature ovarian failure, primary biliary cirrhosis, vitiligo or “systemic autoimmune diseases” as
4 rheumatoid arthritis, multiple sclerosis, systemic lupus erythematosus, systemic sclerosis, urticaria, and angioedema [12].
5 Thus GD can be part of a complex autoimmune reaction against thyroid tissue, due to a generalized dysregulation of the
6 immune system. Finally, several autoimmune endocrinopathies can cluster in Polyglandular Autoimmune Syndromes
7 (PAS). The first classification of polyglandular failure divided two broad categories: PAS type I and PAS type II. PAS I
8 also known as APECED is characterized by mucocutaneous candidiasis, autoimmune hypoparathyroidism and adrenal
9 insufficiency; the more common type II, also known as Schmidt syndrome, comprises the obligatory occurrence of
10 autoimmune Addison disease in combination with thyroid autoimmune diseases and/or type 1 diabetes mellitus. An
11 additional group, PAS type III (PAS III), was subsequently described and is the co-occurrence of autoimmune thyroid
12 disease with two other autoimmune disorders, including diabetes mellitus type 1, pernicious anemia, or a nonendocrine,
13 organ-specific autoimmune disorder in the absence of Addison disease.

14 Autoimmune thyroid disorders (AITD) are the main manifestation of PGA II and III. In particular, the most frequent
15 disease combinations in PGA II are type 1 diabetes and AITD (41%) followed by AITD and Addison’s disease.

16 The close temporal relationship between the onset of GD and GO suggests that these two conditions might share the same
17 etiology. Autoreactive T lymphocytes directed against one or more antigens shared by the thyroid and orbit are assumed
18 to infiltrate the orbital tissue and the perimysium of extraocular muscles; however, the putative autoantigens and the exact
19 link between thyroid and orbit are still unclear.

20 The TSHR was the first structure considered as a candidate autoantigen.

21 TSHR transcripts have been reported in adipose retro-orbital tissues of healthy people and GD patients using polymerase
22 chain reaction, northern blot and liquid hybridization [13]. It is generally accepted that TSHR expression increases during
23 adipogenesis in any fat depot (ref). Zhang L. et al [14] investigated the biological effects of TSH-R activation in cultures
24 of orbital adipose tissues and showed that it stimulates early preadipocyte differentiation and favours formation of brown
25 adipose tissue; in contrast, it seems to render preadipocytes refractory to PPAR γ induced adipogenesis. Kumar S and
26 coauthors [15] demonstrated that a human monoclonal TSAB enhances adipogenesis by signaling via PI3K. The immune
27 response to TSH-R is also involved in the production of cytokines in orbital tissue. Both Th1 (e.g. IFN γ , TNF α , IL-1 β ,
28 and IL-6) and Th2 cytokines (e.g. IL-4 and IL-10) may play a role, although the former are found primarily in eye muscles
29 and the latter in orbital fat. Even serum cytokine levels are elevated in GO patients compared to controls [16].

30 The role of TSH-R is also supported by the correlation between GO activity and TRAb levels and the fact that GD patients
31 who relapse retain high TRAb levels and are the most likely to develop GO [17]. However, the TSH-R is expressed in

1 several other tissues not involved in GD and GO (such as ovary, testis, kidney, skin, bone marrow, white and brown
2 adipose tissue, bone), and, at lower levels, in normal orbital fibro-adipose tissue samples and cultures. Finally, Banga's
3 group have developed a murine GO model, using immunization with human TSH A-subunit by in vivo electroporation
4 and this has been reproduced in another laboratory [18, 19].

5 The presence of increased insulin-like growth factor 1 receptor (IGF-1R) levels in orbital fibroblast, B and T lymphocytes
6 from GO patients prompts a possible role of IGF-1R in the pathogenesis. Moreover, the colocalization of TSH-R and
7 IGF-1R on fibroblasts and thyrocytes shown by some researchers, suggests a possible functional link between them [20].
8 Interestingly, only a minority of GO patients have circulating antibodies to the IGF-1R. This could be related to the low
9 sensitivity and specificity of detection tests used or it may suggest a relevant role only for antibodies locally produced in
10 the orbit [21] or, again, the cross-talk between the TSH-R and IGF-1R may be important, rather than direct activation of
11 the surface IGF-1R [22]. Other autoantigens, including several eye muscle antigens, acetylcholinesterase,
12 thyroperoxidase, thyroglobulin, alpha-fodrin, have been proposed, but their true role is uncertain.

13 ❖ **GENETIC PREDISPOSITION**

14 The relatively high incidence of GD and GO in families and among siblings (sibling risk ratio 11.6) [23] indicates a strong
15 genetic influence. Studies on twins suggest that genetics accounts for 79% of the liability to developing the disease, and
16 environmental factors for the remaining 21% [24].

17 Predisposition to autoimmunity in general results from variation (polymorphisms) in genes implicated in the acquisition
18 of central and peripheral tolerance by regulatory T cells (Treg) and also the co-stimulation of T cells and APCs in the
19 immunological synapse [25].

20 A prime example is mutation in the autoimmune regulator (*AIRE*) gene, expressed in thymic medullary epithelial cells,
21 which induce loss of self-tolerance and lead to autoimmune polyglandular syndrome type 1. However, *AIRE* mutations
22 are rarely present in AITD patients (in about 0.3-0.6% of autoimmune hyperthyroidism) and are not implicated in the
23 more common autoimmune endocrinopathies [26].

24 There are several studies characterizing Tregs in autoimmune thyroid disease (AITD). Nakano et al [27] showed decreased
25 proportion and apoptotic Tregs in thyroid tissue of GD patients, Marazuela [28] and Glick [29], on the other hand, found
26 increased T infiltration in thyroid tissue, but impaired T cell function. Thus, whether the number or the function of Tregs
27 is altered in GD remains uncertain.

28 T cells react against epitopes complexed in human leukocyte antigen (HLA) proteins on antigen presenting cells (APC).
29 HLA-antigen D related (HLA-DR) molecules are the most important as their amino acids sequences determine the shape
30 of the antigen presenting cleft. However, the relationship between DR gene inheritance and GD accounts for about a 2-
31 5-fold increment in risk, which is certainly not enough to explain the marked increase in risk seen in many families.

1 Moreover, the genetic effects of DR genes interact with that of cytotoxic T lymphocyte antigen (CTLA-4) which is
2 expressed by T cells and is the receptor for an adhesion molecule expressed on APC. Specifically, the positive effect of
3 CTLA-4 mitigates in part the negative effect of DRB1*0701, but does not interact with the positive influence of
4 DRB1*0301 [30].

5 Some studies investigated the role of regulators of the immune response. Ban et al. [31] focused their attention on protein
6 tyrosine phosphatase non-receptor type 22 (PTPN22) gene that encodes for a powerful inhibitor of T cell activation. A
7 single-nucleotide polymorphism was shown to inhibit function of the gene, and to be associated with GD and other
8 autoimmune diseases. Since knockout mice deficient in this gene do not develop autoimmunity, the role of PTPN22 gene
9 in the etiology of AITD is unclear.

10 Vitamin D and its receptor are involved in control of immunity and CYP27B1 catalyzes the conversion of 25-OH-D to
11 the active form 1.25-OH-D. An association between polymorphisms in these genes and GD is not surprising even if the
12 mechanism is not clear [32].

13 Results relating to a specific polymorphism of the TSH receptor (PRO52THR) are contradictory [33, 34] but alleles in
14 intron 7 of this gene were found to be associated with GD not only in the Japanese population but also in Caucasian [35,
15 36]. Moreover, two single nucleotide polymorphisms (rs179247 and rs12101255) within TSHR intron 1 exhibited strong
16 association with GD in three independent European cohorts [37].

17 Attempts have been made to distinguish from the pool of all patients with GD those who are most likely to develop GO.
18 Studies have focused on immunomodulatory genes including HLA-DR3, CTLA-4, IL-1, IL-23 receptor (IL-23R), CD40,
19 PTPN22, T-cell receptor b-chain (TCR-b), tumor necrosis factor-b (TNF-b) and various immunoglobulin heavy chain-
20 associated genes. Since both TSH-R expression and adipogenesis are enhanced in the orbit of GO patients, the
21 adipogenesis related gene peroxisome proliferator-associated receptor- γ (PPAR- γ), and the TSH-R gene have been
22 investigated, as have genes encoding thyroglobulin and the glucocorticoid receptor.

23 The only polymorphisms associated with GO, rather than GD are in IL-23R (rs10889677 and rs2201841; OR 1.8), IL-1
24 alpha 889C/T polymorphism (OR of 5.7 for TT genotype) and IL-1 receptor antagonist (IL-1RA) with Mspa-1 11100C/T
25 polymorphism (OR of 6.7 for CC genotype) [38, 39].

26 Genetic factors have been identified in differing GO disease states; e.g. CTLA-4A/G polymorphism at codon 17 and G
27 allele in polymorphic sites Jo31 (rs11571302) and CT60 of CTLA-4 gene were more common in severe GO but
28 Pro(12)Ala PPAR- γ polymorphism in milder and less active ocular disease [40, 41].

29 Finally, no significant association of glucocorticoid receptor polymorphisms ER22/23EK, N363S and Bcl1 with either
30 the therapeutic response to glucocorticoids treatment or the occurrence of side effects was observed [42].

31 ❖ **HUMORAL FACTORS**

1 Further evidence of ongoing autoimmunity in hyperthyroid patients is the increase of adhesion molecules as ICAM-1,
2 that could facilitate the recruitment of T cells to the orbit [43]; and of pro-inflammatory cytokines (interleukin (IL)-17,
3 IL-22, IL-6 and IL-8), chemokines (IFN- γ , TNF- α , CXCL10, IL23, BAFF, etc.) and their receptors suggesting a possible
4 target for new therapies [44, 45].

5 Analysis of T-cell clones from GO orbital tissues has shown both Th1 cytokine (interleukin-2, interferon-gamma, tumor
6 necrosis factor-alpha) and Th2 cytokine (interleukin-4, interleukin-5, interleukin-10) secretory profiles, possibly related
7 to different stages of the disease, with Th1 cytokines predominating early and Th2 cytokines late in the course of GO.
8 Cytokines produced by T cells, macrophages and fibroblasts perpetuate the ongoing inflammatory process through
9 induction of expression of HLA class II antigens, heat-shock proteins, CD40, prostaglandins, adhesion molecules,
10 proliferation of fibroblasts, differentiation of preadipocyte fibroblasts into adipocytes, and stimulation of fibroblasts to
11 synthesize and secrete glycosaminoglycans [46, 47].

12 Understanding that each stage of the natural course of GO has a different inflammatory pattern is essential to select the
13 ideal treatment for each patient. Thus, e.g. corticosteroids are effective in the initial active phase but not in burnout GO
14 when they may elicit harm. Also more innovative drugs, whose use in GO is still matter of research, could have an efficacy
15 influenced by time of administration. For example contradictory results were obtained in two randomized clinical trials
16 employing rituximab, a chimeric mouse-human monoclonal antibody that targets CD20. The rationale for using RTX in
17 GO is the potential blockade of pathogenic autoantibody generation and production of inflammatory cytokines or the
18 depletion of B cells as antigen-presenting cells. Data recently published showed a significantly different efficacy maybe
19 due to a different disease duration and severity of patients recruited, that means a different inflammatory pattern [48].

20 ❖ **ENVIRONMENTAL FACTORS**

21 As mentioned above, not everyone who inherits a sufficient load of genes positively related to the disease will develop
22 an AITD. An environmental factor is necessary to induce the disease and several factors have been.

23 **Treatments and drugs** Firstly, any thyroid injury that leads to exposure of thyroidal antigens can induce an autoimmune
24 reaction. Both irradiation to the neck for Hodgkin's disease and irradiation caused by nuclear accident have a detrimental
25 effect on the thyroid.; even radioactive treatment (RAI) and ethanol injection for toxic multinodular goiter can induce
26 GD. The risk is higher if anti-TPO antibodies are present before the treatment, supporting the importance of a genetic
27 predisposition [49]. Moreover, an increase of TRAb levels is common immediately after RAI treatment, with a high risk
28 of GO worsening, mainly in smokers [50, 51].

29 Administration of immunosuppressant drugs can induce the deterioration or the onset of an AITD, mainly in the period
30 of immune reconstitution. Some of these factors also induce expression of major histocompatibility component (MHC)
31 on thyroid tissue; thyrocytes can then act as APC perpetuating the autoimmune process [52].

1 **Sex** The high rate of autoimmune diseases in women suggests a possible causative role of estrogen receptors. A Polish
2 study showed, in both sexes, an association between ESR2-A allele and GD with a strength comparable to polymorphisms
3 of PTPN22 and CTLA4 CT60 loci [53].

4 Intrathyroidal fetal cell microchimerism is another possible etiologic agent in autoimmunity. Male fetal origin cells were
5 detected in thyroid tissue specimens from patients with GD but their role is still unclear since recent studies suggested a
6 possible protective influence [54].

7 A potential explanation of the higher incidence of GD and GO in women could derive from an epigenetic determinant
8 such as X chromosome inactivation. Yin et al. [55] found more skewed X chromosome inactivation ($\geq 80\%$ inactivation
9 of one X chromosome in the same tissue) in GD when compared to healthy individuals, but the mechanisms through
10 which this inactivation leads to higher risk are not yet known.

11 **Environmental factors** The increased rates of autoimmune disorders reported in urban residential areas in Africa, Asia,
12 Southern and Eastern Europe and Latin America suggests the relevant role of environmental factors more common in
13 Western countries, such as smoking, specific infections, Western nutritional habits, xenobiotics, as well as physical and
14 psychological stress [56].

15 **Psychological stress** Psychological stress has been considered as a risk factor for many years, since GD onset is common
16 after a stressful life event and the GD incidence increased during World War II. Theoretically, stress might cause
17 activation of the adrenal cortex or the sympathetic nervous system and hypercortisolism would tend to suppress
18 autoimmunity. However, studies on this topic showed contradictory results [57, 58].

19 **Smoking** Among proinflammatory factors, smoking is one of the most common. The consistent connection between
20 cigarette smoking and development or worsening of GD and GO firstly described in the end of 80's was then confirmed
21 by further studies. In particular, Brix and co-authors found that the discordant monozygotic twin with GD was more likely
22 to have smoked when compared to the healthy sibling [59].

23 A meta-analysis of studies investigating the association between smoking and thyroid diseases confirmed the increased
24 risk for developing or worsening of GO beyond that associated with GD [60]. Interestingly, the risk for developing GO
25 relates more to the number of cigarettes smoked following development of GD than the life-cumulative smoking burden.
26 In addition, its cessation appears to improve treatment response and to lower risk. The exact mechanism underlying the
27 deleterious effects of smoking remains uncertain. Besides an obvious direct irritative effect on the ocular surface, smoking
28 modulates immune reactions in the orbit, is associated with an increase in the orbital connective tissue volume as assessed
29 by MRI and with an increased adipogenesis and hyaluronic acid production in in vitro cultured orbital fibroblasts [61].

1 **Viral and bacterial factors** Infections could play a role in the development of autoimmune disorders through different
2 mechanisms: molecular mimicry of microorganisms, direct damage to the organ, induction of adhesion molecules and
3 stimulation of immune cells response.

4 For years, viral infections have been thought to have an etiological role in autoimmune diseases. Some studies detected
5 DNA from human foamy viruses in peripheral DNA of GD patients and proteins of human foamy virus in diseased thyroid
6 tissues. Unfortunately, others failed to confirm these findings, thus it remains unclear whether human foamy viruses
7 infection might be associated with GD [62]. In addition, enterovirus capsid protein and RNA have been identified in
8 thyroid tissue from GD patients more often than in people without autoimmune disease; thus, a low-grade chronic
9 enteroviral infection might be involved GD pathogenesis [63].

10 A transient increase of thyroid autoantibodies is possible after subacute thyroiditis, a virus-associated syndrome, maybe
11 due to exposure of thyroid antigens after viral damage. Virus infection might also augment autoimmunity by causing non-
12 specific secretion of IL-2, or by inducing MHC class II expression on thyroid cells. That could be the mechanism used
13 by Human T lymphotropic virus-1, repeatedly associated to AITD [64]. Moreover, a recent study suggested a possible
14 contribution of Epstein - Barr virus in TRAb production following reactivation of the disease after viral infection [65].

15 As concerns bacteria, several possible links between thyroid tissue and *Yersinia enterocolitica* (*YE*) have been found.
16 The presence of peptides having sequence similarity within and on the surface of this intestinal parasite and the TSH-R
17 was shown about 20 years ago. Moreover, the same authors recognized that immunoglobulins of patients recovering from
18 *YE* infections exhibit GD-like activity in human thyroid membranes and a higher proportion of GD patients have been
19 infected by *YE* compared to the general population. More recent data obtained by Hargreaves and coauthors, suggest that
20 *YE* porins could induce B cells somatic hypermutation to acquire a cross-reactive pathogenic response to TSHR [66].

21 Another bacteria that may have an effect on the development of autoimmune thyroid disease is *Helicobacter pylori* (*HP*)
22 infection of the gastric mucosa. An increased rate of prevalence of *HP* expressing the cytotoxin-associated gene A antigen
23 (Cag-A) was found not only in ongoing autoimmune hyperthyroidism, but also in all GD patients compared to healthy
24 controls [67]. A study on the Chinese population confirmed this and found that patients with CagA-positive *HP* and
25 negative HLA-DQA1 0201 or positive HLA-DQA1 0501 were more likely exposed to GD compared with those with
26 only one of these indices [68].

27 Finally, thyroid antigens can also interact by molecular mimicry with Clostridium botulinum neurotoxin which shares
28 amino acid homology with thyroid autoantigens with some of the homologous regions containing HLA-DR3 and/or HLA-
29 DR7 binding motifs [69].

30 **Dietary habits** The increased rate of autoimmune disorders in urban residential areas of some countries could also be
31 related to a change in dietary habits. People from urban areas could have a different food intake, in terms of calories and

1 diversity, and a higher exposure to xenobiotics, like food additives and preservatives, compared to rural dwellers.
2 Interestingly, a recent paper showed a low risk of hyperthyroidism in people following a vegan, lacto-ovo and pesco
3 vegetarian diets compared with omnivores [70].

4 Thus, if considered one by one all these environmental factors could be involved in triggering the disease in genetically
5 predisposed people, but the link between all of them is unclear.

6 **The gut microbiota** In this scenario, a group of researchers from different backgrounds started the INDIGO project, a
7 multicentre European funded project to investigate the role of the gut bacteria in GD and GO pathogenesis.

8 There are several reasons for focusing on the gut. It is the first and the widest area of bacteria access that is why the
9 highest concentration of T cells in the human body is located in the intestinal mucosa. It is even home to an enormous
10 and complex community of commensal bacteria, known as the gut microbiota.

11 The ‘normal’ adult human microbiota is extremely diverse and consists of hundreds of bacterial species reaching densities
12 of up to 10^{12} bacteria per gram content in the large intestine. Since the intrauterine environment is sterile, bacteria do not
13 colonize fetal body surfaces and intestine until the delivery. In the natural delivery, colonization occurs through contact
14 with the maternal fecal and vaginal microbiota. In contrast, babies born by caesarean section have the first microbial
15 contact from other sources such as mother’s skin.

16 Establishment of a stable microbiota takes several years and events occurring during early life are much more relevant in
17 defining the richness and the diversity of the gut microbiota than those of adult life.

18 Neonatal microbiota is very similar to the maternal and it is then shaped by physiological and pathological events such
19 as feeding practice, introduction of solid food, diet, hygienic living conditions and use of antibiotics. Thus, for instance
20 babies that are breast-fed harbour a different microbiota than babies that are formula-fed [71].

21 Gut microbiota provides benefits to its host in many ways, including digestion, production of nutrients, detoxification,
22 protection against pathogens and, especially, regulation of the immune system. In fact, in the gut mucosa, our lymphocytes
23 are trained to react to microorganisms and the gut microbiota can regulate not only the local intestinal immune system
24 but also systemic immune responses.

25 As described above, the microbiota and gut immune system co-evolve and creates an interaction useful for both. Thus, a
26 key feature of intestinal APCs is their ability to protect the body against infection while still maintaining immune tolerance
27 to the normal gut microbiota. For example, gut macrophages develop a unique phenotype, so called “inflammation
28 anergy,” referring to the non-inflammatory profile of intestinal macrophages when they encounter microbial stimuli in
29 homeostatic conditions [72].

30 Interestingly, all the environmental factors listed so far can induce dysbiosis, an altered balance within the gut microbiota.

31 Clearly, bowel infection, such as triggered by *Yersinia enterocolitica* or *Clostridium botulinum*, have a direct effect on

1 the gut microbiota. Even if infection does not involve the gastrointestinal tract, dysbiosis can arise after administration of
2 antibiotics or antivirals.

3 The discovery of the enteric nervous system in the nineteenth century confirmed and explained the existence of an intimate
4 connection between gut and nervous system. Stress may affect different physiologic functions of the gastrointestinal tract
5 including gastric secretion, gut motility, mucosal permeability and barrier function, visceral sensitivity and mucosal blood
6 flow [73]. Interestingly, stress induces changes in neurotransmitter and proinflammatory cytokine levels, which can alter
7 the growth, motility and virulence of pathogenic and commensal bacteria. For example, norepinephrine increases the
8 virulence of some bacteria such as *E. coli* or *C. jejuni* [74].

9 Cigarette smoking has a proinflammatory effect on several tissues and it is fascinating the direct role it can have on the
10 gut, even in healthy people. In fact, intestinal microbiota composition changes after smoking cessation as characterized
11 by an increase in key representatives from the phyla of Firmicutes and Actinobacteria as well as a decrease in
12 Bacteroidetes and Proteobacteria [75]. Moreover, in healthy controls recruited in a clinical trial on Crohn's disease,
13 smokers also had higher Bacteroides-Prevotella (34.8%) than non-smokers (24.1%) (P = 0.038) [76].

14 Finally, several recent studies have shown that dietary factors alter the microbial community beyond the postnatal period
15 both in animals and in humans. Trials on mice indicate that diet has a dominating role in shaping the gut microbiota and
16 changing key populations may transform healthy gut microbiota into a disease-inducing entity. For example, in mice fed
17 a low-fat, plant polysaccharide-rich diet and then switched to a "Western" diet, the microbiota composition shifted to an
18 overgrowth of Firmicutes including *Clostridium innocuum*, *Eubacterium dolichum*, *Catenibacterium mitsuokai* and
19 *Enterococcus spp.*, as well as a significant reduction in several Bacteroides spp [77].

20 Vegetarianism alters intestinal microbiota in humans because high amounts of fibre result in increased short chain fatty
21 acid production by microbes, which decrease the intestinal pH. This prevents the growth of potentially pathogenic bacteria
22 such as *E. coli* and other members of the Enterobacteriaceae [78]. A clinical trial comparing the gut microbiota of children
23 from different regions, in particular Europe and rural Africa, confirmed these findings. Interestingly, European children
24 have a microbiota depleted of Bacteroidetes and enriched in Enterobacteriaceae, which the authors attributed to low
25 dietary fibre intake [79].

26 The complex interplay between immune cells and microbiota explain the relevance of any disequilibrium in the gut.
27 The main actors in this process are the dendritic cells (DC), specialized antigen presenting cells located in intraepithelial
28 pockets, that play a "sentinel" role to protect our body from putative aggressors and to induce tolerogenic responses toward
29 harmless antigens. DCs quickly adapt to changes in microenvironment and their functions are dictated by several factors
30 including the encounter with external cues [80]. DCs are recruited to the lamina propria of the small intestine after
31 bacterial infection, and their number depends on the pathogenicity of the microorganisms encountered.

1 Germ-free animals are experimental animals reared in a sterile environment and never exposed to any microorganisms,
2 and they show a reduced number of intestinal but not systemic dendritic cells. Monocolonization of germ-free animals
3 with *E.coli* was sufficient to recruit DCs to their intestines [81]. While some microorganisms can directly bind to epithelial
4 cells, DCs can also monitor the contents of the intestinal lumen sending periscope-like dendrites outside the epithelium.
5 They then produce a variety of soluble factors, including chemokines and cytokines, which promote the recruitment and
6 activation of other DC. They even migrate into the mesenteric lymph nodes where they induce the differentiation of naïve
7 CD4+ T cells into four major subtypes: T helper 1 (Th1), Th2, Th17, or regulatory T cell. The proper regulation and
8 balance of T-cell subtypes is a crucial factor in determining health status. Treg are key mediators of immune tolerance
9 and its dysfunction can lead to autoimmune disorders with specific bacterial species even being associated with
10 development of particular T-cell subtypes. *Bacteroides fragilis* was shown to induce the development of a systemic Th1
11 response through its polysaccharide A molecules. In contrast, segmented filamentous bacteria (SFB) were found to be
12 potent inducers of Th17 cells in the lamina propria. [82]. Recently, Clostridia, particularly those of cluster IV and XIVa,
13 were shown to be capable of promoting the induction of colonic Tregs [83].

14 Although not required to shape the systemic CD8+ T cell repertoire, the gut microbiota plays an important role in
15 conditioning CD8+ T cells to modulate other peripheral immune cells, such as marginal zone B cells [84].

16 Gut-associated B cells are mostly immunoglobulin (Ig) A secreting plasma cells located in the Peyer's patches.
17 Germ free animals have a reduced number and cellularity of the Peyer's patches and, consequently lower IgA levels and
18 reduced plasma cells numbers in their intestine. Bacterial-specific IgA, produced following colonization, are both likely
19 to adapt to changes in the microbial composition and to shape it [85].

20 Thus, there's a constant collaboration and interplay between gut microbiota and immune system, not only in new-borns,
21 whose immune system is immature, but lifelong. It is clear that every condition that alters the gut microbiota's balance
22 can influence the systemic immune system. Several studies evidenced the link between bowel disease and autoimmune
23 disorders. One of the most complete is that of Shor DB's group who aimed to determine the prevalence of gastrointestinal
24 autoantibodies in patients with several autoimmune disorders, such as antiphospholipid syndrome (APS), rheumatoid
25 arthritis (RA), systemic lupus erythematosus (SLE), diabetes mellitus type 1 (DM), autoimmune thyroid disease,
26 pemphigus vulgaris, polyarteritis nodosa (PAN), Sjögren's syndrome, cryoglobulinemia, Wegener's granulomatosis,
27 Churg–Strauss syndrome, giant cell (temporal) arteritis, microscopic polyangiitis, Crohn's disease, ulcerative colitis (UC)
28 and systemic sclerosis [86].

29 Prevalence of IgA anti gliadin was significantly higher in APS (7.1 %, P=0.012) and in pemphigus vulgaris (25%, P
30 =0.008) patients, as compared with healthy controls. Presence of IgG anti gliadin was more common among Crohn's
31 disease (20.5%, P = 0.023) and RA (6.5%, P=0.027) patients and finally, IgG anti tissue transglutaminases were frequently

1 observed in APS (6.1%, P=0.012), in giant cell arteritis (11.5%, P=0.013) and in UC (11.1%, P=0.018) patients.
2 Interestingly, they also reported an association also with autoimmune thyroid disease; GD patients showed a higher
3 prevalence of IgG anti *Saccharomyces cerevisiae* (highly specific for Crohn's disease) compared to healthy controls
4 (5.7% in Graves' disease vs 0.5% in controls P = 0.018). Some preliminary results obtained in the INDIGO project confirm
5 this point. In fact, faecal samples of GD patients with severe ocular involvement showed the highest presence of yeast
6 (expressed as colony forming unit/gram). The association between both Hashimoto's thyroiditis and GD and
7 inflammatory bowel disease, mainly Crohn's disease and UC, has been long known whilst a very recent paper reviewed
8 the literature on cases of concomitant inflammatory bowel disease (IBD) and thyroid disorders. After the first case of
9 concomitant GD and UC in 1968, the authors identified a further 16 cases but there was no clear tendency in the order of
10 GD/UC diagnosis nor in the time interval between the two disorders or in the type/severity of colitis. As for Crohn's
11 disease, the author identified three case reports [87].

12 Except case reports, no prospective studies have been done to assess the association between inflammatory bowel disease
13 and autoimmune thyroid disorders. Moreover, there are even fewer data regarding GO patients.

14 Kahaly's group analysed about 1000 records of AITD patients in a retrospective cross-sectional study and found a positive
15 association between GD and celiac disease (10.8% of celiac disease in GD group, p value <0.001). Moreover, multivariate
16 analysis showed that celiac disease was associated positively with ocular involvement (13.3% of GO versus 4.3% without
17 ocular involvement, p value <0.001) [88].

18 The Indigo project (<http://www.indigo-iapp.eu/>) hypothesizes that in people with GD/GO, either microbial species
19 favouring development of inflammatory TH17 cells predominate or species leading to increased Treg cells are under-
20 represented. It will apply 16S rRNA sequencing to analyse the gut microbiomes of GD/GO patients and compare them
21 with healthy controls from the same geographic region. It also aims to seek prognostic biomarkers of GD and GO in order
22 to facilitate early preventative intervention and will assess how probiotics may help to avoid or reduce disease progression
23 The project will compare antibody responses in GD patients and controls to determine whether microbial or food derived
24 antigens are involved in triggering disease or associated with GO progression. In particular, it will determine in the GD
25 population not only the prevalence of IgA and IgG anti deamidated gliadin and tissue transglutaminases but also assess a
26 possible hypersensitivity against several food antigens, such as cow's milk, egg white and yolk, white fish and shellfish,
27 corn rice, oak and several others. Moreover, the same immune reaction will be tested in the cohort of patients who develop
28 ocular involvement in order to find a possible risk factor link to the gut.

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