Chronic Intestinal Pseudo-Obstruction (CIPO): interplay between Enteric Nervous System, Serotonin and Mucosa-Associated Microbiota

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INTRODUCTION

1. The Chronic Intestinal Pseudo Obstruction (CIPO)

Chronic intestinal pseudo-obstruction (CIPO) is a rare and severe gut motility disease characterized by recurrent symptoms of intestinal mechanic obstruction, in absence of any detectable anatomical causes (1). The symptoms outcome in a severe impairment of propulsive motility, leading to failure of digestive ability, with the requirement of patients' nutritional support. Although gut dismobility can affect the whole gastrointestinal (GI) tract, colon and small intestine are usually the most involved locations (2). CIPO is primarily a pediatric disease, as about 80% of patients show clinical manifestations in their first year of age (2). In the other 20% of patients, the disturb can manifest sporadically in the first 20 years of life (3). The disease can have primary (congenital), secondary (acquired) or idiopathic (of unknown cause) onset, and it can be pathologically categorized as neuropathy, myopathy, mesenchymopathy or a combination of such abnormalities (4). In pediatric CIPO, also known as PIPO (pediatric intestinal pseudo-obstruction), the disturb is principally idiopathic/primary (4, 5), whereas in almost 50% of affected adults, CIPO is secondary to systemic diseases (like infections, mitochondrial disorders, autoimmune disorders, degenerative neuropathies, or Duchenne muscular dystrophy) (6). To date, the diagnosis of CIPO results challenging for clinicians: there is no unique diagnostic tool available, and it could require different months or years before determining the presence of a pseudo-obstruction, undergoing patients to useless and dangerous procedures or surgeries. Physicians should also be aware of the existence of this rare syndrome to allow for an accurate diagnosis (7). Consequently, the incidence of this disturb is often underestimated. Moreover, the most advanced available treatments could only relieve patients' symptoms. This leads to a late diagnosis and increased morbidity and mortality rates for these patients, worsening their quality life (8).

1.1 Symptoms and main characteristics

CIPO patients are characterized by widely variable and non-specific clinical symptoms, coinciding with those of several GI diseases (9, 10). The severity of symptoms depends on the age at disease

onset and on the location and extent of the GI tract involved (10, 11). Clinical manifestations can be detected also in the prenatal status: the most often found is the non-obstructive megacystis, through ultrasound exam (12). After birth, symptoms start mainly within the first year of life (80% of the cases) or have a sporadic onset during the first twenty years (20%) (11–13). A general classification divides symptoms into “upper GI symptoms” and “lower GI symptoms”, depending on which intestinal tract is involved. The symptoms mirror a mechanical sub-occlusion, with periods between sub-occlusive episodes in which patients usually complain of severe digestive symptoms characteristic of delayed transit (1). Specifically, the symptomatology of both adult and pediatric patients includes abdominal pain and distension (80%), vomiting and bloating (75%), constipation (40%) and diarrhea (20%) (6, 9, 11, 12). Other typical symptoms are dysphagia, gastroesophageal reflux and nutrient malabsorption, leading to an involuntary weight loss (4). CIPO clinical course is characterized by exacerbations and remissions of symptoms. Exacerbations are usually triggered by different factors, as viral or bacterial infections, psychological stress, central line sepsis, general anesthesia and malnutrition (1). In patients with severe CIPO, the disease course may lead to intestinal failure (12). In these patients, the regular gut transit is altered by intestinal malabsorption and the associated dilated bowel loops. Additionally, intestinal dilation and slow food transit influence the small intestinal bacterial overgrowth (SIBO), and SIBO can influence malabsorption and diarrhea symptoms (9). Malnutrition is also a recurrent disturb in CIPO, as ingestion of food generally worsens the patients’ food intake and nutrient absorption (20). All these wide specters of symptoms lead to an association of CIPO with a poor quality of life and high morbidity and mortality (9, 14, 15).

1.2 Etiology

Etiologically, CIPO is generally classified in primary (familiar or sporadic), secondary or idiopathic, depending on the disease origin (16).

Primary CIPO is usually diagnosed in childhood, often of familiar onset and present at birth (17, 18), but most cases result to be sporadic. This condition is characterized by degeneration or inflammation of the enteric nervous system (ENS), resulting in defects or loss of function of GI smooth muscle. Some cases of hereditary CIPO have been identified, underlining the presence of

gene mutations, both at autosomal and sex chromosomes level (19). In adults, CIPO onset is generally secondary, or acquired.

Secondary CIPO is linked to an underlying organic, systemic, or metabolic pathology that results in secondary digestive impairment (20). The disease origins by a wide variety of conditions affecting the intestinal smooth muscle, enteric neurons and the interstitial cells of Cajal (ICCs) network (21). ICCs are dispersed throughout the GI tract, from the oesophagus to the internal anal sphincter. They act as electrical pacemakers initiating slow spontaneous electrical waves in the intestine, provoking intestinal contractions, and regulating peristalsis and segmentation. Their integrity is crucial for proper motility function. Several systemic diseases can impact the GI tract and may be implicated in the development of CIPO, such as rheumatological, endocrinological and metabolic diseases, as well as infections and autonomic nervous system abnormalities that, affecting the ENS, can also induce the onset of CIPO.

In the majority of paediatric patients, CIPO is defined as idiopathic, as neither a primary nor secondary aetiology is identified (18).

1.2.1 Genetics

The etiological classification of CIPO evolved with the discoveries of genetic implications, in particular through the study of familiar forms (20). These forms are included in the primary CIPO group. These cases can appear in adulthood, without previous digestive history, probably following an infection that leads to a dysimmune cascade. The familiar transmission is rare, and several researches tried to identify the genes involved, even if the effective role has not yet been explained (22). The genes identified imply autosomal recessive, autosomal dominant or sex chromosome associated inheritance. Mutations of the gene encoding the intestinal smooth muscle actin (*ACTG2*), a protein crucial for the enteric muscle contraction, were identified in CIPO. In these patients, dominant variants of *ACTG2* were found associated with congenital or late visceral myopathy, or intestinal megacystis-microcolon-hypoperistalsis syndrome (MMIS) (22). Other two specific mutations were identified in the *SOX10* gene, codifying for a transcriptional factor. These autosomal dominant variations were associated with the Waardenburg-Shah syndrome, that leads to pigmentation abnormalities and sensorineural deafness, contributing to widespread dysmotility gut

disorder (23). CIPO disease is often associated with mitochondrial encephalomyopathies, a heterogeneous group of genetic disorders, caused by abnormalities in the mitochondrial respiratory chain, usually affecting highly energy-dependent tissues, such as the brain and muscles. Among them, CIPO is most frequently associated with the mitochondrial neuro-gastrointestinal encephalomyopathy (MNGIE), an autosomal recessive syndrome due to mutations in the thymidine phosphorylase gene *TYMP*. MNGIE diagnosis is based on the presence of severe GI dysmotility and a family history with autosomal recessive inheritance (24). Regarding mutations on sex chromosomes, two variants were identified on the X chromosome; the two genes involved were filamin A (*FLNA*) and cell adhesion molecule L1 (*L1CAM*). The *FLNA* mutation was identified studying an Italian family composed of 10 males CIPO diagnosed in 4 generations, all related through healthy females. These males were also affected by several comorbidities as platelet thrombocytopenia. Of these patients, 9 of them died in the first month of life. So, the condition appeared clearly recessive X-linked, resulting in a chronic X-linked intestinal pseudo-obstruction (25). An overview of the mutations above described and other variants found in CIPO and associated with several pathologies is described in Table 1.

Genetic mode (Chromosome)	Mutant gene/disease
Autosomal dominant inheritance	SOX10/Waardenburg-Shah syndrome[60]
Autosomal recessive inheritance	*ACTG2*/Megacystis–microcolon–intestinal hypoperistalsis syndrome
	SGOL1/Chronic atrial and intestinal dysrhythmia syndrome
	POLG/Alpers' disease[116]
	TYMP, *POLG*/Mitochondrial neurogastrointestinal encephalomyopathy
	8q23-q24: A new chromosomal localization related to CIPO[117]
X-linked recessive	Xq28: *Filamin A* and *L1CAM* genes[118]

Table 1. Examples of CIPO forms with genetic etiology (19).

1.3 Histopathology

Based on abnormal histopathological findings along with the GI system, CIPO was classified into three different groups: neuropathic, myopathic and mesenchymopathic (2). Frequently, more than one histopathological alteration coexists in a single CIPO patient (condition defined as neuromesenchymopathy or neuromyopathy) (2).

The neuropathic form is characterized by malfunctioning of intrinsic intestinal innervation and/or extrinsic autonomic innervations (neuropathy). It is considered the most frequent cause of dysregulation that leads to CIPO disease. Usually, neuropathic CIPOs present two different forms: inflammatory and degenerative (26). Inflammatory neuropathy can be influenced by autoimmune diseases and paraneoplastic syndromes (27), neurological disease and infections, resulting in ganglionitis and/or leiomyositis disrupting signaling within the enteric nervous system (ENS) (8). Degenerative neuropathy can be triggered by endogenous or exogenous insults, leading to ENS damage mediated by a non-inflammatory response, whose causes include mitochondrial dysfunction (28), where free radicals formation leads to neuronal loss and often to diabetes mellitus (4).

The disruption of smooth muscle cells is implicated in the myopathic form, with an altered distribution of smooth muscle cell morphology. Differently from enteric neuropathies, enteric myopathies typically occur in infants and are congenital or genetic; they are mostly characterized by severe visceral dilatation, often involving other organs, and long-term outcomes and prognosis are usually unfavorable (16). Systemic disorders such as scleroderma, muscular dystrophies, and amyloidosis can result in visceral myopathy, and subsequently cause CIPO disease in affected patients (4). Specifically, scleroderma is related to the loss of smooth muscle and increased proliferation of fibroblast, caused by a chronic inflammatory process (27). Intestinal pseudo-obstruction can also result from amyloidosis plaque formation in the *muscolaris propria* of small bowel, due to light chain protein and beta 2-microglobulin amyloid proteins (29); in contrast, the amyloid A protein deposition in the GI myenteric plexus results in neuronal disruption, highlighting the pathological affection of ENS (8). Myopathic diseases include progressive hereditary skeletal muscle disorders, like muscular dystrophy, Duchenne or myotonic dystrophy, which are also associated to GI smooth muscle atrophy (8, 27). Previous researches identified the absence of alpha actin gene as a potential biomarker of myopathic CIPO, as 24% of patients analysed had alterations (deficiency of absence) of this gene (30). However, the possible use of alpha actin as a CIPO biomarker was abandoned when the gene was found to be involved also in other disorders, such as megacystitis microcolon and intestinal hyperperistalsis syndrome (31).

Mesenchimopatic CIPO involves anomalies of ICCs (19). Structural aberration, deficiency or damage of ICCs result in an impairment of the peristalsis regulation and consequently in an abnormal propulsion of GI content (32).

1.4 Diagnosis

The diagnosis of CIPO is currently a challenge for clinicians, as the disturb has non-specific and heterogeneous signs and symptoms, and no easy detectable cause for a significant percentage of patients (19). To date, there isn't a single diagnostic test for the assessment of CIPO. The first step is to exclude the presence of a mechanical obstruction in patients with symptoms mimicking an intestinal block, like abdominal distension, vomiting, constipation and malnutrition, trying to avoid useless surgery for these patients. The first diagnostic tools used are: plain abdominal X-ray, imaging techniques and contrast medium exams.

Plain abdominal X-ray is a simple and inexpensive screening test used to identify dilated loops of small bowel and air fluid levels which may suggest an obstruction (2, 11). For the imaging tools, computed tomography (CT) or magnetic resonance imaging (MRI) can also identify intra and extraluminal causes of intestinal obstruction (6). MRI is most frequently used as is a noninvasive, radiation-free technique able to assess small intestinal motility disfunction in CIPO patients, showing non-specific obstructions of the digestive tract. MRI technology offers also the possibility of acquiring cine-MRI sequences, that can directly assess GI motility (19). Contrast studies are important to rule out organic cause of obstruction (e.g., gut malrotation or a lumen-occluding lesions) (3). This diagnostic test is not expensive and widely available, but its application is limited by the possible inability of patients to ingest large amounts of water-soluble contrast.

An additional tool for the diagnosis of CIPO is manometry. The small bowel manometry (SBM) provides information regarding the ENS and muscular system activity of the small bowel and the stomach. This technique can differentiate mechanical from functional forms of sub-occlusion, even when the organic cause is at an early stage (2). Manometry also allows to highlight the underlying histopathologic mechanism causing dysmotility. In the enteric neuropathy, contractions are uncoordinated, but of normal amplitude, whereas in the enteric myopathy the contractions are well organized but their amplitude in fasting and fed period is low. A careful interpretation of manometry findings is required to differentiate CIPO into neuropathic and myopathic etiologies, since manometric catheter could not be able to detect a non-occlusive contraction when bowel loops are dilatated (2). Additional studies, including esophageal, colonic and anorectal manometries, can be utilized to determine which parts of the GI tract are involved (2). Unfortunately, conventional manometry is invasive, complex and expensive. A recent study, comparing small bowel manometric

abnormalities with histopathological findings of full-thickness intestinal biopsy in patients with severe dysmotility, showed that manometric results are not really diagnostic for CIPO and have poor correlation with its histopathology (33), requiring the support of other diagnostic tools.

A further tool for CIPO diagnosis is the endoscopy, specifically upper GI endoscopy (or gastroduodenoscopy) and colonoscopy. Upper GI endoscopy is used to rule out a mechanical small intestine occlusion and allows to collect duodenal biopsies in patients with suspected celiac disease or eosinophilic gastroenteropathy (2). Colonoscopy may be useful to exclude mechanical obstruction and decompress the large intestine, albeit this maneuver doesn't provide long-term results (34). Endoscopy, in addition to other techniques, is an important tool in the diagnosis of neuromuscular gut disorders as it provides, in a minimally invasive way, the ability of full-thickness biopsy sampling for histologic, histochemical, and immunohistochemical analysis (3). This technique is not completely diagnostic for CIPO: its utilization could be hazardous when a mechanical obstruction has not been clearly ruled out (35).

In addition, scintigraphy is a well-tolerated and noninvasive method that allows the determination of gastric emptying and establishment of the optimal route of feeding (gastric or jejunal) (36). As an alternative to scintigraphy, the lactulose breath test can be performed (37). Breath test is used to diagnose small intestinal bacterial overgrowth (SIBO), frequently present in CIPO patients and important target for subsequent therapies (38).

Lastly, laboratory exams are performed, with the general aim to rule out secondary causes of CIPO related to systemic diseases. It is crucial to highlight the causes of secondary CIPO before treating these patients with appropriate therapy. Laboratory tests frequently performed are a complete blood cells count, electrolytes, albumin, liver enzymes, vitamin B12, fasting cortisol, inflammatory indexes, blood tests for diabetes mellitus, celiac disease, connective tissue and skeletal muscle disorders and hypothyroidism (2). The most useful laboratory analysis is genetic testing, that can find out a familiar or sporadic gene mutation in patients who show symptoms in the neonatal period (20).

In summary, CIPO's diagnosis is still challenging, although several tools are currently available. Diagnosis is mainly based on clinical presentation, intestinal dilation, imaging techniques and atypical manometric findings. The results of all these exams, lead to the exclusion of mechanical obstruction and the identification of an intestinal pseudo-obstruction.

1.5 Treatments

Regarding CIPO treatments, the use of a single pharmacological agent to treat CIPO patients is rarely effective, and usually several tools are required (3). CIPO patients undergo therapies aimed to reduce symptoms severity, prevent unnecessary surgery, improve the nutritional status by maintaining an adequate caloric intake, promote intestinal motility and treat SIBO (20). Recent advances in pharmacological, nutritional, and surgical treatment are improving the management of CIPO patients and reducing their hospitalizations (2).

Currently, the most diffuse pharmacological therapy for CIPO patients are prokinetic drugs, aimed to improve GI dysmotility, relieving symptoms and allowing oral feeding toleration (3). Subcutaneous octreotide, a somatostatin analog, exerts GI prokinetic effects by inducing antrum-duodenal phase III of the migrating motor complex, consequently accelerating the small bowel transit (39). Among prokinetics, erythromycin, a macrolide antibiotic, exerts GI prokinetics effects inducing antro-duodenal contractions and accelerates the small bowel transit (40). The association between erythromycin and octreotide showed a possible synergic effect of these two drugs, which stimulate both antral and duodenal contractions (2). A group of prokinetics also used for CIPO treatment affects the serotonin (5-hydroxytryptamine or 5-HT) pathway. Among them, prucalopride is a 5-HT4 receptor agonist, that accelerates gastric emptying and small-bowel and colonic transit, alleviating patients' symptoms (41–43). Other agents helpful in improvement of GI motility are acetyl-cholinesterase inhibitors (ACIs), like neostigmine and pyridostigmine. Their action is well described in children and adults with CIPO refractory to standard therapies (44, 45). However, these prokinetics agents are only able to improve management of some symptoms of CIPO, not to resolve them.

The second crucial point of CIPO management is to prevent and treat SIBO, condition characterizing about the 30% of CIPO patients. SIBO presents a heterogeneous condition with a bacterial overgrowth and/or an abnormal presence of bacterial species in the small bowel, requiring the treatment with different class of antibiotics (46). Amoxicillin-clavulanate, ciprofloxacin, doxycycline and metronidazole are the most used agents that improve abdominal distention and pain in CIPO patients (3). Nevertheless, the most recent recommended agent to treat hydrogen-dominant SIBO is rifaximin, a poorly absorbed antibiotic that exerts non-traditional effects on the gut microbiota in addition to bactericidal/bacteriostatic activity, producing lower bacterial resistance than traditional

agents (47). Its administration improves SIBO-associated symptoms and breath test results (48). A combination of rifaximin and metronidazole is often used to treat methane-dominant SIBO.

Diet modification is also a useful strategy to treat SIBO: an elemental diet, in which amino acids, glucose and fats do not require digestion, decrease the time of nutrients availability in the GI tract, with a more quick elements absorption and a consequent decrease of bacterial growth (3). Moreover, diet of patients with a sufficient intestinal absorptive function is usually characterized by small and frequent meals, liquid or soft, associated to multivitamin supplementation (49). It has been described that a diet low in lactose, in fructose, in fiber and in fat improves gut motility and lowers SIBO risk (26). In addition to oral feeding, nutritional management tools used for CIPO treatment include also enteral and/or parenteral nutrition (43, 50, 51). Enteral feeding is required in patients with oral feeding intolerance, corresponding about at one-third of cases (6). In the worst cases, when enteral nutrition fails or in patients with refractory CIPO, only parenteral nutrition guarantees an adequate nutrition and hydration to patients, although it is often associated with important complications (49, 52).

Strategies aimed at intestinal deflation, as intermittent nasogastric suction, venting procedures or decompressive colonoscopy, can also give symptoms' relief in about 50% of CIPO patients (19).

Only a restrict group of patients may have relief from surgical treatment. Surgery results always a risk and its efficacy is uncertain (53). Eventually, multivisceral or isolated small bowel transplantation have been suggested in patients developing an end-stage CIPO and with nutrition-related complications (54). However, the prognosis of this intervention is critical, as a 69% 5-year survival after gut transplantation (55): so, surgery is a life-saving option but only in end-stage patients with severe symptoms (3).

Finally, fecal microbiota transplantation (FMT) has been recently proposed as a new therapeutic option for CIPO patients. A pilot study, conducted by Gu and collaborators, demonstrated that FMT significantly improves patients' conditions, alleviating pain and bloating symptoms and eliminating SIBO in 71% of patients after only two weeks of treatment (56).

1.6 CIPO and gut microbiota

CIPO exacerbations can be triggered by bacterial or viral infections, psychological stress or malnutrition (57); all of these factors are somehow bidirectionally linked to the structure of the microbiota and its variations, which could potentially have a negative impact on the gut microbiota and clinical diseases (58). The most common infectious cause for secondary CIPO is Chagas' disease, caused by the protozoan *Trypanosoma cruzi* (59). In CIPO, intestinal dilation and slow transit contribute to SIBO, defined as an excessive presence of bacteria in the small intestine. To date, in patients with CIPO, there are limited studies aimed at assessing whether a specific microbiota may be related to the disease, focusing both on characterizing mucosa-adherent and luminal microbiota. In particular, mucosa-associated (or mucosa-adherent) microbiota (MAM), given its close proximity to the intestinal epithelium and the underlying mucosal immune system, can be considered a risk factor influencing the onset and course of specific diseases. In addition, altered gut microbiota, could lead to intestinal epithelial barrier dysfunction and immune dysregulation, also affecting intestinal neuromuscular homeostasis, a risk factor that could triggers gut dysmotility (60). Finally, gut microbiota can modulate a wide range of human neurotransmitters, including serotonin, which is also involved in the intestinal motility.

An interesting study by Gu and co-workers demonstrated the probable involvement of the gut microbiota in the intestinal motility of CIPO (56). In this study, with the aim of restoring the gut microbiota, nine patients with CIPO underwent FMT treatment (56, 61). After 8 weeks from the FMT, a relief of symptoms for pain and bloating was observed in CIPO patients.

2. The gut microbiota

The human gut microbiota reached a considerable interest in the last years, and the knowledge of its composition and function increased with the development of metagenomic studies (62). The term microbiota refers to a microbial community cohabiting the same ecosystem. In the human body, all the surfaces in contact with the external environment, as the oral cavity, eye sockets, epidermis, GI tract, and others, are naturally colonized by several microbes. The gut microbiota

encompasses an area between 250 and 400 m^2, representing on average the second largest body surface area, after the respiratory tract, and the most densely populated microbial ecosystem on earth (63). The gut microbiota includes bacteria, archea, fungi, parasites and viruses (including bacteriophages, which represent 90% of the human virome). These microorganisms colonize the digestive tract after birth and establish relationships that can influence physiological and pathological states of the host (64); however, recent studies showed the presence of microbes' genome even in the uterus, an environment usually considered sterile. The whole set of genes of the microbiota, named microbiome, has been estimated to have a coding potential 150 times greater than the human genome, providing functional characteristics that the host did not evolve (65). The number of bacteria populating the GI tract has been estimated to be of the order of 10^{14}, thus exceeding the number of human cells by about 10 times (66); more recently it has been reported that the ratio of bacterial cells to human cells approaches 1:1 (67). The Human Microbiome Project (HMP) has allowed a better characterization of the gut microbiota, showing that each individual possesses a specific composition at the taxonomic level of species and subspecies (68), comparable to a "fingerprint". This can be easily explained by the fact that the composition of the gut microbiota is influenced not only by the genetic background of the host, but also by several other factors defined as environmental, such as type of delivery, diet, aging, geography, smoking, stress, and drug intake.

2.1 Gut microbiota composition

The GI tract is colonized by bacteria, archeae, viruses, fungi, and parasites. Focusing on bacteria, the gut microbiota is constituted of almost 1500 species, distributed in about 50 different *phyla* (69); at the taxonomic level of *phyla*, *Firmicutes* and *Bacteroidetes* are the most dominant, representing about the 90% of gut microbiota (62). The other important *phyla* present in the gut are *Proteobacteria*, *Fusobacteria*, *Tenericutes*, *Actinobacteria* and *Verrucomicrobia*, which relative abundances are significantly lower (70). Concerning the virome, the 90% consist of bacteriophages, or phages, which greatly contribute to the microbial ecosystem balance participating in horizontal genetic transfers between bacteria, and through predation on bacteria (71). To date, the microbiota has been defined as a "fingerprint", specific to each individual, but the composition of a healthy microbiota has not yet been characterized. In fact, when considering the taxonomic level of species

and subspecies, the similarity of the microbiota among individuals is lost. In addition, the gut microbiota changes and evolves during the life of the host, depending on age and other factors. To date, several studies indicate that to maintain a healthy status, it is essential to maintain the balance of the gut microbial ecosystem, highlighting its importance (72). Several strategies have been developed for this purpose, including the use of probiotics, prebiotics, symbiotics (73), and fecal transplantation (74). Recently, phage therapies are also gaining popularity, showing good effects on host health (75).

2.2 Establishment and development of gut microbiota

Recently, the dogma of the sterile environment in utero has been questioned by several studies that have provided evidence of the presence of bacterial genome in the placenta (76), umbilical cord (77) and amniotic fluid (78) of healthy full-term pregnancies. So, it has been hypothesized that microbial exposure may begin before birth, allowing colonization of the fetus with early pioneer microbes derived from the maternal microbiota.

In any case, colonization of infants' gut lumen after birth is a dynamic, non-random and evolving process during the first three years of life (Figure 1). The composition of the new-born gut microbiota is influenced by different factors, as mode of delivery, maternal microbiota, prematurity, type of feeding, antibiotic therapy, and environmental hygiene (79). An altered bacterial transmission between mother and child could expose the newborn to an alteration in the correct development of the microbiota, which can successively affect the physiological and health status (64). In a healthy context, a mutualistic relationship is established between microbes, and microbes and host. For the first colonization, contact and interaction with the maternal vaginal and fecal microbiota are fundamental (80). Only microbes capable of forming permanent communities will colonize the infant's GI tract. At birth, the human intestine is an aerobic environment. The first neonatal flora consists of tolerant aerobes, mainly belonging to the family *Enterobacteriaceae* (*phylum*: *Proteobacteria*). Within a few days, these organisms deplete oxygen levels so that the intestinal lumen becomes anaerobic. Subsequent colonization by strict anaerobes, such as *Bifidobacterium* (*phylum*: *Actinobacteria*), *Clostridium* (*phylum*: *Firmicutes*) and *Bacteroides* (*phylum*: *Bacteroidetes*), occurs (81).

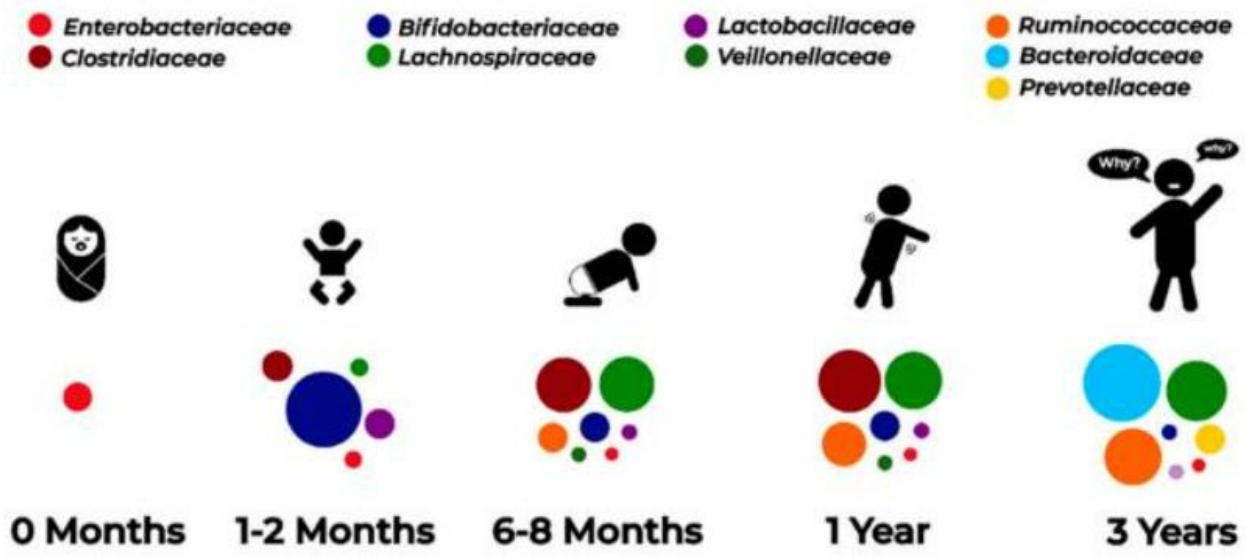

Fig 1. Gut microbiota composition during the first years of life (81).

Vaginal birth is associated with an increase in *Bacteroides*, while caesarean delivery leads to a greater presence of opportunistic pathogens, consequently associated with an increased risk of contracting infections. Infants' gut microbiota differs according to vaginal or caesarean delivery: vaginal delivery is associated with an increase in *Bacteroides* and an abundance of *Lactobacilli* (*Firmicutes*) in the first days of the baby's life, whereas in caesarean delivery the microbiota is depleted, resulting in a greater presence of opportunistic pathogens at the expense of the *Bacteroides* genera, and consequently an increased risk of infections (82). During the first month of life, *Bifidobacteria* and *Escherichia coli* are the prevalent bacteria, followed by *Lactobacillus* spp., *Bacteroidetes*, and Gram-positives, and are all present in comparable quantities (79). Gradually, this initial microbiota reduces the oxygen level, creating a condition favourable for the proliferation of anaerobic bacteria in the intestinal lumen, such as *Clostridium* (*phylum*: *Firmicutes*) and *Bacteroides* (*phylum*: *Bacteroidetes*). The anaerobic microbial community establishes itself in the newborn's gut during the second month of life (79). During the first year of life, the microbial composition of the gut is relatively simple. Changes in the proportions of the dominant members of gut microbiota appear after about one year of life, mainly as a result of the introduction of new food into the diet of the infant: *Lactobacillus* spp., *Bacteroides* spp., and *Clostridia* increase, while *Bifidobacteria* and *E. coli* decrease (79). The composition of the microbiota begins to stabilize after the third year of

age, resembling that of the adult; stabilization will therefore be lost in old age as the variability between bacterial communities increases (83) (Fig 2). It is possible to notice a difference between the microbiota of healthy children (7-12 years) and healthy adults: in fact, the characterization of the metagenomic profiles of the intestinal microbiome has shown that children have an enrichment of genes necessary for development, such as genes involved in vitamin synthesis, folate synthesis and amino acid metabolism. In childhood, the immune system is permissive for microbial colonization and, at the same time, the microbiota influences and educates the immune system to its presence (84). On the other hand, adults show an enrichment of inflammation-related genes, including genes involved in oxidative phosphorylation, lipopolysaccharide biosynthesis, and steroid hormone biosynthesis (82). The microbiome of healthy children and preadolescents is species-rich and functionally complex, and is dominated by *Bacteroidetes* (B) and *Firmicutes* (F), with an abundance of *Firmicutes* and *Actinobacteria* higher than that observed in healthy adults, while the F/B ratio is lower than in healthy adults (82).

A proper microbial colonization during the first years of life has an important role in health and disease later in life and is associated with the development of a functioning immune system. In addition to the host's genetic background, important factors that influence, positively or negatively, the composition of the microbiota include diet, immune system, colonization history, aging, disease, antibiotic/drugs treatment, and stress (85). The use of antibiotics early in life has been associated with the development of dyspnoea, an increased risk of developing inflammatory bowel diseases (IBDs), increased susceptibility to being overweight and/or eczema. Therefore, an adult's microbiota may therefore reflect in part the history of exposure to microbes and environmental factors in the early years of life (86) (Fig. 2).

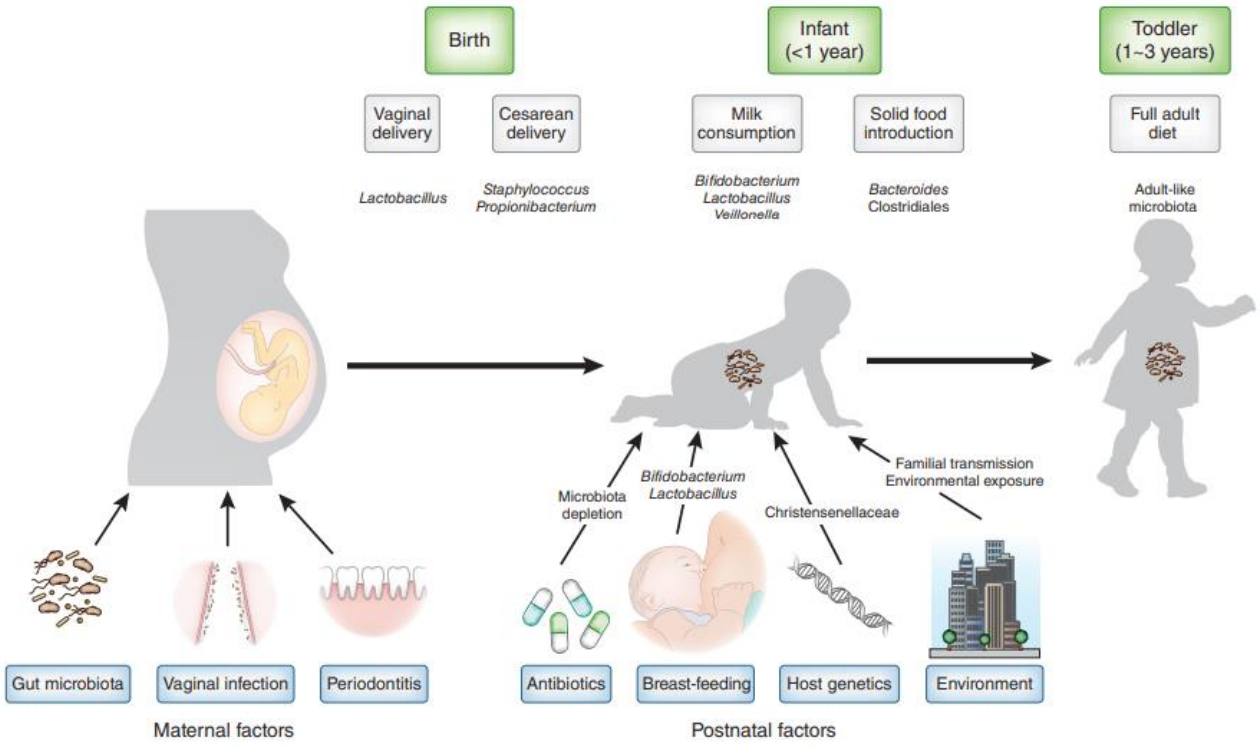

Figure 2. Factors shaping the neonatal gut microbiota (85).

The virome also appears to be highly dynamic during microbiota development, with the highest bacteriophage diversity in the first few months after birth; thereafter, bacteriophages undergo a loss of diversity. Interestingly, the virome contraction occurs at the same time that the infant microbiota adopts a composition similar to that of adults. In fact, the development and diversification of the gut microbiota appear to occur at the expense of the phage community, indicating that the resulting reduction in the number of predators facilitates the establishment of a bacterial community with greater biodiversity in the GI tract. In contrast, eukaryotic viruses, showing low diversity during the first few days of life (2.6 days), were rarely detected before 3 months with increased richness, suggesting that the source of the eukaryotic virome is the environment. Like the gut bacterial community, the composition of the intestinal virome is also influenced by several factors, among which geography and diet seem to have an important impact (Fig. 3) (87, 88).

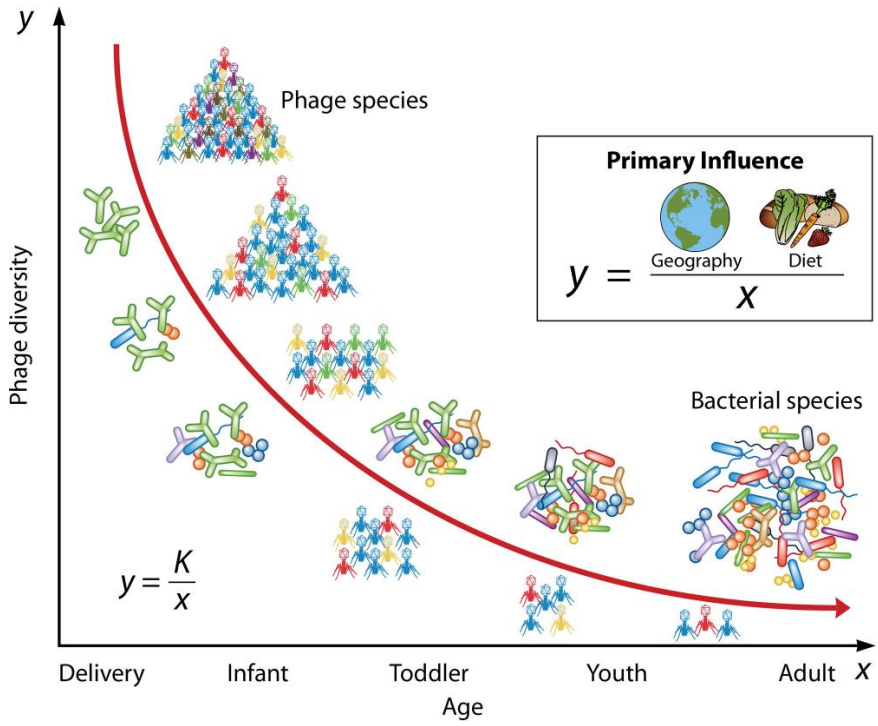

Figure 3. Contribution of phages to gut microbiota development through human aging. Factors influencing the virome biodiversity from infant to adult are schematically represented as factors of the curve's formula (x axis). Phage and bacterial loads are represented to express the concept that while the phage load decreases during aging, the gut microbial population increases in complexity and abundance (y axis). The number of bacterial or phage particles schematically represents the number of species and complexity of the gut population (82).

2.3 Microbial diversity in the GI tract

Gut microbial composition varies along the GI tract. The GI tract is a compartmentalized system that consists of distinct anatomical regions, extending from the stomach to the rectum. These anatomical sections are characterized by variable physicochemical features, such as transit rates of the luminal content, local pH, availability of diet-derived compounds, redox potential, and host secretions (e.g., hydrochloric acid, digestive enzymes, bile, and mucus) (89). That results in a variety of microbial habitats in which the intestinal microbiota varies in richness and biodiversity of microbial species, proceeding in the cephalo-caudal direction. Starting from stomach, where there is a low microbial density, 10^2 CFU/ml, arriving in the colon, where there is a density of 10^{11} CFU/ml and the number of colonizing species increases (Fig. 4) (90).

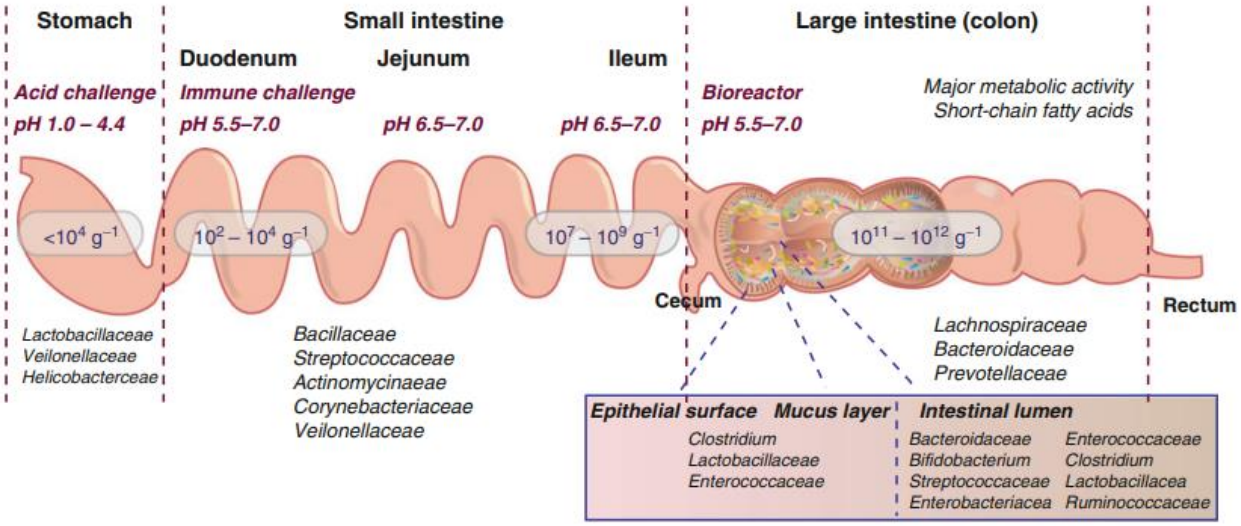

Figure 4. Variations of the gut microbiota composition and numbers along the GI tract. Major features shaping the gut microbiota into the different anatomical regions are indicated (89).

2.3.1 Stomach

The stomach is part of the upper GI tract and is characterized of a low microbial concentration (less than 10^4 CFU/ml). The microbial population residing in this GI tract must be able to counteract acid stress of this site (89). The genera *Prevotella*, *Streptococcus*, *Veillonella*, *Rothiae*, and *Haemophilus* normally populate the healthy human stomach (91). More than 65% of the phylotypes identified in the stomach have been described in human mouth samples, indicating these bacteria as transient in this tract. Transient bacteria are not able to colonize the gastric mucosa and do not dialogue with the host, forming small colonies only for short period. Examples of these species of bacteria are *Veillonella*, *Lactobacillus* and *Clostridium*, found in gastric juice (91).

2.3.2 Small intestine

The small intestine, encompassing duodenum, jejunum, and ileum, is involved in the digestion and absorption of food and nutrients and is an acid environment with high concentrations of antimicrobial substances. In addition, the fast flow of food also causes a rapid wash out of the bacteria. This GI tract is dominated by fast-growing facultative anaerobic bacteria, tolerant to the

combined effects of bile acids and antimicrobials, which compete effectively with both host and other bacteria for available simple carbohydrates. The microbial load increases from 10^{3-4} CFU/mL in the duodenum, to 10^{3-7} CFU/mL in the jejunum, and to 10^{9} CFU/mL in the ileum. The dominant *phyla* in the duodenum, with low diversity (due to the fast transit of food), are *Firmicutes* and *Actinobacteria*; the jejunum is dominated by Gram-positive aerobic and facultative anaerobes such as *Lactobacilli*, *Enterococci* and *Streptococci*; while in the ileum, the bacterial density is like that of the large intestine, with a predominance of aerobic species (92).

2.3.3 Large intestine

The large intestine comprises colon, cecum, and rectum. It constitutes the last part of the gut, where the absorption of water and the fermentation of undigested food take place. The quantity of anaerobes outnumbers aerobes by a factor of 100-1000. The bacterial density reaches 10^{12} CFU/mL and is mainly dominated by *Firmicutes* and *Bacteroidetes*. In this tract, the F/B ratio can change in different phases of life and in different pathophysiological conditions, becoming a predictive index of health and disease (93). It has been observed that the F/B ratio increases significantly from childhood to adulthood, and from adulthood to old age, and then decline markedly. More specifically, the F/B ratio is 0.4 in infants (3 weeks at 10 months), 10.9 in adults (25-45 years), and 0.6 in the elderly (70-90 years) (94). So, the F/B ratio is described as an important factor in the maintenance of intestinal homeostasis: its decrease has been associated to IBDs, whereas its increase with obesity (95).

2.3.4 Cross sectional variation

The microbial composition varies not only longitudinally, in the different anatomical regions of the GI tract, but also latitudinally, within the cross-sectional axis of the gut. The colon wall folds back on itself, creating compartments between the folds (folded regions) that are distinct from the luminal compartment. The inner epithelial surface of the gut is covered by a mucus layer. Figure 5 shows

the different composition of the microbiota according to region of residence. We can observe luminal, mucus-resident, epithelium-resident and lymphoid tissue-resident bacteria.

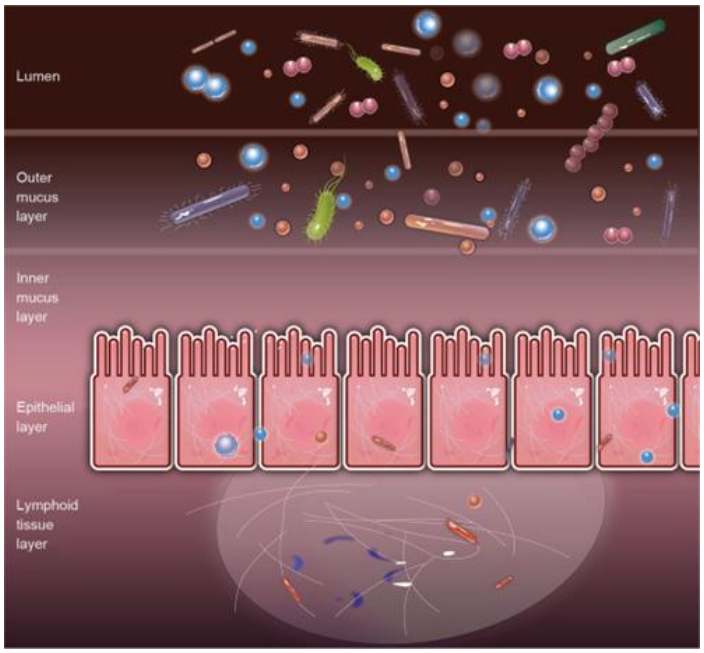

Figure 5. The 4 major cross-sectional subdivisions of the gut microbiota (96).

The luminal content, present in the central region of the colon (lumen or digesta), is composed of dietary fibers that are digested passing through the GI tract. Compared to the lumen, the folded regions contain higher amounts of mucus, which may serve as a food source for some bacteria. *Firmicutes* families *Lachnospiraceae* and *Ruminococcaceae* are enriched between folds regions, and the *Bacteroidetes* families *Prevotellaceae*, *Bacteroidaceae* and *Rikenellaceae* are prominent in the lumen (97). At the level of the colonic mucosa, humans seem to have a noticeable proportion of *Actinobacteria* and *Proteobacteria*, when compared with the community of the colonic lumen (Fig. 6).

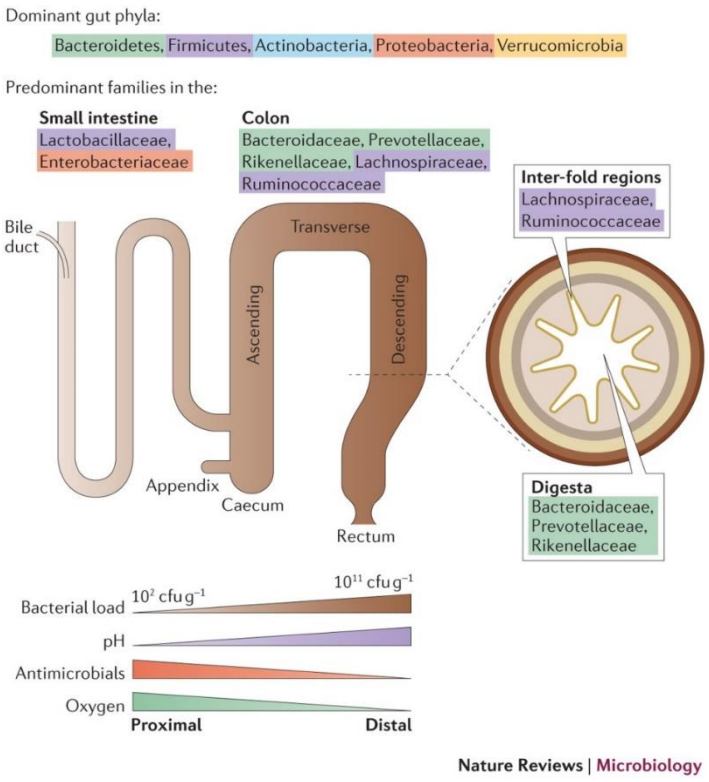

Figure 6. Microbial habitats in the lower GI tract (97).

The mucus layer is produced by the Goblet cells, present in both small intestine and colon. The thickness of mucus layer is variable and partially or completely covers the epithelium, creating a boundary between the intestinal lumen and the host tissue. The small intestine is characterized by a single mucus layer, whereas the colon has a double layer: a more liquid outer layer and a denser inner layer firmly attached to the epithelium. The inner one seems to be sterile when compared to the more populated outer layer (98). The mucus density serves as a physical obstacle for microorganisms, and the epithelium secretes antimicrobial molecules and oxygen, which, as they accumulate in high concentrations within the mucosa, especially in the small intestine, greatly limit potential microbial inhabitants. Consequently, there are more mucus-associated bacteria in the proximal region than in the distal sites (99). In this way, the mucus layers of the GI tract create environments that are distinct and protected habitats for specific bacterial ecosystems that thrive in proximity to the host tissue. Analysis of human colonic biopsies revealed a distinct mucosal community enriched in species of the *phyla Actinobacteria* and *Proteobacteria*, compared to the luminal community (100) (Fig. 7). These observations indicated that microorganisms can be in contact with mucosal surfaces not only in disease states, revealing that lifelong physical associations

between specific members of the microbiota and their hosts represent symbioses generated over millennia of coevolution (101).

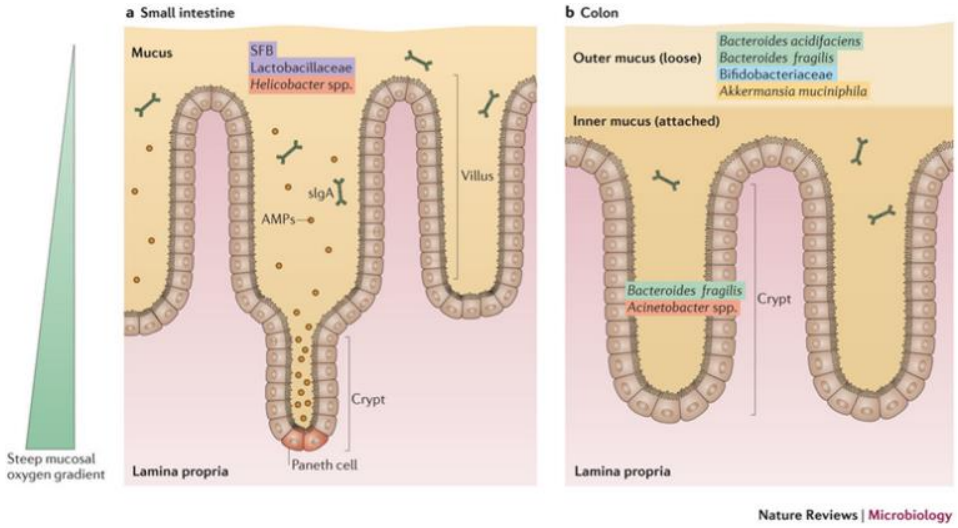

Figure 7. Mucosal layers of the small intestine and colon (97).

MAM plays a very important role in maintaining host cellular homeostasis or in activating inflammatory mechanisms. This is due to its proximity to the intestinal epithelium and the underlying mucosal immune system (102). The stability of the intestinal microbial ecosystem is sufficiently elastic to allow the intrusion of any pathogens with allochthonous flora (derived from food, water, and various environmental components). In this environment, only a relatively small number of pathogens (opportunists/pathobionts) are considered members of the gut microbiota; these pathogens reside unperturbed within the host's enteric microbiota and could pose a health risk to the host when the gut ecosystem loses its balance, resulting in a loss of homeostasis.

2.4 Microbiota functions

The gut microbiota plays an important role in the normal functioning of the host, carrying out protective, metabolic, and trophic/immune functions. The microbiota acts like an independent organ, counting a large number of metabolic pathways useful for the host organism: this type of interaction between host and microbiota has been defined as a mutualistic relationship. The

different bacterial species composing the gut microbiota have the ability to communicate with each other and with the host, mediating physiologically important chemical transformations, storing and redistributing energy, and maintaining themselves through replication.

2.4.1 Protective function

One of the most important functions of the gut microbiota is so-called "colonization resistance," by which commensal organisms prevent colonization of the intestinal epithelium by potential pathogens through competition for any available nutrient or site in the intestinal habitat and through the production and secretion of antimicrobial products such as natural antibiotics and bacteriocins (103, 104). In addition, the intestinal microbiota can help the epithelial barrier in its protective role. Some bacterial communities can fortify the barrier at the level of the tight junctions, which hold the cells of the intestinal epithelium together, through clusters of proteins that form an occlusion between the *lamina propria* and the intestinal lumen (105). It was observed that treatments with probiotics increased the expression of tight junction proteins, while potential pathogenic microorganisms, such as enteropathogenic *E. coli* and *Clostridium difficile*, as well as the proinflammatory cytokines, are able to deteriorate the barrier functions of the tight junctions (106). Disruption of tight junctions increases intestinal permeability often associated with GI disorders (107) and the onset of diseases affecting remote organs. Moreover, resident microorganisms can break down non-digestible compounds by producing short-chain fatty acids (SCFAs). SCFAs are an important source of energy for intestinal epithelial cells (IECs), strengthen the intestinal mucosal barrier (108), and have promising anti-inflammatory and chemo-preventive properties, to the point of being considered tumor suppressors (109). The dynamic relationship between the immune system and the microbiota occurs mainly at the level of the intestinal mucosa, which is the main contact surface between the two. The mutualistic/symbiotic relationship between the host and gut microbiota is made possible by the host's ability to discriminate between pathogenic and non-pathogenic bacteria; the intestinal mucosa plays an important role in this discrimination capacity. The recognition of microbial molecules, called pathogen-associated molecular patterns (PAMPs), is critical and selectively drives immune responses to infectious agents (110). The receptors recognizing PAMPs are the pattern recognition receptors (PRRs). Among PRRs, Toll-like receptors (TLRs) recognize and bind specific microbial macromolecules (111), including lipopolysaccharide,

flagellin, peptidoglycan and N-formylated peptides, and coordinate the immune response. It should be remembered that molecules associated with non-pathogenic bacteria, called microbes-associated molecular patterns (MAMPs), are similar to PAMPs. Host PRRs are constantly exposed to these molecules, even in the absence of infection, but the binding between MAMPs and PRRs does not generate an inflammatory immune response, only a physiological inflammation (as explained below). To maintain intestinal homeostasis, it is important where the interaction between bacterial structures and receptor systems takes place, i.e., whether it occurs on the apical or basolateral side of the intestinal epithelium or directly in the component cells of the epithelium. In general, when the interaction happens in areas where human/bacterial coevolution has not happened, thus in not colonized districts, the host will not tolerate the microorganisms, and commensal bacteria will also be perceived as pathogenic and inducing consequently an appropriate immune response (112).

The IECs, as enterocytes, Goblet cells, and Paneth cells, can limit the access of microbes to intestinal epithelia through the production of antimicrobial peptides (AMPs). Interestingly, also the microbiota can interact with potential pathogens either directly, by producing antimicrobial molecules directed at other microbes, or indirectly, by stimulating host cells to produce antimicrobial products. Several studies indicate that bacteria such as *Lactobacillus*, *Bacillus* (113) or *Bifidobacterium* (114) are able to produce antimicrobial substances to defend occupied niches: usually, these molecules target species also producing AMPs that could occupy their niche. There is a different concentration of these molecules between the lumen and intestinal epithelia. In fact, to discriminate between pathogens and non-pathogens, AMPs have a specific spatial distribution along the GI tract, with maximum antimicrobial activity occurring in the intestinal crypts and in the mucus layer along the mucosa, while there is reduced inhibition of microbial growth in the lumen. This shows that colonization of the mucosa is more controlled than that of the intestinal lumen (by both the host and the microbiota). AMPs include defensins, cathelicidins, and C-type lectins, which are a group of small proteins held in the mucus layer, whereby they can bind to bacterial cell membranes to destroy the outer or inner membrane (115). Defensins are major antimicrobial peptides classified into α, β, and θ defensins. The C-type lectins are a superfamily consisting of more than 1000 proteins having one or more characteristic C-type lectin domains. C-type lectins play an important role in both innate and adaptive immunity to control intestinal bacterial infections (116).

Moreover, the importance of the gut microbiota in avoiding pathogen colonization has been demonstrated by several studies conducted in germ-free (GF) mice that showed that these animals

are more easily colonized by pathogens respect to animals with resident microbial communities (117).

2.4.2 Metabolic function

Humans contain a very limited number of enzymes for processing common polysaccharides (118). These enzymes are provided by the gut microbiota, which, due to its metabolic potential, is able to digest many otherwise indigestible substances, such as various polysaccharides, including plant-derived ones such as pectin, cellulose, hemicellulose and resistant starches. Therefore, the metabolic activities of the microbiota are aimed at retrieving energy and substrates that the host can then reabsorb and, at the same time, providing energy and nutritional products for the growth and proliferation of the bacteria themselves.

Hence, the gut microbiota acts, due to its large gene pool, as a functional organ that performs functions that the host cannot perform on its own. The human gut is considered as an anaerobic bioreactor with a metabolic activity comparable to that of the liver. Functionally, most people possess the same number of bacterial genes involved in metabolic pathways, suggesting that the microbiota, viewed as a functional organ, is similar among individuals, as the number of bacterial genes involved in the metabolic pathways remains the same (119).

Furthermore, there is a functional redundancy in the microbiota, useful to guarantee that, in case of loss of a species involved in a specific metabolic pathway, the biochemical functions related to that species are maintained by other bacteria, even if taxonomically distant. Intestinal microorganisms possess a number of enzymes necessary for the digestion of non-digestible carbohydrates that, by anaerobic fermentation, produce SCFAs. The fermented products represent a significant energy source for IECs and strengthen the mucosal barrier (108). SCFAs have several regulatory functions and are involved in both physiology and host immune processes. They are able to stimulate intestinal motility and intestinal transit: under physiological conditions, they induce an eight- to ten-fold increase in serotonin release (120). The SCFAs produced by microorganisms are acetic acid, butyric acid, propionic acid and lactic acid. The main producers of butyrate belong to the *phylum Firmicutes*, including *Clostridia*, *Eubacteria* and *Roseburia*. Butyrate is involved in numerous processes: it regulates neutrophil migration and function, inhibits inflammatory cytokines

induced by VCAM-1 expression, increases tight junction protein expression in colonic epithelia, and exhibits anti-inflammatory effects by reducing the release of cytokines and chemokines from human immune cells (121). Propionate and acetate are transported from the colon to various organs via the bloodstream, where they are used as substrates for oxidation, lipid synthesis, and energy metabolism (122). Moreover, propionate and butyrate have been observed to have promising anti-inflammatory and chemo-preventive properties (109). In fact, in patients with colon cancer, butyrate-producing microorganisms were decreased respect to healthy ones (123). These properties are due to their ability in reducing the activity of histone deacetylase in colonocytes and immune cells (124).

Gut microbiota is also able to degrade dietary and endogenous proteins, not hydrolysed by the host, via proteases and peptidases. The released peptides and amino acids can then be reused in the colon itself or be transported to other organs via the bloodstream. The most abundant amino acid fermenting microorganisms in the human small intestine are bacteria belonging to the *Clostridium*, *Bacillus*, *Lactobacillus*, *Streptococcus* and *Proteobacteria* groups. In the large intestine, *Clostridia* and *Peptostreptococci* are the species most involved in the fermentation of amino acids. In this tract, the amino acids are not significantly absorbed by the colon mucosa, but there is a high degradation activity by the microbiota. This increased rate of bacterial protein fermentation has been related to high pH and low carbohydrate availability in the large intestine (125). Bacteria also seem to play an important role in the production of amino acids by *de novo* biosynthesis: *in vivo* studies have demonstrated that lysine of microbial origin, which is an essential amino acid, is absorbed and incorporated into host proteins. Hence, modulation of dietary protein or amino acid intake may provide a strategy to shape amino acid fermenting bacteria and their metabolic pathways, thereby potentially influencing host metabolism (126).

Microbiota can also synthesize some vitamins, especially K and B vitamins, including biotin, cobalamin, folic acid, nicotinic acid, pantothenic acid, pyridoxine, riboflavin and thiamine (127). For example, vitamin K deficiency has been observed in patients fed low vitamin K diets only if they were treated with a broad-spectrum antibiotic for the microbiota. The microorganisms belonging to the *phyla Bacteroidetes*, *Fusobacteria* and *Proteobacteria* possess genes for the biosynthetic pathways of riboflavin and biotin (128). Instead, less significant is the involvement of *Firmicutes* and *Actinobacteria* in the production of vitamins belonging to group B.

2.4.3 Trophic/Immune function

Several studies demonstrated the gut microbiota implication in the immune response. As an example, a study of Gordon and collaborators showed that GF mice had impaired expression of antimicrobial proteins, immature gut-associated lymphoid tissue (GALT), reduced IgA production, low intestinal lymphocyte counts, and an imbalance in effector T cell subsets (129). These alterations were lost with the colonization of GF mice, indicating the relevance of host and microbiota interactions in these important immune processes. Therefore, contact with microbes is important for the development of the immune system: the microbiota promotes the maturation of intestinal lymphoid tissue and, as a result, the composition of the colonizing microbiota influences individual immune variations (117). The immune system via the PRRs is able to distinguish beneficial from pathogenic bacteria by linking microbial molecules to the PRRs, which include lipopolysaccharides, flagellin, capsular polysaccharides, unmethylated bacterial DNA CpG motifs and more. PRRs are heterogeneous families of proteins, which can be membrane-bound, secreted or cytosolic. Among them, the most studied are TLRs, membrane-bound receptors, and the nucleotide-binding oligomerization domains (NODs), proteins located in the cytoplasm with an important role against invasive or microbial molecules entering the cytoplasm. TLRs are expressed in cells of innate (mononuclear phagocytes, dendritic cells), specific immunity (B and T lymphocytes) and in IECs.

When bacterial ligands interact with PRRs, the intestinal innate immune system is triggered, and a complex cascade of signalling molecules lead to the activation, by the nuclear transcription factor (NF-kB), of genes encoding for cytokines, chemokines, and other components of the immune system, with the release of pro-inflammatory molecules. In the healthy host, the continuous stimulus to the immune system comes from the resident microbiota and its metabolic products, and these are generally pro-inflammatory signals; continuous activation occurs, named as “physiological inflammation”. Feed-back mechanisms control physiological inflammation through tight regulation of pro-inflammatory signalling, which underlies homeostasis (130). In addition, it should be considered that the location where the interaction takes place is important: in areas where human/bacteria coexistence has not evolved, microorganisms are perceived as pathogenic and consequently an appropriate inflammatory response is induced. The strategic distribution of PRRs also contributes to the discrimination between pathogens and commensals (112).

2.5 Dysbiosis

Gut microbiota coexists in a complex symbiosis with its host, with great variability and plasticity among individuals. A healthy microbiota is in a state of homeostasis, defined "eubiosis" or "normobiosis". Disruption of eubiosis and of the complex symbiosis between humans and their microbiota can have detrimental effects on both. A wide range of factors can cause alterations in this balance, resulting in the so-called state of "dysbiosis". Dysbiosis is usually associated with an imbalance of the microbial ecosystem with a significant decrease in biodiversity and an increase in opportunistic, potentially pathogenic bacteria (57). Pathogens are also permanent residents of the microbiota and their presence is required to have a balanced ecosystem, but they have the potential to induce diseases (for this reason, they are defined as "potential" pathogens or pathobiont) (117). In general, the term eubiosis refers to a healthy, disease-free host status. When the host becomes sick, eubiosis is transformed into dysbiosis, which is usually measured with reference to particular taxa or biomarkers.

All the factors affecting the microbiota balance can contribute to dysbiosis (131). Dysbiosis compromises host health by loosening local homeostasis and increasing intestinal permeability. This status allows the development of chronic inflammations, such as IBDs, metabolic syndromes, cardiovascular disorders, neuropsychiatric diseases, asthma, and tumours. Other diseases caused by disrupting the balance of the gut ecosystem include celiac disease, obesity, type 1 diabetes, allergies, schizophrenia, diabetes, and more (Fig. 8). Dysbiosis also affects the maturation of the innate immune system, leading to less control of pathogens' growth and triggering mechanisms that cause inflammation (132). The state of non-inflammatory homeostasis in the gut may be due to both the host immune system and the gut microbiota; therefore, the imbalance between their interactions increases the risk of immune-related diseases.

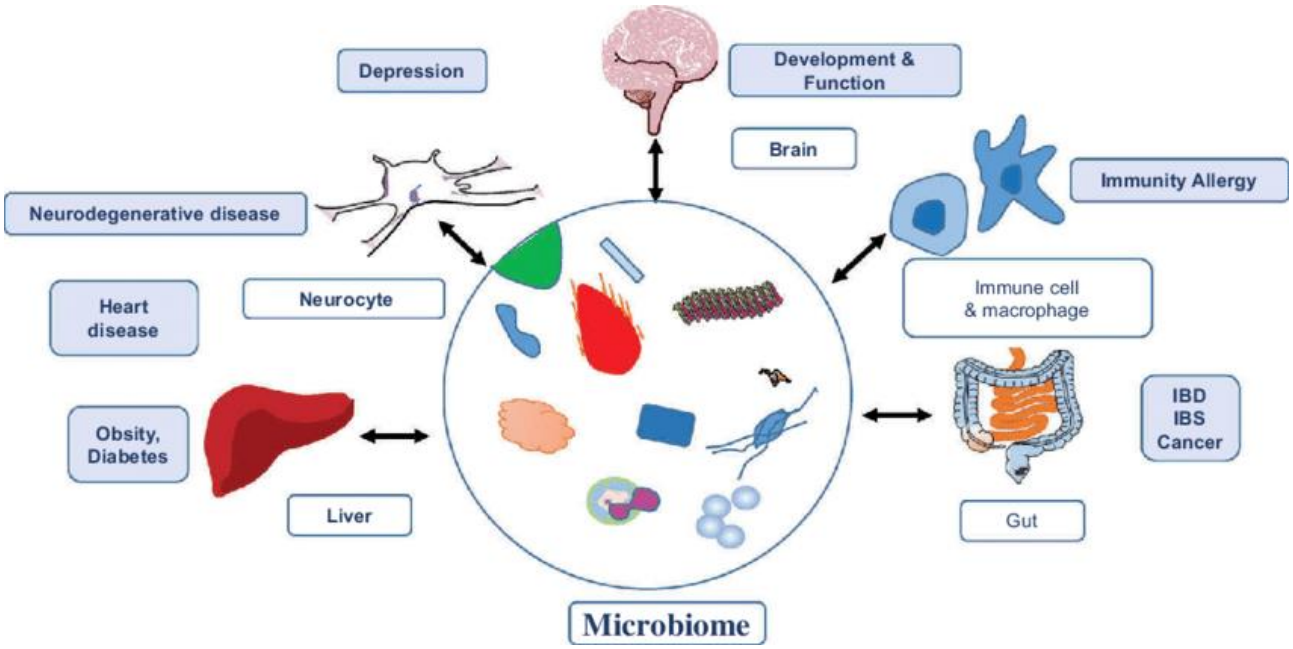

Figure 8. Examples of pathologies with dysbiotic contributions (133).

Dysbiosis is usually associated with harmful, even long-term effects, with consequences leading to disorders or diseases, including obesity, diabetes, and IBDs (134). By comparing the diversity of microbial populations among different individuals, many studies have been able to identify their association with different pathological conditions and, in particular, the associations between the presence/absence of one or more microbial species and the disease, helping to build hypotheses that may link dysbiosis to the aetiology or course of different diseases (72). The mechanisms linking the composition of the gut microbiota and its impact on health and disease phenotypes have recently been better understood through metabolomics approaches, aimed at assessing how metabolites derived from the microbiota may influence the metabolism and physiology of host cells through their activities. Bacterial metabolites, including folate, indoles, secondary bile acids, trimethylamine-N-oxide (TMAO), 5-HT, gamma-aminobutyric acid (GABA), and SCFAs, have host-interacting activity and, at different concentrations, play an important role in the regulation of host physiological phenotypes. To give a more specific causal attributions, a shift to functional definitions of dysbiosis is essential, considering not just the microbiota composition. In fact, although the microbiota changes rapidly in its composition in response to habitat variations, its functionality may remain stable, due to the existence of functional redundancy of microbial genes. Similarly, it is

important to understand the different interactions among the microorganisms in the ecosystem (synergistic or antagonistic), i.e., the ecology of the gut microbiota, because even if it does not change in its composition, the relationships among the gut microorganisms could change, losing the mutualistic relationship.

The functioning of the "organ microbiota" is closely related to the perfect balance among its constituent microbes. Today, there are numerous therapeutic strategies aimed at rebalancing the gut microbial ecosystem: introducing new members into the community through the use of probiotics or by promoting the growth of certain microbes through the use of prebiotics or synbiotics (73); eliminating unwanted members with antibiotic treatment; diet (135); and, more recently, fecal transplantation or bacterial consortium transplantation (136). Finally, phage therapies (75) and the use of bacterial predators (137) are now being intensively studied.

3. The enteric nervous system (ENS)

The GI tract is the only organ that has developed its own nervous system, known as the enteric nervous system (ENS), which can function independently of the central nervous system (CNS). ENS is a nerve network that regulates the motor and secretory functions of the GI tract and consist of approximately 600 million neurons (enteric neurons) embedded within smooth muscle, organized in microcircuits, with interneurons and intrinsic primary afferent neurons (IPANs), which are capable of triggering reflexes. Enteric neurons communicate with each other through more than 50 neurotransmitters (NTs), from small ones (such as acetylcholine or serotonin) to neuropeptides (such as calcitonin gene related peptide, somatostatin, substance P, vasoactive intestinal peptide) and gases (e.g., nitric oxide). The interaction of NTs with specific receptors generates excitatory or inhibitory responses. The anatomy and physiology of the ENS have been studied since the 19th century, demonstrating that the peristaltic reflex is a local nerve mechanism that occurs in the absence of external nerve involvement. Because of this independence and its complexity, Michael D. Gershon compared the ENS to a second brain (138). However, the autonomy is relative because the bidirectional communication between the ENS and the CNS is always active. In fact, the CNS can regulate or alter the normal functioning of the ENS and *vice versa* (139). Therefore, the intestinal motility is coordinated by the CNS that involves an interaction between the ENS, the smooth muscle

cell contractile system, the ICCs and the afferent and efferent nerve fibers. Disruption or impairment of any of the mechanisms of GI motility can result in intestinal motility disturbance. The complex microenvironment of the GI wall includes different cell types (neurons, glia, Cajal cells, muscle cells, and immune cells) capable of communicating with each other in synaptic or paracrine ways. Changes in diet, perturbations in the gut microbiome, its metabolites and neuroactive compounds, impact the functioning of the ENS and its connections with the CNS by altering mucosal permeability and the secretion of hormones and immune cells (140).

3.1 ENS and neurotransmitters

To regulate GI motility, the ENS acts in concert with the intestinal endocrine system (IES), which controls functions by secreting hormones. The GI tract is the largest endocrine organ in the body. It includes enteroendocrine cells (EECs), dispersed throughout the intestinal mucosa, both in the villi and crypts. EECs secrete NTs, bioactive messengers/hormones, to regulate various intestinal functions, including motility and to monitor the intraluminal ecosystem (141). Hormones are secreted in response to specific stimuli in the lumen of the digestive tract and their secretion ceases when the stimuli are no longer present. The apical border of EECs is in contact with the lumen and this allows them to analyse the luminal ecosystem and respond appropriately. NTs, such as acetylcholine, dopamine, GABA, and 5-HT, are key players in GI motility and the connection of neurons in the gut-brain axis.

Acetylcholine is the main neurotransmitter of peripheral nerve fibers, and its major functions are: stimulate contraction of smooth muscles, dilate blood vessels, increase bodily secretions, and slow heart rate. Dopamine is a precursor for other catecholamines, such as norepinephrine and epinephrine, which have various roles in sensory signal detection, behaviour and conditions such as attention, memory and learning (142). GABA is the major inhibitory neurotransmitter of the CNS and, together with its receptor, is extensively disseminated through the mammalian host. Altered GABA level is linked with many CNS disorders, counting behavioural disorders, pain, sleep (143) and interferences with ENS functions, such as gastric emptying, intestinal motility and acid secretion (144). Lastly, serotonin regulates several physiological processes, such as GI secretion and peristalsis, vasoconstriction, respiration, behaviour, and neuronal functions (40, 145).

3.2 The serotonin

The enterochromaffin cells (ECs), a subset of EECs, upon mechanical, chemical or neural stimulation, release the amine 5-hydroxytryptamine (5-HT or serotonin). This NT acts binding to specific receptors, thereby eliciting peristaltic reflexes and propulsive motility (146). 5-HT is a pleiotropic amine that has long been believed to play an important role in several intestinal functions. Its biosynthesis is modulated by different factors, such as variations in luminal glucose levels (147), increases in luminal SCFAs derived from bacteria (148), neuromodulators agents derived from the CNS and/or the ENS (149). The stimulating or inhibiting effect of 5-HT in different parts of the body is related to the site and type of serotonergic receptor involved. Membrane receptors are present in both the central and peripheral nervous systems, as well as in non-neuronal tissues, such as blood or GI, endocrine, sensory, cardiovascular, and other systems (150).

Most endogenous 5-HT is synthesized within the ECs in the GI mucosa via the enzyme tryptophan hydroxylase-1 (TpH1); a minimal amount of 5-HT is synthesized in the enteric neurons via the enzyme TpH-2 (151). 90% of serotonin is produced in the gut, which releases this NT following external stimuli, such as the introduction of food, or internal, such as emotions. Imbalances in the peripheral serotonin have been linked to diseases such as irritable bowel syndrome (IBS), cardiovascular disease and osteoporosis (152, 153).

ECs release 5-HT, which acts by binding to different subtypes of receptors to regulate normal intestinal functions. The 5-HT receptors are subdivided into seven classes, based on pharmacological and structural characteristics: 5-HT1, 5-HT2, 5- HT3, 5-HT4, 5-HT5, 5-HT6 and 5-HT7. In the GI tract, the two receptors 5-HT3 and 5-HT4 are expressed by functionally distinct enteric neurons, smooth muscle cells and EECs and are able to promote peristalsis and induce 5-HT secretion (154).

Since no intercellular degrading enzymes of 5-HT are present, its elimination occurs through a specialized, sodium- and chloride-dependent transport mechanism that allows the molecules to cross the plasma membrane and be degraded by intracellular enzymes. The selective serotonin reuptake transporter (SERT) is found in both brain and intestinal mucosal epithelial cells (155). SERT therefore is a key molecule for the local availability of 5-HT in the gut, and this has also been demonstrated by studies in the gut during postnatal development and in adulthood in which

pharmacological blockade of SERT led to an extracellular increase in 5-HT (156). Serotonin, which is not taken up by epithelial cells, is taken up by SERT-expressing platelets (151). The transcription of neuronal SERT, differs from intestinal SERT: this allows to differentially regulate intestinal 5-HT signalling with drugs that target only the intestinal epithelium (157). Moreover, the reuptake of 5-HT in epithelial cells is required to deactivate its action, whereas accumulation of 5-HT in the interstitial cleft can cause receptors desensitization (158).

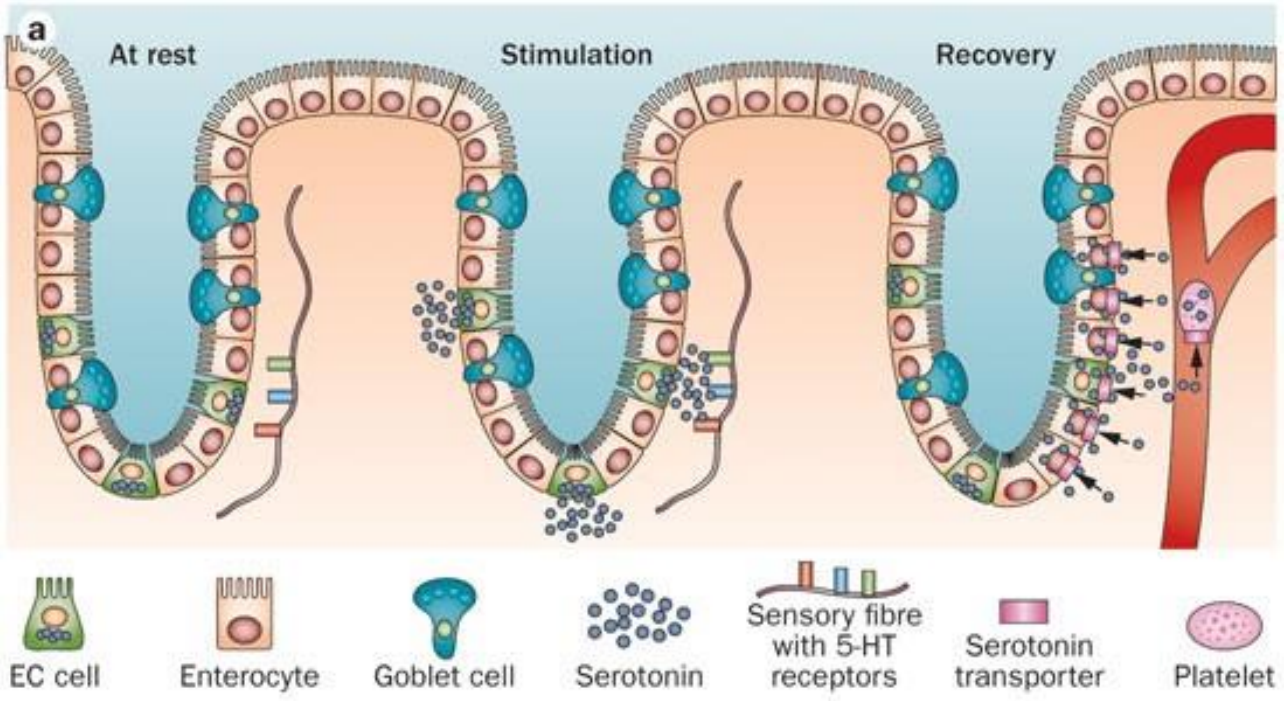

Figure 9. The sequence of events involved in 5-HT signalling in the intestine. At rest, 5-HT is synthesized by EC cells. After mechanical or chemical stimulation, 5-HT is released into the interstitial space of the lamina propria and binds to receptors of nearby nerve fibers. 5-HT signalling ends during the recovery phase: 5-HT is either transported by SERT into epithelial cells, where it is degraded enzymatically, or enters the bloodstream where it is transported to platelets and stored for future use (151).

5-HT is involved in several functions in the gut, including motility. The concept that 5-HT plays a role in peristalsis was proposed by Edith Bülbring and colleagues in the late 1950s. They observed that intraluminal infusion of 5-HT restored peristalsis when pharmacologically interrupted (159). Other studies, supporting 5-HT involvement in peristalsis, show that 5-HT3 and 5-HT4 receptor antagonists slow the intestinal motility (160, 161). Another movement generated by enteric neurons is

segmentation, which consists of alternating contractions of the muscles in a specific region, without forward propulsion of the luminal contents. The whole process serves to mix the contents of the small intestine and maximize its exposure to digestive enzymes during digestion. Ellis and co-workers have shown that mucosal 5-HT is probably involved in segmentation movement (162). In addition, 5-HT is involved in the pathways of activation of reflex-mediated secretory responses. In the intestinal epithelium, active secretion can be initiated by intrinsic enteric reflexes consisting of primary afferent (sensory) and secretomotor neurons. The afferent neurons receive signals from projections to the *lamina propria* and, when stimulated, synaptically activate the secretomotor neurons, which in turn release NTs, such as acetylcholine and vasoactive intestinal peptide (VIP) to activate secretion from the epithelial cells (163). 5-HT can stimulate secretion through a direct paracrine action on adjacent enterocytes, allowing neutralization of luminal contents and elimination of luminal pathogens (164). Serotonin is involved in satiety signals derived from the gut (165). It also performs neuroprotective and trophic functions; indeed, it has been observed that enteric neurons, in mice lacking TpH2, showed reduced cell density. These actions of 5-HT could involve the 5-HT2B receptor, which has been shown to influence enteric neuronal expansion, particularly in development (166). The 5-HT2B receptor is also expressed by ICCs and is important for the integrity of the ICC network (167). From studies in 5-HT4 receptor knockout mice, it has been observed that the density of neurons at birth does not appear to be altered, but there is a postnatal reduction in enteric neurons. The correct number of neurons can be restored by treating adult mice with 5-HT4 agonists (41).

The 5-HT3 and 5-HT4 receptors are the most extensively studied receptors in the intestines, especially regarding the treatment of constipation and diarrhoea. 5-HT4 antagonists have been shown to be efficient in the treatment of diarrhoea. 5-HT4 receptor agonists are effective against constipation and relieve pain, so they are prokinetics, such as prucalopride (151).

The importance of NTs function in the ENS of CIPO patients was confirmed by the effectiveness of drug treatments that affected the acetylcholine and 5-HT systems. For example, cisapride, a 5-HT4 receptor agonist and 5-HT3 antagonist with prokinetic properties, binds to 5-HT receptors in the myenteric plexus inducing acetylcholine release and smooth muscle contractions, resulting in increased postprandial duodenal contractions in CIPO patients (168). In addition, prucalopride, another 5-HT4 receptor agonist, facilitates acetylcholine release resulting in activation of cholinergic neurotransmission that accelerates gastric, small intestinal, and colonic transit (43). Based on the

success of treatments with intestinal 5-HT receptors antagonist drugs, it could be hypothesized that, in patients with CIPO, intestinal dysmotility may also be related to a malfunction of the serotonergic pathway.

3.3 Interaction between gastrointestinal motility, neurotransmitters, and gut microbiota

The gut microbiota appears to affect the ENS and the CNS development and disorders, including neurodegenerative diseases, cerebrovascular accidents and behavioural, neuroimmune-mediated, or motility disorders (169). Interaction pathways along the gut-brain axis include those determined by the immune system, the vagus nerve or variation in neuroactive compounds in the microbiota (170). Two recent studies showed that GF animals have significant alterations in several ENS/CNS-related functions, including intestinal motility (168, 171). Gut bacteria can influence production and secretion of an extensive variety of mammalian NTs, including serotonin, norepinephrine, dopamine or GABA (170). Several bacteria are also able to directly produce these neurotransmitters (172). Also, bacteria like pathogenic *E. coli* O157:H7 (EHEC), *Klebsiella pneumoniae*, and *Staphyloccoccus aureus* are able to respond to dopamine and noroeprine, and to produce them. Although it has not been confirmed that the microbiota modulates norepinephrine or dopamine *in vivo*, evidence is accumulating to suggest that it could play a role in host biosynthesis/catabolism (170). A study by Asano and collaborators suggests that microbiota influences norepinephrine production, in fact GF mice had reduced amounts of norepinephrine in the caecal lumen, which were restored by colonizing the animals with a *Clostridia* mixture. However, it has not been determined whether the bacteria directly produce norepinephrine or modulate host production (172). The gut microbiota appears to influence also circulating GABA levels, as GF animals have substantially reduced serum and luminal levels of GABA (170). It has been reported that several commensal organisms produce GABA, including members of the genera *Bifidobacterium* and *Lactobacillus*; in a recent study, oral supplementation of *Bifidobacterium brevis* NCIMB8807 pESHgadB, a strain engineered to produce GABA by overexpression of glutamate decarboxylase B, reduced sensitivity to visceral pain in a rat model (173).

The microbiota can also influence the formation of the serotonergic system by regulating the production of 5-HT. The gut microbiota can influence host 5-HT biosynthesis through microbial-

derived metabolites. A series of experiments was conducted on GF mice, mice colonized by bacteria but lacking specific pathogens (specific-pathogens free mice, SPF mice), and cell cultures. Compared with SPF mice, GF mice showed reduced levels of 5-HT in the colon, but not in the small intestine, thus suggesting a specific role of microbiota in the regulation of 5-HT in the colon (171). It was also evidenced that there is reduced expression of *TpH1* and elevated expression of *SERT* in the colon. Moreover, it was found that specific sporigenic bacteria (dominated by *Clostridia*), from human and mouse microbiota samples, regulate 5-HT levels, coinciding with increased *Tph1* expression. The researchers also provided evidences that microbiota-dependent changes in 5-HT levels appear to influence GI motility (transit time and activation of enteric neurons) and platelet function (activation and aggregation). Finally, the researchers determined that microbial metabolites confer the serotonergic effects of gut bacteria. Indeed, metabolomic profiling revealed that colonization of GF mice with mouse-associated sporigenic bacteria leads to significant alterations in 75% of the 416 metabolites detected; similar changes were also observed during colonization with human-associated sporigenic bacteria. Specific metabolites produced by the bacteria were tested for their ability to induce 5-HT production *in vitro* and *in vivo*. Several metabolites (including butyrate, deoxycholate, and α-tocopherol) increased 5-HT production in ECs in vitro, and this corresponded to an increase in *Tph1* expression. A similar transient increase in colon and serum levels of 5-HT was observed after injection of these metabolites into GF mice (171). Alterations in mucosal 5-HT levels have been reported in intestinal biopsies of patients with IBS, related to changes in the expression of *TpH1* and *SERT* genes or 5-*HT3* and *5-HT4* genes for intestinal 5-HT receptors (174). Studies in animal models or humans have demonstrated the therapeutic potential of microbiota-targeted interventions in modulating neurotransmitter levels (170). In table 2 are summarized several studies corroborating the idea that gut bacteria impact a wide variety of neurotransmitters, including those involved in the GI motility. Clarifying how the gut microbiota can modulate gene expression, synthesis and/or function of these neurotransmitters could be of great help in better understanding the role of each factor involved in this network.

Author of the Study and Year of Publication	Results
Asano et al., 2012	Bacteria are able to produce neurotransmitters
Strandwitz et al., 2018	In human and mouse models, interventions centered on microbiota composition modify the levels of neurotransmitters
Dey et al., 2015; Yano et al., 2015	Germ-free animals show alterations in different ENS- and CNS-related functions
Gershon et al., 2007; Berger et al., 2009	Serotonin regulates several physiological processes, e.g., peristalsis

Table 2. Impact of gut microbiota on neurotransmitters (2).

OBJECTIVES

To date, CIPO disease is considered a rare disorder involving GI motility with an apparently unknown cause of dysmotility, having limited diagnostic and treatment tools.

The purpose of this thesis was to characterize the mucosa-adherent microbiota (MAM) and the expression of serotonin-related gut genes in CIPO patients and, as a secondary objective, to discover possible correlations between MAM and serotonin- related genes expression.

To this purpose: i) MAM from colon, ileum, and duodenum biopsies of paediatric CIPO patients was characterized, evaluating the presence of a specific disease-associated microbiota; ii) changes in the expression of serotonin pathway genes in the same biopsies were evaluated, focusing on the genes *TpH1* (coding for tryptophan hydroxylase 1), *SLC6A4* (coding for serotonin transporter - SERT), *HTR3A* (coding for serotonin 5-HT3 receptor) and *HTR4* (coding for serotonin 5-HT4 receptor); iii) correlations between MAM and serotonin-related genes expression were assessed.

MATHERIALS AND METHODS

1. Patients

In this study, seven well-characterized CIPO paediatric patients, diagnosed in accordance to the international recommendation (2), and seven controls, sex- and age-matched, were enrolled at "SS. Antonio e Biagio e Cesare Arrigo" Hospital of Alessandria (Italy). To document the diagnosis of CIPO, each patient underwent a thorough diagnostic work-up that included: abdominal radiography, as first screening to identify dilated small intestinal loops; intestinal transit contrast study of the small intestine, to exclude malrotation and organic lesions occluding the gut; entero-MRI, instead or in addition to contrast studies; antro-duodenal manometry, in order to clarify the nature of underlying impairment (either myogenic or neurogenic) and optimize clinical management; upper GI endoscopy, to exclude a mechanical occlusion of the proximal small intestine, rule out peptic disease presenting with severe upper GI symptoms and collect duodenal biopsies in cases with suspected celiac disease or eosinophilic gastroenteropathy; colonoscopy, to exclude mechanical obstruction and collect tissue biopsy specimens throughout the colon and ileum to perform histopathological diagnosis. The characteristics of CIPO patients involved in the study are reported in Table 3. Briefly, they were five males and two females (age 4-14 years old) and, based on histological features, they all had a neuropathic diagnosis, with predominant involvement of enteric neurons. All patients had idiopathic CIPO (of unknown origin). The locations of the gut involved in the disease were both the small intestine and the colon for six of the patients, and only the small intestine for one patient. Regarding the nutrition of CIPO patients, five with the disease affecting both the small intestine and the colon were on oral intake, the sixth was on enteral nutrition (EN), and the last subject, where only the small intestine was affected, was on 50% parenteral and 50% enteral feeding. Almost all patients were on pyridostigmine therapy, while the recent diagnosis of CIPO in the last patient implied a pharmacological therapy not yet prescribed. Regarding the symptoms, all patients suffered of bloating, three had abdominal pain and no one had nausea or vomiting. The frequency of defecation was one time a week for two patients, every two days for other two patients and 1-2 time a day for the remaining three patients (Tab. 4). The stool shape and consistency were assessed by the Bristol Stool Chart (175) (Fig.10). Clinical parameters analysed include blood ferritin, faecal

calprotectin, blood albumin, blood haemoglobin, blood protein C-reactive (PCR), and the growth parameter weight and height percentile (Ht %). These values are reported in Table 5.

Patient	Age	Sex	CIPO	Location involved	Nutrition	Therapy
1	9	M	Idiopathic	Small intestine - Colon	Oral intake	Pyridostigmine
2	12	F	Idiopathic	Small intestine	50% EN + 50% PTN	Pyridostigmine
3	8	M	Idiopathic	Small intestine - Colon	Oral intake	Pyridostigmine
4	4	F	Idiopathic	Small intestine - Colon	Oral intake	Pyridostigmine
5	4	M	Idiopathic	Small intestine - Colon	EN	Pyridostigmine
6	4	M	Idiopathic	Small intestine - Colon	Oral intake	Pyridostigmine
7	14	M	Idiopathic	Small intestine - Colon	Oral intake	No

Table 3. Clinical characteristics of CIPO patients.

The subjects enrolled for the control group had all a negative CIPO diagnosis. They underwent an endoscopic exam due to rectal bleeding symptom, but the workup revealed rectal inflammatory polyps, excluding a diagnosis for motility disorders or IBDs. Control group's symptoms and clinical parameters are described in Table 4 and Table 5. Briefly, the control group was composed of 5 males and 2 females (4-17 years old). They had no symptoms of abdominal pain, bloating or vomiting. The frequency of defecation was 1 time per day and the Bristol Stool Chart described a score of 3 for all controls, except a score of 5. At sampling time, control patients were not under pharmacological treatment, and they did not present altered food intake (Table 4). Clinical parameters derived from laboratory analysis are reported in Table 5.

	Abdominal pain	Bloating	Vomit	N° evacuation/day	Bristol Stool Scale
Patients					
1	No	Yes	No	1 ev/ day	3
2	Yes	Yes	No	1 ev/ day	6
3	No	Yes	No	1 ev/ 2 days	1
4	No	Yes	No	1 ev/ 2 days	3
5	Yes	Yes	No	1-2 ev/ 7 days	6
6	No	Yes	No	1 ev/ 7 days	6
7	yes	Yes	No	1-2 ev/ 1 day	3
Controls					
1	No	No	No	1 ev/day	3
2	No	No	No	1ev/day	3
3	No	No	No	1 ev/day	3
4	No	No	No	1 ev/day	5
5	No	No	No	1 ev/day	3
6	No	No	No	1 ev/day	3
7	No	No	No	1 ev/day	3

Table 4. Symptoms comparison in CIPO patients and controls.

	Proteins (g/dl)	PCR (mg/dl)	Hb (g/dl)	Ht (%)	Ferritin (ng/ml)	Calprotectin (mg/kg)	Albumin (g/dl)
Patients							
1	6,5	0,5	13	42	60	49	4
2	6,2	0,62	12	35	48	67	3,6
3	6,5	0,2	12,5	38	42	51	4,2
4	6,9	0,03	13,5	41	12	61	4,6
5	/	/	/	/	/	/	/
6	6,5	0,01	12,3	36	6	72	4,6
7	6,2	0,01	15,5	83	23	86	4,4
Controls							
1	5,9	0,02	13,8	41	82	11	4.2
2	6,1	0,07	12,1	35	73	42	4.3
3	6,7	0,05	12,3	36	91	169	4.7
4	7,8	0,04	14,1	40	43	76	4.8
5	6,8	0,05	14,1	44	90	82	4.6
6	6,4	0,28	12,5	37	72	77	4.6
7	6,9	0,76	12,2	36	51	34	4.5

Table 5. Clinical parameters comparison in CIPO patients and controls.

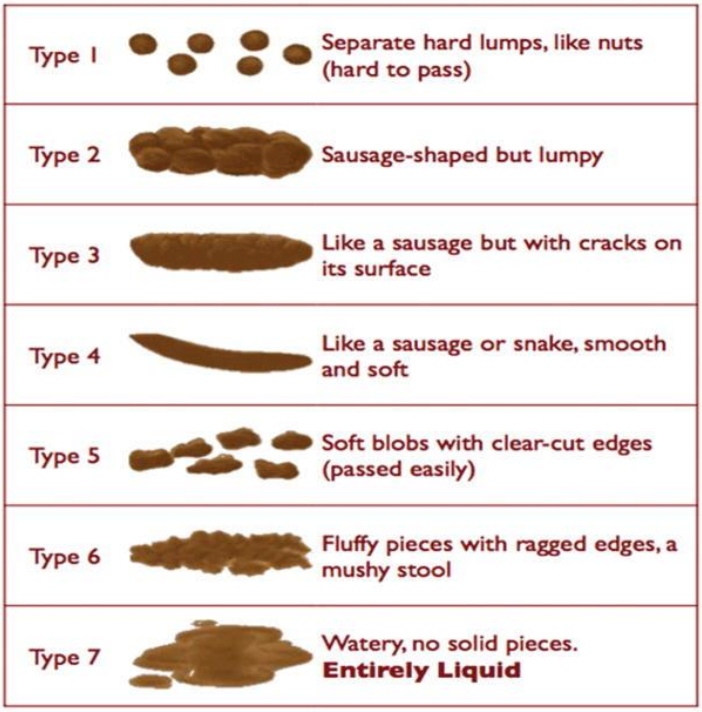

Figure 10. Bristol Stool Chart, stools classification according to shape and consistency (175).

1.1 Exclusion criteria

Patients and control subjects were enrolled according to the following exclusion criteria: acute and chronic IBDs, metabolic disorders, diabetes mellitus, celiac disease, food allergies, anti-inflammatory drugs within 2 weeks prior to recruitment, probiotics/prebiotics/symbiotics in the past 30 days, antibiotics in the past 60 days, concomitant infectious diseases.

1.2 Ethics approval and consent to participate

All procedures achieved in the present study were performed in accordance with the ethical standards of the institutional and national research committee. The study was approved by ethical committee (Prot. n° ASO.Ped.22.03, CE 21/04/2022 for the SS. Antonio e Biagio e Cesare Arrigo" Hospital of Alessandria). Parents of both CIPO and control patients signed the informed consent, giving their consent to participate to the study and to proceed to the subjects' sample collection by

means of endoscopy. All study procedures were performed in accordance with the ethical committee,

1.3 Sample size

The study should be considered have an appropriated statistical power, with a 95% confidence interval, with the enrolment of at least 20 CIPO paediatric patients (age 3-18 years) and 20 sex- and age-matched healthy controls. Nonetheless, CIPO being a rare disturb and of difficult diagnosis, it was challenging to find the appropriate number of subjects for this study. Moreover, the SARS-CoV2 pandemic lived during these years made the enrolment harder, reducing physicals and hospitalisations. Nevertheless, the results of the study are statistically significant and show clear indications for continuing studies in this direction.

2. Sample collection

At Cesare Arrigo Children's Hospital (Alessandria), duodenal, ileal, and colonic biopsies were collected from each enrolled CIPO patient and control subject, during gastroduodenoscopic and colonoscopic examinations. From each GI tract, two tissue specimens, anatomically close and of about 15 mg, were picked. In order to preserve the DNA and RNA's structure, the samples were stored at -80°C in *All protect Tissue Reagent* (Qiagen, Hilden, Germany). At that point, the biopsies were sent to the microbiology laboratory of Public Health and Infectious Diseases department at Sapienza University of Rome. For every location, using dedicated extraction kits, one biopsy was subjected to total DNA extraction, and the other was subjected to total RNA extraction.

3. DNA extraction

In order to characterize the mucosa-adherent microbiota (MAM), total DNA was extracted from each sample. To isolate mucosa-adherent bacteria, biopsies were washed with 500 µL of

physiological saline solution (PBS, Thermo Fisher Scientific, Waltham, Massachusetts, USA), added with dithioerythritol 0.016% (DTT, Sigma Aldrich, Germany). DTT owns a sulfhydryl group that allows the specific reduction of disulfide bonds of mucoproteins, principal component of the gastric mucosal barrier, allowing the removal of non-mucosa adherent bacteria. Biopsies were then washed other three times with 500 µL of PBS, prior to proceed to the DNA extraction by the DNeasy Blood & Tissue Mini kit (Qiagen, Hilden, Germany), following the manufacturer's instruction, with the addition of a four hours incubation with lysozyme (Sigma-Aldrich, Milan, Italy) (20 mg/mL) at 37°C, in order to increase the yield for Gram-positive bacteria. DNA concentration and quality were then checked using the Eppendorf BioPhotometer Spectrophotometer (Eppendorf, Germany) and its integrity was verified by electrophoresis on 1% agarose gel containing SYBR® Safe DNA GelStain (Invitrogen by Thermo Fisher Scientific, Waltham, Massachusetts, USA).

We focalized our research on the characterization of MAM, due to the increasing demonstration that it is crucial for host-bacteria interaction. Many models of inflammation demonstrated the important role of MAM in different inflammatory diseases, evidencing an abnormal bacterial penetration of the usually almost sterile inner mucus layer (102, 176). At mucosa level, the mucosal adaptive immune system is in close contact with the MAM and, in a healthy intestine, it prevents the invasion of bacteria in the mucus layer. Respect to faecal microbiota, the MAM is close to the mucosa and shows a bidirectional communication with the mucus layer, showing an influence in mucosa-adherent dysbiosis and pathological states.

4. NGS sequencing

To evaluate the absence of PCR inhibitors in the extracted DNA, the amplification of the housekeeping gene Beta-globuline was performed for each sample. Once the absence of PCR inhibitors was confirmed, as a requirement for subsequent sequencing, the amplification of V3-V4 region of bacterial 16S was performed, thus assessing the presence of bacterial genetic material in our samples. The characterization of MAM was performed by 16S rRNA gene sequencing. This gene is present in all bacteria and constituted by highly conserved evolutionarily regions and hypervariable and species-specific regions. Conserved regions act as targets for primers, while hypervariable regions are sequenced and, comparing them in specific 16S rRNA databases, give

information about the species diversity and abundance in the sample analysed. In the study, 16S rRNA gene was sequenced by an external service. Briefly, the V3-V4 variable region of 16S rRNA gene was amplified using the primer pairs 16S_F 5'-(TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG)-3' and 16S_R 5'-(GTC TCG TGG GCT CGG AGATGT GTATAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C)-3' and then sequenced via Next Generation Sequencing (NGS), on an Illumina MiSeq 2x300bp platform, generating paired-end reads of 300 base-length. The Illumina MiSeq system provides an end-to-end sequencing, integrating cluster generation, amplification and sequencing processes. After demultiplexing, reads were merged by using USEARCH v11 (177), with a minimum percentage identity of 90% between aligned sequences. Afterward, the primer sequences were removed by using Cutadapt 2.1 (178) and the sequences were filtered in order to keep only those presenting a total expected error ≤0.8 and a size over a range of 400–460 bp. Sequences possessing the required characteristics were imported in the software package Quantitative Insights into Microbial Ecology 2 (QIIME2) V18.6 (179) and passed to the Dada2 algorithm (180) for chimera-checking. QIIME2 was used for all downstream analyses, except those for which an alternative software package is clearly indicated. Operational taxonomic units (OTUs), defined by a 97% of similarity, were picked by clustering sequences with an open reference approach against the 97% clustered Greengenes rDNA reference database v13_8. OTUs found in only one sample and/or presenting <10 sequences across the whole population were filtered out, in order to minimize artefact (181). This system can obtain an efficient identification of microbial communities up to species level. OTUs not identified at species level or identified with a confidence <0.75 were assigned to the deepest taxonomic level, on the base of BLAST results obtained by querying sequences against available published data and taking only those results in agreement to the taxonomy assigned by the Naive Bayes classifier approach if a confidence >0.75 were determined. The sequencing allowed to characterize the mucosa-associated bacteria and, through multivariate/non-parametric statistics and ecological index analysis, significant differences in bacterial community structure were quantified and cross-correlations were made of all collected data.

5. Bioinformatic analysis

Using ecological diversity measures and multivariable/non-parametric processing, α and β diversity were then evaluated. The α-diversity (within sample diversity) was measured in terms of Shannon index and species richness (number of observed OTUs). The β-diversity (between samples diversity) was calculated using Bray-Curtis dissimilarity measure and represented in Principal Coordinate Analysis (PCoA). PCoA was performed to compare the global composition of the bacterial community between samples. The statistically significance of partitions between groups was evaluated with the Permutational Analysis of Variance (PERMANOVA).

Specifically, α-diversity measures the mean diversity of species within a microbial ecosystem and it is proportional to the number of species and the evenness of their relative abundance (or equitability). The α-diversity can be associated with different indices, like the number of different species in a sample (richness indices), the species relative abundances (evenness indices) and diversity indices, considering both species' total number and their relative abundances (182). The β-diversity describes the similarity or dissimilarity present in an ecosystem, indicating the diversity between samples (182). This diversity can be measured with the Bray-Curtis analysis, describing the dissimilarity between bacterial communities, or with the Unifrac distance, that give information on the relative similarity of microbial components of a community incorporating also the phylogenetic distances between microbes. These analyses are simply presented by PCoA, that allows the representation of multivariate data in a relatively small number of dimensions.

Moreover, the relative abundance of taxa between CIPO patients and controls were analysed through the Linear discriminant analysis (LDA) effect size (LEfSe). The relative abundance analysis of taxa was performed at genus and species levels by univariate statistical methods (Mann-Whitney U test).

6. Network analysis

Interactions between members of gut microbiota are fundamental in the crosstalk of the ecosystem, complex and carefully balanced. These interactions can be synergic or antagonist, and it is important

to analyse the interactions' network to better comprehend the role of microbes in health and disease. For each group (CIPO patients and controls), a correlation network was computed independently. Briefly, a first filtering step was achieved by removing OTUs present with a mean relative abundance of <0.01% across all populations. Then, OTUs with a count of zero were filtered out and the remaining unique entries were tested for their correlations by using CoNet v1.1.1 (183), as an application in Cytoscape program (184). Combination of the following methods, Pearson correlation, Spearman rank sum correlation, Bray-Curtis dissimilarity, and Kullback-Leibler divergence, was used with a cut off threshold of 0.4 for both positive and negative values for all considered metrics (correlation ≤ −0.4 for negative interactions, correlation ≥ 0.4 for positive interactions), in order to overcome weakness presented by the use of a single metric respect to a compositionality, matching zeros and sample size. Only correlations supported by at least two different correlation metrics were considered. The statistical significance of each pair was tested using 500 row shuffle randomizations followed by 100 bootstraps. The p-values relative to multi-edges connecting the same node pair were merged using the Fisher's method and the merged p-values were corrected for multiple comparisons. In each randomization round, a sample-wise normalization step was performed for each item pair, in order to account for compositionality bias. Topological parameters were calculated for each computed network using the Network Analyzer plugin included in Cytoscape. From this kind of analysis, we could deduce if a specific connection between two species is involved in the pathology, by observing the dissimilarity with the control network. Finally, the synergistic or competitive relationships between species and the co-occurrence, namely the frequency with which two species appear together in the gut microbiota within a certain cohort, can also be detected.

7. RNA extraction and expression analysis

In order to assess if the expression of genes related to serotoninergic pathway in the gut was altered in CIPO patients, RNA was extracted from the second biopsy collected. The RNA extraction was completed using the RNeasy Mini Kit (QIAGEN, Hilden, Germany), following the manufacturer's instructions. The mRNA extracted was then converted into cDNA by the high efficiency retro-transcription kit "Quanti Nova Reverse Transcription Kit" (QIAGEN, Hilden, Germany). The expression of selected genes was evaluated via qRT-PCR. The genes analysed were: *TpH1*, *SLC6A4*,

HTR3A and *HTR4*. These are all fundamental genes in the intestinal serotonin pathway, in particular: *TpH1* codes for Tryptophan hydroxylase 1, responsible for 5-HT synthesis in the gut district; *SLC6A4* codes for the Serotonin transporter (SERT), that regulates the recaption of serotonin at luminal level; *HTR3A* and *HTR4* code for the intestinal receptors of serotonin.

Quantitative Real Time PCR was assessed using StepOnePlus Real Time PCR System (Applied Biosystem). Specific primers for the selected serotonin-related genes were provided by Qiagen (QuantiNova LNA PCR assay) and the Quantitect RT-PCR kit (QIAGEN, Hilden, Germany), comprehensive of SYBR Green mix (fluorescent dye), was employed for the qPCR reaction. Manufacturer protocol was followed to perform the procedure. In particular, parameters set for the qPCR were: 95°C for 2 minutes, then 40 cycles at 95°C for 10 seconds and at 60°C for 30 seconds. Each reaction was repeated in triplicate and the absence of contaminants was assessed substituting cDNA with Ultrapure water (negative control).

The relative expression of each gene was determined via the ΔΔCT method (185). The ΔΔCT is a widely used, and relatively simple, method that allows the quantitative analysis of qPCR data. In order to obtain significant data, the expression level of target genes (the serotonin-related genes investigated in the study) is normalized to the expression levels of a reference gene, usually housekeeping genes which expression is not altered from the experimental conditions (in this study the reference gene was the GAPDH, coding for glyceraldehyde 3-phosphate dehydrogenase).

When using the ΔΔCT method, the following components are required:

- Target (tar): the genes to be analysed (serotonin-related genes in CIPO patients);
- Calibrator (cal): the same genes to be used as a reference for the comparative analysis (serotonin-related genes of control subjects);
- Referent (ref): a housekeeping gene, constitutively expressed in all samples, necessary to normalize the data (Gapdh genes)

This method assumes that target and reference genes are both amplified with a 100% efficiency (with a 5% of variability accepted). Each target gene's CT was normalized to the reference gene for both CIPO (test) and control subjects (cal) samples:

$\Delta CT\ test = CT\ (target, test) - CT\ (ref, test)$ $\Delta CT\ cal = CT\ (target, cal) - CT\ (ref, cal)$;

Then, the ΔCT of test sample was normalized to ΔCT of calibrator, extrapolating the relative expression of our target genes:

ΔΔCT = ΔCT test − ΔCT cal ;

Lastly, the expression of the ΔΔCT ratio was calculated:

Ratio = 2^(−ΔΔCT) .

The ratio obtained quantifies the increment or decrement of the target gene in CIPO patients with respect to the control samples and normalized with regards to the reference gene expression. The normalization discards any possible difference due to the imbalances in the quantity of analysed cDNA after repeated PCR reactions and gives a high accuracy to the results.

If ΔΔCT > 0, it means that ΔCT (test) > ΔCT (cal), so ratio <1, indicating an under expression of the test target gene respect to control (cal). On the contrary, if ΔΔCT < 0, it means that ΔCT (test) < ΔCT (cal), so ratio >1, indicating an over expression of the test target gene respect to control. Nevertheless, the ratio value indicates a considerable over expression or under expression only if its ratio is >2 or <0.5, respectively.

8. Statistical analysis

A descriptive analysis of the samples was performed with tables and graphs corresponding to the type of qualitative or quantitative variables. For what concerns discrete variables, the presence of statistically significant differences was determined by using the X^2 test. The Mann-Whitney U test has been used for pairwise comparisons while the Kruskal-Wallis test followed by the Dunn's post hoc test was employed for multiple comparisons.

The statistically significance of partitions between groups was evaluated with the Permutational Analysis of Variance (PERMANOVA), while correlations between the relative abundances of taxa at species levels, the considered clinical variables and the expression of 5-HT related genes and have been determined by the Spearman's rank correlation computed with the Vegan package in R.. When necessary, the computed p values have been corrected by using the Benjamini-Hochberg procedure

to take into account for multiple comparisons. In each case, a p value ≤ 0.05 was considered statistically significant.

RESULTS

1. Clinical parameters

Evaluation of clinical parameters showed that only the ferritin value was significantly lower in CIPO patients than in controls (p-value<0.01). Thus, we can describe a homogeneous population in terms of patient characteristics. Median, interquartile range (IQR) and significance of all clinical parameters and demographic variables, such as sex, age and weight, are shown in Table 6.

	CIPO patients (No. 7)	*Control patients (No. 7)*	*Significance*
Sex male n (%)	5 (71%)	2 (29%)	No
Age (years) Median (IQR)	8.0 (14 – 5)	10.0 (14 – 5)	No
Weight (Kg) Median (IQR)	25 (35 – 15)	42 (69 – 12.5)	No
PCR (mg/dl) Median (IQR)	0.115 (0.53 – 0.01)	0.05 (0.28 – 0.04)	No
Hb (g/dl) Median (IQR)	12.75 (14 − 12.225)	12.5 (14.1 − 12.2)	No
Ht (%) Median (IQR)	39.5 (42 – 36)	37 (41− 36)	No
Ferritin (ng/ml) Median (IQR)	32.5 (48 – 12)	73 (90 – 51)	Yes (p <0.01)
Calprotectin (mg/kg) Median (IQR)	64 (72 – 51)	76 (82 – 34)	No
Total proteins (g/dl) Median (IQR)	6.5 (6.5 – 6.2)	6.7 (6.9 – 6.1)	No
Albumin (g/dl) Median (IQR)	4.3 (4.6 – 4)	4.6 (4.7 – 4.3)	No

Table 6. Demographic and clinical variables relative to the studied population of subjects.

2. Mucosa-adherent microbiota characterization

All results reported are relative to the colon district, the only one with significant differences between CIPO patients and control group.

This pilot study enrolled seven paediatric CIPO patients with the aim to determine the presence of a disease-associated MAM in this cohort. The bioinformatic analysis led to the determination of 159 OTUs, which were assigned to 44 bacterial genera and 29 different species. In the colon district, evaluation of α diversity by the Shannon index and the number of observed OTUs showed a significant lower biodiversity in CIPO patients than in controls (Fig 11.a). The β diversity analysis showed statistically significant partition between CIPO and controls population, but only on the basis of the microbial composition present in the colon district (Fig. 11.b).

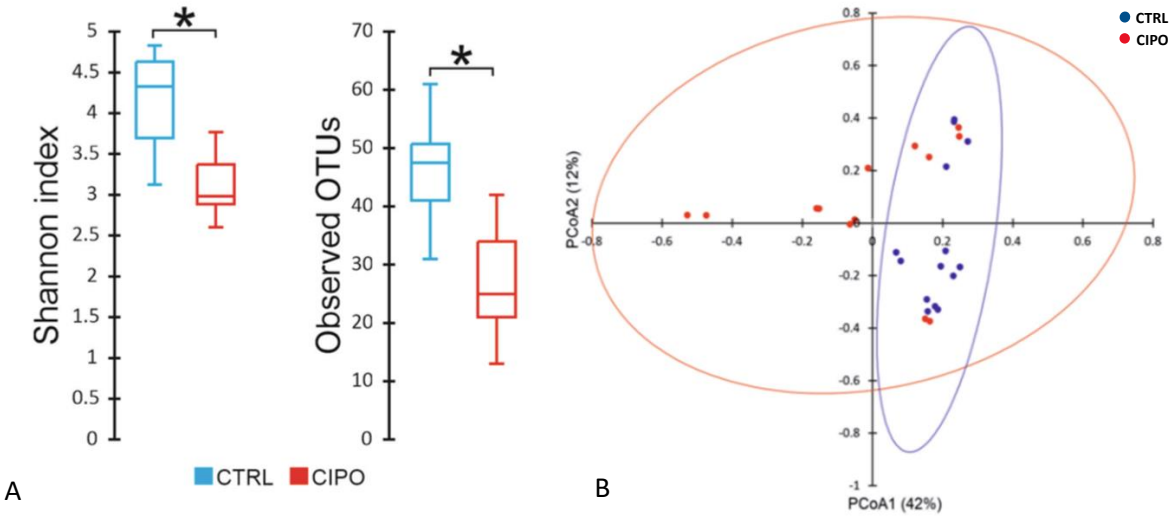

Figure 11. A) Color-coded box and whisker plots showing the distribution of the alfa-diversity estimators among CIPO and Non CIPO patients. * Statistical significance at alpha level 0,05. B) PCoA analysis performed for β diversity based on the Bray–Curtis measure of dissimilarity. For each principal coordinate, the percentage of variance explained is reported between parentheses. Ellipses represents 95% confidence intervals.

For colon associated population, the differences in the relative abundance of taxa, determined by taking into consideration only taxonomic group having a mean relative abundance ≥0.5% in at least one of the analysed groups, evidenced that bacterial species commonly considered beneficial, and

generally associated to health status, as *Akkermansia muciniphila*, *Ruminococcus bromii*, *Bacteroides uniformis* and *Faecalibacterium prausnitzii*, were significantly more abundant in controls respect to CIPO patients. On the other side, bacteria considered potentially pathogens, as *Clostridium difficile*, *Janthinobacterium lividum* and *Pseudomonas veronii*, were significantly more enriched in the CIPO group respect to controls, where they were totally absent (Fig. 12).

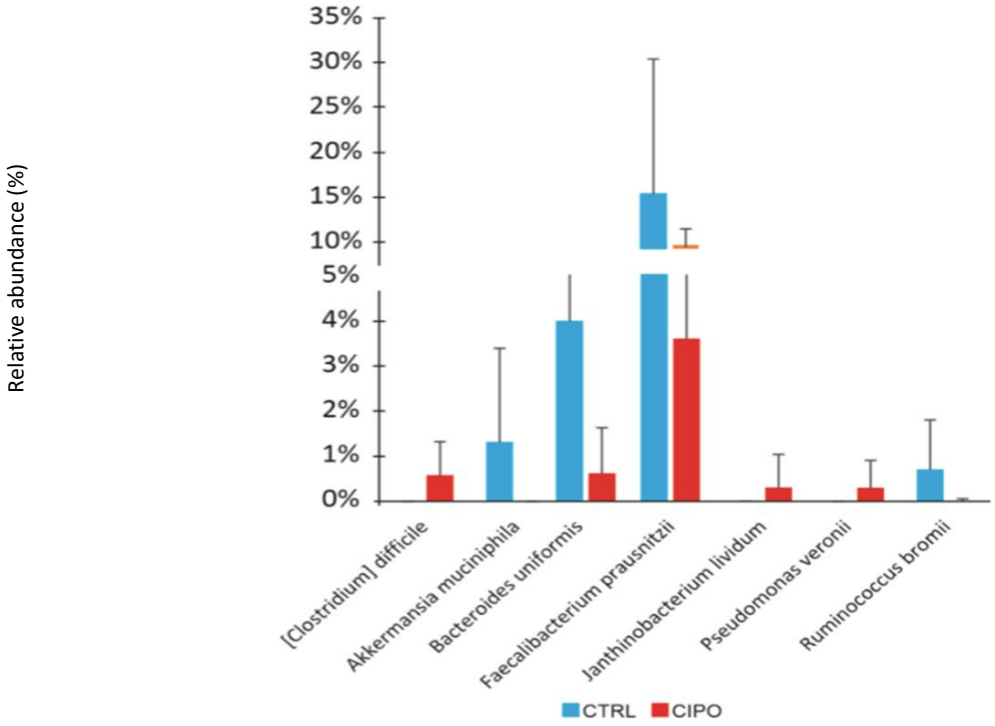

Figure 12. Color-coded bar plot showing differential abundance analysis at genus and species levels performed by Mann–Whitney U tests. Only taxa whose abundances were significantly different between groups at alpha level 0,05 were reported.

3. Network Analysis

Microbial networks were represented for CIPO patients and control subjects separately, investigating the interactions between taxa in the two groups (Fig. 4), always considering MAM composition of the colon district. The correlation networks described the positive and negative relationships between microbes, respectively drawn as green and red edges. A positive relation between two bacteria could reflect a synergic interaction, such as a metabolic interdependency;

conversely, a negative relation could represent an antagonistic interaction, such as a competition for ecological niches. In both patients' and controls' networks, results evidenced the presence of two microbial modules, in which taxa correlated with synergistic interactions, while these two modules correlated with each other with antagonistic interactions. Observing the microbial networks, the key species, important in the antagonistic interaction between modules, are the same in CIPO and control group. However, these taxa differ in their relative abundance between the two groups; in particular, *C. difficile* and *J. lividum* are significantly more abundant in CIPO patients, while *B. uniformis* and *F. prausnitzii* are significantly more abundant in the control group. Still within the key species in the networks, but not correlating the two modules present, there are several species in common between the two groups analysed, while some seem to be lost in the CIPO patients: for example, *Bacteroides fragilis*, *Bifidobacterium bifidus*, and *Veillonella dispar* are species present only in the control network.

Moreover, CIPO patients showed a microbial network characterized by the participation of a more limited number of species, as well as, by a reduced number of connections with respect to the one determined for controls (Fig 13). Negative interactions were also more abundant when compared to controls (Fig 13), with less mutualistic/synergic relationships among bacteria. This altered balance in CIPO network could indicate the presence of a microbial dysbiosis in the gut ecosystem. The characteristics of the networks are described in Table 7.

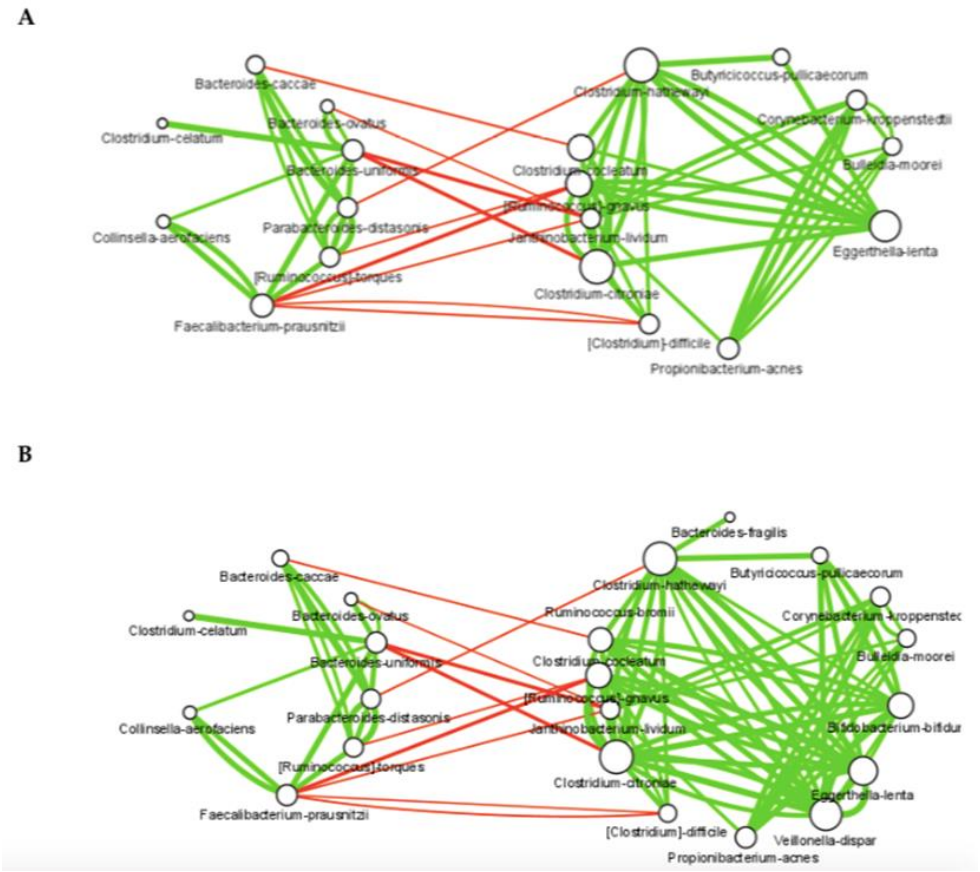

Figure 13. Co-occurrence networks in CIPO patients relative to the colon district (A) and control patients (B). Every bacterial species is represented as a node in the network, and the size of the node is proportional to the number of connections with other nodes in the ecosystem. The connections between nodes are represented by lines (or edges). The thickness of the lines is proportional to the number of links observed between the two species correlated within the considered cohort. If correlation ≥ 0.4, there is a positive connection between two bacterial species (green). If correlation< 0.4, the connection between two species is negative (red).

	Control subjects	CIPO patients'
Number of nodes	23	18
Number of edges	87	50
Edges to nodes ratio	3.78	2.7
Positive to negative edges ratio	7.7	4.1
Network density	0.32	0.25

Table 7. Topological properties of interactions' networks obtained for CIPO patients and control subjects.

4. Serotonin pathways genetic expression

The results of qRT-PCR revealed a general decrease in the expression of serotonin-related genes for CIPO patients when compared to controls in almost all the anatomical locations analysed. The expression profiling of up- and down-regulated genes is represented in Figure 14, and the specific averages of fold changes are reported in Table 8. Our results evidenced an increased expression of *TpH1* gene in the duodenum, while the *SLC6A4* expression was decreased. The 5-HT receptors genes *HTR3A* and *HTR4* were not detected in the same intestinal tract. In the ileum, all the genes had a significant decrease in their relative expression, except *HTR3A*. Regarding the colon, the relative expression of all genes analysed decreased in CIPO patients compared with controls, but statistical significance was found only for the expression of *SLC6A4* and *HTR3* genes. Hence, the expression analysis revealed a general alteration in the serotonin pathway in CIPO patients respect to the control group.

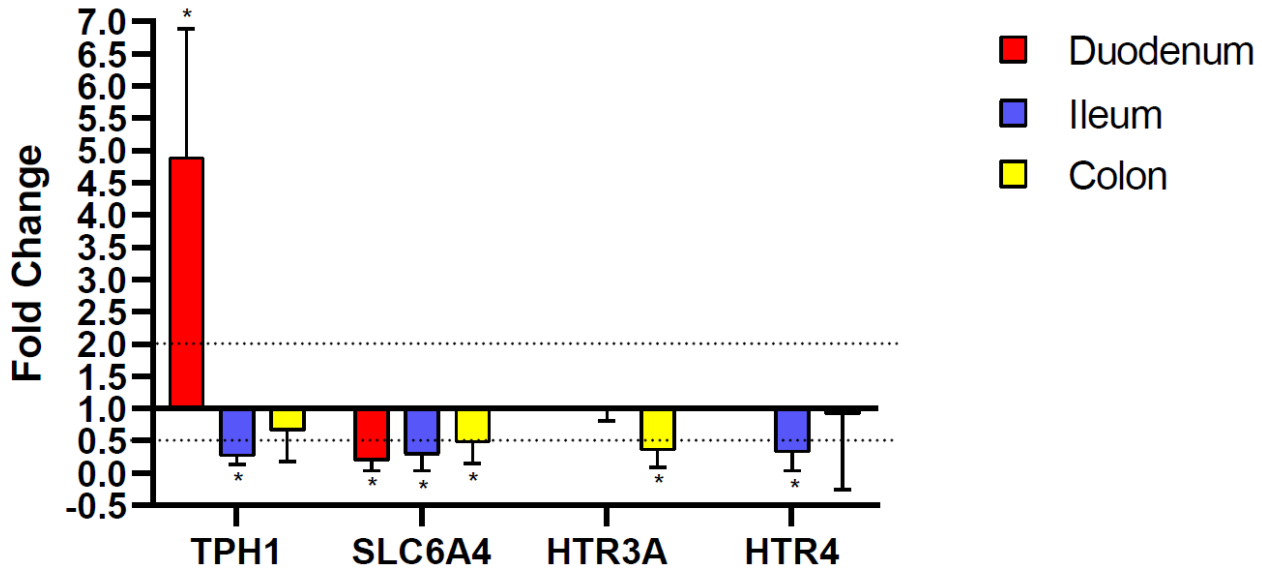

Figure 14. Relative expression of serotonin-related genes (*TpH1*, *SLC6A4*, *HTR3A* and *HTR4*) in CIPO patients compared to controls in different intestinal districts.

Gene	Duodenum	Ileum	Colon
TpH1	4,351843918	0,274784465	0,667919067
SLC6A4	0,203447837	0,299733093	0,476693965
HT3RA	n.d.	0,981906522	0,361638167
HT4R	n.d.	0,327973728	0,92170625

Table 8. Average fold changes of selected serotonin-related genes in CIPO patients compared to controls in different intestinal districts. N.d.: non-detected.

5. Correlation analysis

Correlation analysis between the expression of serotonin-related genes and the gut microbiota showed some significant but weak correlations, probably due to the low number of samples analysed. These correlations are shown in Table 9. It is possible to highlight a negative correlation between *P. veronii*, more abundant in CIPO patients, with the expression of *SLC6A4*, a gene under-expressed in all the GI tract analysed of these patients. Otherwise, the *B. uniformis*, resulted to be a network key species in both contests studied, but more abundant in control group, is positively correlated with *TpH1* and *SLC6A4*, indicating its possible importance in the expression of these genes. The strongest correlations were in *Ruminococcus gnavus* and *Bacteroides ovatus*, key species in the networks of both groups, but with no significant differences in their relative abundances, which were positively correlated with the *HTR3A* gene.

Gene	**Bacteria**	**Correlation**
TpH1	*Bacteroides uniformis*	0,061
SLC6A4	*Bacteroides uniformis*	0,066
	Pseudomonas veronii	-0,057
HTR3A	*[Ruminococcus] gnavus*	0,110
	Bacteroides ovatus	0,075
	Clostridium celatum	0,061
	Eggerthella lenta	0,064
HTR4	*[Ruminococcus] gnavus*	0,060
	Clostridium celatum	0,074
	Parabacteroides distasonis	-0,065

Table 9. Significant correlation values between gut microbes and serotonin-related genes expression.

DISCUSSION

CIPO is a rare disturb of the intestinal motility, which diagnosis and treatments still require investigation. Its incidence and prevalence are not well estimated, but the onset is usually paediatric. CIPO represents the most severe form of GI dysmobility and can affect any GI tract, although the small intestine and colon seem to be more frequently involved (2). The pathogenic mechanisms underlying paediatric CIPO are poorly understood, the management of this highly disabling condition is challenging and frustrating for patients, their families, and physicians. Medical therapies are primarily aimed at avoiding complications such as sepsis or intestinal bacterial overgrowth and, where possible, restoring intestinal propulsion. To date, management of intestinal motility disorders is treated with the use of prokinetic agents, which interact with 5-HT receptors (such as *5-HT3* and *5-HT4*) or with its reuptake enzyme (*SLC6A4*). The present study aimed to evaluate whether a specific MAM could be related to this pathology and whether dysfunctions in the serotonin pathway were present in paediatric CIPO patients. Finally, through correlation analysis, we examined the relationships between MAM and the expression of serotonin-related genes.

At the colon level, we could assess that a specific MAM was associated with CIPO patients. Significant differences in α-diversity and β-diversity were highlighted between CIPO and control groups. Always in the colon tract, we evidenced differences in the relative abundances of some bacterial taxa. Particularly, a significant major relative abundance of taxa potentially pathogens, like *C. difficile* and *P. veronii,* was reported in CIPO group. On the contrary, beneficial species, considered as probiotics and generally associated with a healthy status, such as *A. muciniphila*, *R. bromii*, *B. uniformis* and *F. prausnitzii*, were significantly less abundant in CIPO group respect to controls. Reduced levels of *A. muciniphila* have already been observed in patients with IBDs and metabolic disorders, suggesting its potential anti-inflammatory role (186). Some studies also defined the anti-inflammatory bacterium *F. prausnitzii* as a healthy microbial ecosystem index (187). These bacteria are producers of butyrate and other SCFAs through fermentation of dietary fiber (188). *A. muciniphila* is inversely associated with obesity, diabetes, cardiometabolic disease, and inflammation (189). *B. uniformis* reduces cholesterol and triglyceride concentrations, repress blood glucose, insulin and leptin (190).

Microbial connections in MAM ecosystems were analysed by network analysis for the CIPO and control groups. The results obtained showed the presence of two modules, in which the species within appeared positively correlated. The two modules were related to each other by antagonistic connections, mediated by bacteria found to be key species in both groups. Among them, some species showed significant differences in their relative abundance in the two groups: for example, *C. difficile* and *J. lividum* were significantly more abundant in CIPO patients, while *B. uniformis* and *F. prausnitzii* were significantly more abundant in the control group. Interestingly, the antagonistic connections between the two modules were mostly between bacterial species known to be beneficial, and potentially pathogenic bacteria (Fig. 13.).

For example, in CIPO patients, when the relative abundances of *C. difficile* and *J. lividum* species increased, the relative abundances of their negatively related beneficial species, *F. prausnitzii* and several species of *Bacteriotedes*, decreased. (Fig.13). In contrast, in the control group, the increases in *B. uniformis* and *F. prausnitzii* species leaded to the decrease in *C. difficile* and *J. lividum* species. Therefore, although the key species linking the two modules are the same in the CIPO and control groups, their different relative abundance will exert different selective pressure and the composition of the resulting MAM will be different.

Moreover, differences between the two networks were found in the number and kind of connections. In CIPO network we found a lower number of connections, particularly in the synergistic ones, and few species represented respect to control. Loss of mutualistic/synergistic connections is indicative of a dysbiosis status, as the loss of biodiversity and potentially beneficial species. We report for the first time in CIPO patients, at least at the colonic level, a characteristic MAM that seems to be dysbiotic. The results, which show differences only in the colonic district in paediatric CIPO patients, are in line with literature data reporting the colon as one of the first segments affected by the onset of disease (2).

Analysis of the expression of serotonin-related genes showed altered 5-HT pathway in CIPO patients compared with controls. In almost all sites considered, we observed a lower expression of the selected genes. Only in duodenum the *TpH1* gene was overexpressed with parallel under-expression of *SLC6A4*, indicating a regional specificity in the expression of these genes. The results indicated that an alteration in the serotonergic pathway is linked to paediatric CIPO patients. Our results support a study conducted in GF mice reporting serotonin deficiency in the distal, medial, and

proximal colon, but not in the small intestine, suggesting a specific role of the microbiota in the regulation of 5-HT in the colon (171).

Moreover, in the same study, decreased expression of the *TpH1* gene and elevated colonic expression of the 5-HT transporter *SLC6A4* (171) were reported in GF mice; the hypothesis of a compensatory effect between these two genes expression was confirmed through chemical inhibition of TpH1 expression on mice, which was able to modulate the expression of *SLC6A4*. In our results, we can observe this compensatory effect only in the duodenal district where, as already described, the *TpH1* gene is overexpressed and *SLC6A4* under-expressed, whereas in the other GI districts considered, this peculiarity is not evidenced. Since there are no enzymes for intercellular degradation of 5-HT in the intestinal tract, its elimination occurs only through a specialized transport mechanism (SLC6A4), thus the lack of this modulatory effect between the two genes (*TpH1* and *SLC6A4*) in the main districts involved in CIPO pathology (colon and ileum), could be an important aspect in the malfunction of the serotonin pathway.

The expression of *5-HT3* and *5-HT4* receptor genes, which are essential for proper peristalsis function (150, 164), was found to be generally reduced in all districts analysed. Our results, showing a decreased expression of serotonin receptor genes, support our initial hypothesis of serotonergic pathway malfunction in CIPO patients.

Probably due to the limited number of patients involved, correlation analysis evidenced only weak but significant correlations between bacterial species and serotonin-associated genes expression (Tab. 9). The negative correlation evidenced between *P. veronii*, more abundant in CIPO patients, and *SLC6A4* gene expression, is corroborated by the detected under-expression of this gene in this group, indicating a possible link between gut microbiota and 5-HT reuptake. In contrast, *B. uniformis*, the bacterial species that was found to play an important role in the MAM ecosystem of the control group, was positively correlated with the expression of the *TpH1* and *SLC6A4* genes: result supported by the detected lower expression of these genes in the patients group compared with controls. The strongest significant positive correlation was between *R. gnavus* and the serotonin receptor genes *HTR3A* and *HTR4*. *R. gnavus* is a mucosa-associated microbe found in 90% of people (191), reported as a mucus-degrading bacterium that is able to modulate mucins expression and intestinal glycosylation (192). In our CIPO and control groups, *R. gnavus* was equally present in MAM, resulting in both being a key species in the network. Correlation analysis results,

although showing significant but weak correlations, support the idea that the microbiota influences the expression of serotonin-related genes.

Concerning clinical parameters, only intestinal ferritin was significantly lower in CIPO patients than in controls, indicating serum ferritin as a possible marker of the disease. Notably, a low ferritin level indicates a low iron store in patients and could be the consequence of impaired iron absorption or blood loss: this condition has been reported in subjects with IBDs, where iron absorption is described as down-regulated (193). Furthermore, several studies indicate that microbiota can influence iron level, and *vice versa* (193–195). The correlation analysis between MAM and clinical parameters showed no significant correlations, but we should probably increase the number of CIPO patients involved in the study.

CONCLUSIONS

Our initial hypothesis was that abnormalities in serotonergic signalling pathways, which might be triggered/linked by alteration of the gut microbiota, could underlie the dysfunction of intestinal motility in patients with CIPO. Our results clearly indicate, for the first time in CIPO patients: i) a specific, colon-associated MAM; ii) an altered expression of serotonin-related gut genes; and (iii) significant, but weak, interconnections between the microbiota and serotonin-related genes expression, suggesting that alterations in this pathway seem to be related to the altered MAM. Further studies will be needed to improve and confirm our results, with the involvement of a larger number of patients. However, our results represent a first step in delineating the pathophysiology of CIPO, indicating variations of serotonergic pathways, triggered by or consequence of MAM alterations, which could symbolize the mechanism underlying the GI motility of CIPO.

In addition, our results could be the starting point for the selection of new disease markers and new therapeutic targets for alternative drug treatments aimed at modulating the gut microbiota, or to complement existing therapies to improve the management of CIPO patients.

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