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Impact of gut Microbiome on clinical management of critical patients

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Introduction

The role that the intestinal bacterial microflora or microbiota plays in maintaining the homeostasis of the human organism has been known for many years, but only recently, through the use of increasingly efficient cultivation techniques and molecular biology, have there been significant progress in the study of endogenous microflora(1,2).

The interest of the scientific community has been focused on the interactions between the intestinal microbiota and the host1, with particular regard to the physiological and/or pathological conditions that can cause an alteration of the composition and number of microorganisms present in the human intestine(3). On the other hand, numerous studies have already proposed the use of bacteria with beneficial effects on human health or probiotics, in the treatment of some pathological conditions, such as infectious diarrhoea, irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD)(4). In this regard, the deepening of studies on the role of the intestinal microbiota in the carcinogenesis of the colon mucosa, from the formation of adenomatous polyps to that of colorectal carcinoma, assumes considerable importance, with the aim of reducing its occurrence in humans(5).

Gut Microbiota

Definition

By intestinal microbiota we mean the set of microorganisms, their genes and their metabolites, present in the human gastrointestinal tract(6,7). Depending on the type of relationship that the microbiota establishes with its host, we consider symbiotic, commensal and/or pathobiont microorganisms: in the first case, both the microbiota and the individual benefit from their association, in the second case, it is only the microorganisms to take advantage of the association with the host, without causing damage or benefits to it and finally, in the third case, the bacteria, harmless in normal conditions, can cause damage to the host in pathological conditions(8). Within the gastrointestinal tract, the sources

of nourishment for the microbiota consist essentially of the substances ingested with the individual's diet and of various components present at the level of the intestinal mucosa, such as mucus and flaking epithelial cells. Normally, the individual's immune system and the intestinal microflora are in a condition of dynamic equilibrium which, in the event of an alteration of the composition of the microflora, can be disturbed and would be associated with the appearance of gastrointestinal pathologies, such as irritable bowel, inflammatory diseases chronic, diverticulitis and/or colon cancer and systemic, such as allergies, obesity, type 2 diabetes and atherosclerosis(2).

Composition

With development of new molecular biology techniques based on the sequencing of the 16S subunit of ribosomal RNA, researchers have been able to identify and classify the constituents of the intestinal microflora(9). This complex ecosystem includes both autochthonous species, commensal microorganisms acquired at birth or, as recently hypothesized, transferred in the prenatal phase from the mother to the fetus since they are already present in the amniotic fluid, in the placenta or in the cord blood, as well as transitory species of environmental origin(10,11).

The gut microbiota is composed by multiple commensal microbial species including 100 trillion (10^{14}) bacteria, quadrillion viruses, fungi, parasites, archeas and yeasts, reaching an overall biomass of about 1 kg and more than 3 million of genes. The different gastrointestinal regions are characterized by a different bio-compartmentalization with a distinct and stable microbial community(12). This is influenced by the acid environment, the presence of bile and pancreatic secretion and the high peristaltic activity in the stomach and small intestine, which do not allow a stable bacterial colonization, differently from the colon where bacterial colonization is favored by the low redox potential and the slow transit. The majority of microbes forming the human microbiota can be assigned to five major phyla: Bacteroidetes, Firmicutes, Proteobacteria Actinobacteria and Verrucomicrobia.

Gut microbiota is pivotal in the maintenance of intestinal barrier functions, increasing tight junctions expression, regulating mucin biosynthesis and catabolism, providing energy for epithelial cells

proliferation and stimulating the immune system. In this scenario, Actinobacteria are absolute players in maintaining gut barrier homeostasis.

Firmicutes and Bacteroidetes represent more than 90% of the relative abundance of the gut microbiome and their relationship plays a pivotal role in the maintenance of gut homeostasis. Actinobacteria and Proteobacteria represent the remaining 10%.

Bacteroidetes are the largest Phylum of Gram-negative bacteria inhabiting our gastrointestinal tract and are considered the leading players of the healthy state and sophisticated homeostasis safeguarded by gut microbiota.

Bacteroidetes are involved in immune modulation (activation of inflammation, autoimmune diseases), in metabolic syndrome and also in the regulation of the gut–brain-axis, with interesting therapeutic implications in mood impairment and neurologic disorders.

Firmicutes play a significant role in the relationship between gut bacteria and human health. Many of the members of this Phylum break down carbohydrates in the gut that can't be digested by the body's enzymes, such as dietary fiber and resistant starch.

Proteobacteria constitute a small percentage of the gut microbiota in healthy individuals and are characteristically facultative anaerobes but an increasing amount of data identifies Proteobacteria as a possible microbial signature of disease.

Verrucomicrobia resides in the mucous lining of the intestinal tract, where they can be found in high abundance in healthy individuals; this discovery suggests that Verrucomicrobia aid in glucose homeostasis of the human gut. Verrucomicrobia is not known to cause gastrointestinal related problems in the human gut(13).

In total, the bacterial cells that make up the intestinal microflora are 10^{14}, a number 10 times higher than that of human eukaryotic cells: this is why the human intestinal microbiota contains a number of genes at least 100 times higher than that of the genes that make up the human genome and can be considered a real supplementary genome for the host, called microbiome. The intestinal microbiota, which includes over 70% of the microorganisms present in the human body, varies considerably in

terms of quantity and quality in the different portions of the gastrointestinal tract and in particular, the bacterial density increases exponentially passing from the upper to the lower portion of the intestine. A lower bacterial density is recorded in the stomach and duodenum, while in the small intestine (jejunum and ileum), this density increases, to then reach its peak in the colon. The qualitative and quantitative variations that are recorded along the entire digestive tract depend on bacterial factors (adhesion capacity and metabolic activity) and on host-related factors, which are subdivided into extrinsic factors (diet, drug intake and environmental factors) and intrinsic (pH, motility and transit time, mucus, gastric acids and gastrointestinal secretions and presence of oxygen). Thus, the colon has a high bacterial density due to the slow transit of the ingested material, but also to the low redox potential characteristic of this tract and is therefore the only portion of the intestine in which the microflora stabilizes permanently(14,15). Conversely, the bacterial population is scarce in the stomach due to the extremely acidic pH of the gastric environment and is mainly made up of Lactobacilli, Streptococci and Yeasts, which reside in the mucosal layer lining the gastric epithelium. The duodenum is also poorly colonized by microflora due to the rapid transit of ingested food, the secretion of biliary and pancreatic fluids, which eliminate most of the microorganisms present and the effect of the propulsive motor activity of the intestine which prevents stable colonization of the light.

Functions

The intestinal microflora performs various functions that contribute to the maintenance of a good state of health of the individual, including metabolic, trophic and defence functions and also plays an important role in the regulation of immunity and systemic inflammation. As far as metabolic activity is concerned, it is known that the flora contributes to the production of vitamins K and B12, biotin and folic acid, as well as intervening in the metabolism of bile acids and bilirubin, forming secondary bile acids through deconjugation and dihydroxylation of the primary ones(16).

The microbiota also participates in the saccharolytic fermentation process, through which non-digestible polysaccharides such as cellulose, gums and pectins are converted into volatile substances

(carbon dioxide) and short-chain fatty acids or SCFAs (propionic acid 5 and butyric). SCFAs improve the absorption of calcium, magnesium and iron, are essential in maintaining optimal acidification of the intestinal pH, which constitutes an efficient defence system against pathogens, and their production stimulates cell growth and proliferation/differentiation epithelial intestines. Butyric acid is the main energy source of colonic epithelial cells and has an antitumor action (modulating growth and differentiation in epithelial tumor cell lines in vitro and favouring the return to a non-neoplastic phenotype, through blocking the expression of cyclooxygenase-2 and the induction of apoptosis in colon adenomas and carcinoma). As regards the defence and modulation activity of the immune system, the resident flora constitutes a valid line of resistance to colonization by exogenous bacteria, indirectly, by competing for their attachment sites on the brush border of intestinal epithelial cells and directly, by producing antibacterial substances. Immune system and microbiota are in constant equilibrium with each other: when the intestinal mucosa triggers the innate immune system via the Toll-like receptors or TLRs, which recognize and bind various microbial macromolecules (lipopolysaccharide, flagellin and peptidoglycan), initiates a cascade of signals that lead to the production and release of peptides, cytokines and phagocytes that contribute to the defence of the organism. It is therefore useful that the intestinal immune system does not react uncontrollably to the microbiota, so as to avoid the possibility of developing autoimmune diseases. The study of the microbiota and the role it plays in human health has prompted researchers to try to modify this microhabitat, through the use of or the variation of the diet, but above all the administration of probiotic bacteria(16,17).

Studies published in the last decade confirmed that the gut microbiome is implicated in the pathogenesis of various diseases, such as cancer and autism, depression, *Clostridium difficile* infection, inflammatory bowel disease, irritably bowel syndrome, colorectal carcinoma, infectious and non-infectious chronic liver diseases, obesity, diabetes mellitus type 2, atherosclerosis, and chronic kidney diseases(18).

Gut microbiome as a “virtual metabolic organ” makes axis with a number of extraintestinal organs, such as kidneys, brain, cardiovascular, and the bone system, but the gut-liver axis attracts increased attention in recent years (19).

Gut – Liver Axis

The crosstalk between the gut and liver is increasingly recognized, strengthened by the parallel rise in liver diseases and gastrointestinal (GI) and immune disorders(20,21). The most common type of liver disease, non-alcoholic fatty liver disease (NAFLD), alone affects more than 65 million Americans with a cost burden of $103 billion annually within the US itself(22). Intestinal dysbiosis and increased intestinal permeability leads to translocation of microbes and microbial products including cell-wall components (endotoxins from gram-negative bacteria, β-glucan from fungi) and DNA, together referred to as microbial- (or pathogen-) associated molecular patterns (MAMPs/PAMPs). These patterns are recognized by immune receptors on liver cells such as Kupffer cells and hepatic stellate cells and lamina propria (an immune cell-rich tissue beneath the intestinal epithelium) which initiate and maintain inflammatory cascades that ultimately lead to liver damage in the form of fibrosis. This damage can progress from cirrhosis (severe fibrosis) to hepatocellular carcinoma (HCC), the most predominant form (more than 80%) of primary liver cancers (Figure 1). Previously demonstrated associations between intestinal health and several different types of neoplasia suggest a potential role of the microbiome in HCC. Additionally, the liver and microbiome engage in co-metabolism of xenobiotics including carcinogens, which can independently predispose the host to HCC. These findings encourage microbiome-oriented therapeutic modalities to treat liver-associated as well as other metabolic diseases (23).

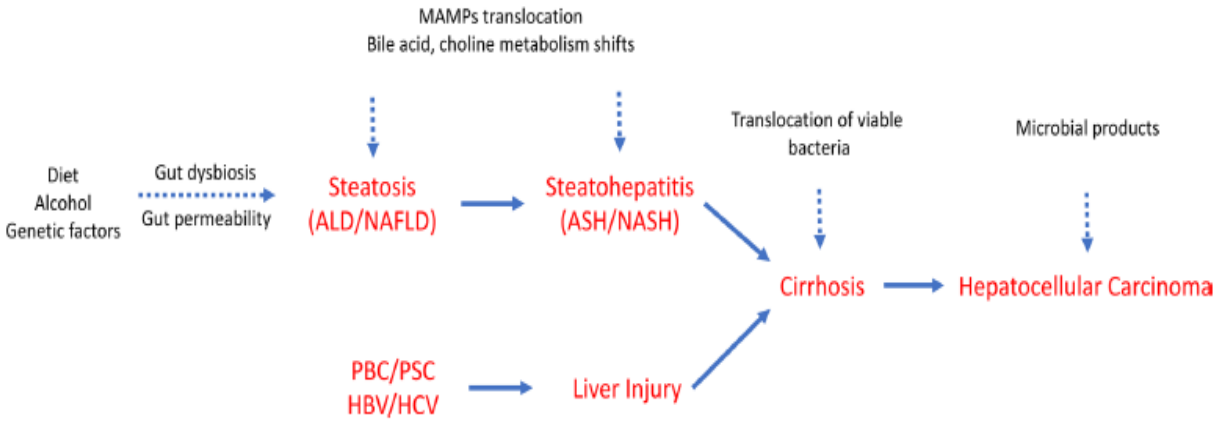

Figure 1. Physiological manifestations of liver injury along a spectrum of progression.

Risk factors such as alcohol abuse, unbalanced diet, infection (HBV/HCV) or immune dysfunction (PBC/PSC) can independently lead to liver injury. Alcohol-abuse patients and obese individuals often develop steatosis (fatty liver), which is characterized by increased intestinal permeability and dysbiosis. Subsequently, bile acid and choline homeostasis are disturbed along with increased translocation of MAMPs across the gut-barrier, leading to steatohepatitis, the progressive form of liver damage. Both, steatosis-dependent and steatosis-independent liver damage can progress to cirrhosis (end-stage liver damage), which is marked by translocation of viable bacteria to the liver and severe inflammation. As liver function is progressively compromised, tumor-promoting metabolites and xenobiotics accumulate. These could activate oncogenic pathways causing hepatocellular carcinoma, the most predominant form of primary liver cancers.

MAMPs: Microbial-associated molecular patterns; ALD: Alcoholic liver disease; NAFLD: Nonalcoholic fatty liver disease; ASH: Alcoholic steatohepatitis; NASH: Nonalcoholic steatohepatitis; HBV: Hepatitis B virus; HCV: Hepatitis C virus; PSC: Primary sclerosing cholangitis; PBC: Primary biliary cholangitis.

How do the liver and gut communicate

The gut and liver communicate via tight bidirectional links through the biliary tract, portal vein and systemic circulation (Figure 2). Liver communicates with the intestine by releasing bile acids and many bioactive mediators into the biliary tract and the systemic circulation. In the intestine, host and microbes metabolize endogenous (bile acids, amino acids) as well as exogenous substrates (from diet and environmental exposure), the products of which translocate to the liver through the portal vein and influence liver functions(24,25).

Enterohepatic circulation of bile acids

Bile acids (BAs) are amphipathic molecules synthesized from cholesterol in the pericentral hepatocytes(26). These are conjugated to glycine or taurine and released in the biliary tract. On reaching the small intestine through the duodenum, BAs, together with other biliary components, facilitate emulsification and absorption of dietary fats, cholesterol, and fat-soluble vitamins(27). About 95% of the BAs are actively reabsorbed in the terminal ileum and transported back to the liver. The remaining five percent are deconjugated, dehydrogenated and dehydroxylated by the intestinal microbiota to form secondary bile acids, which reach the liver via passive absorption into the portal circulation. The liver recycles BAs and secretes them back to the biliary tract completing the “enterohepatic circulation” a system of exchange between the gut and the liver (28). A carrier-mediated process transports hydrophilic primary bile acids across cell membranes for uptake into intestinal epithelial cells. An imbalance in BAs and gut bacteria elicits a cascade of host immune responses relevant to the progression of liver diseases(29,30).

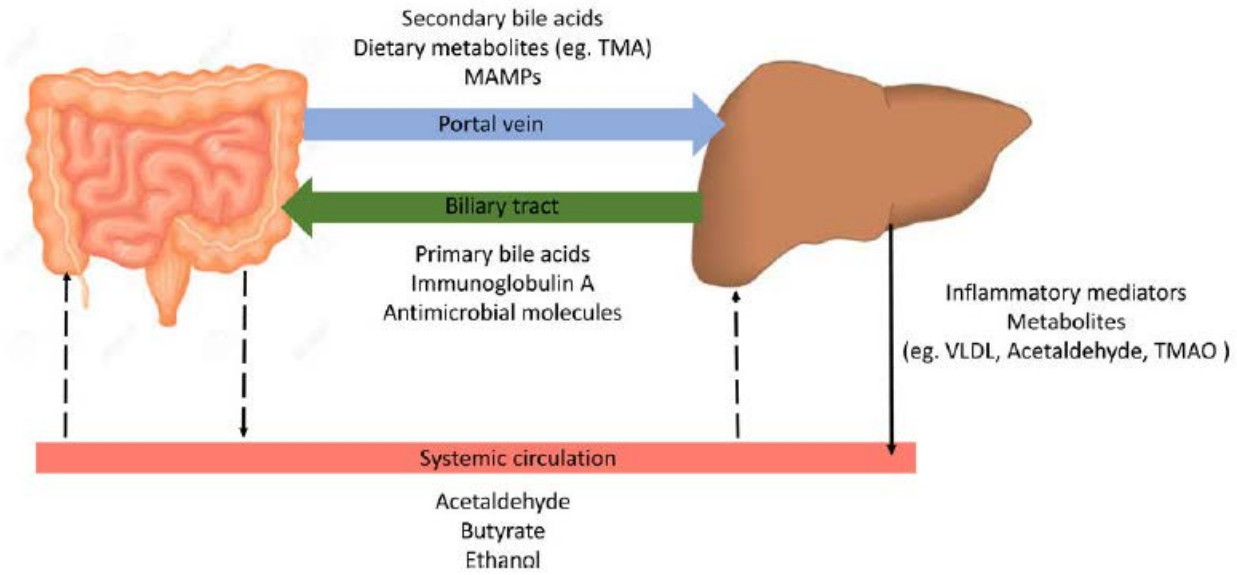

Figure 2: Bidirectional communication between gut and liver.

The liver transports bile salts and antimicrobial molecules (IgA, angiogenin 1) to the intestinal lumen through the biliary tract. This maintains gut eubiosis by controlling unrestricted bacterial overgrowth. Bile salts also act as important signaling molecules via nuclear receptors (such as FXR, TGR5) to modulate hepatic bile acid synthesis, glucose metabolism, lipid metabolism and energy utilization from diet. On the other hand, gut-products such as host and/or microbial metabolites and MAMPs translocate to the liver via the portal vein and influence liver functions. Additionally, systemic circulation extends the gut-liver axis by transporting liver metabolites from dietary, endogenous or xenobiotic substances (eg. FFAs, choline metabolites, ethanol metabolites) to the intestine through the capillary system. Owing to this medium of transport and ease of diffusion of systemic mediators across blood capillaries, these could affect the intestinal barrier both, positively (butyrate) or negatively (acetaldehyde). TMA: Trimethylamine; TMAO: Trimethylamine N-oxide; MAMPs: Pathogen-associated molecular patterns; VLDL: Very low-density lipoprotein; FXR: Farnesoid X receptor; TGR5: Takeda G-protein coupled receptor 5; FFA: Free fatty acid

Intestinal permeability

The central components of the intestinal barrier are enterocytes that are tightly bound to adjacent cells by apical junctional proteins that include claudins, occludins, junctional adhesion molecules (JAMs) and E-cadherins. This barrier restricts movement of microbes and molecules from the gut lumen, while allowing permselective, active transport of nutrients across the tight junctions(31,32). The intestinal barrier is further strengthened by several additional lines of defence:

- Mucins (heavily glycosylated protein aggregates) form a physical barrier between luminal bacteria and the underlying epithelial layer.
- Antibacterial lectins, such as regenerating islet-derived protein III-gamma (REG3G), which are produced by intestinal Paneth cells to target bacteria associated with mucosal lining.
- Immunoglobulins, specifically sIgA, produced by plasma cells and transported into the lumen through the intestinal epithelial cells that neutralize microbial pathogens by blockading epithelial receptors(33).
- Commensal bacteria are closely associated with the gut mucosa, and reinforce barrier integrity by stimulating cell-mediated immunity via toll-like receptor mediated signaling or by producing metabolites that directly strengthen tight junctions (short chain fatty acids) and inhibit other microbes.

Breakdown of one or more of these barrier components compromises gut-barrier integrity(34). The major drivers of increased permeability include gut inflammation and dysbiosis which have been linked to consumption of high-fat, Western diet, chronic alcohol consumption, prolonged antibiotic usage, and immune-mediated inflammatory diseases such as IBD. An important association between the gut microbiota, inflammation and gut-barrier integrity is provided by *Akkermansia muciniphila*, a gram-negative anaerobe that colonizes the intestinal mucus layer. Reduced abundance of *A. muciniphila* has been associated with thinning of mucus layer and increased inflammation promoting both, alcoholic and non-alcoholic liver damage(35–37). When the gut barrier is compromised, microbes and microbe-derived molecules can translocate to the liver through the portal system,

causing inflammation and hepatic injury. Some translocated intestinal products may also directly interact with host factors and contribute to exacerbation of liver disease(38,39).

Bacteria and MAMPs

Intestinal permeability is characterized by compromised tight junctions between enterocytes, and is consistently seen across the spectrum of liver diseases. Liver damage is associated with small intestinal bacterial overgrowth (SIBO) and microbial dysbiosis of the lower gastrointestinal tract. Together, these lead to increased translocation of MAMPs into the portal circulation. On reaching the liver, MAMPs induce localized inflammation through pattern recognition receptors (PRRs) on Kupffer cells and hepatic stellate cells. Endotoxin-mediated activation of Toll-like Receptor-4 (TLR4) along with TLR9 (activated by methylated DNA and TLR2 (activated by gram-positive bacteria) are the primary drivers of immune response in liver disease(40). TLR signaling in Kupffer cells activates downstream proinflammatory cascade, leading to MyD88 mediated activation of NF-kB. Additionally, TLR4 signaling also promotes fibrosis by down regulating Bambi, a decoy receptor for TGF-β. These lead to expression of inflammatory cytokines, oxidative and endoplasmic reticulum (ER) stress, and subsequent liver damage(41).

Choline metabolites

Choline is a macronutrient that is important for liver function, brain development, nerve function, muscle movement, and maintaining a healthy metabolism. Choline is processed into phosphatidylcholine (lecithin) by the host, which assists in excretion of very-low density lipoproteins (VLDL) particles from the liver(38,39). This prevents hepatic accumulation of triglycerides (liver steatosis). Additionally, choline can also be converted to trimethylamine (TMA) by intestinal bacteria. TMA can translocate to the liver through the portal circulation where it is converted to trimethylamine N-oxide (TMAO)(42,43). The significance of methylamines is increasingly being recognized with respect to liver, cardiometabolic and more recently, mental disorders. Increased systemic circulation of TMAO is concomitant with reduced levels of host-produced phosphatidylcholine, an imbalance characteristic of intestinal dysbiosis. This has been linked with liver damage due to increased

triglyceride accumulation (hepatic steatosis) and consequently, non-alcoholic fatty liver disease and liver tumorigenesis.

Free fatty acids

Free fatty acids include short-chain fatty acids (SCFA) and saturated long-chain fatty acids (LCFA). Butyrate and propionate (products of bacterial fermentation) are the dominant short chain fatty acids in the large intestine. Butyrate is an energy source for the enterocytes and facilitates maintenance of intestinal barrier(44,45). Alcohol-induced liver injury is suggestively marked by reduced butyrate and propionate and increased acetate (possibly produced by ethanol metabolism in the lumen, but predominantly derived from ethanol metabolism in the liver). Increased acetaldehyde can weaken gut barrier and induce hepatic stress on translocation of intestinal antigens to the liver. Butyrate supplementation in the form of a glycerol ester, tributyrin, reduced ethanol-induced intestinal permeability and subsequent liver injury in mice on a short-term alcohol diet(46–48).

Ethanol and acetaldehyde

The mucosa of the GI tract absorbs ethanol by simple diffusion. Within the GI tract, the majority of ethanol from food and beverages is absorbed by the stomach (~ 20%) and small intestine (~ 70%). Although, microbial fermentation contributes to luminal ethanol concentration, the biggest share of alcohol in the large intestine comes from the systemic circulation. Gut microbiota and enterocytes express alcohol-metabolizing enzymes such as alcohol dehydrogenase, aldehyde dehydrogenase co-metabolizing ethanol into acetaldehyde and, to a lesser-studied extent, acetate(49). The liver also responds to circulating levels of ethanol by upregulating its ethanol metabolism pathway. The importance of microbes for xenobiotics metabolism was underscored by a study that demonstrated an increase in hepatic expression of ethanol metabolizing genes in germ-free mice, and subsequent liver damage. Non-alcoholic and alcoholic liver diseases are characterized by increased luminal and circulating levels of ethanol and its metabolites, acetaldehyde and acetate(50,51). These metabolites have independently been associated to liver damage. Acetaldehyde has been implicated in weakening the intestinal tight junctions compromising the gut barrier and allowing translocation of microbial

products(52,53). It has also been associated with downregulating the expression of antimicrobial peptides (AMPs) in the intestine, and eliciting inflammatory and adaptive host immune responses. Additionally, ALD is marked by reduced intestinal butyrate (an energy source for enterocytes) which is linked to weakening of intestinal tight junctions and hence, permeability.

Links between the microbiome and specific liver diseases

Non-alcoholic fatty liver disease (NAFLD)

NAFLD refers to a spectrum of liver disease that can be broadly classified into two categories: non-alcoholic fatty liver (NAFL), the non-progressive form of NAFLD, and non-alcoholic steatohepatitis (NASH), the progressive form of NAFLD (54). NASH is generally linked to type 2 diabetes, cardiovascular risk factors and obesity, although incidences have also been reported in lean individuals, emphasizing that genetic and environmental factors also contribute to disease development. Several studies have stressed on the role of the gut microbiota in NAFLD however, causality is yet to be established(55–57). Patients with NAFLD have a higher prevalence of small intestinal bacterial overgrowth (SIBO) and microbial dysbiosis. Using 16S amplicon sequencing, bacterial genera, Bacteroides and Ruminococcus were significantly increased, and Prevotella was reduced in NASH patients with stage 2 fibrosis or higher. Loomba et al.(58) utilized whole genome metagenomics to characterize the gut microbiota in NAFLD patients with and without advanced fibrosis (stages 3 and 4) and showed an increased abundance of *Escherichia coli*, and *Bacteroides vulgatus* in advanced fibrosis patients. An enrichment of *Escherichia spp.* (genera) was also seen in pediatric NASH patients compared to obese controls. Consistent with preclinical studies, these studies indicate an association between gram-negative bacteria and progression of liver fibrosis. Genetically modified mouse models have been used to study NAFLD-associated gut dysbiosis and permeability for mechanistic insights in liver disease progression. Rahman et al.(59) used JAM-A (junctional adhesion molecule-A protein) knockout mice to demonstrate that deficiency in this tight junction protein is linked to increased intestinal permeability and liver inflammation(60,61). This

inflammation could be alleviated by administering antibiotics, underscoring the importance of microbial translocation in promoting immune response in the liver. Another group used muc-2 knockout mice and found that there was a compensatory increase in intestinal levels of Reg3b and Reg3g genes leading to an overall protective response against NAFLD(60). The contribution of liver-damaging inflammation in response to translocation of microbes and MAMPs was elucidated by Henao-Mejia and colleagues(61). Using NLRP3- and NLRP6- (inflammasome-) deficient mice models, they demonstrated an increase in influx of TLR4 and TLR9 in portal circulation, which enhanced the expression of hepatic tumor-necrosis factor (TNF)-α driving NASH progression. Furthermore, cohousing inflammasome-deficient mice with wild-type controls exacerbated hepatic steatosis and obesity in healthy cage mates, suggesting transferability of disease via the microbiome.

Alcoholic liver disease (ALD)

The manifestation of ALD in chronic alcohol abuse patients is a consequence of multifactorial interactions involving genetics, immune system, gut microbiome and environmental factors. Like NAFLD, non-progressive form of ALD is characterized by accumulation of fat inside liver (fatty liver or steatosis), while its progressive form is marked by inflammation and liver injury (alcoholic steatohepatitis or ASH)(62). Our understanding of the compositional and mechanistic contributions of the gut microbiota in ALD is improving with the increasing number of studies investigating this link. As in NAFLD, SIBO has been demonstrated as an important hallmark of alcohol-associated liver disease in humans and mice. Intestinal dysbiosis in alcohol-abuse patients is characterized by significant enrichment of Enterobacteriaceae and reduction in Bacteroidetes and *Lactobacillus spp.* It has also been demonstrated that alcohol-induced dysbiosis is only partially reversible by alcohol-withdrawal or probiotic treatment. Interestingly, alcohol-dependent patients also displayed reduced fungal diversity and *Candida spp.* overgrowth, presenting the first evidence of the role of gut mycobiome in pathogenesis of liver diseases. Genetically-modified murine models have advanced our mechanistic understanding of the contribution of various components of the gut-barrier in the etiology and progression of ALD. Furthermore, IgA knockout in mice led to increased levels of IgM

and a net protective effect against ASH progression(63). In response to ethanol-induced gut-barrier dysfunction and translocation, TLRs and other pathogen recognition receptors activate hepatic Kupffer cells and macrophages, as was demonstrated in male Wistar rats. This starts inflammatory cascades releasing TNF-alpha, IL-1, IL-10, IL-12, and TGF-beta. Using TLR-4 chimeric mice, it was shown that endotoxin-induced release of TGF-beta is mediated by MyD88-NF-kappaB-dependent pathway providing explanatory mechanism of inflammation-induced liver damage(64). Concomitant with immunological responses to barrier dysfunction, ALD is also marked by system-wide changes in many bioactive compounds. Alcohol consumption leads to an increase in hepatic bile acid synthesis humans and mice.

Hepatocellular carcinoma (HCC) and end-stage liver disease

The etiology of non-viral HCC follows a “multiple-hit” pathway, whereby liver steatosis followed by oxidative stress, endoplasmic reticulum stress together with intestinal dysbiosis and inflammation contribute to the final manifestation of cancer (65). The gut microbiota changes in composition dramatically in hosts suffering from HCC(66). *Clostridium spp.* have been found to be enriched in obesity-induced mouse models of HCC, but clinical studies with HCC patients detect an overgrowth of intestinal *Escherichia coli*. To get insights into the molecular events explaining the progression of liver disease to HCC, various murine models (diet-based, toxin plus diet-based and genetic plus diet-based models) have been explored. Accumulating evidence suggests that HCC-associated dysbiosis is accompanied by gut-barrier dysfunction, bacterial translocation, systemic circulation of their tumor-promoting metabolites and activation of proinflammatory and oncogenic signaling pathways(67). The intestinal poly-immunoglobulin receptor (PIgR) regulates the transport of IgA into the intestinal lumen and maintains microbial homeostasis. A recent study showed that PIgR−/− mice modelling NASH-induced HCC had increased systemic and liver IgA, and a concomitant increase in hepatic tumorigenesis due to localized inhibition of liver cytotoxic T cells that prevent HCC development(68). Further, the application of broad spectrum antibiotics has been shown to attenuate

liver inflammation and HCC-development in mice highlighting the role of the intestinal microbiome in liver tumorigenesis. End-stage liver disease (or cirrhosis) is an extreme manifestation of chronic liver injury characterized by loss of liver cells, thick fibrous scar, and regenerating nodules. This topic has been extensively reviewed recently so we only provide a brief discussion here. NAFLD, ALD, primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) or hepatitis can each progress to cirrhosis and constitute its subtypes. Currently, NASH is the second leading cause of adult cirrhosis in the USA. Depending upon the etiology of cirrhosis, there is a variable risk of developing HCC. Alterations in the gut microbiome including dysbiosis and SIBO have been associated with cirrhosis and its complications. Treatment for portal systemic encephalopathy and decompensated cirrhosis includes treatment with non-systemic antibiotics to reduce intestinal microbiota overgrowth. Gut microbiome alterations were observed in alcohol- and hepatitis-associated cirrhotic patients in a Chinese cohort, which observed an invasion of the lower intestinal tract by oral bacteria. Concordant with these findings, Chen and colleagues also found an overrepresentation of genera including Veillonella, Megasphaera, Dialister, Atopobium, and Prevotella in the duodenum of cirrhosis patients. The genera, Neisseria and Gemella were discriminative between hepatitis-B-virus- and PBC-related cirrhosis. All experimental models of liver fibrosis result in gut microbial dysbiosis and increased intestinal permeability and treatment of GI tract with nonabsorbable antibiotics decreases liver fibrosis(69–71).

Cirrhosis

Cirrhosis is the endpoint in patients who have chronic progressive liver disease(72). Patients with abnormal liver function who develop ascites, variceal hemorrhage, hepatic encephalopathy, or renal impairment are considered to have end-stage liver disease (ESLD). While liver transplantation is a viable treatment option for ESLD, with increasing waiting times for organ transplantation, nearly 17% of patients on the transplant wait list die annually; others are not candidates for a liver transplant.

Patients with ESLD have a constellation of symptoms and disease-related complications that affect survival and health-related quality of life.

When a substance or disease attacks and damages the liver, liver cells are killed and scar tissue is formed. This scarring process is called fibrosis and it happens little by little over many years. When the whole liver is scarred, it shrinks and gets hard. This is called cirrhosis, and this damage is irreversible.

Any illness that affects the liver over a long period of time may lead to fibrosis and, eventually cirrhosis. Some common causes are heavy drinking, viruses, a build-up of fat in the liver, inherited diseases, toxic effects from drugs and autoimmune diseases.

Cirrhosis has numerous causes. In the United States, heavy alcohol consumption and chronic hepatitis C have been the most common causes of cirrhosis. Obesity is becoming a common cause of cirrhosis, either as the sole cause or in combination with alcohol, hepatitis C, or both. Many people with cirrhosis have more than one cause of liver damage(73).

Cirrhosis is not caused by trauma to the liver or other acute, or short-term, causes of damage. Usually, years of chronic injury are required to cause cirrhosis. Common causes of cirrhosis are:

- Alcohol-related liver disease. Most people who consume alcohol do not suffer damage to the liver. But heavy alcohol use over several years can cause chronic injury to the liver. The amount of alcohol it takes to damage the liver varies greatly from person to person. For women, consuming two to three drinks-including beer and wine-per day and for men, three to four drinks per day, can lead to liver damage and cirrhosis. In the past, alcohol-related cirrhosis led to more deaths than cirrhosis due to any other cause. Deaths caused by obesity-related cirrhosis are increasing.
- Chronic hepatitis C: the hepatitis C virus is a liver infection that is spread by contact with an infected person's blood. Chronic hepatitis C causes inflammation and damage to the liver over time that can lead to cirrhosis.

- Chronic hepatitis B and D: the hepatitis B virus is a liver infection that is spread by contact with an infected person's blood, semen, or other body fluid. Hepatitis B, like hepatitis C, causes liver inflammation and injury that can lead to cirrhosis. The hepatitis B vaccine is given to all infants and many adults to prevent the virus. Hepatitis D is another virus that infects the liver and can lead to cirrhosis, but it occurs only in people who already have hepatitis B.
- Non-alcoholic fatty liver disease (NAFLD): in NAFLD, fat builds up in the liver and eventually causes cirrhosis. This increasingly common liver disease is associated with obesity, diabetes, protein malnutrition, coronary artery disease, and corticosteroid medications.
- Autoimmune hepatitis: this form of hepatitis is caused by the body's immune system attacking liver cells and causing inflammation, damage, and eventually cirrhosis. Researchers believe genetic factors may make some people more prone to autoimmune diseases. About 70% of those with autoimmune hepatitis are female.
- Diseases that damage or destroy bile ducts. several diseases can damage or destroy the ducts that carry bile from the liver, causing bile to back up in the liver and leading to cirrhosis. In adults, the most common condition in this category is primary biliary cirrhosis, a disease in which the bile ducts become inflamed and damaged and, ultimately, disappear. Secondary biliary cirrhosis can happen if the ducts are mistakenly tied off or injured during gallbladder surgery. Primary sclerosing cholangitis is another condition that causes damage and scarring of bile ducts. In infants, damaged bile ducts are commonly caused by Alagille syndrome or biliary atresia, conditions in which the ducts are absent or injured.
- Inherited diseases: cystic fibrosis, alpha-1 antitrypsin deficiency, hemochromatosis, Wilson disease, galactosemia, and glycogen storage diseases are inherited diseases that interfere with how the liver produces, processes, and stores enzymes, proteins, metals, and other substances the body needs to function properly. Cirrhosis can result from these conditions.

- Drugs, toxins, and infections: other causes of cirrhosis include drug reactions, prolonged exposure to toxic chemicals, parasitic infections, and repeated bouts of heart failure with liver congestion.

Liver cancer, also known as hepatocellular carcinoma (HCC), can also develop in cirrhosis when some of the damaged liver cells start to multiply out of control. As liver function deteriorates, one or more complications may develop, often the first signs of the disease.

- Edema and ascites: when liver damage progresses to an advanced stage, fluid collects in the legs, called edema, and in the abdomen, called ascites. Ascites can lead to bacterial peritonitis, a serious infection.
- Bruising and bleeding. when the liver slows or stops producing the proteins needed for blood clotting, a person will bruise or bleed easily.
- Portal hypertension: normally, blood from the intestines and spleen is carried to the liver through the portal vein. But cirrhosis slows the normal flow of blood, which increases the pressure in the portal vein. This condition is called portal hypertension.
- Esophageal varices and gastropathy: when portal hypertension occurs, it may cause enlarged blood vessels in the esophagus, called varices, or in the stomach, called gastropathy, or both. Enlarged blood vessels are more likely to burst due to thin walls and increased pressure. If they burst, serious bleeding can occur in the esophagus or upper stomach, requiring immediate medical attention.
- Splenomegaly: when portal hypertension occurs, the spleen frequently enlarges and holds white blood cells and platelets, reducing the numbers of these cells in the blood. A low platelet count may be the first evidence that a person has developed cirrhosis.
- Jaundice. jaundice occurs when the diseased liver does not remove enough bilirubin from the blood, causing yellowing of the skin and whites of the eyes and darkening of the urine. Bilirubin is the pigment that gives bile its reddish-yellow colour.

- Gallstones: if cirrhosis prevents bile from flowing freely to and from the gallbladder, the bile hardens as gallstones.
- Sensitivity to medications: cirrhosis slows the liver's ability to filter medications from the blood. When this occurs, medications act longer than expected and build up in the body. This causes a person to be more sensitive to medications and their side effects.
- Hepatic encephalopathy: a failing liver cannot remove toxins from the blood, and they eventually accumulate in the brain. The build-up of toxins in the brain-called hepatic encephalopathy-can decrease mental function and cause coma. Signs of decreased mental function include confusion, personality changes, memory loss, trouble concentrating, and a change in sleep habits.
- Insulin resistance and type 2 diabetes: cirrhosis causes resistance to insulin-a hormone produced by the pancreas that enables the body to use glucose as energy. With insulin resistance, the body's muscle, fat, and liver cells do not use insulin properly. The pancreas tries to keep up with the demand for insulin by producing more, but excess glucose builds up in the bloodstream causing type 2 diabetes.
- Liver cancer: hepatocellular carcinoma is a type of liver cancer that can occur in people with cirrhosis. Hepatocellular carcinoma has a high mortality rate, but several treatment options are available.
- Other problems: cirrhosis can cause immune system dysfunction, leading to the risk of infection. Cirrhosis can also cause kidney and lung failure, known as hepatorenal and hepatopulmonary syndromes.

Initially, patients may have no symptoms at all, this is called compensated cirrhosis. As cirrhosis progresses, the most common symptoms are: weakness, fatigue, loss of appetite, nausea, vomiting, weight loss, abdominal pain and bloating when fluid accumulates in the abdomen, itching, spiderlike blood vessels on the skin.

Cirrhosis progresses from compensated to decompensated cirrhosis when serious conditions develop as it worsens. These complications can be life-threatening and requires a new liver to replace the diseased one through a liver transplant. As discussed earlier, another serious complication of cirrhosis is liver cancer, which may occur in the compensated or decompensated stage. There may be no signs of liver cancer until the tumor is large and causing pain.

- Bleeding varices. internal bleeding from large blood vessels in the esophagus
- Ascites: a build-up of fluid in the belly.
- Encephalopathy: confusion from the build-up of toxins in the blood.

The model for end-stage liver disease (MELD) score measures the severity of cirrhosis. The MELD score was developed to predict the 90-day survival of people with advanced cirrhosis. The MELD score is based on three blood tests:

- international normalized ratio (INR)-tests the clotting tendency of blood
- bilirubin-tests the amount of bile pigment in the blood
- creatinine-tests kidney function

MELD scores usually range between 6 and 40, with a score of 6 indicating the best likelihood of 90-day survival.

The diagnosis of cirrhosis is usually based on the presence of a risk factor for cirrhosis, such as alcohol use or obesity, and is confirmed by physical examination, blood tests, and imaging.

A liver biopsy can confirm the diagnosis of cirrhosis but is not always necessary. A biopsy is usually done if the result might have an impact on treatment. The biopsy is performed with a needle inserted between the ribs or into a vein in the neck. Precautions are taken to minimize discomfort. A tiny sample of liver tissue is examined with a microscope for scarring or other signs of cirrhosis. Sometimes a cause of liver damage other than cirrhosis is found during biopsy.

Treatment for cirrhosis depends on the cause of the disease and whether complications are present. The goals of treatment are to slow the progression of scar tissue in the liver and prevent or treat the complications of the disease. Hospitalization may be necessary for cirrhosis with complications.

A liver transplant is considered necessary when complications cannot be controlled by treatment. Liver transplantation is a major operation in which the diseased liver is removed and replaced with a healthy one from an organ donor. Survival rates have improved over the past several years because of drugs that suppress the immune system and keep it from attacking and damaging the new liver.

The number of people who need a liver transplant far exceeds the number of available organs. A person needing a transplant must go through a complicated evaluation process before being added to a long transplant waiting list. Generally, organs are given to people with the best chance of living the longest after a transplant. Survival after a transplant requires intensive follow-up and cooperation on the part of the patient and caregiver.

Considering all the above evidences, it is therefore surprising that in the literature it is not possible to find substantial piece of works investigating the role of the enteric microbiome and intestinal permeability in influencing the different outcomes of liver transplantation.

Gut Microbiota and liver transplant

To date, there are few studies on how the gut microbiota changes following liver transplantation. In a study by Bajaj et al.(74) 45 patients were enrolled and the characteristics of the intestinal microbiota were evaluated before and after liver transplantation. Microbiota diversity was lower in transplant patients than in healthy controls with a trend towards significant increase within six months after transplantation. These results observed changes in microbial composition with an increase in Ruminococcaceae and Lachnospiraceae and a decrease in Enterobacteriaceae following transplantation.

In a similar study, Kato et al.(75) analyzed the intestinal microbiota of 38 liver transplanted patients and showed an increase in the post-transplant microbial diversity index with a significant peak after the first seven days post-transplant. The authors also demonstrated that poor pre-transplant liver function was associated with a prevalence of Enterobacteriaceae and Enterococcaceae compared to

Ruminococcaceae and Lachnospiraceae; less diversity associated with post-operative complications such as acute rejection and infections was observed following transplantation.

Alterations in gut microbial diversity following liver transplantation were also observed in a study by Sun et al.(76), who demonstrated an important difference in gut microbiota composition both before and after liver transplantation, particularly in post-transplanted patients the authors showed a profile similar to that of healthy controls.

Another study, conducted by Annavajhala et al.(77) , 177 transplanted patients were enrolled and authors analyzed changes in the gut microbiota up to one year post-transplant; the authors associated high MELD scores with poor pre-transplant diversity and associated some gut microbial profiles (decreased presence of the genus Bacteroides and the family Lachnospiraceae and prevalence of the genus Enterococcus) with an increased risk of post-transplant colonization from multiresistant microorganisms. Poor pre-transplant microbial diversity was therefore significantly associated with colonization by multidrug-resistant bacteria and post-transplant complications.

Currently, knowledge on how the intestinal microbiota changes following liver transplantation is still limited and the need for new studies is of utmost importance. A better understanding of these aspects is very useful for the early recognition of critical patients and can allow for a possible modulation of the composition of the intestinal microbiota aimed at favoring protective taxa.

Objectives of the proposed study

Our study aims to study the intestinal microbiome in critically ill patients, in particular patients on the waiting list and undergoing liver transplantation.

We propose to study longitudinally the stool microbiome of liver transplant patients and candidates and their intestinal permeability, both before and after the transplant at clinically useful time-points and in combination with a large panel of clinical, laboratory and functional parameters.

With this study we propose to:

- Understand and analyze the composition and any alterations of the intestinal microbiome before transplantation and therefore in conditions of liver disease.
- Understand and analyze the composition and any alterations of the intestinal microbiome following transplantation, considering the patient's conditions and the pharmacological treatments used in the post-operative period.
- Compare sequencing results to identify significant differences between subjects.
- Evaluate the role played by the enteric bacteria on the transplanted liver and understand the mechanisms underlying the gut-liver axis.
- Identify possible microbiome-based markers for the early stratification of patients, even before the transplant itself, in terms of risk of adverse outcomes.
- Evaluate the obtained results and define the potentially most effective and patient-tailored flora-modulating strategies.
- Improve the outcomes of liver-transplanted patients with new strategies.

In synthesis, the main objective of this study is to allow a better risk stratification of the patients undergoing liver transplantation through the identification of early enteric microbiome-based markers correlated to different clinical outcomes. This could possibly lead to the set-up of novel diagnostic and therapeutic algorithms allowing a better management of liver-transplanted patients.

Materials and Methods

Patients

The study included patients from the two main Italian liver transplantation centres (Ospedale Le Molinette, Torino and Azienda Ospedaliera Pisana, Pisa), allowing to enrol more than 200 patients in over the period of study of three years. More in details, all >18-years-old patients listed for and undergoing liver transplantation were included in the study after signing an informed consent. Each patient was then be prospectively followed one year. A second cohort of 55 patients was enrolled in the following 6 months as internal validation sample.

Study plan

We evaluated biological samples at three different time points for each transplant procedure:

1. Pre-transplantation assessment of the recipient (T0, at listing and then every three months on the waiting list): stool and blood
2. Early post-transplantation assessment of the recipient (T1, 7 days from surgery): stool and blood
3. Late post-transplantation assessment of the recipient (T2, 90 days from surgery): stool and blood

Starting from stool samples, we performed microbiome profiling and gut permeability analysis.

Starting from blood samples, a permeability analysis (hematic zonulin) and the evaluation of circulating catecholamines were performed. Moreover, the concentration of bacterial endotoxin and bacterial metabolites (short chain fatty acids, SCFAs) was evaluated (Figure 3).

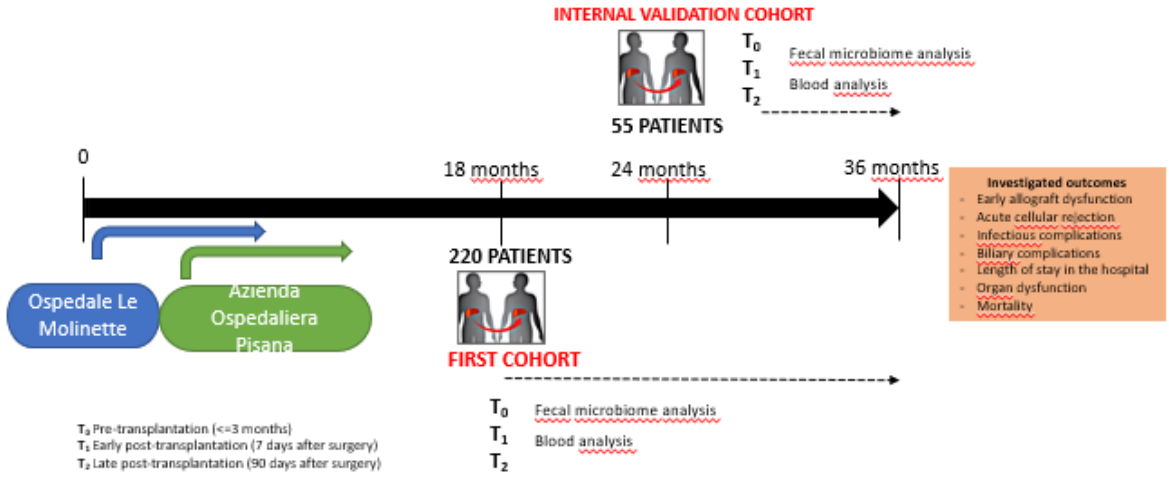

Figure 3. Study plan

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Starting from blood samples, a permeability analysis (hematic zonulin) and the evaluation of circulating catecholamines were performed. Moreover, the concentration of bacterial endotoxin and bacterial metabolites (short chain fatty acids, SCFAs) was evaluated. Child-Pugh and MELD scores were assessed at all time points, as well as relevant clinical complications (such as cirrhosis complications, post-transplant allograft dysfunction, acute rejection, infection, biliary complication) and graft/patient survival.

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The Child-Turcotte-Pugh (CTP) and the MELD (Model for End-Stage Liver Disease) scores were designed to predict the outcome of decompressive therapy for portal hypertension. They were prospectively validated to predict mortality risk in patients with a wide spectrum of liver disease etiology and severity. Unlike the CTP score, the MELD score was derived from prospectively gathered data. Its calculation was based on serum bilirubin, serum creatinine, international normalized ratio (INR) and etiology of liver disease (Figures 4 and 5) (78).

Child-Turcotte-Pugh Classification for Severity of Cirrhosis			
Clinical and Lab Criteria	Points*		
	1	2	3
Encephalopathy	None	Grade 1 or 2	Grade 3 or 4
Ascites	None	Mild to moderate (diuretic responsive)	Severe (diuretic refractory)
Bilirubin (mg/dL)	< 2	2-3	>3
Albumin (g/dL)	> 3.5	2.8-3.5	<2.8
Prothrombin time Seconds prolonged *or* International normalized ratio	<4 <1.7	4-6 1.7-2.3	>6 >2.3
*Child-Turcotte-Pugh Class obtained by adding score for each parameter (total points)			
Class A = 5 to 6 points			
Class B = 7 to 9 points			
Class C = 10 to 15 points			

Figure 4. The CTP calculation

The CTP scoring system incorporates five parameters: serum bilirubin, serum albumin, prothrombin time, severity of ascites, and grade of encephalopathy. Based on the sum of the points from these five parameters, the person is categorized into one of three CTP classes: A, B, or C.

Model for End-Stage Liver Disease (MELD) Score

MELD = 3.78 x $\log_e$ serum bilirubin (mg/dL) +
11.20 x $\log_e$ INR +
9.57 x $\log_e$ serum creatinine (mg/dL) +
6.43 (constant for liver disease etiology)

NOTES:

- If the patient has been dialyzed twice within the last 7 days, then the value for serum creatinine used should be 4.0
- Any value less than one is given a value of 1 (i.e. if bilirubin is 0.8, a value of 1.0 is used) to prevent the occurrence of scores below 0 (the natural logarithm of 1 is 0, and any value below 1 would yield a negative result)

Figure 5. The MELD score calculation

The MELD score, which estimates the survival probability of a patient with end-stage liver disease, is based on three commonly obtained laboratory tests: serum bilirubin, serum creatinine, and international normalized ratio (INR).

Collected routine laboratory and clinical data

Clinical data regarding anthropometric features, etiology of liver disease, presence of hepatocellular carcinoma, diabetes, renal function, ascites and encephalopathy were collected at all time points. Hematologic (leucocytes with formula, erythrocytes, hemoglobin, hematocrit, platelets), biochemical (glucose, creatinine, urea, sodium, potassium, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total Bilirubin, gamma-glutamyl transferase (GGT), alkaline phosphatase (ALP), albumin, ammonium), and coagulation parameters were also evaluated. Child-Pugh and MELD scores were assessed at all time points, as well as relevant clinical complications (such as cirrhosis complications, post-transplant allograft dysfunction, acute rejection, infection, biliary complication) and graft/patient survival. Main immunosuppressant drug levels were recorded at the post-transplant time points.

Fecal microbiome analysis

Stool samples from a single bowel movement were collected by patients in sterile containers. Each sample was stored at 4°C until transport to the collecting laboratory of each participating centre, and then rapidly aliquoted into three vials and stored at - 80°C until further processing and analysis. All microbiome analyses were performed at the Laboratory of Medical Microbiology and Virology, Ospedale San Raffaele, Milan.

Bacterial DNA was extracted from 100-300 mg of fecal material using the QIAamp PowerFecal Pro DNA Kit (Qiagen) following the manufacturer's instructions, with only one minor modification in lyses time, 15 min at 95°C instead of 5 min, to try to retrieve all difficult-to-lyse bacteria. The concentration of extracted DNA was measured using a Qubit double-stranded DNA (dsDNA) HS Assay Kit (Qubit) on a Qubit 2.0 Fluorometer (Qubit; Life Technologies Corporation, Carlsbad, CA). Variable regions 3 and 4 (V3–V4) of 16S ribosomal ribonucleic acid (rRNA) gene were amplified starting from 500 ng of total extracted genetic material using gene-specific sequences. Illumina adapter overhang nucleotide sequences were added to the gene-specific sequences. Amplification

primers used were as follows: 16S_FW: TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG and 16S_Rev: GTC TCG TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATCC. The AccuPrime Taq DNA Polymerase (Invitrogen) and the following cycling conditions were used: 94°C for 2 min, 35 cycles of (94°C for 30'', 56°C for 30'' and 68°C for 1 min) and the samples were stored at 4°C until usage.

Generated amplicons were loaded on 1.5% agarose gel in order to check whether the amplification is successful.

Amplicons were purified using the AMPure XP beads (Beckman Coulter, Brea, USA), following the steps described in the "16S Metagenomic Sequencing Library Preparation" protocol by Illumina. A second PCR step was performed for indexing and for adding Illumina sequencing adapters to each sample. The Nextera XT Index Kit (Illumina) and the KAPA HiFi HotStart PCR Kit (KAPA Biosystem) were used along with the following amplification protocol: 95°C for 3 min, 8 cycles of (95°C for 30", 55°C for 30", 72°C for 30"), 72°C for 4 min and then 4°C until usage. A second purification step with AMPure XP beads was performed to clean up the samples after PCR reaction.

The purified DNA was quantified using the Qubit double-stranded DNA (dsDNA) HS Assay Kit on a Qubit 2.0 Fluorometer (Thermo Fisher). Subsequently, using a mathematical formula, samples were diluted to a concentration of 4nM and then pooled in a sequencing library, following "16S Metagenomic Sequencing Library Preparation" protocol by Illumina.

Subsequently, library pool was denatured and diluted to a 20pM concentration. Another dilution was necessary to generate a 12pM library pool which was ready to be loaded on a MiSeq reagent cartridge, as reported in the "Denature and dilute libraries guide" by Illumina.

Sequencing was performed using the MiSeq Illumina platform with a 600 (2 X 300)-base pairs (bp) paired-end read protocol. Data quality trimming and reads demultiplexing were performed on the MiSeq instrument.

Bioinformatic and statistical analysis of metagenomic data

Data analysis of the Illumina sequence data was performed using phylogenetic and Operational Taxonomic Unit (OTU) methods in the QIIME (Quantitative Insights into Microbial Ecology) software (version 1.9) (http:/qiime.org). All sequence reads obtained in this study were pre-processed for quality filtering using the following stringent criteria:

1) Removal of primer sequence;

2) Truncation of sequence reads before three consecutive low-quality bases (of 20 over a 30 bp) and re-evaluation for length;

3) No ambiguous base calls;

4) Minimum sequence length of 150 bp after trimming.

Sequences were binned according to sample-specific barcode clustered with u-clust into operational taxonomic units (OTUs) at 97% sequence similarity. Representative sequences were picked for each OTU and aligned with Pynast using the Greengenes. Taxonomy assignment of each OTU was performed according to RDP (Ribosomal Database Project) taxonomy followed by construction of OTU tables at different taxonomic levels. For analysis of diversity within samples (alpha-diversity), OTU tables were rarefied at 30,000 reads and diversity indices including Chao 1, Shannon, Simpson, and the number of observed species was determined. To assess differences between microbial communities (beta-diversity) weighted Unifrac analysis on rarefied data with 97% (equivalent to species-level) and 95% (equivalent to genus-level) OTU clustering was performed and followed by principal coordinates analysis (PCoA).

Results

Patient data

The study allowed to analyse the complete results of 58 patients of centre A, 54 patients of centre B (both at time T0 and T1) and 36 patients of centre B at time T2.

For each centre, a database was created with clinical and biochemical information relating to patients undergoing liver transplantation.

Centre B enrolled patients with liver cirrhosis, whose etiology includes: Wilson disease, alcoholism, dysmetabolic syndrome, post HCV, post HBV, sclerosing cholangitis, hepatorenal polycystosis, non-alcoholic steatohepatitis (NASH), primary biliary cholangitis, autoimmune, fulminant hepatitis HBV-related, fulminant hemangioendothelioma.

For each of these patients, the presence of concomitant diseases was reported in the database, and in about 50% of the patients, one or more diseases were present, such as: diabetes mellitus, dyslipidemia, short intestine, osteoporosis, chronic renal failure, renal insufficiency in previous kidney transplant, sigmoid resection for diverticula, epilepsy, osteopenia, hypertension, ulcerative colitis, patent foramen ovale, autoimmune thyroiditis, previous liver transplant, portopulmonary hypertension, sclerosing cholangitis, history of splenectonima due to anemia, coronary heart disease hypertension, history of prostate cancer, autoimmune myelitis, coronary artery disease, hypothyroidism, hepatocellular carcinoma.

For each liver disease, the MELD score was evaluated and the values range from a minimum score of 6 to a maximum of 42.

Time points comparison

The sequencing analyses made it possible to analyse the composition of the microbiome, in particular for the 58 patients of centre A and for the 54 patients of centre B, the results of the bacterial ecosystem at T0 and at T1 were available; T2 results for 36 patients from Centre B.

The sequencing analyses did not allow to go into the genus and species details, due to the difficulties in distinguishing small variations in the genome, for this reason the sequencing stopped at the Phylum and Family levels.

Phyla and Families obtained were reported in the following table (Table 1).

PHYLUM	FAMILY	PHYLUM	FAMILY	PHYLUM	FAMILY
Actinobacteria	Actinobacteria_other	Firmicutes	Firmicutes_other	Proteobacteria	Proteobacteria_other
	Actinomycetales_other		Bacilli_other		Caulobacteraceae
	Actinomycetales_unassigned		Bacillales_other		Rhizobiales_other
	Actinomycetaceae		Bacillales_unassigned		Rhizobiales_unassigned
	Brevibacteriaceae		Bacillaceae		Brucellaceae
	Corynebacteriaceae		Listeriaceae		Methylobacteriaceae
	Geodermatophilaceae		Planococcaceae		Rhizobiaceae
	Gordoniaceae		Staphylococcaceae		Rhodobacteraceae
	Microbacteriaceae		Gemellales_other		Rickettsiales_mitochondria
	Micrococcaceae		Gemellales_unassigned		Sphingomonadaceae
	Micromonosporaceae		Gemellaceae		Betaproteobacteria_other_other
	Nocardiaceae		Lactobacillales_other		Betaproteobacteria_unassigned
	Propionibacteriaceae		Lactobacillales_unassigned		Burkholderiales_other
	Bifidobacteriaceae		Aerococcaceae		Burkholderiales
	Coriobacteriaceae		Carnobacteriaceae		Alcaligenaceae
Bacteroidetes	Bacteroidales_other		Enterococcaceae		Burkholderiaceae
	Bacteroidales_unassigned		Lactobacillaceae		Comamonadaceae
	Bacteroidaceae		Leuconostocaceae		Oxalobacteraceae
	Porphyromonadaceae		Streptococcaceae		Neisseriaceae
	Prevotellaceae		Turicibacteraceae		Nitrosomonadaceae
	Rikenellaceae		Clostridia_other_other		Desulfovibrionaceae
	Barnesiellaceae		Clostridia_unassigned		Campylobacteraceae
	Odoribacteraceae		Clostridiales_other		Gammaproteobacteria_other_other
	Paraprevotellaceae		Christensenellaceae		Aeromonadaceae
	Flavobacteriaceae		Clostridiaceae		Succinivibrionaceae
	Weeksellaceae		Dehalobacteriaceae		Alteromonadaceae
	Balneolaceae		Eubacteriaceae		Shewanellaceae
Chloroflexi	Anaerolinaceae		Lachnospiraceae		Cardiobacteriaceae
Cyanobacteria	Streptophyta_unassigned		Peptococcaceae		Enterobacteriaceae
Deferribacteres	Deferribacteraceae		Peptostreptococcaceae		Halomonadaceae
Euryarchaeota	Methanobacteriaceae		Ruminococcaceae		Pasteurellaceae
Fusobacteria	Fusobacteriaceae		Veillonellaceae		Moraxellaceae
	Leptotrichiaceae		Mogibacteriaceae		Pseudomonadaceae
Lentisphaerae	Victivallaceae		Tissierellaceae		Xanthomonadaceae
Synergistetes	Synergistaceae		Erysipelotrichaceae		Dethiosulfovibrionaceae
Tenericutes	Anaeroplasmataceae	Verrucomicrobia	Cerasicoccaceae		Mycoplasmataceae
Thermus	Deinococcaceae		Verrucomicrobiaceae	Unassigned	Unassigned

Table 1. Phyla and Families obtained with sequencing.

Many Phyla and many Families were extremely rare in each sample for this reason we evaluated only the Phyla and Families with relative abundances greater than 1%, in particular we selected for the analysis of the Phylum: Unassigned, Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, Verrucomicrobia and for Family analysis: Unassigned, Bifidobacteriaceae, Coriobacteriaceae, Bacteroidaceae, Porphyromonadaceae, Prevotellaceae, Rikenellaceae, Enterococcaceae, Lactobacillaceae, Streptococcaceae, Clostridiales other, Clostridiaceae, Lachnospiraceae, Ruminococcaceae, Veillonellaceae, Erysipelotrichaceae, Enterobacteriaceae and Verrucomicrobia.

The first step of the analysis of the results involved the comparison at the Phylum level of the T0 and T1 for the patients of centre A and centre B; results are reported in Table 2 and Figure 6.

	Phylum T0	Phylum T1	p value
Unassigned	1,20%	1,30%	n.s.
Actinobacteria	6,90%	5,20%	n.s.
Bacteroidetes	19,30%	15,60%	n.s.
Firmicutes	54,50%	48,40%	n.s.
Proteobacteria	15,00%	22,70%	0,01
Verrucomicrobia	2,80%	6,40%	0,02

Table 2. Relative abundance of both Proteobacteria and Verrucomicrobia increased significantly; n.s. not significant. Statistical test: Student's t-test.

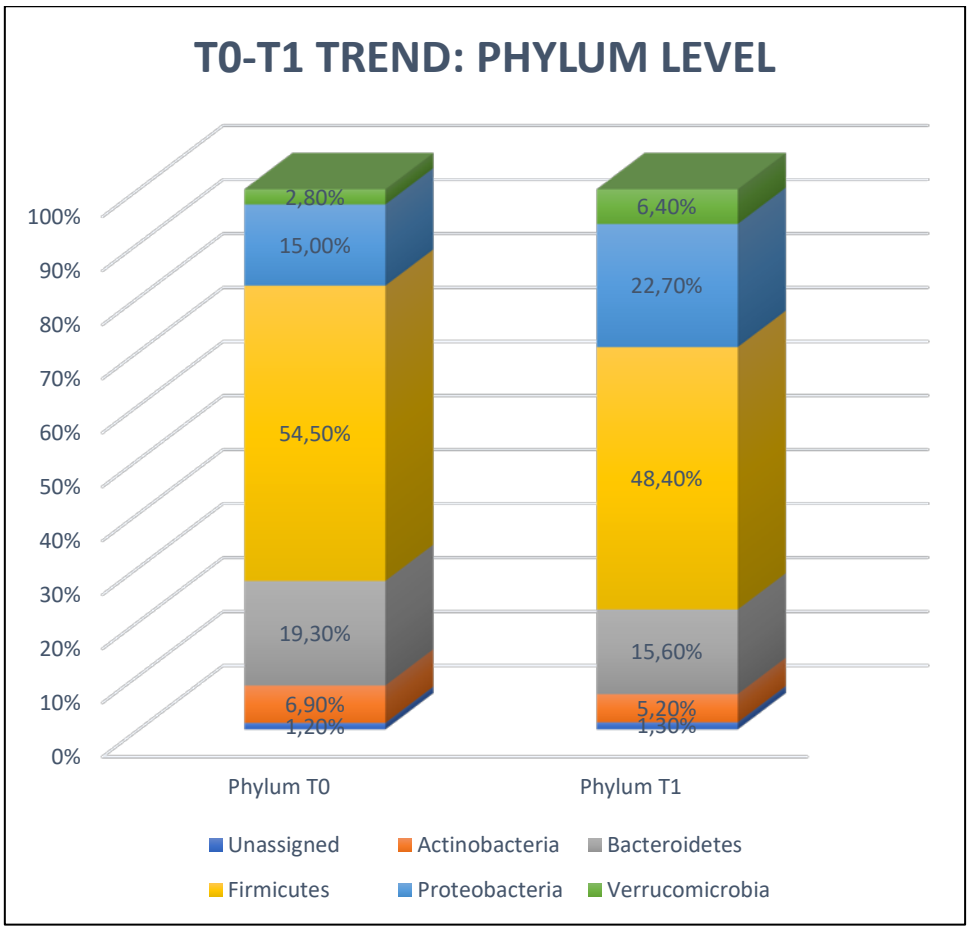

Figure 6. Comparison at the Phylum level of the T0 and T1 for the patients of centre A and centre B

Proteobacteria and Verrucomicrobia had statistically significant alterations, in particular Proteobacteria had an average relative abundance of 15% at T0 and 22.7% at T1 with a p value of 0.01, therefore highly significant, while Verrucomicrobia passed from a relative abundance of 2.8% at T0 to 6, 4% at T1 with a p value of 0.02, again highly significant.

At the Phylum level, but only for centre B, the comparison between T0, T1 and T2 was performed, as shown in Figure 7; results (reported in Table 3) show that there were extremely significant variations between T1 and T2, 90 days after liver transplantation.

	Phylum T0	Phylum T1	Phylum T2	p T0 - T1	p T1 - T2
Unassigned	0,80%	0,90%	1,60%	n.s.	0,00
Actinobacteria	7,50%	5,60%	6,90%	n.s.	n.s.
Bacteroidetes	19,00%	18,00%	0,80%	n.s.	0,00
Firmicutes	57,30%	51,30%	84,60%	n.s.	0,00
Proteobacteria	13,50%	16,70%	5,20%	n.s.	0,00
Verrucomicrobia	1,70%	7,00%	0,90%	0,02	0,03

Table 3. Unassigned Phylum with Firmicutes, underwent an increase in relative abundance at T2, Verrucomicrobia increased at T1 and Bacteroidetes, Proteobacteria and Verrucomicrobia decreased at T2; n.s. not significant. Statistical test: Student's t-test.

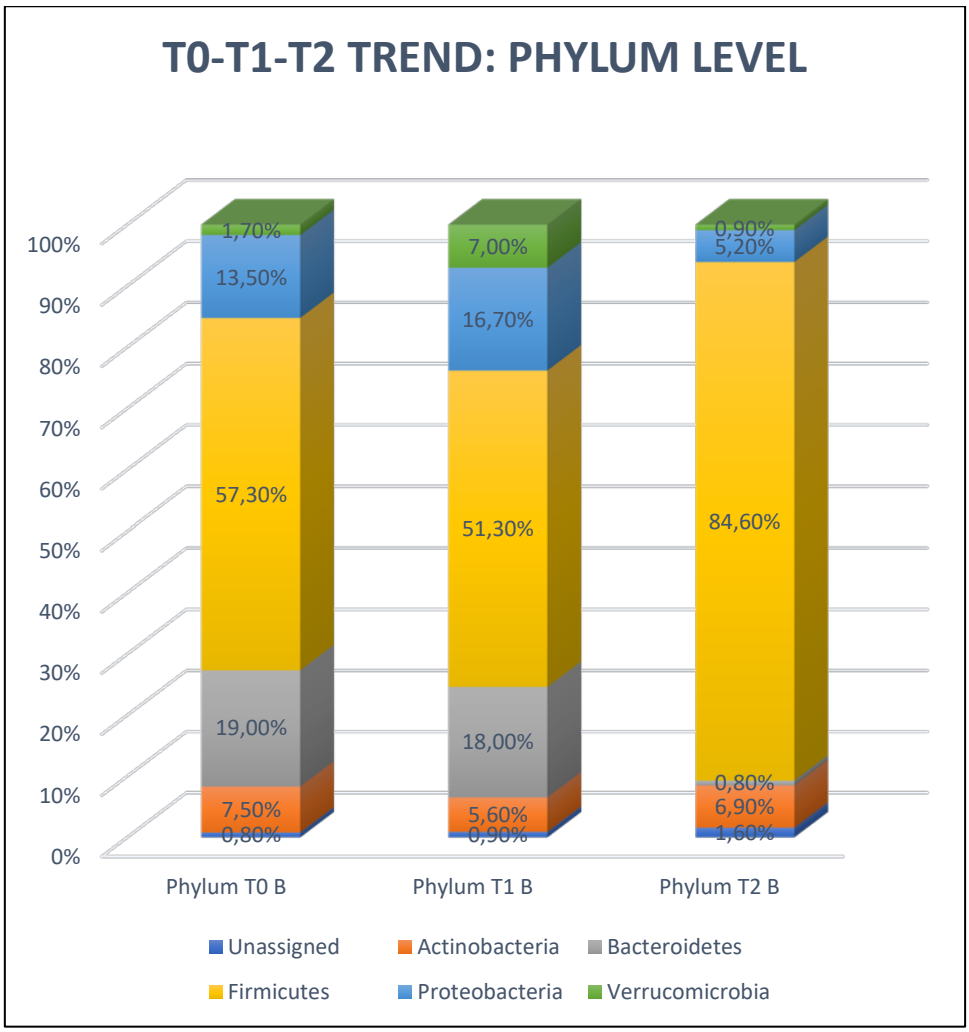

Figure 7. Comparison between T0, T1 and T2 only for centre B, Phylum level

Bacteria called unassigned had an increase in relative abundance from 0.9% at T1 to 1.6% at T2 with a p value of 0.00, therefore extremely significant; the same values of statistical significance were observed for Bacteroidetes, which passed from 18% at T1 to 0.8% at T2, Firmicutes which grow in relative abundance from 51.3% at T1 to 84.6% at T2 and Proteobacteria which decreased from 16.7% at T1 to 5.2% at T2. For Verrucomicrobia statistical significance was observed both between T0 and T1 and between T1 and T2 in fact they passed from 1.7% in relative abundance at T0 to 7% at T1 and decreased again to 0.9% at T2 with p value respectively of 0, 02 between T0 and T1, and 0.03 between T1 and T2, both highly significant.

The same type of analysis, therefore comparison between T0 and T1 for patients from centre A and centre B, and comparison between T0, T1 and T2 only for patients from centre B, was performed at the Family level (Figure 8); results are reported in Table 4.

	Family T0	Family T1	p value
Unassigned	1,2%	1,30%	n.s.
Bifidobacteriaceae	6,3%	4,3%	n.s.
Coriobacteriaceae	0,5%	0,4%	n.s.
Bacteroidaceae	10,9%	12,5%	n.s.
Porphyromonadaceae	3,0%	1,1%	0,049
Prevotellaceae	3,2%	0,5%	0,00
Rikenellaceae	0,7%	0,6%	n.s.
Enterococcaceae	3,4%	5,4%	n.s.
Lactobacillaceae	5,9%	3,5%	n.s.
Streptococcaceae	6,8%	6,9%	n.s.
Clostridiales_other	2,3%	1,5%	n.s.
Clostridiaceae	1,4%	1%	n.s.
Lachnospiraceae	14,3%	10,6%	0,02
Ruminococcaceae	11,9%	9,3%	n.s.
Veillonellaceae	6,3%	7,6%	n.s.
Erysipelotrichaceae	1,4%	1%	n.s.
Enterobacteriaceae	13,9%	21,3%	0,01
Verrucomicrobiaceae	2,8%	6,4%	0,02

Table 4. Five Families present statistically significant alteration, in particular Porphyromonadaceae, Prevotellaceae, Lachospiraceae, Enterobacteriaceae and Verrucomicrobia; n.s. not significant. Statistical test: Student's t-test.

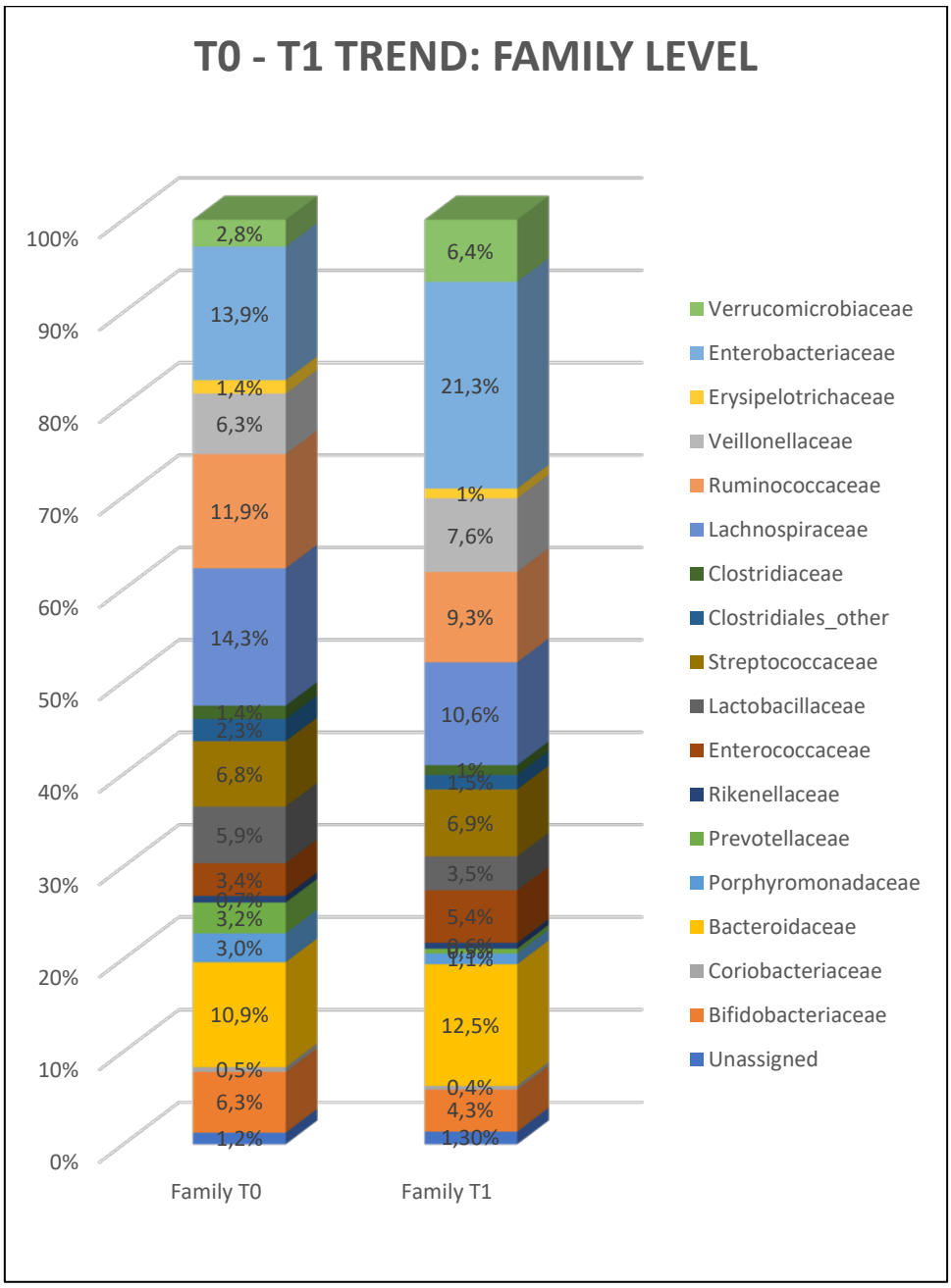

Figure 8. Comparison at Family level of the T0 and T1 for the patients of centre A and centre B

In the analysis between T0 and T1 we observed five Families that present statistically significant alteration, in particular Porphyromonadaceae decreased from 3% at T0 to 1.1 at T1 with a p value of 0.049 therefore at the limits of significance, Prevotellaceae passed from 3 .2% at T0 to 0.5% at T1 with a p value of 0.00, therefore highly significant. Lachospiraceae decreased from 14.3% at T0 to 10.6% at T1 with a p value of 0.02, also in this case highly significant, Enterobacteriaceae increased from 13.9% at T0 to 21.3% at T1 and the p value is 0.01, very significant; Verrucomicrobia increased from 2.8% at T0 to 6.4% at T1, with a highly significant p value of 0.02.

The analysis at Family level (Figure 9) of the three times demonstrated that also in this case, the most evident variations occured at T2, as indicated in Table 5.

	Family T0	Family T1	Family T2	P T0 - T1	P T1 - T2
Unassigned	0,8%	0,9%	1,6%	n.s.	0,00
Bifidobacteriaceae	6,9%	4,9%	6,3%	n.s.	n.s.
Coriobacteriaceae	0,4%	0,3%	0,4%	n.s.	n.s.
Bacteroidaceae	11,2%	15,0%	0,6%	n.s.	0,00
Porphyromonadaceae	3,7%	1,0%	0,1%	n.s.	0,01
Prevotellaceae	2,8%	0,5%	0,0%	n.s.	0,02
Rikenellaceae	0,5%	0,4%	0,1%	n.s.	n.s.
Enterococcaceae	5,8%	9,7%	2,6%	n.s.	n.s.
Lactobacillaceae	10,0%	5,1%	7,7%	n.s.	n.s.
Streptococcaceae	7,0%	6,7%	12,0%	n.s.	0,01
Clostridiales_other	1,9%	1,5%	2,5%	0,03	n.s.
Clostridiaceae	1,0%	0,6%	3,4%	n.s.	0,00
Lachnospiraceae	13,4%	9,7%	43,1%	n.s.	0,00
Ruminococcaceae	11,0%	7,1%	10,2%	n.s.	n.s.
Veillonellaceae	4,7%	8,3%	0,3%	n.s.	0,00
Erysipelotrichaceae	1,5%	0,8%	2,0%	n.s.	0,01
Enterobacteriaceae	12,7%	14,9%	5,2%	n.s.	0,00
Verrucomicrobiaceae	1,7%	7,0%	0,9%	0,02	0,03

Table 5. Results indicate a significant variation in relative abundance at T2; n.s. not significant. Statistical test: Student's t-test.

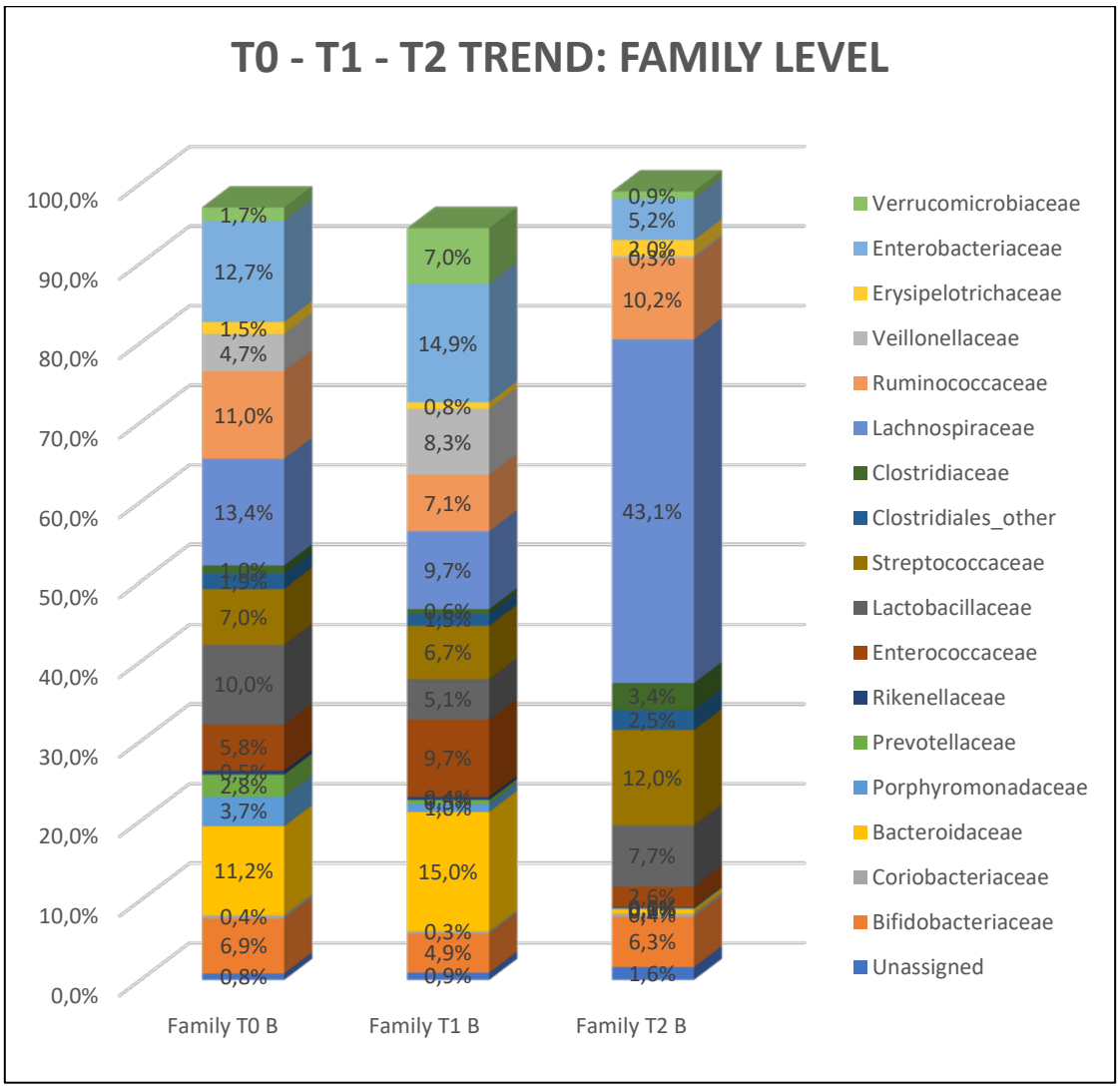

Figure 9. Comparison between T0, T1 and T2 only for centre B, Family level

The analysis demonstrated that the most evident variations occur at T2, unassigned bacteria passed from 0.9% at T1 to 1.6% at T2, with a p value of 0.00; the same significant values occurred for Bacteroidaceae which decreased from 15% to 0.6%, Clostridiaceae which increased from 0.6% to 3.4%, Lachnospiraceae which increased from 9.7 % to 43.1%, Veillonellaceae decreased from 8.3% to 0.3% and Enterobacteriaceae decreased from 14.9% to 5.2%. Porphyromonadaceae decreased from 1.0% to 0.1% with a p value of 0.01, Prevotellaceae decreased from 0.5% to 0.0% and the p value was 0.02. Streptococcaceae increased from 6.7% to 12% with a p value of 0.01, as did Erysipelotrichaceae which increased from 0.8% to 2%. Verrucomicrobiaceae decreased from 7% to 0.9% with a p value of 0.03. Only Clostridiales and Verrucomicrobiaceae showed statistically significant differences between T0 and T1, with p values of 0.03 and 0.02, respectively.

Outcomes comparison

Subsequently to the analysis of the comparison between the different time points, the microbial compositions of the cases with adverse outcomes, such as Early Allograft Dysfunction (EAD), rejection, infections and biliary complications, were evaluated for the patients of centre B; in particular, the main Phyla and the main Families of the patients with these adverse events were compared with those of the patients who did not present any abnormality and, for this reason, they were considered as negative controls.

Eleven patients developed EAD (Figure 10) and the comparison of the intestinal bacterial populations with the negative control group showed a statistical significance at the Phylum level for Actinobacteria, as reported in Table 6.

	EAD	NEGATIVE	p value
Unassigned	0,6%	0,8%	n.s.
Actinobacteria	**16,9%**	5,3%	0,03
Bacteroidetes	18,5%	18,0%	n.s.
Firmicutes	58,3%	58,1%	n.s.
Proteobacteria	4,4%	15,5%	n.s.
Verrucomicrobia	1,4%	2,0%	n.s.

Table 6. Decrease in relative abundance for Actinobacteria at Phylum level in Early Allograft Disfunction (EAD); n.s. not significant. Statistical test: Student's t-test.

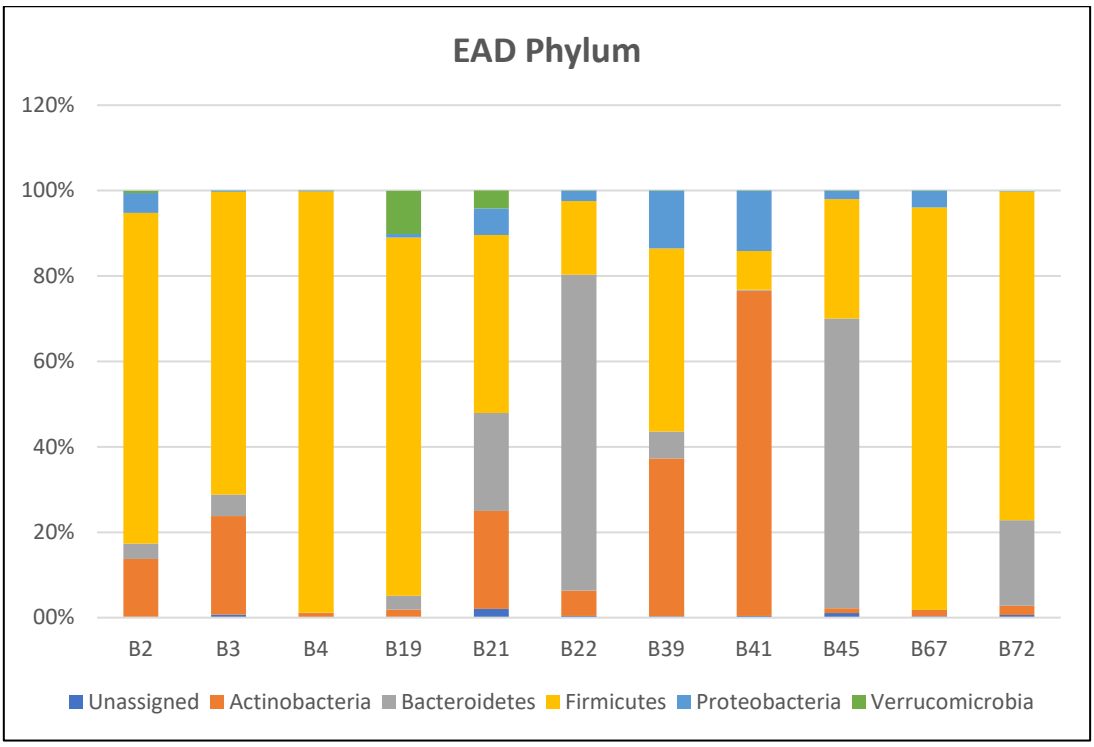

Figure 10. Outcomes comparison: Early Allograft Dysfunction, Phylum level

Eleven patients developed EAD and the comparison of the intestinal bacterial populations with the negative control group showed a statistical significance at the Phylum level for Actinobacteria, which has a relative abundance of 16.9% compared to 5.3% of the negatives, with a p-value of 0.03.

The same category of patients presented at Family level (Figure 11) an increase in Bifidobacteriaceae (Table 7).

	EAD	NEGATIVE	p value
Unassigned	0,6%	0,8%	n.s.
Bifidobacteriaceae	**16,4%**	4,6%	0,02
Coriobacteriaceae	0,3%	0,4%	n.s.
Bacteroidaceae	10,4%	11,5%	n.s.
Porphyromonadaceae	1,6%	2,2%	n.s.
Prevotellaceae	5,9%	2,3%	n.s.
Rikenellaceae	0,2%	0,6%	n.s.
Enterococcaceae	9,4%	4,9%	n.s.
Lactobacillaceae	13,7%	10,1%	n.s.
Streptococcaceae	3,9%	6,5%	n.s.
Clostridiales_other	1,4%	2,2%	n.s.
Clostridiaceae	1,3%	0,9%	n.s.
Lachnospiraceae	11,2%	13,4%	n.s.
Ruminococcaceae	10,4%	12,3%	n.s.
Veillonellaceae	5,7%	4,8%	n.s.
Erysipelotrichaceae	0,9%	1,6%	n.s.
Enterobacteriaceae	4,0%	14,5%	n.s.
Verrucomicrobiaceae	1,4%	2,0%	n.s.

Table 7. Patients with EAD presented at Family level an increase in Bifidobacteriaceae; n.s. not significant. Statistical test: Student's t-test.

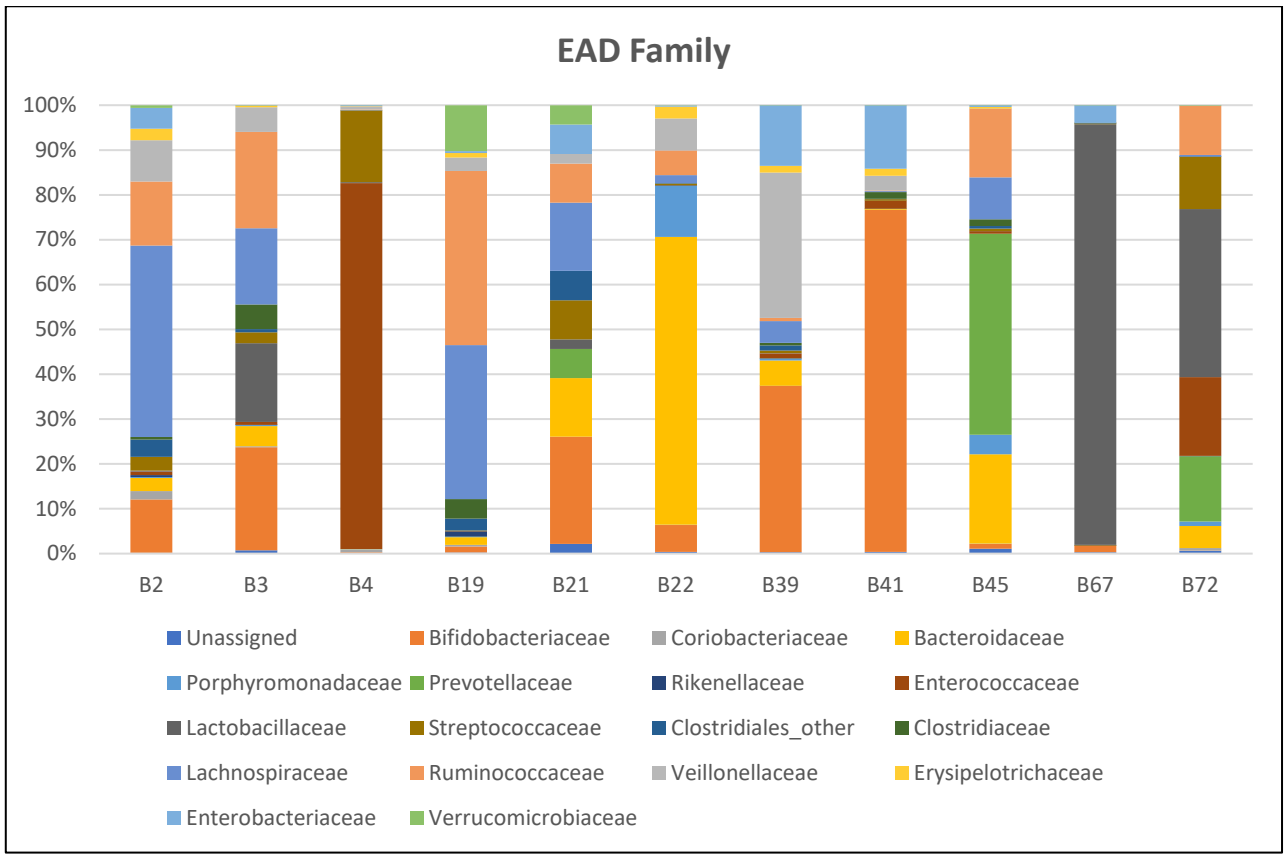

Figure 11. Outcomes comparison: Early Allograft Dysfunction, Family level

Patients with EAD presented at Family level an increase in Bifidobacteriaceae which rise to 16.4% compared to 4.6% of the negative controls (p 0.02).

Three patients developed organ rejection (Figures 12 and 13); although the number is low, a significance was found in the composition of the intestinal microbiome at the Phylum level for Actinobacteria (Table 8).

	REJECTION	NEGATIVE	p value
Unassigned	0,3%	0,8%	n.s.
Actinobacteria	**29,0%**	5,3%	0,01
Bacteroidetes	10,3%	18,0%	n.s.
Firmicutes	54,7%	58,1%	n.s.
Proteobacteria	5,2%	15,5%	n.s.
Verrucomicrobia	0,2%	2,0%	n.s.

Table 8. Patients with rejection had a significant reduction in Actinobacteria Phylum; n.s. not significant. Statistical test: Student's t-test.

Family level analysis was performed for these three patients which also reported an increase in Bifidobacteriaceae, as indicated in Table 9.

	REJECTION	NEGATIVE	p value
Unassigned	0,3%	0,8%	n.s.
Bifidobacteriaceae	**28,4%**	4,6%	0,01
Coriobacteriaceae	0,5%	0,4%	n.s.
Bacteroidaceae	7,9%	11,5%	n.s.
Porphyromonadaceae	2,1%	2,2%	n.s.
Prevotellaceae	0,0%	2,3%	n.s.
Rikenellaceae	0,1%	0,6%	n.s.
Enterococcaceae	1,2%	4,9%	n.s.
Lactobacillaceae	4,1%	10,1%	n.s.
Streptococcaceae	9,9%	6,5%	n.s.
Clostridiales_other	1,2%	2,2%	n.s.
Clostridiaceae	1,6%	0,9%	n.s.
Lachnospiraceae	27,6%	13,4%	n.s.
Ruminococcaceae	4,5%	12,3%	n.s.
Veillonellaceae	1,7%	4,8%	n.s.
Erysipelotrichaceae	2,7%	1,6%	n.s.
Enterobacteriaceae	5,0%	14,5%	n.s.
Verrucomicrobiaceae	0,2%	2,0%	n.s.

Table 9. Comparison between rejection and negative patients demonstrated a significant increase in Bifidobacteriaceae; n.s. not significant. Statistical test: Student's t-test.

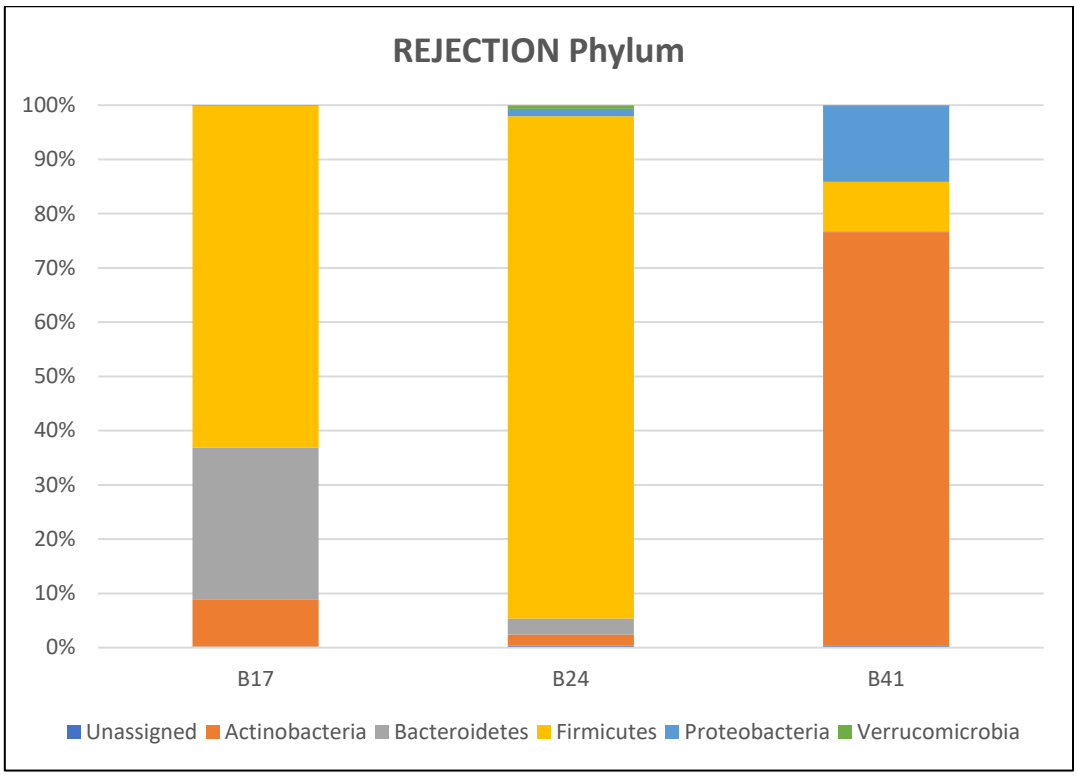

Figure 12. Outcomes comparison: Rejection, Phylum level

Three patients developed organ rejection; although the number is low, a significance was found in the composition of the intestinal microbiome at the Phylum level for Actinobacteria which rose to 29% compared to 5.3% of the negative controls with P equal to 0.01.

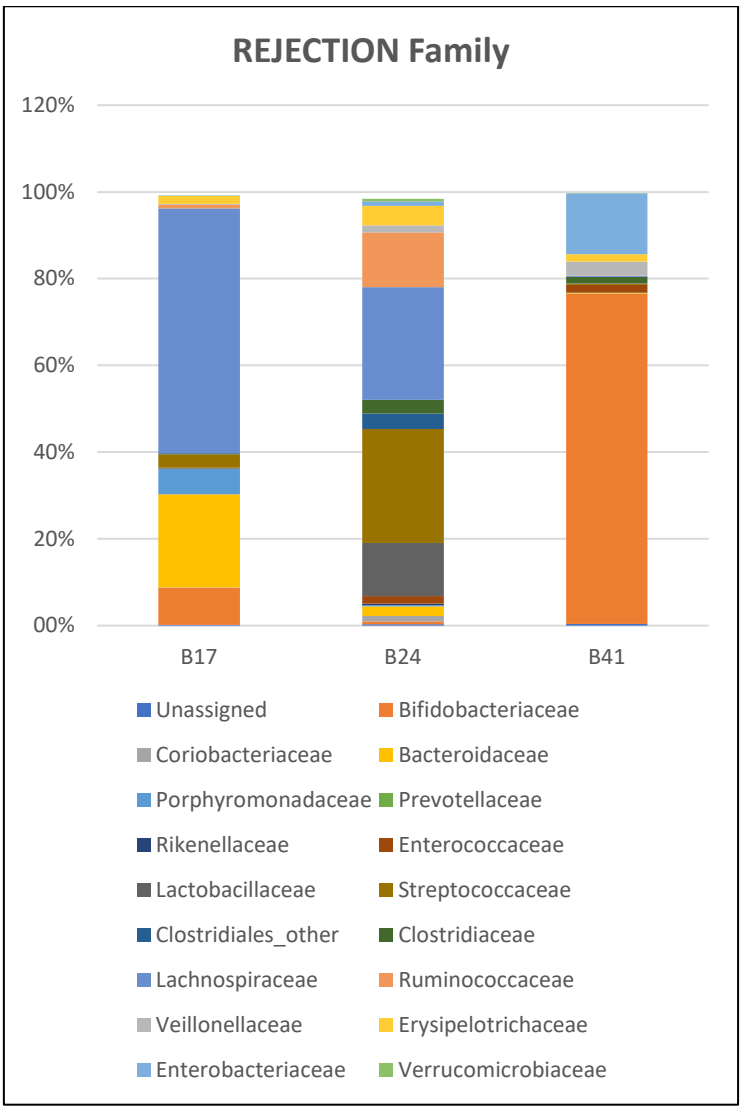

Figure 13. Outcomes comparison: Rejection, Family level

Family level analysis was performed for three patients with organ rejection, which reported an increase in Bifidobacteriaceae to 28.4% compared to negatives which have a relative abundance of 4.6%; the statistical significance is extremely high with $p = 0.01$.

The development of various types of post-transplant infections was found in six patients (Figures 14 and 15), however comparative analyses of the intestinal microbial community versus negative controls did not provide significant data (Tables 10 and 11).

	INFECTIONS	NEGATIVE	p value
Unassigned	0,8%	0,8%	n.s.
Actinobacteria	4,9%	5,3%	n.s.
Bacteroidetes	25,5%	18,0%	n.s.
Firmicutes	61,0%	58,1%	n.s.
Proteobacteria	6,8%	15,5%	n.s.
Verrucomicrobia	0,9%	2,0%	n.s.

Table 10. Comparative analyses at Phylum level of the intestinal microbial community in infections versus negative controls did not provide significant data; n.s. not significant. Statistical test: Student's t-test.

	INFECTIONS	NEGATIVE	p value
Unassigned	0,8%	0,8%	n.s.
Bifidobacteriaceae	4,7%	4,6%	n.s.
Coriobacteriaceae	0,1%	0,4%	n.s.
Bacteroidaceae	14,5%	11,5%	n.s.
Porphyromonadaceae	3,3%	2,2%	n.s.
Prevotellaceae	7,4%	2,3%	n.s.
Rikenellaceae	0,2%	0,6%	n.s.
Enterococcaceae	3,1%	4,9%	n.s.
Lactobacillaceae	18,2%	10,1%	n.s.
Streptococcaceae	11,1%	6,5%	n.s.
Clostridiales_other	0,6%	2,2%	n.s.
Clostridiaceae	0,8%	0,9%	n.s.
Lachnospiraceae	16,6%	13,4%	n.s.
Ruminococcaceae	5,9%	12,3%	n.s.
Veillonellaceae	2,3%	4,8%	n.s.
Erysipelotrichaceae	2,1%	1,6%	n.s.
Enterobacteriaceae	6,4%	14,5%	n.s.
Verrucomicrobiaceae	0,9%	2,0%	n.s.

Table 11. Comparative analyses at Family level of the intestinal microbial community in infections versus negative controls did not provide significant data; n.s. not significant. Statistical test: Student's t-test.

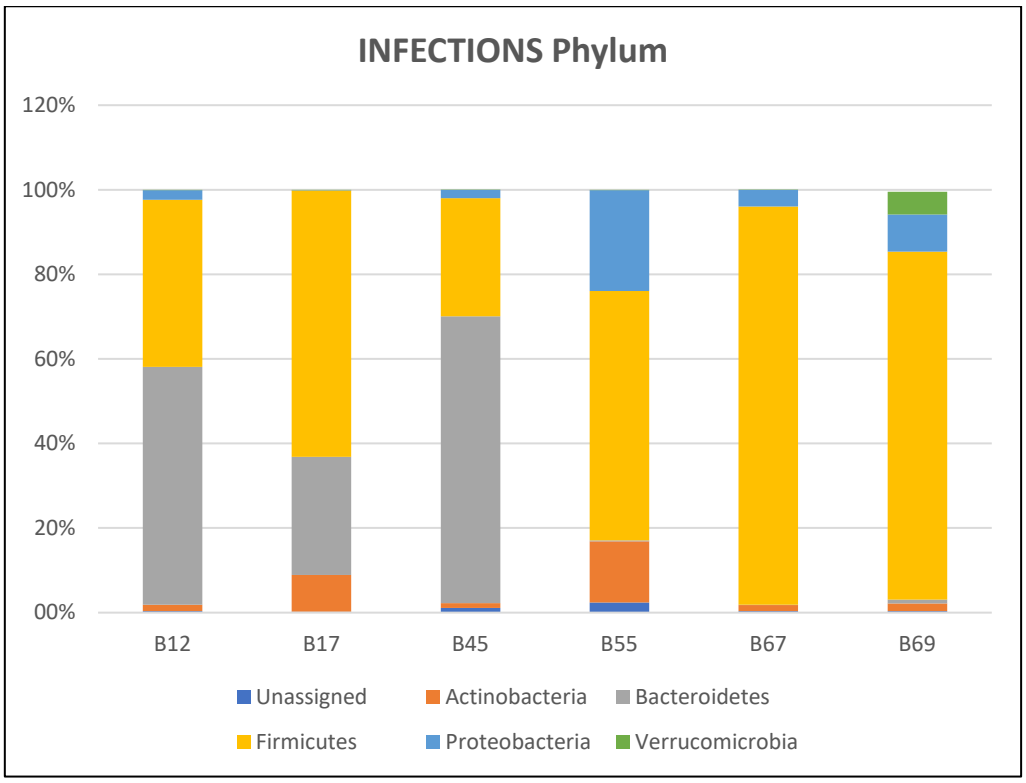

Figure 14. Outcomes comparison: Infections, Phylum level

The development of various types of post-transplant infections was found in six patients however comparative analyses of the intestinal microbial community versus negative controls did not provide significant data.

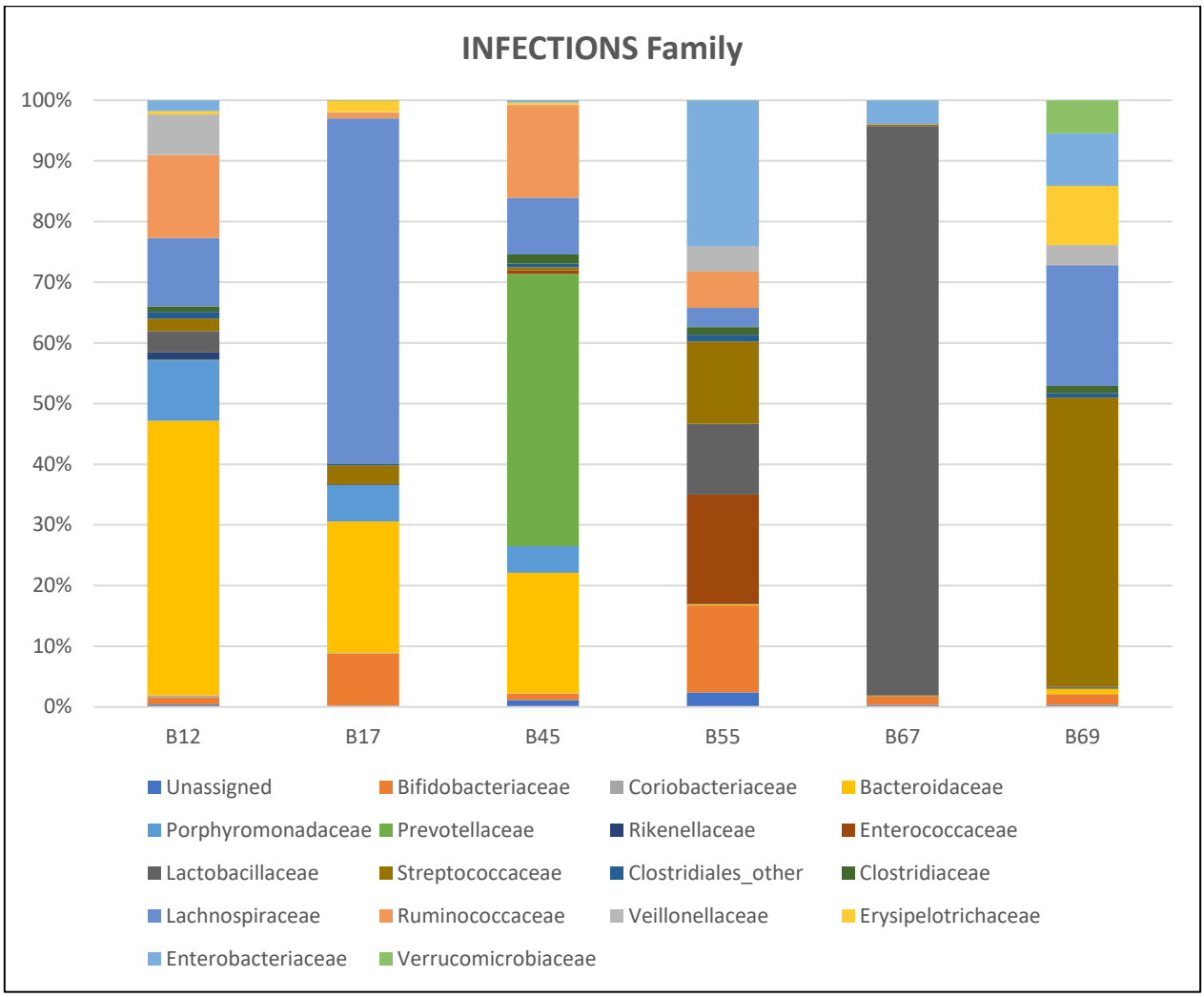

Figure 15. Outcomes comparison: Infections, Family level

The development of various types of post-transplant infections was found in six patients however comparative analyses of the intestinal microbial community versus negative controls did not provide significant data.

Biliary complications were present in two patients (Figures 16 and 17); although analyses and comparisons within this small population were limited, at the Phylum level there were a significant increase in Bacteroidetes and decrease in Firmicutes (as reported in Table 12), while at Family level, Bacteroidaceae and Prevotellaceae increased as indicated in Table 13.

	BILIARY COMPLICATIONS	NEGATIVE	p value
Unassigned	0,7%	0,8%	n.s.
Actinobacteria	3,5%	5,3%	n.s.
Bacteroidetes	**70,9%**	18,0%	0,00
Firmicutes	**22,6%**	58,1%	0,03
Proteobacteria	2,1%	15,5%	n.s.
Verrucomicrobia	0,0%	2,0%	n.s.

Table 12. Phylum level: significant increase in Bacteroidetes and decrease in Firmicutes; n.s. not significant. Statistical test: Student's t-test.

	BILIARY COMPLICATIONS	NEGATIVE	p value
Unassigned	0,7%	0,8%	n.s.
Bifidobacteriaceae	3,4%	4,6%	n.s.
Coriobacteriaceae	0,1%	0,4%	n.s.
Bacteroidaceae	**41,1%**	11,5%	0,01
Porphyromonadaceae	7,7%	2,2%	n.s.
Prevotellaceae	**22,0%**	2,3%	0,00
Rikenellaceae	0,0%	0,6%	n.s.
Enterococcaceae	0,3%	4,9%	n.s.
Lactobacillaceae	0,0%	10,1%	n.s.
Streptococcaceae	0,4%	6,5%	n.s.
Clostridiales_other	0,3%	2,2%	n.s.
Clostridiaceae	0,7%	0,9%	n.s.
Lachnospiraceae	5,5%	13,4%	n.s.
Ruminococcaceae	10,2%	12,3%	n.s.
Veillonellaceae	3,5%	4,8%	n.s.
Erysipelotrichaceae	1,5%	1,6%	n.s.
Enterobacteriaceae	0,3%	14,5%	n.s.
Verrucomicrobiaceae	0,0%	2,0%	n.s.

Table 13. Family level: Bacteroidaceae and Prevotellaceae increased significantly; n.s. not significant. Statistical test: Student's t-test.

These data were preliminary and further updates are required to complete the patient series, which in some cases was still too small but nonetheless indicative of a microbiological trend.

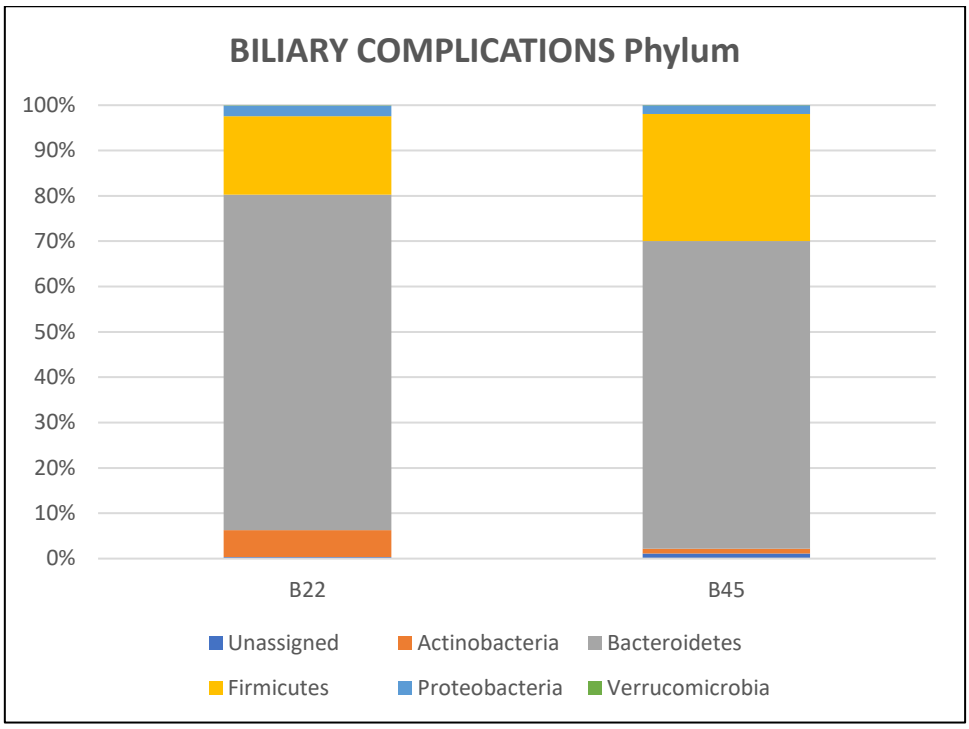

Figure 16. Outcomes comparison: Biliary Complications, Phylum level

Biliary complications are present in two patients; at the Phylum level there was a significant increase in Bacteroidetes which presented a relative abundance of 70.9% compared to 18% of the negatives with p = 0.00. The Firmicutes population instead decreased to 22.6% versus 58.1% of the negatives with P = 0.03.

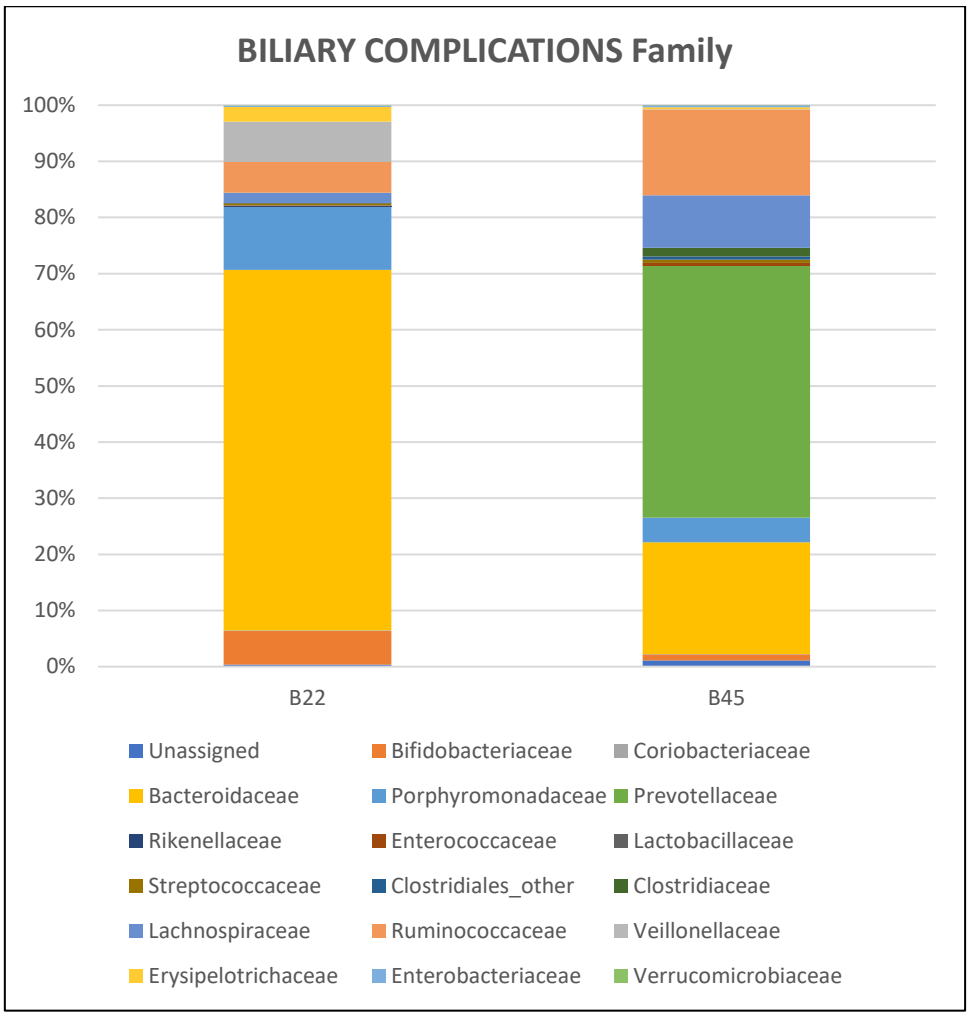

Figure 17. Outcomes comparison: Biliary Complications, Family level

At the Family level, Bacteroidaceae increased by 41.1% compared to 11.5% of the negatives with p = 0.01, as well as the Prevotellaceae which had a relative abundance of 22% compared to 2.3% of the controls with p = 0, 00.

Summary of result

Our study has allowed us to analyse and compare the different microbial populations in critically ill patients, affected by cirrhosis and undergoing liver transplantation, at different times pre and post transplantation and the results have highlighted the alterations of the intestinal microbiome.

The analysis of the patients belonging to both centres under evaluation found an increase in the Phylum Proteobacteria at T1 compared to T0, thus indicating the occurrence of dysbiosis, compatible with the short period of time following the transplant, in a situation in which the patient is subjected to a heavy drug regimen; instead, the Phylum Verrucomicrobia decreased, which should be an indication of intestinal health but whose function is still little known.

As far as the three-point analysis of the patients belonging to centre B is concerned, we observe a decrease in the Phylum Bacteroidetes, responsible for maintaining homeostasis and with metabolic functions, an initial decrease at T1 and then going back to T2 of the Phylum Firmicutes involved in enzymatic and digestive, an increase at T1 of Proteobacteria, associated with dysbiosis, to then decrease at T2, an index of recovery of the physiological functions of the intestinal microbial; a progressive decrease of the Phylum Verrucomicrobia was also observed.

The analysis of adverse outcomes such as EAD, rejection, infections, biliary complications, showed differences in the architecture and microbial composition compared to patients whose postoperative course did not show complications; these differences and alterations require further studies in order to evaluate their significance and allow the setting up of an algorithm for the risks evaluation and predispositions to the development of pathologies and complications for each patient.

Conclusions

The role of intestinal bacterial microflora or microbiota in maintaining the homeostasis of the human organism has been known for many years, but only recently, thanks to the use of increasingly performing cultivation techniques and molecular biology, significant progresses in the study of endogenous microflora have been made. The rapid development of so many methods, however, creates a significant problem in interpreting the results, leading to the need for developing models that are easy to use.

The interest of the scientific world is focused on the interactions between the intestinal microbiota and the host, with particular regard to the physiological and pathological conditions that can cause alteration of the composition and number of bacteria present in the human gut. On the other hand, numerous studies have already suggested the use of bacteria with beneficial effects on human health or probiotics, in the treatment of some pathological conditions.

In this regard, the deepening of studies on the role of the intestinal microbiota in the carcinogenesis of the colon mucosa, from the formation of adenomatous polyps to that of colorectal carcinoma, assumes considerable importance, with the aim of reducing its onset and/or l incidence in humans.

Translational medicine, through the continuous interaction between experimental models and in vivo conditions, can help researchers to better clarify the aspects of the microbiota-host interaction, with particular reference to the relationship between the microbiota and inflammatory and neoplastic processes.

In relation to these considerations, our study aimed to characterize the composition of the intestinal microbiome in patients undergoing liver transplantation; the study highlighted the variations in the structure of the microbiota before and after transplantation and provided preliminary data on the correlation of bacterial composition and various post-transplant complications, although this last part will still have to be implemented.

It is important to remember that both pharmacological therapies and the immune system are involved in the alteration of the intestinal microbial composition, clearly having an impact in this study, but other factors must also be considered such as age, sex, ethnicity, presence of concomitant pathologies, hormones, habits food and the environment; all these factors play a fundamental role in the structure of the intestinal microbiome.

Data from our study are still partial and it will be very useful to evaluate the impact of the composition of the intestinal microbiota in association with clinical conditions; this will allow to understand the correlations between the structure of the microbiota and clinical aspects in pre- and post-transplanted patient.

The pre-transplant analysis will allow us to evaluate how the microbiota and the patient's pathologies are connected to each other, in particular it could be useful to evaluate how the microbiota influences the progression of the patient's pathology.

The post-transplant analysis will give important information on the changes in the microbial composition and on different clinical outcomes, such as rejection, early allograft dysfunction, the development of infections and biliary complications, as we have partially analyzed in our study, but other very important factors will also be evaluated such as microbial diversity and specific microbiome markers capable of providing long-term information in the management of transplant recipients, allowing for better patient stratification.

Future studies will allow for improving knowledge not only on this category of patients but also on other subjects who undergo alterations of the intestinal microbiome, identifying families or genera of microorganisms capable of providing early information about the tendency towards the development of particular conditions; it will therefore be possible to create personalized algorithms for each patient in order to prevent most of the adverse events.

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