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INTESTINAL MICROBIOTA CHANGES TNF-INHIBITORS INDUCED IN IBD-RELATED SPONDYLOARTHRITIS

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Sommario

ABSTRACT

Background: the close relationship between joints and gut inflammation has long been known and several data suggest that the dysbiosis could represents the link between Spondyloarthritis (SpA) and Inflammatory Bowel Diseases (IBD). To date, the manipulation of the intestinal microbiota is considered the key to the cure or control of the natural history of several pathologies sustained or favored by dysbiosis. The introduction of biologic drugs, in particular Tumor Necrosis Factor inhibitors (TNFi), revolutionized the management of both these diseases, thanks to the strong inhibition of inflammation and partially indirectly with mechanisms not yet fully clarified. While the impact of conventional drugs on gut microbiota is well known poor data are available about TNFi.

Aim: to investigate the impact of TNFi on gut microbiota.

Results: we evaluated 20 patients affected by enteropatic arthritis, naïve for biologic drugs, treated with TNFi. After six months of therapy we observed a significant increase in Lachnospiracae family (Δ +10.3, p 0.04) and in Coprococcus genus (Δ +2.8, p 0.003). We also observed a decrease trend in Proteobacteria (Δ -8.0 p 0.095) and Gammaproteobacteria (Δ -9, p 0.093) and an increase trend in Clostridia (Δ +8.2 p 0.083). We didn't find differences between TNFi responders (SpA improvement or IBD remission achieved) and not responders.

Conclusions: our findings are consistent with the hypothesis that TNFi therapy tends to restore the intestinal eubiosis.

INTRODUCTION

THE HUMAN GUT MICROBIOTA

DEVELOPMENT AND COMPOSITION

The microbiota consists in the pool of microbes that collectively inhabit a given ecosystem[1].

In the 2007 a USA project called The Human Microbiome Project (HMP) was established to better understand the microbial components of the human genetic and metabolic landscape and how they contribute to normal physiology and predisposition to disease.

Thank's to the new methods of analysis colture free, the study of microbiome, the bacterial genome, become easier and faster and recently less expensive than in the past. In particular the increasing speed of DNA sequencing, coupled with advances in the computational approaches used to analyze complex data sets, have encouraged several researchers of many different areas to study the microbiota throught the analysis to small-subunit (16S) ribosomal RNA . The 16S rRNA

gene is found in all microorganisms and has enough sequence conservation for accurate alignment and enough variation for phylogenetic analyzes[2].

The human microbiota is composed of the whole of microorganism, commensal, symbiotic, and pathogenic, hinabiting sufaces and cavities communicting with the environments such as mouth, upper respiratory and bowel and vaginal tract and it is composed mostly of bacteria and to a lesser extent from fungi (mycobiota)[3].

The microbiota shows remarkable variability within and among individuals. In addition to external exposures such as host lifestyle including diet and environment (home/work), ecological relationships (both oppositional and symbiotic) between microorganism are important contributors to this variation as well as combination of factors such as host genotype, host physiological status (including the properties of the innate and adaptive immune systems), host pathobiology (disease status), and the presence of transient populations of microorganisms that cannot persistently colonize a habitat[4].

It was extimated that the number of bacterial living in the human body is much higher of the body's human cells, extimated around 3.72×10^{13}, by 2-3 orders of magnitude and approximately 400-500 bacterial species make up the gut microbiota[5,6]. The microbiome consists in the collective community of bacteria and their total genome there is approximately 150 times larger than the human gene complement, with an estimated 3.3 million microbial genes[7],[8]. Qin et al etablished that each human individual carries approximately 600,000 microbial genes in their gut. Around 300,000 were common and present in 50% of individuals of the cohort. They identified 1,150 prevalent bacterial species, with at least 160 species per individual[7].

The microbiota contributes to provision of essential nutrients, metabolism of inigestible compounds, defence against colonization by opportunistic pathogens, contributions to the development of intestinal architecture[9],[10].

To explore the microbiota role and the complex interaction with the host, germfree models were colonized with specific species (one or more) of bacteria in experimental model (gnotobiota). Even though these models were artificials, comparisons between germfree and wild animals have helped to better understand the effects of the indigenous GI microflora on its host. Germfree rodents showed changed in morphological, physiological and immunological aspects. In particular increased cecum size, decreased weight and thickness of intestinal wall, thinner intestinal villi and decreased rate of villus epithelial cell renewal with consequent intestinal surface area reduction, smaller size of internal organ such as liver and heart with a cardiac output reduction as well as blood volume, altered mucosal enzyme patterns with an increased trypsin and decreased g-glucuronidase, a greater oxidation-reduction potential consequent to pH reduction, decreased synthesis of vitamin K and vitamin 8 complex, absent bile acid transformation, lack of short chain fatty acids or coprostanol, and finally an overall reduction of basal metabolic rate[11].

Contrary to the sterile womb paradigm from the past[12], there are many reports that corroborate the hypothesys that the uterine environment is not sterile, so know it is well known as the first contact with microorganisms that will contribute to the human body colonization begins into the

uterus where the fetus is exposed to commensal bacteria and their metabolites that originate from the commensal microbiota of the mother which cross the placenta and infiltrate the amniotic fluid[13],[14],[15]. The colonization beginning into the maternal uterus continues at the birth during the passage in the maternal vaginal tract. Therefore it is not surprising that there is a profound difference between cesarean section, wich also requires antibiotics administration to the mother which can be found into breast milk, and vaginal delivery.

The first gut colonization is also influenced by the neonatal feeding (breastfeeding vs formula); the matenal colostrum and milk are a source of microflora witch contributes to the first GI colonization that have a pivotal role in modulating and influencing the newborn's immune system. Indeed, bacteria in both colostrum and mature milk can promote anti-inflammatory responses, by stimulating the production of anti-inflammatory cytokines, reducing the risk of developing diseases such as inflammatory diseases and preventing the expression of immune-mediated diseases[16]. The maternal milk microbiota comprises more than 200 different bacterial species with a pivotal role in the formation of the first gut microbiota[17].

The specific mechanisms that lead to the formation of the human milk microbiota are still unknown. It has been widely believed that parts of these bacteria is composed by oral newborn bacteria and maternal skin population, such as Streptococcus spp. and Staphylococcus spp. that can flow back into mammary ducts during lactation[18],[19],[20]. Also, maternal gut bacteria can be found in the milk probably carried by dendritic cells (DCs) and CD18 through the mechanism of traslocation[20]. In addition to the first gut colonization contribute also vaginal and maternal intestinal bacteria ingested during delivery[21]. Human milk can stimulate the proliferation of Bifidobacterium and Lactobacillus, the main probiotic microorganisms in the gut, creating an acidic environmental rich in short chain fatty acids (SCFAs) with a protective and nutritive role at intestinal level[22].

In the first period of life lactic acid bacteria and coliforms are predominats. However, during the weaning drastic changes of the microflora occur and obligately anaerobic bacteria became prevalent. The first 2 years of life are crucial for the development and achievement of a complete colonization of the gastrointestinal tract. The transient gut microbiota strongly impacts on the newborn’s development, acting mainly on the maturation of his immune system, promoting the switch from a Th2 prevailing response in utero to a Th1/Th2 balanced one[21].

During the same period, the infant develops appropriate intestinal host defenses against infections and immune-mediated diseases. Since intestinal bacteria influence not only metabolic but also immunologic gut function, the fluctuations in bacterial colonization at the time when immune homeostasis is developing has a profound effect on the general health and on the prevention of diseases in particular infections and immune-mediated diseases[13]. In this regard the development of immune tolerance versus innocuous gut microbical antigens is pivotal to avoid a systemic immune response.

The GI tract is the seat of the body most populated by microorganisms. It is an ecosystem occupied by indigenous microorganism that remain remarkably constant over time.

A study in a female and a male healthy people, evalueted stool sampled daily for 6 months and 15 months respectively, has shown permanent fluctuations in the composition of the faecal microbiota over time[23]. However, the faecal microbiota tends to return to its typical compositional pattern, in a phenomenon termed resilience. The gut micobial mucosal-associated community differs from the colonic lumen coomunity and it is highly stable from the terminal ileum to the large bowel in a given individual[24].

Transient species derived from food and water, in a health system can't colonize the tract. The variability is higher in the colon because slower peristalsis and a more suitable pH. In the mouth there are aboundant transient bacteria due to food and drink, in order to 10^9 species and also about 200 species of indigenous flora[11]. The bacterial population is less represented in the stomach and the first intestinal tract because a faster peristalsis and also a lower pH. The indigenous microflora population is represented by Helycobacter species in the stomac and acid-tolerant lactobacilli and streptococci in the duodenum and jejunum able to survive the passage through the stomac[25].

The ileum is considered a transition zone characterized by a lower oxidation-reduction potential, with a bacterial population amount to 10^8. The large intestine is the most inhabiting side of the human body with a bacterial population amounting between 10^{10}-10^{11} and it is the dominant contributor to the total bacterial population. The 99.9% of the indigenous GI microflora is made up obligate anaerobes that are 100-1000 fold more numerous than facultative anaerobes.

More than 90% of all phylogenetic types (phylotypes) of colonic bacteria belong to just 2 of the 70 known divisions (phyla) in the domain Bacteria: the Firmicutes and the Bacteroidetes[26],[27]. The other 5 most fequent phyla are Proteobacteria, Fusobacteria, Verrucomicrobia, Cyanobacteria and Actinobacteria[28],[29]. Recent studies on healthy patients revealed a prevalence of Firmicutes (50-75%), followed by Bacteroidetes (10-50%), then Actinobacteria (1-10%), and Proteobacteria (<1%)[24]. The Firmicutes phylum is composed mainly by Gram+, aerobic and anaerobic bacteria. The largest population is composed by Clostridia strains, with variable activity ranging from beneficial and protective (e.g. C. scindens, cluster IV-XIVa) to pathogenic (e.g. C. difficile, C. perfrigens). The Firmicutes phylum also includes other well known potentially pathogenic streptococci, enterococci and staphylococci. They play an essential role in fermentation of carbohydrates producing SCFAs. Actinobacteria are Gram+ bacteria with beneficial potential including the Bifidobacterium genus, considered as a major probiotic bacteria. The Proteobacteria phylum comprises Gram-bacteria, in particular the family Enterobacteriaceae, including E. coli and K. pneumoniae. These are poorly represented in normal conditions, but use to increase upon dysbiosis[30].

The predominant genera are Bacteroides, Eubacterium, Collinsella, Bifidobacterium, Clostridium, Fusobacterium, Ruminococcus, Peptococcus, and Peptostreptococcus[31]. Other more recent studies identified as most abundant genera, Bacteroides, Faecalibacterium and Bifidobacterium with high variability between individuals[32]. Data suggested that the overall structure of the gut microbiota in each individual shows distinct patterns defined by interactions between members of the microbial community. Three different enterotypes were indentified by metagenomic sequences of faecal samples analysis from North American, European and Japanese adult

individuals[24]. The most interestingly found is that the distribution into these clusters was not related to the apparent phenotypic characteristics such as gender, age, body mass index, race, or country or continent of residence. Three enterotypes were identified based on the variation in the levels of the following genera: Bacteroides (enterotype 1), Prevotella (entero type 2) and Ruminococcus (enterotype 3) proving di existence of a limited number of well-balanced host–microbial symbiotic patterns. These findings might indicate that different enterotypes generate profile dissimilarities not related to the host phenotype, and differences between groups not necessarily mean abnormalities or disease-associated patterns[24],[33].

IMMUNOLOGICAL IMPLICATIONS

In the last decades the gut is recognized as the largest and the most influential immune organ in the body. It actively respond to potentially harmful pathogens and antigens, also create and maintain the tolerance towards other antigens and to potentially beneficial commensal and symbiotic bacteria[34].

It is now established that intestinal microflora helps to prevent disease-associated aberrant immune responses through the influence and relathionship with the host's immune system. The microbiota effect on regulatory cells influences the development of both Th1- and Th2-mediated diseases. Indeed the microbiota play an integral role in the development of Th1 and Th2 balance as showed by studies on germ free laboratory mice that tend to have Th2 dominant immune responses to the detriment of Th1[35]. Therefore microflora play a foundamental role in anti-inflammatory immune response through induction of regulatory T cells that help guide Th1 and Th2 balance[36].

The gastrointestinal mucosal barrier is selective for molecules and signals able to cross it. Its role is not only physical but also immunoloigcal. Transmembrane toll like receptors (TLRs) and cytoplasmic Nucleotide binding oligomerization domains (NOD) receptors are components of the innate immune system that act as microbial pattern recognition receptors[37]. These receptors play an important role in the interaction between luminal microbes and host-immune defense, leukocytes recruitment, and mucosal inflammation. Indeed Podolsky et al. back in 2002, demonstrated the association of some NOD2 gene mutations with Crohn's Disease (CD)[38], while Cario et al in 2007 showed the development of more severe experimental colitis in various TLR knockout mice[39]. NOD1 also recognize the intestinal microbiota-derived peptidoglycan enhancing the systemic innate immunity[40]. The symbiotic relationship between some microbes and the host is also supported by the evidence that the intestinal homeostasis is sustained by the recognition of specific commensal bacteria by TLR. The interaction of the microflora and immune cells of the gastrointestinal tract also supports the development and maturation of both the innate and adaptive immune system[34,41]. Other data showed that the commensal gut microflora DNA(gfDNA) reduces regulatory T-cell conversion through the interaction with TRL9 in vitro[42].

A study focused on Bacteroides Fragilis, an ubiquitous Gram-negative anaerobe mammalian gut colonizer, showed that bacterial zwitterionic polysaccharides (ZPSs) are unique T-cell dependent antigens that is able to mediate the proliferation of CD4+ Tcells in vitro[43] and a monocolonization with B. Fragilis is sufficient to correct several immunologic defects encountered in germ free

animals. In particular, the polysaccharide A (PSA), the most immunodominant ZPS, is internalized and processed by antigen processing cells (APCs) that presented by major histocompatibility complex class II to the CD4+ activated T-cells with expansion of these cells. Finally PSA play a role not only in Th1/Th2 imbalance but also in immune maturation by directing lymphoid organogenesis[44].

Other data showed that the commensal bacterium Bacteroides thetaiotaomicron can induces the production of a microbicidal protein, angiogenin 4 (Ang4), that is a mediator of host defense in the intestine[9]. These findings highlight the importance of mutual interactions between microbes and the host[34].

LABORATORY METHODS OF MICROBIOTA'S ANALYSIS

GENERAL CONSIDERATIONS

Until 1990 the study of the microbiota was dependent on coltures and approximately the 40% of species was never coltured in laboratory because difficulties due to intrinsic characteristic of bacteria (e.g. anaerobes obligate population) or difficulties in managing samples, therefore the microbioma analysis was unfeasible. In the past colture was the only methods to analyze the gut microflora. The main advantage of coltures methods is represented by the low cost but it is labour intensive and it gives only a limited view of the diversity of the gut microbiota, in fact <30% of gut microbiota population has been cultured, not necessarily because unculturable but rather because permissive growth conditions for these organisms have not yet been developed or discovered[45].

However, we must consider that some gut bacteria have a symbiotic relationship with each other and some elements depend on the metabolic activity of other for growth, thus limiting the usefulness of new pure-culture techniques[46].

Recentely, thanks to the culture-independent techniques for phylogenetic investigation and quantification, based on sequence divergences of the small subunit ribosomal RNA (16S rRNA), we are able to demonstrate the microbial diversity of the gut microbiota, qualitative and quantitative analysis of bacterial species and changes in the gut microbiota in relation to diseases. Available techniques are denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), fluorescence in situ hybridization (FISH), DNA microarrays, and next-generation sequencing (NGS) of the 16S rRNA gene or its amplicons[45]. The latter is the most frequentely and commonly used.

16S rRNA

70S ribosomes comprise a complex macromolecular machine present in Prokaryotes (bacterium), mitochondria and chloroplast, dispersed throughout the cytoplasm of a bacterial cell. They consists of 2 subunits, 50S and 30S, composed of 60% RNA and 40% proteins.. The 50S subunit (or large subunit) contains two RNA molecules: 5S and 23S, complexed with approximately 34 proteins. The 30S subunit (or small subunit) contains one RNA molecule: 16S ribosomal rRNA (16S rRNA). One of the functions of 16S rRNA is the initiation and extension of protein synthesis. As

rRNA (5S, 16S and 23S) is highly conserved between bacterial species, yet contains variable regions that yield a phylogenetic signal, it is a useful target for phylogenetic identification (bacterial identification). Of the three bacterial rRNA genes, the 16S rRNA gene provides the most tractable combination of conserved sites for PCR primers and variable regions that act as evolutionary chronometers, and it is, therefore, usually used in preference to 5S or 23S rRNA genes for phylogenetic identification[47],[48],[49].

The major part of contemporary culture-independent techniques for the analysis of the gut microbiota are based on analysis of the 16S rRNA gene.

qPCR (QUANTITATIVE PCR)

The Polymerase chain reaction is a method widely used to rapidly make millions to billions of copies of a specific DNA sample. The quantitative PCR (qPCR) or real-time PCR can also quantifies the amount of DNA present in the sample thanks to a compound that fluoresces when binding the double-stranded DNA. By plotting the level of fluorescence in a test sample against the number of PCR cycles using a logarithmic scale, the amount of DNA in the test sample can be quantified by reference to a standard curve derived from parallel amplification of known target copy numbers[50]. The signal intensity reflects the amount of DNA in the sample. The main PCR limits in the study of microbioma consist of bias caused by different lysis capacity from Gram+ and Gram-[49], the availability of primers able to check any phyla; last each step from the collection of the samples until the 16S rRNA amplicons represents a potential source of distortion[51].

qPCR can be used alone or in combination with other techniques. It is used to improve results from semiquantitaive techniques as microarrays DGGE o DNA[52].

SEQUENCING

Sequencing is the gold standard for taxonomic identifietion of species level, but this approach requires information from the full-length 16S rRNA gene, almost 1,500 base pairs long, which can only be practically sequenced from a clone library insert. The sequence is compared to an available databases which contain nucleotide sequences for more than 380,000 organisms[45]. Specialist databases for ribosomal RNA genes are increasingly being used. A comparison of the sequences to the database identifies the organism, provided that the full-length 16S rRNA gene has been sequenced. Sequencing can be performed directly on the 16S rRNA amplicon or using DGGE, TGGE or T- RFLP with reamplification of bands excised by PCR. Sanger sequencing was used until the 1990s, but it as been replaced by the faster and cheaper next-generation sequencing technologies[53].

Applied to the gut microbiota the Sanger method was been able to demonstrate interindividual variability[26]. This method quantifies and phylogenetically identifies the gut microbiota, and is useful for the analysis of uncultured bacteria. However, this approach is expensive and labour intensive, because difficulties related to the large number of clones to analyzed[45],[54].

NEXT-GENERATION SEQUENCING (NGS)

Next-generation sequencing (NGS) allows the sequencing of large genomes of millions of base pairs of DNA in a short time, in the order of weeks. It represents an approximately 2,000-fold increase in throughput over Sanger sequencing. The possibility to read shorter sequences (in order to one half of the read lengths generated in Sanger sequencing), allows to detect many more bacteria sequences that are in low abundance[45],[55].

The main challenge of this technology is the required infrastructure, such as computer capacity and storage and the management of the amount of produced data that required informatic advanced methods and bioinformatics and biostatistics skills[56].

With NGS the base are identified during the addition to the nascent chain. The process starts from a primer combining with a DNA polymerase and single labeled nucleotides. Once the double-stranded DNA synthesis reaction has started, every nucleotide inserted by the DNA polymerase, it is detected thanks to a specific fluorescence signal released by each nucleotides.

The newly formed cluster serves to have millions of close and identical sequences positioned on the flow cell. The primers used are Rd1 SP and Rd2 SP (SP = sequencing primer) which will allow the DNA polymerase to proceed with the synthesis of nucleotides. Each added nucleotide corresponds to a particular fluorescence emission, a detector records the fluorescence emitted at each point of the flow-cell, and an output system provides the exact sequence of the nucleotides.

However, all NGS platforms perform sequencing of millions of small fragments of DNA in parallel. Bioinformatics analyses are used to piece together these fragments by mapping the individual reads to the reference genome.

Several competing methods of Next Generation Sequencing have been developed by different companies.

To date there are a number of different NGS platforms using different sequencing technologies such as fluorescent sequencing (Illumina, Genereader NGS system-QUIAGEN), single molecule sequencing (Pacific Biosciences SMRT, Oxford Nanopore), pH sequencing (Life Technologies) and others. All NGS platforms perform sequencing of millions of small fragments of DNA in parallel and all of these methods need a DNA library to identify the genome[57].

The most common is Illumina also used by BMR-genomics laboratory for this study.

REVERSIBLE TERMINATOR SEQUENCING (ILLUMINA)

Reversible terminator sequencing differs from the traditional Sanger method in that, instead of terminating the primer extension irreversibly using dideoxynucleotide, modified nucleotides are used in reversible termination. Whilst many other techniques use emulsion PCR to amplify the

DNA library fragments, reversible termination uses bridge PCR, improving the efficiency of this stage of the process.

Reversible terminators can be grouped into two categories: 3'-O-blocked reversible terminators and 3'-unblocked reversible terminators.

3'-O-blocked reversible terminators

The mechanism uses a sequencing by synthesis approach, elongating the primer in a stepwise manner. Firstly, the sequencing primers and templates are fixed to a solid support. The support is exposed to each of the four DNA bases, which have a different fluorophore attached (to the nitrogenous base) in addition to a 3'-O-azidomethyl group.

3'-unblocked reversible terminators

The reversible termination group of 3'-unblocked reversible terminators is linked to both the base and the fluorescence group, which now acts as part of the termination group as well as a reporter. This method differs from the 3'-O-blocked reversible terminators method in three ways: firstly, the 3'-position is not blocked (i.e. the base has free 3'-OH); the fluorophore is the same for all four bases; and each modified base is flowed in sequentially rather than at the same time.

The main disadvantage of these techniques lies with their poor read length, which can be caused by one of two phenomena. In order to prevent incorporation of two nucleotides in a single step, a block is put in place, however in the event of no block addition due to a poor synthesis, strands can become out of phase creating noise which limits read length. Noise can also be created if the fluorophore is unsuccessfully attached or removed. These problems are prevalent in other sequencing methods and are the main limiting factors to read length.

This technique was pioneered by Illumina, with their HiSeq and MiSeq platforms[58].

ENTEROPATIC SPONDYLOARTHITIS

The close relationship between joints and gut inflammation has long been known[59],[60],[61]. Subclinical gut involvement was detected in up to 70% of patients with seronegative spondyloarthritis (SpA)[62],[63],[64] showing two forms of histological gut lesions: an "acute" inflammation mimiking an acute bacterial enteritis, and a "chronic" inflammation resembling the picture of inflammatory bowel diseases (IBD), especially Crohn disease (CD)[65].

In the long term, approximately 6% of patients with SpA will develop overt IBD[66], in particular in patients that showing chronic gut inflammation from the onset.

On the other hand, articular involvement is the most common extraintestinal manifestation of IBD, and SpA symptoms are present in up to 50% of patients with inflammatory bowel disease[67], and affects significantly the quality of life of patients[68].

The presence of joint involvement is second only to IBD severity itself in determining the reduction of quality of life[69]. Overall, literature reported that the prevalence rates of arthritis ranging from 16% to 33%[70,71,72,73,74,75,76,77,78,79,80].

Enteropatic arthritis are included in a cluster of inflammatory joint diseases including ankylosing spondylitis (AS), undifferentiated SpA, arthritis associated with acute anterior uveitis, psoriatic arthritis (PsA), reactive arthritis and idiopathic arthritis[81] according to the classification criteria of the European Spondyloarthropathy Study Group (ESSG)[82].

Articular manifestations in IBD should be clinically divided into peripheral and axial involvement. Peripheral arthritis is usually asymmetrical, predominantly in the lower extremities.

CLINICAL FEATURES

PERIPHERAL INVOLVEMENT

Peripheral Arthritis

Peripheral arthritis without axial involvement could be divided essentially into two groups even if overlaps are common: an oligoarticular large joint arthritis (Type 1), and a bilateral symmetrical polyarthritis (Type 2). Type 1 is the most common presentation and is predominantly seen in CD[72,73,76,83,78], and it seems most frequent in colonic localization[84]. The onset may precede the onset of bowel symptoms although it usually coincides with or presents after the onset of IBD[73,85]. Type 1 joint involvement results in joint pain with evidence of inflammatory signs, such as swelling or effusion, affecting fewer than 5 joints and mainly the large joints of the lower limbs. Symptoms are usually acute and self-limiting, persisting for a maximum of 10 weeks, without permanent articular damage and mostly occur during IBD flares. Type 2 peripheral arthritis affects more than five joints, minly the small joints of the hands, with a symmetrical distribution. Symptoms can persist for months to years and the evolution is independent of the activity of IBD. Both types are associated with the presence of other comrbidities suc as uveitis, and arthritis with erythema nodosum (mainly type 1)[73].

The prevalence in IBD patients ranges from 7 to 16% - 5–14% in UC and 10–20% in CD depending on study design[73,76,83,84,86]. Studies using the type 1 and 2 subdivision show a higher prevalence of type 1 vs type 2 (4–8% vs 1–3%)[72,73,78].

Enthesitis and Dactylitis

Enthesitis and dactylitis are common manifestations in SpA but are less extensively studied in BD patients. Enthesitis is the inflammation of the site of insertion of tendons in bones tha can cause severe pain, tenderness and swelling. Deending on the studies, the prevalence of enthesitis in IBD ranges from 5 to 10%[72]. Dactylitis, is a characteristic feature of SpA known also as sausage-like finger or toe characteryzed by a painful and diffuse swelling of the entire digit due to flexor

tenosynovitis and sometimes synovitis of small joints. The prevalence of dactylitis in IBD range from 2% to 4%[72],[86].

AXIAL INVOLVEMENT

Axial arthropathy IBD-related includes isolated sacroiliitis, inflammatory back pain (IBP) and AS. Peripheral involvement may also occur (Type 3 entheropatic arthritis). The onset of axial involvement frequently precedes the onset of IBD and follows a course independent of the IBD course and it is not influenced by bowel surgery[87].

Isolated Sacroiliitis

Isolated monolateral or bilateral inflammation of the sacroiliac (SI) joints can be asymptomatic[88] or symptomatic with pain and/or decreased mobility and morning stiffness. The prevalence varies widely from 2 to 32%[76],[88],[89],[90],[91],[92]. Radiological grading of sacroiliitis seems to be indipendent to the localization, extent, and duration of bowel disease[89].

Inflammatory Back Pain

Inflammatory back pain (IBP) is a consequence of inflammation of the SI, and it is the major clinical symptom of AS. IBP is characterized by an insidious onset, it improves with exercise and usually worse with rest, and is associated with morning stiffness. It may also present as pain during the second half of the night and/or alternating buttock pain. The prevalence of IBP in IBD patients ranges from 5 to 30%[72],[73],[75],[82], [90],[93].

Ankylosing Spondylitis

AS is a chronic inflammatory disease involving the axial. AS is prevalent in white males with an age between 15 and 40 years. Symptoms include inflammatory low back pain, loss of the lumbar lordosis and a limitation in spinal mobility due to bone neoapposition and fusion. The diagnosed is supported by evidence of radiological sacroiliitis.

The last modification of the New York criteria are useful to identify spine involvements in IBD[94]. Prevalence in IBD patients varies from 1 to 10%[73],[90],[92],[93]. No correlation was found between localization or extent of the intestinal inflammation and AS[90].

Non-inflammatory Pain

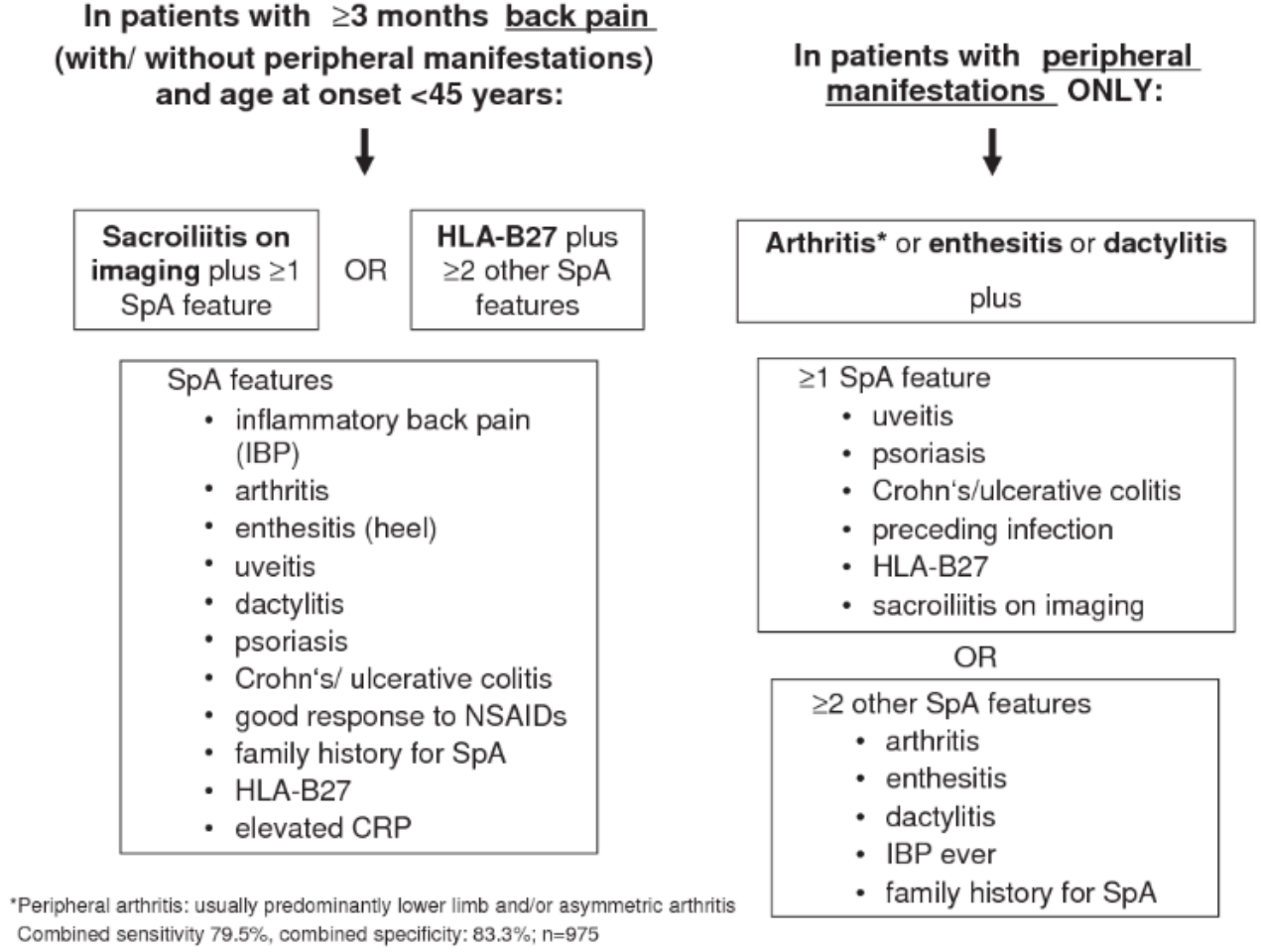
Joint pain without signs of inflammation ranged between 22% of CD patients and in 14% of UC patients[68], and in 14.3% in CD and 5.3% in UC[73]; de Vlam et al. reported a higher prevalence (30%)[93]. Arthralgia is a major medical problem in IBD patients[68]. Pain in IBD can be also related with altereted central processing and perception, such as fibromyalgia, but the exact prevalence of cental painful is debated[95] and ranged between 49% in CD patients and of 19% in UC patients ans 3.0% in CD, and 3.7% in UC[96],[97].

CLASSIFICATION CRITERIA

The Assessment of SpondyloArthritis international Society (ASAS) developed and validated new criteria for inflammatory back pain (IBP), which is a cardinal feature of SpA.

The sensitivity is 80% and specificity is 72% in a validation cohort. The ASAS IBP are fulfilled if at least four out of the following five parameters are present: 1) age at onset <40 years, 2) insidious onset, 3) improvement with exercise, 4) no improvement with rest and 5) pain at night (with improvement upon getting up) [98]. ASAS group has published new classification criteria for axial[99] and peripheral[100] spondyloarthritis, summarized in figure 1. Overall, these criteria, with a sensitivity of 79.5%, and a specificity of 83.3%, performed better than the largely adopted ESSG and Amor criteria.

Figure 1. ASAS classification criteria for axial (on the left) and peripheral (on the right) athritis.



LABORATORY ASSESSMENT

There are no helpfull laboratory tests as a diagnostic tool in the management or diagnosis of arthropathy in IBD. Diagnosis is clinical, based on the presence of signs and symptoms of arthritis. Indeed inflammatory markers such as C-Reactive Protein (CRP), Erythrocyte Sedimentation Rate

(ESR), platelet, or white blood cell count, can be elevated due to IBD activity or may be normal in active arthritis.

While a strong association is established between the Human Leukocyte Antigen-B27 (HLA-B27) and AS (positive in more than 90%), the frequency of HLA-B27 in IBD patients was found to be similar to the general population. The 25–78% of IBD patients with AS showed HLA-B27 positivity[90],[101], [102],[103] while isolated sacroiliitis in CD patients is not related to HLA-B27[104]. Currently, HLA-B27 is the only laboratory test included in the ASAS criteria[100].

CLINIMETRY

Several scores are used in the clinical practice to assess functional ability and disease activity in spondyloarthropathies; they are validated for ankylosing spondylitis (AS), but are also used for the evaluation of axial involvement in enteropatic arthritis. The most common used are: the Bath Ankylosing Spondylitis Metrology Index (BASMI)[105], the Bath Ankylosing Spondylitis Disease Activity Index (BASDAI)[106], the Bath Ankylosing Spondylitis Functional Index (BASFI)[107], and the ASAS-endorsed disease activity score (ASDAS score)[108],[109].

The BASMI score is a composite index and includes five clinical measurements that accurately reflect the axial involvement: cervical rotation, tragus-wall distance, lateral flexion, modified Schober's test and intermalleolar distance to evaluate the function of a different part of the spine.

A score between 0 to 2 is assigned to each topic, based on the results. The final BASMI score is calculated by a sum of individual scores; it can range between 0 and 10.

The BASDAI is a self-administered questionnaire defining disease activity in patients with AS. It consists of six 10 cm horizontal visual analogic scales used to measure severity of fatigue, spinal and peripheral joint pain, localized tenderness and morning stiffness. The final BASDAI score ranges from 0 to 10. Due to fast and simplicity, requiring less than 1 minute it is widespread in the clinical practice.

The BASFI explores the functional disability in patients with AS. It is a self-assessment questionnaire and consists of eight questions regarding function and two questions exploring the patient's ability to cope with everyday life. Each question is answered on a 10 cm horizontal visual analog scale; the BASFI total score ranges from 0 to 10 and is calculated as the average of the ten individual answers. Like BASDAI, BASFI score is quick and easy.

The ASDAS is a composite index for disease activity in ankylosing spondylitis. The variables are: four self-reported items (back pain, patient global, duration of morning stiffness and peripheral pain/swelling that are all assessed on a visual analogue scale from 0 to 10 cm or on a numerical rating scale, from 0 to 10) and the value of ESR (mg/L). The ASDAS containing CRP as acute phase reactant is the preferred version and the erythrocyte sedimentation rate version is the alternative one (Table 3).

There are three cut-off to evaluate disease activity

IMAGING

Enteropatic arthritis is usually nondeforming and nonerosive[72]. To detect peripheral involvement, ultrasonography and MRI studies on dactylitis are useful to identify signs of flexor tenosynovitis, soft tissue oedema associated to small joint synovitis. In particular musculoskeletal ultrasonography is the faster cheaper and accurate technique to evaluate enthesal abnormalities, erosions, calcification and enthesophytes, tendinopaty and bursal involvement[110]. Also power Doppler can reveal entheseal neoangiogenesis, increasing the specificity of ultrasonography in the diagnosis of SpA[111]. Recently, this technique was validated in entheropatic SpA[112].

Regarding the axial involvement, the demonstration of sacroiliitis on X-Ray is necessary to diagnose AS according to modified New York[94], while evidence of MRI bone oedema is a topic of ASAS criteria for the diagnosis of SpA[100]. X-Ray can highlight sclerosis, erosions, syndesmophytes and ankylosis. Radiographic spinal changes of AS are sclerosis and syndesmophytes, vertebral bridging and fusion (bamboo spine)[113].
A delay of 5–10 years may occur in the diagnosis of AS according to the modified New York criteria because a radiographic sacroiliitis of at least grade 2 bilaterally or grade 3 unilaterally is needed[114].
Thanks to more sensitive imaging techniques sacroiliitis was revealed in patients With silent X-Ray[92].
MRI shows acute inflammation, abnormalities of bone including edema (STIR sequences - short tau inversion recovery) and soft tissues and detects active sacroiliitis, especially in the early phase of disease; it is the only technique able to indentify sacroiliitis at te onset and then il all stages of evolution[113],[115],[116] therefore is useful to monitor the efficacy of treatment.

However bone dema can be detected at MRI also in patients with non-inflammatory diseases even if the severity is significantely lower[115].
In 2009, the ASAS/OMERACT MRI working group described the MRI findings of sacroiliitis and proposed ti defined active sacroiliitis if bone marrow edema or osteitis is present.
In the earliest active phases, MRI shows T1-hypointense, T2- and STIR-hyperintense signal abnormalities at one or more vertebral body corners representing oedema or focal osteitis, corresponding to X-Ray shining corners, that precede Romanus lesions.

Nowdays CT and scintigraphy have a marginal role.

PATHOPHYSIOLOGY

The exact pathogenetic mechanisms linking gut and joint inflammation remain unclear but shared genes and shared environmental factors are presumably involved[87]. Overlaps of several loci, including IL23R, rs2872507, and STAT3 are defined[117],[118],[119] which might have a significant pathogenetic role.

Increased intestinal mucosal permeability has been demonstrated both in IBD and ankylosing spondylitis patients[120]. This finding was not attributable to drug treatment[121] but it was shown to be independent from NSAIDs[122]. Furthermore, intestinal permeability was significantly increased only in patients with the chronic gut lesions, not in acute[123].
As T cells reactive to bacterial antigens have been detected in the joints, naive T cells are possibly primed by bacterial antigens in inflamed gut mucosa, recirculate, home to the synovium, and induce joint inflammation[124]. Lymphocyte trafficking to various tissues depends on various adhesion molecules and such as, for gut homing, α4β7 and αEβ7 integrins and MadCAM-1 mucosal vascular receptor. It has been speculated that lymphocytes from the gut may migrate to the synovium, leading to inflammatory arthritis; in fact identical T cell clones have been indentified both in synovium and gut mucosa from a patient with SpA[125] and macrophages expressing the scavenger receptor CD163 have been found in gut mucosa and synovium from patients with CD and SpA[126],[127]. Other studies revealed a pro-angiogenic intestinal mucosal phenotype was shown in spondyloarthritis patients with subclinical intestinal inflammation, with marked overexpression of VEGF-A, VCAM-1, and PlGF[128]. However the inciting antigen or immune trigger remains unclear.
Studies regarding TNF proposed a mechanism inolving overexpressing TNFΔARE [129]. Mice model of SpA-like disease has suggested that mesenchymal gut and joints cells may be targets for TNF mediated inflammation[130]. This suggests another cell type linking intestinal and joint pathologies.

A microbiota-host interaction is also fundamental to maintining gut wall integrity thanks to epithelial fucosylation that helps the commensal colonization, and also resisting pathogens in the mucosal lining. Fucosylated proteins are shed into the lumen and gut microbiota liberated and metabolized the fucose[131].

Also short chain fatty acids (SCFAs) are involved in intestinal permeability. In particular butyrate, regulates intestinal permeability[132]. While low doses of butyrate enhance barrier function high doses increase intestinal permeability, probably due to cell death[133].

A common feature of SFB and other intestinal microbes able to potentiate Th17 responses, such as Citrobacter Rodentium, is the strict attachment to gut epithelial cells. These findings support the notion that mucosa associated bacteria may be particularly relevant to IBD and SpA pathogenesis.

The NOD2 gene plays a role in the innate immune response by activating nuclear factor-kB (NF-κB), a regulator of the expression of a large number of genes encoding proinflammatory cytokines, adhesion molecules, chemokines, growth factors, and inducible enzymes[134]. Mutations in the NOD2 gene (R702W, G908R, and 1007 fs) found in about 20–30% of CD patients results in a altereted cellular response to bacterial components such as muropeptide Nacetylmuramic-L-Ala-D-isoGln and lipopolysaccharides, leading to intracellular persistence of pathogens[135],[136].

Some studies identified an association between NOD2 and isolated sacroiliitis with an high percentage of NOD2 variant in these patients[137] or a high prevalence of NOD2 polymorphisms in a CD-SpA population[119] but other studies do not confirm this data in AS[138],[139],[140]. Thus, NOD2 could play a role in the genetic link between intestinal and joint inflammation.

Another gene complex associated with IBD is the pathway leading to T helper 17 (Th17) cell development[141]. Th17 produces a specific cytokine pattern including IL17A, IL17F, IL22, TNF-α and IL6. TGFb and IL21 induce the Th17 differentiation while the perpetuation of the Th17 cell program is mediated by IL23R signaling. The differentiation is also regulated by retinoic acid receptor-related orphan receptor gamma (ROR gamma) and ROR alpha[142]. Variants in genes involved in IL23R signalling and Th17 differentiation have been found in both IBD and SpA. Th17 effector cytokines are aberrantly expressed in affected tissues of patients with CD and AS. IL23 gene expression is found increased in terminal ileum of AS patients, but unlike in CD, IL17 is not induced[143]. IL17 differentiation and effector cytokines are increased in intestinal mucosa of IBD patients mostly in active ulcerative colitis[144]. Findings suggest that a neutralisation of Th17 effector cytokines as therapeutic target or a neutralization of ROR gamma deserves attention, with possibly different effects in colonic and ileal inflammation. Recently it has been postulated that the treating of SpA with secukinumab, an IL-17 drug, could be trigger of gut inflammation even if data are not conclusive[145].

A strong relationship between HLA-B27 and arthritis is found for AS (90% of patients), while only the 25-78% of patients with AS and IBD was HLA-B27 positive, the 7% of patients with IBD and sacroiliitis and only the 7% of patients with isolated IBD[90],[146]. The role of HLA-B27 in IBD has not yet been clarified; some proposed, the persistence of bacteria because of altered or defective intracellular killing by HLA-B27 positive cells and misfolding of the HLA-B27 beta-pocket.

Another hypothesis proposes a cross reaction between bacterial peptides or arthritogenic selfpeptides due to HLA-B27 restricted T lymphocytes that present the antigens[147],[148],[149].

In HLA-B27 expressing macrophages, IL23 was found up-regulated in response to lipopolysaccharide stimulation. In addition a strongly expression of IL23 and IL17 in the colon of transgenic rats has been proven[150].

Results from HLA-B27 transgenic rat model studies highlighted the tendence of HLA-B27 heavy chain to misfold during the assembly of class I complexes in the endoplasmic reticulum (ER) and to form aberrant disulfide-linked dimers after transport to the cell surface[151], inducing profound changes in the cellular metabolism thought the activation of the unfolded protein response (UPR)[152]. These changes include inhibition of the translation and the transcription of molecular chaperone genes resulting in a downstream inflammation[153].

ER seems to have an important role in IBD in particular an association between variants in a key components of ER stress (XBP1) with CD and UC has been demonstrated and a deletion of XBP1 in intraepithelial cells induces spontaneous enteritis caused by Paneth cell dysfunction and over-reactive epithelium to bacterial products and TNF-α[154].

TREATMENT

The introduction of biologic drugs revolutionized the management of both spondyloathritis and IBD. In addition, during last 5 years new molecules target options non anti-TNF alpha are available for the treatment of seronegative spondloarthritis and IBD.

Since Type 1 peripheral arthritis is strictly related with exacerbations of IBD, the treatment of active intestinal flare is often enough to improve the arthritis. Conversely type 2 arthritis activity is independent of the activity of IBD and the options of therapy derive from the therapy of SpA including physiotherapy[155] that remains an essential part of the management plan.

NON-STEROIDAL ANTI-INFLAMMATORY DRUGS

Non-steroidal anti-inflammatory drugs (NSAID), including Coxibs, is still recommended as first-line drug treatment for AS in patients with pain and stiffness. The treatment with NSAIDs should be continuous other than on demand[156]. However an increased intestinal permeability was found in patients treated with NSAIDs, which could enhance mucosal susceptibility to luminal toxins including bacteria. Furthermore, NSAIDs inhibit prostaglandin synthesis, which usually improves gut integrity.

The intestinal toxicity of NSAIDs is well known[157],[158],[159],[160] and several studies confirmed the NSAIDs role in inducing intestinal flares[161],[162],[163].

Nimesulide and low-dose aspirin seem to be well tolerated[164], and limited evidence shows that the use of COX-2 inhibitors is not associated with IBD flares[165],[166],[167],[168]. However, since some studies reported IBD flares during COX-2 inhibitors therapy and data shown the role of COX-2 in the maintenance of small-bowel mucosal integrity[169],[170] NSAIDs use should be reserved to selectionned cases[171].

DISEASE MODIFYING ANTIRHEUMATIC DRUGS (DMARDS)

According to the 2019 American College of Rheumatology recommendations for the management of spondyloarthritis[156], at present there is no evidence that non-biologic DMARDs are effective for the treatment of axial involvement and few evidences support their use in peripheral involvement.

In particular, trials on methotrexate showed negative results on symptoms controlling regardless of the dosage. A mild improvement has been shown in patients with peripheral involvement so methotrexate could be considered for patients with predominately peripheral arthritis[172],[173],[174],[175].

To date Sulfasalazine is recommended primarily for patients with peripheral arthritis only. Although exist evidences for the efficacy of sulfasalazine in peripheral arthritis, other trials showed modest or nil response both in clinical and imaging[176],[177]. However, data strongly suggest that TNFi are a better option for these patients. Sulfasalazine may have a role for patients who can't take TNFi for any reasons. There are no evidence for other DMARDs. Preliminary data on tofacitinib are promising but studies are still ongoing[178].

In conclusion Sulfasalazine, and in few cases methotrexate, should be considered only in patients with peripheral arthritis or when tumor necrosis factor inhibitors (TNFi) are not available[156]

TNF-A INHIBITORS (TNFI)

There is strong evidence that the pro-inflammatory cytokine TNFα plays a role in the pathogenesis of AS[179]. The efficacy of TNFi in patients with active AS has been demonstrated in more than 24 randomized controlled trials with improvements in patient-reported outcomes, composite response criteria, and bone inflammation on magnetic resonance imaging (spine and sacroiliac MRI). According to the 2019 American College of Rheumatology recommendations, TNFi treatment is strongly reccomended for patients with active AS that failed 2 NSAIDs (at least 4 weeks of treatment)[156]. Indirect comparisons in meta-analyses of clinical trials have not showed clinically meaningful differences among TNFi[180] and only few direct comparisons are available, limited to a trial of infliximab versus its biosimilar, and a very small open-label trial of infliximab versus etanercept[181].

Randomized controlled trials have shown the efficacy of the TNF-α inhibitors etanercept[182],[183],[184],[185], infliximab[186],[187], adalimumab[188] and golimumab[189] in the treatment of axial disease and also in the treatment of peripheral and entheseal involvement[179],[187],[190],[191],[192].

Only few data are available on efficacy of anti-TNFα in the treatment of entheropatic SpA. In these reports signs and symptoms of axial and peripheral joint involvement in IBD were improved by infliximab. It also improved gastrointestinal symptoms and was well tolerated[193],[194],[195],[196],[197].

Adalimumab has proven to be effective and well tolerated in CD patients[198], but no studies are available on the effectiveness of adalimumab on arthropathies IBD related.

Despite etanercept is well tolerated and efficacious in the treatment of SpA[199],[200] it is not reccomended in IBD because it showed no efficacy in controlling CD colitis[201],[202] and seems to favor inflammation in the bowel mucosa probably trought the production of TNF-α and IFN-γ[203].

Regarding new molecules approved for SpA, IL-17 inhibitors (secukinumab and ixekizumab), despite their efficacy on articular involvement they are not recommended for IBD patients because a possible association with the new onset, or exacerbation, of CD [156],[204],[205],[206],[207].

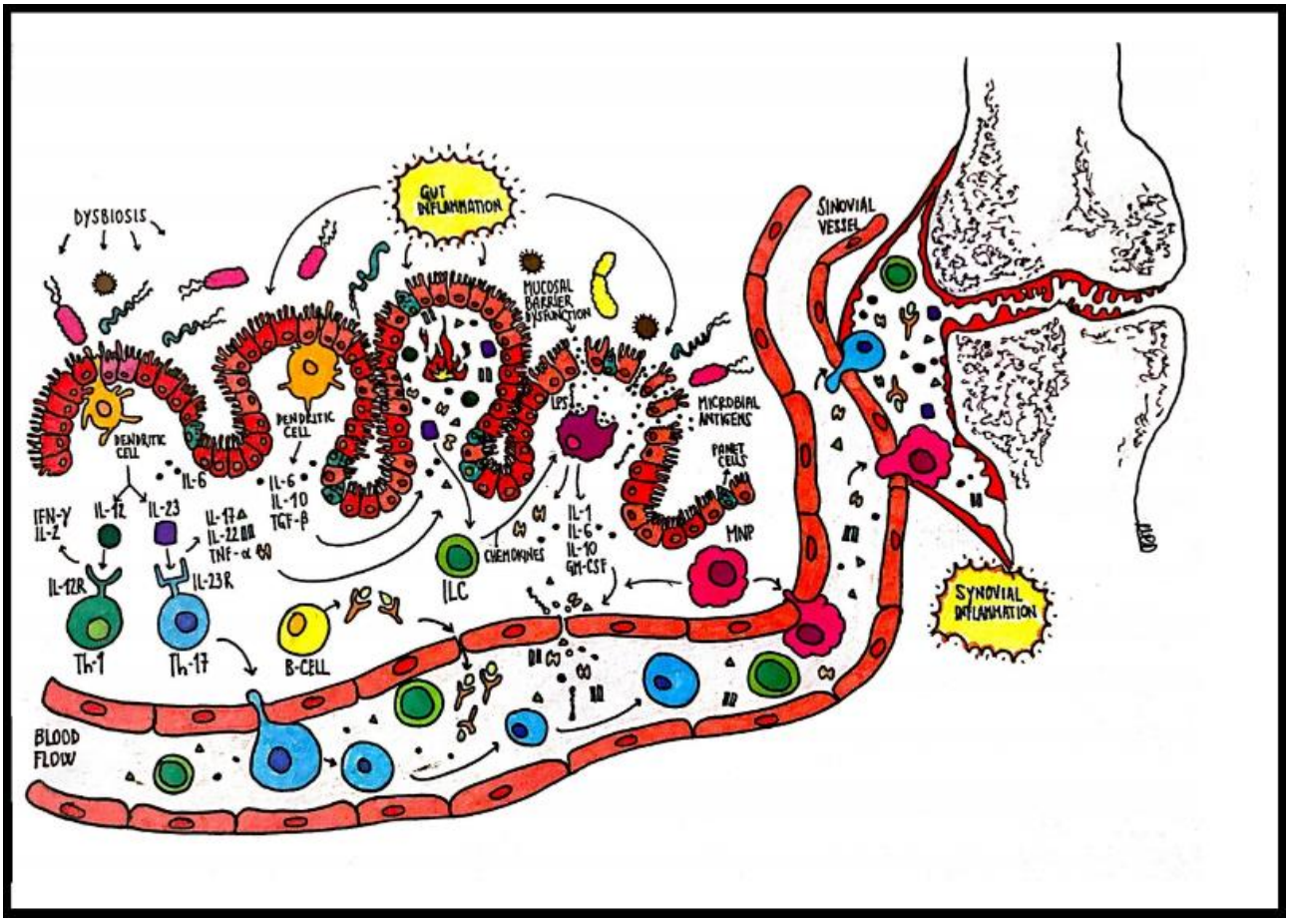
In summary infliximab, adalimumab, and certolizumab (the latter in USA only) are approved for the treatment of CD disease, and infliximab, adalimumab, and golimumab are approved for the treatment of UC, while etanercept is not approved for either condition[208],[209] so the 2019 recommendations favor TNFi monoclonal antibody use in patients with AS and coexisting IBD while IL-17 inhibitos are not recommended[156].

MIROBIOTA, SPONDYLOARTHRITIS AND IBD

Several studies showed the relationship between dysbiosis and diseases including periodontal disease[210], cancer[211], obesity[212],[213], diabetes[214],[215], IBD[216],[217], chronic fatigue syndrome[218]. Evidences on dysbiosis and Spondyloarthritis are published. Interestingly data shown a close

relationship between gut inflammation, SpA and dysbiosis that could be a link between SpA and IBD. In particular, a decreased in a major phyla of Firmicutes, in particular Faecalibacterium prausnitzii and Clostridium leptum species, have been found in several inflammatory diseases including SpA and IBD[219] (Figure 2).

Figure 2. Relationship between dysbiosis, IBD and SpA. Abbreviations: *IL* Interleukin, *ILC* innate lymphoid cell, *IFN* interferon, *TNF* tumor necrosis factor, *LPS* lipopolysaccharide, *MNP* mononuclear phagocytes, *GM-CSF* granulocyte monocyte colony stimulating factor.



ANIMAL MODELS OF SPA

As mentioned above, the gut microbiota plays a crucial role in the education and costitution of human immune system as proven in sperimental animal germ free models[220]. So is not surprising that dysbiosis can promote autoimmune diseases. Back in 1994 Taurg et al showed for the firts time as B27 transgenic rats raised in a germfree environment do not develop gut and joints inflammatory disease, but re colonization was sufficient to drive the disease. Interestingly, in germ free condition only joint and gut but not skin and genital manifestation were avoided, again supporting the close relationship between gut an joints[221].

Lin et al[222] compared the microbiome studied on histological of gut mucosa of HLA-B27 transgenic rats and wild type (non-transgenic); they found an increase of Prevotellaceae and a decrease of

Rikenellaceae combined with gut inflammation. Gill et al[223] investigated the effect of HLA-B27 on inflammation and the relationship with gut microbiota. Based on evideces that HLA-B27 transgenic rats raised in germ free conditions developped key feaures of SpA after recolonization[221],[224] they compared 3 different genetics rats backgroung (dark agouti - DA, Lewis, and Fischer, were compared, using wild-type littermates and HLA–B7– transgenic Lewis rats as controls). Trasgenic Lewis and Fischer developed gut inflammation with a production of proinflammatory cytokines such as IL-17, IL-23, IFN, TNF,IL-1 while DA were restistant to the HLA-B27 effect. Moreover authors observed differences in HLA-B27 mediated dysbiosis between rats but the immunological inflammatory pathway was the same for Fischer and Lewis, indicating that despite the effect of HLA-B27 on gut microbiota was dependent on genetic background, immune dysregulation was similar in both groups.

Regarding microbial population, HLA-B27 transgenic Fischer rats showed an increases in the abundance of Akkermansia and Bacteroides uniformis, an increase in Roseburia and Anaerotruncus and a decrease in Coprococcus, while no diferences was found in these population in HLA-27 transgenic Lewis or DA rats compared with their respective WT controls. Contrary, in HLA-B27 transgenic Lewis rats authors found a decrease of f_Christenellaceae, Mucispirillum schaedleri and an unknown species of genus, Mucispirillum and an increase in Prevotella but not in DA or Fischer rats. Lewis rats and DA rats shared an increase in the frequency of an unclassified genus of f_Barnesiellaceae and Sutterella not found in Fischer[223]. An interesting finding was the absence of segmented filamentous bacteria (SFB), in particular Candidatus Arthromitus, in DA, indipendent of HLA-B27 in contrast with both wild and transgenic Lewis and Fischer. SFB has been related with hight levels of [219]by Th-17 in several reports[225],[226],[227]. Other studies focused on SFB such as Citrobacter rodentium, showed that the colonization of germ free K/BxN mice was sufficient to induce arthritis Th17 cell mediated[228],[229]. SFB antigens are presented by intestinal dendritic cells (CD11c +), evoking a highly SFB-specific Th17 response proving that mucosal T cells are modulated by gut bacterial components. The microbiota Th17 induced response and its intimate association and attachment to intestinal epithelial cells support the pathogenesis microbiota mediated of IBD and SpA[219].

The frequency of SFB was very low also in HLA-B27 transgenic Lewis. In these two groups lacking SFB, two major contributors to this niche were f_Clostridiaceae (unknown genera) and Lactobacillus. Despite the paucity of shared dysbiotic microbiota HLA-B27 related in contrast with similar inflammatory mediators involved, these data attested that the background has a crucial role on HLA-B27 induced dysbiosis. Moreover, even if microbiota abundance differed between Fischer and Lewis (Akkermansia muciniphila, p_Verrucomicrobia vs Prevotella, p_Bacteroidetes respectively) both of these microbes have pro-inflammatory activity trought the disruption of mucosal homeostasis. Similarities between cecum and colon flora of Lewis and Fischer rats were found, in particular an increase in short-chain fatty acid producers such as Clostridium and Coprobacillus, that may play a role in disease development, because in short-chain fatty acid are able to influence regulatory Tcell homeostasis. Also a dicrease in Ruminococcus was detected[230].

Other bacteria such as Chlamydia trachomatis, fequentely identified as etiologic pathogen of reactive arthritis, has been associated with IL-23 and polymorphisms in the IL-23 receptor (IL23R) was found both in AS and IBD[231]. IL-23 interaction with IL23R promotes the expansion of Th17 cells and stimulates Th17 cytokine production[232]. Rehaume et al. confirmed the close relathionship between genetic factors and microbiota and the crucial role of IL-23 in flares of diseases. They studied SKG mice carring a mutation in ZAP-70, wich are a model of SpA. This mutation results in the development and expansion of CD4+ Th17 T cells with T cells activation in SKG but not in WT. When these mice was treated with microbe-associated molecular patterns (MAMPs), mice showed a hight grade of inflammation Th17 mediated IL-23 induced, hesitating in SpA-like phenotype. Mice in germfree conditions showed a mild arthritis and ileitis. Cohousing SKG mice with WT mice suppressed the ileitis but had not influence on arthritis, suggesting that host microbiome interactions play a role in IL-23-dependent loss of mucosal function in SKG mice, triggering ileitis in response to MAMPs[233].

DISBYOSIS IN SPA

Several studies on microbiota and spondyloarthrits revealed a status of dysbiosis in these patients. Some studies found a distinct microbial colonization in the terminal ileum of a patients with AS vs healthy controls. In particular an increase in the abundance of Lachnospiraceae, Ruminococcaceae, and Prevotellaceae in AS patients was found that are the same species observed in the drug-induced colitis in experimental mice[234]. Authors also described a decreased of Streptococcus and Actinomyces. Similar findings discovered Breban et al. that compared 3 groups of patients: rheumatoid arthrtitis (RA), SpA and healty control (Hc). Ruminococcus Gnavus was found to be 2 or 3 times more abundant in SpA that in Hc and RA. Moreover HLA-B27+ patients showed a different microbiota compared with HLA-B27 negative. Germfree growth condition was enough to avoid both IBD and arthritis[235]. Analyzing differences between the 3 groups, despite the profile of gut microbiota appeared dominated by Firmicutes and Bacteroidetes at the phylum level and by Lachnospiraceae, Ruminococcaceae and Bacteroidaceae families, consistent with the usual human microbiota composition, they found differences in distribution between groups. In particular regarding SpA the analysis showed more Lachnospiraceae less Prevotellaceae and Paraprevotellaceae. Analysis showed an increase in Firmicutes belonging to the Lachnospiraceae family, including Ruminococcus, Dorea, Coprococcus and Blautia genera and in Actinobacteria from the Coriobacteriaceae family in SpA patients and increased species included R. gnavus, Blautia pruducta and Bifidobacterium longum, while Roseburia faecis was decreased. The most important variation between groups that distinguished SpA from RA and Hc was R. Gnavus. Interestingly authors found a relationship between R. Gnavus and BASDAI in SpA patients in particular with concomitant IBD or history of IBD, while no correlations with NSAIDs were highlighted. R. Gnavus may substein infammation trought its beta-Glucuronidase activity wich plays a major role in the generation of toxic and carcinogenic metabolites in the large intestine[236].

Its increased abundance has been shown also in Crohn's disease, especially after ileocolonic resection with a hight risk of pouchitis[237],[238],[239],[240],[241]. This mucolytic, anaerobic, Gram-positive bacteria may play a role in the triggering or maintenance of gut mucosa inflammation[242].

Other study on AS patients confirmed an increase in sulphate reducing Bacteroides. Similar findings were discovered in juvenile SpA classified as enthesitis-related arthritis (ERA), exhibit decreased abundance of Clostridium leptum[243],[244],. Rath et al studied HLA-B27 transgenic rats models in germfree conditionts and after recolonization: Bacteroides were able to drive gut inflammation, whereas Lactobacillus and fusiform were not[224].

Several species including Bacteroides vulgatus and Akkemansia muciniphila, linked by mucolitic activity, have been related to joint and gut inflammation. Such property could be a trigger of disease, facilitating access of the gut epithelium to other bacteria and their invasion of the mucosa that may contribute to joint inflammation[222],[245],[246].

Other reports studied gut mucosa in SpA confirmed an enrichment of the Firmicutes phylum in particular Lachnospiracae family, including Coprococcus and Ruminococcaceae in these patients. This study reports an increased biodiversity in SpA patients in contrast with the theory of a poor microbiota in dysbiosis[247]. However studies on biopsies found no significative differences in SpA microbial composition except for incrising in Dialister genus[248].

Environmental factors, such as breastfeeding, have been related to SpA susceptibility[249]. Last the role of genetic components is supported by comparison between siblings with reports that the HLA-B27+ healthy siblings of patients with SpA exhibited a different microbiota composition from their HLA-B27− healthy siblings, with increased R. mucilaginosa and E. lenta, two bacterial species related with IBD[250],[251], and low levels of Bifidobacterium and Odoribacter, as reported in patients with ileal Crohn's disease and ulcerative colitis[250],[252].

Subclinical gut inflammation was also detected in many patients affected by PsA[253]. Microbial alterations were also detected in both psoriatic and psoriatic arthritis patients. In particular a global reduction of diversity of taxa[254] and a decrease in Coprococcus whereas a decline in relative abundance of Ruminococcus and Akkermansia, unique to PsA, similar to IBD[239].

Another commensal, Alistipes, was lower in abundance in both PsA[254] and Crohn's disease[255]. Many of these microorganisms play a role in degrading mucus and producing SCFAs that influencing gut homeostasis.

DISBYOSIS IN IBD

The reduction of biodiversity has been detected also in both CD and UC patients, with a lower proportion of Firmicutes, and an increase in Gammaproteobacteria[256]. In particular, studies of twin couples with one element affected by UC, evaluating biopsy samples, showed less bacterial diversity with more Actinobacteria and Proteobacteria and less Bacteroidetes in UC than in their healthy twin[257]. This loss of the biodiversity in patients with UC is associated with temporal instability of the dominant taxa; in faecal samples sequentially collected from UC patients in stable remission and with stable medication for 1 year, only one-third of the dominant taxa were persistently detected[258].

In CD several studies showed alterations in Clostridia, in particular a decrease in Roseburia and Faecalibacterium genera of the Lachnospiracae and Ruminococcaceae families, in the contrast

with increase in Ruminococcus gnavus[240],[259],[260]. Morgan et al analyzed 231 fecal and biopsies samples of IBD patients. Interestingly the most differences in microbial population were related with the sample origin (stool or biopsy) as highlighted by other reports[252],[257],[261]. Overall they found a decrease aging related in Bifidobacterium and a significant reduction in Roseburia and Phascolarctobacterium, in both UC and CD, while Clostridium was increased. Roseburia is associated with anti-inflammatory regulatory T cell production[262], and it is a butyrate producers[263], while Phascolarctobacterium is succinate consumer, and propionate producer when cocultured with Paraprevotella[264] with a resulting decrease in butyrate and propionate production. The Ruminococcaceae, were decreased in CD, while the Leuconostocaceae were decreased in UC. In the contrast the Enterobacteriaceae Escherichia/Shigella were increased in CD.

The microbiome of CD patients with ileal involvement and pancolic UC showed a dramatic decrese in Ruminococcaceae family and of Faecalibacterium compared to other involvement. These data were similar to previous studies[265],[266].

Faecalibacterium prausnitzii is a major butyrate producer and exhibits anti-inflammatory effects in a colitis setting[260] which was found decreased in several reports[267]. Reduction of Roseburia and Ruminococcaceae may have consequences on the ability of the host to repair the epithelium and to regulate inflammation[263],[264]. Other studies documented an increase in E. Coli known to activate TLR4, upregulated in intestinal epithelium of IBD patients; mutations in TLR4 are associated with both CD and UC[38],[268],[269].

The involvement in IBD pathogenesis of bacteria able to produce damaging substances was proved by Rowan et al that analyzed colon biopsy samples from patients with UC demonstrating an increase in Desulfovibrio subspecies, a Gram-negative, anaerobic and sulphate-reducing bacteria involved in the pathogenesis of UC substeined by the capacity to generate sulphides[270],[271]. Moreover high density of Fusobacterium was found in biopsy of colonic mucosa showing inflammatory activity in several studies[272],[273] showing citotoxic power against Vero cells in vitro[274] and invasive power in a Caco- 2 cell assay[275].

TNF INHIBITORS AND MICROBIOTA

The interactions between gut microbiota and drugs are complex. It is easy to understand how difficult is to investigate the impact of drugs in unhealty people, such as IBD or autimmune disorders, because the disease itself impacts on microbiota. Moreover in the major part of cases the patient's therapy consist of multiple drugs with consequent multiple drug-interactions. Differences in microbial composition of sick people could influence the occurrence of side effects or alter the mechanism of action of certain drugs[276].

The impact of antibiotics on gut microbiota is well known[277],[278],[279],[280] in particular on Bifidobacterium species[281]. Bacteria are the therapeutic target of antibiotics but they can also act on the resident microbiota. For instance, treatment with ciprofloxacin can influence not only the abundance of bacterial taxa but also causes a decrease in taxonomic richness, diversity and evenness of the host microbiota but the recover after treatment is often incomplete after 6

months[282]; vancomycin reduces the microbial diversity and the absolute number of gram-positive such as Firmicutes; amoxicillin seems to have unsignificant impact on total bacterial numbers and diversity; finally data showed that a combination of antibiotics (ampicillin, gentamicin, metronidazole, neomycin, and vancomycin) may reduce the total number of bacteria and radically shifted the composition of the gut microbiota[283],[30]. Some authors showed that the microbiota imbalance may persist for two years after antiobiotic interruption[284] probably due to the interdependence of different bacterial taxa explaining why , for instance, vancomycin that targeted Gram+, can deplete Gram− commensals[285]. In the light of this, several studies focused on antibiotics in IBD were performed and the effect of antibiotics treatment was exstensively studied with different results[286],[287]. Even if antibiotics, probiotics and fecal transplantation may play a role in reestablishing a microbial balance, the real efficacy against the disease is controversial and seems to be modest[24],[30],[288],[289].

Moreover, several studies on the effect of a variety of common drugs on gut microbiota were published[277],[290],[276],[291]. Is well known the impact on gut microbiota of PPI, associated with an increase of oral bacteria in the gut[292],[293] and metformina related with an increase of SCFA producers[294],[295]. Vich Vila and collegues published a metanalysis evaluating three Dutch cohorts of patients including general population, IBD and IBS undergoing several therapies (almost 50 different drugs). They identify 537 combination of drugs showing an increase in total antibiotic resistance genes in drug users compared to people not using any drugs. They also observed that PPIs use consistently altered the gut composition probably due to the changes in acidity that facilitate the growth of upper intestinal bacterial in the gut. Also a combination of different drugs induced alteration in composition but this effect could reflect either severe health conditions that impact the microbiome composition or a direct effect on microbiota caused by multiple drug intake. For all cases of multi-drug combination no change in the microbial richness was detected, suggesting no clear depletion or colonisation of bacteria multi-drug induced. Oral steroids increased Methanobrevibacter smithii[276] while metformine induced significant changes in the bacterial composition and metabolic potential with an increase of E. Coli[294],[295]. A recent study, demonstrated that PPI may altered the microbiota not only through the action on pH but also directly exhibiting inhibitory effects on bacterial species in particular Dorea and Ruminococcus[290].

While the impact of TNF inhibitors(TNFi) on restoring gut permeability is well known[296],[297],[298], poor data are available on microbiota and TNFi. A study on mice proteoglycan(PG)-induced arthritis revealed a lack of diversity in PG group compared with mice treated with TNFi and the controls. Moreover PG-induced mice showed a decreased abundance of Bacteroidetes (38.4%) and an increased abundance of Firmicutes (54.8%) and Proteobacteria (5%) compared controls while the relative abundances of these three phyla in mice treated with TNFi did not significantly differ, and the ratio of bacteroidetes to Firmicutes was similar to that of its control group. The TNFi group showed high abundances of Alloprevotella, Barnesiella, and Lactobacillus than the PG and control groups. TNFi also restored the relative abundances of Alloprevotella, Barnesiella, Odoribacter, and Clostridium XIVa in PG patients, and the relative abundance of Lactobacillus was increased in TNFi treated mice compared with PG and control group[299]. In addition Noth R et al. found that TNFi

decreased the intestinal permeability in patients with CD but is not cleare if this effect depends directly on TNFi or by indirect regulation of the gut microbiota[300].

A recent study published by Aden and collegues focusing on IBD and rheumatic disorders-RD (AS and Rheumatoid Arthritis), showed an increase in richness and phylodiversity in IBD patients treated with TNFi, whereas there were no differences after treatment in microbiota of patients with RD. However, the authors didn't identify differences between remitting and non-remitting patients based on a-diversity restorations. The evaluation of b-diversity showed increased interindividual differences among IBD patients after TNFi treatment confirming that the treatment induces an increase of heterogeneity between patients with IBD. Once again these findings were not be confirmed in RD patients, in fact in this group the treatment was associated with a decrease of dissimilarity, indicating an overall constriction of microbial b-diversity among these patients. By the comparison of differences between healthy subjects and the two patient groups (IBD, RD) before and during TNFi, they found a shift TNFi-induced of the microbial communities of both groups toward healthy group, proving a subtle corrective effect of anti-TNF treatment on microbial dysbiosis of both disease entities, IBD and rheumatic diseases. Again, no differences between remitting and non-remitting subjects was found. Indicator bacterial phylotypes were different between Healthy, IBD (14 phylotye identified) and RD (5 phylotypes identified) patients losting the indicator species status after TNFi treatment, suggesting that these phylotypes are normalized with therapy; in particular Coprococcus and Roseburia in IBD patients and Erysipelotrichaceae and Dorea in RD group[301].

Bazin and collegues investigated the predictive role of microbiota composition in the TNFi response. They evaluated 19 patients divided in two groups, responders and not responers. They showed a hight percentage of Firmicutes. Authors found that patients non-responders to TNFi were patients with high disease activity and unstable microbiota composition. Moreover patients with reduced microbiota diversity at the baseline were more likely to fail to the TNFi treatment. Interestingly the treatment abolished the differences among patient groups, suggesting that, independently to the clinical response, TNFi treatment was able to restore the fecal microbial diversity in all patients[302].

AIM OF THE STUDY

To investigate the impact of TNFi on gut microbiota.

MATERIALS AND METHODS:

PATIENTS RECRUITMENT AND STUDY DESIGN

Twenty patients were recruited at the Rheumatologic-Gastroenterologic Diseases clinic of the Città della Salute e della Scienza di Torino, Italy, from June 2017 to December 2018.

Inclusion criteria were established as it follows:

- at least 18 years old, able to provide informed consent to the partecipation in the study;

- patients on a typical Mediterranean diet;
- Crohn's or UC diagnosis in patients fulfilling criteria for axial or peripheral SpA (ASAS 2009[303],[304]);
- patients able to perform the sample's own test after appropriate training as per BMR genomics protocol;
- naïve to TNFi, with the introduction of treatment for at least one of arthritis or IBD or both, according to current guidelines for RD[305] and for IBD[306],[307];
- stable concomitant treatment during the previous three months and for the duration of the study.

Exclusion criteria were as follows:
- history of major bowel resection;
- history of minor bowel resection or digestive stoma during the preceding five years;
- any contra-indication to TNFi therapy;
- refusal to sign the informed consent or linguistic or cognitive difficulties that did not allow a full understanding of the consent form;
- pregnancy or breastfeeding.
The non-interventional character of this study has been approved by the ethical committee.

Clinical history, physical examination, instrumental examinations, biochemical examination including C-reactive protein (CRP), erhytrocyte sedimentation rate (ESR), HLA-B27 and fecal calprotectin at the baseline and after 6 months were performed.

All patients were screened for HBV, HCV and Mycobacterium Tuberculosis (HBsAg, HBcAb, HBsAb; HCV-Ab; quantiferon-TB gold assay, chest X-ray) before starting biologic, as per normal clinical practice. Fecal samples were collected for every partecipant, the first 24 hours before starting TNFi and the second after 6 months of therapy. Treatment decisions were made due to clinical requirements after discussion at the interdisciplinary rheumatic/gastroenteric board.

All patients filled out a diet diary to verify the mediterrean diet and the absence of food intolerance with consequent bias due to food restrictions.

The design of prospective study had no influence on treatment or other clinical actions. Patients were naïve to TNFi treatment or other biologic.

The study was approved by the ethics committee of the Città della Salute e della Scienza di Torino (approval code 0056924), Italy and subjects provided written informed consent. TNFi included infliximab and adalimumab.

SAMPLES COLLECTION, PROCESSING AND ANALYSIS
Fecal samples were collected using feces collection tubes 2 ml with screw caps and transport buffer (prefilled DNA stabilizer) provided by BMR genomics, and stored at -80°C until further use. All patients were trained for fecal sample collection, to reduce as much as possible contaminating contacts of the sample. All samples were found to comply with the quality control.

The processing performed through metagenomic NGS (next generation sequencing or sequencing in parallel) by BMR Genomics laboratories included:

- DNA extraction with Qiagen automated method and pre-treatment with Tissue Lyzer (Qiagen®);
- the amplification of the V3 and V4 regions of the 16S (V3 and V4) with universal primers according to Takahashi et. al 2014[308];
- DNA extraction with Qiagen automated method and pre-treatment with Tissue Lyzer (Qiagen®);
- the second amplification step for the attachment of adapters and index (Nextera XT®) for the sequencing and identification of the samples;
- multiplexing of samples;
- sequencing on the Illumina MiSeq platform (San Diego, CA, USA) in the Paired End 2x300 bp format;
- verification of sequencing results.
- standard bioinformatic taxonomic analysis of the 16S NGS

A simple food daily diary was distributed to all participants, with the request to write in detail about all the food they consumed to check strict dietetic regimens and the intake of drugs different from standard therapy, for any reason.

PATIENT ASSESSMENT

All patients were scheduled for biologic therapy for medical reasons and received drug at least until week 24. Clinical disease indices were assessed at baseline and at the end of the study using Visual Analogue Scale (VAS)-pain and Visual Analgue Scale(VAS)-disease activity for all patients, Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) for axial involvement, clinical disease activity index (CDAI) and Health Assessment Questionnaire-Disability Index (HAQ-DI) for peripheral involvement, Harvey-Bradshaw Index for CD (HBI)[309], or partial Mayo (pMAYO) score for UC. The BASDAI clinical response was defined as a BASDAI <4[107]. The improvement in HAQ-DI was defined as a minimum clinically important difference (MCID) of 0.22[310]. Responders in the HBI for CD were defined by a decrease in the HBI score greater than or equal to 2 (or HBI ≤ 4 at six months), corticosteroid-free therapy. For UC patients remission was defined as reduction of the pMAYO[311] by at least 2 points, or final score ≤ 1, in the absence of corticosteroids.

STATISTICAL ANALYSIS

Continuous characteristics are presented as median and interquartile range (IQR) and mean and standard deviation (SD). For proportions, absolute and relative frequencies are reported.

Mc-Nemar test was performed to analyze qualitative differences and Wilcoxon test to analyze mean differences (between paired samples).

Univariate regression models are implemented to test the microbiota differences between baseline and after 6 months (linear model for clinimetric variables and logistic model for IBD success or SPA improvement).

A p value <0.05 is considered to indicate statistical significance. The analysis was performed using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Twenty patients met inclusion criteria and accepted to partecipate to the study. Informed consent was obtained from study participants.

Overview of patient characteristics are summarized in table 1. Patients were 12 males and 8 females and the mean age was 51(±12.8) years old; 4(20%) patients were smokers; 17(85%) patients were affected by CD, 3(15%) by UC; arthritis involvement was AS in 11(55%) patients, peripheral SpA in 8(40%), psoriatic arthritis in 1(5%); extra-articular manifestations included psoriasis in 4(20%) patients and uveitis in 6(30%) patients; HLA-B27 was positive in 3 (15%) patients.

The IBD localization was colon in 12(60%) patients, ileum in 8(40%). Surgery (ileocecal resection) had been performed in 9(45%) patients before the last 5 years.

At the baseline 90% of patients received mesalazine, 60% of patients took systemic corticosteroids and 20% took an immunosuppressant (azathioprine). All patients followed a mediterreanean diet.

Table 1. Demographic and biometrics characteristics.

Characteristics	Value
Median age(IQR)	53(43.8-61.5)
Sex n(%) - Male	12 (60%)
Smokers n(%)	4 (20%)
Family History of RD/IBD n(%)	3 (15%)
Years of illness (years)	14.5 (1–38)
Arthritis n(%) - PsA	1 (5%)
Arthritis n(%) - AS	8 (40%)
Arthritis n(%) - SpA	11 (55%)
Axial n(%)	10 (50%)
Peripheral n(%)	12 (60%)
IBD n(%) - Crohn's	17 (85%)
IBD n(%) - UC	3 (15%)
Skin involvement n(%)	4 (20%)
Eye involvement n(%)	6 (30%)
HLA-B27 n(%)	3 (15%)
Endoscopic score n(%) - 1	2 (10%)
Endoscopic score n(%) - 2	13 (65%)
Endoscopic score n(%) - 3	5 (25%)
Gut localization n(%) - E3	2 (10%)
Gut localization n(%) - L1	8 (40%)
Gut localization n(%) - L2	3 (15%)
Gut localization n(%) - L3	6 (30%)
Gut localization n(%) - L4	1 (5%)

CLINICAL OUTCOMES

After six months of therapy, no patient discontinued TNFi due to adverse events; 100% of the patients achieved clinical IBD remission, but the success of the therapy (corticosteroids free) was achieved in 65% of patients (13 out of 20) only. Improvement of rheumatic manifestations was achieved in 70% of patients.

CRP decreased from a median value of 8.2 mg/L (4.8–20.8 mg/L) at T0 to a median value of 2.9 mg/L (1–4 mg/L) at T6 (p = 0.001). Similarly, erythrocyte sedimentation rate (ESR) decreased from the median value of 21.5 mm/h (10.8–34 mm/h) at T0 to 11 mm/h (7.8–21 mm/h) at T6 (p = 0.003). Calprotectin decreased from a median value of 207.5 µg/g (125.5–446.2 µg/g) at T0 to a median value of 81 µg/g (50–197.2 µg/g) at T6 (p = 0.004).

Regarding clinimetry we observed a BASDAI decrease from 5.2(4.1-5.6) to 2.8(2.5-4.3, p 0.013), CDAI decrease from 13(10.5-16) to 7(5.2-11, p 0.004)(see table 2).

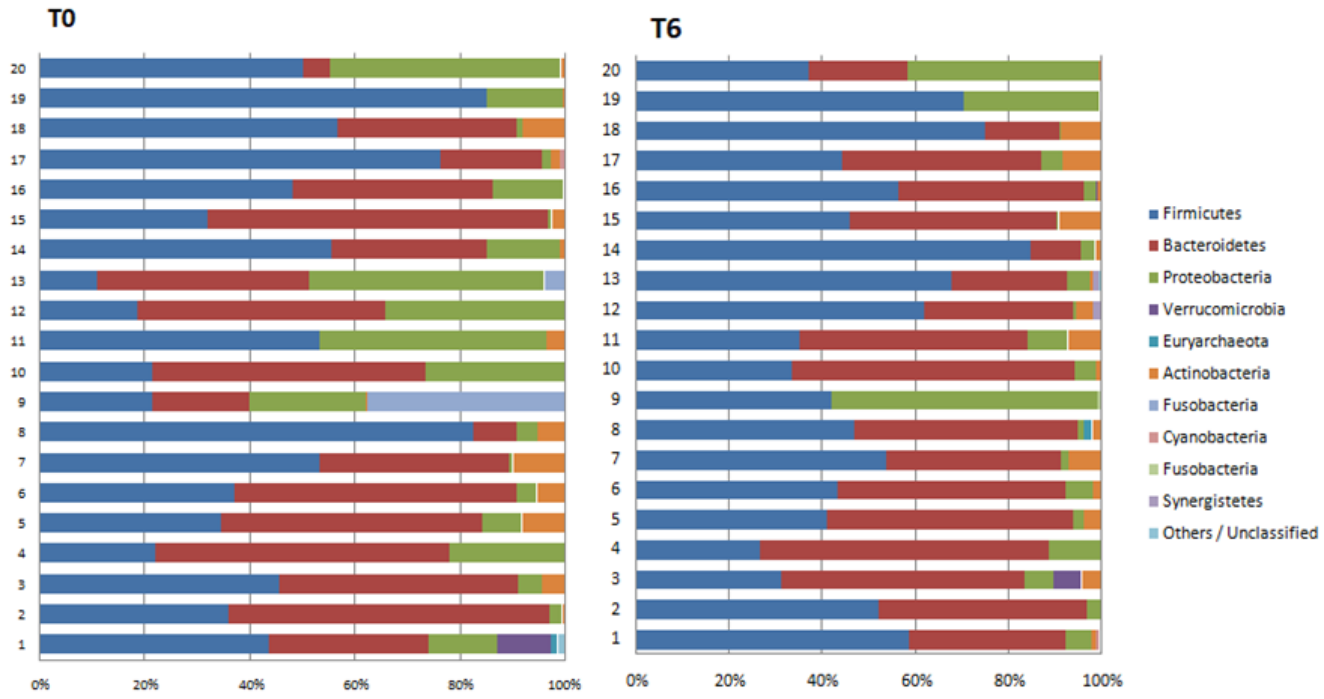
Table 2. Comparison between clinical variables between baseline and T6 (six months)

Clinical variables	T0	T6	P_value
Fecal Calprotectin(µg/g)- median (IQR)	207.5(125.5-446.2)	81(50-197.2)	0.004
CRP(mg/L)- median(IQR)	8.2(4.8-20.8)	2.9(1-4)	0.001
ESR(mm/h)- median(IQR)	21.5(10.8-34)	11(7.8-21)	0.003
VAS_pain- median(IQR)	50(38.8-60)	35(10-42.5)	0.001
VAS_disease- median(IQR)	50(38.8-50)	37.5(25-42.5)	0.006
HAQ- mediana(IQR)	0.6(0.1-0.8)	0.2(0.1-0.6)	0.004
BASDAI_score- median(IQR)	5.2(4.1-5.6)	2.8(2.5-4.3)	0.013
CDAI_activity- median(IQR)	13(10.5-16)	7(5.2-11)	0.004
IBD activity n(%) - 0	11 (55%)	20 (100%)	0.174
IBD activity n(%) - 1	6 (30%)	0 (0%)	
IBD activity n(%) - 2	2 (10%)	0 (0%)	
IBD activity n(%) - 3	1 (5%)	0 (0%)	

MICROBIOTA COMPOSITION DESCRIPTION AND EFFECT OF TNFI ON MICROBIOTA

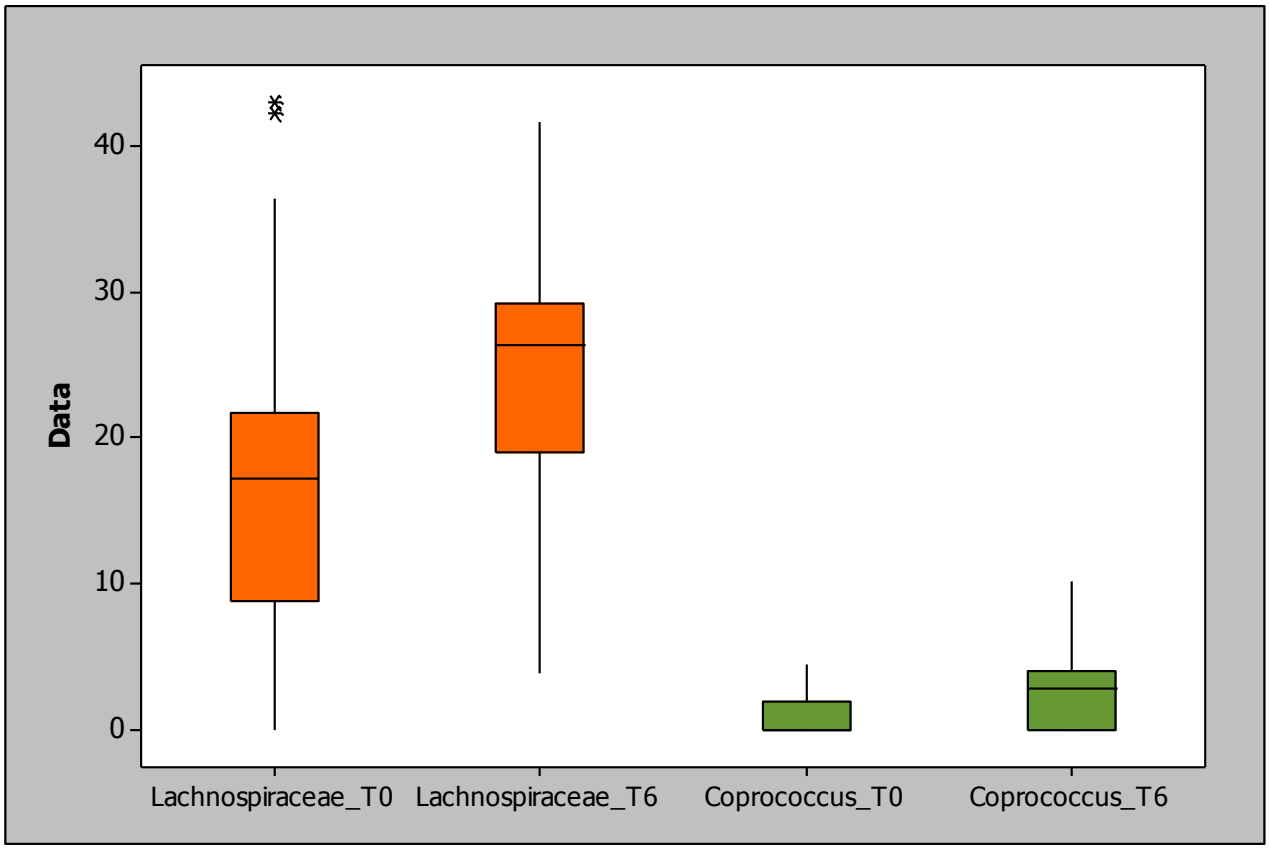
The fecal microbiota at baseline was characterized by a very high proportion of Firmicutes [43.6(29.5-53.8)] followed by Bacteroidetes [35.5(19.1-50.3)], Proteobacteria [12.6(3.4-23.3)], Actinobacteria [0.8(0.1-4.5)] (see figure 3).

Figure 3. Distribution of phylum at the baseline (T0) and after six months (T6).



We compared the global composition of the fecal microbiota at T0 and T6 (table 3-8). After 6 months of therapy we observed significant differences among family of Lachnospiraceae (Δ +10.3, p 0.04 - table 6) and genus of Coprococcus (Δ +2.8, p 0.003 - table 7). We also observed an increase in unclassified species group (Δ +10.3, p 0.015).

Figure 4. Comparison between Lachnospiraceae and Coprococcus at the baseline (T0) and after 6 months (T6) of therapy.



Despite no statistical significant diefferences, we observed a trend in Proteobacteria phylum (Δ -8.0 p 0.095 - table 3), class of Clostridia (Δ +8.2 p 0.083) and Gammaproteobacteria (Δ -9, p 0.093 - table 3).

The univariate analysis of comparison between clinimetric variables (covariates) and the delta (T6-T0) of the significant microbiota variables (outcome) didn't show significant results except for CRP (p 0.033) (table 9).

The univariate analysis of comparison between clinimetric variables (covariates) and the delta (T6-T0) of the microbiota variables (covariates) and the IBD success/SpA improvement outcome didn't show any significant correlation (table 10).

Table 3. Differences between microbiota phylum at baseline and after 6 months.

PHYLUM	**T0**	**T6**	**P_value**
Firmicutes- median(IQR)	43.6(29.5-53.8)	46.4(40-59.6)	0.261
Bacteroidetes- median(IQR)	35.5(19.1-50.3)	41.5(23.9-49)	0.952
Proteobacteria- median(IQR)	12.6(3.4-23.3)	4.6(2-6.9)	**0.095***
Verrucomicrobia- median(IQR)	0(0-0)	0(0-0)	1
Euryarchaeota- median(IQR)	0(0-0)	0(0-0)	1
Actinobacteria- median(IQR)	0.8(0.1-4.5)	1.4(0.5-4.7)	0.433
Fusobacteria- median(IQR)	0(0-0)	0(0-0)	0.371
Cyanobacteria- median(IQR)	0(0-0)	0(0-0)	1
Fusobacteria- median(IQR)	0(0-0)	0(0-0)	1

Synergistetes- median(IQR)	0(0-0)	0(0-0)	0.346
Unclassified- median(IQR)	0(0-0.1)	0(0-0.1)	0.533

Table 4. Differences between microbiota class at baseline and after 6 months.

CLASS	**T0**	**T6**	**P_value**
Clostridia- median(IQR)	34(18.9-47.2)	42.2(34.3-52.1)	**0.083***
Bacteroidia- median(IQR)	36.2(24.5-50.8)	42.9(28.6-49.1)	1
Gammaproteobacteria- median(IQR)	10.8(0-22.8)	1.8(0-6)	**0.093***
Verrucomicrobiae- median(IQR)	0(0-0)	0(0-0)	1
Betaproteobacteria- median(IQR)	0.4(0-1.7)	0(0-1.9)	0.286
Erysipelotrichi- median(IQR)	0(0-0.7)	0(0-0.9)	0.919
Bacilli- median(IQR)	1.2(0-6.4)	4.5(0-8.8)	0.813
Actinobacteria.1- median(IQR)	0.6(0-4.1)	1.4(0-5.7)	0.295
Alfaproteobacteria- median(IQR)	0(0-0)	0(0-0)	1
Fusobacteriia- median(IQR)	0(0-0)	0(0-0)	0.371
Coriobacteriia- median(IQR)	0(0-0)	0(0-0)	1
Deltaproteobacteria- median(IQR)	0(0-0)	0(0-0)	0.371
Methanobacteriales- median(IQR)	0(0-0)	0(0-0)	1
Synergistia- median(IQR)	0(0-0)	0(0-0)	0.346
Unclassified- median(IQR)	0.7(0.4-1.4)	1.7(0.5-2.5)	0.26

Table 5. Differences between microbiota order at baseline and after 6 months

ORDER	T0	T6	P_value
Clostridiales- median(IQR)	34(18.9-47.2)	38.2(33.4-49.1)	0.247
Bacteroidales- median(IQR)	35.5(19.1-50.3)	41.5(23.9-49)	0.952
Enterobacteriales- median(IQR)	7.7(0-22.8)	0.8(0-6)	0.132
Verrucomicrobiales- median(IQR)	0(0-0)	0(0-0)	1
Burkholderiales- median(IQR)	0.4(0-1.7)	0(0-1.9)	0.266
Erysipelotrichales- median(IQR)	0(0-1.2)	0(0-0.9)	0.824
Lactobacillales- median(IQR)	1.9(0.1-9.4)	4.5(0.2-8.7)	1
Fusobacteriales- median(IQR)	0(0-0)	0(0-0)	0.371
Bifidobacteriales- median(IQR)	0.1(0-3.7)	1.1(0-4.3)	0.625
RF32- median(IQR)	0(0-0)	0(0-0)	1
Pasteurellales- median(IQR)	0(0-0)	0(0-0)	0.371
Actinomycetales- median(IQR)	0(0-0)	0(0-0)	0.371
Turicibacterales- median(IQR)	0(0-0)	0(0-0)	1
Coriobacteriales- median(IQR)	0(0-0)	0(0-0)	1
Desulfovibrionales- median(IQR)	0(0-0)	0(0-0)	1
Methanobacteriales.1- median(IQR)	0(0-0)	0(0-0)	1
Synergistales- median(IQR)	0(0-0)	0(0-0)	0.346
Unclassified- median(IQR)	1(0.4-1.7)	1.8(0.8-2.5)	0.33

Table 6. Differences between microbiota family at baseline and after 6 months

FAMILY	T0	T6	P_value
Ruminococcaceae- median(IQR)	3.6(0-11.7)	0(0-12.7)	0.263
Bacteroidacea- median(IQR)	30(13.6-42.1)	23.6(16.3-40.6)	0.92
Enterobacteriaceae- median(IQR)	7.7(0-22.8)	0(0-6)	0.142
Verrucomicrobiaceae- median(IQR)	0(0-0)	0(0-0)	1
Lachnospiraceae- median(IQR)	17.1(9.2-21.1)	26.5(20-29.1)	**0.044****
Prevotellaceae- median(IQR)	0(0-0)	0(0-0)	1
Veillonellaceae- median(IQR)	1.9(0-5.9)	1.2(0-12.5)	0.17
Porphyromonadaceae- median(IQR)	0(0-0)	0(0-4.3)	0.126
Bifidobacteriaceae- median(IQR)	0(0-0.8)	0(0-0)	0.529
Alcaligenaceae- median(IQR)	0(0-0)	0(0-0)	1
Streptococcaceae- median(IQR)	0(0-0.8)	0(0-6.1)	1
Fusobacteriaceae- median(IQR)	0(0-0)	0(0-0)	0.371
Enterococcaceae- median(IQR)	0(0-0)	0(0-0)	1
Pasteurellaceae- median(IQR)	0(0-0)	0(0-0)	0.371
Clostridiaceae- median(IQR)	0(0-0)	0(0-0)	1
Peptostreptococcaceae- median(IQR)	0(0-0)	0(0-0)	0.181
Erysipelotrichaceae- median(IQR)	0(0-0)	0(0-0)	0.423
Rikenellaceae- median(IQR)	0(0-0)	0(0-0)	0.423
Lactobacillaceae- median(IQR)	0(0-0)	0(0-0)	0.281
Paraprevotellaceae- median(IQR)	0(0-0)	0(0-0)	1
Unclassified- median(IQR)	8.4(3.9-12.1)	12.2(6.1-13.9)	0.305

Table 7. Differences between microbiota genus at baseline and after 6 months

GENUS	T0	T6	P_value
Faecalibacterium- median(IQR)	0(0-5.2)	0(0-3.3)	0.262
Bacteroides- median(IQR)	30(13.5-42.1)	24.3(18.1-41.4)	0.828
Escherichia- median(IQR)	4.2(0-20.9)	0.2(0-5.6)	0.209
Akkermansia- median(IQR)	0(0-0)	0(0-0)	1
Parabacteroide- median(IQR)	0(0-2)	0.3(0-3.9)	0.724
Oscillospira- median(IQR)	0(0-0.4)	0(0-2.4)	0.906
Blautia- median(IQR)	3.8(1.2-7.3)	8.1(2.6-12.4)	**0.087***
Ruminococcus- median(IQR)	2.1(0.9-3.6)	2.1(1.5-4.9)	0.828
Roseburia- median(IQR)	0(0-1.7)	0(0-0.2)	0.813
Sutterella- median(IQR)	0(0-1.7)	0(0-1.9)	0.683
Prevotella- median(IQR)	0(0-0)	0(0-0)	1
Paraprevotella- median(IQR)	0(0-0)	0(0-0)	1
Lachnospira- median(IQR)	0(0-0)	0(0-0)	0.673
Phascolarctobacterium- median(IQR)	0(0-0)	0(0-0)	0.584
Coprococcus- median(IQR)	0(0-1.6)	2.8(0-4)	**0.003****
Coprobacillus- median(IQR)	0(0-0)	0(0-0)	1
Acidaminococcus- median(IQR)	0(0-0)	0(0-0)	0.855
Dorea- median(IQR)	0(0-0.8)	0.6(0-3)	0.152
Streptococcus- median(IQR)	0.9(0-3.1)	1.6(0-6.1)	0.589
Dialister- median(IQR)	0(0-0.1)	0(0-0.2)	0.813
Bifidobacterium- median(IQR)	0(0-3.1)	1.3(0-4.3)	0.379
Phascolarctobacterium.1- median(IQR)	0(0-0)	0(0-0)	1
Megasphaera- median(IQR)	0(0-0)	0(0-0)	0.787
Peptostreptococcus- median(IQR)	0(0-0)	0(0-0)	1
Fusobacterium- median(IQR)	0(0-0)	0(0-0)	0.371
Veillonella- median(IQR)	0(0-0)	0(0-0)	0.418
Klebsiella- median(IQR)	0(0-0)	0(0-0)	0.59
Clostridium- median(IQR)	0(0-0)	0(0-0)	0.272
Enterococcus- median(IQR)	0(0-0)	0(0-0)	1
Lactobacillus- median(IQR)	0(0-0)	0(0-0)	1
Serratia- median(IQR)	0(0-0)	0(0-0)	1
Haemophilus- median(IQR)	0(0-0)	0(0-0)	0.371
Morganella- median(IQR)	0(0-0)	0(0-0)	0.789
Actinomyces- median(IQR)	0(0-0)	0(0-0)	0.371
Granulicatella- median(IQR)	0(0-0)	0(0-0)	1
Acidaminococcus.1- median(IQR)	0(0-0)	0(0-0)	0.423
Succinaclasticum- median(IQR)	0(0-0)	0(0-0)	0.371
Turicibacter- median(IQR)	0(0-0)	0(0-0)	1
Eubacterium- median(IQR)	0(0-0)	0(0-0)	1
Pediococcus- median(IQR)	0(0-0)	0(0-0)	0.789
Proteus- median(IQR)	0(0-0)	0(0-0)	0.371
Collinsella- median(IQR)	0(0-0)	0(0-0)	1
Bilophila- median(IQR)	0(0-0)	0(0-0)	1
Porphyromonas- median(IQR)	0(0-0)	0(0-0)	1
Megamonas- median(IQR)	0(0-0)	0(0-0)	1
Epulopiscium- median(IQR)	0(0-0)	0(0-0)	1
Unclassified- median(IQR)	12.1(8.3-22.7)	16.9(9.9-27.9)	0.36

Table 8. Differences between microbiota species at baseline and after 6 months

SPECIES	T0	T6	P_value
Faecalibacterium_prausnitzii- median(IQR)	0(0-5.2)	0(0-3.3)	0.286
Escherichia_coli- median(IQR)	4.2(0-20.9)	0(0-5.6)	0.184
Akkermansia_muciniphila- median(IQR)	0(0-0)	0(0-0)	1
Bacteroides_uniformis- median(IQR)	1.1(0-5.9)	1.1(0-4)	0.514
Bacteroides_plebeius- median(IQR)	0(0-0)	0(0-0)	1
Bacteroides_ovatus- median(IQR)	0.6(0-3.2)	0.8(0-3.5)	0.625
Bacteroides_fragilis- median(IQR)	0(0-1.8)	0(0-0.8)	0.944
Bacteroides_Caccae- median(IQR)	0(0-0)	0(0-0)	0.586
Parabacteroides_distasonis- median(IQR)	0(0-2)	0(0-0.2)	0.919
Prevotella_copri- median(IQR)	0(0-0)	0(0-0)	1
Ruminococcus_bromii- median(IQR)	0(0-0)	0(0-0)	1
Ruminococcus_gnavus- median(IQR)	0(0-1.8)	0(0-2.2)	0.563
Ruminococcus_Torques- median(IQR)	0(0-0)	0(0-0)	0.789
Ruminococcus_callidus- median(IQR)	0(0-0)	0(0-0)	1
Dorea_formicigenerans- median(IQR)	0(0-0)	0(0-0)	1
Bifidobacterium_adolescentis- median(IQR)	0(0-1.5)	0(0-0.4)	1
Bifidobacterium_longum- median(IQR)	0(0-0)	0(0-0)	0.181
Blautia_producta- median(IQR)	0(0-0)	0(0-0)	1
Blautia_Obeum- median(IQR)	0(0-0)	0(0-0)	1
Roseburia_Faecis- median(IQR)	0(0-0)	0(0-0)	1
Veillonella_Dispar- median(IQR)	0(0-0)	0(0-0)	0.855
Streptococcus_Luteciae- median(IQR)	0(0-0)	0(0-0)	0.588
Clostridium_Butyricum- median(IQR)	0(0-0)	0(0-0)	1
Clostridium_piliforme- median(IQR)	0(0-0)	0(0-0)	0.361
Clostridium_neonatale- median(IQR)	0(0-0)	0(0-0)	1
Haemophilus_Parainfluenzae- median(IQR)	0(0-0)	0(0-0)	0.371
Morganella_Morganii- median(IQR)	0(0-0)	0(0-0)	0.789
Eubacterium_dolichum- median(IQR)	0(0-0)	0(0-0)	1
Lactobacillus_mucosae- median(IQR)	0(0-0)	0(0-0)	0.371
Lactobacillus_zeae- median(IQR)	0(0-0)	0(0-0)	1
Coprococcus_eutactus- median(IQR)	0(0-0)	0(0-0)	1
Pyramidobacter_piscolens- median(IQR)	0(0-0)	0(0-0)	1
Unclassified- median(IQR)	66.8(48.5-76)	77.1(68.6-80.5)	**0.015****

Table 9. Univariate analysis of comparison between clinimetric variables (covariates) and the delta (T6-T0) of the significant microbiota variables (outcome)

	Lachnospiraceae		Coprococcus	
Variable	**Coeff**	**P-value**	**Coeff**	**P-value**
Fecal Calprotectin	-0.003	0.408	-0.000	0.413
CRP	-0.157	0.540	-0.099	0.033**
ESR	0.062	0.652	-0.022	0.409
VAS pain	-0.044	0.783	0.002	0.946
VAS disease	-0.028	0.868	0.013	0.679
HAQ	1.717	0.774	1.208	0.287
BASDAI	2.669	0.507	-0.512	0.560
CDAI	-1.366	0.062	0.017	0.867

Table 10. Univariate analysis of comparison between the delta (T6-T0) of the microbiota variables (covariates) and the IBD success / SPA improvement (outcome)

	IBD success		SPA improvement	
Variable	**Exp(Coeff)**	**P-value**	**Exp(Coeff)**	**P-value**
Delta Lachnospiraceae	1.053	0.201	0.925	0.100
Delta Coprococcus	1.412	0.234	1.251	0.394

DISCUSSION

The human gut microbiota is composed of approximatelly 100 trillion bacteria, belonging to several hundreds of different species[6]. More than 90% of all colonic bacteria belong to just 2 of domain Bacteria: the Firmicutes and the Bacteroidetes, followed by Actinobacteria and Proteobacteria; other less represented phyla include Verrucomicrobia and Fusobacteria[26]. The distribution of these groups varies along the gastrointestinal tract, according to different microenvironments and nutrient availability[30].

The healthy microbiota contributes to the health of the host. It plays a fundamental role in mantainance in the nutrient metabolism of indigestive compounds, drugs metabolism, contribution in development and maintenance of structural integrity of the intestinal mucosal barrier, regulation of immune system, and defence against pathogens[9],[10].

It was strongly established the relationship between dysbiosis and several diseases including periodontal disease[210], cancer[211], obesity[212],[213], diabetes[214],[215], chronic fatigue syndrome[218].

Evidences on dysbiosis in Spondyloarthritis and in IBD were found and it has been suggested that it represents a link between these two inflammatory diseases. For instance germ free rats HLA-B27+ developed not only gut but also joints inflammation after recolonization[220]. While the effect of

some drugs on microbiota, such as metphormine, PPI and antibiotics is well known, poor data are available on TNF inhibitors.

The efficacy of these drugs on SpA and IBD has now been widely demonstrated so it is not suprising how as expected we found a significant improvement in inflammatory parameters both in acute phase index (CRP, ESR) and fecal calprotectin.

The aim of our study was to check microbiota modification after TNFi in a population affected by enteropatic arthritis. While some data are available on IBD or SpA alone no literature data exist on this cluster of patients. TNFi can impact on the disease improvement not only trought modifying intestinal permeability but also with a direct action on microbiota. For instance, animal models of PG-induced arthritis showed an impaired abundance on the three most representative phyla while TNFi treated animals do not and TNFi was able to restore the relative abundance of such species including Lactobacillus and Clostridium[298], a decrease in a major phyla of Firmicutes, in particular Faecalibacterium prausnitzii and Clostridium leptum species in both SpA and IBD[219], and an imbalance between Firmicutes and Gammaproteobacteria, with an increase of the latter has been observed in CD[255]. So first we investigated the microbiota composition before and after treatment at the taxonomic levels (phylum, order, family, genus and species) comparing the faecal samples before and after six months of TNFi (adalimumab or infliximab) therapy. Focusing on the phylum level we observed a reduction in Proteobacteria from 12.6(3.4-23.3) to 4.6(2-6.9) consistent with literature even if without statistical significance (p 0.09). Similarly we observed the same trend for the class of Gammaproteobacteria with e reduction from 10.8(0-22.8) to 1.8(0-6, p 0.09) belonging to Proteobacteria phylum while an increase in Clostridia from 34(18.9-47.2) to 42.2(34.3-52.1, p 0.08) belonging to Firmicutes. These results are consistent with the increase of Firmicutes/Proteobacteria ratio in IBD patients and seems to indicate a shift versus eubiosys after TNFi.

SpA and IBD share the decrease of Roseburia and the increase of Ruminococcus Gnavus belonging to Lachnospiraceae family but while in SpA an increase of the overall Lachnocpiraceae has been shown this population was found globally reduced in CD[266]. From the analysis at the family level we observed a significative increase of Lachnospiraceae from 17.1(9.2-21.1) to 26.5 (20-29.1, p 0.04) and in particular for Coprococcus genus switching from 0(0-1.6) to 2.8(0-4, p 0.003) even if we can't demonstrate variation on Ruminococcus Gnavus. Our data suggest again an impact of TNFi on gut microbiota (figure 4).

The typical dysbiosis in our cohort, based on these findings, appears to be more similar to IBD dysbiosis, probably indicating a major impact of IBD respect to SpA on the microbiota of these patients. Moreover since reports about dysbiosis in SpA are often antithetical, probably depending on the samples (mucosal or fecal) and SpA and IBD share some but not all microbial alterations, this could explain why we have observed such trends not always real statistical significance as well as the reason for which we have not highlighted well-known and verified bacterial alterations and encoded in particular for IBD (e.g. Ruminococcus Gnavus and Fecalibacterium Praunantzii).

Our data are consistent with findings from Aden and collegues that evaluated two groups of patients affected respectively by IBD and rheumatic diseases and treated with TNFi. They observed that the treament restored the microbiota with an increase both in α and β diversity only in IBD patients but not in rheumatic patients. No differences was found between responders and not responders[301]. Another study concluded that the response to TNFi was influenced by high disease activity and unstable microbiota composition and patients with reduced biodiversity at the baseline were more likely to fail to the TNFi treatment. TNFi abolished the differences among patient groups, suggesting that, independently to the clinical response, the treatment can restore the microbiota[302]. So we performed further analysis to verify the impact of the inflammatory status on the change of the microbiota and to check some relationship between the movement of the microbiota and the course of the disease.

The univariate analysis showed that CRP trend, that is a parameter of inflammation both for SpA and IBD, is consistent with the Coprococcus trend; this is consistent with the hypothesis that TNFi therapy, by decreasing inflammation, tends to restore the intestinal eubiosis.

We didn't find differences between responders and not responders suggesting that indipendentely to clinical response TNFi are able to restore fecal microbiota. Also these findings are consistent with published data[301].

STRENGTH AND WEAKNESS OF THE STUDY

The sample size of our population was small, so the power of our statistical analysis is limited.

Our study did not compare intestinal microbiota of patients with those of healthy people, so it does not establish the nature of the dysbiosis that is highly suspected to play a key role in the pathogenesis of SpA. Furthermore, we did not compare groups of patients treated either by TNFi or by other concomitant treatment, and the potential effect on these changes by the drugs regularly used by patients as maintenance therapy (such as, mesalazine, azathioprine, steroids and antibiotics) is not known[24] so we cannot strongly affirm that modifications of microbiota are specifically related to TNFi. To partially solve this bias patients mantained the same therapy before and during the study period. Moreover all patients were treated with infliximab or adalimumab (etanercept was excluded) and their ability to mediate complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity, and to increase the proportion of cells undergoing apoptosis and the level of granulocyte degranulation in vitro is comparable[312].

We analyzed microbiota on fecal samples used as a surrogate for the entire gut microflora and it is well known as the composition and function of the fecal microflora differs from mucosal microflora[26]. However, evaluation of stool samples is the easiest way to assess intestinal microbiota, and they are easily to collect.

Our study is based on 16S rDNA sequencing, which allows to investigate bacteria only excluding the virobiota, the mycobiota and eucaryota ihnabiting intestinal tract that play a consistent role in physiopathology of several diseases[313].

We take into account differing diets of patients and included into the study only patients getting a typical mediterrean diet but we didn't perform any specific evaluation such as percentages of carbohydrates, fats and proteins intake that may influence the results[314].

Although enteropathic arthritis is a well-known nosological entity, there are no clinimetric systems to evaluate the progress of this disease itself; the trend of both IBD and arthritis are actually assessed separately. Furthermore, literature concerning dysbiosis in these two pathologies often differs thus leading to a distortion of our results.

Despite these limits, our results unequivocally reveal (independently of the achievement of clinical success) a modification of the microbiota towards eubiosis related to TNFi.

CONCLUSIONS

The decrease of Proteobacteria and the increase of Lachnospiraceae and Coprococcus is consistent with the hypothesis that TNFi therapy, by decreasing inflammation, tends to restore the intestinal eubiosis. However further studies on larger cohort incuding the evaluation of gut virota and micobiota will be necessary to definitively clarify the effects of TNFi on the composition and function of the gut microbiota.

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