Evaluation of the intestinal microbiota and correlation with functioning in patients suffering from knee osteoarthritis: a cross-sectional study

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ABSTRACT

Purpose: The aim of this study was to evaluate the composition and metabolic activity of the gut microbiota (GM) in a cohort of patients suffering from knee osteoarthritis (OA) and its impact on their functional status.

Methods: We recruited patients with a radiological diagnosis of mild-moderate knee OA (grade II or III according to the Kellgren and Lawrence classification). The primary outcome was the analysis of the intestinal microbiota (with stool sampling, DNA extraction and PCR and sequencing of the microbiota). The secondary outcomes were: Western Ontario and McMaster University Osteoarthritis index (WOMAC), Visual Analog Scale (VAS), Short Physical Performance Battery (SPPB), Timed Up and GO (TUG), Hand Grip Strength (HGS) test, and Berg Balance Scale (BBS).

Results: This preliminary study included 17 patients aged 72.1±8.2 years. In these patients suffering from knee OA, in addition to alterations on the main physiatric scales administered (BBS: 40.727±11.22; HGS test: 18.73± 7.15; SPPB: 7.1± 3.13; WOMAC: 41.0±16.4; VAS 6/10), we were able to highlight an alteration in the composition of the microbiota, and in particular an increase in the physiological ratio, in favor of the *Firmicutes* vs. the *Bacteroidetes phylum* (v: 2.56 ± 1.78).

Conclusions: These results, showing an alteration in the composition of the microbiota with an increase in the *Firmicutes*/*Bacteroidetes* ratio, point to a potential correlation between intestinal dysbiosis and arthrosis. There is therefore a need for more specific understanding of the role of the intestinal microbiota in the pathogenesis of OA, and of the pathogenetic mechanism of the gut-joint axis.

KEYWORDS

Gut microbiota, osteoarthritis, *Firmicutes*, *Bacteroidetes*, gut-joint axis.

Introduction

Osteoarthritis (OA) is one of the most common joint disorders in the world, affecting, approximately 9.6% of men and 18% of women aged > 60 years [1]. The precise etiology of osteoarthritis is still unknown, but various risk factors have been identified, such as age, sex, obesity, diet, and joint conditions such as trauma, misalignments, and abnormal weight-bearing loads [2,3]. In this scenario, the high prevalence of synovitis in OA contributes substantially to the development of inflammation and pain [4], which lead to reduced joint mobility with loss of function, and subsequently pain and disability [5].

The International Society for the Study of Osteoarthritis (OARSI) recommends several interventions as appropriate treatment options for individuals with knee OA [5]. In recent years, there has also been a growing interest in the use of non-pharmacological techniques in subjects affected by knee OA: it has in fact been suggested that physical exercise could play a key role not only in improving the functioning of patients affected by arthrosis, but also in modulating the composition of the gut microbiota (GM) [6,7].

A hypothesis has recently been proposed according to which the GM may be a major risk factor for the development of OA [8]. Specifically, it has been hypothesized that changes in the composition and metabolic activity of the GM could can alter the host's immune response and metabolism, leading to a constant state of low-grade inflammation with consequent weakening and fragility of the musculoskeletal system [9].

The microbiota colonizes the entire gastrointestinal tract and represents a real ecosystem, weighing approximately 1.5 kg, and composed of more than 10^{14} bacteria and more than 1000 species [10]. The main phyla of the healthy intestinal micro-

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biota are *Bacteroides* (B) and *Firmicutes* (F), followed by *Actinobacteria, Fusobacteria*, and *Proteobacteria*; The intestinal microbiota has numerous functions, including nutrient absorption, maintenance of metabolic homeostasis, and development of systemic and mucosal immunity [10]. A possible correlation between intestinal dysbiosis and OA has been suggested by the relationship between serum levels of bacterial metabolites in patients with joint degeneration [12]. This correlation has been described in murine and human models of OA with high levels of circulating inflammatory markers and mediators [13,14], suggesting that in the pathogenesis of OA (as well as most chronic musculoskeletal conditions), it is plausible that specific features may manifest in terms of alteration of the composition of the intestinal microbiome and its metabolic products [9,15].

Recent scientific evidence shows that changes in the composition of the microbiota, leading to altered intestinal permeability and consequent leakage of bacteria and their components into the bloodstream, can trigger a systemic inflammatory reaction, and thus lead to progression of joint degeneration [12,16].

Nonetheless, physical exercise could play a key role not only in improving the functioning of patients suffering from arthrosis, but also in terms of modulating the composition of the microbiota, and thus strengthening the immunity of the intestinal mucosa.

It is important to underline that a correlation has also been observed between the role of the intestinal microbiota and osteoporosis; indeed, a recent review of the literature [12] showed that the intestinal microflora influences the activity of osteoclasts by regulating serum levels of IGF-1 and acting on intestinal absorption of calcium. Intestinal dysbiosis can have a negative impact on bone health by influencing the RANK/RANKL/OPG pathway; in this context, it has been hypothesized that food supplements, prebiotics and probiotics can support an adequate balance of the intestinal microbiota, and consequently promote bone health [12,17].

Therefore, a better understanding of the possible association between the intestinal microbiota and OA could facilitate the development of new diagnostic and therapeutic approaches in affected patients. More specifically, a clear understanding of the role of the intestinal microbiota in the pathogenesis of OA could have crucial implications for not only the treatment of the disease, but also its prevention (through manipulation of the nutrient-intestinal microbiota-bacterial metabolites axis) [11].

Literature data concerning changes in the intestinal microbiota and its metabolic activity in patients with OA and the potential correlation between a condition of low-grade inflammation and the pathogenesis of OA still seem to be insufficient.

Therefore, the aim of this pilot study was to assess the composition of the GM (in terms of the number of *Firmicutes* and of *Bacteroidetes*) and its relationship with functioning in a cohort of patients suffering from knee OA.

Methods

Participants

We recruited patients aged between 40 and 80 years and with a radiological diagnosis of knee OA (Grade II or III according to the Kellgren and Lawrence classification); we excluded participants with: injuries to the knee ligaments, knee arthroplasty surgery, previous knee surgery, fractures of the tibia and/or fibula, or previous intra-articular therapy with corticosteroids or hyaluronic acid. The patients included consented to the processing of their personal data, after having read and understood the written information they were given and also understood the supplementary information provided orally by the practice staff. They were also informed, in writing and orally, of the rights that can be exercised pursuant to Article 7, Italian Legislative Decree no. 196 of 30/06/2003 (the so-called Privacy Code). The study was approved by the local ethics committee and performed according to the STROBE guidelines, and in accordance with the Declaration of Helsinki.

Intervention

All the participants were assessed — assessment consisted of GM analysis, biochemical analyses, assessment of genetic profile — at the Internal Medicine Unit of the University Hospital "Renato Dulbecco" of Catanzaro, Italy.

The collected samples were labeled with progressive numbers and without any reference to patients' sensitive data; patients' first and last names were reported by initials only to protect their privacy.

Outcome measures

First, we assessed the composition and the phylogenetic profile of the GM in terms of number of *Firmicutes*, number of *Bacteroidetes*, and F/B ratio. We only evaluated families with a population greater than 300 units.

For analysis of the intestinal microbiome (fecal sampling, DNA extraction and PCR), fecal samples were collected in sterile stool containers. Within four hours of delivery to the healthcare provider, these samples were stored at a temperature of –80°C until processing. Extraction of bacterial DNA from fecal samples was performed using the QIAamp DNA Stool Mini kit (Qiagen, Hilden, Germany). Then, PCR amplification (ABI2720 Thermal Cycler, Thermo Fisher Scientific, USA) of the V3-V4 hypervariable gene region of 16S rRNA was performed in three replicate reactions with the primer pairs:

- F (Illumina adapter sequence 1 + CCTACGGGNGGCWGCAG)
- R (Illumina adapter sequence 2 + GACTACHVGGGTATCTAATCC)

The replicate products were pooled and purified using Agencourt AMPure XP magnetic beads (Beckman Coulter, USA; TopTaq DNA Polymerase kit, Transgen, China). The purity and concentration of the DNA sample was tested using a nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

Under physiological conditions, the composition of the intestinal microbiota presents a normally conserved ratio between Firmicutes and Bacterioidetes; the human intestinal microbiota undergoes maturation from birth to adulthood and is further altered with ageing. it was observed that the ratio of Firmicutes to Bacteroidetes evolves during different life stages. For infants, adults and elderly individuals we measured ratios of 0.4, 10.9 and 0.6, respectively [18].

Microbiota sequencing and sequence quality control

PCR products were sequenced with the Illumina MiSeq platform using the 2 x 250 bp paired method and TrimGalore was used with a quality threshold of Q20 and remove lengths <100 bp. Subsequently, read pairs of the original DNA fragments were merged using FLASH2 and low-quality sequences were removed. Next, Mothur was used to remove primers from the sequences and eliminate N bases/homopolymers >6 bp. Finally, reads with an error rate >2 were removed and reads with <100 bp were assigned operational taxonomic units (OTUs) by clustering sequences with 97% base pair identity. Chimeras, however, were removed using UPARSE. To assign taxonomies to OTUs we used Mothu's RIBOSOMAL Database project and applied a confidence threshold of 80%.

Functional profile analysis

To estimate metagenome composition, the "Phylogenetic Investigation of Communities by Reconstruction of Unobserved States to estimate metagenome composition and Kyoto Encyclopedia of Genes and Genomes (KEGG)" protocol was followed. A statistically significant difference in the abundance of KEGG orthologs was defined as p-value <0.05.

We then assessed the following outcome measures: age in years; body mass index (BMI) expressed as kg/m^2; Visual Analog Scale; Timed up and go (TUG) assessment; Hand Grip Strength (HGS); Berg Balance Scale (BBS); Western Ontario and McMaster University Osteoarthritis Index; Short Physical Performance Battery; Mini-Nutritional Assessment.

Statistical analysis

Statistical analysis was performed using JASP (JASP project, Amsterdam, Netherlands 17.0.1). Data were tested for normal distribution according to the Shapiro-Wilk test. Categorical or dichotomous variables were described as frequencies. Continuous data were defined as means ± SD. Moreover, we performed a partial plot, and heatmaps of pairwise correlations among outcome measures were calculated using Pearson's correlation coefficient. As this is a proof-of-concept study, sample size calculation was not planned.

Results

This preliminary study included 17 patients (M/F: 5/12) aged 72.09±8.20 years. We found an alteration in the composition of the microbiota and in particular an increase in the physiological ratio, in favor of the *Firmicutes* compared with the *Bacteroidetes phylum* (v: 2.56 ± 1.78).

Table I shows the main results of the analyses conducted in the patients under study.

Furthermore, linear regression analysis highlighted a statistically significant difference between the F/B ratio and muscle strength (Pearson's r: 0.702, p<0.016), between the TUG and the BBS values and these patients' BMIs (Pearson's r: 0.770; p<0.006; Pearson's r: –0.895; p<0.001 respectively), and between the HGS values and the age of the population examined (Pearson's r: –0.799; p<0.003).

Table 1 Study participants characteristics.

	MEAN	± SD
Age	72.0	± 8.2
BMI	29.3	± 4.6
VAS	6.1	± 1.1
TUG	16.8	± 5.1
HGS	18.7	± 7.1
BBS	40.7	± 11.2
SPPB	7.1	± 3.1
WOMAC	41.0	± 16.4
MNA	25.3	± 2.8
Firmicutes	19442.1	± 4578.85
Bacteroidetes	13981.01	± 10717.09
F/B	2.5	± 1.7

Abbreviations: **BMI** = body mass index; **VAS**= Visual Analog Scale; **Timed Up and Go** (TUG); **HGS** = Hand Grip Strength test; **SPPB** = Short Physical Performance Battery; **WOMAC** = Western Ontario and McMaster University Osteoarthritis Index; **MNA** = Mini Nutritional Assessment.

Discussion

This pilot study aimed to examine the composition of the intestinal microbiota in patients suffering from knee OA and the possible correlation with these patients' functional status. We observed a significant increase in the F/B ratio in favor of *Firmicutes* [19-21], and a statistically significant difference between this ratio and muscle strength in our study patients. Chronic low-grade inflammation seems to be the primary feature linking the GM with OA, a finding that supports the emergence of a novel OA phenotype referred to as "metabolic OA" [18,19,22].

Physical exercise may play an important role in both preventing and treating numerous chronic ill-nesses, including OA [5], having a significant impact in terms of improving the functionality of affected patients, and also modulating the composition of the GM [23,24], thereby influencing the F/B ratio [6,25,26]; it is recommended, by multiple guidelines, as the primary therapeutic option for the management of this condition [27,28].

Thus, a potential correlation between increased levels of bacterial metabolites in serum, particularly *Firmicutes*, and joint degeneration suggests that there may be a correlation between gut dysbiosis and OA.

Nevertheless, the existing literature on these subjects is still limited, and therefore further observational studies are needed to determine whether modulating the GM through therapeutic exercise can reduce chronic inflammatory status (improving the gut dysbiosis and the resulting leaky gut) and consequently enhance patient functionality. Recent research indicates that engaging in physical activity has the potential to alter the composition of the GM [29,30], enhance immune defenses within the intestinal lining, elevate the F/B ratio, alter the profile of bile acids, and enhance the synthesis of short-chain fatty acids [29,30].

How can the GM be modified in patients undergoing intense physical activity? At present, the question remains unan-

swered. Nevertheless, studies have shown that the GM has the ability to regulate oxidative stress and inflammatory reactions, ultimately enhancing metabolism and energy expenditure [31].

Conclusions

In conclusion, dysbiosis of physiologic gut homeostasis is followed by both adaptive and innate immune responses caused by the migration of bacteria and bacterial metabolites into the bloodstream and into the joint, a cycle that eventually ends in low-grade inflammation in the joint, which contributes to the development of inflammatory joint disease.

Our study, finding an alteration in the composition of the microbiota, specifically an increase in the F/B ratio, sheds light on the potential role of the GM in knee OA. However, there still a need for greater understanding of the role of the intestinal microbiota in the pathogenesis of OA and the pathogenetic mechanism of the gut-joint axis, in addition to the potential role of physical exercise combined with diet and probiotics.

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