Intestinal microbiome composition and its relation to joint pain and inflammation

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Macrophage-mediated inflammation is thought to have a causal role in osteoarthritis-related pain and severity, and has been suggested to be triggered by endotoxins produced by the gastrointestinal microbiome. Here we investigate the relationship between joint pain and the gastrointestinal microbiome composition, and osteoarthritis-related knee pain in the Rotterdam Study, a large population based cohort study. We show that abundance of Streptococcus species is associated with increased knee pain, which we validate by absolute quantification of Streptococcus species. In addition, we replicate these results in 867 Caucasian adults of the Lifelines-DEEP study. Finally we show evidence that this association is driven by local inflammation in the knee joint. Our results indicate the microbiome is a possible therapeutic target for osteoarthritis-related knee pain.
Osteoarthritis (OA) is a degenerative joint disease and the most common form of arthritis: an estimated 22% of the adult population has at least one joint affected by osteoarthritis and this prevalence increases to 49% in individuals over 65 years of age. The hallmark clinical symptom of OA is pain, which is one of the leading causes of disability in OA. Pathological changes in OA affect all joint tissues: degradation of cartilage and bone, abnormal bone formation (osteophytes), and inflammation of the synovial membrane, (synovitis). Although OA is often described as predominantly caused by mechanical factors and genetic predisposition, the existence of inflammation in OA, locally or systemic, is widespread. In addition, it has become apparent that, by promoting or exacerbating OA symptoms, predominantly OA joint pain is this local or systemic inflammation has a causal role in OA pathology.

Obesity, a well-known risk factor for OA, is thought to increase OA risk through increased mechanical loading on weight-bearing joints. However, obesity also increases the risk for OA in non-weight-bearing joints. The increased risk of OA in non-weight-bearing joints seen in obese individuals might be directed through low-grade systemic inflammation. The gastrointestinal microbiome has emerged as one of the factors triggering obesity associated low-grade systemic inflammation. Obesity is associated with changes in gastrointestinal-microbiome composition, which can lead to an increased intestinal absorption of immunogenic bacterial products. Gastrointestinal bacteria produce a wide range of biologically active molecules, such as metabolites, short-chain fatty acids, proteins and enzymes, of which some are secreted in, outer membrane or membrane vesicles (OMVs/MVs). All gram-negative bacteria, archaea, fungi and several gram-positive bacteria can constitutively produce these vesicles. These vesicles are insensitive to proteases, suggesting they can transport their content over long distances from their sites of origin. The content of these vesicles can be delivered to different organs in a concentrated manner. Also, some of these biologically active molecules can affect intestinal mucosal permeability (short-chain fatty acids) or activate the immune system (lipopolysaccharide). Specifically, these molecules can affect macrophage activation and Toll-like-receptor (TLR) pathways, which have recently been shown to be the predominant inflammatory responses seen in OA. Indeed, elevated levels of the bacterial endotoxin LPS (lipopolysaccharide), in the blood or in the synovium of OA patients, is associated with more severe knee OA, knee pain and inflammation. This and other studies link gut-microbiome composition to low-grade systemic and local inflammation seen in OA.

In the present study we examine stool microbiome as a proxy for the gastrointestinal-microbiome composition in relation to knee OA severity, OA-related knee pain, measured by the WOMAC-pain score, and obesity, in a large population-based cohort. We report a significant association between Streptococcus species (spp.) abundance in stool microbiome samples, knee WOMAC pain, knee inflammation, and replicate this finding in an independent cohort. Our results suggest a true relationship between the gastrointestinal microbiome, low-grade inflammation in the knee, and knee OA pain, independent of obesity.

**Results**

**Rotterdam Study Microbiome cohort profile.** For 1427 participants from the Rotterdam Study (RSIII) we determined the gastrointestinal-microbiome composition by taking the stool microbiome as a proxy for the intestinal microbiome. In the Rotterdam Study Microbiome cohort, we sequenced two hypervariable regions of the bacterial 16S rRNA gene, hypervariable regions V3 and V4. After quality control, the 16S reads were directly mapped against the Silva 16S sequence database (v128) using RDP classifier for taxonomic classification. Classification prediction was done on multiple taxonomic levels: domain, phylum, class, order, family and genus. Gastrointestinal-microbiome composition of the 1427 participants in the Rotterdam Study Microbiome cohort is schematically presented Fig. 1. In total, there are 596 single taxonomies in our cohort, with unknown and unclassified bacteria excluded, as these could not be identified for clinical therapeutic relevance. At phylum level, the dominant phyla are Firmicutes (77.8%) and Bacteroidetes (12.5%), followed by Proteobacteria (4.9%) and Actinobacteria (4.1%, Fig. 1). This is in concordance with other large-scale population-cohorts of Caucasian adults.

**Streptococcus abundance is associated with OA knee pain.** First, we examined whether the overall microbiome composition was different across knee WOMAC pain scores and OA severity (Kellgren-Lawrence radiographic OA severity scores). We found that knee WOMAC pain significantly contributes to the intestinal microbiome β-diversity as evaluated at genus level (Aitchison distance, \( r^2 = 0.0014, P\text{-value} = 0.005, \text{PERMANOVA, Supplementary Fig. 1} \)). This association was attenuated when BMI was added to the model and significance was lost (\( r^2 = 0.00088, P\text{-value} = 0.143, \text{PERMANOVA} \)). The intra-individual gastrointestinal-microbiome diversity (α-diversity, Shannon index and inverse Simpson) was not associated with knee WOMAC pain. For knee OA severity (KLsum, \( n = 941 \)), we did not identify an association with gastrointestinal microbiome α- and β-diversity (Supplementary Table 1).

To gain more insight into which gut-microbiome taxonomies drive the association with knee WOMAC pain, we performed multivariate association analysis on the 256 taxonomies remaining after additional QC. After adjusting for age, sex and technical covariates, we found four microbiome abundances significantly (FDR < 0.05) associated with knee WOMAC-pain severity (Table 2). These were all in the same clade; from class, order, family leading to the bacterial genus of Streptococcus (genus: coefficient = 5.0 × 10^{-03}, FDR P-value = 1.2 × 10^{-05}, MaAsLin, Table 2; See Supplementary Data 1 for the full summary statistics). After additional correction for possible confounders (smoking and alcohol consumption) Streptococcus spp. abundance remained significantly associated with knee WOMAC pain (coefficient = 4.8 × 10^{-03}, FDR P-value = 3.8 × 10^{-05}, MaAsLin, Supplementary Table 2). This association is largely independent of BMI (coefficient = 4.1 × 10^{-03}, FDR P-value = 2.1 × 10^{-03}, MaAsLin, Supplementary Table 2). Ethnicity has recently been put forward as a possible confounder for gastrointestinal-microbiome composition. However, after exclusion of individuals with non-European ancestry (n = 163) from our analysis, Streptococcus spp. remained associated with knee WOMAC pain (coefficient = 5.0 × 10^{-03}, FDR P-value = 5.8 × 10^{-05}, MaAsLin, Supplementary Table 3), also, after adjustment for confounders (coefficient = 4.3 × 10^{-03}, FDR P-value = 1.9 × 10^{-03}, MaAsLin). Altogether, we found that the gut microbiome, and in particular a greater relative abundance of Streptococcus spp., is significantly associated with higher knee WOMAC pain independent of smoking, alcohol consumption and BMI.
Proton Pump Inhibitors (PPI) are among the most widely used over-the-counter drugs in the world. They are used to treat gastro-esophageal reflux and prevent gastric-ultcers. Recent research has shown that the gastrointestinal-microbiome composition of PPI users is profoundly different from non-PPI users, mainly due to a strong increase in *Lactobacilli* abundance, driven by *Streptococcus spp.*. Another potential over-the-counter medication frequently taken by patients with joint complaints are non-steroidal anti-inflammatory drugs (NSAIDs), which also affect the gastrointestinal microbiome. To investigate, whether the association between *Streptococcus spp.* abundance and knee WOMAC pain is mediated by use of these drugs, we included PPI and NSAID use as covariates in our model (in addition to age, sex, technical covariates, smoking, alcohol consumption, and BMI). After adjustment for these covariates, the coefficient was attenuated, but the association remains significant (coefficient $= 3.4 \times 10^{-03}$, $P$-value $= 2.4 \times 10^{-04}$, MaAsLin, Supplementary Table 4). In line with these results, excluding all current PPI users ($n = 265$) from the analysis, greater *Streptococcus spp.* abundance was significantly associated with higher knee WOMAC pain ($n = 1104$, coefficient $= 2.4 \times 10^{-03}$, $P$-value $= 4.0 \times 10^{-03}$, MaAsLin). Thus, the association between *Streptococcus spp.* and knee WOMAC pain was not due to the confounding effect of PPI use.
Since knee WOMAC-pain scores were not normally distributed (left-skewed with an overabundance of zeros), we also assessed the association using a Poisson-regression model, which can account for the non-normal distribution. Here as well, a significant association with *Streptococcus* spp. abundance was observed ($\beta = 1.85$, $P$-value $= 1.4 \times 10^{-6}$, Poisson-regression). Further sensitivity analysis excluding individuals who did not report pain ($n = 1142$) or whose knee WOMAC-pain scores were deemed as outliers (WOMAC-pain score $> 10$, $> 4$ SD, $n = 56$) resulted in slight changes in the association coefficients. The association, however, remained significant (Supplementary Table 5). Altogether, these analyses show that the association between relative abundance of *strepotococcus* spp and knee WOMAC pain is robust.

**Quantitative determination of *Streptococcus* spp.** As 16S rRNA-sequencing derived relative microbiome data cannot provide information about the extent or directionality of changes in microbiome taxa abundance\(^{30}\), we determined the absolute amount of *Streptococcus* spp. in the individuals of our study population. For each sample in our cohort ($n = 1427$), we quantified the number of *Streptococcus* spp. using genus specific qPCR and the total microbial load using 16S rRNA qPCR. We calculated the absolute quantity of *Streptococcus* spp. and normalized for the total bacterial load in each samples as measured by 16S rRNA qPCR. The 16S rRNA-sequencing results and qPCR *Streptococcus* spp. quantity yielded similar results (Spearman correlation, $r = 0.80$, $P$-value $= 2.2 \times 10^{-16}$, Supplementary Fig. 2). Using the absolute abundance of *Streptococcus* measured by qPCR instead of the relative abundance derived from the 16S rRNA-sequencing profiles, we again found a significant association between higher knee WOMAC pain and greater absolute *Streptococcus* spp. abundance ($\beta = 0.10$, $P$-value $= 7.4 \times 10^{-03}$, Poisson regression), also after adjustment for smoking, alcohol consumption, and BMI ($\beta = 0.074$, $P$-value $= 4.5 \times 10^{-02}$, Poisson regression).

To adjust for possible spurious collinearity between microbe abundancies\(^{31}\), we have used the isometric log-ratio transformation (ILR). Using ILR, we have compared the relative *Streptococcus* spp. abundance against the geometric mean of the abundance of all other genera. Results show that the ILR transformed *Streptococcus* spp. abundance is associated with knee WOMAC pain ($P$-value $= 9.9 \times 10^{-06}$, MaAsLin, Supplementary Table 6). The association remained significant after adjusting for smoking, alcohol consumption and BMI ($P$-value $= 8.4 \times 10^{-04}$, MaAsLin, Supplementary Table 6), and NSAID and PPI use ($P$-value $= 8.5 \times 10^{-03}$, MaAsLin, Supplementary Table 6). The qPCR results and ILR transformation demonstrate that the association between *Streptococcus* spp. abundance and knee WOMAC pain is not an artefact of the 16S rRNA sequencing.

**Independent replication of *Streptococcus* association.** We sought replication for all four associations with WOMAC pain, i.e., class, order, family and, the bacterial genus of *Streptococcus*, in an independent Dutch cohort, LifeLines-DEEP (LLD) (Supplementary Table 7). LLD has a lower sample size ($n = 867$), younger age (mean age = 45.6) and fewer individuals with OA-related knee pain (WOMAC pain $> 0$, $n = 197$), however, the average knee WOMAC pain was very similar compared with the Rotterdam Study cohort (RS = 0.9 and LLD = 0.9, Table 1 and Supplementary Table 7). Despite lower power in the replication cohort we observed a significant association ($P$-value $< 0.05$) between knee WOMAC-pain scores and all four taxonomies to the genus of *Streptococcus* ($\beta_{\text{replication}} = 3.3 \times 10^{-03}$, $P$-value$_{\text{replication}} = 3.7 \times 10^{-02}$, MaAsLin, Table 2). Also, in the
meta-analysis of RS and LLD, greater Streptococcus spp. abundance was significantly associated with higher knee WOMAC pain ($P_{\text{meta}} = 1.3 \times 10^{-08}$, MaAsLin, $n = 2256$, Table 2). After adjusting for BMI, we found significant replication on class, order and the family level of Streptococaceae (coefficient$_{\text{replication}} = 2.7 \times 10^{-03}$, $P_{\text{value, replication}} = 3.5 \times 10^{-02}$, MaAsLin, Supplementary Table 8). In the replication study, additional adjustment for BMI slightly attenuated the association, a 9.1% decrease in coefficient, for the genus of Streptococcus (Supplementary Table 8). This was in concordance with the 14.5% decrease in coefficient for streptococcus seen in the discovery cohort after BMI adjustment (Supplementary Table 2). In the BMI adjusted meta-analysis for RS and LLD, the association between knee WOMAC pain and Streptococcus spp. was highly significant ($P_{\text{meta}} = 1.1 \times 10^{-06}$, METAL, $n = 2256$, Supplementary Table 8).

Streptococcus association with knee joint inflammation. If Streptococcus spp. abundance is causally related to higher knee WOMAC pain, a possible mechanism might be through local priming of macrophages in the synovial lining resulting in inflammation\textsuperscript{22}. For a random subset of the Rotterdam Study Microbiome cohort, knee magnetic resonance images (MRI) were available ($n = 373$, all females)\textsuperscript{32} at the time of microbiome measurements. Knee joint inflammation was assessed by scoring the amount of effusion in the tibiofemoral joint and in the patellofemoral joint for both knees. We found that higher WOMAC-pain scores were significantly correlated with more knee effusion (Spearman correlation $r = 0.14$, $P_{\text{value}} = 9.0 \times 10^{-10}$). In addition, we observed that greater Streptococcus spp. abundance was significantly associated with more effusion in the knee joints (coefficient $= 1.0 \times 10^{-02}$, $P_{\text{value}} = 1.3 \times 10^{-02}$, MaAsLin, Table 3). When WOMAC pain was added to the model, the association of knee effusion with Streptococcus spp. disappears (coefficient $= 3.3 \times 10^{-03}$, $P_{\text{value}} = 0.21$, MaAsLin), indicating that the association of Streptococcus spp. with knee pain severity is driven by knee inflammation severity.

Discussion

Using a large, deeply phenotyped population-based cohort, we identified a significant association between greater relative and absolute Streptococcus spp. abundance and higher OA-related knee pain. These results were validated by replication in an independent cohort and by meta-analysis. Finally, we presented evidence that this association was driven by local inflammation in the joint.

We observed that the intestinal microbiome $\beta$-diversity was significantly associated with knee WOMAC scores. After testing 256 taxonomies individually, we found a microbiome-wide association with knee WOMAC pain and Streptococcus spp., where greater Streptococcus spp. relative abundance is associated with higher knee WOMAC pain. We found this association to be robust, not be caused by outlier observations, or due to the confounding effects of smoking, alcohol intake, oral medication usage or BMI\textsuperscript{28,29}. Neither was the association an artefact of the microbiome profiles as relative fractions of the 16S rRNA sequencing, or due to possible co-linearity in the data\textsuperscript{30,33}.

Although, the results of the sensitivity analyses were in line with a true association between Streptococcus spp. and knee WOMAC pain in our cohort, validation in an independent cohort is essential. Replication of microbiome abundance, however, is difficult, because data might not be similar between studies if sample preparation and data analysis are not done in the exact same way\textsuperscript{34}. We sought replication in the LLD cohort, which has a different study population and data preparation method than our cohort does\textsuperscript{27}. Despite these differences, we could replicate our association in LLD for all taxonomic levels, class, order, family and for the genus of Streptococcus. Also, in the meta-analysis of RS and LLD, Streptococcus spp. was significantly associated with knee WOMAC pain.

Obesity-mediated gastrointestinal-microbiome changes are postulated to affect low-grade systemic and local inflammation in OA\textsuperscript{23–25}. Nevertheless, in our study the effect of Streptococcus spp. on knee WOMAC pain is not fully driven by BMI. This suggests a direct role for the gastrointestinal microbiome in OA-related knee pain and inflammation. We postulate that greater Streptococcus spp. abundance leads to higher knee WOMAC pain through local joint inflammation. This is in line with our observation that Streptococcus spp. abundance was significantly associated with effusion severity in the knee joints. This leads us to believe that Streptococcus spp. might also be involved in other inflammatory joint pain disorders. This is not unlikely since several Streptococcus spp. have been linked to osteomyelitis,\textsuperscript{35,36} rheumatic fever,\textsuperscript{37,38} and, post-streptococcal reactive arthritis.\textsuperscript{39,40} The last two are disorders in which due to molecular mimicry with group A Streptococcus, cross-reactive antibodies are produced against joint tissues, leading to rheumatic joint inflammation and damage.\textsuperscript{38} However, these disorders involve mainly pathogenic species, such as S. pyogenes. Yet, most Streptococcus spp. are commensal species and have been found throughout the human oral-gastro-intestinal microbiome\textsuperscript{41–43}, still, these can produce immunogenic bacterial products. Several Streptococcus spp. have been shown to constitutively produce MVs\textsuperscript{18}. These MVs may present Streptococcus spp. epitopes and/or may contain immunogenic products\textsuperscript{17,24}. Such bacterial products can trigger macrophage activation through TLR pathways. This type of macrophage activation is predominantly seen in OA-related joint inflammation,\textsuperscript{16,21} and is thought to be related to pain\textsuperscript{3–7}.

We therefore propose that greater Streptococcus spp. abundance may lead to an increase of bacterial products in the circulation through increased production of metabolites that pass the gut-blood barrier or through immunogenic products that prime local or systemic macrophages. We have summarized this hypothetical model in Fig. 2.
Our results show a difference in microbiome $\beta$-diversity in individuals with higher knee WOMAC-pain scores, which is driven by greater abundancy of *Streptococcus spp*. This association is highly robust. Moreover, we replicated our findings in an independent cohort. However, our study has some limitations. First, a cross-sectional study design was used and cannot unequivocally establish causality. For this, longitudinal studies are required. Second, other follow-up studies are needed to better elucidate the molecular pathway connecting *Streptococcus spp.*, knee inflammation and WOMAC pain, to validate or reject our postulated hypothesis (Fig. 2). Blood and joint tissue could be examined for the presence of *Streptococcus* MVs, metabolites, and their possible association to knee inflammation and WOMAC pain severity. Third, to examine whether the association found in this study is unique for knee OA pain, other quantitative pain measurements should be examined, as well as measurements of pain at other joints. In addition, other inflammatory joint disorders could also be examined. Forth, due to the limited resolution of 16S rRNA-sequencing methodology, we were unable to identify whether a specific *Streptococcus* species or strain was driving the association. Last, we find no association between knee OA severity (KLsum) and gastrointestinal-microbiome composition. Knee OA severity, however, was measured by radiographic OA severity, and this does not include the clinical symptom of joint pain. Although considering our proposed mechanism, an effect on knee OA severity could be expected, as inflammation can lead to joint damage. It is possible that our study currently lacks the power to detect such an association, or requires a longitudinal design to detect such effects on OA severity or disease progression.

In sum, we demonstrate an association between greater abundance of *Streptococcus spp.* and higher osteoarthritis-related knee pain, but the causality of this association needs to be established. A possible explanation for the found association is the induction or exacerbating of local joint inflammation by *Streptococcus spp.*. The precise mechanisms by which...
**Methods**

**Rotterdam study cohort.** The Rotterdam study (RS) is a large population-based prospective study population (14,926 participants) ongoing since 1990 to study determinants of chronic disabling diseases. The first cohort RS-I, started in 1990 and includes individuals of 55 years and older living in the Ommoord district of Rotterdam in the Netherlands. In 2000, a second cohort was started RS-II with individuals who had become 55 years of age or moved into the study district since the start of the study. In 2006, the third cohort was initiated, RSIII, of individuals aged 20+ living in the Ommoord district. The participants are predominantly Caucasian and a further detailed description of the design and rationale of the Rotterdam Study has been published elsewhere.

The Rotterdam Study has been approved by the Medical Ethical Committee of the Erasmus MC, University Medical Center Rotterdam, the Netherlands (MEC 02.1015). All subjects provided written consent prior to participation in the Rotterdam Study.

Stool sample collection started in 2012 during the second visit of the Rotterdam Study III population (RSIII-2). A random group of 2440 participants was invited to provide a stool sample. In total 1691 (response rate 69%) stool samples were received via mail at the Erasmus MC for analysis of stool microbiome composition and another 41 samples were received prior to sequencing.

**Taxonomic profiling of gastrointestinal microbiota in RS.** Sample collection: Stool samples were collected at home by the participant using a Commode Specimen Collection System (Cobidiend, Mansfield, MA) and –1 g aliquot was transferred to a 25 × 76 mm fesce collection tube, which was sent via the regular mail to the Erasmus MC. Participants also filled out a short questionnaire addressing date and time of defecation, current or recent antibiotics use, current probiotics use, and recent dietary modifications. Samples arriving samples were rejected (≤ 20 °C), samples arriving 3 days to 4 weeks at the Erasmus MC were rejected from further analysis, for the remaining samples TimelnMail (in days) was registered as a technical covariate. DNA isolation: For each participant, frozen stool samples were allowed to thaw for 10 min at room temperature prior to DNA isolation. An Aliquot of 200 mg was homogenized with 0.1 mm silica beads (MP Biomedicals, LLC, Bio Connect Life Sciences BV, Huisien, The Netherlands) and DNA was isolated from the samples using the Arrow stool DNA kit according to the manufacturer’s protocol (Arrow Stool DNA; Iogen Life Science, de Meern, The Netherlands). 16S rRNA gene sequencing: The V3 and V4 hypervariable regions of the 16S rRNA gene were sequenced using the primer pair 319F (ACTCTACACGGGGCACGCA) – 806R (GGACTACHVGGGTWTCTAAT) primer pair and dual indexing. A full list of all primers used can be found in Supplementary Table 9. Amplicons were normalized using the SequAmp Preparation Normalization Plate kit (Thermo Fisher Scientific) and pooled. Amplicon pools were purified prior to sequencing (Agencourt Ampure XP, Beckman Coulter Life Science, Indianapolis, IN) and size and quantity was assessed (LabChip GX, PerkinElmer Inc, Groningen, The Netherlands). A Control library was added to ~10% of each amplicon pool as a positive control (Phix Control v3 library, Illumina Inc., San Diego, CA). The hypervariable V3 and V4 regions of the 16S rRNA gene were sequenced in paired-end mode (2 × 300 bp) using the MiSeq platform (Illumina Inc., San Diego, CA) with an average depth of 50,000 paired reads per sample. Data processing and quality control: Sequence read quality control and taxonomic classification, was done using an in-house pipeline (μRAPtor) based on QIME version 1.9.0 and UPARSE version 8.1.22. Low-quality, merged, and chimeric reads were excluded. Duplicate samples, samples with <10,000 reads, and samples from participants that have used antibiotics (self-reported) in the 6 months prior to sample production were excluded. The remainder of the reads (~93%) were normalized using random 10,000 read subsampling (rarefaction). To reconstruct taxonomic composition a direct classification of 16S sequencing reads using RDP classifier (2.12) and the SILVA 16S rRNA database (release128). This was done for each taxonomic level available: domain, phylum, class, order, family, and genus, with binning posterior probability cutoff of 0.8. The microbial Shannon diversity index was calculated on the taxonomic level of genera, using vegan package in R. Relative abundances were calculated for each taxonomic level prior to any additional QC (domain, phylum, class, order, family and genus).

**Lifelines-DEEP replication cohort.** The LifeLines-DEEP (LLD) cohort is a sub-cohort of the LifeLines cohort (167,729 participants) that employs a broad range of investigative procedures to assess the biomedical, socio-demographic, behavioral, physical and psychological factors that contribute to health and disease in the general Dutch population. A standard Bacterial DNA Standard (Zymo Research) was included in LLD subcohort. For these participants, additional biological materials were collected, including analysis of the gut-microbiome composition. The collection, phenotyping, and processing of LLD have been described in detail. Briefly, microbiome data was generated for 1179 LLD samples. Fecal samples were collected at home within two weeks of hospital sample collection and stored immediately at −20 °C. After transport on dry ice, fecal samples were stored at −80 °C. Aliquots were made and DNA was isolated with the AllPrep DNA/RNA Mini Kit (Qiagen; cat. #50304). The 16S rRNA gene of the isolated DNA was sequenced at the Broad Institute, Boston, using Illumina MiSeq 250 16S rRNA high-throughput sequencing protocol. 16S rRNA gene sequencing was done using the primer pair 338F (ACTCTACACGGGGCCGAGGCAGCCAGCGGCTATAACT) and reverse primer 806 R (GGACTACHVGGGTWTCTAAT) (Supplementary Table 9). Closed-reference qPCR picking has been done with 97% similarity cutoff using UCLUST and GreenGenes 13.5 reference database from QIIME software. Overall, for 878 samples, both WOCAM scores and microbiome information was available. The library of microbial sequencing was rarefied to 10,000 read-depth using the rarely functional in R package (version 2.5.2). At this depth, 11 subjects were excluded. After the exclusion step, we had 867 samples (362 men and 505 women) remained for the final analysis.

Phenotype data. Osteoarthritis knee joint pain was determined by the WOMAC questionnaire, which is a disease specific questionnaire to assess the severity of hip and knee OA, consisting of 24 items covering three domains: stiffness, pain, and function. The WOMAC includes five items that measure OA joint specific pain, scored on a 5-point Likert scale, 0–4. The five specific knee WOMAC-pain scores were summed to create the knee WOMAC pain score, ranging from 0 to 20, where higher scores represent worse OA-related knee joint pain. Knee OA severity was determined by the radiographic Kellgren and Lawrence scale (KL-score).

Phenotype description. Osteoarthritis knee joint pain was determined by the WOMAC questionnaire, which is a disease specific questionnaire to assess the severity of hip and knee OA, consisting of 24 items covering three domains: stiffness, pain, and function. The WOMAC includes five items that measure OA joint specific pain, scored on a 5-point Likert scale, 0–4. The five specific knee WOMAC-pain scores were summed to create the knee WOMAC pain score, ranging from 0 to 20, where higher scores represent worse OA-related knee joint pain. Knee OA severity was determined by the radiographic Kellgren and Lawrence scale (KL-score). Using radiographs of both knee joints, left and right, the KL-score was determined for each joint. Scores were subsequently summed for the left and right knee to form the Knee KL-sum score. Knee joint effusion could be determined, for an all-female random subset of the Rotterdam Study III cohort (RSIII), by knee US (version 8.147). Low-quality, merged, and chimeric reads were excluded.

**qPCR replication of 16S rRNA-sequencing results.** To validate the 16S rRNA-sequencing results we determined the absolute quantitative amount of Streptococcus spp. using qPCR. We determined the amount of Streptococcus spp. for each fecal DNA sample using the BactoReal qPCR assay (Ingenix GmbH, Vienna, Austria) based on the Streptococcus 23S rRNA gene. A standard curve of a Plasmid standard containing the 23S rRNA gene (Ingentix GmbH, Vienna, Austria) was included in each plate to calculate the amount of Streptococcus spp. present in each sample. Each sample was run in duplo in a 384-wells PCR plate containing 40 ng fecal DNA, 1x primers and probes (Ingentix GmbH, Vienna, Austria), 1x TaqMan Gene Expression Master Mix (Life technologies) in a total volume of 5 μl. The qPCR was performed in a QuantStudio 7 Flex (Thermofisher) with an initial denaturation at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 s for 60 s. To normalize the amount of Streptococcus spp. for the total amount of bacteria in each sample, a 16 S qPCR was performed (U16SRT-F: ACTCCT ACGGAGGACGAGGAT and U16SRT-R: TATATTGCGGGTCTGCG), a standard curve of a Bacterial DNA Standard (Zymo Research) sample was included in each PCR plate to calculate the total amount of bacteria. Each sample was run in duplo in a 384-well plate containing 200 pg fecal

Streptococcus spp. may trigger joint inflammation, are not known. We hypothesized that it may involve metabolites or MVs produced by Streptococcus spp. in the gastrointestinal tract. As joint pain is one of the most impacting clinical OA symptoms and pain in OA has a high socioeconomic burden, it is of crucial importance to identify novel treatments that reduce clinical symptoms, in particular pain, and decrease the socioeconomic burden. Our results point to the microbiome as a therapeutic target for OA-related joint pain and possibly for other inflammatory joint pain disorders. The gastrointestinal microbiome is a promising therapeutic target, because it is sensitive to change through (diet) interventions. This could offer an easily accessible and safe treatment options for OA associated joint pain. Therefore, it is pivotal to explore the causal mechanism and possible translation of the present findings into clinical practice.
DNA, 200 pmol forward and reverse primers, 1x SYBR Fast ABI PrismTM Mastermix (KapaBiosystems) in a total volume of 5 μl. The qPCR was performed in a QuantStudio 7 Flex (ThermoFisher) with an initial denaturation at 95°C for 3 min followed by 40 cycles of 95°C for 5 s and 60°C for 20 s. Absolute abundancies were calculated from the standard curves. We adjusted the total absolute abundancy of bacteria for the average number of 165 copies (4.2 copies per bacterium)\(^6\). We adjusted the absolute abundance of Streptococcus spp. for the average number of 235 reads. The corresponding data on Streptococcus spp. (346 copies per Streptococcus spp. based on S. pneumoniae, S. thermopile, S. pyogenes, S. parasanguinis, S. dysgalactiae, S. salivaruis, S. suis, S. mutans, and S. agalactiae). We normalized the absolute abundance of Streptococcus spp. by calculating the log transformed value of the number of Streptococcus spp. per 1000 bacteria in each sample: 

\[
\log_{10}(\text{absolute abundance}) = \log_{10}\left(\frac{\text{number of reads}}{1000}\right)
\]

### Statistical analysis

Statistical analyses were performed in R: A Language and Environment for Statistical Computing\(^9\). Inter-individual microbial composition (β-diversity) was calculated using the Aitchison distance calculated from the CLR (centered log-ratio) normalized data. CLR normalization was calculated on the counts of the directly taxonomic classified reads. The full dataset, including unknown and unclassified taxonomies was used for the CLR. To all read counts was added to cope with the overabundance of zero’s in the data, for CLR\(^8\). Subsequent statistical analysis was done through permutation analysis of variance (PERMANOVA) to inspect the global effect of WOMAC-pain score and KLsum score on the overall microbiome profiles, using the adonis PERMANOVA function in VEGAN. PERMANOVA models included age, sex, TimeInMail, DNA isolation batch, BMI and qPCR technical covariates. ILR transformed data was used. The full dataset, including unknown and unclassified taxonomies was used. Multivariate linear regression model adjusted for age, sex, and cohort-specific technical covariates was performed on the ILR transformed data. Replication analysis in a separate cohort was performed. For each analysis we forced the following cofactors: age (years), sex (0/1), use (y/n), and NSAID use (y/n). Statistical significance was determined by Poisson-regression model adjusted for age, sex, and qPCR technical covariates. Adonis, nor was automatic QC of the metadata, WOMAC pain score and cov-ariates performed on the ILR transformed data. Replication analysis in a separate cohort was performed.

### Data availability

All relevant data supporting the key findings of this study are available within the article and its Supplementary information files. DNA files are available in the Supplementary figures 1 and 2 are provided as Source Data file. Other data that are available from the corresponding author upon reasonable requests. Due to ethical and legal restrictions, individual-level data of the Rotterdam Study cannot be made publicly available. Data are available upon request to the data manager of the Rotterdam Study Frank van Rooij (f.vanrooij@erasmusmc.nl) and subject to local rules and regulations. This includes submitting a request to the management team of RS, where upon approval, analysis needs to be done on a local server with protected access, complying with GDPR regulations.

**Received:** 18 December 2018; **Accepted:** 3 October 2019; **Published online:** 25 October 2019

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