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Inflammatory molecules produced by meniscus and synovium in early and end-stage osteoarthritis: a coculture study

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Abstract

The aim of this study was to identify the molecules and pathways involved in the cross-talk between meniscus and synovium that may play a critical role in osteoarthritis (OA) pathophysiology. Samples of synovium and meniscus were collected from patients with early and end-stage OA and cultured alone or cocultured. Cytokines, chemokines, metalloproteases, and their inhibitors were evaluated at the gene and protein levels. The extracellular matrix (ECM) changes were also investigated. In early OA cultures, higher levels of interleukin-6 (IL-6) and IL-8 messenger RNA were expressed by synovium and meniscus in coculture compared with meniscus cultured alone. RANTES release was significantly increased when the two tissues were cocultured compared with meniscus cultured alone. Increased levels of matrix metalloproteinase-3 (MMP-3) and MMP-10 proteins, as well as increased release of glycosaminoglycans and aggrecan CS846 epitope, were observed when synovium was cocultured with meniscus. In end-stage OA cultures, increased levels of IL-8 and monocyte chemoattractant protein-1 (MCP-1) proteins were released in cocultures compared with cultures of meniscus alone. Chemokine (C-C motif) ligand 21 (CCL21) protein release was higher in meniscus cultured alone and in coculture compared with synovium cultured alone. Increased levels of MMP-3 and 10 proteins were observed when tissues were cocultured compared with meniscus cultured alone. Aggrecan CS846 epitope release was increased in cocultures compared with cultures of either tissue cultured alone. Our study showed the production of inflammatory molecules by synovium and meniscus which could trigger inflammatory signals in early OA patients, and induce ECM loss in the progressive and final stages of OA pathology.

KEYWORDS

coculture, inflammation, knee, meniscus, osteoarthritis, synovium

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1 | INTRODUCTION

Osteoarthritis (OA) is the most common joint disorder and is the major cause of disability in the adult population (Loeser, Goldring, Scanzello, & Goldring, 2012). It is now well established that OA is not only a disorder of cartilage homeostasis but a whole-joint disease involving all tissues, including the subchondral bone (S. R. Goldring, 2012), meniscus (Favero, Ramonda, Goldring, Goldring, & Punzi, 2015), synovial membrane (Scanzello & Goldring, 2012), and infrapatellar fat pad (Belluzzi et al., 2017; Favero et al., 2017). Though not normally considered a classical inflammatory arthropathy, OA is often associated with low-grade synovitis characterized by the presence of mononuclear cell infiltrates and production of inflammatory mediators (Robinson et al., 2016). Synovial inflammation correlates with joint pain and dysfunction (Scanzello & Goldring, 2012). Currently, evidence indicates that the pathogenesis of synovial inflammation involves damage-associated molecular patterns (DAMPs), including cartilage extracellular matrix (ECM) breakdown products (Sellam & Berenbaum, 2010). However, recent studies have given attention to the roles of other joint tissues such as the meniscus in inducing synovial inflammation (Belluzzi et al., 2018; Edd, Giori, & Andriacchi, 2015). The meniscus plays a critical protective role in joint stabilization, shock absorption, and transfer and distribution of mechanical load (Englund, 2008). Macroscopic and histopathologic analyses of meniscus tissue from OA joints have revealed that fibrillation and disruption are first seen at the inner rim, spreading to the articular surfaces of the meniscus over time and progressing to total disruption or loss of meniscus tissue (Pauli et al., 2011). In moderate or severe OA, there is severe matrix disruption in one or more regions of the meniscus associated with abnormal cell clusters (Pauli et al., 2011). Several studies have also established that there is a strong relationship between traumatic, degenerative, or surgical meniscal damage and the risk for subsequent development of OA (Edd et al., 2015). It is possible that articular cartilage may be exposed to abnormal biomechanical forces as a consequence of meniscal damage exacerbating the inflammation (Edd et al., 2015). The literature shows that synovial inflammation is often present in patients with meniscal tears without evidence of OA (Scanzello et al., 2011). The presence of synovial effusion has been noted also by magnetic resonance imaging in patients with meniscal damage (Roemer et al., 2009). In this view, meniscal changes would precede the alterations of articular cartilage. Interestingly, Scanzello et al. (2011) showed that inflamed synovial tissue displayed a unique pattern of chemokines compared with noninflamed synovial tissue in patients with traumatic meniscal injury undergoing meniscectomy. These findings suggest that there could be cross-talk between meniscal tissues and synovial membrane and it may play a role in the pathophysiology of OA. Moreover, recently it has been showed that conditioned medium from OA meniscus can induce inflammation in human fibroblast-like synoviocytes cell line (K4IM) (Belluzzi et al., 2018). The present study was focused on understanding the interactions between the meniscus and synovial membrane in early and end-stage OA. To this end, we cocultured meniscal and synovial tissue to evaluate gene expression and protein release pattern of cytokines, chemokines, metalloproteases, and metalloprotease inhibitors. Moreover, we evaluated the status of ECM components including glycosaminoglycans (GAGs) and collagen fragments.

2 | MATERIALS AND METHODS

2.1 | Patients and sample collection

Patients with symptomatic meniscal tears undergoing arthroscopic partial meniscectomy were enrolled after providing written informed consent in accordance with the approval of the Local Ethical Committees, within the framework of a multicenter prospective cohort study, funded by the Italian Ministry of Health (GR-2010–2317593; Trisolino et al., 2017). Patients with previous surgery, evidence of anterior ligament rupture, or other pathologies (tumors, infections, coagulation disorders, inflammatory, rheumatic, and metabolic diseases) were excluded.

Biopsies of synovium from suprapatellar pouch and meniscus from the inner-superficial zone were collected as discarded tissues from meniscectomy surgery from five patients (five males, median age 34 years with interquartile range (IQR) = 41.5–25.5, median of the body mass index (BMI) = 29.54 kg/m^2, and IQR = 33.96–24.45. During surgery cartilage changes were assessed intraoperatively using the Outerbridge scoring system (Outerbridge, 1961) and all patients were scored as Grade I or II. Since Outerbridge Grades I and II signify early OA (Favero et al., 2015), all five enrolled patients undergoing partial meniscectomy were considered as early OA patients.

End-stage OA tissue samples of synovium from suprapatellar pouch and meniscus were harvested from five patients undergoing total knee replacement (TKR) (one male and four females, median age 62 years with IQR: 80–57, median of BMI 29.41 kg/m^2, and IQR 31.70–25.83).

2.2 | Histological evaluations of synovial membrane

Synovial samples from early and end-stage OA patient were fixed in 4% formaldehyde (Kaltek, Padova, Italy). After fixation, tissues were embedded in paraffin, and sections of 5 μm were prepared. The sections of synovium were processed for morphological evaluation by hematoxylin/eosin staining and inflammation was graded based on perivascular mononuclear cell infiltration, as described in Scanzello et al. (2011).

2.3 | Cocultures from OA tissues

All tissue samples were cut into pieces of 2 mm by 2 mm and placed in 24-well plates with ultralow attachment (Corning, NY) to avoid tissue adhesion. The tissues were maintained separate during coculture using a Transwell® device (pore 0.4 μm; Costar Corning Inc, Kennebunk, ME; Supporting Information Figure 1). Meniscus and synovial membrane tissues, which were cultured alone or together in coculture, were obtained from the same patient for a total of five patients. Explants were cultured in Dulbecco's modified Eagle's

medium (DMEM)/Nutrient Mixture F-12 (Gibco, Thermo Fisher Scientific, Waltham, MA) 1:1 with 1% Insulin-Transferrin-Selenium (ITS) Universal Cell Culture Supplement Premix (BD bioscience, San Jose, CA) up to 7 days at 37°C with 5% CO_2. At Day 7, supernatants were collected and stored at −80°C until analyzed, while tissues were frozen and stored in liquid nitrogen.

2.4 | Cell viability assay

The tissues collected from early and end-stage OA patients were cultured alone or in coculture and cell viability from the same tissue was analyzed at the beginning (Day 0) and end (Day 7) of culture by AlamarBlue© Assay (AbD Serotec-Biorad, Hercules, CA). 500 µl volume of AlamarBlue© Solution (1:10 in DMEM/F12 + 1% ITS) was added to each sample and incubated for 4 hr. After collection of supernatants, tissues were washed with phosphate-buffered saline (PBS) and fresh medium was added. AlamarBlue© fluorescence was detected using an excitation wavelength of 530 nm and emission wavelength of 590 nm by Mithras LB 940 Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany).

2.5 | Gene expression

Frozen tissues were pulverized using Mikro-Dismembrator S (Sartorius AG, Goettingen, Germany) at a shaking frequency of 2.000/min for 1 min. Then, pulverized tissues were solubilized using 500 µl of TRIzol® (Invitrogen, Thermo Fisher Scientific, Waltham, MA) and RNA was isolated according to the manufacturer's protocol. RNA was treated with DNAse Kit (AMBION, Thermo Fisher Scientific, Waltham, MA) and complementary DNA (cDNA) was transcribed using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen). Quantitative Real time PCR (qPCR) was performed on LightCycler Instrument (Roche, Basel, Switzerland) using the SYBR® Premix Ex Taq™ (Tli RNaseH Plus; Takara Bio Inc., Shiga, Japan) and specific primers for each target gene (Supporting Information Table 1). The levels of messenger RNA (mRNA) expression for each target gene were normalized to glyceraldehyde-3 phosphate dehydrogenase (*GAPDH*) as housekeeping gene according to the $2^{-\Delta\Delta C_t}$ method (Schmittgen & Livak, 2008) and we expressed the gene expression as "Number of molecules every 100000 GAPDH." Gene expression levels of cytokines (*IL6*, *CXCL8*, *TNFA*, Chemokine (C-C motif) ligand 5 [*CL5*], *IL1B*, and *IL15*), chemokines (*CCL2*, *CCL19*, and *CCL21*), metalloproteinases (*MMP3*, *MMP10*, and *MMP13*), and metalloproteinase inhibitors (*TIMP1*, *TIMP2*, *TIMP3*, and *TIMP4*) were evaluated by qPCR.

2.6 | Multiplex enzyme-linked immunosorbent assay (ELISA)

Cytokines (interleukin-6 [IL-6], IL-1β, transforming growth factor α [TNF-α], and IL-15), chemokines (IL-8, CCL2/MCP-1, CCL5/RANTES, CCL19/MIB-3-b, and CCL21/6kine), metalloproteinases (MMP-3, MMP-10, and MMP-13), and metalloproteinase inhibitors (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) were simultaneously evaluated in coculture supernatants of early and end-stage OA samples. Commercially available multiplex bead-based sandwich immunoassay kits (Bio-Rad Laboratories, Hercules, CA) were used by means of the Bio-Plex Protein Array System (Bio-Rad Laboratories, Hercules, CA). Data were analyzed by using the Bio-Plex Manager software version 6.0 (Bio-Rad Laboratories Hercules, CA). Standard levels between 70 and 130% of the expected values were considered accurate and were used. A value half of the lower limit of quantification (LLQ) was assigned to analyte concentrations less than the LLQ, while a value equal to the upper limit of quantification (ULQ) was assigned to analyte concentrations over the ULQ.

2.7 | Evaluation of GAGs, collagen, and aggrecan epitopes

The GAGs released into the culture medium were quantified by dimethylmethylene blue (DMMB) assay modified from Farndale, Buttle, and Barrett (1986). GAGs were measured by reaction of 5 µl of medium in 300 µl of DMMB solution for end-stage OA cocultures. By reaction of 40 µl of medium in 125 µl of DMMB solution due to the low GAGs concentration in early OA samples. Chondroitin sulfate sodium salt from shark cartilage (Sigma-Aldrich, St. Louis, MO) was used as standard. GAG content was measured as the absorbance at 524 nm with NanoQuant InfiniteM200 (Tecan Systems Inc., San Jose, CA) microplate reader. Collagen Type I (C1) and collagen Type II (C2) fragments (carboxy terminus of neoepitope for 3/4 C1, 2C or C2 3/4 short) were evaluated in supernatants using a commercially-available ELISA Kit (IBEX Pharmaceuticals, Montreal, Quebec, Canada). Aggrecan chondroitin sulfate epitope 846 (CS846 was quantified in coculture supernatants using a commercially ELISA Kit (IBEX Pharmaceuticals, Montreal, Quebec, Canada).

2.8 | Statistical analysis

Statistical analysis was performed with GraphPad Prism 6.0 (GraphPad Software, Sand Diego, CA). Differences of mRNA expression levels between different conditions were evaluated by paired *t* test. Differences of protein levels between different conditions were evaluated by paired *t* test. Data were expressed as mean ± standard error. A alue of $P < 0.05$ were considered significant.

3 | RESULTS

3.1 | Inflammation synovial score and cell viability

As previously described in Scanzello et al. (2011), a moderate inflammatory cell synovial infiltration was present in two patients with meniscal tear and in all the biopsies from end-stage OA patients (data not shown).

All tissues collected cultured alone contained viable cells after 7 days with no significant variation in cell number compared with Day 0. Moreover, cocultures of meniscus with synovial membrane from end-stage OA but not early OA showed a significant increase in cell number after 7 days compared with Day 0 (Figure 1a,b).

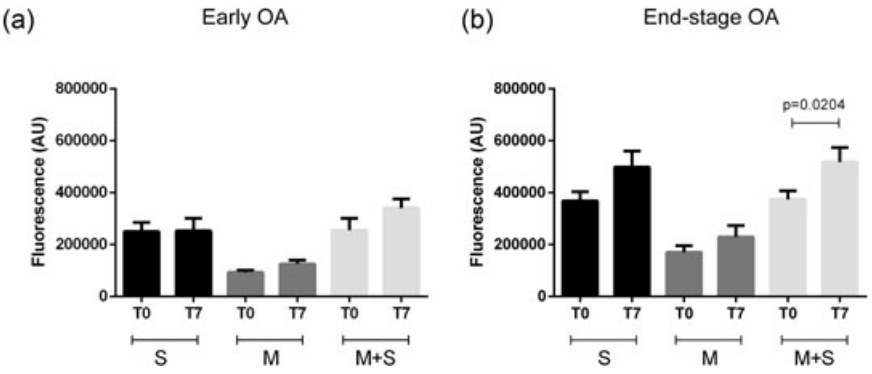

FIGURE 1 Cell viability evaluated by AlamarBlue© assay at the beginning of culture (*T0*) and after 7 days (*T7*): early (left) and end-stage OA (right) tissues culture. Values are expressed as mean ± standard error of the five experiments for each group. Significant differences are reported in the graphs and were calculated by paired *t* test. M: meniscus; M + S: meniscus in coculture with synovium; S: synovial membrane; OA: osteoarthritis

3.2 | Inflammatory mediators in early OA cocultures

The cross-talk between different tissues was evaluated by analyzing a panel of inflammatory mediators implicated in OA pathology. The cytokines IL-1β, TNF-α, and IL-15 were not detectable in any supernatant and the gene expression was also not detected in any sample with the exception of *IL1B* mRNA expressed only by synovium biopsies cultured alone and in coculture with meniscal tissue (data not shown).

In early OA cultures IL-6 protein level was close to the upper limit of detection in supernatant from both tissues cultured alone. The supernatants from cocultures of synovium and meniscus showed similar protein levels of IL-6 compared with the synovium cultured alone (Figure 2). *IL6* gene expression in meniscal tissue in the cocultures revealed a significant increase in *IL6* mRNA levels compared with meniscus cultured alone ($p = 0.0161$, Supporting information Figure 2).

We also analyzed the release of selected chemokines. The protein level of IL-8 in early OA cultures was close to the upper limit of detection in supernatants from synovium from two patients cultured alone. Meniscus biopsies cultured alone released very low levels of IL-8; in contrast, the coculture of synovium and meniscus produced similar IL-8 protein levels compared with the synovium cultured alone (Figure 3).

The qPCR of early OA samples highlighted a significant role of synovium in promoting the *CXCL8* gene expression in meniscal tissue in the coculture ($p = 0.0460$; Supporting Information Figure 3).

CCL2/MCP-1 was produced mainly by synovial membrane (Figure 3). All samples of meniscus cultured alone showed low or undetectable CCL2/MCP-1 protein release. The two tissues in coculture showed no significant difference compared with synovial tissue cultured alone. Gene expression of CCL2 was very low in all culture conditions (Supporting Information Figure 3).

Low protein levels of CCL5/RANTES were detected in supernatants from synovium or meniscus cultured alone, whereas significantly higher levels ($p = 0.0459$) were observed when the tissues were cocultured (Figure 3). Gene expression of CCL5 was increased only in meniscus cultured alone (Supporting Information Figure 3).

CCL21/6kine levels were low in cultures of synovium or meniscus from early OA patients with a no significant increase in coculture (Figure 3) and CCL21 gene expression was detectable only in synovial biopsies cultured alone and in coculture (Supporting Information Figure 3).

The chemokine CCL19/MIB-3-b and its gene expression were not detectable in any sample (data not shown).

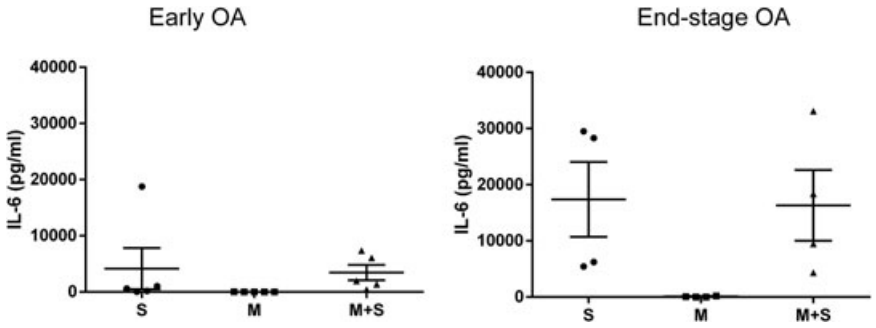

FIGURE 2 Cytokine IL-6 protein levels evaluation in early and end-stage OA cocultures supernatants. Values are expressed as mean ± standard error of the five experiments for each group. Paired *t* test was performed to analyze statistical differences. IL: interleukin; M: meniscus; M(M + S): meniscus tissue in coculture with synovial membrane; OA: osteoarthritis; S: synovial membrane; S(M + S): synovial membrane in coculture with meniscus

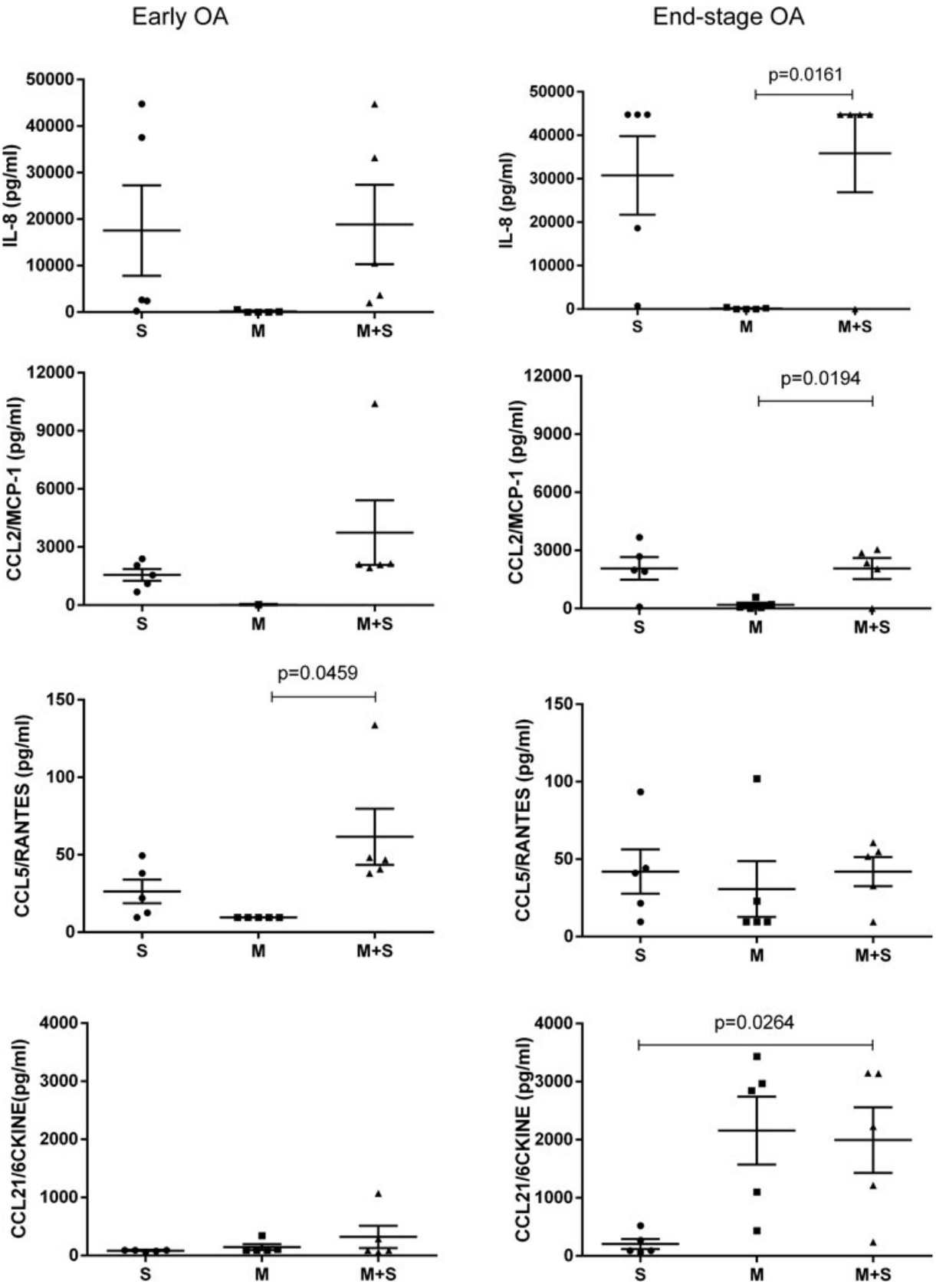

FIGURE 3 Chemokines (IL-8, CCL2/MCP-1, CCL5/RANTES, and CCL21/6kine) evaluation in cocultures of early and end-stage OA tissues. Values are expressed as mean ± standard error of the five experiments for each group. Significant differences are reported in the graphs and calculated by paired *t* test. CCL2/MCP-1: chemokine (C-C motif) ligand 2/monocyte chemoattractant protein 1; CCL5/RANTES: chemokine (C-C motif) ligand 5/regulated on activation normal T cell expressed and secreted; CCL21/6kine: chemokine (C-C motif) ligand 21; M = meniscus; M(M + S) = meniscus tissue in coculture with synovial membrane; OA: osteoarthritis; S: synovial membrane, S(M + S) = synovial membrane in coculture with meniscus

3.3 | Matrix metalloproteinases (MMP) and their inhibitors evaluation in early OA cocultures

MMP-3 protein release was significantly higher in cocultures of synovium and meniscus collected from early OA patients compared with the tissue cultured alone ($p = 0.0348$ and 0.0351, respectively; Figure 4). MMP-10 protein release was significantly higher in cocultures of synovium and meniscus compared with the meniscus cultured alone ($p = 0.0314$; Figure 4). However, there was no difference in *MMP3* and *MMP10* gene expression comparing cocultures and tissue cultured alone (Supporting Information Figure 4). MMP-13 protein was little in the supernatants collected from meniscus and synovial membrane cultured alone and an increase of protein levels were found in coculture even if not statistical significant (Figure 4). *MMP13* gene expression in early OA samples was low in synovium or meniscus cultured alone and in the meniscus in coculture; only few patients showed increased *MMP13* mRNA in synovium cocultured with meniscus (Supporting Information Figure 4).

TIMP protein release showed that early OA samples produced high levels of TIMP-1 and 3 and low levels of TIMP-4 in tissues cultured

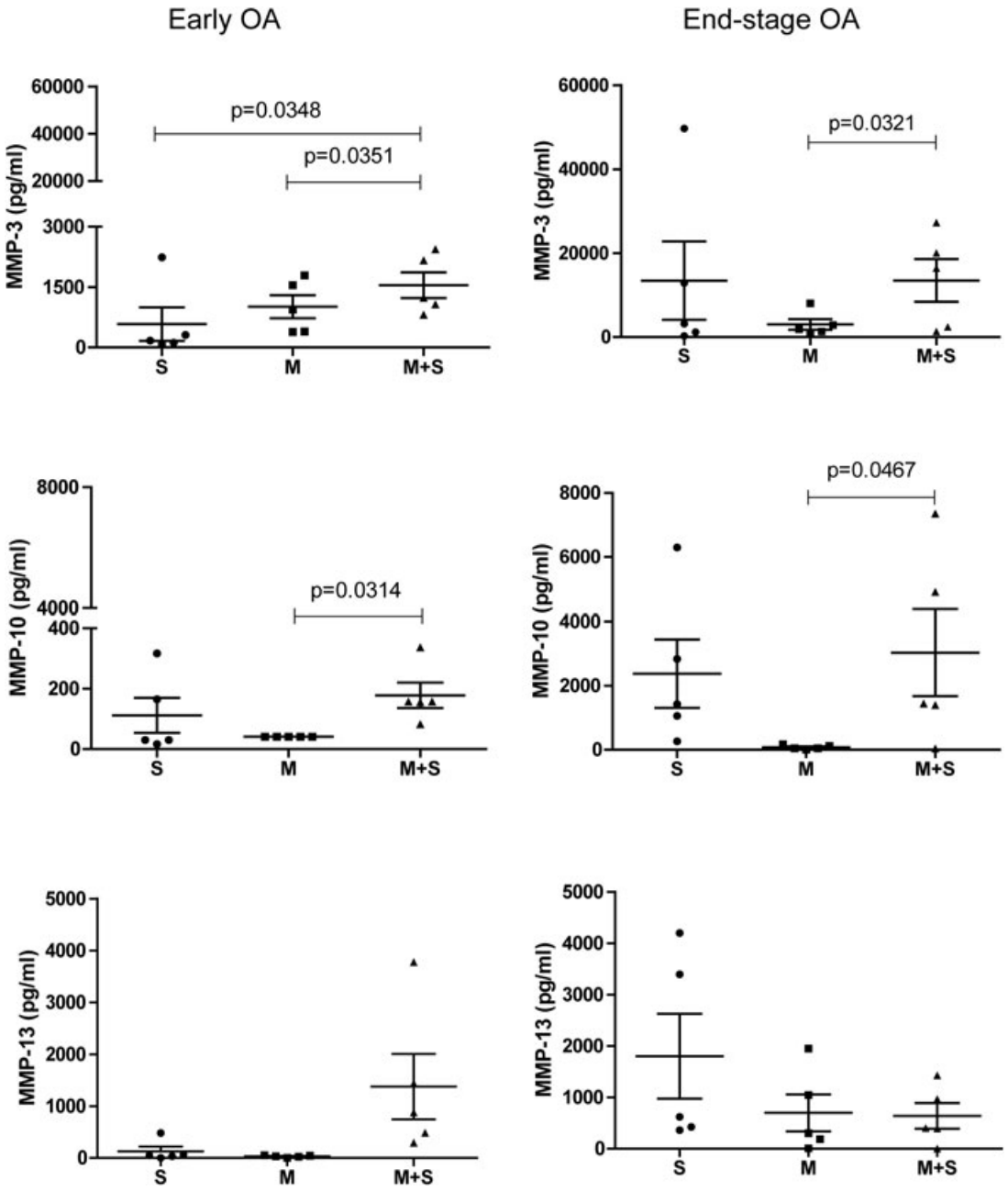

FIGURE 4 Metalloproteinases (MMP-3, MMP-10, and MMP-13) protein evaluation in cocultures of early and end-stage OA samples. Values are expressed as mean ± *SE* of the five experiments for each group. Significant differences are reported in the graphs and calculated by paired *t* test. MMP-3: matrix metalloproteinase-3; MMP-10: matrix metalloproteinase-10; MMP-13: matrix metalloproteinase-13; M: meniscus; M (M + S) = meniscus tissue in coculture with synovial membrane; OA: osteoarthritis; S: synovial membrane; S(M + S): synovial membrane in coculture with meniscus

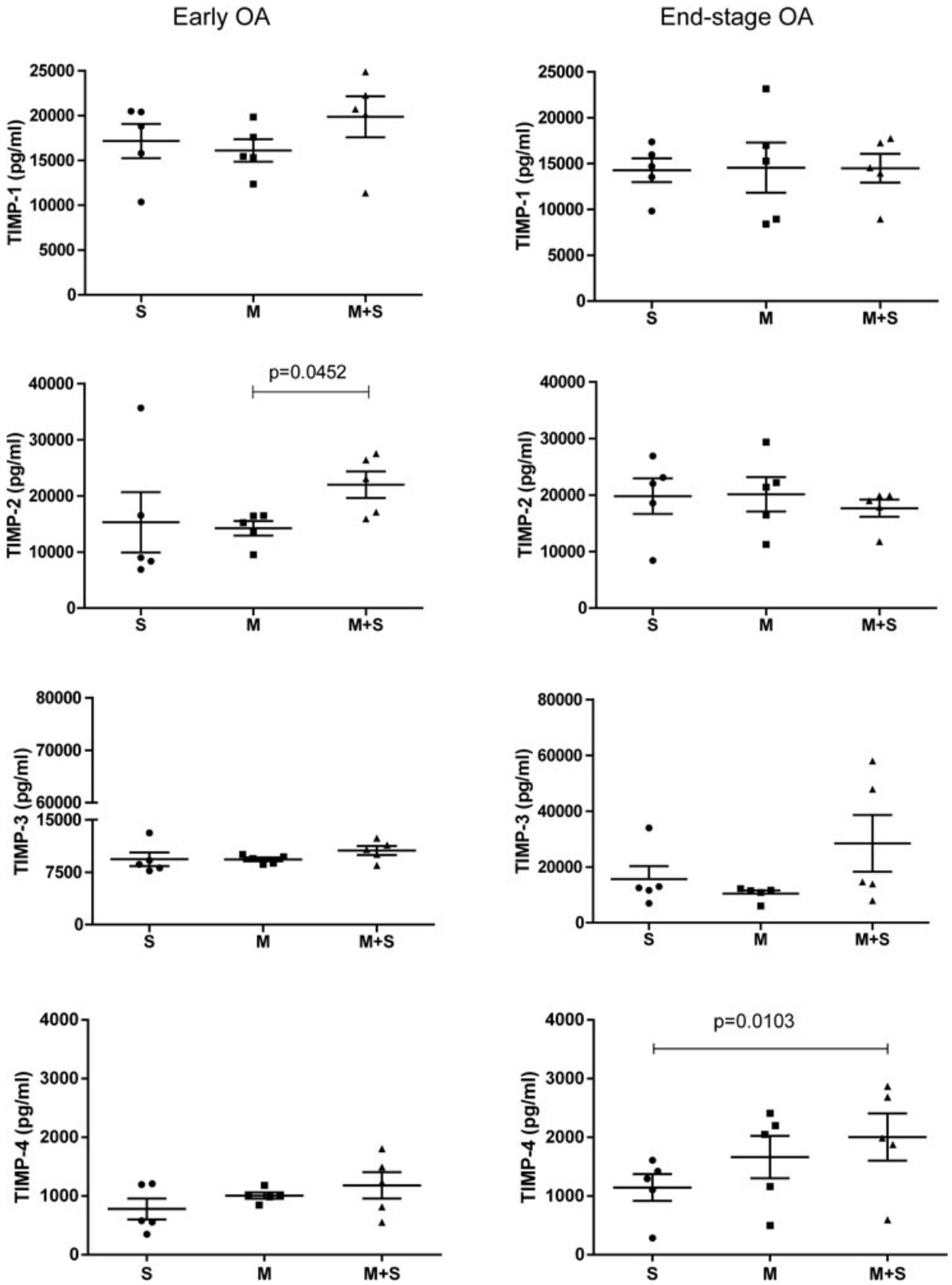

FIGURE 5 TIMPs protein evaluation in cocultures of early and end-stage OA. Values are expressed as mean ± *SE* of the five experiments for each group. Significant differences are reported in the graphs and calculated by paired *t* test. M: meniscus; M(M + S): meniscus tissue in coculture with synovial membrane; OA: osteoarthritis; S(M + S): synovial membrane in coculture with meniscus; TIMP: tissue inhibitor of metalloproteinases

alone and in coculture with no significant difference between culture conditions (Figure 5). TIMP-2 protein release was significantly higher in cocultures of synovium and meniscus compared with the meniscus cultured alone ($p = 0.0452$; Figure 5). TIMP gene expression did not show any trend (Supporting Information Figure 5).

3.4 | Inflammatory mediators in end-stage OA cocultures

Similar to early OA cultures, IL-1β, TNF-α, and IL-15 were not detectable in the supernatants of any end-stage OA cultures. *IL1B* mRNA was expressed only by synovial biopsies cultured either alone or in coculture with meniscus (data not shown).

In end-stage OA cultures, the trend of IL-6 protein release was similar to that found in early OA patients but the levels were higher. Indeed, the protein was detected only in supernatants of synovium cultured alone and in cocultures with meniscus (Figure 2). *IL6* gene expression in end-stage OA cultures was detected in all samples but without statistic differences (Supporting Information Figure 3).

In the end-stage OA cultures, we observed high IL-8 protein release from synovial membrane cultured alone and statistically significant levels in cocultures compared with that in meniscus alone ($p = 0.0161$; Figure 3). *CXCL8* mRNA was present at higher levels in synovium cultured alone or in coculture compared with meniscus alone or in coculture (Supporting Information Figure 3).

CCL2/MCP-1 was produced mainly by synovium cultured alone or in coculture with meniscus. with a significantly increased levels in cocultures compared with meniscus cultured alone ($p = 0.0194$; Figure 3). *CCL2/MCP-1* gene expression was very low in all samples (Supporting Information Figure 3).

The protein level and the mRNA expression of *CCL5/RANTES* in synovium or meniscus cultured alone or in coculture did not show any difference in comparisons of all samples (Figure 3 and Supporting Information Figure 3).

In end-stage OA samples, the protein release of CCL21/6kine from synovium cultured alone was low, but the release from meniscus cultured alone was higher in four patients. The cocultures showed significantly increased CCL21/6kine compared with the synovium cultured alone ($p = 0.0264$; Figure 3). *CCL21* gene expression was detectable only in synovial biopsies cultured alone with the exception of samples from three patients that showed *CCL21* gene expression in meniscus cultured alone (Supporting Information Figure 3).

Similar to results from early OA samples, the chemokine CCL19/MIB-3-b release and the gene expression were undetectable in any sample (data not shown).

3.5 | Matrix metalloproteinases and their inhibitors evaluation in end-stage OA cocultures

We found significant differences in MMP-3 protein release only in cocultures compared with meniscus cultured alone ($p = 0.0321$; Figure 4). Gene expression did not show variation in any cultures (Supporting Information Figure 4), except that *MMP3* mRNA was increased in synovial membrane cocultured with meniscus compared with tissues cultured alone.

MMP-10 protein release was significantly higher in cocultures of synovium and meniscus compared with the meniscus cultured alone ($p = 0.0467$; Figure 5). On the contrary, MMP-10 gene expression did not show any variation (Supporting Information Figure 4).

Despite the high concentration of MMP-13 in synovial supernatants from two end-stage OA patients, there was no significant difference among the different culture conditions (Figure 4). The same trend was observed both in *MMP-13* gene expression and protein release (Supporting Information Figure 4).

Both early and end-stage OA samples produced high levels of TIMP-1, 2, and 3 when the tissues were cultured alone or in coculture with no significant difference among culture conditions (Figure 5). In contrast, we found significantly different TIMP-2 protein release in cocultures compared with meniscus cultured alone ($p = 0.0452$; Figure 5) and TIMP-4 protein release in cocultures compared with synovium cultured alone ($p = 0.0103$; Figure 5). TIMPs gene expression did not show any difference, except for TIMP1 mRNA, which was highly expressed in synovium cultured alone ($p = 0.0419$; Supporting Information Figure 5).

3.6 | ECM products evaluation in both OA patients

GAGs, C1,2C fragments and aggrecan CS864 were quantified in the culture supernatants. Early OA meniscus or synovium cultured alone released less GAG compared with the cocultures ($p = 0.084$ and 0.0193, respectively; Figure 6).

The release of collagen neoepitopes C1,2C did not show any statistical differences in any culture condition for both the OA patients groups. Finally, aggrecan CS864 levels showed that synovium as well as meniscus cultured alone produced less aggrecan compared with the cocultures, but there was a statistical difference only between synovium cultured alone and the cocultures ($p = 0.0324$; Figure 6).

In end-stage OA cultures, the meniscus cultured alone showed the highest levels of GAG release (Figure 6). The release of C1,C2 fragments was similar from the tissues cultured alone or cocultured. The aggrecan CS864 production was increased in cocultures of meniscus and synovium compared with either tissue cultured alone ($p = 0.0261$ and 0.0380, respectively; Figure 6).

4 | DISCUSSION

OA is now considered a whole joint disease, but little is known about the role of the meniscus in the pathogenesis of OA and the cross-talk between this tissue and the synovium. Therefore, the aim of this study was to investigate the interactions between meniscus and synovium from patients undergoing partial meniscectomy and total knee replacement. The ultimate goal was to identify the common targets between early and late OA that may provide a basis for identifying the mechanisms contributing to the development of OA associated with meniscal tear.

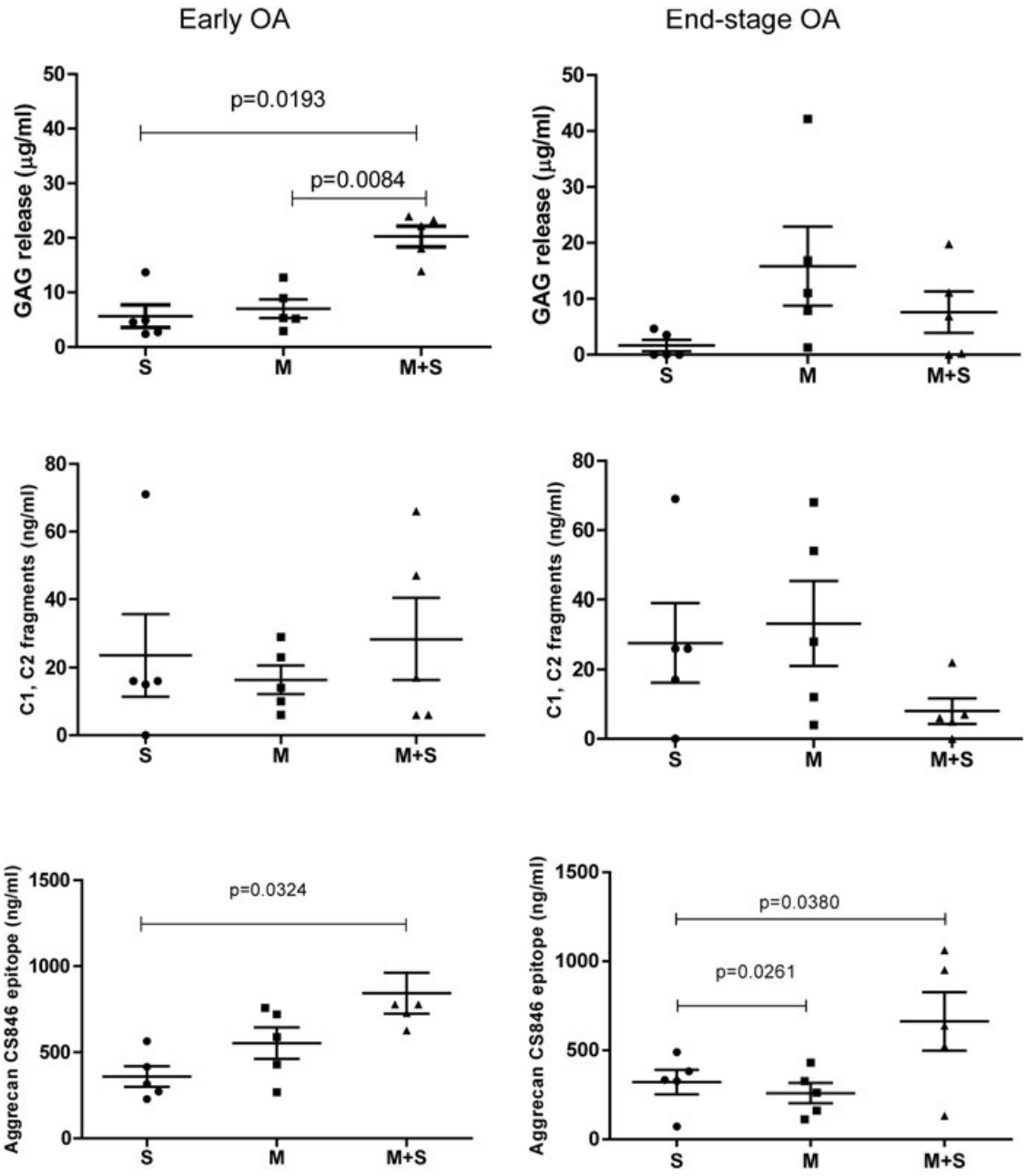

FIGURE 6 Extracellular matrix products (GAGs; C1,2C fragments and aggrecan CS846 epitope) quantification in early and end-stage OA cultures. Values are expressed as mean ± *SE* of the five experiments for each group. Significant differences are reported in the graphs and calculated by paired *t* test. GAGs: glycosaminoglycans; M: meniscus; M(M + S) = meniscus tissue in coculture with synovial membrane; S(M + S): synovial membrane in co-culturecoculture with meniscus

A panel of cytokines, chemokines, MMPs, and TIMPs, involved in OA pathology (Kapoor, Martel-Pelletier, Lajeunesse, Pelletier, & Fahmi, 2011; Sambamurthy et al., 2018; Scanzello & Goldring, 2012; Sokolove & Lepus, 2013), was evaluated in supernatants of cultures and the corresponding mRNAs encoding the same markers were quantified in the tissues (Supporting Information Table 2).

Interestingly, we found a discrepancy between protein levels detected in the culture supernatants and mRNA expression in the tissues. This is not surprising, since genomic and proteomic studies have shown that mRNA abundance correlates weakly with protein production. Besides a multifaceted regulation of gene transcription, it is necessary to consider posttranscriptional regulation (Mata, Marguerat, & Bähler, 2005). Moreover, the protein synthesized can undergo posttranslational modifications, which regulate its function, localization, and degradation. The discrepancy that we have found points out the need for future studies focused on tissue-specific posttranslational modifications and at different time points.

In our analysis IL-1β protein levels were not detectable in any culture supernatants, while *IL1B mRNA* was expressed only in synovial biopsies from early and end-stage OA samples cultured

alone or in coculture with meniscus. IL-1β protein is synthesized as a cytosolic inactive precursor, pro-IL-1β, which is cleaved by caspase-1 and then released into the extracellular space by several mechanisms (Lopez-Castejon & Brough, 2011). There are published data showing IL-1β protein evaluated by immunohistochemistry in synovium (Farahat, Yanni, Poston, & Panayi, 1993) and in synovial fluid (SF) of OA patients (Leung, Huebner, Haaland, Wong, & Kraus, 2017) with a significant variability in the concentration (Blasioli & Kaplan, 2014).

TNF-α and IL-15 were also not detectable at the protein or mRNA level in any condition both in early and end-stage OA. Our results on synovium from early OA patients contrast with those obtained by Scanzello et al. (2009), who found that the release of these two cytokines in SF and their gene expression in the synovial membrane correlated with the grade of synovitis. The discrepancy may be related to the variability of the patients selected among the studies, the reduced sample size used in this study, or a possible bias of our "in vitro" system compared with the analysis of synovial biopsies and SFs.

In early OA cocultures *IL6* and *CXCL8 mRNA* levels significantly increased in the meniscus in coculture compared with the meniscus cultured alone, supporting the occurrence of cross-talk between these two tissues.

In the end-stage OA tissues, the trend for IL-6 and IL-8 proteins was comparable with early OA. In particular, the proteins were produced mainly by synovium cultured alone and, in a statistically significant manner for IL-8, by cocultures compared with meniscus cultured alone. Our analysis showed a major role of the synovium in gene expression of both *IL6* and *IL8*, suggesting its important role as a source of inflammatory molecules that will affect the surrounding joint tissues (Mabey & Honsawek, 2015; Wang, Hunter, Jin, & Ding, 2018).

Both in early and end-stage OA patients, CCL2/MCP-1 and CCL5/RANTES were produced at higher levels by synovium compared with meniscus cultured alone. This chemokine was produced at higher levels in end-stage OA cocultures compared with meniscus cultured alone. It is interesting that meniscus cultured alone expressed *CCL2* mRNA, but protein secretion was not detected in supernatants in early OA cultures. This contrasts with the end-stage OA cultures, where *CCL2* mRNA expression levels were very low in synovium cultured alone but protein levels were high.

CCL5/RANTES protein release was significantly increased in cocultures from early OA patients, showing a possible mutual stimulation of the two tissues. As OA articular chondrocytes produce CCL5/RANTES mRNA and protein (Alaaeddine, Olee, Hashimoto, Creighton-Achermann, & Lotz, 2001), meniscal fibroblast-like cells may also be a source of inflammatory chemokines. We confirmed the *CCL5/RANTES* mRNA expression in synovium and meniscus biopsies from both early and end-stage OA patients in accordance with published data (Sambamurthy et al., 2018; Scanzello et al., 2009).

In contrast to Scanzello et al. 2011 data, we did not show any difference in *CCL21/6CKINE* gene expression in early or end-stage OA patients, likely related to the lower sample size evaluated. Interestingly, examination of late OA samples showed an active role of the meniscus in CCL21/6CKINE protein release when cultured alone and in coculture with synovial membrane, as shown in animal model of OA (Loeser, Olex, et al., 2012).

The secretion of MMPs by the meniscus contributes to the development and progression of OA (Fuller, Smith, Little, & Melrose, 2012; Stone et al., 2014). Among the MMPs, MMP-3 is important because it is upregulated in cartilage in early OA (M. B. Goldring & Otero, 2011); moreover, MMP-3 protein was higher in degenerated menisci compared with normal (Ishihara, Kojima, Saito, & Ishiguro, 2009). We confirmed that the MMP-3 protein release, in both early and end-stage OA patients, was significantly higher when meniscus was cocultured with synovium. Proinflammatory molecules produced by the synovium and by the meniscus itself may therefore stimulate MMPs secretion in a vicious cycle.

MMP-10, which contributes to the catabolism of proteoglycans (Barksby et al., 2006) and activation of MMP13 (Olivotto et al., 2013), showed the same trend as MMP-3.

An increase in the release of TIMP-1, 2, and 4, was shown in early OA tissues in coculture compared with the tissues cultured alone, suggesting a mutual action of the two tissues. In end-stage OA samples, the protein levels of all TIMPs analyzed were high but only TIMP-4 was increased in cocultures of synovium and meniscus compared with either tissue cultured alone.

Proinflammatory molecules and proteinases released in the joint may induce ECM turnover (Silverstein et al., 2017; Sorokin, 2010). In particular, in early OA samples both GAGs and aggrecan CS846 epitope were increased in supernatants when synovium was cocultured with meniscus, likely related to the stimulation of the meniscus by the inflammatory molecules produced by the synovium. In end-stage OA samples, the aggrecan CS846 epitope release was increased when synovium was cocultured with meniscus, confirming that inflammatory molecules released from synovium may influence the imbalance in the ECM turnover (Lotz et al., 2013).

The principal limitations of our study is the limited number of patients and the analysis performed at one timepoint. However, our study represent a first attempt to characterize the cross-talk between meniscus and synovial tissues using a coculture model comparing both mRNA expression and protein release. In the future, it will be interesting to characterize the cultures at different time points and investigate also other markers.

In conclusion our study suggest that the cross-talk between synovium and meniscus is common in early and late OA patient only for MMP3 and MMP10, while different TIMPs were involved in the two groups (TIMP-2 for early OA patients and TIMP-4 for late). All these proteins can trigger inflammatory signals and, consequently induce ECM turnover. In end-stage OA patients, we also demonstrated an additive proinflammatory role between meniscus and synovium for CCL21 protein. We confirm the main role of the synovium in inflammation also in early OA patients.

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CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

AUTHOR CONTRIBUTIONS

M. F., E. B., G. T., A. P., A. G., and E. O were included in the conception and design of the study, or acquisition of data, or analysis and interpretation of data. M. F., E. B., M. B. G., S. R. G., P. R., R. R., B. G., L. P., and E. O. drafted the article or revised it critically for important intellectual content. All authors approved the final version of the manuscript. Final approval of the version to be submitted.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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