Supplementary Material to Alunno et al. “Platelets Contribute to the Accumulation of Matrix Metalloproteinase Type 2 in Synovial Fluid in Osteoarthritis” (https://doi.org/10.1160/TH17-06-0379)

Supplemental Methods and Results

Methods

Study design

Patients with knee OA and with joint pain, stiffness and/or functional impairment interfering significantly with normal daily activities, were enrolled. Inclusion criteria were: mono or bilateral knee OA, time of onset >5 years, absence of any recent intraarticular therapy (corticosteroids for at least 3 months and/or HA for at least 6 months prior to enrolment), presence of joint effusion confirmed by ultrasonography (US), and ability to fill a self-assessment questionnaire [1]. Exclusion criteria were: inflammatory rheumatic disorders (e.g. RA), dry OA, oral therapy with steroids, intake of non-steroidal anti-inflammatory drugs or antiplatelet agents during the 15 days prior to enrolment, intra-articular (i.a.) corticosteroids in the 30 days prior to enrolment, cognitive impairment, hemoglobin levels <12 g/dl in males and <11g/dl in females in the 3 months prior to enrolment, oral anticoagulant therapy, pregnancy, infectious diseases.

All patients underwent 5 weekly i.a. injections of sodium hyaluronate (HA, 500-730 kDa, Hyalgan®, Fidia S.p.A, Abano Terme, Italy) of 20 mg/2 mL each. SF was collected by US-guided articular puncture at T0 (at the 1st injection of HA), and at T1 (at the time of 5th injection). Clinical and US scan assessment were performed at T0 and T1, and clinical re-evaluation at T2 (three months after the 5th injection).

Demographic and clinical data (weight, height, current therapies), the Western Ontario and McMaster University (WOMAC) self-assessment clinical index, and the visual analogic scale (VAS) for pain were recorded at enrolment. The study was approved by the local Institutional review board (CEAS Umbria) and all patients gave their written informed consent in accordance
with the Declaration of Helsinki. Investigators performing laboratory studies were blinded as to patient number and time of observation.

**Cell and microparticles count in SF**

SF was collected in sterile Vacutainer tubes containing 0.18% K$_3$EDTA and an aliquot was incubated with hyaluronidase (250 U/ml at 37°C for 10 min) to allow accurate cell count [2]. Ten μl SF was added to 190 μL of Stromatol® solution (Mascia Brunelli srl, Milan, Italy) and total cell number was counted by optical microscopy using a Bürker chamber (Neuroprobe Inc, Gaithersburg, MD, USA) with a 40x objective. Differential count was determined in smears of cytocentrifuged, hyaluronidase-treated SF after May-Grünwald staining [3].

**In vitro co-culture experiments**

In selected experiments, platelets were co-cultured with FLS in the presence of HA (100, 500 and 1000 μg/ml), or of a blocking anti-CD44 antibody (Thermo Scientifics, Waltham, MA USA) (10 μg/ml), or of a blocking anti-P-selectin antibody (Monoclonal Mouse IgG1 Clone #9E1, R&D system, Inc., Minneapolis, USA) (20 μg/ml), or of an isotype-control IgG. In order to establish the effective concentrations of the blocking antibodies, we performed preliminary studies. The inhibitory effect of different concentrations of the anti-P-selectin antibody on P-selectin expression by platelets activated with ADP or TRAP-6 was assessed by flow cytometry. The inhibitory effect was maximal at 20μg/ml and this concentration was chosen for subsequent experiments.

The effect anti-CD44 blocking antibody at a concentration largely used in the literature (10μg/ml) [4], was tested in FLS adhesion assay on fibrinogen and found to be effective in reducing FLS adhesion.
Results

Comparing P-selectin expression on platelets prior 24h incubation (basal) vs platelets after 24h incubation in wells without FLS (our control), we observed that P-selectin exposure tended to decrease, although not significantly (Figure 1).

![Figure 1](image)

**Figure 1.** P-selectin expression on platelets before and after 24h incubation in control wells (without FLS). p=ns

We examined the possible role of platelets in the accumulation of MMP-2 in SF of OA patients by in vitro co-incubation experiments using FLS or lympho-monocytes, the two most abundant cell type present in OA SF. Our in vitro co-incubation experiments demonstrated that MMP-2 production by FLS was significantly enhanced in the presence of platelets while co-incubation of platelets with lympho-monocytes, isolated as previously described [5], at the same average MNC to platelets ratio (~1:37) found in the SF of OA patients, did not increase MMP-2 in the supernatant (lympho-monocytes=1.20±0.4 ng/ml, lympho-monocytes and platelets=1.35±0.77 ng/ml, n=3, p=ns). These data are in agreement with previous evidence demonstrating that the principal cellular source of MMP-2 in several inflammatory conditions is represented by fibroblasts rather than lympho-monocytes [6,7].
Co-incubation of platelets with FLS induced a significant increase of the release of platelet-derived microparticles (PMPs) in the supernatant, showing activation of platelets upon contact with FLS. Moreover, a significative fraction of PMPs recovered after incubation with FLS was positive for CD62P (Figure 2).

![Figure 2: P-selectin expression on PMP recovered after incubation with FLS.](image)

References


Figure S1. Flow-chart of the study. Out of a total of 60 screened patients with OA, all cases who satisfied the inclusion criteria were enrolled. Out of 38 enrolled patients 27 were reassessed after 4 weeks of treatment for clinical evaluation and 24 for SF analysis, because in 3 patients SF was absent at T1.
Figure S2. Total cell, differential cell count and synoviocytes count in SF, before and after i.a. treatment with HA. (A) After HA treatment (T1, black bars), total cell count was significantly different from baseline (T0, white bars). (*= p<0.05) (n=24). (B) After HA injection, the percentage of different white cells in the synovial fluid, determined by light microscopy in smears of cyt centrifugate synovial fluid after May-Grunwald staining, was not statistically different from T0. (C) After HA treatment number of synoviocytes in SF tend to be different from baseline (p=0.052). L-M= lympho-monocytes, N=neutrophils, E=eosinophils.
Figure S3. Effect of HA on platelet activation. Treatment with increasing concentration of HA (100-1000 µg/ml) does not influence platelet activation during coincubation with synovial fibroblasts (n=6, p=ns).
Table S1. Demographic characteristics of the study subjects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Gender (M/F)</td>
<td>6/21</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>78.9±2.8</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>165.3 ±1.42</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>78.9±2.9</td>
</tr>
<tr>
<td>BMI (kg/m$^2$)</td>
<td>28.9±0.97</td>
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Values are reported as mean±SEM.
Table S2. Effect of i.a. treatment with HA on clinical parameters.

<table>
<thead>
<tr>
<th></th>
<th>T0</th>
<th>T1</th>
<th>T2</th>
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<tbody>
<tr>
<td>WOMAC total</td>
<td>133.2 ± 9.6 (152.5; 13-228)</td>
<td>80 ± 9.1 (83; 14-177)*</td>
<td>81.2 ± 9.9 (83.5; 0-146)#</td>
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<tr>
<td>WOMAC pain</td>
<td>25.6 ± 2.2 (28; 5-46)</td>
<td>16.8 ± 1.7 (16; 3-38)*</td>
<td>17.5 ± 1.8 (16; 3-40)#</td>
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<tr>
<td>WOMAC stiffness</td>
<td>11 ± 1 (12; 0-20)</td>
<td>8 ± 1.1 (8; 0-20)*</td>
<td>8.2 ± 1.1 (8; 0-20)</td>
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<tr>
<td>WOMAC function</td>
<td>96.5 ± 7 (96.5; 108.5)</td>
<td>69 ± 7 (70; 7-123)*</td>
<td>70 ± 7 (75; 7-130)#</td>
</tr>
<tr>
<td>VAS pain</td>
<td>68 ± 4.3 (78; 10-100)</td>
<td>46.3 ± 4.7 (50; 2-90)*</td>
<td>48 ± 7.2 (40; 2-100)</td>
</tr>
<tr>
<td>Joint Effusion (mm)</td>
<td>5.3 ± 0.6 (5.3; 0.3-12)</td>
<td>3.4 ± 0.6 (3; 0-11.7)*</td>
<td>N.A.</td>
</tr>
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T0 (at the 1st injection of HA); T1 (prior to the 5th injection); T2 (three months after the 5th injection). Values are reported as means±SEM; within brackets median and minimum-maximum. n=27.

A significant reduction of the total WOMAC score, of all the individual WOMAC domains and of the VAS pain score was observed at the end of the i.a. HA cycle. Clinical benefit lasted for up to 3 months after the end of treatment, as all parameters at T2 were comparable to T1 and most of them still significantly different from T0. No adverse events were observed during the study period.

* p<0.01 T1 vs T0, # p<0.01 T2 vs T0 by One way ANOVA followed by Bonferroni post test. All the WOMAC domain and VAS pain score at T2 did not differ significantly from T1. N.A.= not assessed; mm= millimeter.