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FULL-LENGTH ARTICLE

Basic Research

Release kinetic of pro- and anti-inflammatory biomolecules from platelet-rich plasma and functional study on osteoarthritis synovial fibroblasts

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ABSTRACT

Background aims: This study evaluated the release kinetics of numerous representative and less studied platelet-rich plasma (PRP) cytokines/chemokines with regard to the effects of various cellular compositions and incubation times. In addition, the biological effects of different PRPs on osteoarthritis synovial fibroblasts *in vitro* were tested.
Methods: Peripheral whole blood was collected from healthy donors, and pure platelet-rich plasma (P-PRP), leukocyte-rich platelet-rich plasma (L-PRP) and platelet-poor plasma (PPP) were prepared for the analysis of the following biomolecules: IL-1β, IL-4, IL-6, IL-10, IL-17a, IL-22, MIP-1α/CCL-3, RANTES/CCL-5, MCP-3/CCL-7, Gro-α/CXCL-1, PF-4/CXCL-4, ENA-78/CXCL-5, NAP-2/CXCL-7, IL-8/CXCL-8, Fractalkine/CX3CL-1, s-CD40L P-PRP, L-PRP and PPP. Their effect on osteoarthritis synovial fibroblasts *in vitro* was tested by analyzing changes induced in both gene expression on a panel of representative molecules involved in physiopathology of joint environment and synthesis of IL-1β, IL-8 and hyaluronic acid.
Results: This study demonstrated that among the 16 analyzed biomolecules, four were undetectable, whereas most of the detected biomolecules were more concentrated in L-PRP even when concentrations were normalized to platelet number. Despite the pro-inflammatory boost, the various PRP preparations did not alter synovial fibroblast gene expression of specific factors that play a pivotal role in joint tissue homeostasis and are able to induce anti-inflammatory (TIMP-1) biomolecules.
Discussion: This study provides a set of reference data on the concentration and release kinetics of some less explored biomolecules that could represent potential specific effectors in the modulation of inflammatory processes and in tissue repair after treatment with PRP.

Introduction

Platelet-rich plasma (PRP) is the liquid fraction of autologous peripheral blood with platelet concentration above the baseline [1]. Upon activation, platelets release a cocktail of biologically active molecules from their alpha-granules that enhance the reparative and regenerative processes by cellular activities, morphogenesis and inflammatory responses [2]. The use of orthobiologics has been widely studied in various fields of medicine [3]. This therapy has raised hopes for the treatment of musculoskeletal disorders [4–6] by showing encouraging results in terms of regenerative capacity, and it is currently considered a suitable option with clinical benefits [4].

However, discrepancies in the final outcome has significantly challenged the usefulness of PRP in clinical applications [7-9]. Strong evidence suggests that the release of bioactive molecules from PRP differs significantly depending on formulations (presence or absence

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of leukocytes), duration of incubation, methods of activation and centrifugation parameters [10,11]. In addition, although many studies have focused on anabolic effects of growth factors contained in platelet concentrates, there is insufficient evidence available on the soluble factors mix and their pro- and anti-inflammatory balance in PRP due to different preparation methods [12-14]. Data in the literature indicate that biological profiles in terms of the amount and release kinetics associated with different platelet concentrates need to be investigated more thoroughly to better understand the use of soluble biomolecules potentiality in clinical applications.

To address this concern, the present study aimed to compare two products based on different preparation procedures (already used in clinical practice) that represent two opposite approaches, making the comparison of particular interest for therapeutic implication. We compared biomolecules released by various PRPs in different compositions (i.e., containing only platelets [P-PRP], leukocytes and platelets [L-PRP] or platelet-poor plasma [PPP]) and with time-dependent delivery.

Various biomolecules contained primarily in platelet alpha granules were simultaneously analyzed up to 7 days after PRP activation, with particular focus on the balance of pro- and anti-inflammatory biomolecules. The molecules analyzed were as follows: interleukin (IL)-1β, IL-4, IL-6, IL-10, IL-17a and IL-22; macrophage inflammatory protein-1α (MIP-1α/CCL-3), regulated upon activation, normal T-cell expressed, and secreted (RANTES/CCL-5), monocyte chemoattractant protein-3 (MCP-3/CCL-7), growth-regulated oncogene-α (Gro-α/CXCL-1), platelet factor 4 (PF-4/CXCL-4), epithelial neutrophil activating peptide-78 (ENA-78/CXCL-5), neutrophil-activating peptide-2 (NAP-2/CXCL-7), IL-8/CXCL-8, fractalkine/CX3CL-1 and soluble CD40 ligand (s-CD40L). In addition, the biological effects of P-PRP, L-PRP and PPP on osteoarthritis synovial fibroblasts *in vitro* were tested by analyzing changes induced both in gene expression of a panel of representative molecules involved in physiopathology of the joint environment (pro- and anti-inflammatory cytokines, ECM, matrix-degrading enzyme and their inhibitors) and in the synthesis of hyaluronic acid (HA).

Methods

Blood samples

Blood samples were collected from 10 healthy males (age range 26–38 years), enrolled on a voluntary basis. Subjects with systemic disorders, infections, hemoglobin concentration <11 g/dL, platelet number $<150 \times 10^3/\mu$L; individuals who had taken nonsteroidal anti-inflammatory drugs in the 5 days before blood collection and smokers were excluded from the study. The Institutional Ethic Committee approved the protocol (Protocol no. 0008705/2014), and each donor signed informed written consent. The samples were univocally coded and appropriately processed to obtain two different PRP products from each donor. A one-step centrifugation PRP preparation (containing P-PRP) and a two-step centrifugation PRP preparation (containing a higher platelet number and leukocytes—L-PRP) were obtained using a standard laboratory centrifuge.

Preparation of one-step centrifugation PRP

P-PRP was prepared similarly to a previous description [15,16]. Briefly, a venous blood sample was collected in five tubes containing 1 mL of sodium citrate solution (3.8 %) (9 mL/tube) and centrifuged for 8 min at 460*g*. The platelet-rich supernatant above the red blood cell layer was collected (1 mL/tube), carefully avoiding the underlying leukocytes.

Preparation of two-step centrifugation PRP

A venous blood sample (150 mL) was collected in a standard transfusion bag containing 21 mL sodium citrate and centrifuged for 15 min at 730*g*. Plasma and buffy-coat layers were transferred in a new bag by a closed circuit. A second centrifugation for 10 min at 3800*g*, allowed the stratification of PPP in the upper part and of L-PRP over the red blood cells [16]. PPP and L-PRP fractions were separately collected.

Determination of platelet and white blood cell concentrations

Platelet and white blood cell (WBC) concentrations were determined by an automated hematology analyzer (Coulter LH 750, Beckman Coulter, Milan, Italy). Linearity was $5–1000 \times 10^3/\mu$L and $0.1–100 \times 10^3/\mu$L for platelet and for WBC counts, respectively.

Activation of different preparations and collection of supernatants containing soluble factors

Each preparation (PPP, P-PRP and L-PRP) was divided in five tubes (500 μL/tube), activated by the addition of 10% $CaCl_2$ (22.8 mmol/L final concentration, corresponding to activation system and concentration used for clinical applications) that induced the formation of a clot, without the addition of medium. Tubes were then incubated for 1 and 18 h and for 2, 3 and 7 days at 37°C in 5% CO_2. The first and the last time points were chosen in agreement with the clinical protocol that foresees the injection of PRP within 1 h from the $CaCl_2$ activation and schedules the injections at weekly intervals. At the end of each incubation time, samples were centrifuged for 15 min at 2800*g* at 20°C, and the supernatants were collected and frozen at −80°C until tested.

Determination of released soluble factors by multiplex-beads immunoassay and data analysis

Collected supernatants were assayed in duplicates and soluble factor concentrations (IL-1β, IL-4, IL-6, IL-10, IL-17α, IL-22, MIP-1α/CCL-3, RANTES/CCL-5, MCP-3/CCL-7, Gro-α/CXCL-1, PF-4/CXCL-4, ENA-78/CXCL-5, NAP-2/CXCL-7, IL-8/CXCL-8, Fractalkine/CX3CL-1 and s-CD40L) were evaluated using bead-based multiplex sandwich immunoassay kits, following the manufacturer's instructions (Bio-Rad Laboratories, Hercules, CA, USA; Millipore, Billerica, MA, USA) [17].

The immunocomplexes formed on distinct beads were quantified by using the Bio-Plex Protein Array System (Bio-Rad Laboratories). Values presenting a coefficient of variation beyond 10% were discarded before the final data analysis. Data were analyzed using the Bio-Plex Manager software version 6.0 (Bio-Rad Laboratories). Standard levels between 70% and 130% of the expected values were considered accurate. In general, at least six standards were accepted and used to establish standard curves following a five-parameter logistic regression model. Sample concentrations were immediately interpolated from the standard curves.

Synovial fibroblast isolation and culture

Synovial fibroblasts were isolated by enzymatic digestion from patients with osteoarthritis (n = 3 Kellgren-Lawrence grade II–III) undergoing joint surgery (Institutional Ethic Committee Protocol no. 8342/2010) as previously described [18]. Briefly, the synovial tissue underwent trypsin (Sigma-Aldrich, Milan, Italy; 0.1 % in phosphate-buffered saline for 30 min at 37°C in 5% CO_2) and collagenase P (Roche, Basel, Switzerland) (0.1 % for 1 h at 37°C) digestions under constant rotation. The resulting cell suspension was cultured in flasks with OPTIMEM culture medium (Gibco-BRL, Life Technologies Grand Island, NY, USA) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) at 37°C, 5% CO_2 in a humidified atmosphere.

Synovial fibroblasts were grown to subconfluence, detached by the culture flasks, seeded at a concentration of 25000/cm^2 in 12-well

tissue culture plates and then maintained with serum-free medium for 24 h. P-PRP, L-PRP or PPP activated with $CaCl_2$, as previously reported, were then added to the culture medium at 10% (vol/vol). The dilution of PRP preparations (not only the released supernatant) underwent clotting directly in a Transwell device (0.4-μm porosity; Costar, Corning, Corning, NY, USA) inserted in the culture plates to avoid direct cell–cell contact but favoring interaction with synovial fibroblast monolayers adherent to the plate bottom. Plates were incubated up to 7 days without medium changes. Cell viability, analyzed by Alamar blue colorimetric assay (AbD Serotec, Kidlington, UK) on day 0 and 7, was ≥90 %.

At the end of the incubation time, Transwell devices containing clots were discarded, culture supernatants were collected and maintained at −80°C until tested and synovial fibroblast monolayers were directly lysed in the well for RNA extraction.

Synovial fibroblasts gene expression analysis

Samples were assayed with real-time quantitative reverse transcriptase polymerase chain reaction (RT-PCR) for IL-1β, IL-4, IL-6, IL-8/CXCL8, IL-10, metalloproteinase (MMP)-13, tissue inhibitor of metalloproteinase (TIMP)-1, TIMP-3, TIMP-4, HA synthases (HAS)-1, HAS-2 and HAS-3 gene expression.

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommended protocol. RNA was reverse-transcribed using superscript first-strand kit (Invitrogen). RNA-specific primers for PCR amplification (Table 1) were generated from GeneBank sequences using Primer 3 Software. Real-time PCR was run on the LightCycler Instrument (Roche) using the SYBR Premix Ex Taq (TaKaRa biotechnology).

Technical specifications of light cycler instrument used to perform PCR provide a uniform temperature for all samples during each run of PCR, increasing the reproducibility of the data. The crossing point values were determined for each sample, and specificity of the amplicons was confirmed by melting curve analysis. Amplification efficiency of each amplicon was evaluated using 10-fold serial dilutions of positive control cDNAs and calculated from the slopes of log input amounts plotted versus crossing point values. They were all confirmed to be high (>92 %) and comparable; micro-RNA (mRNA) levels for each target gene were calculated and normalized to glyceraldehyde-3 phosphate dehydrogenase (GAPDH, reference gene); according to the ΔΔCt method, the data were calculated as the ratio of each gene to GAPDH and expressed as number of molecules per 100 000 GAPDH.

Table 1
List of primers used in real-time PCR.

RNA template	Primer sequences (5′–3′)	Annealing temperature (°C)	References
GAPDH	5′-CCTGGCCAAGGTCATCCATG 3′-CGGCCATCACGCCACAGTT	60	Primer 3
IL-1β	5′-GTGGCAATGAGGATGACTTGTT 3′-TGGTGGTCGGAGATTCGTAG	60	Primer 3
IL-6	5′-TAGTGAGGAACAAGCCAGAG 3′-GCGCAGAATGAGATGAGTTG	60	Primer 3
IL-8	3′-ACTTCTCCACAACCCT 3′-CTGGTTGGCTTCCTTCACA	60	Primer 3
IL-4	5′-CAGTTCCACAGGCACAAG 3′-CTGGTTGGCTTCCTTCACA	60	Primer 3
IL-10	5′-CTTTAAGGGTTACCTGGGTTG 3′-CTTGATGTCTGGGTCTTGG	60	Primer 3
MMP-13	5′-TCACGATGGCATTGCT 3′-GCCGGTGTAGGTGTAGA	60	Primer 3
TIMP-1	5′-CCGACCTCGTCATCAG 3′-GTTGTGGGACCTGTGGAA	60	Primer 3
TIMP-3	5′-CCTTGGCTCGGGCTCATC 3′-GGATCACGATGTCGGAGTTG	60	Primer 3
TIMP-4	TIMP-4 was purchased from Qiagen (Hilden, Germany; cat no. PPH00889E)		
HAS-1	5′-TGGTGCTTCTCTCGCTCTACG 3′-GAACTTGGCAGGCAGGAGG	60	[17]
HAS-2	5′-AAATGGGATGAATTCTTTGTTTATG 3′-GGCGGATGCACAGTAAGGAA	60	[17]
HAS-3	5′-CAGCTGATCCAGGCAATCGT 3′-TGGCTGACCGGATTTCCTC	60	[17]

Measurement of HA and TIMP-1 levels

HA and TIMP-1 in synovial fibroblast culture supernatants were evaluated using commercial DuoSet ELISA kit (R&D Systems) following the manufacturer's instructions. The three molecular weight forms of hyaluronan (low 15–40 kDa, medium75–350 kDa, high >950 kDa) were all detected in this assay.

A six-point standard curve using threefold serial dilutions and high standards (HA: 90 000 ng/mL and TIMP-1: 70 ng/mL) were performed and run in replicates (coefficient of variation average 18%). The accuracy of the method was assessed by evaluating the agreement between the expected and measured values by Bland-Altman plot (all differences between repeated measures and expected values did not exceed 95 % confidence interval). Reliability of the test was estimated by Cronbach's alpha coefficient (>0.99).

A four-parameter logistic curve-fit based on standard optic density values was used to calculate hyaluronan and TIMP-1 concentrations considering three decimals.

Statistical analysis

Data obtained by soluble factor dosages presented a skewed distribution that did not fulfill the hypothesis of normality; thus, appropriate $\log_{10}$ transformations were applied. All resulting data fulfilled the normality assumption as verified by the Kolmogorov-Smirnov test.

Results were analyzed by the general linear model (GLM). The GLM was used according to incubation times (1 and 18 hours; 2, 3 and 7 days), preparation types (PPP, P-PRP and L-PPP) and their association as fixed effects and subjects as a random effect. Partial eta squared (η^2_p) was considered evidence of the strength of the association (effect size) between the fixed effects and concentrations of soluble factors. The Sidak correction was applied for multiple comparisons.

Results obtained by gene expression analysis and assessment of IL-1β, IL-8, HA and TIMP-1 production in culture supernatants were analyzed by Friedman's test for multiple comparison of paired data, and, when significant, Dunn's post hoc test was subsequently carried out.

Data were expressed as medians, interquartile ranges, minimum/maximum values, and a P value <0.05 was considered significant. Statistical analysis was carried out using SPSS v.19.0 (IBM Corp., Armonk, NY, USA).

Results

Platelet and WBC concentrations

Platelets and WBCs were not detectable in PPP (not shown). Platelets were significantly more enriched in L-PRP than in P-PRP (P< 0.005), whereas WBC, nearly absent in the P-PRP preparation, showed concentrations similar to the peripheral blood ones in L-PRP (Table 2).

Analysis of soluble factor release kinetics

Sixteen cytokines/chemokines (IL-1β, IL-4, IL-6, IL-10, IL-17α, IL-22, MIP-1α/CCL-3, RANTES/CCL-5, MCP-3/CCL-7, Gro-α/CXCL-1, PF-4/CXCL-4, ENA-78/CXCL-5, NAP-2/CXCL-7, IL-8/CXCL-8, Fractalkine/CX3CL-1 and s-CD40L) were quantified at different time points (from 1 h up to 7 days). Of these, 11 biomolecules (IL-1β, IL-6, MIP-1α/CCL-

Table 2
Platelet and WBC concentrations in PRP preparations.

Preparations	Platelets × 103/μL Median [interquartile ranges]	WBC × 103/μL Median [interquartile ranges]
P-PRP	178 [100–343]	0.1 [0.1–0.2]
L-PRP	912 [713–995]	5.5 [4.3 – 7.5]
	$P < 0.005$	$P < 0.005$

P values determined by Wilcoxon matched pair test.

3, RANTES/CCL-5, Gro-α/CXCL-1, PF-4/CXCL-4, ENA-78/CXCL-5, NAP-2/CXCL-7, IL-8/CXCL-8, Fractalkine/CX3CL-1 and sCD40L) were detectable from the initial incubation time; IL-10 was barely detectable (on average ≈50% of subjects did not release the cytokine after 7 days), and 4 biomolecules (IL-4, IL-17α, IL-22 and MCP-3) were almost undetectable (data not shown).

GLM analysis of the concentrations of detected biomolecules (Table 3) showed variations in all release kinetics with time (P = 0.014, at least) excluded IL-10 and PF-4/CXCL-4, whereas preparation significantly influenced the concentration of all detected factors (at least P = 0.043) (Table 3). In addition, the combined analysis of time and preparation type–associated parameters (reported in the Table 3 as Time*Preparation) evidenced the influence of them in IL-10, RANTES/CCL-5, PF-4/CXCL-4, ENA-78/CXCL-5, Fractalkine/CX3CL-1 and sCD40L (at least P = 0.019) (Table 3).

Inflammatory IL-1β, IL-6 (Figure 1), IL-8/CXCL-8, Gro-α/CXCL-1 and ENA-78/CXCL-5 (Figure 2) biomolecules were more concentrated in L-PRP than in P-PRP (at least $P < 0.005$) and PPP ($P < 0.0005$). P-PRP and PPP concentrations were similar except for ENA-78/CXCL-5, which showed increasing concentrations from PPP to P-PRP and L-PRP (at least $P < 0.01$). The release of these molecules presented similar trends, which were sustained over a period of 7 days' incubation, and the concentrations obtained in the first hour of incubation were lower than all the subsequent concentrations (at least $P < 0.005$) (Figure 1 and 2).

In addition, IL-1β and IL-6 concentrations detected after 18 h were lower than the 7-day concentrations ($P < 0.05$ and $P < 0.001$, respectively) (Figure 1). IL-8/CXCL-8, Gro-α/CXCL-1 and ENA-78/CXCL-5 time-related increase was progressive for almost all consecutive times (at least $P < 0.05$) (Figure 2).

PF-4/CXCL-4 showed progressively increasing concentrations from PPP to P-PRP and L-PRP ($P < 0.0005$) (Figure 3), whereas NAP-2/CXCL-7 was similarly concentrated in L-PRP and P-PRP, and both were greater than in PPP ($P < 0.001$ at least) (Figure 3). Fractalkine/CX3CL-1 (Figure 3) was more concentrated in L-PRP compared with PPP but not P-PRP. PF-4/CXCL-4 and NAP-2/CXCL-7 releases were almost stable. Fractalkine/CX3CL-1 (Figure 3) showed an initial burst release decreasing as the incubation time extended up to 7 days (at least $P < 0.01$).

MIP-1α/CCL-3 was more concentrated in L-PRP than in P-PRP and PPP ($P < 0.01$), whereas P-PRP and PPP concentrations were similar (Figure 4). In contrast, RANTES/CCL-5 concentrations were similar in L-PRP and P-PRP, and both were more concentrated than in PPP ($P < 0.0005$) (Figure 4). MIP-1α/CCL-3 and RANTES/CCL-5 (Figure 4) releases were sustained over a period of 7 days' incubation, and the concentrations obtained at the first hour of incubation were lower than those at all the subsequent times (at least $P < 0.005$). In addition, RANTES/CCL-5 progressively increased from the 18 h of incubation up to 7 days ($P < 0.0005$). sCD40L (Figure 5) showed increasing concentrations from PPP to P-PRP and L-PRP ($P < 0.0005$) and an initial burst release that progressively decreased as the incubation time extended (at least $P < 0.02$).

Finally, IL-10 (Figure 5), the only anti-inflammatory factor released among the explored molecules, was more concentrated in L-PRP ($P < 0.02$) than in the other formulations.

The concentrations of soluble molecules, normalized to 100 000 platelets, generally showed a similar trend to that displayed in the previous GLM analysis (Supplementary Table 1). For the same number of platelets, L-PRP had a higher release capacity than P-PRP for IL-1β, IL-8, sCD40L, ENA-78 and Fractalkine, which reached statistical significance at different incubation times (Supplementary Table 1). In contrast, RANTES and NAP-2 were released more from P-PRP. The other biomolecules showed similar concentrations in P-PRP and L-PRP after normalization (Supplementary Table 1).

Table 3
Biomolecules: GLM analysis was used according to incubation times, preparations and their association.

Biomolecules	Effects	*P* value	η_p^2
IL-1	Time	0.001	0.509
	Preparation	0.000	0.778
	Time*preparation	0.063	0.223
IL-6	Time	0.000	0.611
	Preparation	0.000	0.462
	Time*preparation	0.708	0.088
IL-10	Time	0.253	0.174
	Preparation	0.043	0.189
	Time*preparation	0.019	0.268
MIP-1α/CCL-3	Time	0.000	0.534
	Preparation	0.010	0.263
	Time*preparation	0.848	0.067
RANTES/CCL-5	Time	0.000	0.902
	Preparation	0.000	0.696
	Time*preparation	0.000	0.396
Gro-α/CXCL-1	Time	0.000	0.702
	Preparation	0.000	0.425
	Time*preparation	0.264	0.156
PF4/CXCL-4	Time	0.297	0.178
	Preparation	0.000	0.882
	Time*preparation	0.012	0.309
ENA 78/CXCL-5	Time	0.000	0.657
	Preparation	0.000	0.650
	Time*preparation	0.019	0.276
NAP 2/CXCL-7	Time	0.014	0.360
	Preparation	0.000	0.483
	Time*preparation	0.466	0.122
IL-8/CXCL-8	Time	0.000	0.892
	Preparation	0.000	0.655
	Time*preparation	0.063	0.222
Fractalkine/CX3CL-1	Time	0.005	0.414
	Preparation	0.005	0.299
	Time*preparation	0.001	0.368
sCD40L	Time	0.000	0.761
	Preparation	0.000	0.880
	Time*preparation	0.007	0.303

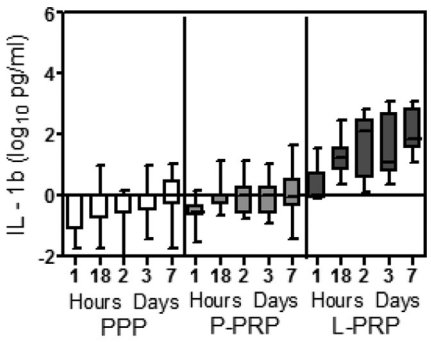
Gene expression and protein analysis of factors involved in joint physiopathology

IL-1β and IL-8/CXCL8 gene expression were significantly enhanced in cultured synovial fibroblasts by L-PRP compared with both P-PRP and PPP ($P < 0.05$). Conversely, no difference among preparations was found in IL-6 and IL-10, and the IL-4 gene expression was not detectable (Table 4).

Expression of MMP-13 (one of the most important matrix-degrading enzymes), as well as the tissue inhibitors of degrading matrix enzymes (TIMP-3 and TIMP-4), were similarly induced by the three preparations. In contrast, TIMP-1 expression was significantly increased by L-PRP incubation. Finally, no effect of the different PRP preparations was observed on HA production or on gene expression of its synthases (Table 4).

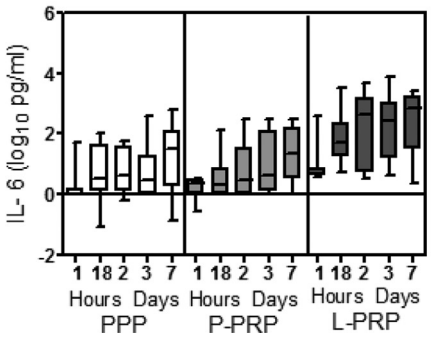
Protein analysis was also performed for IL-1β, IL-8 and TIMP-1, which displayed significantly modulated gene expression (Table 5).

IL-1β production in sinoviocyte culture supernatants was not modulated by the various PRPs. In contrast, IL-8 production was similarly induced by PPP and P-PRP but significantly up-regulated by



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	<0.005	<0.001	<0.0005	<0.0005
18 hour		ns	ns	<0.05
2 days			ns	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.0005



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	<0.001	<0.0005	<0.0005	<0.0005
18 hour		ns	ns	<0.001
2 days			ns	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.0005

Figure 1. Concentration of IL-1β and IL-6 in PPP, P-PRP and L-PRP. Results are reported as medians (bar), 25th and 75th percentiles (boxes), minimum to maximum values (whiskers). GLM statistical analysis is summarized in Table 3, whereas comparisons between incubation times and between preparations are reported in tables on the right side of each graph. ns, not significant.

L-PRP compared with both PPP and P-PRP ($P< 0.02$). TIMP-1 production was modulated in all culture conditions (at least $P< 0.05$).

Discussion

Besides the classical catabolic/pro-inflammatory molecules (IL-1β, IL-6, IL-8) [19,20], most of the soluble factor we studied, such as chemokines, are less explored platelet-released molecules. They are involved in several physiopathological responses of joint tissues [21-24], and, similar to the preceding interleukins, they are more concentrated in L-PRP preparations.

Previously published results on IL-1β do not appear substantially different from ours, in both the wide variability among the three analyzed subjects and the concentrations within the range we obtained up to 7 days [25]. However, the evaluation methods were different (enzyme-linked immunosorbent assay versus multiplex dosage on beads). The multiplex test has a different dynamic range and a superior sensibility, which may allow reliable IL-1β evaluation from samples of lower concentrations.

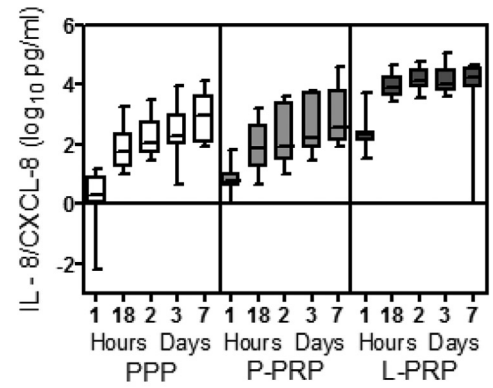
IL-6, a pleiotropic cytokine described both as a pro- and anti-inflammatory mediator [26], was present at a concentration on average 25-fold lower in L-PRP preparation than in osteoarthritis synovial fluids [27]; therefore, IL-6 contribution whether pro- or anti-catabolic, would probably be negligible. GRO-α shares with IL-8 and NAP-2 the homologous properties of activating neutrophils [22,28,29]. GRO-α was more concentrated in L-PRP, probably contributing to this effect, whereas the stable elevated release of NAP-2 was similar to the stable concentration of HA in the culture supernatant and to the expression of its synthases. Fractalkine has been described as promoting the induction of MMP-3 mRNA in osteoarthritis synovial fibroblast but not other MMP mRNA, including MMP-13 (involved in osteoarthritis progression together with MMP-3) [30]. Although we have not analyzed MMP-3 gene expression, FKN concentration in L-PRP [30] was probably not sufficient to induce MMP-3 gene expression. In agreement with other studies [30,31], RANTES did not demonstrate an inducing effect on MMP-13 in human synovial fibroblasts. Elevated ENA-78, PF4 and MIP-1α (chemotactic for inflammatory cell targets involved in bone remodeling and angiostatic response) in L-PRP could be involved in arthritis progression [28,32,33]. sCD40L is a potent immunomodulator [34,35], potentially contributing to the the inflammatory cascade, even if MMP-13 gene expression was not induced.

Finally, among anti-inflammatory cytokines analyzed, only IL-10 was detected in L-PRP fractions. IL-10 is an immunosuppressive chondroprotective cytokine, involved in collagen II and aggrecan synthesis and inhibiting the production of MMP [36,37]. Given the low concentration of IL-10, it is impossible to speculate on its effective anti-inflammatory contribution in this study.

The experimental evidence of *in toto* influence of P-PRP and L-PRP preparations was partially modified after normalization of the release concentrations to the number of platelets at some incubation times. The obvious difference of platelet number between preparations alone cannot explain these data. Rather than absolute platelet concentration, the key point seems to be the optimal relative combination of PRP components (platelets, leukocytes, fibrin and growth factors) that should be tailored to the targeted pathology [38,39]. Indeed, therapeutic effects stem from multiple, synergistic and combined biological responses of all platelet concentrate components [38,39]. In addition, it is possible that many clinical uses do not foresee the normalization to a fixed number of platelets; therefore, the number of factors potentially available after injection mirrors the "not normalized" condition of our study.

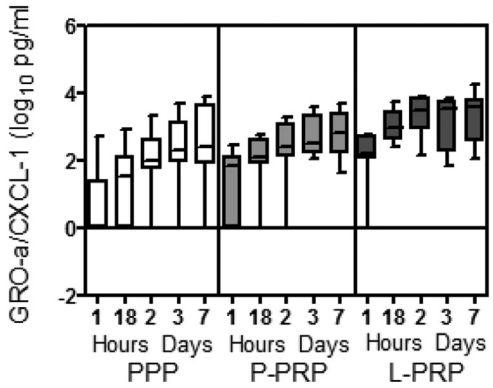
In agreement with published data [18], our study demonstrates that L-PRP is able to sustain long-term up-regulation of IL-1β and IL-8/CXCL8 gene expression levels in osteoarthritis synovial fibroblasts compared with P-PRP and PPP. The up-regulation of these genes may be due to the IL-1β-induced up-modulation of its own and IL-8 production; however, the contribution of other synergistic molecules, such as PDGF and TGF-β, cannot be excluded [33,40,41].

Despite the pro-inflammatory impulse, L-PRP preparation did not alter gene expression of specific factors playing a pivotal role in joint tissue remodeling. In particular, neither gene expression of MMP-13 (one of the most important matrix-degrading enzymes strongly involved in cartilage matrix breakdown in osteoarthritis) [41] nor



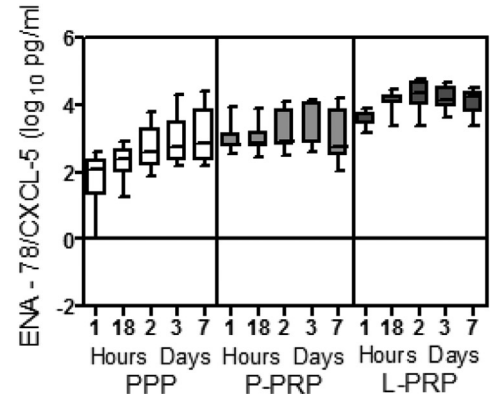
	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	<0.0005	<0.0005	<0.0005	<0.0005
18 hour		<0.0005	<0.0005	<0.001
2 days			ns	<0.05
3 days				ns

Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.0005



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	<0.00′	<0.0005	<0.0005	<0.0005
18 hour		<0.0005	<0.0005	<0.0005
2 days			ns	<0.030
3 days				ns

Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.005



	Time			
Time	18 hours	2 days	3 days	7 days
′ hour	<0.00′	<0.0005	<0.0005	<0.0005
′8 hour		<0.0005	<0.0005	<0.002
2 days			ns	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	<0.01
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.0005

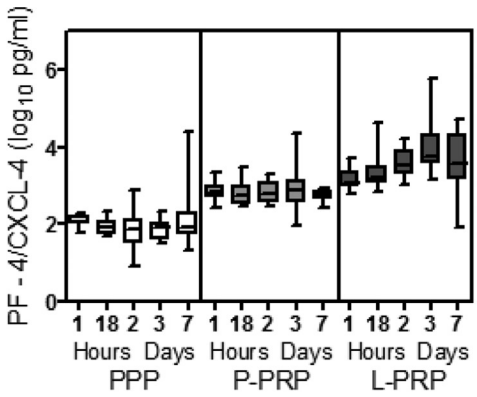
Figure 2. Concentration of IL-8, Gro-α and ENA-78 in PPP, P-PRP and L-PRP. Results are reported as medians (bar), 25th and 75th percentiles (boxes), minimum to maximum values (whiskers). GLM statistical analysis is summarized in Table 3, whereas comparisons between incubation times and between preparations are reported in tables on the right side of each graph. ns, not significant.

tissue inhibitors of matrix metalloproteinases TIMP-3 and TIMP-4 were triggered by inflammatory stimuli, in agreement with previously reported data [42]. In addition, the L-PRP–induced TIMP-1 gene expression in synovial fibroblasts could be of particular interest given the ability of TIMP-1 in attenuating ECM degradation [43]. Data showed that TIMP-1 and IL-8 but not IL-1β were differently modulated by P-PRP and L-PRP also at the protein level. The presence of leukocytes in the PRP was able to induce both pro-inflammatory (IL-8) and anti-inflammatory (TIMP-1) biomolecules, supporting the relevance of catabolic–anabolic balance in promoting the resolution of the inflammatory phase and the switching to the regenerative phase.

The lack up-regulation of MMP in synovial fibroblasts, even if exposed to L-PRP inflammatory factors, suggests that PRP use may be not associated with increased cartilage and extracellular matrix catabolism. Indeed, a preliminary study in a group of osteoarthritis patients demonstrated that the presence of leukocytes in PRP preparation injected in knee joints did not up-modulate synovial fluid concentration of classical pro-inflammatory cytokines (IL-1β, IL-6 and IL-8) [26], highlighting once again the complex interplay among several pro- and anti-inflammatory molecules that contribute to the final effect on the target tissues.

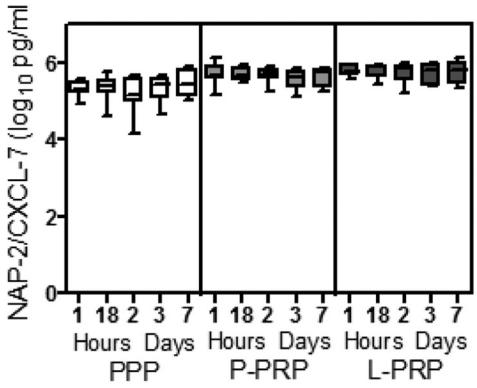
We can speculate that the respective amount of the different chemokines (which *in vitro* indicate relatively rigid patterns of target cell selectivity) [34] could remodel intra-articular trafficking response depending on the different inflammatory status of the disease.

Finally, no modification on the expression of the three HAS transmembrane isoforms and on HA production by synovial fibroblasts was observed. HA is an important component of cartilage extracellular matrix and synovial fluid production, relevant for joint homeostasis and L-PRP seems not to interfere with its synthesis. Similar catabolic pathways were promoted in chondrocyte cultures, although MMP13 expression was not induced, also HA and lubricin production were not down-modulated [44]. Analogous data on MMP-13 were reported on tendon explants [45].



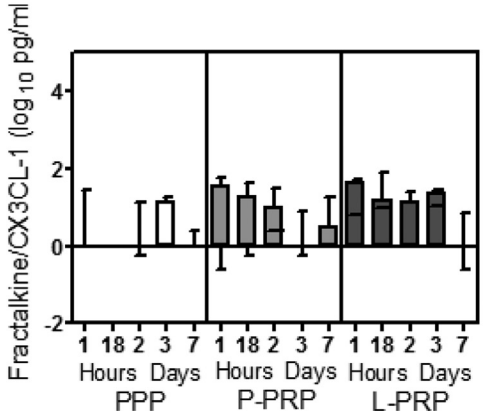
	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	ns	ns	<0.05	ns
18 hour		ns	ns	ns
2 days			ns	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	<0.0005
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.0005



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	ns	ns	ns	ns
18 hour		<0.005	ns	ns
2 days			ns	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	<0.001
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	ns



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	ns	ns	ns	<0.01
18 hour		ns	ns	<0.002
2 days			ns	ns
3 days				<0.005

Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	<0.001
P-PRP Vs L-PRP	ns

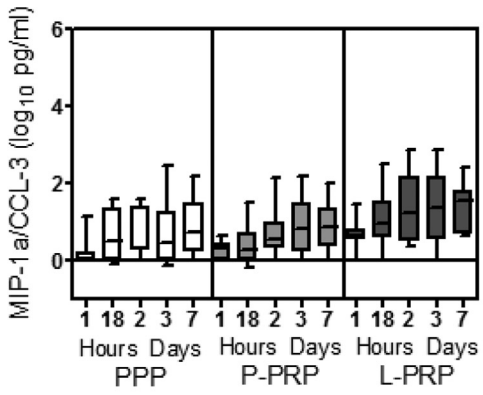
Figure 3. Concentration of PF-4, NAP-2 and Fractalkine in PPP, P-PRP and L-PRP. Results are reported as medians (bar), 25th and 75th percentiles (boxes), minimum to maximum values (whiskers). GLM statistical analysis is summarized in Table 3, whereas comparisons between incubation times and between preparations are reported in tables on the right side of each graph. ns, not significant.

The inclusion of leukocytes in platelet concentrate preparations remains a widely debated concern [18,45-48,22] given that both beneficial and detrimental effects have been suggested. Leukocyte presence (three times more concentrated than in peripheral blood) within PRP was reported to spontaneously induce a pro-inflammatory environment [47]. However, the reported amount of soluble factors is oversized compared with, for example, the joint inflammatory response occurring in osteoarthritis patients [27].

It must be considered that in our PRP preparation, leukocyte concentration was not highly enriched, mirroring the number normally present in the peripheral blood. PRP preparations [13] contain multiple mediators with concentrations that can vary depending on manifold factors, and it is therefore difficult to foretell whether the overall effect of PRP treatment will be catabolic or anabolic *in vivo*. As a consequence, leukocyte-rich preparations are not univocally described as good or bad: leukocytes in PRP may be important to increase the antibacterial effect [49,50] and immunological resistance [51]; on the other hand, the presence of neutrophils, in particular, can be significantly associated with catabolic cytokines and the consequent negative influence on tissue healing [32].

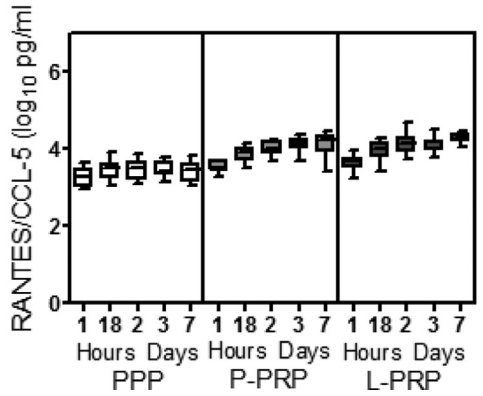
Clinical studies have indicated that a positive or negative effect of leukocytes cannot be generalized and that the type of PRP should be matched to the specific field of application [5,6,52]. In addition, even if both pain and swelling reactions were more frequently reported in the L-PRP treated patients as minor adverse event [53], we cannot exclude that the symptoms may reflect the effect of a cell boost capable of reactivating endogenous resolution programs and the biosynthesis of specialized pro-resolving mediators in non resolving inflammatory diseases [54].

The present study does, however, have some limitations. First, the effect of different activators was not measured, and the influence of the different protocols cannot be ignored because the release kinetics of biomolecules could be greatly affected by the activation



	Time			
T me	18 hours	2 days	3 days	7 days
1 hour	<0.005	<0.0005	<0.0005	<0.0005
18 hour		ns	ns	ns
2 days			ns	ns
3 days				ns

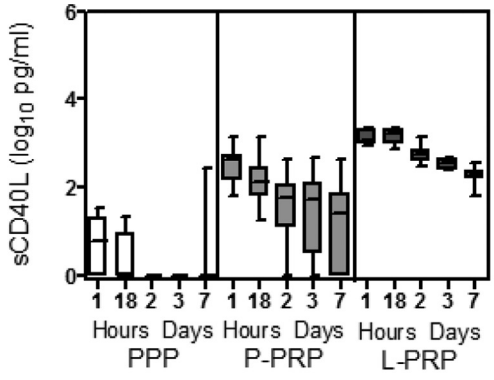
Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	<0.01
P-PRP Vs L-PRP	<0.01



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	<0.0005	<0.0005	<0.0005	<0.0005
18 hour		<0.0005	<0.0005	<0.0005
2 days			ns	ns
3 days				ns

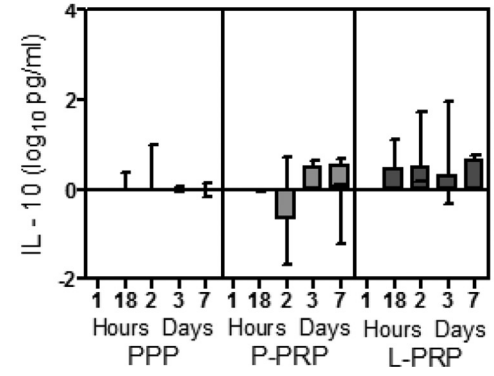
Preparation	p-value
PPP Vs P-PRP	<0.0005
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	ns

Figure 4. Concentration of MIP-1α and RANTES in PPP, P-PRP and L-PRP. Results are reported as medians (bar), 25th and 75th percentiles (boxes), minimum to maximum values (whiskers). GLM statistical analysis is summarized in Table 3, whereas comparisons between incubation times and between preparations are reported in tables on the right side of each graph. ns, not significant.



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	<0.005	<0.0005	<0.0005	<0.0005
18 hour		<0.0005	<0.0005	<0.0005
2 days			<0.02	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	<0.0005
PPP Vs L-PRP	<0.0005
P-PRP Vs L-PRP	<0.0005



	Time			
Time	18 hours	2 days	3 days	7 days
1 hour	ns	ns	ns	ns
18 hour		ns	ns	ns
2 days			ns	ns
3 days				ns

Preparation	p-value
PPP Vs P-PRP	ns
PPP Vs L-PRP	ns
P-PRP Vs _-PRP	<0.02

Figure 5. Concentration of sCD40L and IL-10 in PPP, P-PRP and L-PRP. Results are reported as medians (bar), 25th and 75th percentiles (boxes), minimum to maximum values (whiskers). GLM statistical analysis is summarized in Table 3, whereas comparisons between incubation times and between preparations are reported in tables on the right side of each graph. ns, not significant.

Table 4
Gene expression of pro- and anti-inflammatory cytokines, matrix degrading enzyme, their inhibitors and HA synthesis.

Factors	PPP	P-PRP	L-PRP	P
IL-1 beta	2574.00 (25.81–5292.00)	37.00 (14.32–3516.00)	2758 (464.50–32988.00)	P-PRP vs. L–PRP $P < 0.05$[a]
IL-6	982.00 (300.20–7536.00)	232.20 (100.40–2739.00)	4737.00 (825.80–6337.00)	NS[b]
IL-8	2628.00 (2062.00–23005.00)	6037.00 (292.00–11188.00)	23488.00 (13679.00–100000.00)	P-PRP vs. L-PRP $P < 0.05$[a]
IL-10	4.99 (1.93–56.48)	3.31 (0.91–39.3.02)	8.07 (3.77–16.61)	NS[b]
MMP-13	2.69 (1.66–108.40)	9.19 (0.06–25.45)	9.25 (0.24–71.99)	NS[b]
TIMP-1	23326 (9539.00–70222.00)	32760 (9605.00–145397.00)	81225 (44442.00–123971.00)	P-PRP vs. L-PRP $P < 0.05$[a]
TIMP-3	6.23 (0.85–10.48)	1.13 (0.26–8.22)	0.26 (0.14–3.05)	NS[b]
TIMP-4	7.41 (4.72–12.73)	5.90 (3.65–63.54)	3.45 (1.72–13.36)	NS[b]
HAS-1	159.70 (46.84–718.90)	66.70 (15.24–315.10)	166.50 (92.39–342.40)	NS[b]
HAS-2	369.60 (30.69–1105.00)	74.01 (35.25–298.10)	639 (119.40–2646.00)	NS[b]
HAS-3	155.60 (56.56–697.80)	74.06 (34.34–379.70)	112 (51.78–575.90)	NS[b]
HA	827.00 (232.40–1393.00)	261.90 (112.60–1971.00)	986.20 (548.50–2545.00)	NS[b]

Results are reported as median (interquartile range) of no. mol RNA × 100 000 GAPDH for all factors except HA,0 which is reported as median and (interquartile range) of ng/10^6 cells.
[a] Dunn's post hoc test.
[b] Friedman test for paired data multiple comparisons.

Table 5
Protein analysis.

Factors	PPP	P-PRP	L-PRP	*P* value
IL-1 beta	0.35 (0.03–0.56)	0.42 (0.00–1.44)	0.00 (0.00–122.2)	NS[b]
IL-8	31.33 (2.98–33.70)	35.34 (30.70–39.14)	48.17 (38.24–52.44)	P-PRP vs. L-PRP $P < 0.02$[a]
TIMP-1	434.16 (390.92–676.72)	751.04 (428.56–877.52)	2213.44 (1357.28–2590.16)	P-PRP vs. L-PRP $P < 0.05$[a]

Results are reported as median (interquartile range) of ng/10^5 cells. IL-1β and IL-8 were determined by multiplex-beads immunoassay and TIMP-1 by enzyme-linked immunosorbent assay.
[a] Dunn's post hoc test.
[b] Friedman test for paired data multiple comparisons.

procedures. Recently, Cavallo *et al.* [55] showed that different activation modalities can largely influence releasate concentrations and kinetics obtained from PRP clot ($CaCl_2$ inducing a gradual accumulation of factors, contrary to thrombin-inducing an immediate and stable boost). Second, the inter-individual variability is still a concern since the release kinetics of the biomolecules is also influenced by the population differences. Third, the biomolecule release in *in vitro* studies measure the total accumulation over time in a solution and do not account for the denaturation, proteolysis or the biomolecule uptake by synovial fibroblasts. Under *in vivo* conditions, tissues' degradative processes can make biomolecule release differently available due to a multitude of complex interactions among tissue components.

In conclusion, this study demonstrated that (i) the selected biomolecules had time-dependent variations in their release with kinetics and concentrations mostly increasing up to 7 days of incubation; (ii) the release dynamic was largely biomolecule-dependent, as also reported by other authors [56] for TGF-β1, B-FGF and IGF-I; (iii) most of the detected biomolecules (including the most important inflammatory ones) were more concentrated in L-PRP than in P-PRP, and for five biomolecules, the higher concentration in L-PRP was evident also when releasates were normalized to platelet number; (iv) the optimal relative combination of PRP components is relevant and (v) the pro-inflammatory boost of L-PRP preparation did not alter gene expression of specific factors playing a pivotal role in joint tissue remodeling in synovial fibroblast cultures and was able to induce anti-inflammatory (TIMP-1) biomolecules.

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Declaration of Competing Interest

GF received institutional support and support from Fin-Ceramica Faenza SpA, Fidia Farmaceutici SpA, CartiHeal (2009) Ltd., EON Medica SRL, IGEA Clinical Biophysics, Biomet Inc. EK received support from CartiHeal (2009) Ltd.; Fin-Ceramica Faenza SpA; FidiaFarmaceutici SpA, IGEA Clinical Biophysics, Biomet Inc., and KenseyNash Corp. The other authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Study conception and design: EM, GF; analysis and interpretation of the data: EM, LP, EA; Acquisition of data: LC, LP, EA; provision of study materials after patient selection: AR, AC, GF, GSK; obtaining funding: EK, EM. All authors were involved in drafting the article or revising it critically for important intellectual content and approved the final version.

Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.02.006.

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