

## ORIGINAL ARTICLE

## Growth-promoting action and growth factor release by different platelet derivatives

F. Passaretti<sup>1,2</sup>, M. Tia<sup>3</sup>, V. D'Esposito<sup>1</sup>, M. De Pascale<sup>1,2</sup>, M. Del Corso<sup>4</sup>, R. Sepulveres<sup>1</sup>, D. Liguoro<sup>5</sup>, R. Valentino<sup>5</sup>, F. Beguinot<sup>1,5</sup>, P. Formisano<sup>1,5</sup>, & G. Sammartino<sup>3</sup>

<sup>1</sup>Department of Translational Medical Sciences, Federico II University of Naples, Naples, Italy, <sup>2</sup>Department of Pharmaceutical and Biomedical Sciences, University of Salerno, Salerno, Italy, <sup>3</sup>Department of Neurosciences, Reproductive and Odonto-stomatological Sciences, Federico II University of Naples, Naples, Italy, <sup>4</sup>Academy of Non-Hemotransfusional Blood Components, Turin, Italy, and <sup>5</sup>Institute of Experimental Endocrinology and Oncology, National Council of Research (CNR), Naples, Italy

## Abstract

Platelet derivatives are commonly used in wound healing and tissue regeneration. Different procedures of platelet preparation may differentially affect growth factor release and cell growth. Preparation of platelet-rich fibrin (PRF) is accompanied by release of growth factors, including platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF) and transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), and several cytokines. When compared with the standard procedure for platelet-rich plasma (PRP), PRF released 2-fold less PDGF, but >15-fold and >2-fold VEGF and TGF $\beta$ 1, respectively. Also, the release of several cytokines (IL-4, IL-6, IL-8, IL-10, IFN $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$  and TNF $\alpha$ ) was significantly increased in PRF-conditioned medium (CM), compared to PRP-CM. Incubation of both human skin fibroblasts and human umbilical vein endothelial cells (HUVECs) with PRF-derived membrane (mPRF) or with PRF-CM enhanced cell proliferation by >2-fold ( $p < 0.05$ ). Interestingly, PRP elicited fibroblast growth at a higher extent compared to PRF. At variance, PRF effect on HUVEC growth was significantly greater than that of PRP, consistent with a higher concentration of VEGF in the PRF-CM. Thus, the procedure of PRP preparation leads to a larger release of PDGF, as a possible result of platelet degranulation, while PRF enhances the release of proangiogenic factors.

## Keywords

Cytokine, endothelial cell, fibroblast, growth factor

## History

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## Introduction

Platelet products have been extensively used for clinical and surgical applications which require tissue regeneration [1]. Thus, platelet derivatives represent promising therapeutic tools offering opportunities for periodontal, oral, maxillo-facial, orthopedic and dermatological procedures [2, 3]. Indeed, platelets represent a known source of cytokines and growth factors involved in wound healing and tissue repair [4, 5]. Many platelet-derived factors are considered important players in wound healing processes. In particular, beside their known functions in hemostasis and clot formation, platelet granules contain growth factors, including platelet-derived growth factor (PDGF), transforming growth factor  $\beta$  (TGF $\beta$ ), IGF-1, involved in cell proliferation and differentiation [6]. It should also be considered that, among platelet-released factors, no individual growth factor has proven *per se* effectiveness both in soft and in hard tissue regeneration [7]. Therefore, most probably, the mixture of more platelet-derived factors contained in platelet releasates, could be responsible for the tissue-regeneration potential of platelet derivatives [8]. There is also a large body of evidence that the cross-talk between factors released by platelets and those released by recipient cells mediates the propagation of the tissue repair

mechanisms [9]. Indeed, many cell types, including blood cells, fibroblasts and endothelial cells participate to the final healing process and each cell type may specifically affect the function of the other cell types, both by cell-cell and cell-matrix contacts and by producing and releasing soluble factors [10]. Thus, understanding the complex mechanisms regulating tissue repair and regeneration, is still incomplete.

Platelet-rich plasma (PRP) has long been used as a source of platelet growth factors [11]. Several different products have been developed in the last years [6]. All available PRP procedures have some points in common. A first centrifugation step is needed to separate red blood cells (RBC), buffy coat and platelet-poor plasma (PPP). Then, different procedures have been used to discard RBC and PPP and to collect the buffy coat. The platelets could be eventually activated by thrombin and calcium and applied to the injured/surgical site [12]. More recently, an alternative approach has also been adopted. It requires blood collection in tubes without anticoagulant and immediate centrifugation for the formation of a fibrin clot, which includes platelets and leucocytes (L-PRF) [13].

Leucocytes are also a significant source of cytokines and growth factors which may synergistically interact with those released by platelets [14]. However, whether the combined use of platelets- and leucocytes-derived products is beneficial still represents a matter of controversy.

Here, we have compared the effect of two different procedures on the release of cytokines and growth factors and on the ability to induce the growth of fibroblasts and endothelial cells.

Correspondence: Prof. Pietro Formisano, Dipartimento di Scienze Mediche Traslazionali, Federico II University of Naples, Via Pansini 5, 80131 Naples, Italy. Tel: +39 0817464450. Fax: +39 0817464334. E-mail: [fpietro@unina.it](mailto:fpietro@unina.it)

## Methods

### Subject recruitment and preparation of biomaterials

Fourteen healthy blood donors (M/F:6/8; age 24–40 years) were enrolled in the study. All were non-smokers, non-obese (BMI range: 20.4–26.3) and with a platelet count  $>180,000/\text{mm}^3$ . None of them was under any medication for the last 21 days. Informed consent was obtained from every subject before blood drawing. The protocol has been approved by the Ethical Committee of the University of Naples.

Blood was drawn from each individual and two 9-ml aliquots were obtained. One aliquot was collected in tubes without anticoagulant for the preparation of leukocyte- and platelet-rich fibrin (L-PRF). The other was collected in a vacutainer tube (Vacutainer; Becton Dickinson, East Rutherford, NJ) containing 10% trisodium citrate anticoagulant solution for the preparation of PRP.

L-PRF was prepared through a single 12-min step of centrifugation of whole blood (PRF production kit, Process, Nice, France) according to manufacturer's instruction. Each 9 ml tube produced one L-PRF clot. Where indicated, the L-PRF clot was separated from the RBC base and condensed through sterile gauzes in order to obtain a membrane-like structure (mPRF) [13].

For PRP preparation, the whole blood was initially centrifuged at  $350 \times g$  for 15 min. The supernatant was transferred into another tube and a second centrifugation step was performed for 10 min at  $980 \times g$ . After centrifugation, the upper fraction containing PPP was discarded and the lower fraction containing PRP was used for the experimental procedures [9, 15, 16]. For platelet gel preparations, autologous thrombin (0.1 NIH unit/ml final concentration) and calcium gluconate (10 mg/ml final concentration) were added to PRP for 5 min at room temperature to allow clot formation.

Conditioned media (CM) were obtained by incubating the platelet preparations for 24 hours with serum-free Dulbecco's modified Eagle medium (DMEM)-F12 (1:1). 0.25% BSA was added to the medium in order to prevent osmotic cell death. After the incubation, the medium was collected and centrifuged at  $14,000 \times g$  to remove cellular debris and analyzed for cytokines and growth factor content or placed onto recipient cells for different times, as described below.

### Determination of cytokines and growth factors released by platelet-based biomaterials

PRP, PRF and mPRF CM were screened for the concentration of IL-2, IL-4, IL-6, IL-8, IL-10, GM-CSF, IFN $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES, TNF $\alpha$ , bFGF, PDGF, vascular endothelial growth factor (VEGF) using the Bioplex Multiplex human cytokine assay kit and the Bioplex Multiplex human growth factor kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions.

### Cell culture and growth

Skin fibroblasts were obtained by punch biopsy and cultures established as described previously [17]. The cells were grown at 37 °C in DMEM supplemented with 10% fetal calf serum in a 5% CO $_2$ -95% air-humidified atmosphere. For the experimental procedures, the cultures were used between the 8th and 15th passage, and, for each individual experiment, cells were maintained in culture for an equal number of generations. Primary human umbilical vein endothelial cells (HUVECs) were obtained and cultured as previously described. HUVECs were cultured under 37 °C and 5% CO $_2$ -95% air-humidified atmosphere in the endothelial cell medium (ECM, ScienCell) according to the manufacturer's instruction. The ECM was consisted of 500 ml of basal medium, 25 ml of fetal bovine serum, 5 ml of endothelial

cell growth supplement and 5 ml of penicillin/streptomycin solution. For all experiments HUVEC up to passage five were used [18].

For cell growth determination, the studies were performed as previously described [19]. Briefly, either skin fibroblasts or HUVEC cells were seeded in 6-well culture plates in a complete medium. The following day, the cells were starved in serum-free DMEM 0.25% BSA for 16 hours and incubated with either platelet preparations or CM obtained as described above for different times. Cell count was performed by both Bürker chamber counting and the TC10™ Automated Cell Counter (Bio-Rad, Hercules, CA) according to the manufacturer's instruction.

### Statistical analysis

Data were analyzed with Statview software (Abacus concepts) by one-factor analysis of variance. *p* values of  $<0.05$  were considered statistically significant.

## Results

### Release of cytokines and growth factors by PRF

We have first evaluated the ability of PRF, obtained as described in "Methods" section to release cytokines/chemokines. To this end, PRF has been allowed to release factors into serum-free medium for 24 hours. PRF released detectable amounts of IL-2, IL-4, IL-6, IL-8, IL-10, IFN $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , RANTES and TNF $\alpha$  (Table I). Similarly, detectable levels of bFGF, PDGF, VEGF and TGF $\beta$ 1 were found in PRF-CM (Table II).

### Comparison of PRF- and PRP-released factors

Next we have compared the release of cytokines/chemokines and growth factors by PRF (as membrane, mPRF – see "Methods" section) and PRP (as platelet gel). The use of mPRF was preferred to that of PRF since, when the latter was incubated in serum-free

Table I. Cytokines released by PRF. PRF was incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex cytokine assay kit as described in "Methods" section.

	Concentration (pg/ml)
IL-2	5.39 $\pm$ 0.67
IL-4	9.63 $\pm$ 0.77
IL-6	13423.71 $\pm$ 3192.81
IL-8	51497.47 $\pm$ 7724
IL-10	38.57 $\pm$ 3.20
IFN $\gamma$	539.74 $\pm$ 89
MIP-1 $\alpha$	641.21 $\pm$ 70
MIP-1 $\beta$	595.16 $\pm$ 58
RANTES	1837.97 $\pm$ 190
TNF $\alpha$	85.54 $\pm$ 7.90

Table II. Growth factors released by PRF. PRF was incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex growth factor assay kit as described in "Methods" section.

	Concentration (pg/ml)
bFGF	7.66 $\pm$ 0.65
PDGF	2189.09 $\pm$ 225
VEGF	1376.3 $\pm$ 129
TGF $\beta$ 1	265667.50 $\pm$ 39851.60

medium, many cells (mainly RBC) and cellular debris were found floating, possibly interfering with the evaluation of the function of PRF-released factors. PRF and PRP were obtained by equal amounts of blood drawn by the same individual. mPRF and PRP gels were then applied onto culture dishes and incubated with serum-free medium for 24 hours to obtain CM. The amount of several inflammatory cytokines such as IL-6, IL-8, IL-10, IFN $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF $\alpha$ , was higher in the mPRF-CM compared to PRP-CM. At variance, the levels of RANTES were  $\sim$ 3-fold higher in PRP-CM compared to mPRF-CM (Table III). Concerning the concentration of growth factors, the levels of bFGF were detectable in small amounts compared to the other growth factors both in mPRF-CM and in PRP-CM (Table IV). VEGF and TGF $\beta$ 1 levels were 11- and 2.6-fold higher, respectively, in mPRF-CM compared to PRP-CM. Instead, the amount of PDGF was  $\sim$ 2-fold lower in mPRF-CM compared to PRP-CM (Table IV).

### Induction of cell growth by PRF- and PRP-released factors

In order to address whether PRF and PRP may have different effects on different cell types, we tested the ability of those preparations to induce the growth of primary cultures of human fibroblasts and vascular endothelial cells (HUVEC). To this end, PRP gel and mPRF were directly applied onto the culture plate containing either skin fibroblasts or endothelial cells. Indeed, clinical applications of PRF and PRP often require fibroblast proliferation and angiogenesis [1, 11]. Interestingly, PRP gel and mPRF-induced proliferation of both cell types. However, PRP was significantly more effective than mPRF in inducing fibroblast growth (Figure 1). Very similar results were also obtained with mesenchymal stem cells obtained from human subcutaneous

Table III. Cytokines released by mPRF and PRP. mPRF and PRP (as platelet gel) were incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex cytokine assay kit as described in ‘‘Methods’’ section.

	mPRF (pg/ml)	PRP (pg/ml)
IL-2	3.05 $\pm$ 0.45	2.34 $\pm$ 0.30
IL-4	10.56 $\pm$ 0.1	1.78 $\pm$ 0.43***
IL-6	10093.55 $\pm$ 1064.03	6.20 $\pm$ 0.55**
IL-8	43407.34 $\pm$ 5011	1303.01 $\pm$ 126***
IL-10	79.01 $\pm$ 7.9#	3.91 $\pm$ 0.42***
IFN $\gamma$	484.04 $\pm$ 57.6	37.30 $\pm$ 3.40*
MIP-1 $\alpha$	479.56 $\pm$ 28.6	0.25 $\pm$ 0.03**
MIP-1 $\beta$	822.18 $\pm$ 82.2#	6.90 $\pm$ 0.57***
RANTES	2818.57 $\pm$ 281#	5505.29 $\pm$ 530*
TNF $\alpha$	73.55 $\pm$ 5.3	2.21 $\pm$ 0.35*

\*denotes statistically significant differences (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001). # denotes statistically significant differences of mPRF vs. PRF conditioned media (# $p$  < 0.05).

Table IV. Growth factors released by mPRF and PRP. mPRF and PRP (as platelet gel) were incubated with serum-free DMEM-F12 (1:1). After 24 hours, the media were collected and tested by using the Bioplex multiplex growth factor assay kit as described in ‘‘Methods’’ section.

	mPRF (pg/ml)	PRP (pg/ml)
bFGF	7.29 $\pm$ 0.80	1.40 $\pm$ 0.14**
PDGF	2225.69 $\pm$ 333.85	4708.19 $\pm$ 601.89*
VEGF	2330.89 $\pm$ 233#	125.48 $\pm$ 12.5***
TGF $\beta$ 1	261886 $\pm$ 9282.9	102817.60 $\pm$ 15422.64*

\*denotes statistically significant differences of PRP vs. mPRF conditioned media (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001). # denotes statistically significant differences of mPRF vs. PRF conditioned media (# $p$  < 0.05).

adipose tissue (data not shown). At variance, mPRF effect on proliferation of endothelial cells was slightly higher than that of PRP, suggesting a prevalent pro-angiogenic function (Figure 2).

In order to verify if released factors rather than the particulate fraction of mPRF and PRP were responsible for the growth promoting action, mPRF-CM and PRP-CM were obtained as previously described and added onto cultured human fibroblasts for 24 hours. Again, both mPRF- and PRP-released factors induced fibroblast growth with PRP significantly more effective than mPRF in inducing fibroblast growth (Figure 3). Next, we have evaluated the effect of mPRF-CM and PRP-CM on the growth of endothelial cells. Interestingly, mPRF induced growth of HUVEC to a significantly greater extent compared to PRP releasate (Figure 4).

### Discussion

It is well established that platelet derivatives may play a key role in soft and hard tissue regeneration and in enhancing hemostasis in patients receiving anti-coagulating agents [1, 3, 8, 11, 13, 20–22]. Nevertheless, many procedures have been used to obtain platelet factors alone or in combination with other factors eventually derived from white blood cells or circulating stem cells [12, 23]. Much less is known about the biological activity of

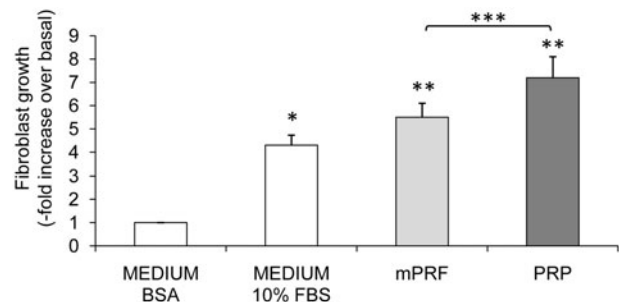


Figure 1. Effect of mPRF and PRP on fibroblast cell growth. mPRF and PRP gels were directly applied for 24 hours onto the culture plates containing serum-starved skin fibroblasts, previously obtained by punch biopsy ( $n$  = 10). As a control, fibroblasts have been incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS). The cells were then counted as described in ‘‘Methods’’ section. Results are presented as fold-increase over basal (cell count in MEDIUM BSA). \* denotes statistically significant values (\* $p$  < 0.05; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001).

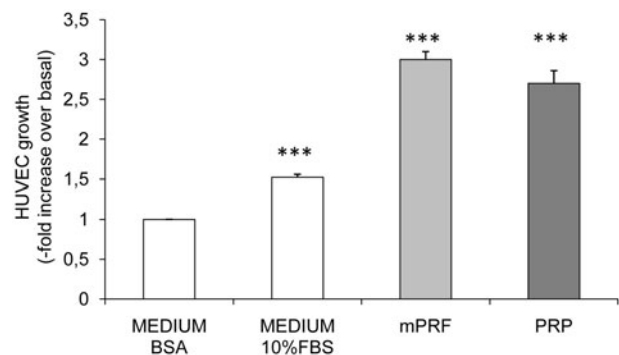


Figure 2. Effect of mPRF and PRP on endothelial cell growth. mPRF and PRP gels were directly applied onto the culture plates containing serum-starved primary human umbilical vein endothelial cells (HUVEC) for 24 hours. As a control, endothelial cells were incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS). The cells were then counted as described in ‘‘Methods’’ section. Results are presented as fold-increase over basal (cell count in MEDIUM BSA). \* denotes statistically significant values (\*\*\* $p$  < 0.001).



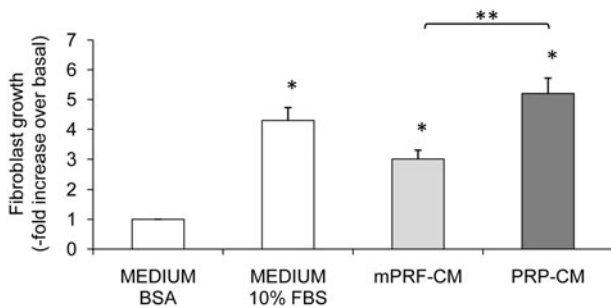


Figure 3. Effect of mPRF- and PRP-released factors on fibroblast cell growth. mPRF and PRP (as platelet gel) were incubated with serum-free DMEM F12 (1:1). After 24 hours, media were collected and added to serum-starved skin fibroblasts for 24 hours ( $n=10$ ). As a control, fibroblasts were incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS). The cells were then counted as described in ‘‘Methods’’ section. Results are presented as fold-increase over basal (cell count in MEDIUM BSA). \* denotes statistically significant values ( $*p<0.05$ ;  $**p<0.01$ ).

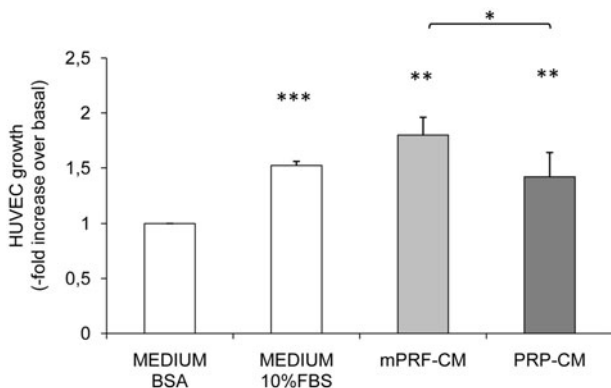


Figure 4. Effect of mPRF- and PRP-released factors on endothelial cell growth. mPRF and PRP (as platelet gel) were incubated with serum-free DMEM F12 (1:1). After 24 hours, media were collected and added for 24 hours to serum-starved HUVEC cells. As a control, HUVEC cells were incubated with DMEM F12 (1:1) without serum supplementation (MEDIUM BSA) or with 10% fetal bovine serum (MEDIUM 10% FBS). The cells were then counted as described in ‘‘Methods’’ section. Results are reported as fold-increase over basal (cell count in MEDIUM BSA). \* denotes statistically significant values ( $*p<0.05$ ;  $**p<0.01$ ;  $***p<0.001$ ).

the individual platelet preparations, in particular with the aim of defining their involvement in selected clinical applications.

In this work, we have addressed whether different procedures to obtain platelet products may lead to the release of a differential spectrum of molecules and may differentially control the growth of specific cell types. To this end, we have determined the concentration of several growth factors and cytokines/chemokines in the media exposed to either PRP gels or PRF membranes. Indeed, platelet gels were obtained by treating PRP with calcium and thrombin and PRF membranes were obtained by condensing PRF clots (see ‘‘Methods’’ section). Interestingly, while PRP released a significantly higher amount of PDGF and RANTES, the amount of several cytokines, typically involved in wound healing and re-vascularization, was more abundant in the release of PRF membranes. It is conceivable that the enrichment of platelets obtained in the standard procedure for PRP is responsible for the higher release of PDGF [9, 24]. Also, RANTES, a pro-inflammatory chemokine, is very abundant in platelets [25]. On the other end, the clot obtained by the PRF procedure is most

likely enriched in white blood cells, which may represent major producers of inflammatory cytokines (IL-6, IL-8, IL-10, IFN $\gamma$ , MIP-1 $\alpha$ , MIP-1 $\beta$ , TNF $\alpha$ ) and of pro-angiogenic factors (VEGF and TGF $\beta$ 1).

It should also be noticed that mPRF released higher levels of IL-10, MIP-1 $\beta$ , RANTES and VEGF, compared to PRF clot. The mechanism responsible for these events has not been completely explained, but it may involve activation of the cells embedded in the clot, following condensation and mechanical stress.

The differences in the release of specific cytokines and growth factors prompted us to investigate whether mPRF and PRP may have differential growth effect in fibroblasts and endothelial cells. According to previous reports, platelet derived products can increase cellular survival and proliferation [26–28]. Indeed, both preparation procedures were able to induce cell growth (either in fibroblast or in endothelial cells). Interestingly, however, direct application of PRP gel was significantly more effective in inducing fibroblast growth, compared to mPRF. Similarly, when PRP was enabled to release factors and CM added to fibroblasts, greater effectiveness than with mPRF CM was observed. On the other hand, growth of endothelial cells was slightly more effective following direct application of mPRF, compared to PRP, while, the effect of mPRF CM on HUVEC growth was significantly more pronounced than that of PRP CM.

These data indicate that different procedures to obtain platelet products display quantitative differences in the content of growth factors and cytokines. This may be relevant for the choice of the appropriate tool. For example, standard procedures for PRP, more efficiently release PDGF and RANTES, most likely because of the higher enrichment in platelets occurring through the preparation and, perhaps, following the activation with thrombin and calcium which facilitates platelet degranulation. On the other hand, PRF procedures allow a better yield of several growth factors (bFGF, VEGF, TGF $\beta$ 1) and cytokines, which derive from white blood cells and, possibly, circulating progenitor cells, which are embedded in the fibrin clot.

This is a relevant issue, since it may recommend the use of one or the other method, based on the specific goal to achieve. Indeed, if the expansion of connective tissue is mainly required, the use of a higher concentration of PDGF, to increase the number of the fibroblast component, is more indicated. Alternatively, if reduced angiogenesis is a major obstacle to tissue regeneration, one may choose preparations containing higher levels of bFGF and VEGF, thereby stimulating endothelial cell recruitment and vessel formation. In addition, procedures for PRF production enable the achievement of an higher concentration of several cytokines, which may play important roles in many events involved in the tissue regeneration process.

## Conclusion

We have reported that different procedures to obtain blood derivatives for regenerative medicine applications may yield different products. When platelet activation is achieved via thrombin stimulation, platelets release greater amounts of PDGF and other factors, mainly involved in fibroblast growth. At variance, when clot is directly and rapidly obtained without anticoagulation, the enrichment of VEGF and pro-angiogenic cytokines, possibly released by the embedded white blood cells, facilitates endothelial cell growth.

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## Declaration of interest

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