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Platelet-rich plasma (PRP) and platelet derivatives for topical therapy. What is true from the biologic view point?

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Background PubMed accessed on January 23 revealed 160 items about 'platelet gel or releasate' associated to topical therapy. Yahoo! provided 8580 items; Altavista 8650; Google 25 300. Companies providing blood separators are going to offer devices to prepare platelet-rich plasma (PRP) for topical therapy. Several devices are filling the marketplace aiming to produce platelet gels for human therapy. Never-ending lists of clinical conditions supposed to benefit from platelet gel application are published. Clinical benefits include bactericidal activity, pain reduction, tissue repair and regeneration. Are platelet derivatives the magic bullet for topical therapy?

Methods Many *in vitro* studies account for clinical benefit from platelet gel. Several *in vivo* studies provide clinical evidence about healing of tissue repair induced by platelet derivatives. Nevertheless, systematic reviews reveal inadequate studies providing enough methodological strength to confirm evidence-based efficacy. At present we must deal the subject with care using mostly inductive criteria. Only reproducible scientific data are to be considered. Every effort should be made for commercial, private and personal popularity-related scenarios to be rejected from our consideration. Sometimes, this is not so simple to be done.

Results There is a list of more than 60 biologically active platelet-derived factors directly involved in tissue repair mechanisms: chemotaxis, cell proliferation, angiogenesis, extracellular matrix deposition and remodelling. Biological functions are also indirectly mediated by platelet-derived growth factors; such functions are triggered by chemokines and cytokines produced by bystander cells such as fibroblasts, macrophages, endothelial cells, lymphocytes, under platelet-derived factor stimulation. All of this is well demonstrated. Clinical studies endorsed with stringent randomized controlled trials are lacking. However, several serious studies have been published reporting clinical efficacy of platelet derivatives in many clinical areas. Considering these papers seriously, we maintain that in most cases, clinical efficacy is by far more than just a suggestion.

Discussion Although we consider evidence-based medicine (EBM) highly meaningful, we emphasize that medicine moved forwards also before EBM was conceived. We do not consider platelet gel and releasate such as a 'magic bullet', but we are strongly impressed by the results our group, and other groups have obtained treating a variety of tissue lesions in a variety of clinical conditions. Clinical benefits are a composite result of the lesion state, severity and duration, coexisting pathologies, patient's age and product characteristics. From our point of view, the last is a pivotal variable that has strong influence over the clinical outcome. Platelet-rich plasma, platelet gel and platelet releasate need stringent definitions. Too many methods are used to prepare these products. Both methods and product need definition, validation, specific quality parameters and clinical indications. Further biologic and biochemical studies are needed as well to understand (if possible to modulate) the inner mechanisms of healing induced by topical treatment with platelet derivatives.

Introduction – a little bit of history

The popularity of platelet gel rose in the late 1990s after the publication (1998) of a paper about the usefulness of the platelet-rich plasma (PRP) for bone regeneration in dental care [1]. This paper was shortly preceded by the first published report (1997) about the use of platelet gel in maxillofacial surgery [2]. Besides dental care, one of the most interesting areas to employ the platelet-derived growth and chemotactic factors is wound healing. Because diabetes is by far one of the most critical area for difficult ulcers to be healed, diabetic ulcers were those lesions where platelet-derived factors where firstly challenged for [3] (1998). Where this idea was coming from?

The haemostatic and tissue repair property of fibrin clot and platelets is known since long ago. Nevertheless, scientists need evidence and experimental data to infer new hypotheses and rationale-based practical treatments. As far as we know, the first published paper conceptually relating to the platelet gel is dated 1975: 'use of platelet-fibrinogen-thrombin mixture as a corneal adhesive' [4]. A few years later (1979), a very exiting report was published about platelet gel (gel foam) used to obtain sutureless nerve anastomosis [5]. An elegant model showing the role of fibrin to drive fibroblast migration and collagen deposition leading to granulation tissue formation was published in 1980 [6]. In a rabbit cornea model, it was demonstrated (1982) that platelets and fibrin trigger a process necessary for tissue lesion to heal: cell migration, collagen synthesis, fibroplasia and angiogensis [7].

The clinical application of platelet gel or platelet releasate, such as the platelet derived wound healing formula (PDWHF) and the activated platelet supernatant CT-102® (Curative Technologies, Setauket, NY), begun to spread in the mid-1980s after the first report published by Knigthon *et al.* in 1986 [8–17]. During those years, new exciting experiences were carried out in different and quite distant clinical areas. The effect of the PDWHF, alone or associated to the nerve growth factor (NGF), on experimental axon lesions was studied in animal models [18,19]. The axonal growth-stimulating activity of NGF was more effective than that of PDWHF. Nevertheless, PDWHF was shown to be strongly neoangiogenetic, thus providing extracellular microenvironment highly favourable to NGF stimulatory action. It was also demonstrated that low amount of PDWHF is sufficient to induce a long-term healing acceleration, hence suggesting, for the first time, that platelet-derived growth factor (PDGF) may induce a positive autocrine feedback loop for new synthesis of growth factors from resident macrophages [20]. This was also suggested by clinical results in burn lesion treated with PDWHF [21].

Along with the clinical use, more basic experimental studies were carried out to understand the biology of the wound-healing process. This insight made possible new expectation about biotechnological composites, possibly including cell, growth factors and scaffolds. A pivotal study was published in 1996 on the relationship between mesenchymal cells, fibrin and growth factors to arrange the establishment of granulation tissue [22]. It was demonstrated that formation of the granulation tissue is strongly enhanced by platelet releasate. The platelet releasate provides continuous stimulation for mesenchymal-derived cells to interact with fibrin, thus accelerating the development of granulation tissue. In the absence of such stimulation, slower development of the granulation tissue was observed.

Another fundamental paper published in the late 1990s provided numerous insights on the basic mechanisms of the physiologic process of healing after injury have occurred [23]. Some of the results provided by this paper are the following: fibroblasts are recruited at the injury site by soluble factors; platelet releasate contains high level of chemotactic factors that recruit fibroblast in a strictly dose-dependent manner. Fibroblast migrates along fibrin-rich matrix; the presence of fibronectin is necessary for such migration to occur, with fibronectin providing a conduit for transmigration. In their migration, fibroblasts adhere to fibronectin fibres through $\alpha5\beta1$ and $\alpha5\beta3$ receptors. Proteases are necessary for fibroblast transmigration through fibrin-rich matrix (the role of metalloproteases was depicted some years later). Fibrin/fibronectin fibres retain growth factors released from platelets within the injured site. Nowadays, all of this sounds quite familiar but in the late 1990s, such complexity was quite new.

Are there clinical evidences to say that platelet gel really works?

During the last 1950s, many papers have been published maintaining that platelet gel or platelet releasate is effective in clinical and experimental settings, both in humans and in animals. A few papers maintained dubitative concern. From a scientific viewpoint, a vast majority of positive papers are not proofs for platelet gel to be considered fully effective (science is not democracy). Recently, evidence-based medicine (EBM) gained a major role for objective literature-based evaluation of the effectiveness of treatments or therapeutic strategies. It needs stringent criteria for clinical trials to be designed and carried out [24]. In conditions characterized by many important clinical variables, it is quite difficult if not impossible to design clinical trials that can meet the EBM acceptability criteria, e.g., number of patients, epidemiological stratification, randomization, blind evaluation of the results and so on. This is the case of the treatment with platelet gel. Among hundreds of published papers about the clinical use of the platelet derivatives, very few belong to the class of the prospective randomized clinical trials [10,11,18,25,26] or to the category of retrospective clinical trials (RCTs) [16,27–29]. The great majority belongs to the category of the case report or to that of the pilot study. In spite of this paucity of RCTs

we maintain that many of the published papers, taken together and skillfully analysed, support the evidence that platelet gel is clinically effective.

The application of platelet gel spam through many clinical settings including skin, bone, dental care, maxillofacial surgery, diabetic foot and diabetic leg, vascular and cardiac surgery, tympanic lesions, ocular and corneal lesions, nerve lesions, spinal fusion, skin lesion owed to congenital haemoglobin defects, burns, aesthetic surgery and lifting.

Most of these papers disclaim positive or encouraging results. Just few papers show clinical ineffectiveness of the platelet gel or releasate. In both cases – favourable and not – the results need some more discussion. Papers disclaiming dubious or null effectiveness belong to two categories. Some of them neither considered nor reported the platelet concentration or the enrichment of the platelet suspension used to prepare the platelet gel or the platelet releasate. In these few cases, it is likely that the platelet concentration was not high enough for the healing activity to be exerted [10,30,31]. The second category typically refers to the spinal fusion [32–34]. This singleness is quite intriguing. Nevertheless, it is likely that in spinal fusion – and the related diseases – some anatomical, pathophysiological or microenvironment peculiarities play a significant role impeding the healing action that platelet-derived factors provide.

Also, considering a limited reference list such that reported here, the articles claiming for the effectiveness of platelet gel are the great majority [1–5,9–18,21,22,25–29,35–56]. Some criticism is needed also in this case. In some instances, simple case reports are described. With some exceptions [11,16,18,25–29], case-control studies are lacking. Sometimes the platelet gel treatment is considered vs. placebo and not vs. gold standard treatment. It should be emphasized that in many cases the treatment was targeted to the diabetic lesions. In these case it is emphasized that platelet gel, to be effective, must be use in the context of a comprehensive treatment [8,9,11,13,16].

Considering the existing literature and our own experience, we are prone to maintain that platelet gel is effective in about 85% of the lesions. However, platelet gel must be used as adjunctive therapeutic means in the context of a comprehensive care of both the lesion and the patient. Generally speaking, our experience shows that the younger the patient, the faster the healing; acute lesions heal faster than chronic ones; complications and copathologies such as diabetes, vasculo-neuropathies, autoimmune disease and infections are prognostic negative factors in order to gain fast or complete remission. In our experience, a major advantage of platelet-gel-driven healing is the complete absence of keloid formation, as far as pathologic hyperplasia, metaplasia or dyschromia of the healed lesion. This very important evidence, which is very much appreciated by the patients, is rarely reported [8,39] and it is quite surprising that this effect was underscored through the current literature. Other advantages are comfort and pain diminution [38,39,51]; less oedema and ecchymosis [38,41,44,45]. Particularly valuable advantages are reported through literature: reduced mortality after cardiac surgery [29], reduced infection rate after cardiac and orthopaedic surgery [28,50,56], reduced transfusion supply and hospital stay after orthopaedic surgery [50,51,57], reduced amputation rate in diabetic foot [9,16].

All these data are strongly suggestive for a true effectiveness of platelet gel and platelet releasate in many clinical conditions. If this is true, we must consider if and how the current knowledge about the platelet-derived factors and about the physiology of tissue healing can help us to find out rationale for the clinical use and quality assurance parameters for clinical grade products.

Are there biologic data supporting the evidence that platelet gel should work?

Knowledge about platelet factors that are topically delivered through the administration of platelet derivatives is growing with time. Nowadays, such knowledge has reached a quite impressive magnitude. Albeit not fully comprehensive, an up-to-date survey on this knowledge is summarized in Table 1.

A large number of stimulatory effects as far as many regulatory functions are shown therein. Generally speaking, such functions are directed to cells, extracellular matrix, tissue organization. Most actions concern chemotaxis, cell adhesion, mitogenesis, proliferation, angiogenesis, antimicrobial defence. Enhancing and regulatory functions concern proliferation of mature cells and maturation or differentiation of precursor cells; extracellular matrix synthesis and remodelling; amplification or inhibitory loops; inflammation; immune response; signal transduction at the extracellular, cytoplasmatic and nuclear levels. All these functions are carried out by a single-factor activity, directly or indirectly, or by engendering autocrine and paracrine mediator production by resident and chemoattracted cells.

All these functions have been demonstrated through specifically designed *in vitro* experiments [14,58–73]. An extremely complex network of coordinately interacting functions occur *in vivo* [74,75]. So far, no instrument exists for such extreme complexity to be dissected into single (or simple) causal effects; just a global biologic effect can be observed, which is generated by the synergic activity of platelet-derived factors [4,6,17,18,20–22,26,28,36,40,41,46,49,76–78].

When preparing platelet concentrate, platelet gel, and platelet releasate, membrane debris and actively formed microparticles are generated. The platelet membrane-derived microparticles (PMMs) have intriguing biological properties. Aside from their procoagulant and thrombogenic properties, PMMs exhibit peculiar properties strictly related with tissue repair mechanisms. Transcellular signalling and PDGF-independent mitogenic activity associated with PMMs were

Table 1

Platelet factors	Tissue healing related functions[a]
PDGF-A, -AB, -B	Chemotactic, mitogenic, gene up-regulation
Connective tissue GF (CTGF) (CCN family)	Collagen synthesis, angiogenic
CTAP-3 connective tissue-activating protein-3	Chemotactic, mitogenic, glycosaminoglycan synthesis
basic-fibroblast GF	Wide-spectrum cell growth stimulator
Transforming GF-β1	Modulation (inhibition) of EGF, PDGF, bFGF
PF4	Chemotactic for inflammatory cells and fibroblasts; inhibits the migration of vascular endothelial cells
Platelet-derived angiogenic factor (PDAF)	Angiogenesis
Endothelial cell growth inhibitor	Function exerted by clived PF4
Early pregnancy factor (EPF)	Member of heat shock protein family; survival factor for many cells
Epithelial growth inhibitor (EGI)	Modulate (inhibit) epithelial cell growth
Keratinocyte growth factor (KGF)	Potent growth factor for keratinocyte proliferation
Angiopoietin-like-6 (ANGPTL6)	Epidermal cell proliferation
Insulin-like GF (IGF)	Mitogenic for mesodermal cells; stimulates matrix synthesis by bone cells
IGFBP-3	Negative regulator of cell growth
Transforming GF-β2	Modulation (inhibition) of EGF, PDGF, bFGF.
Estrogen receptor-related protein	Member of HSP27; pro-angiogenic dubitative function
VEGF	Mitogenenic for vascular endothelial cells; induces the synthesis of the metalloproteinase
Fibroblast-derived endothelial cell GF (f-ECGF)	Supports the growth of endothelial cells
Hepatocyte GF (HGF)	Migration and proliferation of endothelial cells; neoangiogenesis *in vitro* (3D gel cultures)
Histamine-releasing factors	Striking homology with CTAP-3 mitogenic
Human collagenase inhibitor	Modulation of collagenase and some MMPs
Fibronectin	Regulation of cell migration and differentiation
Platelet microbicidal protein-1 (PMP-1)	Antimicrobial kinocidin
Thrombin-induced platelet microbicidal protein-1 (t-PMP)	Antimicrobial kinocidin
Thrombocidin-1 (TC1)	Antibacterial protein
Thrombocidin-2 (TC2)	Antibacterial protein
Vitronectin	Prevention of thrombin degradation
Thrombospondin	Extracellular matrix component driving cell adhesion and migration
Serotonin	Chemotactic for neutrophils; vascular permeability
Cathepsin	IL8 converting enzyme; chemotactic for neutrophils
Platelet basic protein-PBP (CXCL7)	Antimicrobial kinocidin
Neutrophil-activating protein-2 and -4 (NAP-2; 4)	Chemotactic for neutrophils; induction of elastase release from neutrophils
Somatostatin (SST)	Modulator of immune activities
RANTES	Chemotactic for inflammatory cells; proliferation and activation of killer cells; kinocidin
CTAP-3	Mitogenetic: induces extracellular matrix synthesis in fibroblast cultures
Placental protein 14 (PP14)	Immune suppression
SCUBE1	Adhesive matrix-binding molecule; cell migration dubitative function
CCN family	At least six different molecules with different activities: stimulate mitosis, adhesion, cell death by apoptosis, extracellular matrix production, growth arrest and migration of multiple cell types; promote the integrated growth of the bone; promote or modulate angiogenic events
Annexin 11	Mediator of cell–cell adhesion
Heat shock protein 27 (HSP27)	Modulator of endothelial cell migration dubitative function
Heat shock protein 60 (HSP60)	Inducer of pro-inflammatory cytokine expression in macrophages
von Willebrand factor	Collagen binding protein sharing a domain with PDGF and with TGFβ
Albumin	Antioxidant; fatty acid delivery to cells; modulation of Zn^{++} and Cu^{++} concentration;
Immunoglobulins IgG, IgM, IgA	Immunocompetence
Coagulation factors V, VII, XI, XIII	Coagulation
Fibrinogen	Inducer of platelet-derived growth factors from activated platelets
Histamine	Modulation (inhibition) of keratinocyte proliferation

Table 1 *Continued*

Platelet factors	Tissue healing related functions[a]
ATP, ADP, GPT, GDP	Amplification of IL-1-mediated signals; purine receptor (P2) stimulation; stimulation of Ca^{++} efflux from cells; stimulation of osteoclast formation and bone remodeling; throphic action on nerve cells; stimulation of mesenchymal and haemopoietic stem cell proliferation; stimulation of extracellular signal-regulated kinase ERK; stimulation of the FGF-induced chondrocyte proliferation
Ca^{++} , Mg^{++} , Zn^{++}	Proactivator of protein and enzyme function
Enzymes	
Collagenase	Analogous to matrix metalloproteinases
ADAMTS-13	Metalloprotease multimer cliving
Superoxide dismutase (SOD)	Inhibitor of cycling and growth factor-induced responses
Heparinase	Procoagulant: heparin inhibitor
α1-α2 anti/trypsin	Protease inhibitor
α2-antiplasmin	Modulator of coagulation cascade
α2-macroglobulin	Inhibitor of proteases such as collagenase, elastase, plasmin, thrombin; inhibitor of autocrine TGF-β loop; α2-macroglobulin binds and transports cytokines such as IL1, IL2, IL6, PDGF, FGF, VEGF; in other instances the biological activity of bound cytokines are suppressed
C1-INH	Mediator of inflammatory response
Trace amounts enzymes: aldolase, carboxypeptidases, acid phosphatase, arylsulphatase, β-galactoidase, β-glucoronidase, β-glycerolphosphatase, α/β-glucosidases, α/β-fucosidases, α-mannosidase, α-arabinosidase.	Substrate specific enzymatic activity
Inducible factors	promotion
SIG, JE, KC (inducile genes)	Chemokine gene induced after cell activation; involved in inflammation and wound healing
Inducible BMP-2, -6, -7 (TGF-β superfamily)	Stimulation of cartilage and bone formation and neurone cell differentiation
Metalloprotease MMP-1, -2, -9, -13	Remodelling of collagenous extracellular matrix; facilitating tubulogenesis and angiogenesis; modulation of several chemokines; sustain tissue remodelling and wound healing
ECM remodelling factors	See: induced MMP, TNF-α, elastase
Autocrine ERK (ext. cell. reg. kinase)	
Autocrine and paracrine protein C (PC)	Supporting cell survival, growth and migration
Autocrine and paracrine lysophosphatidic acid (LPA)	Prolongs wound-induced growth-factor activity
HMGB1 (amphiregulin)	Nonhistone chromosomal architectural protein; it is induced by cell damage; signal transduction protein; extracellular HMGB1 induces angioblast migration and proliferation; required for neurite outgrowth and neuronal migration during development of the nervous system

[a]Most information from cope.cgi version 18·0, November 2006; http://www.copewithcytokines.de/

reported [79,80]. Platelet membrane-derived microparticles are chemoattractant for endothelial cells through vascular endothelial growth factor (VEGF) and PDGF and they display angiogenetic capacity through VEGF, b-FGF, and PDGF [81]. Other properties have been reported: PMMs can enhance cell–cell contact engaging cell-surface receptors simultaneously; they exchange and share membrane and cytosol proteins and receptors between different cells; they influence adherence, migration and tube formation of endothelial cells inducing an increase in ICAM-1 expression; they deliver metalloproteases, hence facilitating neovascular structure formation and cell invasion; they can transfer pathogens from the environment to phagocytes [69].

Several *in vitro* and *in vivo* evidences demonstrate that platelet derivatives and mesenchymal cells engage powerful co-operation accelerating cell proliferation and tissue forming and that combination of mesenchymal cells and platelet derivatives can be prepared and administrated contemporaneously achieving clinical effectiveness [22,26,37,49,52,54,63,65,82]. We have arranged a very simple method to prepare high-yield (50–70%) and high-concentration bone marrow-derived mesenchymal cells and to deliver them in combination with highly enriched (five to eight times) autologous PRP (manuscript in preparation). We obtained the improvement of mesenchymal cell expansion and differentiation in bone-forming cells by means of platelet-derived factors [83] (Fig. 1).

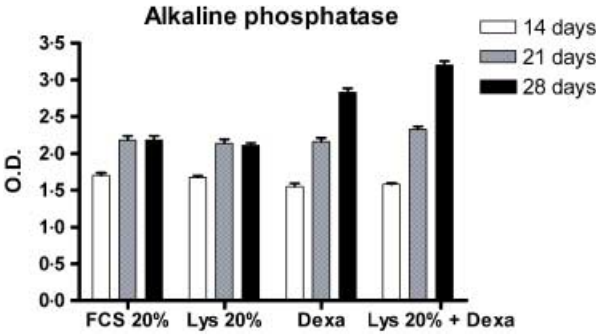

Fig. 1 Bone marrow-derived mesenchymal cells were cultured in medium supplemented with: FCS20%; platelet lysate (20%) produced by three freeze-thaw cycles of washed platelets at the concentration of 1×10^6/ml; dexamethasone 10 nM; platelet lysate 20% + dexamethasone 10 nM. Cultures were run up to 28 days. Alkaline phosphatize activity, a marker for osteogenic differentiation, was determined by colourimetric assay using pNPP as substrate.

We maintain that all this is very suggestive for platelet derivatives to play a significant biologic role in wound healing and tissue repair.

Of course, even if effective, platelet gel and derivatives can not be use such as a panacea. The platelet derivatives are anyway an adjuvant therapy to be used in a comprehensive treatment such as stated clearly in the case of diabetic patients [7,8,11,13,16,84].

Can technical weakness or trouble affect the clinical efficacy of platelet gel?

Besides reasoning about the biological mechanisms that sustain the clinical efficacy of platelet gel, one must consider that the technical aspect of the preparation of both the PRP and the gel are intrinsically and strictly related to its clinical effect.

Since our first trials we perceived poor clinical response after treating patients with scantly enriched PRP (platelet count less than 1×10^6/ml). Accordingly, since late 1999, we adjusted the platelet concentration of the PRP to $1–2 \times 10^6$/ml.

Several papers maintaining poor clinical effect of platelet gel did neither report the platelet count of the PRP or reported narrow platelet enrichment. On the contrary, most papers sustaining the effectiveness of the platelet gel reported good PRP enrichment: four- to eight-folds the basal count or more than 1×10^6/ml. The first rough conclusion is that to obtain clinical effectiveness a cut-off limit of approximately 1×10^6/ml must be adopted. This is a roughly mechanistic concept. In fact, one can argue that albeit not strictly proportional, the amount of platelet-derived factors delivered by platelet gel are somehow proportional to that of the platelets used to prepare it. Furthermore, one must consider the dose–effect relationship evidenced in many *in vitro* and *in vivo* studies about cell proliferation or recruitment.

Concentrated PRP may be produced within blood banks according to standard blood banking methods and criteria or through commercially available dedicated devices developed for the use at the point of care as well. Products prepared using different methods and devices cannot have overlapping properties. Heterogeneous production generates heterogeneous products which, in turn, have distinct biological properties and healing capacity [75,85–93]. Heterogeneity is conceived for platelet concentration; white blood cells or buffy-coat inclusion in the product; kind, age, storage conditions of the starting material; means of activation. It is quite obvious that these variables determine fundamental differences among the final products. Last but not the least, the impressive interindividual variability of the PDGFs must be stressed (we also argue the high interindividual variability of their receptors) such as demonstrated by some authors [87,93] and by ourselves (Fig. 2).

We stress here another variable that was poorly, if ever, investigated in this context: the 'activation' phase. In general, gel is formed providing Ca^{++} and thrombin to the PRP. In the United States, the use of bovine thrombin is allowed; in European countries and in Japan, bovine derivatives are still considered to bring unacceptable risks. For this reason, autologous thrombin is frequently used [47,94]. Thrombin cleaves fibrinogen, and polymerized fibrin produces the clot. Then clot retraction occurs: in this phase, platelet bearing thrombin receptors play a primary role. In this critical phase, activated platelet secretes the α-granule content. Thus, platelet-derived factors are released as well. It is quite obvious that such activation makes considerable amount of platelet-derived factors and MMPs promptly available for wound healing and tissue regeneration. Another scenario is that of batroxobin-based activation. Fibrin is similarly produced by batroxobin-mediated fibrinogen digestion. Nevertheless, clot retraction and platelet activation do not occur because platelets are batroxobin insensitive [95–97]. In this context, one can argue that most platelet-derived factors are retained within the platelet bodies and that they are released slower, making it available for the tissue to be healed for a longer time. It is likely that such differences play a significant clinical role. Unfortunately, no data are still available and new *in vitro* and *in vivo* studies are needed do evaluate the clinical impact of this variable.

Need for standardization?

Owing to the high heterogeneity of the products, a need for standardization is a commonly shared sentiment. Nevertheless, what we have shown in Table 1 is quite impressive; on this basis everyone is legitimated to think that, if this is the situation, standardization is a crazy idea. However, some kind of standard must be found because standards are basic instruments for science-based (and evidence-based) clinical trials to be carried

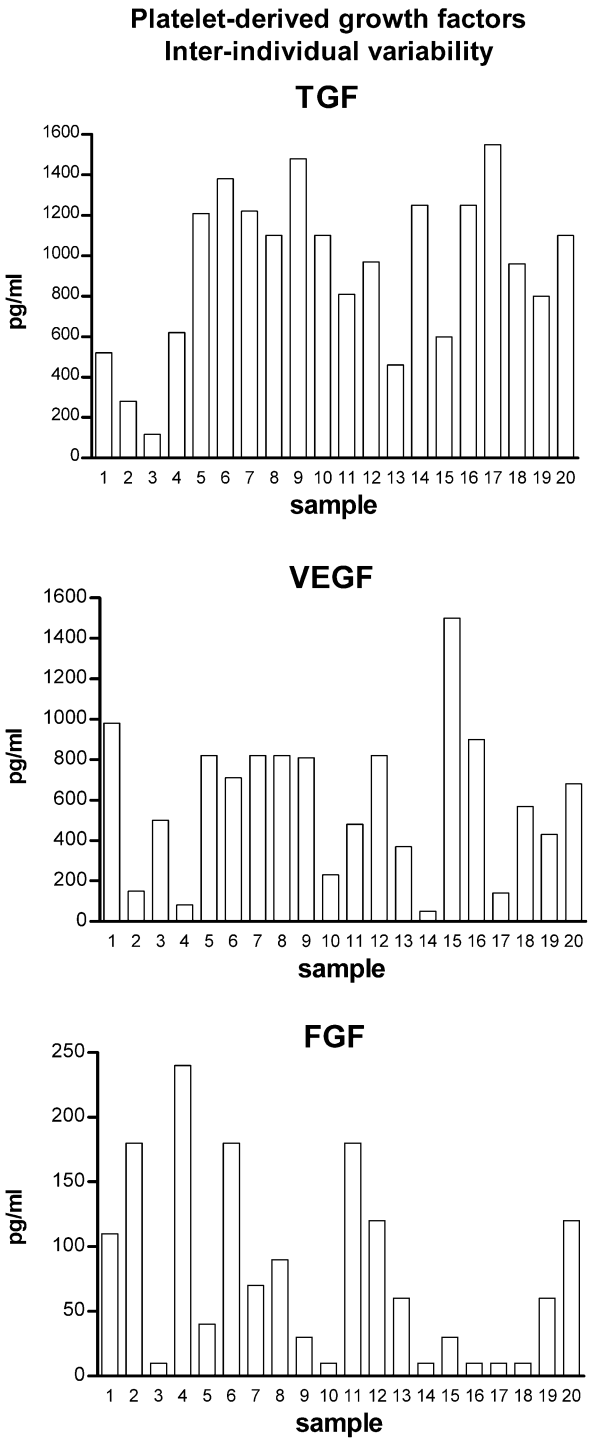

Fig. 2 Platelet concentrates were obtained from 20 blood donors. The platelet count of each sample was adjusted to 2×10^6/ml. TGF, VEGF, FGF were determined using commercial kit (Quantikine, R & D System, Minneapolis, USA) after Triton-x 100 solubilization (see ref. [86]).

out. In the absence of shared standards, a certificate of analysis for each delivered product is highly desirable, such that one we have proposed recently [98]. Finally, considering that heterogeneity involves products, clinical conditions, tissues to be cared, treatment protocols and probably many other minor variables, we strongly support the project for establishment of a clinical database necessary collect enough data to make it possible multivariate analysis of the clinical results [99].

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