Comparative Clinical Pathology
https://doi.org/10.1007/s00580-025-03674-x

RESEARCH

Growth factor concentration in canine platelet-rich plasma and platelet lysate is correlated with platelet number

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Received: 20 December 2024 / Accepted: 30 April 2025

Abstract

Platelet lysate (PL) and platelet-rich plasma (PRP) are blood derivatives that have gained increasing clinical relevance in both human and veterinary medicine in recent years. These derivatives exhibit anti-inflammatory and regenerative potential due to the presence of multiple growth factors (GFs) and cytokines stored in platelet cytoplasmic granules, which can be disrupted and harvested. Currently, there are no standardized protocols or specific consensus for the preparation and production of canine PRP and PL. This study aims to compare and correlate platelet concentration in various platelet concentrates (PCs) obtained through an in-house method, with the presence of three different GFs: vascular endothelial growth factor A (VEGF-A), transforming growth factor beta-1 (TGF-β1), and platelet-derived growth factor BB (PDGF-BB). Eight healthy adult dogs that met pre-established inclusion criteria were enrolled in the study, with assessments conducted at two time points, separated by a 4-month interval. Research was conducted in accordance with experimental ethics protocols established by the University. Blood samples were collected into EDTA and sodium citrate tubes in order to obtain whole blood samples as well as various plasma derivatives: plasma, leukocyte-reduced PRP, platelet-poor plasma, and PL. The presence and concentration of GFs were analyzed using canine-specific ELISA kits. Results show that TGF-β1 and PDGF-BB concentrations were significantly higher in PRP and PL samples. Furthermore, GF concentrations were higher in PL samples compared to PRP, suggesting that the in-house method employed may be able to increase both platelet and growth factor concentrations. In addition, a strong positive correlation ($r^2 = 0.7195$) was established between platelet concentration and TGF-β1 growth factor levels. The findings of this study suggest that the in-house method used is able to concentrate platelets and growth factors such as TGF-β1 and PDGF-BB in platelet-concentrated products like PRP and PL. In conclusion, platelet concentration may serve as a predictive parameter for estimating specific GF concentrations such as TGF-β1. Due to the fact that platelet counts are easily and rapidly obtainable, this parameter could direct the selection of GF-rich plasma samples to be used in interventions requiring enhanced bone regeneration and may also help to estimate the quantity of GFs delivered during the treatment.

Keywords Platelet lysate · Platelet-rich plasma · Growth factors · Platelet-derived growth factor · Transforming growth factor · Canine

Introduction

Platelet-rich plasma (PRP) and other platelet concentrates (PCs) have become increasingly prevalent in veterinary medicine over the past decades, with applications in the treatment of various medical conditions. Therapies based on regenerative medicine can help avoid extreme and radical procedures, such as amputations, and in challenging cases of non-union fractures or fractures with massive bone loss (i.e., bone osteolysis, cysts, or tumors) (Arnhold and Wenisch 2015;Franklin and Birdwhistell 2018).

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Published online: 08 May 2025

Autologous cancellous bone grafting has been considered the gold standard for treating large bone defects in veterinary small-animal orthopaedics and human medicine alike. However, this type of therapy has limitations due to the high morbidity associated with cancellous bone collection at the donor site and its limited availability (Akagi et al. 2015; Gens et al. 2023).

Tissue engineering and cumulative research have employed different biomaterials to enhance bone augmentation (Black et al. 2015), focusing on providing additional support to naturally occurring healing mechanisms through the addition of the essential functional elements for bone reconstructions: mineral scaffolds, building cells, and growth factors (GFs) (Gothard et al. 2014; Ho-Shui-Ling et al. 2018; Poldervaart et al. 2015).

Many of these GFs can be obtained from platelets through concentrated subproducts such as PRP, platelet lysate (PL), and other PCs (Wang et al. 2019a). PRPs and PLs are biological products rich in platelets at concentrations higher than physiological levels, directly implying a higher concentration of GFs in these subproducts, thus increasing clinical interest as treatments or supplements for various health disorders (Harrison 2018).

Platelets contain three types of granules, and when they degranulate, they release proteins, growth factors, and chemokines, which promote tissue regeneration (Pavlovic et al. 2016). PCs, such as PRPs and PLs, remain popular regenerative therapies or co-therapies in both human and veterinary medicine, mainly because of the infrequent secondary effects and the ease of preparation and in-house application. Nevertheless, several controversies persist. Some studies show that PCs are not effective when applied to canine joints in the management of cranial cruciate rupture ligament (Sample et al. 2018). Other studies indicate potential beneficial effects of PCs in canine osteoarthritis and for enhancing fracture healing in dogs when combined with mesenchymal stem cells (Lee et al. 2019; Sharun et al. 2021). Furthermore, some investigations have shown improvement in fracture healing through the application of PCs (Dehghan et al. 2015; López et al. 2019; Zhang et al. 2021).

These contradictory results may be due to the high variability in the preparation and functional definition of the different available PCs. Many procedures used for obtaining PCs have not been qualitatively or quantitatively assessed. Blood heterogeneity among donors may also contribute to the already mentioned diversity of final PC products, an issue that pool sampling may ameliorate when heterologous PCs are considered (Gilbertie et al., 2018; Franklin and Birdwhistell 2018; Sharun et al. 2021; Liebig et al. 2020). In conclusion, the quantity of final GFs in PC preparations can be highly variable, thus affecting their effectiveness after application.

The most important GFs contained in platelet concentrates are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), transforming growth factor beta-1 (TGF-β1), basic fibroblast growth factor (b-FGF), and insulin-like growth factor 1 (IGF-1) (Franklin et al. 2017; Sharun et al. 2021).

GFs would be addressed to solve bone regeneration problems such as bone defects, delayed unions, and nonunions. Bone regeneration is regulated by several growth factors, but it has been demonstrated that TGF-β1, bone morphogenetic protein (BMP), b-FGF, PDGF, and VEGF are the main ones promoting bone regeneration (Behr et al. 2010; Ehrhart et al. 2005; Hu and Olsen 2016; Kaigler et al. 2011; Poldervaart et al. 2015; Wang et al. 2019b; Yamamoto et al. 2000).

Although bone regulation is complex, TGF-β1 plays a major role in bone maintenance and repair upregulation. BMP, which plays a major role in the regulation of bone formation and osteoblast differentiation, is a signaling cytokine from the TGF-β superfamily (Rahman et al. 2015).

During bone formation, PDGF-BB, secreted by preosteoclasts, enhances bone formation by stimulating angiogenesis, particularly in CD31 Emen vessels, which subsequently promotes osteogenesis through increased vascular development. (Xie et al. 2014).

Throughout the bone repair process, VEGF plays a critical role in promoting macrophage recruitment in the first stage of bone healing (inflammation). Furthermore, it stimulates endothelial cells to induce angiogenesis, as well as osteoblast proliferation and differentiation, thus enhancing intramembranous ossification (Hu and Olsen 2016).

In vitro degranulation of platelets, also known as PRP activation, can lead to the release of growth factors from platelet granules, increasing the concentration of GFs in the releasate. PRP activation can be achieved through different chemical and physical processes that mimic the in vivo process (Franklin et al. 2017). Some compounds, such as $CaCl_2$ and thrombin (Franklin and Birdwhistell 2018), are also used to activate PRP but can induce PRP coagulation and rapid fibrin formation (Toyoda et al. 2018). Freezing/thawing cycles are a physical method used to activate platelets without the need of adding exogenous products (Franklin et al. 2017).

The main objective of this study is to quantitatively and qualitatively compare the presence of three GFs (TGF-β1, VEGF-A, and PDGF-BB), which play a critical role in bone healing, in two PC samples—PRP and PL—produced using an in-house method. Additionally, the study aims to investigate the correlation between platelet concentration and GF levels, evaluating the potential use of platelet count as a predictive parameter for estimating GF concentrations.

Materials and methods

Donors

This study was conducted between July and November 2023 at the animal and experimental facilities of the Autonomous University of Barcelona (UAB). Eight adult Beagle dogs were authorized as blood donors in accordance with the Animal Research Ethics Committee of the UAB (Authorization procedure number #5075).

The inclusion criteria for donors in this study required a complete health and immunization record, normal physical examination results, up-to-date deworming status, absence of systemic diseases, negative serology for Leishmania and Rickettsia, and no haematological abnormalities. Additionally, baseline platelet concentration within the standard range (Schneider and Mischke 2016) for whole blood was mandatory for inclusion in platelet level analysis.

Whole blood

The process to obtain the plasma (*P*) consisted in the collection of blood from eight canine donors at two different times (collections were carried out 4 months apart). Blood extraction was performed by a veterinarian using a jugular venipuncture technique with a butterfly catheter system and vacuum extraction (blood collection set safety lock 21 G—18 cm; BD Vacutainer. Franklin Lakes, USA). Seven sodium citrate tubes (buffered sodium citrate blood collection tubes 4.5 ml 1.29 M; BD Vacutainer. Franklin Lakes, USA) of 4.5 ml each were used, resulting in a total of 31.5 mL of blood collected from each canine donor. Additionally, 1 ml of blood per donor was collected and preserved in an EDTA tube (EDTA K3 1 ml tube Aquisel, Everest Tecnovet; Barcelona. Spain), which was rotated for 2 min to obtain the basal complete blood count (CBC).

PRP and PL

Blood samples stored in citrate tubes were processed within 40 to 50 min after collection to ensure sufficient interaction between platelets and citrate, thereby preventing aggregation. The technique described by Cowper et al. (2019) was modified to generate canine PLs. Sterile conditions were strictly maintained throughout sample processing by working under a laminar flow hood with sterile equipment and cleanroom garment protection. An initial soft spin (800 G; 5 min; automatic acceleration and braking with an approximate acceleration time of 30 s and braking time of 40 s; Rotofix 32 A centrifuge, 1624 swing bucket rotor, and 1369 bucket; Hettich Tuttlingen. Germany) was performed on all whole blood samples (7 tubes of 4.5 ml per donor) to separate *P* from red blood cells (RBCs) and leukocytes. The P was carefully aspirated while ensuring the buffy coat remained intact to minimize aspiration of white blood cell (WBC) contamination. The collected *P* was then transferred into a 10-ml Falcon tube. A second, hard centrifugation step (1500 G; 15 min; 20-s acceleration, 21-s braking; 5804 centrifuge and A-4–44 swing bucket rotor; Eppendorf, Hamburg, Germany) was performed to obtain a platelet pellet and platelet-poor plasma (PPP). The platelet pellet was resuspended in a calculated volume of autologous PPP to achieve a theoretical platelet concentration of 1×10^9 plt/µl, thus obtaining PRP. Additionally, a pooled PRP sample was prepared by mixing 0.1 ml of each donor's PRP sample collected during the second sampling time point.

After that, PRP was activated by disrupting platelets throughout thawing cycles in order to lyse them and obtain the PL. The first cycle of freezing was performed for 10 min at − 80 °C, followed by thawing at 37 °C for 7 min in a water bath. A second freezing cycle was conducted at − 80 °C for 15 min, followed by thawing at 37 °C for 7 min. The final freezing cycle involved freezing at − 80 °C for 10 min and thawing at 37 °C for 7 min. Finally, a 300 µl sample of each PL was individually stored in 0.5-ml Eppendorf tubes to analyze GF levels.

Hemogram analysis

Basal blood counts, including platelets, RBCs, and WBCs, were performed for each canine donor sample stored in EDTA tubes using an automated hematological analyzer (XN-1000, Sysmex. Kobe, Japan). During the PRP manufacturing process, basal blood counts were analyzed for *P* (*P* is obtained from whole blood samples through centrifugation in order to perform GFs analysis), PPP, and PRP samples. Also, six PL samples were also analyzed at the obtention time by the automated analyzer to determine the actual platelet concentration, as it could differ from the theoretical value previously calculated. However, due to insufficient sample volume, it was not possible to assess all PL samples for platelet concentration. These samples were processed within 10 min of obtention and maintained at room temperature (25 °C).

Growth factor analysis

GF analysis was performed immediately after PL obtention process. All samples were maintained at room temperature and analyzed within 40 to 150 min of obtention. The following kits were used according to manufacturers' instructions to measure GFs: PDGF-BB, (#ECPDGFB PDGF-BB Canine ELISA Kit; Invitrogen, Thermo Fisher ® Waltham, USA), VEGF-A (# EC18RB Canine VEGF-A ELISA Kit;

Invitrogen, Thermo Fisher ® Waltham, USA), and TGF-β, (#DB100 C Human/Mouse/Rat/Porcine/Canine TGF-beta 1 Quantikine ELISA; Bio-Techne R&D Systems ®). After that, data was collected and analyzed by Microsoft excel (Microsoft 365 MSO (2502 Build 16. 0. 18,526. 20,168 version, Redmont, Washington, USA)), and SigmaPlot 10.0. (Systat Software, Inc San Jose, CA, USA) software.

Statistics and data analysis

Tables and statistical tests were performed using Prism GraphPad software (*GraphPad Prism software 10.1.2 version Boston, USA. RRID:SCR_002798*) and "R" (*R project for statistical computing org; R 4.3.3 version. Vienna, Austria. RRID:SCR_001905*).

Statistical analysis for the correlation between platelet concentration and GFs levels was conducted using the Pearson correlation test. The significance level for differences in GF concentration between groups was analyzed through a Kruskal–Wallis test and was set at a 0.05 *p*-value ($\alpha = 0.05$).

Results

From the initial eight dogs sampled at two time points, two samples from different donors at the first time point were excluded from the platelet statistical analysis for not meeting the inclusion criteria for minimum platelet levels in the baseline hemogram. A pooled sample was added at the PRP stage, resulting in a total of 15 samples included in the platelet analysis (P $n = 14$; PPP $n = 14$; PRP $n = 15$; PL $n = 8$). The target platelet concentration was achieved in all PRP samples, and all 16 samples, along with the pooled sample, were included in the GFs analysis.

Mean platelet concentration in PRP was 3.87 ± 1.13-fold higher than that in whole blood (*P*), with a minimum increase of 1.96-fold and a maximum increase of 5.36-fold (Fig. 1).

Media platelet concentration was $165.5 \pm 41.12 \times 10^3$ plt/µl in P samples, $5.43 \pm 3.34 \times 10^3$ plt/µl in PPP, $613.43 \pm 168.78 \times 10^3$ plt/µl in PRP, and $528.71 \pm 117.76 \times 10^3$ plt/µl in PL. Comparative statistical analysis revealed significant differences between PRP and both P and PPP groups. However, no statistical difference was observed between PRP and PL. Notably, P demonstrated statistical significance when compared to PRP and PL. The PPP group showed significant differences with all other groups except when compared to P (Fig. 2).

WBC analyzed resulted in a decrease during the PRP obtention process (mean: whole blood $= 6.85 \pm 1.538 \times 10^3$/µl; P $= 0.056 \pm 0.035 \times 10^3$/µl; PPP $= 0.005 \pm 0.005 \times 10^3$/µl; PRP $0.347 \pm 0.296 \times 10^3$/µl) while RBC followed the same tendency (mean: whole blood $= 6.971 \pm 0.766$

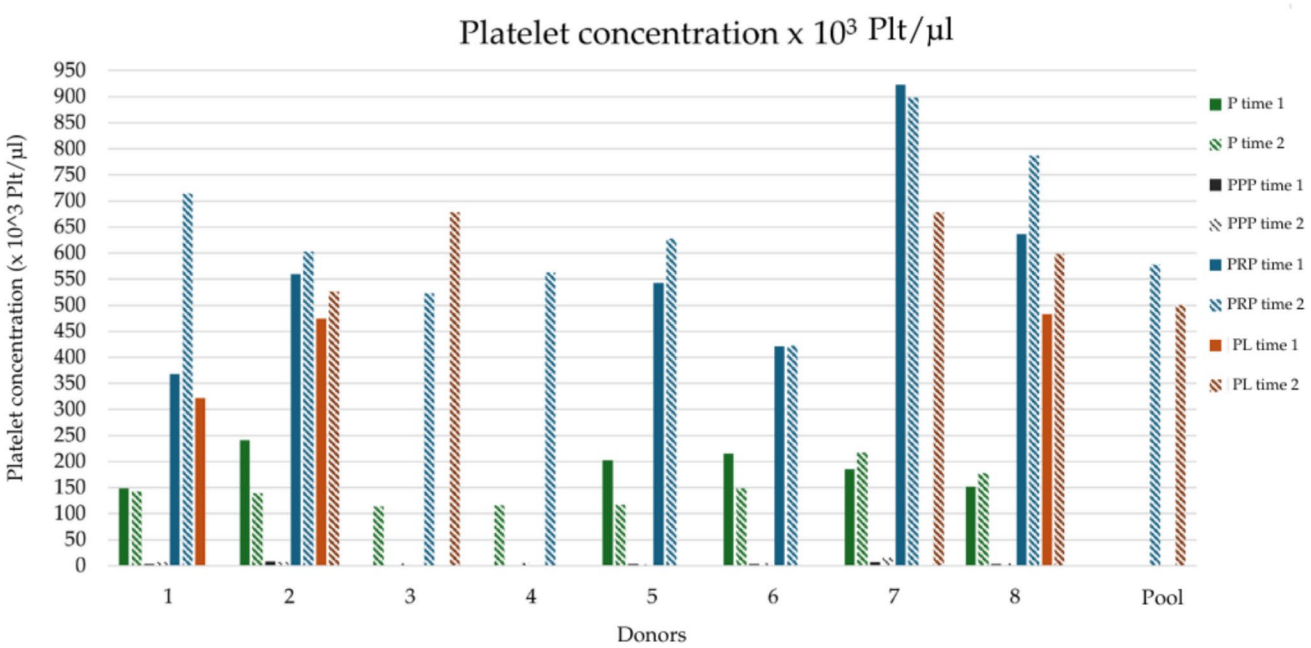

Fig. 1 Platelet concentration ($\times 10^3$ plt/µl)throughout sample processing (plasma, PPP, PRP, and PL). The X axis represents samples from eight different donors included in the study and the PRP poolsample(Time 1: P $n = 6$, PPP $n = 6$, PRP $n = 6$, PL $n = 3$. Time 2: P $n = 8$, PPP $= 8$, PRP $= 9$, PL $n = 5$). The different colorsrepresented on the graph are associated with sample groups (plasma, PPP, PRP, and PL). The Y axis represents platelet concentration obtained by the analyzer ($\times 10.^3$ plt/µl)

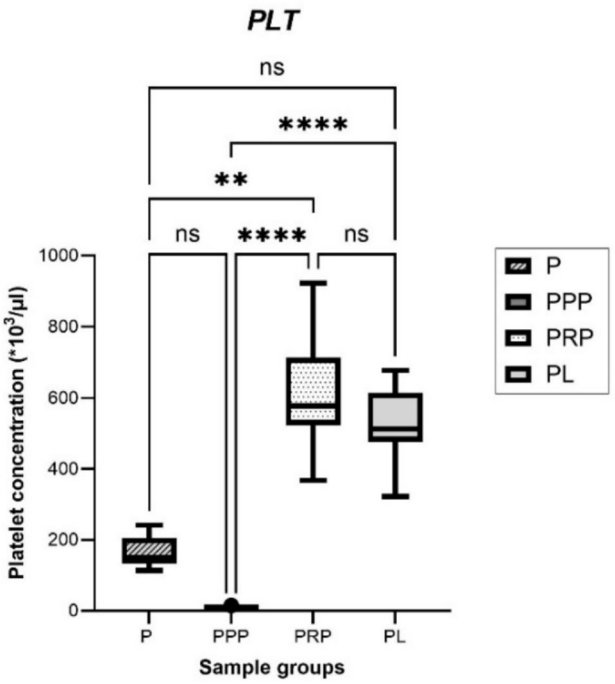

Fig. 2 Platelet concentration and its difference between groups (P, PPP, PRP, and PL). The X axis represents platelet concentration ($\times 10.^3$ plt/µl) obtained by the analyzer. The Y axis represents samples from different groups (P $n=14$; PPP $n=14$; PRP $n=15$; PL $n=8$). Different shades of grey and patterns represented on the graph are associated with sample groups (**$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. Data is presented as mean value ± standard deviation)

$\times 10^6$/µl; P = 0.007 ± 0.006 $\times 10^6$/µl; PPP = 0.000 ± 0.000 $\times 10^6$/µl; PRP 0.065 ± 0.039 $\times 10^6$/µl).

The three different GFs analyzed in this study were detected in varying concentrations in PL and PRP samples (Fig. 3). TGF-β1 was detected in PL ($n=17$), PRP ($n=17$), PPP ($n=16$), and P ($n=16$) samples. Its median concentration increased sequentially from PPP (7676 ± 8834.62 pg/µl), P (21,332.50 ± 14,106.07 pg/µl), PRP (100,690.17 ± 55,344.71 pg/µl), and finally, PL (124,869.23 ± 59,135.17 pg/µl) (Fig. 3a). The concentration in the PRP pool was 144,120 pg/µl, and 168,240 pg/µl in the PL pool.

PDGF-BB was detected in most of PL ($n=13$) and PRP ($n=16$) samples. The median concentration of PDGF was 169.09 ± 76.77 pg/µl in PRP and 607.00 ± 566.42 pg/µl in PL (Fig. 3b). The PDGF concentration in the PRP was 120 pg/µl, and in the PL pool, it was 250 pg/µl.

VEGF-A was detected only in five donor samples; in the remaining samples, it was below the detection limit (6 pg/ml). The mean concentration of VEGF-A was 36.30 ± 64.83 pg/µl in PPP, 39.15 ± 64.21 pg/µl in P, 46.20 ± 70.38 pg/µl in PRP, and 39.55 ± 75.11 in PL (Fig. 3c).

Correlation between platelet concentration and GFs was calculated for all study-included samples, except for PL samples since platelets had been previously lysed. Correlation between platelets and TGF-β1 was 0.7195 with a p-value of 7.111×10^{-10} (Fig. 4). Correlation between platelets and PDGF-BB concentration was 0.204 with a p-value of 0.0016 (Fig. 5). Correlation between VEGF and platelets was −0.019142 with a p-value of 0.9853.

Correlation between different GFs resulted in 0.204 for TGF-β1 and PDGF-BB in PRP samples and 0.09561 in PL samples. The p-value for PRP samples was 0.2272, and for PL samples, it was 0.2175.

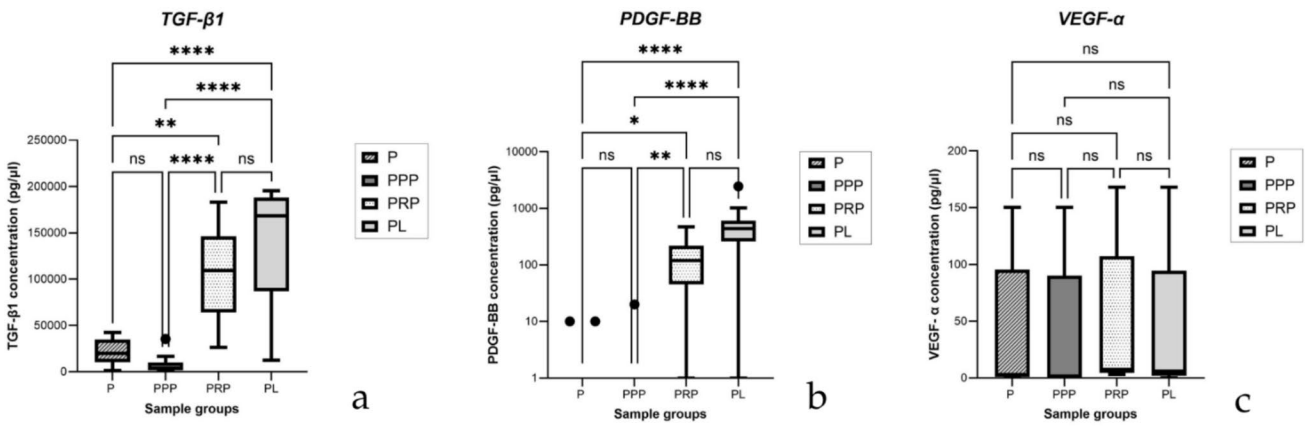

Fig. 3 Statistical analysis and Pearson correlation test (**a**) TGFb-1 concentration (pg/µl)found in plasma (P), PPP, PRP, and PL groups and its significance between them. **b** PDGF-BB concentration found in P, PPP, PRP, and PL groups and its significance between them. **c** VEGF-A concentration found in P, PPP, PRP, and PL groups and its significance between them. Different shades of grey represented on the graph are associated with sample groups (*$p<0.05$, **$p<0.01$, ***$p<0.001$, and ****$p<0.0001$. Data is presented as mean value ± standard deviation)

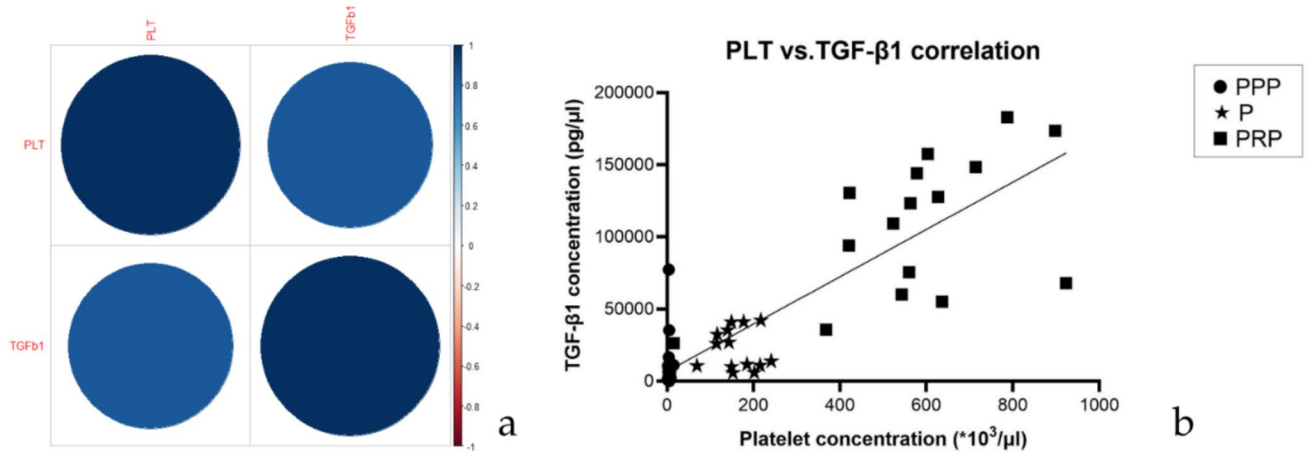

Fig. 4 Positive correlation of platelet concentrationand TGF-β1. **a** Circle correlogrambetween TGF-β1 and platelets *b* TGF-β1 and platelet scatter plot with regression line. X axis represents platelet count (× 10.^{3} plt/µl) and Y axis represents TGF-β1 concentration (pg/µl) (circle represents PPP samples, star represents plasma, and square represents PRP)

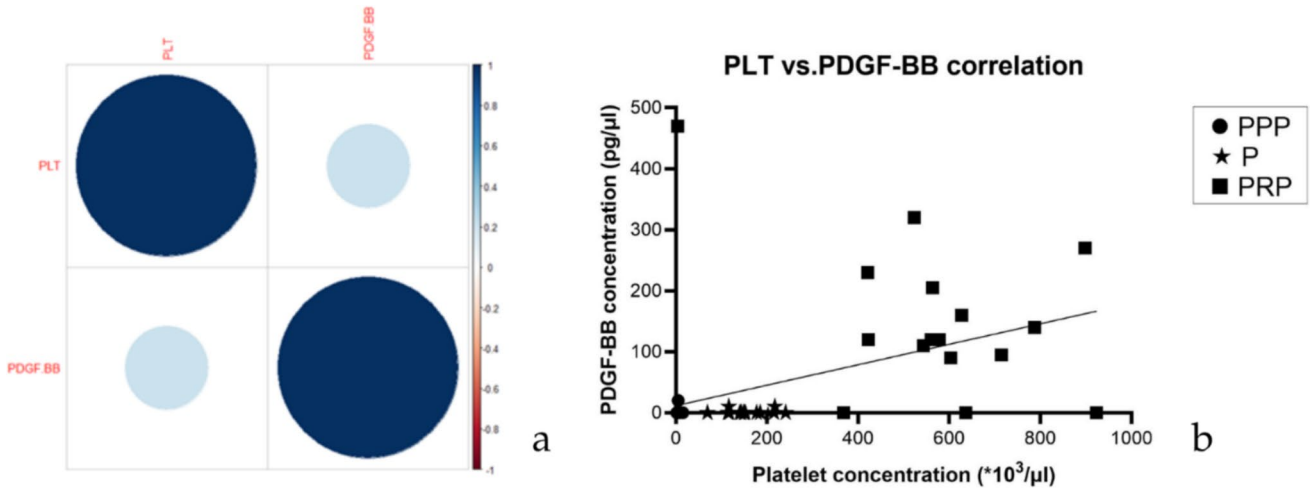

Fig. 5 Positive correlation of platelet concentration and PDGF-BB. **a** Circle correlogram between PDGF-BB and platelets and **b** PDGF-BB and platelet scatter plot with regression line. X axis represents platelet count (× 10.^{3} plt/µl) and Y axis represents PDGF-BB concentration (pg/µl) (circle represents PPP, star represents plasma, and square represents PRP)

Discussion

The three different GFs analyzed in this study were detected in PL and PRP samples. However, GFs were not detected in all samples. As hypothesized, platelet concentrations increased during the process, with the highest concentrations in PRP samples. Platelet concentration in PL decreased compared to PRP, as expected, due to the lysate process of cells during the freezing–thawing cycles.

Despite the fact that PDGF-BB was not detected in PPP and in just one plasma sample, it was detected in most of the PL ($n = 16$) and PRP ($n = 13$) samples, where platelet concentration was higher. PDGF-BB concentration was found to increase after the lysate process, suggesting that GFs are enhanced in PC preparations when platelets are lysed. In a previous study on kit-made PRP by Franklin et al., PDGF-BB was only detected in PRP samples activated by $CaCl_2$, which is comparable to our freeze–thaw degranulation system.

Furthermore, TGF-β1 concentrations seemed to increase in PL compared to PPR, which was expected due to the lysate process of platelets and the release of different GFs after the freezing cycles. However, the difference in TGF-β1 levels was not statistically significant due to the limited number of samples. Compared to the previous study mentioned, performed in 2017 by Franklin et al., TGF-β1 concentrations analyzed with the same ELISA kit and obtained

by our in-house method are higher than those obtained by commercial kits. The importance of high TGF-β concentrations lies in its proven bone augmentation effect in critical bone defects in rabbits (Ehrhart et al. 2005) and in in vitro studies (Yamamoto et al. 2000).

VEGF-A was only detected in five donor samples and did not show an increase in concentration in PL compared to PRP. There was no statistically significant difference in concentration between groups. This may be due to an insufficient number of donors, as VEGF-A concentration could only be detected in a small number of samples.

PL samples were excluded from the platelet correlation analysis, as most platelets in the PL were likely lysed and therefore not accurately detected. Platelet volume could not be effectively assessed, and lysis could not be evaluated due to the limited sample volume. A positive correlation between platelet count and GF concentration has been demonstrated for TGF-β1 with strong confidence ($R^2 = 0.7195$, $p < 0.05$), indicating that platelet concentration and TGF-β1 concentration are correlated, and this GF could potentially be predicted from platelet concentration. The concentration of TGF-β1 in samples is higher than that of PDGF-BB and VEGF-A, contributing to this high correlation.

The correlation between platelets and PDGF-BB showed a low positive correlation, although the *p*-value suggested significance. Conversely, the correlation between VEGF-A and platelets was not supported by the *p*-value obtained. To confirm this correlation, more samples need to be included in the study. VEGF-A was above the detection limit (6 pg/µl) in only a few samples, indicating that this GF is present in PRP and PL obtained but in lower concentrations. This finding is similar to the results obtained by Franklin et al. in 2017, where VEGF-A could not be detected in PRP samples obtained by various commercialized systems.

A correlation between the different GFs concentration was investigated, but this correlation could not be established. It showed a low positive correlation for TGF-β1 and PDGF-BB in PRP and PL sample groups, but this correlation was not statistically significant.

These findings, combined with a recent study published in 2025 by Lee et al., suggest that GF levels can be predicted based on platelet concentrations in PRP. Lee and colleagues could demonstrate a strong correlation between PDGF, TGF-β1, and epidermal GF (EGF) in human PRP. Therefore, it is likely that a similar correlation exists across mammal species.

The principal limitation of this study was the low amount of PL obtained per donor and the limited number of donors. As the donors were dogs from the university facilities, their maximum allowable blood donation volume was restricted. When blood extraction is performed on a healthy punctual patient, this volume can be larger, resulting in a higher volume of PL obtained. Additionally, GF bioactivity should be analyzed in further studies to standardize this method and the storage times of PRP and PL. More donors will be needed to obtain more conclusive results for VEGF-A, as these GFs are found in small quantities near the detection limit of available methods.

This study reports that a GF-rich PC can be obtained without the use of commercial kits. Furthermore, platelet quantification—a simple, cost-effective, and widely accessible method—can serve as a reliable predictor of GF concentrations, particularly TGF-β1. While another study (Lee et al. 2025) has suggested that other GFs may also be predictable based on platelet counts, further research is needed to standardize these correlations across species. This approach not only reduces costs but also streamlines the selection process for the most suitable PC for clinical use, eliminating the need for time-intensive and expensive ELISA assays. However, strict adherence to good manufacturing practices is crucial when preparing PCs for clinical application to prevent the risk of product contamination during the preparation process (Brecher and Hay 2005).

Conclusions

Although similar studies have been performed comparing different commercialized systems, to our knowledge, no other studies have evaluated canine GFs in PRP versus PL obtained by an in-house method. The relevance of this study lies in demonstrating that any veterinary professional having access to a lab equipped with basic lab devices and working under good manufacturing practices can produce a standardized plasma preparation rich in GFs without the need of a commercial system.

The correlation between TGF-β1 and platelet counts suggests a possible prediction method for the GFs present in PC through platelet analysis. However, more research performed using more donors is needed in order to validate this prediction.

As TGF-β can enhance bone healing, the high levels of this GF in our product suggest that this method may represent an approach for enhancing bone healing in canines. It has been demonstrated that the described in-house method can achieve a high-quality PC rich in GFs without the need for a commercial kit. Canine GF-rich PL can be obtained by an in-house method for orthopaedic use as well as a cell culture supplement. However, it is mandatory to work under good manufacturing practices in order to ensure the safety of the product when PC is used in patients.

Author Contribution All authors contributed to the design of the study. M. M. wrote the introduction, results, conclusions and elaborated the figures of the manuscript. D. H. wrote material and methods section. J. F. wrote introduction and conclusions, and supervised the protocol

research. And A.R. write introduction and results. All authors reviewed the manuscript.

Funding Open Access Funding provided by Universitat Autonoma de Barcelona. This study was not supported by any funding.

Data Availability No datasets were generated or analysed during the current study.

Declarations

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent For this type of study, informed consent is not required.

Consent for publication For this type of study, consent for publication is not required.

Conflict of interest The authors declare that they have no conflict of interest.

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