



Original Article

Abundant proteins in platelet-rich fibrin and their potential contribution to wound healing: An explorative proteomics study and review of the literature



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Abstract *Background/purpose:* It is well-known that diverse types of blood proteins contribute to healing process via different mechanisms. Presence and potential involvements of blood-derived abundant proteins in the platelet-rich fibrin (PRF) to its regenerative capacity have not been sufficiently emphasized in the literature. The aim of this paper was to analyze the abundant proteome content of PRF and summarize previously reported effects of identified proteins on wound healing via a literature review.

Materials and methods: The PRF samples obtained from non-smoking, systemically healthy volunteers were subjected to 2D gel electrophoresis after extracting the proteins from fibrin matrices. All matching spots were excised from the gels and identified by MALDI TOF/TOF MS/MS analysis. A literature review was conducted to reveal possible contributions of identified proteins to wound healing.

Results: Totally, thirty-five blood proteins were commonly identified among all studied samples. These proteins included serine protease inhibitors, such as alpha-1-antitrypsin, alpha-1-antichymotrypsin, alpha-1-acid glycoprotein, inter-alpha-trypsin-inhibitor, protease C1 inhibitor, and complement proteins. In addition, abundant presence of immunoglobulin G was observed. The abundance of albumin, haptoglobin, ceruloplasmin vitronectin, fetuin-A, ficolin-3 and transthyretin was also detected.

Conclusion: The results of this study indicated that PRF abundantly contains blood-origin actors which were previously reported for their direct contribution to wound healing. Further

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studies exploring the protein content of PRF are needed to reveal its undisclosed potential roles in the healing process.

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Introduction

The quality of postoperative wound healing is crucial for the clinical success of surgical treatments. Some important biological processes, such as cell migration/differentiation, angiogenesis and extracellular matrix synthesis are regulated by various growth factors and cytokines which predominantly originate from platelets and leukocytes in the fibrin clot during the wound healing process.¹ Adjunctive application of platelet-rich concentrates to the surgical wound sites is done with an expectation that artificially increasing platelets in the wounds may consequently increase the concentration of growth factors, thus accelerate wound healing and improve regenerative potential.² Platelet-rich concentrates became popular as surgical adjuvants in clinics due to their beneficial effects on the healing process. Accordingly, different types of platelet concentrates and their variations have been developed depending on their preparation protocols.³ In addition, some fibrin-based products including pure fibrin gel or fibrin matrices have been used as supplementary surgical materials.^{4,5}

Among the autologous platelet concentrates, platelet-rich fibrin (PRF) is widely used because it is easy to prepare and is devoid of any foreign agents in its structure.⁶ Numerous clinical studies have investigated its possible advantages in various oral surgical treatment modalities. Accordingly, application of PRF into in 3-wall infrabony defects and mandibular degree II furcation lesions was found to be beneficial with respect to bone filling and clinical attachment gain comparing with open flap debridement alone.^{7,8} Besides, combination of PRF with some bone graft materials has been found to increase their regenerative potential.^{9,10} As a sole material or in combination with other biomaterials, using PRF for sinus lifting reportedly improves clinical outcomes.^{11,12} Beneficial results of PRF application were also reported in other treatment modalities such as socket preservation, alveolar ridge augmentation, and peri-implant guided bone regeneration.^{13,14}

According to the current knowledge, PRF comprises platelets, leukocytes, some circulating stem cells, growth factors, cytokines, and matrix metalloproteinases (MMPs); these are considered to contribute the regenerative potential.¹⁵ However, the most accentuated molecules contributing to its regenerative potential are platelet-derived growth factor, transforming growth factor (TGF), vascular endothelial growth factor (VEGF), and insulin-like growth factor.¹⁶ Its fibrin network also acts as a biological scaffold and a reservoir for growth factors as it post-operatively enables sustained release of these molecules to an extent.^{17,18} Some *in vitro* studies have investigated the

biological effects of PRF.¹⁹ Accordingly, Dohan Ehrenfest et al.¹⁸ reported that PRF stimulates the proliferation and osteoblastic differentiation of human bone mesenchymal cells.²⁰ PRF reportedly promotes bone regeneration by enhancing attachment and proliferation of osteoblasts and production of collagen-related proteins.²¹ In addition, PRF increases the fibroblast activity in periodontal ligaments by triggering the expression of the *in vitro*-phosphorylated extracellular signal-regulated protein kinases osteoprotegerin and alkaline phosphatase.²² Moreover, beneficial effects of PRF on the proliferation of human dermal fibroblasts, endothelial cells, keratinocytes, and gingival fibroblasts have also been reported.^{23–26}

Besides being a source of various cell types and growth factors, blood also contains a great variety of proteins that contribute to a broad array of biological processes, including wound healing.^{27,28} Enhanced wound healing after their separate applications has been demonstrated by experimental studies.^{29,30} Since PRF is derived from blood, it is highly possible that some of these proteins in the blood may also exist in PRF and its releasate to some extent and may contribute to regenerative potential. However, to our knowledge, proteome profile of PRF has not been documented in the literature. The aim of this exploratory study was to investigate the total protein content of PRF releasate via 2DE-based proteomics analysis and to identify the most abundant proteins in PRF. The potential contribution of the identified proteins to the wound healing process was discussed considering the existing literature on the subject.

Materials and methods

The study was approved by the Ethics Committee of Kocaeli University (GOKAEK 2016/45). Venous blood samples were obtained from eight systemically healthy, non-smoker volunteers (six female and two male; mean age: 29.4 ± 5.6 years) who had not taken any medication during the last month. Approximately 10-mL of venous blood sample was collected without anticoagulants in vacuum plain glass tubes and was immediately centrifuged at approximately 400 g for 10 min.^{31–35}

Preparation of protein samples for 2D

After centrifugation, the PRF fibrin clot in the middle of the tube was separated from the red blood cell base using sterile scissors and tweezers, and the red blood cell layer was also removed to evade proteomic dominance of hemoglobin. The collected PRF fibrin clot was transferred into a new tube without separating the liquid part. To remove the fibrin proteins and to extract PRF releasate, additional

centrifugation at 20,000 g for 30 min at 4 °C was performed. The resulting supernatant, PRF releasate, rather than the fibrin pellet was then transferred into a new tube and used for further analysis. All materials were stored at –80 °C between all experimental steps.

2D gel electrophoresis

One milligram protein from each protein pool was loaded onto immobilized 17-cm, pH gradient strips (IPG) (pH 3–10NL) by passive rehydration. Separations based on isoelectric points were achieved using a Protean isoelectric focusing cell (Bio-Rad, USA). The strips were run through a stepwise incremental voltage program [250V for 30 min (linear), 4000V for 2.5 h (linear), and 40000 V for 1 h (rapid)]. The plate temperature was maintained at 20 °C. The strips were then subjected to a two-step equilibration in equilibration buffers containing 6M urea, 2% SDS, 0.375M Tris–HCl (pH 8.8), 20% glycerol, and 2% DTT for the first step and the same buffer without DTT but with iodoacetamide (2.5%) for the second step with 20 min of gentle shaking. Following equilibration steps, IPG strips were rinsed with SDS-PAGE running buffer and loaded directly onto 1-mm-thick 12% in-house-made SDS-polycrylamide gels. The second dimension was accomplished using Dodeca gel running system (BioRad, USA) to minimize gel-to-gel variation. Electrophoresis was carried out at 16 °C at a current intensity of 45 mA/gel until the front dye reached the bottom of the gel. After separation, the gels were fixed in 40% methanol and 10% acetic acid and were then stained with colloidal Coomassie blue G250 (Bio-Rad, USA).

Image analysis

Gel images were captured using VersaDoc MP 4000 (Bio Rad, USA). PDQuest Advance 2D-analysis software (BioRad, USA) was used for comparative analysis of protein spots. The quantity of each spot was normalized by a linear regression model. All matching spots among the gels were selected and excised using ExQuest spot cutter (Bio-Rad, USA) for protein identification.

Protein identification

Protein identification experiments were performed at Kocaeli University DEKARTproteomics laboratory using ABSCIEX MALDI-TOF/TOF 5800 system. In-gel tryptic digestion of proteins was performed using an in-gel digestion kit following the recommended protocol (Pierce, USA). The data obtained from MALDI-TOF/TOF were searched against the MASCOT database version 2.5 (Matrix Science) using a streamline software, ProteinPilot (ABSCIEX, USA), with the following criteria: National Center for Biotechnology Information non-redundant (NCBI nr), species restriction to *R. norvegicus*, enzyme of trypsin, at least five independent matching peptides, at most one missed cleavage site, MS tolerance set to ± 50 ppm and MS/MS tolerance set to ± 0.4 Da, fixed modification being cysteine International carbamidomethyl (Cys) and variable modification being oxidation (Met), a peptide charge of 1+, and being

monoisotopic. Only significant hits, as defined by the MASCOT probability analysis ($p < 0.05$), were accepted.

Results

The samples used in 2D gel electrophoresis were prepared by centrifugation of the PRF-containing fibrin clot. The supernatants collected from this centrifugation were subjected to separation in a pH range of 3–10 using 17-cm IPG strips. A total of 115 ± 10 protein spots was detected in an *in silico* spot matching experiment (Fig. 1). The overall mean coefficient of variation was 175, indicating that the gels were highly similar in their 2D protein profile. When spot scattering plots were analyzed, an average correlation coefficient value of 0.75 ± 0.15 was obtained. The low correlation coefficient value was due to changes in the quantities of abundant proteins in PRF samples.

A total of 55 spots that were abundantly present in all PRF releasate samples were cut from the gels and identified. Some of the spots were identified twice or more although they were cut from different locations on the gel. This was probably because these proteins are abundant and are thus represented more than one spots or they may be post-translationally modified. After reducing the number of identified proteins by discarding the redundant entries, 35 proteins were identified (Table 1). Some of these proteins were subunits of a protein complex. A detailed literature search revealed that 16 of the identified proteins have been previously reported to be either directly or indirectly linked with the wound healing process (Table 2).

Discussion

Even though it is well-known that the leukocytes, platelets, some growth factors, cytokines and stem cells in PRF are the main actors, exact mechanism of PRF on the wound healing has not been completely revealed.¹⁵ According to the hypothesis of this study, since the blood comprises numerous abundant proteins which can affect the wound healing via different mechanisms, some blood-derived proteins existing in PRF may contribute to the regenerative potential. Therefore, it would be worthy to explore the proteins in PRF to identify their potential involvement in wound healing. To our knowledge, no study has investigated the proteomic profile of PRF thus far. Herein, we aimed to investigate the prominent proteins of PRF releasate and discuss their possible contributions to regenerative potential of PRF. The results indicated an abundance of various types of proteins, including complement proteins, serine protease inhibitors, immunoglobulins, and some acute phase proteins, which have been reported to influence the wound healing process by various mechanisms (Table 2). Some of these identified proteins in the PRF releasate are already being used as clinical therapeutic agents and some of them are still being tested experimentally for their potential therapeutic effects.³⁶

Wound healing is an elaborate process comprising the entire period from hemostasis to tissue maturation. This process can be divided into inflammatory, proliferative, and maturational phases. During the inflammation stage, complement proteins have important functions.³⁷ The

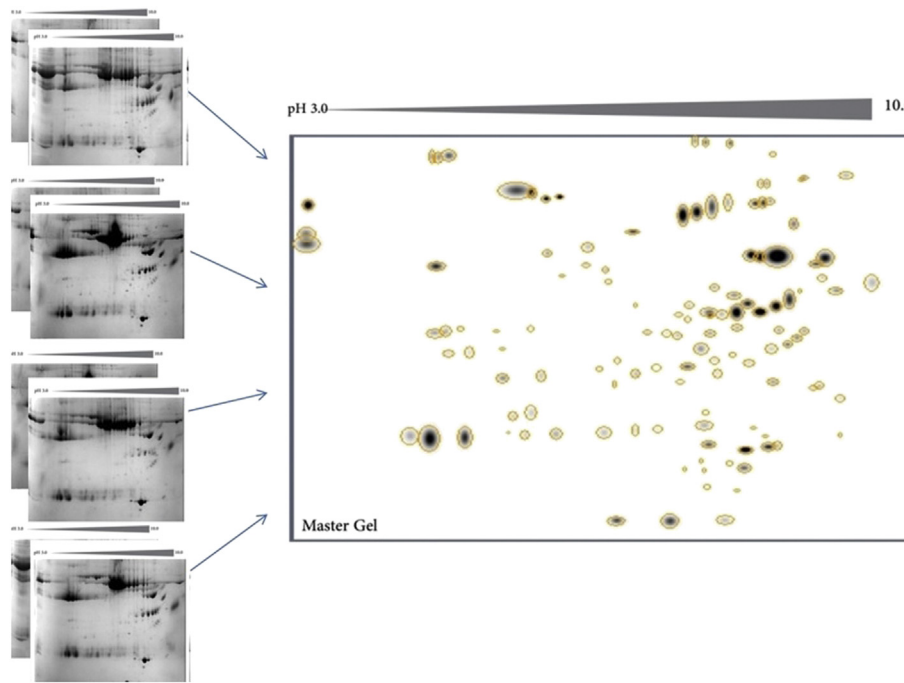


Figure 1 The overlapping protein spots among the proteome maps of PRF samples of the volunteers were subjected to MALDI TOF/TOF analysis.

blood clot limits the hemorrhage and also acts as an extracellular matrix that enables initial cell migration. Platelet-related secretions, including the growth factors, attract fibroblasts and inflammatory cells to the fibrin clot. After the subsequent activation of the complement cascade, some complement proteins, including complement C3, function as chemoattractants during the recruitment of these cells.³⁸ Based on beneficial features on wound healing, Sinno et al.³⁹ topically applied complement C3 protein to experimental cutaneous wounds in rats.³⁹ They found that complement C3 protein application accelerated the healing with enhanced physical endurance of the wounds when compared to the control group. In addition, increased collagen I and fibronectin levels and inflammatory cell and fibroblast infiltration were detected in C3-treated wounds. Beneficial effects of applying complement C5 protein to wounds have also been reported.⁴⁰ The present study revealed the abundance of complement proteins, including complement C3, in PRF. Accordingly, it may be speculated that complement proteins in PRF contribute to the PRF-mediated postoperative wound healing process.

Cell migration/differentiation, angiogenesis, extracellular matrix synthesis, and tissue maturation are regulated by some cytokines and growth factors that interact with various cell types during wound healing.⁴¹ Serine proteases are secreted by neutrophils to control the activity of these molecules. However, prolonged secretion of serine proteases may considerably impair tissue repair.⁴¹ Proteases in wounds may reportedly degrade growth factors and their receptors and consequently prevent complete healing and tissue maturation.⁴² However, serine protease inhibitors suppress these devastating effects of serine proteases in the wound region and positively affect wound healing.

Various types of serine protease inhibitors that directly contribute to tissue repair have been reported.^{29,30,43} This study identified the abundance of these protease inhibitors in PRF releasate; the inhibitors included alpha-1-antitrypsin, alpha-1-antichymotrypsin, alpha-1-acid glycoprotein, inter-alpha-trypsin-inhibitor, and protease C1 inhibitor.

Alpha1-antitrypsin and alpha-1-antichymotrypsin are serum-derived protease inhibitors and affect wound healing by inhibiting the activation of pro-MMP-9, a prominent serine protease.²⁹ Alpha1-antitrypsin is reportedly an anti-inflammatory and anti-apoptotic protein which inhibits pro-cytokine production (IL-1 α , IL-6, IL-8, and TNF- α) and increases anti-inflammatory cytokine (IL-10) production.^{44,45} The therapeutic use of alpha-1-antitrypsin and alpha-1-antichymotrypsin on experimental wound models has been demonstrated. For instance, Hoffman et al.³⁰ used recombinant alpha-1-antichymotrypsin as a therapeutic agent on experimental dermal open wounds in rabbits and found that wound closure was dose-dependently faster in rabbits treated with recombinant alpha-1-antichymotrypsin than in the control group rabbits.³⁰ Interestingly, Zilm and Bartold⁴⁶ reported the presence of alpha-1-antichymotrypsin in porcine enamel matrix derivative (EMD®) and speculated that it contributed to the regenerative potential of EMD.⁴⁶ In this study, alpha-1-acid glycoprotein was another protease inhibitor abundantly found in PRF. It is reportedly a potential therapeutic protein for experimental ischemic stroke and acute liver failure^{47–49} and may accelerate wound healing by stimulating fibroblast proliferation and collagen synthesis.^{43,50} Inter-alpha-trypsin-inhibitor is another protease inhibitor found abundantly in PRF in this study. Its potential additive effects on wound healing have

Table 1 The list of the identified proteins and related MALDI TOF/TOF data.

#	Protein Accession	Best Protein Mass	Best Protein Score	Best Protein Description
1	A1AG2_HUMAN	23588	400	Alpha-1-acid glycoprotein 2
2	A1AT_HUMAN	46707	668	Alpha-1-antitrypsin
3	AACT_HUMAN	68342	672	Alpha-1-antichymotrypsin
4	A1BG_HUMAN	54239	805	Alpha-1B-glycoprotein
5	ACTB_HUMAN	41710	656	Actin, cytoplasmic 1
6	AFAM_HUMAN	69024	609	Afamin
7	ALBU_HUMAN	69321	313	Serum albumin
8	AMBP_HUMAN	38974	360	Protein AMBP
9	APOA1_HUMAN	30759	592	Apolipoprotein A-I
10	APOE_HUMAN	36132	685	Apolipoprotein E
11	APOH_HUMAN	38273	202	Beta-2-glycoprotein 1 (Apolipoprotein H)
12	C1S_HUMAN	76635	344	Complement C1s subcomponent
13	CD5L_HUMAN	38063	601	CD5 antigen-like
14	CERU_HUMAN	122128	633	Ceruloplasmin
15	CFAI_HUMAN	65677	215	Complement factor I
16	CLUS_HUMAN	52461	236	Clusterin
17	CO4A_HUMAN	192650	265	Complement C4-A
18	CO3_HUMAN	187030	393	Complement C3
19	FCN3_HUMAN	32882	322	Ficolin-3
20	FETUA_HUMAN	39300	441	Alpha-2-HS-glycoprotein (Fetuin-A)
21	HEMO_HUMAN	51643	571	Hemopexin
22	HPT_HUMAN	45177	324	Haptoglobin
23	IC1_HUMAN	55119	560	Plasma protease C1 inhibitor
24	IGHG1_HUMAN	36083	353	Ig gamma-1 chain C region
25	IGHG2_HUMAN	35878	115	Ig gamma-2 chain C region
26	IGHM_HUMAN	49276	226	Ig mu chain C region
27	IGKC_HUMAN	11602	321	Ig kappa chain C region
28	ITIH4_HUMAN	103293	192	Inter-alpha-trypsin inhibitor heavy chain H4
29	KV302_HUMAN	11768	223	Ig kappa chain V-III region SIE
30	THRB_HUMAN	69992	533	Prothrombin
31	TPM4_HUMAN	28504	475	Tropomyosin alpha-4 chain
32	TRFE_HUMAN	77000	1030	Serotransferrin
33	TTHY_HUMAN	15877	348	Transthyretin
34	VTDB_HUMAN	52929	848	Vitamin D-binding protein
35	VTNC_HUMAN	54271	482	Vitronectin

been previously demonstrated.^{51,52} As an acute phase protein circulating in the blood, proteinase C1 inhibitor exhibits protective effects against complement-mediated inflammatory tissue breakdown.⁵³ Replacement treatment of C1 inhibitor has been widely used for treating angioedema.⁵⁴ Local administration of C1 inhibitor reduced edema formation and inflammatory tissue damage and increased re-epithelialization in experimental coetaneous burn lesions in animals.^{53,55} Conclusive evidence has proven that systemic administration of C1 inhibitor prevents the formation of secondary ischemic and dermal lesions in the experimental cutaneous burn wounds.^{55,56}

Infection control during periodontal wound healing is crucial due to residual bacteria which make the process more complicated. In addition, postoperatively, improper oral hygiene and subsequent plaque accumulation on the adjacent teeth may influence treatment success.⁵⁷ As demonstrated herein, PRF releasate contains abundant amounts of immunoglobulin G (IgG). Thus, IgGs may provide some infection control against periodontal bacteria during

the postoperative wound healing period and may thus consequently contribute to the favorable effects of PRF. Accordingly, it is important to know whether IgGs in PRF actually exhibit a specific antimicrobial activity to the periodontal bacteria during the healing process. Although this possibility has to be investigated by further studies, many studies have reported that blood contains IgG serotypes specific to periodontal pathogens. Papapanou et al.⁵⁸ reported that compared with the controls, chronic periodontitis patients presented with up to 800-fold higher IgG titers as a response to 19 periodontal species.⁵⁸ In fact, the mere presence of periodontal pathogens can increase serum IgG levels to act against periodontal bacteria, indicating that the occurrence of periodontitis is not an obligatory requirement for immune response.⁵⁹ We speculate that the abundant amount of IgG detected in the PRF samples herein may contain IgGs against periodontitis-specific bacteria and thus provide some antibacterial defense and contribute to uneventful healing from PRF application to regenerate damaged periodontal tissues. Therefore, the effect of IgGs in PRF on wound healing

Table 2 The summarize of reported evidences for possible contributions of some of the identified proteins to the healing process.

Identified protein	Evidence for the possible contribution to healing process	Reference
Albumin	Significantly fast healing after topical albumin application in experimental corneal erosions in rabbits. Albumin application provided an anabolic effect during the <i>in vitro</i> bone healing. Anti-oxidant activity via free radical-trapping properties.	60–62
Alpha1-acid glycoprotein	Accelerating the wound healing via stimulating fibroblast proliferation and collagen synthesis	43,50
Alpha1-antitrypsin	Reducing the activity of MMP-9, anti-inflammatory and anti-apoptotic effects during the healing.	44,45
Alpha1-antichymotrypsin	Similar effects with Alpha1-antitrypsin during the healing. Accelerated wound closure after the application to experimental dermal open wounds in rabbits.	30
Alpha-2-HS-glycoprotein (Fetuin A)	Enhancement of wound closure in primary keratinocyte culture.	79
Apolipoprotein A-I	Promotion of endothelial cell migration. Topical application accelerated the healing of corneal epithelium in experimental dry eye defects.	71,72
Apolipoprotein E	Topical application corrects delayed wound healing in apolipoprotein E deficient mice.	73
Ceruloplasmin	Antioxidant activity in the tissues during the healing. Chaperone activity targeting misfolded proteins during the wound healing.	75
Complement C3	Increased collagen/fibronectin levels and enhanced wound healing were seen in C3 applied experimental coetaneous wounds in rats.	39
Ficolin-3	Participating in the clearance of apoptotic and necrotic cells during the healing.	77
Haptoglobin	Assisting in mitigating the oxidative damage related with neutrophils. Reduces tissue destruction during the inflammation via inhibiting both COX and LOX pathways. Promotes fibroblast migration and inhibits MMP-9 activity during the healing. Stimulation of angiogenesis. Acting as a chaperone targeting misfolded proteins during the wound healing.	64–67
Inter-alpha-trypsin-inhibitor	Anti-inflammatory properties during the healing. <i>In vitro</i> extracellular matrix stabilization thought binding to the hyaluronic acid.	51,52
Protease C1 inhibitor	Local administration of C1 inhibitor provided inhibition of edema formation, reduction of inflammatory tissue damage and increased reepithelialization in experimental cutaneous burn lesions in animals.	53,55
Transthyretin	Regulation of angiogenesis during the healing.	76
Vitronectin	Direct interaction with numerous growth factors and cytokines during the wound healing and thus contributing the wound healing by various aspects. Topical application of vitronectin on experimental corneal defects and tissue cultures resulted in accelerated corneal epithelial wound healing.	68–70

cannot be underestimated and should be scrutinized in further studies.

Numerous studies have emphasized on the contributions of some other blood proteins to the wound healing process; these were also identified in our study. Among them, albumin, the most abundant blood protein, was investigated for its potential effects on wound healing. Some reported contributions are as follows: 1) faster wound healing after its topical application to experimental corneal erosions, 2) anabolic effect during *in vitro* bone healing, and 3) improved wound strength and collagen synthesis.^{60–62} Haptoglobin, another abundant blood protein, acts as a chaperone that targets misfolded proteins during wound healing.⁶³ Furthermore, it assists in mitigating oxidative

damage, reduces tissue destruction, promotes fibroblast migration, inhibits MMP activity, and stimulates angiogenesis.^{64–67} Vitronectin, which is also an abundant blood protein, interacts directly with numerous growth factors and cytokines. Accelerated corneal epithelial wound healing following topical application of vitronectin has also been reported.^{68–70} A specific group of abundant blood proteins, apolipoproteins, have also been investigated for their potential effects in correcting delayed wound healing; topical application of apolipoprotein E was found to be beneficial in this regard.^{71–73} Alpha-2-HS-glycoprotein (fetuin-A), another blood protein identified in PRF, possesses some features that contribute to tissue remodeling.⁷⁴ Like alpha-1-antichymotrypsin, it has also

been found in the proteome of EMD.⁴⁶ Ceruloplasmin, transthyretin, and ficolin-3 were also identified, and their possible associations with wound healing have already been reported.^{75–79}

The possible interactions between some of the identified proteins in PRF releasate and growth factor activity have been documented. Accordingly, albumin reportedly possesses some amino acid sequences which are significantly similar to TGF- β . Concordantly, it has been suggested that albumin activates TGF- β receptor type II and consequent biological pathways owing to its molecular homology with TGF- β .⁸⁰ Vitronectin potentiates VEGF-mediated angiogenesis.⁸¹ Overexpression of clusterin promotes angiogenesis via VEGF.⁸² Fetuin-A reportedly induces VEGF expression and enhances vascular cell growth *in vitro*.⁸³ Alpha1-antitrypsin increases VEGF expression in different cell types.^{84,85} Alpha1-acid glycoprotein alone induces *in vitro* endothelial tube formation.⁸⁶ In addition, the pro-angiogenic properties of alpha-1-acid glycoprotein are attributed to its supportive effect on VEGF.⁸⁶ Complement C3a reportedly increases VEGF expression.⁸⁷

Studying proteins at the tissue level is challenging.⁸⁸ Tissues in the body comprise various abundant proteins related to their extracellular matrix contents; this makes studying proteomics at a tissue level more complicated than studying proteins in cultured cells. After protein extraction, all proteins in the investigated tissue sample remain in the same test tube. Therefore, due to the domination of abundant proteins in the investigated tissue samples, it is difficult to identify proteins which exist in very small amounts. In this study, since the high abundance of fibrin in PRF is indisputable, we aimed to eliminate it during the protein extraction stage. For this purpose, PRF samples were additionally centrifuged to extract the liquid portion, PRF releasate, completely and let noting but fibrin pellet remains at the bottom of the tubes. Therefore, we did not encounter any spots representing fibrin in 2DE gels. One of the concerns regarding PRF extraction practiced in this study may involve the removal of some “key players” along with the fibrin. However, the centrifugation step used in this study tightly sequesters the fibrin clot and clearly separates the soluble proteome, because it has been done at 20,000 g at 30 min. This centrifugation speed and duration have been used in many publications to prepare cell-free extracts and thus were used in this study as well.^{89,90} On the other hand, there will always be a concern for the fibrin attracted proteins (sponge effect) that may be stacked in between fibrin fibers. We think that those proteins should also be present and representative in the soluble phase. A similar phenomenon is also encountered when abundant proteins are reduced from serum samples using proteominor/MARS depletion kits. But the researchers almost always omit those proteins in their analysis.⁹¹

The importance of preserving the red blood cell layer on the PRF surface in obtaining more platelets/leukocytes has been emphasized by Dohan Ehrenfest et al.⁹² However, since hemoglobin is another abundant protein along with fibrin, both of which probably preclude the detection of relatively less abundant proteins and thus reduce proteome coverage,⁹³ we did not include the red blood cell layer of

PRF for analysis. If we had included it during the analysis, we would have potentially been unable to detect some spots representing minor proteins due to the domination of multiple spots representing hemoglobin considering that it is a highly abundant protein. In future studies, identifying the proteome of the hemoglobin-containing layer after hemoglobin depletion may be beneficial to enrich the current proteome map of PRF. Considering that blood is a very rich protein source that represents over 10,000 different proteins,⁹⁴ it is highly possible that PRF may contain a large number of proteins within different quantities in mass. Thus, it would be extremely difficult to reveal the complete proteome map of PRF as it would require step-by-step reduction of abundant proteins and numerous repetitive experiments. Further studies exploring the protein content of PRF are needed to reveal its yet undisclosed potential involvements in the wound healing process.

PRF may be applied directly to the surgical wounds or used as a membrane with most of its liquid part removed. Most of the studies have focused on its fibrin part and growth factors which originate from platelet cells. The results of this study indicated that PRF releasate abundantly contains blood proteins which potentially possess some beneficial effects on wound healing, as discussed above. Therefore, the importance of PRF releasate should not be underestimated.

Considering all the above-discussed data as well as the limitations of our study, it can be speculated that the regenerative potential of PRF may be due to its rich protein content. PRF application may lead to a load of some serine protease inhibitors, complement factors, and immunoglobulins, being unintentionally applied to surgical wounds; these contribute to the overall favorable effects of PRF as discussed. Further studies should confirm if PRF fibrin clot acts as a scaffold and reservoir for these blood proteins and the growth factors.

As this is an explorative research, there is no control group in this study related with its nature. There are numerous similar studies in the literature investigating the proteome profile of targeted biological materials.^{90,95} Accordingly, if we compare the proteome profile of PRF with other biomaterials as control, the identification of various proteins may not be possible due to their possible presence in the control material. The aim of this study was to reveal the prominently expressed proteins in the PRF releasate which potentially contribute to its regenerative potential.

As conclusion, the importance of underestimated and overlooked blood-derived protein factors of the PRF was underlined using the proteomic approach. The beneficial effects of most of the identified proteins have been reported in the literature by *in vitro* and *in vivo* studies. The abundance of protease inhibitors, complement proteins, some acute phase proteins, and immunoglobulins in the PRF releasate probably contributes to its regenerative potential in the wound healing process. Further studies exploring the protein content of PRF are needed to reveal its yet undisclosed potential roles in wound healing. The presence and enrichment of these blood proteins should also be considered while developing new modifications of PRF or other types of blood-derived biomaterials in future.

Conflicts of interest

The authors declare no conflicts of interest related to this study.

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