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Biochemical and cellular markers differentiate recovered, in-line filtered plasma, and plasma obtained by apheresis methods

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Abstract

Background and Objectives: Assessment of plasma quality often focuses on the common safety tests for minimizing the risk of transmitting blood-borne pathogens. Little attention is paid to the possible quality attributes that ensure a consistent biochemical composition of plasma for fractionation. We therefore investigated the suitability of selected biochemical and haematological attributes that could be used as markers of plasma quality obtained by different separation and pre-treatment procedures.

Material and Methods: We characterized six plasma types, including source plasma, plasma recovered by classic means and in-line filtered plasma, by determining the analytical attributes protein content, coagulation factors and markers of coagulation, contact and complement activation. Residual cell content and cell-specific variables were also measured.

Results: We found relevant differences between the plasma types in complement activation, as indicated by C3a measurements, while thrombin antithrombin complex values and, to a minor extent, activated factor XII concentrations indicated only moderate differences in activation levels of coagulation and contact systems. The most striking differences, however, were detected in residual cell content and concentrations of the platelet-associated proteins, platelet factor 4 and β-thromboglobulin. We showed that leucocyte reduction filters disrupt cells. This includes platelets, thereby releasing the platelet-associated proteins platelet factor 4 and β-thromboglobulin, and leucocytes as demonstrated by the release of elastase from polymorphonuclear leucocytes. Furthermore, the filtration processing of whole blood can lead to activation of the complement system.

Conclusion: Our results show that biochemical and cellular surrogate markers are valuable discriminators of plasma types.

KEYWORDS
cell disruption, plasma for fractionation, plasma quality, platelet-associated proteins, platelet factor 4, PMN elastase, β-thromboglobulin

INTRODUCTION

Human plasma is a valuable raw material that produces functional proteins used in the therapy of coagulation, haematological and immunological disorders [1–4]. The quality of this starting material and the final products derived from it depends on two main factors: the blood or plasma of donors [5], and plasma preparation method [3, 6–9]. Current quality control of plasma for fractionation focuses on minimizing the risk of transmitting infectious diseases caused by blood-borne viruses and bacteria [6, 10–12], with testing done on each single plasma unit. Apart from the pharmacopeial requirements regarding, amongst others, residual blood cells, total protein content and minimum content in coagulation factors, where testing is not done on each single unit, little attention has been paid to the biochemical variables that ensure a consistent biochemical plasma composition. Types of plasma vary according to the preparation technique used, with several different types available from plasmapheresis units and transfusion services.

Source plasma [13] is defined as the fluid portion of human blood collected by plasmapheresis intended for further manufacturing use and can be prepared by automated plasmapheresis. This procedure can be carried out, for instance, by the Autopheresis-C or Aurora system (Fresenius Kabi) [14–16], which uses spinning membrane technology, or the PCS-2 system (Haemonetics, Massachusetts) [14, 17], which uses centrifugation.

Plasma can also be recovered by centrifugation from whole blood donations, with and without leucoreduction. Leucoreduction is achieved by filtration. Plasma recovered with leucoreduction is also termed in-line filtered plasma. All the different filter systems used in leucocyte reduction steps aim to reduce the leucocyte load in erythrocyte concentrates and minimize leucocyte-associated side effects [18–21], and the possibility of blood components transmitting prions that cause a variant form of Creutzfeldt-Jakob disease (vCJD) [22, 23].

The various plasma types produced by these methods are the raw and starting material for plasma fractionators, which commonly process plasma into several different products. However, different plasma production methods could have an impact on the composition of the plasma obtained. Therefore, to achieve a controlled, sustained quality of these products, it would clearly be beneficial if the quality attributes for the starting material could be defined. Up until now, apart initiating the common serological and nucleic acid amplification tests [24, 25], there have been few attempts to define the attributes that define plasma quality. Thus, Burnouf et al. [26] compared protein composition and activation markers in plasma collected by three apheresis procedures, while Runkel et al. investigated the quality of apheresis and recovered plasma [27], and the impact of whole blood in-line filtration [28]. The biochemical analyses were conducted on single plasma donations, and were similar in all three studies, focusing mainly on the measurement of various activation markers.

To advance knowledge on this topic, we investigated whether different plasma preparation techniques influence the function and morphology of the plasma obtained. In particular, we compared selected biochemical and haematological attributes using single plasma donations and plasma production pools from different sources. In addition, data on the content of residual cells and cell debris were generated as described [29]. A similar protocol was used to compare source plasma quality in three apheresis protocols [30].

MATERIALS AND METHODS

Plasma samples and freezing protocol

We used six types of plasma obtained by different preparation methods: two were source plasma obtained by Autopheresis-C (Fresenius Kabi) and by the PCS-2 system (Haemonetics), respectively, one was recovered by the classic method (hereafter termed recovered plasma) and three were in-line filtered from whole blood that had been subjected to different filter systems to reduce the leucocyte load. These three different in-line filtered plasma types were designated type A, B, and C. Our aim was to have at least 10 individual samples from each plasma type. Accordingly, 10 individual units each of plasma types Autopheresis-C, Haemonetics, A and B were analysed, while 12 and 26 individual units were available from recovered and type C plasma, respectively. The plasma samples were obtained from Austrian BioLife plasmapheresis centres and from other European Baxter AG plasma suppliers. Source plasma (Autopheresis-C and Haemonetics PCS-2) was collected with 6% trisodium citrate (v/v) as an anticoagulant by use of 4% (w/v) trisodium citrate solution. A citrate-phosphate-dextrose solution was used as an anticoagulant for recovered and in-line filtered plasmas.

All samples were aliquoted directly after preparation (1-ml samples), frozen at -70°C overnight and subsequently stored at -20°C. Before testing, the samples were thawed at $+37^{\circ}$C. Some selected samples were investigated immediately after preparation. These freshly prepared samples were transported to the laboratory at $+4^{\circ}$C and testing started within 2 h of their preparation.

Plasma pool samples (source plasma, $n = 36$, recovered plasma, $n = 24$ and in-line filtered plasma, $n = 22$) consisting of 2000 to 6000 single plasma donations were obtained from the standard manufacturing process at Baxter AG, Vienna, Austria (part of Takeda).

Assays for assessment of biochemical attributes

The protein concentration was measured by Biuret assay [31]. Factor VIII (FVIII) activity was determined by chromogenic assay [32] using reagents supplied by Technoclone (Vienna, Austria). Von Willebrand factor ristocetin cofactor (VWF:RCo) was determined by measuring the agglutination of formaldehyde-fixed human platelets using the standard procedure [33], with a 570-VS whole blood aggregometer (Chrono-Log, Havertown, Pennsylvania) equipped with a chart recorder. The antigen concentration of VWF (VWF:Ag) was determined with sandwich ELISA Asserachrom® VWF (Boehringer Mannheim, Germany), while VWF collagen-binding (VWF:CB) activities were determined by Immunozym VWF:CBA [34] (Technoclone, Vienna, Austria). VWF multimers were analysed by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis

using a 1% gel according to Ruggeri and Zimmerman [35], with minor modifications [36]. Thrombin-antithrombin complex (TAT) and prothrombin fragments F1 + 2 concentrations were determined by commercially available sandwich ELISAs (Siemens, Vienna, Austria), activated coagulation factor XII (FXIIa), a marker for the activation of the contact activation system, was determined by a commercially available sandwich ELISA system (Shield Diagnostics, Dundee, UK). Complement activation was determined by measuring C3a concentrations with a commercially available sandwich ELISA system (Quidel, Mountain View, California). Lactate dehydrogenase (LDH) was measured by a photometric standard method at 25°C (Boehringer Mannheim GmbH, Mannheim, Germany).

Determination of residual cell content

Residual cells (platelets, leucocytes, erythrocytes, and erythrocytes ghosts) were quantified in frozen samples by flow cytometric (FACS) analysis using a no-lyse/no-wash preparation procedure [37]. The manufacturer's instructions were followed for leucocyte enumeration with LeucoCount (BD Biosciences, Sunnyvale, California). To quantify platelets and erythrocytes, 100 µl of plasma were added to TrueCount tubes (BD Biosciences) and incubated with 10 µl of a monoclonal antibody cocktail against CD42a (FITC, BD Biosciences) and glycophorin-A (PE, Dako, Glostrup, Denmark). After 30 min of incubation at 4°C, 300 µl PBS were added before measurement on a FACSCalibur (BD Biosciences). Platelets and erythrocytes were subsequently measured separately from the same tube. Cell debris signals were removed. Logarithmic amplification was used for all variables. Data were evaluated by multi-parameter analysis using the Paint-A-Gate software (BD Biosciences).

Blood cell-associated proteins

Platelet factor 4 (PF4) and β-thromboglobulin (bTG) concentrations were determined in samples frozen and thawed one time by commercially available sandwich ELISAs obtained from STAGO Diagnostics (Roche Diagnostics, Mannheim, Germany). In a separate study, bTG and PF4 were released from platelets. Briefly, platelets were prepared from whole blood by a standard in-house procedure and suspended in physiological HEPES-NaCl buffer, pH 7.35. The platelet content was measured with the Sysmex K-1000 cell counter (Toa Medical Electronics, Kobe, Japan). Suspensions were adjusted to contain 125,000, 250,000 and 500,000 platelets/µl and stabilized by the addition of 5% (w/v) human serum albumin (Baxter AG, Vienna, Austria). The suspensions were deep frozen at −70°C and lyophilised in aliquots of 1 ml. After lyophilisation, the samples were reconstituted with the appropriate amount of water and the bTG and PF4 concentrations determined. Elastase from polymorphonuclear leucocytes was determined in complex with alpha1-proteinase inhibitor by use of an ELISA (Merck, Darmstadt, Germany).

Platelet function analysis with platelet function analyser

The platelet function analyser PFA-100/200 (Siemens, Vienna, Austria) is a high shear stress in vitro system for the detection of platelet dysfunction [38, 39]. The instrument simulates the process of primary haemostasis by aspirating the whole blood sample from a reservoir through a capillary and a microscopic aperture cut in the membrane. This membrane is coated with type I collagen and either epinephrine (EPI) or adenosine 5′-diphosphate (ADP) as a stimulating agent. The time needed to obtain full occlusion of the aperture is defined as closure time. Aliquots of 800 µl of each, taken from the three samples of citrated whole blood obtained from two independent blood donations before and after in-line filtration with in-line filters type A, were applied to collagen-EPI and collagen-ADP cartridges. Closure times were determined by the standard procedure according to the manufacturer's instructions and were expressed as means of six values (three aliquots of two blood donations). The samples' platelet content was determined as described above.

Statistical evaluation

Statistical data evaluation was done with one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism version 8.0.0 for Windows. The significance level was 0.05.

RESULTS

Biochemical variables in different plasma types

Our first step was to analyse biochemical attributes in analytical samples of different plasma types that had been subjected to freezing and thawing. These data are summarized in Table 1. ANOVA revealed significant differences between total protein levels: The lowest protein values were determined for the two source plasma types Autopheresis-C and Haemonetics PCS-2. This was to be expected for source plasma collected from donors undergoing intensified plasmapheresis [40, 41]. In addition, comparison of recovered and the in-line filtered plasmas showed that the level of total protein is not affected by the different in-line filtration procedures (Supplemental Material, Figure 1).

The FVIII activity levels of the different plasma types are summarized in Table 1 and Figure 1a, which show the significant differences found between the individual plasma types. Actual values ranged from 0.79 IU/ml (In-line Filtered Plasma, Type B) to 1.19 IU/ml (recovered plasma), but with high variability between the individual units. [Correction added on 06 September 2021 after first online publication: The range of actual values of individual plasma types was corrected to "0.83 IU/ml (Autopheresis-C)" in the preceding sentence.]. Their levels did not differ significantly between the plasma types, with VWF:RCo showing slightly higher differences (Table 1). All data

TABLE 1 Biochemical variables in different plasma types

Attribute	Source Plasma		Recovered	In-line Filtered Plasma			ANOVA Results[a] ($\alpha = 0.05$)	Reference Range
	Auto-C ($n = 10$)	Haemonetics ($n = 10$)	Classic ($n = 12$)	Type A ($n = 10$)	Type B ($n = 10$)	Type C ($n = 26$)		
Protein (mg/ml)	51.1 ± 2.1	55.9 ± 2.4	58.0 ± 4.1	58.5 ± 2.9	63.6 ± 4.8	56.4 ± 4.3	$p < 0.0001$	60–75
FVIII:C (IU/ml)	0.83 ± 0.29	1.00 ± 0.29	1.19 ± 0.38	0.90 ± 0.15	0.79 ± 0.18	0.99 ± 0.27	$p = 0.0392$	0.6–1.4
VWF:Ag (IU/ml)	1.21 ± 0.46	1.32 ± 0.36	1.02 ± 0.27	1.28 ± 0.22	1.22 ± 0.40	1.04 ± 0.49	$p = 0.2685$	0.6–1.4
VWF:CB (U/ml)	0.96 ± 0.41	0.98 ± 0.23	0.94 ± 0.25	1.05 ± 0.21	0.99 ± 0.28	0.92 ± 0.44	$p = 0.9408$	0.6–1.4
VWF:RCo (IU/ml)	0.82 ± 0.34	0.94 ± 0.23	0.94 ± 0.32	1.31 ± 0.31	0.99 ± 0.26	0.98 ± 0.41	$p = 0.0501$	0.6–1.4
TAT (ng/ml)	1.4 ± 0.7	4.7 ± 11.2	17.4 ± 33.8	3.3 ± 2.9	1.6 ± 1.5	2.4 ± 1.4	$p = 0.0471$	1.0–4.1
F1 + 2 (nmol/l)	0.9 ± 0.4	0.9 ± 0.5	1.0 ± 0.4	1.2 ± 0.3	0.8 ± 0.3	0.8 ± 0.2	$p = 0.0337$	0.4–1.1
FXIIa (ng/ml)	1.9 ± 0.3	1.6 ± 0.8	1.8 ± 0.9	2.0 ± 0.4	1.6 ± 0.3	2.5 ± 1.2	$p = 0.0187$	< 1–2.9
C3a (ng/ml)	4914 ± 1059	250 ± 78	299 ± 133	245 ± 94	621 ± 343	2307 ± 1489	$p < 0.0001$	26–146[b]

Note: Remarks: Means ± standard deviations are shown.
[a]One-way ANOVA was performed followed by Tukey's multiple comparisons test using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California. Results obtained for the individual comparison of groups with the Tukey's multiple comparison test are shown in Supplemental Material.
[b]The reference range for the C3a concentration was determined in EDTA plasma.

showed that the in-line filtration procedures did not affect the VWF levels. Moreover, detailed analysis of the different VWF variables indicated that the multimeric structure of VWF was not affected in the different samples with one exemption: Plasma obtained by the Haemonetics PCS-2 system was devoid of the high-molecular weight multimers, as demonstrated in Figure 1b (lane 2). However, this was not reflected by the functional VWF assays VWF:CB and VWF: RCo, probably as a consequence of their variability or their lower sensitivity in detecting high-molecular weight VWF multimers compared with multimer analysis. Despite this small variation in the different individual plasma samples, there was no evidence that the in-line filtration process affected the multimer composition of VWF.

The concentrations of the coagulation activation marker prothrombin fragment F1 + 2 and TAT differed significantly between the plasma types (Table 1, Figure 2). The lowest average TAT value was found for the source plasma obtained by the Autopheresis-C system, and highest for the recovered plasma (Figure 2a). The large differences could, however, be partly due to outlying abnormal values of single donors that can originate from the venepuncture applied and which is known to lead to activation of the coagulation system. We noticed that TAT concentrations ranged widely (from 0.7 to 122 ng/ml) in the recovered plasma. The values for FXIIa, a marker of contact activation, also differed significantly, mainly between Haemonetics and plasma type C (Supplemental Material Figure 8). FXIIa concentrations higher than 4 ng/ml were measured which were probably due to in-line filtration with the filter for type C (Figure 2c). Significant differences were found for the C3a concentrations. The highest mean concentrations of C3a, indicative of complement activation, were obtained for source plasma prepared with the Autopheresis-C system (4914 ng/ml, Figure 2d). Plasma obtained by the Haemonetics system and by the in-line filtered plasma type A had essentially the same concentration (range 200–300 ng/ml). Slightly higher values were measured for in-line filtered plasma type B (approximately 600 ng/ml) and considerably higher values for in-line filtered plasma type C (2307 ng/ml). We conclude that filtration processes during plasma preparation can induce complement activation, dependent on the filter type used.

As mentioned above, we were especially interested in determining the residual cell contents of different plasma types. For these measurements, we used a FACS procedure [37] which allows linear detection of as low as 6 platelets/µl, 8 erythrocytes/µl, and 1 leucocyte/µl. We found large differences between the plasma types in the platelet content of samples that had been frozen and thawed again. Clearly, this procedure could impact the results obtained. Assuming that these possible effects would affect all samples to the same extent, a comparative data evaluation seemed to be justified. The highest residual cell contents (mean approximately 15,000 cells/µl; Figure 3) were found for plasma obtained with the Haemonetics system and recovered plasma, both of which were prepared by a process based on centrifugation. By contrast, the three plasma types prepared from in-line filtered whole blood, which were also separated by centrifugation, had considerably lower platelet content. We therefore hypothesise that the platelets are affected by the in-line filtration procedure. Plasma prepared with Autopheresis-C had a very low platelet content, which was close to the detection limit of the FACS procedure.

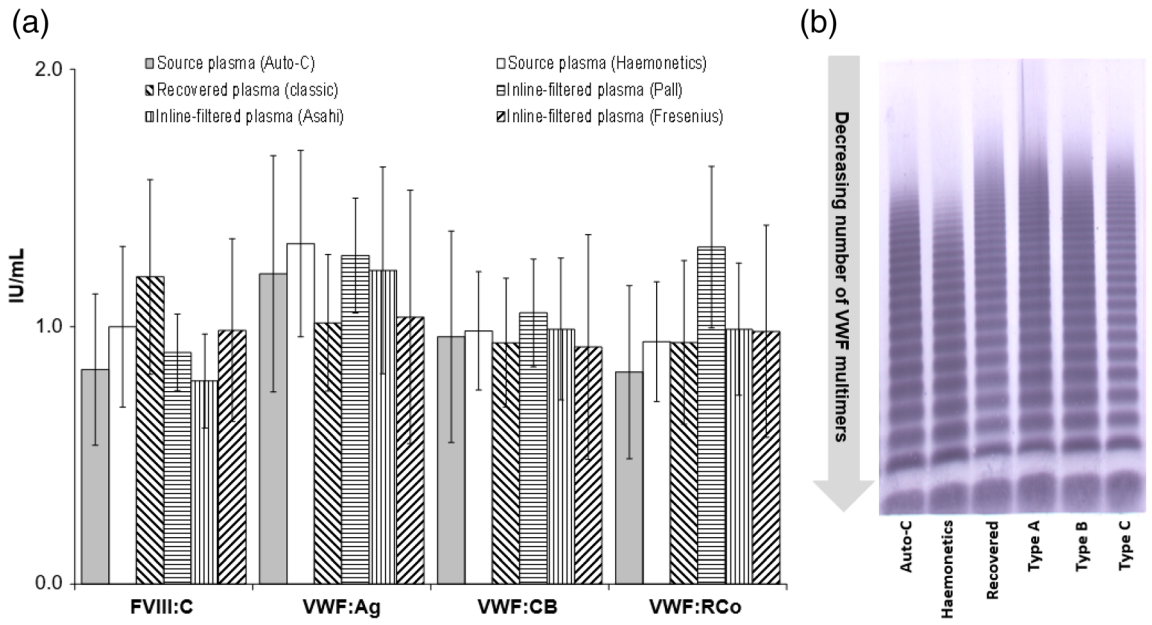

FIGURE 1 FVIII and VWF-related attributes in different plasma types. (a) The mean FVIII activities and the mean VWF-related attributes VWF:Ag, VWF:Co and VWF:CB concentrations for the six different plasma types. Error bars mark the single SD. The following numbers of single plasma samples were averaged: Source plasma (Autopheresis C), $n = 10$; source plasma (Haemonetics), $n = 10$; recovered plasma (classic), $n = 12$; in-line filtered plasma type A, $n = 10$; in-line filtered plasma type B, $n = 10$; in-line filtered plasma type C, $n = 26$. (b) The analysis of the multimeric composition of VWF with a 1% agarose gel

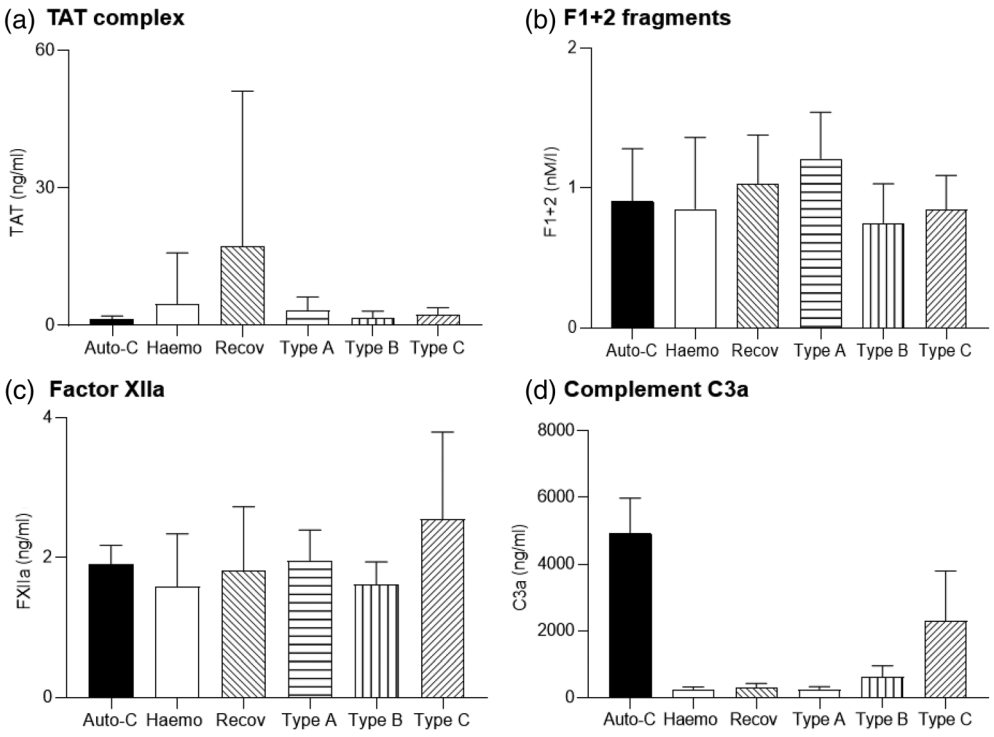

FIGURE 2 Activation markers in different plasma types. The mean concentrations of the activation markers TAT (a), prothrombin F1 + 2 (b), FXIIa (c) and complement C3a (d) are shown. Error bars mark the single SD. The following numbers of single plasma samples were averaged: Source plasma (Autopheresis C), $n = 10$; source plasma (Haemonetics), $n = 10$; recovered plasma (classic), $n = 12$; in-line filtered plasma type A, $n = 10$; in-line filtered plasma type B, $n = 10$; in-line filtered plasma type C, $n = 26$

We also found that recovered plasma had the highest leucocyte content (approximately 50 cells/μl, Figure 3). All the other plasma types showed a very low leucocyte content (<10 cells/μl). No intact erythrocytes could be detected in the different plasma preparations, although we did find damaged cells, referred to as "ghosts", to a varying extent (Figure 3) [37]. The two source plasma types had the lowest content. Recovered plasma and the three in-line filtered plasmas had substantially higher values. Measurement of the residual cell content demonstrated that plasma prepared by the Autopheresis-C system was virtually cell free (Table 2).

To investigate the influence of repeated freezing and thawing on the residual cell levels, we carried out freezing experiments with Autopheresis-C source plasma and in-line filtered plasmas type A and type C. We found that the freezing process only had a minor influence on the platelet content (Figure 4). In addition, the low leucocyte levels found (Figure 4) in the two in-line filtered plasma types before freezing indicated that the in-line filtration procedures are highly effective in reducing the leucocyte content of whole blood. Erythrocytes were destroyed by the freezing and thawing process, resulting in the detection of many erythrocyte "ghosts" after the process (Figure 4). Interestingly, the measurement LDH, a general marker for cell damage [19], demonstrated no differences between the plasma types (Table 3). Thus, the in-line filtration process seemed to have no influence on this variable.

Platelet-associated proteins in different plasma types

The residual platelet content in the plasma samples suggested that platelets were affected by the in-line filtration procedure. This prompted us to measure the content of PF4 and bTG, which are probably indicative for the level of platelet damage, in the different plasma preparations. By exerting stress on platelets by freeze-drying, which is known to disrupt cells, we demonstrated a linear relation between bTG and PF4 concentrations and the number of damaged platelets (Figure 6). In particular, a harsh lyophilisation procedure released PF4 and bTG from platelets, while the process of freezing and thawing alone was not responsible for cell damage (Figure 6).

Figure 5 and Table 3 summarize our results obtained for PF4 and bTG for the frozen and thawed samples (Table 3 also shows the LDH data). Higher values were obtained for the in-line filtered plasma types than for plasma recovered by the classic process, confirming our hypothesis that platelets are disrupted by the filtration process. The lowest PF4 and bTG concentrations were found for source plasma prepared with the Autopheresis-C system. Haemonetics source plasma had much higher values, suggesting that the centrifugation caused platelet damage by shear stress. Values obtained for source plasma (Autopheresis-C) and in-line filtered plasma type C were hardly affected by the freezing and thawing process (Figure 6), which seemed to be essential for the release of platelet-associated proteins

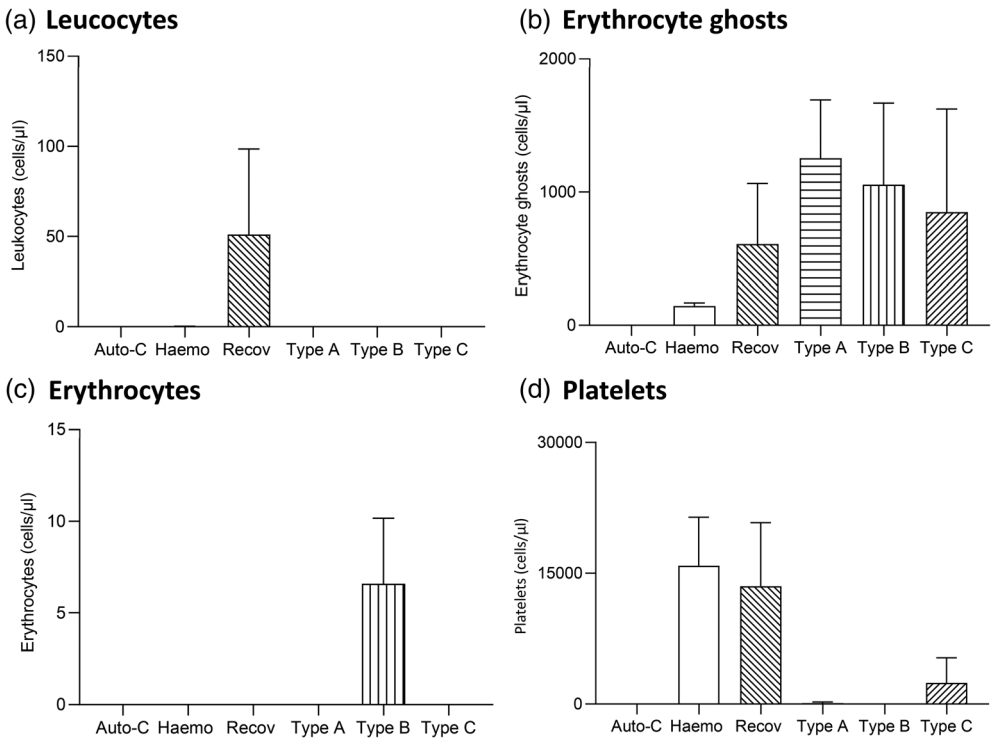

FIGURE 3 Residual cell count in different plasma types. The mean cell counts for leucocytes (a) and erythrocytes (c), while (b) shows the means for erythrocytes (ghosts) and (d) for platelets. The following numbers of single plasma samples were averaged: Source plasma (Autopheresis C), $n = 10$; source plasma (Haemonetics), $n = 10$; recovered plasma (classic), $n = 12$; in-line filtered plasma type A, $n = 10$; in-line filtered plasma type B, $n = 10$; in-line filtered plasma type C, $n = 26$

TABLE 2 Residual cell content in different plasma types

	Cells/µl (mean ± SD)			
Plasma Type	**Leucocytes**	**Erythrocytes[a]**	**Erythrocytes[b]**	**Platelets**
Source plasma (Auto-C), $n = 10$	0	0	0	8 ± 3
Source plasma (Haemonetics), $n = 10$	0.18 ± 0.14	0	143 ± 25	15 860 ± 5590
Recovered plasma (classic), $n = 12$	51 ± 48	0	610 ± 455	13 503 ± 7297
In-line filtered plasma type A, $n = 10$	0.006 ± 0.013	0	1255 ± 436	100 ± 127
In-line filtered plasma type B, $n = 10$	0	7 ± 4	1056 ± 613	3 ± 2
In-line filtered plasma type C, $n = 26$	0	0	849 ± 775	2447 ± 2861
ANOVA results ($\alpha = 0.05$)[c]	$p < 0.0001$	Not done	$p = 0.0247$	$p = < 0.0001$

Note: Remarks: Means ± standard deviations are shown. The abbreviation n.d. stands for not detected.
[a]The column shows the erythrocyte count with normal phenotype.
[b]The number of erythrocyte "ghosts" is shown.
[c]One-way ANOVA was performed followed by Tukey's multiple comparisons test using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California. Results obtained for the individual comparison of groups with the Tukey's multiple comparison test are shown in Supplemental Material.

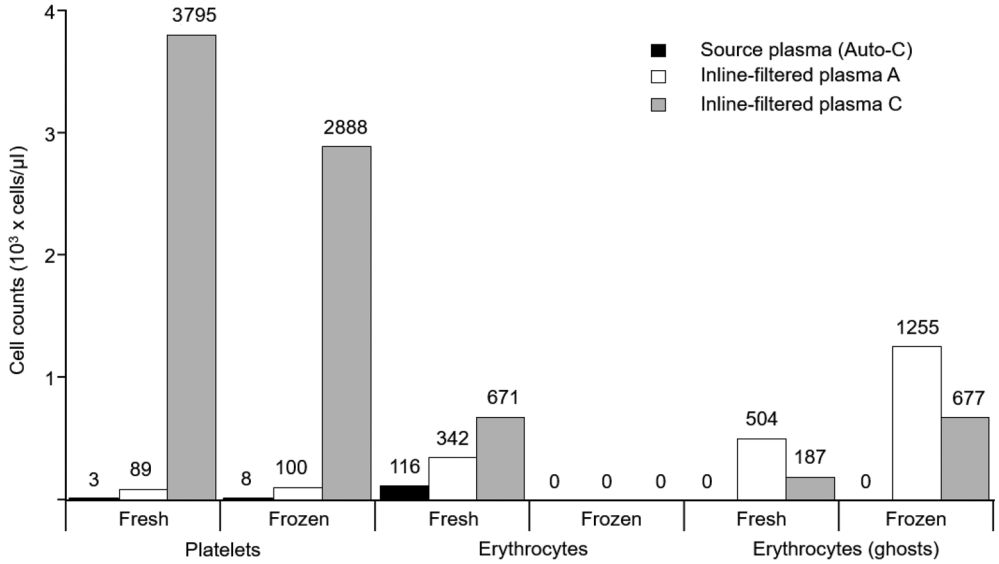

FIGURE 4 Influence of freezing-thawing on residual cell content in different plasma sources. Erythrocytes, erythrocytes (ghosts) and platelets were measured in fresh and frozen samples of source plasma (Autopheresis C) and of in-line filtered plasma types A and C

TABLE 3 Cell-associated proteins in different plasma types

Plasma Type	**LDH (U/l)**	**PF4 (IU/ml)**	**bTG (IU/ml)**
Source plasma (Auto-C), $n = 10$	113 ± 20	161 ± 85	176 ± 62
Source plasma (Haemonetics), $n = 10$	142 ± 28	630 ± 210	2145 ± 804
Recovered plasma (classic), $n = 12$	113 ± 20	239 ± 95	308 ± 116
In-line filtered plasma type A, $n = 10$	139 ± 30	1680 ± 1093	3199 ± 1572
In-line filtered plasma type B, $n = 10$	111 ± 21	420 ± 371	1129 ± 562
In-line filtered plasma type C, $n = 26$	114 ± 26	805 ± 488	1321 ± 1095
ANOVA ($\alpha = 0.05$)[a]	$p = 0.0053$	$p < 0.0001$	$p < 0.0001$
Normal physiological range	<240	0–5	10–50

Note: Remarks: Means ± standard deviations (SD) are shown.
[a]One-way ANOVA followed was performed by Tukey's multiple comparisons test using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California. Results obtained for the individual comparison of groups with the Tukey's multiple comparison test are shown in Supplemental Material.

in in-line filtered plasma type A, indicating a different mechanism of platelet disruption than for in-line filtered plasma type C (Figure 6).

Platelet function analysis with PFA 100

We investigated the influence of whole blood filtration with in-line filter type A to further confirm our hypothesis that platelets are affected by whole blood in-line filtration. We measured whole blood mean closure times of 163 s with collagen-EPI cartridges and of 95 s with collagen-ADP cartridges. These closure times were within the published reference ranges of 98–185 s for collagen-EPI cartridges and 77–133 s for collagen-ADP cartridges [42]. After filtration through the appropriate filter device, no definite closure times (>300 s) could be measured for any of the samples with either of the cartridges. This reflects the reduction in content of intact platelets resulting from the filtration process. The content, as determined by the whole blood cell counter, went down from 290,000 to 30,000 cells/µl.

Determination of blood cell-associated marker proteins in manufacturing plasma pools

The study described above was performed with a limited number of single donor plasmas and showed the variability of platelet-associated proteins PF4 and bTG in different plasma types used for fractionation. Therefore, it was of special interest to investigate if these results could be verified for manufacturing plasma pools used in the plasma

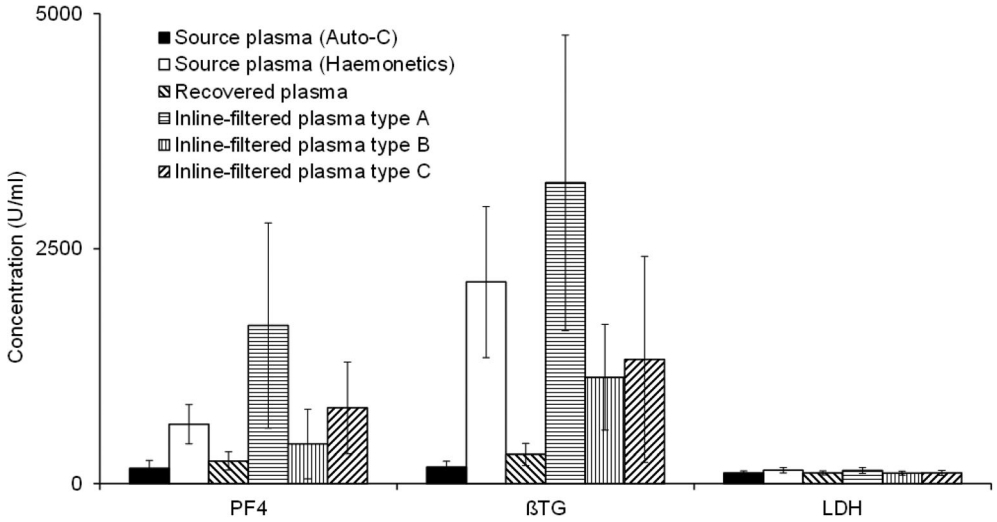

FIGURE 5 Cell-associated protein levels in different plasma types. The mean concentrations of the cell-associated proteins PF4, bTG and LDH are shown. The following numbers of single plasma samples were averaged: Source plasma (Autopheresis C), $n = 10$; source plasma (Haemonetics), $n = 10$; recovered plasma (classic), $n = 12$; in-line filtered plasma type A, $n = 10$; in-line filtered plasma type B, $n = 10$; in-line filtered plasma type C, $n = 26$

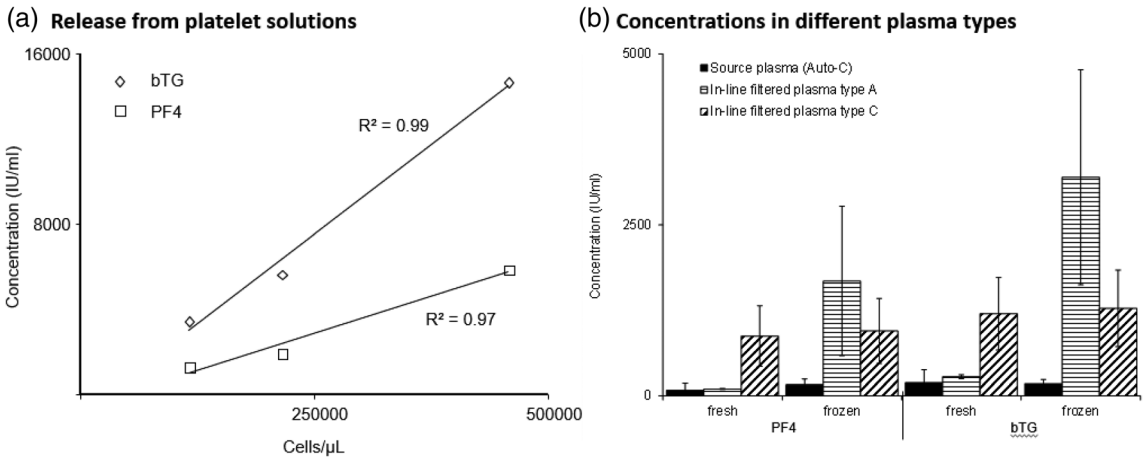

FIGURE 6 Release of PF4 and bTG after freezing and thawing. (a) PF4 and bTG levels in platelet suspensions, adjusted to 125,000, 250,000 and 500,000 cells/µl. PF4 and bTG are released by a procedure including deep freezing at −70°C, lyophilisation and reconstitution of the samples with water. (b) The concentrations of PF4 and bTG in different plasma types

TABLE 4 PMN elastase and PF4 levels in manufacturing plasma pools

Plasma Type	PMN Elastase (μg/ml)	PF4 (IU/ml)
Source plasma (Auto-C), $n = 36$	69 ± 29 (29–136)	114 ± 143 (8–508)
Recovered plasma, $n = 24$	123 ± 87 (32–453)	369 ± 336 (22–1026)
In-line filtered plasma, $n = 22$	329 ± 145 (125–677)	1023 ± 256 (600–1656)
ANOVA ($\alpha = 0.05$)[a]	$p < 0.0001$	$p < 0.0001$
Tukey's multiple comparison test: Source vs. recovered	$p = 0.0651$	$p = 0.0005$
Tukey's multiple comparison test: Source vs. in-line filtered	$p < 0.0001$	$p < 0.0001$
Tukey's multiple comparison test: Recovered vs. in-line filtered	$p < 0.0001$	$p < 0.0001$

Note: Remarks: Means ± SD values are shown. The numbers in brackets give the minimum and maximum values determined. Plasma pools of source and recovered/in-line filtered plasma correspond to 2000 to 2800 and 4200 to 6600 single plasma units, respectively.
[a]One-way ANOVA was performed followed by Tukey's multiple comparisons test using GraphPad Prism version 8.0.0 for Windows, GraphPad Software, San Diego, California.

fractionation process. Thus, we measured PF4 as a selected variable in different plasma pools of source plasma (Autopheresis-C), recovered plasma and in-line filtered plasma consisting of 2000 to 6600 single plasma units, each. As we demonstrated that platelets were affected by in-line filtration of whole blood, we decided to also determine the levels of elastase from polymorphonuclear (PMN) leucocytes as a marker for testing the integrity of leucocytes. Our results are summarized in Table 4. Like the results above, source plasma pools were shown to have very low mean PF4 levels (114 IU/ml). Higher mean values were found for recovered plasma (369 IU/ml) and in-line filtered plasma (1023 IU/ml). A similar relation between plasma type and marker protein level was found for elastase, determined and present as an inactive elastase-alpha1-antitrypsin complex. Thus, we measured mean concentrations of 69, 123, and 329 μg/ml for source, recovered and in-line filtered plasma pools, respectively. The PMN elastase values for in-line filtered plasma were substantially higher than for recovered plasma. This is a strong indicator for the fact that leucocytes are not only depleted from whole blood by the in-line filtration procedure but also that the filtration process obviously affects the integrity of the leucocytes leading to a release of PMN elastase. PMN elastase activity, however, is immediately neutralized by effective complex formation with alpha1 antitrypsin.

DISCUSSION

Currently safety and quality of plasma for fractionation focus on pathogen safety issues, while protein-specific variables (e.g. FVIII levels) in plasma for fractionation are not considered to be related to the basic need for assuring the quality and safety of plasma. In his review [43], Farrugia proposed that such variables should be restricted to arrangements between the supplier of plasma and manufacturer of its products. However, variables and standards need to be set for plasma for fractionation, preferentially on a global level, for regulatory authorities to assess the essential safety features, particularly those contributing to viral safety, independently from issues centred around the need for specific products, such as coagulation factors [6, 10–12, 23]. While for obvious reasons there are numerous publications on pathogen safety, studies on plasma quality assessed by biochemical variables are relatively rare. Consequently, in a comprehensive study, clotting factors and several critical proteins were measured and compared in plasma obtained after whole-blood filtrations with different filters and non-filtered plasma [44]. In our comparative study, we chose six different plasma types used as starting plasma for fractionation: one source plasma prepared with the Autopheresis-C and another with the Haemonetics PCS-2 system, plasma recovered by the classic method and three types of in-line filtered plasmas prepared from whole blood that had been subjected to different filter systems to reduce the leucocyte load.

We selected several biochemical and cellular markers to characterize the plasma types. Our tests for biochemical markers comprised protein content, coagulation factors FVIII and VWF, prothrombin fragment (F1 + 2) and TAT, two activation markers of coagulation [45], FXIIa, a marker of contact activation [46, 47], and complement factor C3a, a marker of complement activation [48]. In addition, we determined the residual cell content (platelets, leucocytes, erythrocytes, and erythrocyte "ghosts") by FACS analysis [37] and measured different cell-specific markers: LDH as a general marker of cell damage [19] and the platelet-associated proteins [49] PF4 and bTG, which are constituents of alpha granules [50] and are known as heparin-neutralizing proteins [51]. In another series of analyses, we measured PF4 and PMN elastase in plasma pools used for fractionation, representing more than 10,000 single plasma units.

By measuring these biochemical and cellular markers, we found that there are substantial differences between plasma types in complement activation, TAT values and, to a minor extent, FXIIa concentrations. Our results for complement activation in source plasma prepared by apheresis technology confirmed previous reports of complement activation on membranes via the alternative pathway [44, 52–54]. The most striking differences between the plasma types we investigated were, however, in the residual cell content and cell-associated proteins. This confirms previous studies reporting that source plasma prepared by the Autopheresis-C system is virtually cell-free [55].

Our results also show that leucocyte reduction of whole blood is highly effective, but the depletion procedure is accompanied by the

release of PMN elastase. This finding contrasts with an earlier study where no increase in PMN elastase levels was found after in-line filtration of whole blood by use of different filter systems [56]. However, our study confirms a more recent finding of strong neutrophil and complement activation, which depended on the type of filter and whole-blood storage conditions [44]. The authors of this study concluded that despite the considerable elastase release by whole-blood filtration, as detected by the increase in elastase-antitrypsin complexes, the plasma showed no sign of proteinase activity due to a sufficient inhibitory antitrypsin activity of a healthy donor. Additionally, our data demonstrate that the integrity of leucocytes is obviously strongly affected by the filtration process with not only activation of neutrophil and the subsequent release of elastase during the filtration process, but also a complete disruption of cells. Several studies investigated how prions, which are believed to cause vCJD, can be eliminated during the manufacture of pharmaceutical products derived from human blood [57, 58]. There is evidence to indicate that any infectivity present may be reduced during these processes [59]. The implication of lymphoid tissues and lymphocytes in the peripheral pathogenesis of prion disease was the rationale for introducing leucodepletion of blood donations to reduce possible vCJD transmission by blood components [60]. However, this practice, although implemented in many countries and mandatory in some, was questioned by some authors who believed at best it was only hypothetically suitable for preventing the transmission of vCJD and there had already been a vague discussion concerning potential risks of leucodepletion such as increased leakages, which had not been investigated empirically [61, 62]. As our results indicate that the in-line filtration process destroys cells instead of or in addition to removing them, it seems questionable if any benefit can be gained by removing leucocytes, which would also remove leucocyte-associated proteins and pathogens including vCJD. The impact of in-line filtration on blood cells is furthermore reflected by both the platelet counts in whole blood and the functional characterization of platelets in the PFA-100 system. Intact platelets were reduced substantially to about 10% of the value before in-line filtration and the reduced number of functionally active platelets was ineffective to obtain full occlusion of a collagen-coated capillary. However, our results show that the passage of blood through leucocyte reduction filters not only removes but disrupts the remaining platelets and promotes the release of the platelet-associated proteins PF4 and bTG. Similarly, an increase in bTG concentrations has been observed after in-line filtration [63]. In our study we used an additional platelet protein marker, PF4, which is an alpha-granule protein [50] that originates from disrupted platelets. In contrast to our findings, another group found no increase in bTG values by in-line filtration of whole blood [56], suggesting differences in filtration conditions might play a role in this process. Despite the effects observed on cellular blood components and marker proteins derived from them, most of the plasma separation methods did not affect the proteins of the VWF complex. This is remarkable because VWF with a maximum molecular size of ≥20 million Dalton [64] is not only the largest circulating soluble plasma protein, and consequently difficult to filter [65], but is also subject to specific shear-dependent degradation by ADAMTS13, a metalloprotease [66–69]. Nevertheless, the largest VWF multimers were not lost due to the separation process. There was one exemption: The PCS-2 system, which showed a somewhat lower content of the high molecular weight VWF multimers. There is currently no explanation for this and to find one further detailed analysis would be required. However, our result is in-line with that found in a previous study, where the VWF content of normal plasma pools was compared with plasma obtained by five different apheresis procedures and where VWF:Ag, VWF:RCo and 11–15 VWF multimers were well preserved in all plasma units from each of the five apheresis procedures [70]. Nevertheless, the highest molecular weight forms of VWF are missing in all plasma products as a consequence of co-purification of VWF with its cleaving protease ADAMTS13, which degrades VWF in plasma starting during apheresis and continuing throughout the fractionation process. In addition, our study shows that the filtration process of whole blood can lead to activation of the complement system, as indicated by an increase in C3a concentrations, regardless of whether the process is performed in-line or by a frequently used filtration-based plasmapheresis system [54].

So far, most studies on starting plasmas for fractionation have concentrated on the freezing process and its possible influence on the levels of coagulation factors [41, 71, 72]. Only a few have investigated the influence different plasma types have on the manufacturing of therapeutic coagulation proteins. With respect to the high sensitivity of the FVIII molecule to proteolysis, the measure of the coagulation activation marker fibrinopeptide A was introduced as a criterion for determining the quality of plasma as a source of factor VIII [73]. Some data suggest that FVIII degradation products present in plasma of different qualities, in addition to other factors, might lead to increased immunogenicity of FVIII concentrates [74], and molecular modifications have been detected in factor VIII concentrates produced from different plasma pools [75]. Other investigators described the effects of the plasma collection systems and processing variables on the quality of factor IX concentrate and found an increased thrombogenic potential in plasma prepared by centrifugation [7]. Furthermore, different qualities of plasma for fractionation were found in one report, where the investigators recommended avoidance of platelet contamination and platelet activation [8]. These publications underline the importance of minimizing the residual cell content, especially platelets, containing negatively charged phospholipids, which can serve as procoagulatory surfaces [76]. If the residual platelet content is not minimized, unwanted side effects such as the promotion of activation phenomena [77] or thrombogenicity problems [7, 8] may occur. In addition, it should be emphasized that the anticoagulant efficacy of heparin added in some manufacturing processes might be impacted by PF4. However, as the neutralization of 1 IU heparin requires about 16,000 IU PF4 [78], this seems to be an important but only theoretical concern, as such high PF4 levels were not observed in our study.

In summary, our findings indicate that besides several specific tests, the platelet surrogate markers bTG and PF4 might be valuable for comparing plasma sources, especially for evaluating new or modified devices for plasma collection and for monitoring biochemical plasma quality.

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CONFLICT OF INTEREST

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SUPPORTING INFORMATION

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