Microparticles variability in fresh frozen plasma: preparation protocol and storage time effects

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Background. Extracellular vesicles or microparticles exhibiting procoagulant and thrombogenic activity may contribute to the haemostatic potential of fresh frozen plasma.

Materials and methods. Fresh frozen plasma was prepared from platelet-rich plasma at 20 °C (Group-1 donors) or directly from whole blood at 4 °C (Group-2 donors). Each unit was aseptically divided into three parts, stored frozen for specific periods of time, and analysed by flow cytometry for procoagulant activity immediately after thaw or following post-thaw storage for 24 h at 4 °C. Donors' haematologic, biochemical and life-style profiles as well as circulating microparticles were analysed in parallel.

Results. Circulating microparticles exhibited a considerable interdonor but not intergroup variation. Fresh frozen plasma units were enriched in microparticles compared to plasma *in vivo*. Duration of storage significantly affected platelet- and red cell-derived microparticles. Fresh frozen plasma prepared directly from whole blood contained more residual platelets and more platelet-derived microparticles compared to fresh frozen plasma prepared from platelet-rich plasma. Consequently, there was a statistically significant difference in total, platelet- and red cell-derived microparticles between the two preparation protocols over storage time in the freezer. Preservation of the thawed units for 24 h at 4 °C did not significantly alter microparticle accumulation. Microparticle accumulation and anti-oxidant capacity of fresh frozen plasma was positively or negatively correlated, respectively, with the level of circulating microparticles in individual donors.

Discussion. The preparation protocol and the duration of storage in the freezer, independently and in combination, influenced the accumulation of microparticles in fresh frozen plasma units. In contrast, storage of thawed units for 24 h at 4 °C had no significant effect on the concentration of microparticles.

Keywords: fresh frozen plasma, microparticles, extracellular vesicles, storage, prothrombotic activity.

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Introduction

Extracellular vesicles or microparticles (MP) are small membrane particles ubiquitously released by cells under physiological or pathological conditions. They are found in biological fluids at concentrations relevant to the functional state of the secreting cells[1,2]. Their composition varies considerably, depending on the type of cell and stimulus, as well as on donor-related factors[3-5]. MP represent an important mode of intercellular communication required in processes such as immune regulation, coagulation and inflammation[6,7]. Plasma MP are generated by all circulating blood cells and by the endothelium. They may feature phosphatidylserine (PS), tissue factor and highly adhesive surfaces through which they bind to endothelium, blood cells and matrix molecules. Their catalytic surface promotes the assembly of circulating procoagulant proteins and stimulates coagulation reactions leading to the production of thrombin[8]. As a result, the majority of released MP are thought to fulfill a haemostatic function under physiological conditions, with possible deviation towards thrombosis when produced in excess.

Microparticles represent an intrinsic component of all the blood products used for transfusion[9]. They accumulate in red blood cell (RBC) and platelet (PLT) concentrates during their storage[10]. On account of their reactivity, they have been implicated in neutrophil activation, immunomodulation and thrombotic complications

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associated with the negative clinical outcome of transfusions[9,11,12]. On the other hand, the objective of fresh frozen plasma (FFP) transfusion is to maintain the coagulation parameters in patients with acquired multiple coagulation factor deficiencies and severe bleeding after injury[13]. The ability of FFP to generate thrombin and form a clot is the cumulative result of many intrinsic components, including coagulation factors, calcium and procoagulant phospholipid surfaces, all of which are primarily involved in the assembly of coagulation complexes and coagulation activation[14].

It has been reported that factor VIII levels do not predict the haemostatic potential of FFP[15]. On the basis of their probable role in haemostatic response, MP might serve as an independent factor for predicting the quality of FFP. The aim of this study was the characterisation of the MP present in the two standard FFP preparations used for transfusions, namely in FFP prepared from platelet-rich plasma at 20 °C and in FFP prepared directly from whole blood at 4 °C. The cellular origin and concentration of MP were determined as a function of FFP storage duration immediately after thaw or following post-thaw storage for 24 h at 4 °C.

Materials and methods

Blood donors and study design

Twelve male regular blood donors were studied both *in vivo* and at FFP level. Six of them donated blood to prepare FFP according to the first protocol studied (Group-1, FFP-1) and 6 of them according to the second protocol (Group-2, FFP-2) (Figure 1). Thorough examination *in vivo* verified that there were no significant base-line differences between groups with respect to their haematologic profile, lifestyle, ABO classification and circulating MP levels. A pair-study strategy (preparation of FFP-1 and FFP-2 products from the same donor) was not followed since the examination of the storage duration effect on MP accumulation presupposes splitting the FFP units. Indeed, to avoid repeated freeze-thaw cycles that affect MP generation[16], the FFP units were aseptically divided into three bags, promptly frozen for 6 (one bag) or for 12 (two bags) months at minus 20-25 °C and examined immediately after thaw or following post-thaw storage for 24 h at 4 °C. To verify that the FFP volume (total or split) had no effect on the MP accumulation inside the post-thaw stored units, eighteen additional whole-volume FFP units (nine for each protocol) already stored for 12 months in the freezer were also examined (insert in Figure 1); *in vivo* haematologic and biological data were not available. The study was conducted in accordance with the principles of the Declaration of Helsinki and was approved by the Research Bioethics and BioSecure Committee of the Faculty of Biology, University of Athens. All subjects gave written consent prior to their participation in the study and filled out a life-style questionnaire.

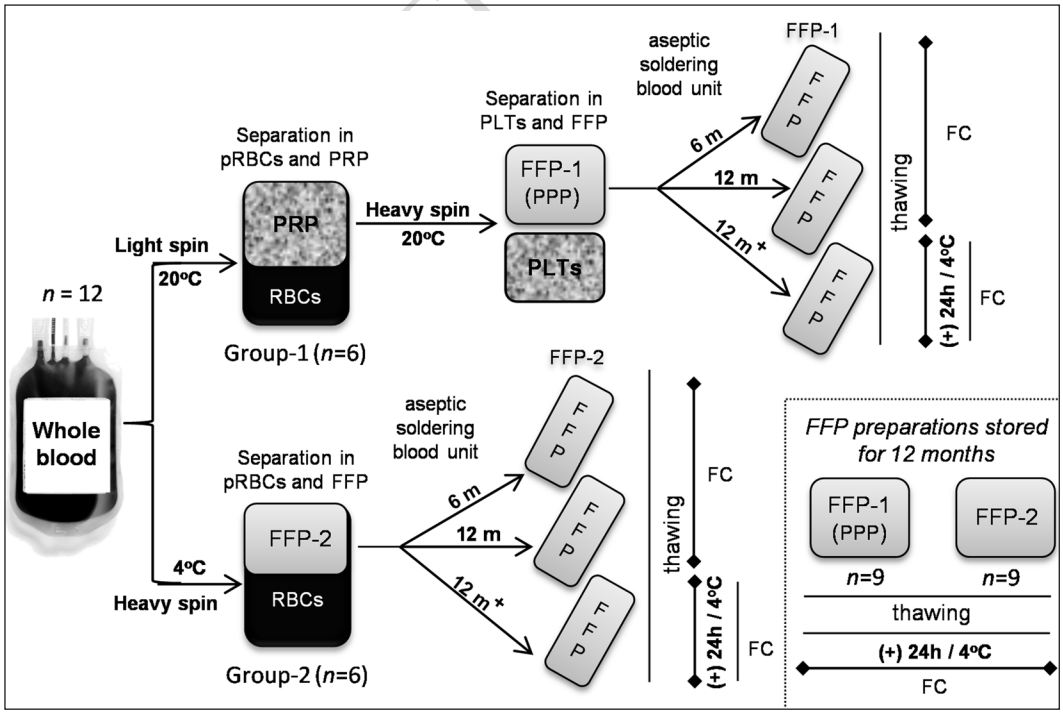

Figure 1 - Study plan and the two FFP preparation protocols examined.
RBCs: red blood cells, FFP: fresh frozen plasma, PLTs: platelets, PRP: platelet-rich plasma, PPP: platelet-poor plasma, FC: flow cytometry.

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Haematologic and serum biochemical analysis

To define the base-line haematologic profile of the donors involved in the study, whole blood was anti-coagulated with either EDTA or citrate standard anticoagulant mixtures. Blood cell counts and indexes were measured using an automatic blood cell counter (Sysmex K-4500; Roche, Indianapolis, IN, USA). Biochemical analysis of serum factors (including lipid and iron homeostasis parameters and electrolytes) was performed using automatic analyzers: Hitachi 902, 9180 and Elecsys Systems Analyzer (Roche).

Fresh frozen plasma preparations

An average 465 mL of blood was collected into 63 mL of CPDA-1 (citrate-phosphate-dextrose-adenine) anticoagulant. Clinical-grade FFP was prepared according to the standard blood banking protocols[17] either after the isolation of PLT concentrates at 20 °C (FFP-1, double-spin protocol: i) 2,000×*g* for 5 min and ii) 4,300×*g* for 10 min) or directly from whole blood units at 4 °C (FFP-2, single spin protocol: 4,500×*g* for 15 min) (referred to as platelet-rich-plasma or platelet-poor-plasma methods, respectively) within 8 h from blood donation (Figure 1). The supernatant plasma was squeezed off using a plasma expressor (Fenwall Laboratories, Deerfield, IL, USA), split in three bags (Figure 1), and promptly frozen for 6-12 months at minus 20-25 °C. FFP was thawed in a water bath for 10-20 min at 30-37 °C according to standard American Association of Blood Banks (AABB) operating procedures, and analysed immediately after thawing (two aliquots) or following 24 h-storage at 4-6 °C (one aliquot). Whole-volume FFP units stored for 12 months in the freezer and for 24 h at 4-6 °C post thaw were also examined (n=18).

Flow cytometry

Flow cytometry analysis of circulating MP was immediately performed in plasma produced from citrated blood, after a double 2,500×*g* spin at 20 °C, within 15 min of venipuncture. To avoid sources of pre-analytical variability[16], and to replicate as far as possible clinical routine, MP analysis in FFP was performed in units subjected to strictly one freeze-thaw step within 15 min from thawing, without an intermediate MP isolation step. MP counts and phenotypes were analysed as previously described[14,18]. Briefly, MP were identified by their size (<1 μm), exposure of cell-specific markers and annexin-V (AnnV) binding (PS^+). Although PS negative MP are also found in circulation[19], it has been shown that the phospholipid-dependent procoagulant activity is limited to the AnnV-binding subpopulation of MP[19]. MP were double stained with AnnV (PE Annexin V Apoptosis Detection Kit I, 559763) and one of three monoclonal antibodies CD235a-FITC (clone GA-R2, HIR2, 559943), integrin-α2b-FITC (CD41a, clone HIP8, 555466) or CD45-FITC (clone HI30, 555482, from BD Biosciences, San Jose, CA, USA) to identify PS^+ RBC-derived MP (RMP), PLT-derived MP (PMP) and leukocyte-derived MP (LMP), respectively. Fresh plasma or FFP aliquots (10 μL) were re-suspended in AnnV binding buffer containing 2.5 mM $CaCl_2$. After addition of 2.5 μL of PE-AnnV plus 2.5 μL of a cell-specific and FITC-conjugated monoclonal antibody, samples were incubated in the dark for 15 min at room temperature and the reaction was stopped by the addition of 400 μL of binding buffer. The samples were analysed within 30 min in a FACScan flow cytometer (Beckton Dickinson, San Jose, CA, USA). Data from at least 100,000 events (1-2 min) were acquired and analysed with the use of CELL Quest Software (Becton Dickinson). Forward scatter (FSC) and side scatter (SSC) were set at a logarithmic gain. To define MP gate, 0.5 μm, 0.9 μm and 3 μm fluorescent bead mix was used according to the manufacturer's instructions (Megamix; Biocytex, Marseille, France) and the recommendations from the International Society on Thrombosis and Haemostasis SSC Collaborative workshop[20]. TruCount™ beads (340334, BD Pharmingen, San Jose, CA, USA) were used for quantification. The specificity of the monoclonal antibodies was verified by using identical concentrations of isotype-matched control antibody (FITC mouse IgG1κ 555748; BD Biosciences, San Jose, CA, USA). CaliBRITE beads were used to adjust instrument settings, set fluorescence compensation, and check for instrument sensitivity (BD CaliBRITE 3 BEADS, 340486).

Microparticle procoagulant activity assay

Microparticle-associated procoagulant activity was estimated through thrombin generation measurement using a functional Elisa assay kit (Zymuphen MP-activity, Hyphen BioMed, Neuville-sur-Oise, France). Briefly, plasma samples supplemented with calcium, Factor Xa, and thrombin inhibitors were added to microplate wells pre-coated with streptavidine and biotinylated AnnV. A factor Xa-Va mixture followed by prothrombin was introduced to the microplate wells. PS^+ MP in the presence of calcium allow FXa-FVa to activate prothrombin into thrombin, which was measured by the detection of a chromogenic substrate at 405 nm (nM of PS equivalents).

Total antioxidant capacity of the plasma

Total antioxidant capacity (TAC) of freshly isolated and FFP plasma was measured by the ferric reducing ability of plasma (FRAP) assay, as previously described[21]. To determine the uric acid-independent antioxidant capacity, plasma aliquots were treated with 0.005 U of uricase and processed, as previously described[22].

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Statistical analysis

Repeated measures analysis of variance (ANOVA) was used to determine statistical differences between the time points tested. Mixed repeated measures ANOVA was used to test the possible interaction between duration of storage and FFP preparation. Bonferroni adjustment for multiple comparisons was applied for each of the abovementioned approaches. Differences between the FFP groups in each time point were identified using one-way ANOVA. Comparisons between samples stored for 12 months and 24 h pre- and post-thaw, respectively, were made by paired *t*-test. After testing all parameters for normal distribution profile, Pearson's and Spearman's (where needed) correlation tests were used to determine correlation coefficients (*r*). $p<0.05$ was considered significant.

Results

Base-line microparticle levels and donor characterisation *in vivo*

Group-1 and Group-2 donors exhibited normal average haematologic and serum biochemical profiles (Table I); however, aberrant lipids, MPV and iron were occasionally detected. In addition, evaluation of the questionnaires revealed a common average lifestyle for Group-1 and Group-2 volunteers without any statistically significant deviation between them at baseline (Table II). Total antioxidant capacity (TAC) of the plasma varied within normal range and there was no significant difference between groups (Table I). Normal levels of circulating MP and MP-associated procoagulant activity (MP-PA) (Table I) were measured in all volunteers under examination. In spite of the considerable interdonor variation, no intergroup deviation was observed in MP levels (Figure 2A). As expected[23], the vast majority of MP were of PLT and RBC origin (Figure 2A). RMP concentration was positively correlated with donor age (Figure 2B) and monocyte count (Table III). PMP count was positively correlated with triglycerides, though this was not significant. Taken together, the two groups of donors used in the study of the FFP-1 and FFP-2 preparations were evenly matched by age, gender, haematologic profile, MP levels and MP-associated procoagulant activity at baseline.

Fresh frozen plasma properties as a function of preparation method and storage duration

Residual cells and antioxidant capacity: analysis of FFP preparations before freezing, revealed a negligible amount of residual RBC and WBC but significantly more residual PLT, especially in the FFP-2 units (43 2×10^3/μL *vs* 9 1×10^3/μL in FFP-1; $p<0.05$). The average cell size of the residual PLT was similar in both settings (MPV 8.1±1.7 and 7.7±0.6 fL and PDW 17.9±0.9 and

Table I - Base-line characteristics of the donors under study.

Characteristic	Group-1 Total n=6	Group-2 Total n=6	Normal range
Gender	*Male*	*Male*	-
Age (years)	34.0±5.8	33.7±3.8	-
ABO blood group	A: 3, O: 3	A: 3, O: 3	-
White blood cells (10^3/μL)	6.7±1. 4	8.8±1.1	4.0-10.0
Neutrophils (10^3/μL)	4.3±1.3	5.4±0.8	1.7-7.7
Lymphocytes (10^3/μL)	2.2±0.6	2.8±0.3	0.4-4.4
Monocytes (10^3/μL)	0.5±0.1	0.6±0.2	0.0-0.8
Red blood cells (10^6/μL)	4.93±0.23	4.65±0.18	3.80-5.30
Haemoglobin (g/dL)	16.2±0.9	15.1±0.5	13.5-18.0
Haematocrit (%)	43.3±1.9	42.1±1.6	42.0-50.0
MCV (fL)	85.7±2.8	86.4±3.2	80.0-100.0
MCH (pg)	32.0±1.2	30.9±1.4	27.0-32.0
MCHC (g/dL)	37.0±0.8	35.8±1.5	32.0-36.0
RDW (%)	13.8±0.4	14.2±0.2	10.0-16.5
Platelets (10^3/μL)	200±37	275±68	150-380
Mean platelet volume (fL)	10.3±0.7	10.0±1.2	5.0-10.0
Platelet distribution width (%)	14.1±1.7	12.2±1.9	12.0-20.0
Glucose (mg/dL)	91±11	91±21	65-110
Urea (mg/dL)	31±9	20±7	10-50
Creatinine (mg/dL)	0.46±0.08	0.36±0.01	0.31-1.11
Uric acid (mg/dL)	6.7±0.2	6.1±1.1	3.5-7.2
Triglycerides (mg/dL)	144±19	187±56	10-150
Cholesterol (mg/dL)	230±47	227±8	140-200
HDL (mg/dL)	51±19	42±8	37-70
LDL (mg/dL)	89±38	132±4	<130
Iron (mg/dL)	138±84	116±70	35-150
TIBC (mg/dL)	344±58	324±45	260-390
Ferritin (ng/mL)	100±38	122±41	18-270
Transferrin (mg/dL)	278±52	289±76	200-400
Albumin (g/dL)	4.4±0.3	4.3±0.2	3.5-5.5
Total proteins (g/dL)	7.7±0.2	7.2±0.1	6.4-8.2
Calcium (mg/dL)	9.5±0.4	9.5±0.2	8.6-10.0
Phosphorus (mg/dL)	3.6±0.5	3.5±0.4	2.5-4.9
Potassium (mmol/L)	3.9±0.3	4.2±0.5	3.6-5.1
Sodium (mmol/L)	141±1	142±1	135-145
AST (U/L)	30±10	24±12	5-40
ALT (U/L)	33±25	26±7	7-56
γGT (U/L)	40±40	46±45	5-85
Alkaline phosphatase (U/L)	107±42	87±43	17-142
Plasma TAC (μM Fe_2^+)	1,069±92	950±50	984±143
MP-PA (nM of PS equivalents)	1.09±0.50	2.63±1.47	<5

Mean±SD. MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; RDW: RBC distribution width; HDL: high density lipoproteins; LDL: low density lipoproteins; TIBC: total iron binding capacity; AST: aspartate transaminase; ALT: alanine transaminase; γGT: gamma-glutamyl-transferase; TAC: total antioxidant capacity; MP-PA: MP-associated procoagulant activity; PS: phosphatidylserine.

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Table II - Life-style characteristics of the donors under study.

Characteristic (n. of donors)	Group-1	Group-2
	Total n=6	Total n=6
Smokers	3	4
3 years of smoking	3	4
20 cigarettes/day	0	1
Athletes	1	0
Physical activities > once/week	4	2
Regular alcohol consumption	3	4
3 beverage serving/week	0	1
3 red meat meals/week	6	3
3 junk food meals/week	0	1

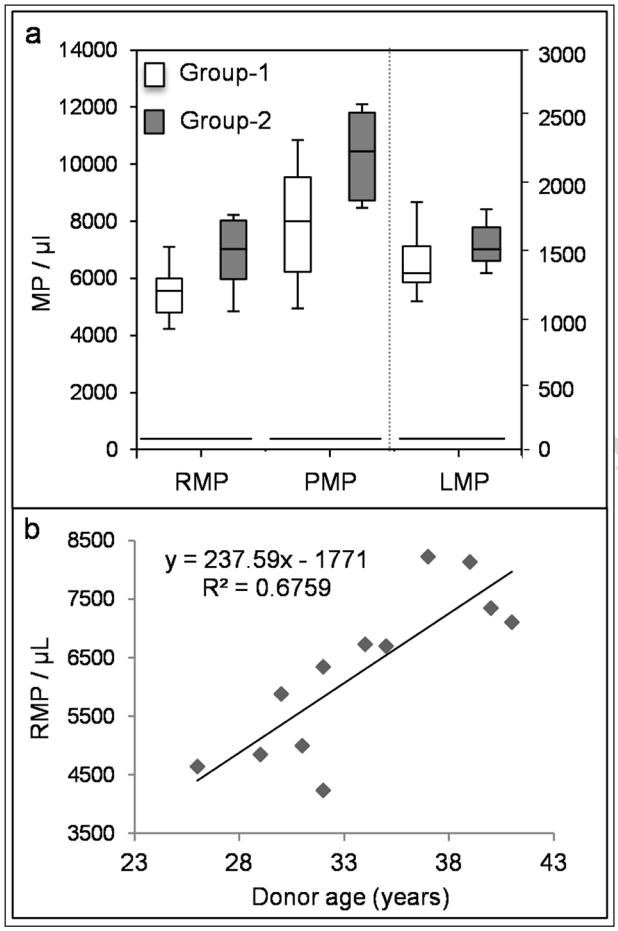

Figure 2 - Circulating MP analysis in donors under study (n=12).

(A) Box plots representing the flow cytometry results of baseline RMP, PMP and LMP concentration in Group-1 (n=6) and Group-2 (n=6) donors. MP/µL = MP counts per µL of plasma *in vivo*. (B). RMP level *in vivo* was strongly correlated with donor age (years). Scatter-plot presentation of the paired-values dispersion for the two parameters.

MP: microparticles, PMP: platelet-derived microparticles, RMP: RBC-derived microparticles, LMP: leukocyte-derived microparticles.

16.3±0.8%, respectively). The average total antioxidant capacity (TAC) of the FFP units (887±43 and 872±38 µM of Fe^{+2} equivalents for FFP-1 and FFP-2, respectively), exhibited a slight reduction compared to *in vivo* levels (Table I). Measurement was below normal range (805 µM Fe^{2+} equivalents). In only a few cases of units that had been stored for a long period of time. The antioxidant capacity of the FFP units was negatively correlated with the levels of circulating MP (Table III).

Microparticle analysis: in order to define the effect of: i) FFP preparation protocol; ii) duration of FFP storage in the freezer; and iii) the combined effect of both variables, we performed mixed repeated measures ANOVA measures using duration of storage and FFP protocol as within and between subjects variable, respectively. Within subjects analysis showed a significant effect of storage duration on total MP [$F(1.3, 13)=49.35$, $p<0.001$], RMP [$F(1.1, 11)=30.03$, $p<0.001$] and PMP [$F(1.32, 13.2)=11.91$, $p<0.01$], but not on LMP accumulation. Between groups analysis showed a significant effect of FFP preparation method on total MP [$F(1, 10)=12.50$, $p<0.01$] and PMP [$F(1, 10)=7.20$, $p<0.05$] but not on RMP and LMP accumulation in FFP. Regarding the combined effect of both variables, mixed analysis revealed a significant interaction between them in terms of total MP [$F(2, 9)=17.98$, $p<0.001$], RMP [$F(2, 9)=4.84$, $p<0.05$ (marginal)] and PMP [$F(2, 9)=4.85$, $p<0.05$], but not on LMP generation. This result represented a statistically significant difference between FFP-1 and FFP-2 units in total MP, RMP and PMP concentration over time.

Table III- Microparticle correlations *in vivo* and in fresh frozen plasma.

Variable 1	Variable 2			Variable 2		
In vivo	In vivo	*r-value*	*p-value*	*FFP*	*r-value*	*p-value*
Total MP	PMP	0.920	0.000	Total MP	0.769	0.003
	RMP	0.816	0.001	PMP	0.713	0.009
				RMP[a]	0.944	0.000
				TAC	−0.766	0.004
PMP				Total MP	0.748	0.005
				PMP	0.818	0.001
				TAC	−0.602	0.038
				RMP[a]	0.741	0.006
RMP	Donor age	0.822	0.027	Total MP	0.727	0.007
	Monocytes	0.755	0.005	RMP	0.757	0.004
				TAC	−0.748	0.005
Monocytes				RMP	0.765	0.004

N=12. [a] long-stored FFP units; r-value: Pearson's correlation coefficient. FFP: fresh frozen plasma; MP: microparticles; PMP: platelet-derived microparticles; RMP: red blood cell-derived microparticles; TAC: Total antioxidant capacity.

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The preparation and storage of FFP promoted the generation of MP compared to the *in vivo* levels, especially those of PLT and white blood cell origin, irrespectively of the FFP storage duration (Figure 3). As expected[24], a wide interdonor variability in the FFP MP concentration was clearly seen and PMP represented the prevalent MP subpopulation in FFPs, especially in the FFP-2 units. In general, FFP units stored for 12 months contained more PMP and RMP compared to those stored for 6 months. With the exception of the RMP concentration in FFP-2 units, however, this increase was not statistically significant. Comparison of the FFP preparation methods over time revealed that the FFP-2 contained more PMP and consequently more total MP compared to the FFP-1 at any time point during storage. In addition, FFP-2 units stored for 12 months contained statistically more RMP ($p<0.05$) compared to FFP-1 units that had been stored for the same period of time (Figure 3). LMP were estimated at similar levels in the two FFP preparations irrespective of the storage period.

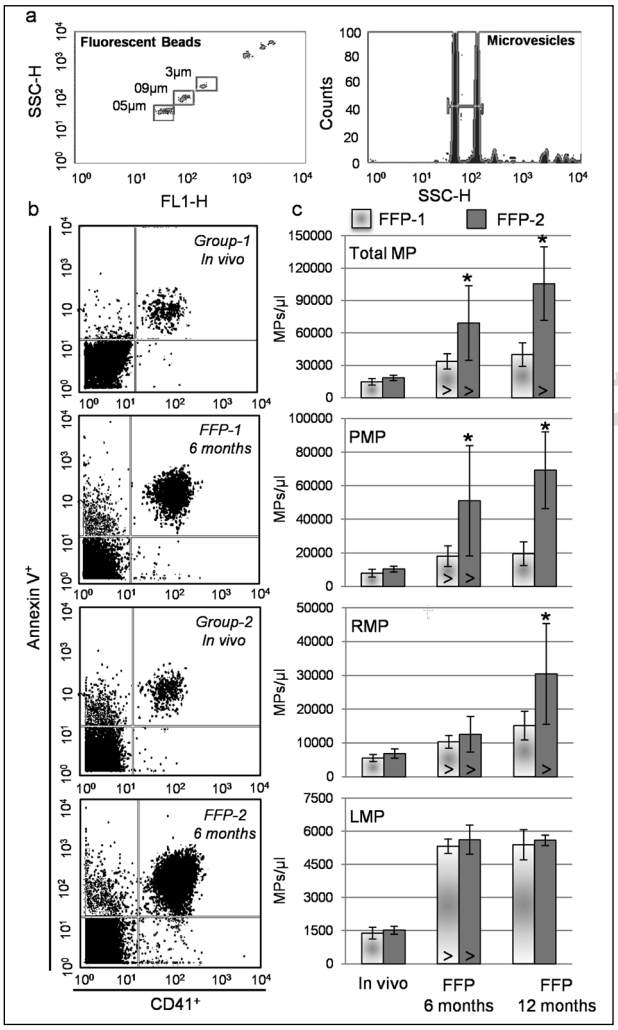

Figure 3 - Flow cytometry analysis of MP in FFP-1 and FFP-2 units after storing for 6 or 12 months in the freezer *vs* circulating (*in vivo*) MP count.

(A) MP gate size was set with the help of fluorescent 0.5, 0.9 and 3.0 μm fluorescent beads. (B) Representative FACS plots of CD41-FITC and AnnV-PE double labelled MP *in vivo* and in FFP. Background level for fluorescence intensity for FITC channel was established under control conditions. (C) Flow cytometry analysis results (n=12). * $p<0.050$ FFP-1 *vs* FFP-2. (>): $p<0.050$ *vs* the previous time point

Consistent with the flow cytometry measurements[18], the FFP-2 units contained significantly more PS equivalents (as measured by ELISA) compared to the FFP-1 units (77.33±24.05 *vs* 29.33±11.83 nM of PS equivalents, respectively, $p<0.010$). A number of interesting correlations between the circulating and FFP MP are shown in Table III. In agreement with our findings in fresh plasma, RMP accumulation in FFP units was positively correlated with the donor-specific monocyte count.

Finally, no statistically significant differences were observed in the MP count of FFP units stored for 12 months when analysed immediately after thawing or following 24 h preservation at 4 °C (n=12), although slightly lower MP levels were measured in a large proportion of units post storage. This result was verified in whole-volume (no split) FFP units stored for the same period of time before and after thaw (n=18) (insert in Figure 1). An overview of the results of these experiments (n=30) is shown in Figure 4.

Discussion

Microparticle release *in vivo*

Circulating MP may vary significantly in number and composition[4] among healthy subjects in relation to factors such as gender, diet or smoking[6]. Although there was no statistical difference between donors in this study according to these and other parameters, the interdonor variability in circulating MP levels was still high, confirming the complexity of the factors orchestrating the MP release. According to our results, increasing donor age might be a factor that promotes RMP generation. Although a statistical correlation does not imply causation, it is physiologically relevant, since RBC exovesiculation has been functionally associated with cellular ageing and oxidative stress lesions that exhibit age-dependence in healthy humans[25,26]. Nevertheless, this finding needs to be verified with a larger donor group, exhibiting a more extended age range than the one currently involved.

Microparticle accumulation in FFP represents the sum of the circulating MP and those derived by the contaminating cells. Our donors exhibited no intergroup differentiation in MP concentration *in vivo* or in haematologic and life-style parameters that might be related to the vesiculation degree. We cannot eliminate the possibility that chylomicrons and lipoprotein particles may also contribute to scatter, especially in

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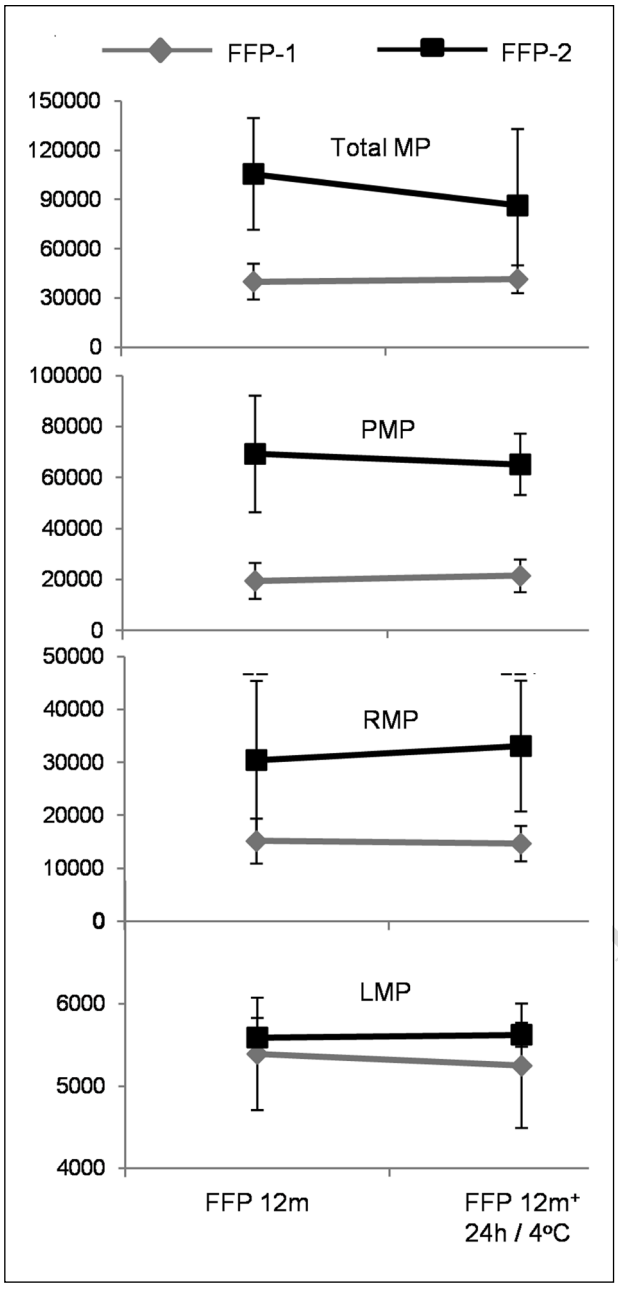

Figure 4 - Flow cytometry results of MP concentration in FFP-1 (n=15) and FFP-2 (n=15) stored for 12 months in the freezer, with or without further storage for 24 h at 4 °C (FFP 12m^{+}).
FFP: fresh frozen plasma; MP: microparticles; PMP: platelet-derived microparticles; RMP: red blood cell-derived microparticles; LMP: leukocyte-derived microparticles. Bars: standard deviation.

donors exhibiting high lactate dehydrogenase lipoprotein or triglyceride levels. Triglyceride levels positively correlated with the PMP level *in vivo*, as previously reported[27], but this correlation was not significant, and furthermore, no intergroup difference in the PMP level was observed at baseline.

Microparticles in fresh frozen plasma preparations

As previously suggested[28], lack of substances normally provided by the vascular endothelium promotes MP shedding from blood cells *ex vivo*. Not only the number, but also the cellular origin of MP in FFP differ from those in peripheral blood in association with the FFP preparation protocol and storage conditions[14,18,29]. In our study, the variability observed between the two FFP preparations cannot be attributed to base-line differences but rather to the protocols followed and protocol-associated biological and non-biological stimuli for MP generation.

Centrifugation conditions considerably affect MP measurements[16,30], mainly through cellular contamination[31] and shear stress, an important mechanical factor inducing MP release from platelets and other cells[32,33]. The single, high-speed centrifugation used to prepare the FFP-2 may have induced microvesiculation of blood cells prior to their removal from the suspension. The two products contained similarly low numbers of residual RBC and leukocytes, but higher[18] and unequal concentrations of residual PLT. According to previous studies, accumulation of MP highly correlates with the number of contaminating platelets in both FFP and cryoprecipitate[33]. The different composition of FFP-2 in residual PLT may account for higher PMP concentration compared to the PLT-poor FFP-1 preparation. Although George *et al.*[34] have reported lower PLT content in frozen plasma prepared from whole blood, in their study the centrifugation was performed at room temperature. The low temperature applied to prepare FFP-2 may synergistically promote vesiculation of the residual PLT through cold activation[35], as previously shown in PLT units stored at 4 °C *vs* room temperature[36]. Room temperature is strongly recommended for blood centrifugations involved in MP enumeration protocols in order to avoid cold-related PLT microtubules modifications[30,37]. In addition to mechanical factors, the freeze-thaw stimuli[23,38] imposed on the residual cells may induce a proportional release of MP[18,34].

Moreover, within donors analysis showed a significant effect of FFP storage duration on PMP and RMP accumulation, leading to more MP in FFP-2 over storage time. It is highly likely that the numerous PMP present in the FFP-2 (in contrast to FFP-1) promoted the generation of RMP through the transfer of PMP bioactive materials to red cells[39]. In our study, this effect seemed to be storage time-dependent. Finally, we have shown that preservation of thawed plasma for 24 h at 4 °C did not significantly affect MP accumulation in either whole-volume or split FFP-1 and FFP-2 units. Previous studies have demonstrated decreased PMP levels after longer storage[18], probably as a result of MP degradation by extracellular phospholipases[40].

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Although the biological activity of the FFP residual cells has not been documented, it has been shown that FFP MP contribute towards clot formation[14]. MP decline after long storage of thawed FFP units, resulting in the concomitant decrease in procoagulant activity, thrombin generation and clotting response[18]. Diminished haemostatic potential of thawed plasma has been accompanied by decreased levels of coagulation proteins[41], while replacement of previously removed MP restored the thrombin generation potential[18]. Our study shows that FFP-2 preparation is more advantageous compared to the FFP-1 in terms of both procoagulant activity and bioactive MP content. The procoagulant and endothelial repair activity of the PMP is well established both *in vitro* and *in vivo*. It has been estimated that PMP exhibit 50- to 100-fold higher procoagulant activity[42] and thrombin generation potential compared to the PLT themselves[43], at least *in vitro*. Insufficient PMP formation *in vivo* is associated with Scott syndrome, a moderate to severe bleeding disorder[44]. Even PMP released from unstimulated PLT retain procoagulant properties and stimulate thrombin generation over a PLT storage time course[45]. In thrombocytopenic rabbits, transfusion of PMP induced significant reduction in bleeding time, while transfused PMP have been identified in lesion sites[46]. Similar results have been reported in a mouse model of haemophilia[47]. Yet the contribution of the additional types of MP to the coagulant activity of the FFP might not be negligible. Indeed, it has been found that RMP exhibit FXI-dependent procoagulant properties and can initiate, propagate and enhance thrombin generation in plasma[48,49]. RMP exhibited broad haemostatic activity (primary and secondary) in bleeding thrombocytopenic animal models[50]. Proteomic analysis of the MP from stored RBC units revealed the presence of coagulation-related proteins[51], while the procoagulant state in sickle cell disease has been found to be positively correlated with the procoagulant properties of circulating RMP[23]. In addition, LMP harbour proteins and bioactive lipids known to be related to coagulation, and as a result may participate in haemostasis[52]. Accordingly, the haemostatic activity of FFP might be a function of the MP component, as seen in cryopreserved platelets[53].

Although polyphosphate and TF-bearing RMP have not been detected in circulating blood or stored blood units[49,50], and only a small percentage of total or PMP express TF in circulation[54,55] and FFP units[14,18], it has been suggested that tissue factor-bearing circulating MP largely of monocyte/macrophage and endothelial origin are potential mediators of blood coagulation[56,57]. In the light of the previously described functional role for PS in the expression of the full procoagulant potential of the MP-TF[58], study of the contribution of MP and non-MP[59] bound functional TF in the haemostatic potential of stored FFP is warranted. Interestingly, a decrease in TF^+ MP has been observed in frozen plasma in the past, regardless of storage duration[31].

Finally, according to our results, TAC of the FFP may be correlated with basal MP levels. The role of MP (as a cause or result) in cellular redox signalling has been increasingly recognised over recent years[6], and their concentration has been linked with plasma oxidation in metabolic syndrome[60]. The statistical linkage of RBC exovesiculation with the monocyte count both *in vivo* and in FFP is probably indicative of the interplay between RBC and macrophages in maintaining the MP, RBC and immune system homeostasis[39,61].

The present study has certain limitations, mainly arising from the FFP preparation protocols that have been tested and the size detection limits of flow cytometry. Indeed, the currently used FFP units have been prepared according to AABB standards for FFP freezing and storage that are quite different from the Council of Europe guidelines. These recommend rapid initial freezing to −30 °C within one hour and subsequent storage for only 3 months at from −18° to −25 °C, while for longer term storage (up to 36 months) a storage temperature of less than −25 °C is recommended. In addition, the flow cytometric method only allows analysis of extracellular vesicles above the detection threshold of the instrument, thus potentially excluding smaller particles. Our numerical data of MP characterisation are markedly different to those of other studies[14,16,18,54], verifying the poor standardisation of the MP methodologies and the close dependence of MP generation on sample handling. Nevertheless, we improved the specificity of our flow set up by applying a 3-point definition of MP (size, PS and cellular marker positivity). Moreover, the primary objective of this study was the comparative analysis of the two FFP preparation protocols that are used in clinical practice. Furthermore, the results were verified by methods that did not involve the use of cytometry and by detection of significant correlations between those results and flow cytometry numbers. Finally, *in vitro* analysis of FFP parameters that might influence its therapeutic potential seems premature, given that the clinical effectiveness of FFP transfusion is still a subject of debate[62]. However, there is a general need for clinical studies to determine how the MP present in all blood labile products might affect the transfusion recipients. Particularly in the case of FFP, it is important to determine not only how MP are involved in the coagulation cascade, but also the effect of each FFP haemostatic component on FFP quality and efficacy as a function of preparation, storage, processing and donor-associated variables. Further integrated studies, including proteomic and coagulant evaluation, are, therefore, warranted to help fill the current gap in knowledge of the clinical effectiveness of FFP.

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Conclusions

The present study provides new evidence on parameters of the MP component of the FFP as a function of the preparation method and duration of storage. The measurements were performed under conditions identical to those used in clinical practice, i.e. after a one freeze-thaw cycle of FFP and without the implementation of additional centrifugation steps that add the risk of pre-analytical variability. Preparation of FFP promotes generation of all MP; however, PMP represent the prevalent MP subpopulation. Preparation protocols that result in a high number of residual PLT also result in MP-rich FFP units. FFP storage duration in the freezer affects PMP and RMP accumulation and, consequently, the MP-associated FFP thrombogenicity *in vitro*. In contrast, preservation of thawed FFP for one day at 4 °C has no effect on FFP MP concentration, composition and thrombogenicity.

Acknowledgements

We would like to thank the Hellenic Society of Blood Transfusion chaired by Prof. Alice Maniatis for their financial support for this study; SafeBlood Bioanalytica Greece chaired by Gomatos Ilias for providing the flow cytometer; all blood donors for volunteering to take part and for their exceptional help and collaboration; and finally, Dr T.G. Ntouroupi for the thorough editing of the manuscript. Our special thanks to Dr Nikolaos Orologas, Eutichia Valasiadi and Ioanna Athanasiadou for their technical assistance in the flow cytometry analysis.

Funding and resources

This study has been financially supported by a research grant offered to ISP, KES, MHA and AGK by the Hellenic Society of Blood Transfusion (HSBT/2011); SafeBlood Bioanalytica Greece, provided the flow cytometer instrument used in the study.

Authorship contributions

AGK and MHA are equally first Authors. KES and ISP are equally last Authors. AGK completed the life-style questionnaires, prepared the FFPs, performed the haematologic and serum biochemical analysis as well as the flow cytometry experiments; MHA performed part of the experiments, analysed the data and wrote the paper; HTG and VLT performed part of the experiments and the statistical analysis of the data; KES conceived the idea of this project, contacted donors, provided medical support for the donations, and contributed to the final draft of the paper; ISP critically commented on the study design and contributed to the final draft of the paper.

All Authors read and approved the final manuscript.

The Authors declare no conflicts of interest.

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Arrived: 9 July 2015 - Revision accepted: 20 October 2015
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