

Experimental effect of different dilutions of blood with human plasma protein fraction and large dose factor one on blood coagulation and chemistry *in vitro*

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ABSTRACT

Background and Aims: Human plasma protein fraction 5% (PPF5%) is an albumin-based colloid used to expand the plasma volume during volume deficiency. The current basic medical experimental study assessed *in vitro* coagulation of PPF5% solution and its effects on blood coagulation and chemistry. **Methods:** The study involved 20 volunteers, and each volunteer donated 20–50 ml of fresh blood. Three dilutions of blood with PPF5% dilutions were prepared (30, 50, and 70%). The fibrinogen dose required to correct coagulation in the 50% diluted samples was assessed (two doses used). The thromboelastogram (TEG) measured the haemostatic parameters (fibrinogen level, initiation of coagulation [R time], kinetics [K], acceleration of coagulation [α angle], maximum amplitude [MA] and coagulation index [CI]), and the ABL gas analyser measured the blood chemistry changes. **Results:** All dilutions showed significant TEG and blood chemistry changes when compared to controls. The two doses of fibrinogen corrected the clot formation speed with no significant difference in speed between the two doses. Acidosis measured by the strong ion gap (SID) and pH were significant for all dilutions when compared with the baseline. The 30% dilution remained within the lower normal acceptable value while 50% dilution was beyond the critical normal values. **Conclusion:** *In vitro* PPF5% to replace blood loss up to 50% dilution did not have significant coagulation and blood chemistry effects while coagulopathy should be expected in extreme dilutions (70%). Fibrinogen in a dose equivalent to 4 gm/70 kg adult improved clot strength at 50% dilution.

Key words: Blood coagulation and chemistry, fibrinogen level, human plasma protein fraction, thromboelastography

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INTRODUCTION

The human plasma protein fraction 5% (PPF5%) solution is an albumin-based colloid solution currently used to expand the plasma volume, resulting in blood dilution and possible dilution coagulopathy. However, the literature is inconsistent in terms of the degree of dilution that results in coagulopathy and in relation to the different types of diluting fluids.^[1,2] Such inconsistencies mean that the effects on coagulation are rarely considered either in the colloid vs. crystalloid

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debate or in the anaesthesiologist's choice of specific resuscitation fluid for a given patient.

A 40% dilution corresponds to that created when resuscitating a patient with severe haemorrhagic shock, and this dilution level is likely to reveal differences between the different resuscitation solutions.^[3]

PPF5%'s combined effects on coagulation and electrolyte and base deficit remain unknown and have not been examined to date. Therefore, the current basic medical experimental study assessed the *in vitro* effects of different dilutions of blood with PPF5% on coagulation (primary endpoint) and on blood chemistry (secondary endpoints). The fibrinogen dose required to correct coagulopathy at 50% dilution was also evaluated as a secondary endpoint of the study.

METHODS

This study (162421/16) was done (May 2017 to March 2017) after ethics and research centre approval and in strict accordance with the principles of the Declaration of Helsinki.

Twenty adult male volunteers participated in this experimental *in vitro* study after signing informed consent. The inclusion criteria included adult volunteers (20–60 years age) with normal differential blood count, a minimum haemoglobin level of 14 gm/dl, with normal vital signs and no medical history of chronic disease or coagulation abnormality.

All volunteers recruited were American Society of Anesthesiologists (ASA) I and II hospital staff, and the study duration was one year. Each volunteer donated 20–50 ml of fresh blood. Wide cannula size 18 G was used and inserted in a large antecubital vein. Three PPF5% dilutions were then prepared as follows:

- a. 30% dilution (3 ml PPF5% added to 7 ml blood)
- b. 50% dilution (2.5 ml PPF5% to 2.5 ml blood)
- c. 70% dilution (7 ml PPF5% to 3 ml blood).

Dilutions were chosen in-line with previous studies that demonstrated that low coagulation occurs at 60% and critically low coagulation at 70% dilutions.^[3,4]

The fibrinogen concentrate dose required to correct clot strength and fibrinogen level in the 50% diluted samples was assessed. Two different doses were

tested, and dose 1 was equivalent to 10% fibrinogen level correction (5 gm of fibrinogen concentrate to an average 70 kg healthy adult) while dose 2 was equivalent to 15% fibrinogen level correction (7.5 gm of fibrinogen concentrate to an average 70 kg healthy adult).

Thromboelastography (TEG) (USA computer-derived TEG® 5000 Haemostasis Analyzer) was used to assess coagulation. The device measures the haemostatic process viscoelastic changes, providing real-time functional evaluation of the coagulation cascade; initiation of coagulation (R time); acceleration of coagulation (α angle [°]); kinetics (K) or clot amplification (time taken to achieve a certain level of clot strength, [20 mm amplitude]); maximum amplitude or clot strengthening and stabilization (MA [mm]); coagulation index (CI) and lysis after 30 min of MA (LY 30).^[5]

A technician trained in operating TEG devices processed the collected samples within 3 min after venepuncture. Two TEG devices with four channels were used. For each of the three dilutions under examination, measurements included *in vitro* fibrinogen levels, fibrinogen function assay, and standard TEG. TEG measurements included: R time in mins, α angle, K time, MA, CI and lysis after 30 min of MA (LY 30). The venous base deficit, pH and strong ion gap (SID) were measured and calculated ($\text{SID} = \text{Na}^+ - \text{Cl}^-$) using a blood gas analyser (ABL 90 FLEX). All results were compared to the zero dilution of each volunteer (control values).

The sample size was calculated using initiation of coagulation (R time) as the primary endpoint and a minimum, clinically significant change of 300 sec (derived from normal values)^[6,7] and the use of the TEG in haemodialysis^[8] with a power of 0.8 and a *P* value of 0.05.

Using the STATA software Version 15.0, findings were reported as mean and standard deviation (SD). Log transformation was undertaken for FLEV correction after adding different dilutions of fibrinogen 10–15%); however; only the graph is presented [Figure 1c] to show the change. Analysis of variance (ANOVA) analysed all parametric data, with 95% confidence intervals, and *P* < 0.05 was considered significant. Generalised linear regression predicted the line fitting the change of the variables with the change in dilutions.

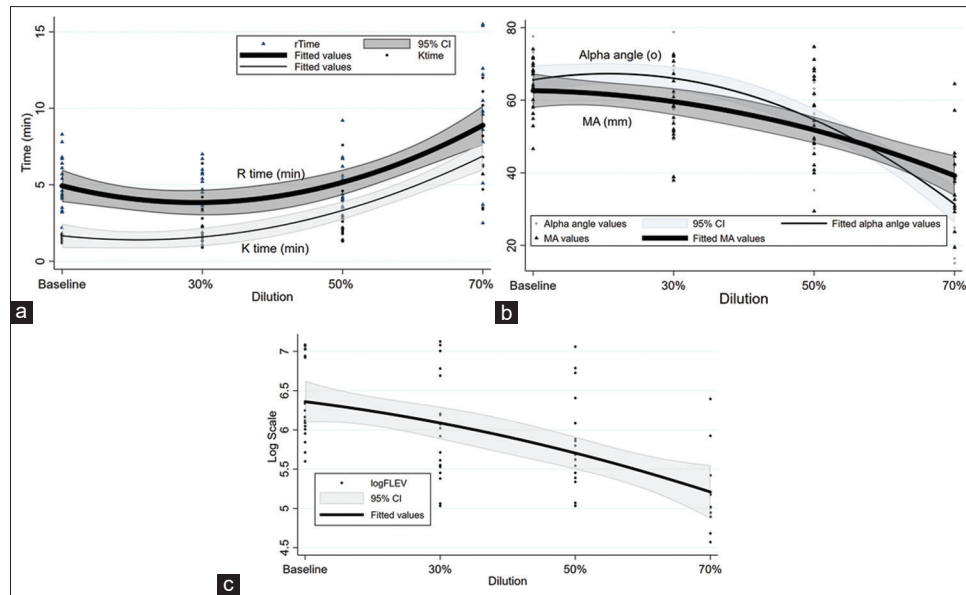


Figure 1: Coagulation changes: Baseline vs. 30%, 50% and 70% dilutions. (a) R Time and K Time. (b) Alpha angle and maximum amplitude. (c) Log fibrinogen level

RESULTS

At baseline, the TEG findings were within the normal range and comparable among all the blood donors (20 male volunteers, 38 ± 12 years old). Table 1 shows that at baseline, all dilutions (30, 50 and 70%) displayed significant TEG and blood chemistry values when compared to controls except LY 30. At 70% dilution, clot initiation (R time) and clot strength (MA) on a regular curve were significantly changed [Table 1]. Functional fibrinogen level assessment showed that fibrinogen level was significantly reduced at 50% and 70% dilutions [Table 1].

Functional fibrinogen assay showed that clot strength (MA) returned to normal values after mixing fibrinogen concentrate dose 1 and 2 with 50% diluted donor blood [Table 2].

Initiation of coagulation (R time) in 30% dilution was shorter than the control in the functional fibrinogen curve when compared to control values because of initial hypercoagulability [Figure 2] while it was longer than controls in 50% and 70% dilutions (3.98 ± 1.03 vs. 4.87 ± 1.75 and 9.48 ± 3.91 , respectively) [Figure 1a and Table 1].

The speed of clot formation (α angle) after initiation of coagulation (R time) decreased when compared to the control values. However, it was significantly decreased in the 70% dilution but not in the 50% dilution [Table 1 and Figure 1b].

Fibrinogen displayed low levels in the 50% and 70% dilutions [Tables 1, 2 and Figure 1c]. Adding fibrinogen to the 50% dilution corrected the fibrinogen level with no statistically significant difference between the two fibrinogen doses (631.6 ± 275.7 vs. 773.7 ± 230.9 mg/dl) [Table 2]. In summary, there was weak and slow clot formation at 50% dilution and significant coagulopathy at 70% dilution [Figure 1 and Table 1].

Adding fibrinogen corrected the speed of clot formation (R time) in the 50% dilution [Table 2], again with no significance between the two fibrinogen doses. The clot strength (MA) in the functional fibrinogen curve decreased significantly when the blood was diluted to 50%. Adding fibrinogen by 10% increased MA, which was insignificant when compared to the baseline while the 15% correction led to a significant increase in MA to above normal baseline value [Table 2].

The Kruskal–Wallis statistic tested the differences between fibrinogen content in the control values and the different dilutions (30%, 50% and 70%). The fibrinogen level decreased significantly in both the 50% and 70% dilutions when compared to the baseline control values. However, there was no significant change between control and 30% dilution. The fibrinogen level increased significantly after correction by 10% and 15% fibrinogen of 50% dilution, with no statistically significant differences in fibrinogen level between the 10% and

Table 1: TEG and chemistry changes of three dilutions vs. control

Variable	Controls M±SD	Dilutions	Value M±SD	P
R (minutes)	3.98±1.03	30%	4.49±1.55	0.124
		50%	4.87±1.75	0.118
		70%	9.48±3.91	<0.001
K (minutes)	1.40±0.18	30%	2.11±1.65	1.000
		50%	2.50±0.74	0.009
		70%	6.13±3.50	0.018
α Angle (O)	67.25±5.11	30%	63.80±8.90	1.000
		50%	55.65±10.53	0.127
		70%	28.76±10.39	<0.001
MA (mm)	63.81±7.27	30%	55.43±10.88	0.132
		50%	54.56±13.87	0.186
		70%	37.83±12.33	<0.001
Functional Fibrinogen mg/dl	685.15±366.46	30%	474.46±346.44	0.195
		50%	332.72±151.79	0.010
		70%	205.83±151.79	0.002
CI (%)	1.55±2.11	30%	-3.02±8.42	0.478
		50%	-1.11±3.55	0.082
		70%	-7.46±5.73	<0.001
LY 30	4.21±4.04	30%	0.42±0.72	0.015
		50%	3.03±5.06	1.000
		70%	0.49±1.29	0.056
BD (base deficit)	2.41±1.72	30%	-9.64±3.81	<0.001
		50%	-13.65±2.21	<0.001
		70%	-18.71±5.22	<0.001
pH	7.35±0.03	30%	7.29±0.03	<0.001
		50%	7.25±0.04	<0.001
		70%	7.15±0.03	<0.001
Na (Sodium) mmol/L	140.5±1.5	30%	145.93±1.33	<0.001
		50%	147.5±0.94	<0.001
		70%	140.64±40.96	1.000
CL (Chloride) mmol/L	104.31±2.06	30%	116.08±2.36	<0.001
		50%	120.85±1.46	<0.001
		70%	126.62±1.26	<0.001
Lactate mmol/L	1.18±0.61	30%	0.55±0.32	<0.001
		50%	0.35±0.21	<0.001
		70%	0.06±0.08	<0.001
SID	35.42±2.59	30%	28.34±1.25	<0.001
		50%	26.29±1.08	<0.001
		70%	22.84±1.28	<0.001

Bolded cells indicate statistical significance. M±SD – Mean±standard deviation, R – Initiation of coagulation, K – Kinetics, MA – Maximum amplitude, α Angle – Acceleration of coagulation, CI – Coagulation index, LY 30 – Lysis after 30 min of MA, SID – Strong ion gap

15% corrections [Figure 3 and Tables 1, 2]. Figure 4 represents the plasma protein fraction transfusion limit to reach haemoglobin <8 and/or double R time.

There was a significant change in the SID and pH for all dilutions when compared to the baseline values; however, the 30% dilution remained within the lower normal acceptable value (pH 7.3, SID 30), and in the 50% dilution, it was above the critical value of pH 7.2 and SID 25 [Table 1].

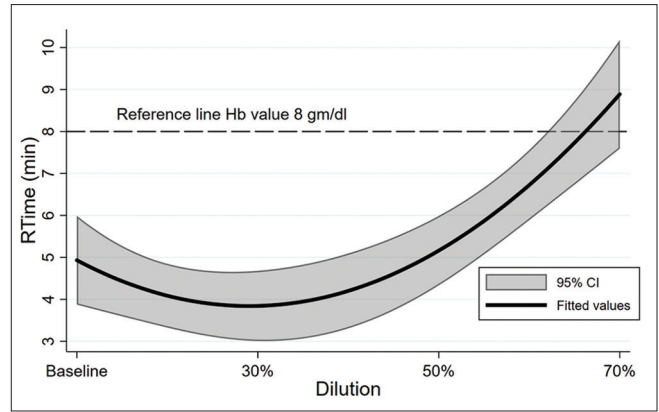


Figure 2: Initial hypercoagulability (R time) at 30% dilution (functional fibrinogen curve)

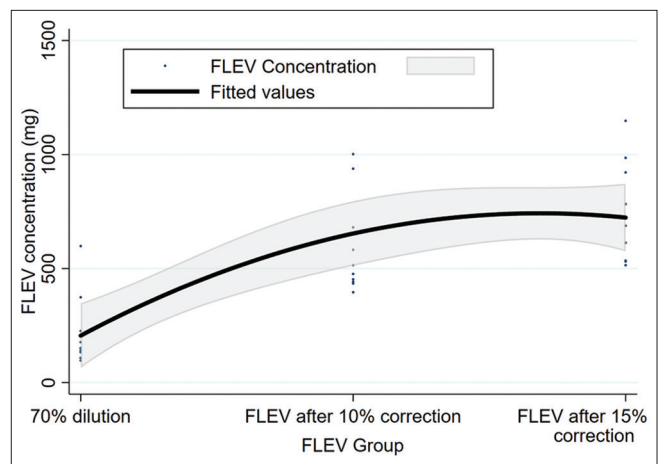


Figure 3: Fibrinogen level after correction with dose 1 and 2 fibrinogen

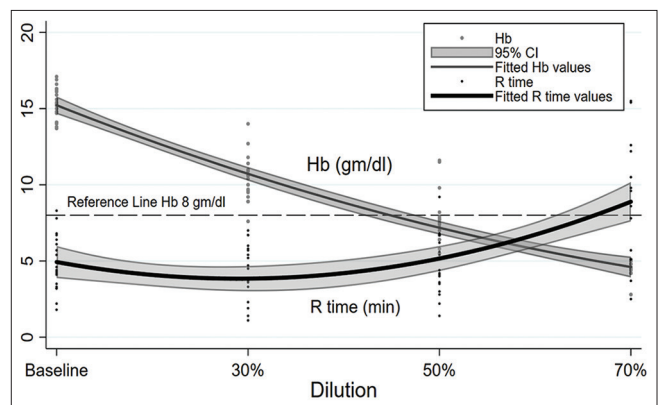


Figure 4: Plasma protein fraction transfusion limit to reach haemoglobin <8 and/or double R time

DISCUSSION

Primary haemostasis function and perioperative coagulopathy occur because of dilution effects resulting from blood loss, fluid resuscitation, and consumption of coagulation factors and platelets. *In vivo/vitro* investigations have demonstrated that

Table 2: Coagulation changes after correction of fibrinogen level (Functional fibrinogen assessment)

Variable	Controls M±SD	Dilutions	Value M±SD	P
Fibrinogen mg/dl	553.5±281.6	50%	325.5±302.2	0.008
		Dose 1	631.6±275.7	0.277
		Dose 2	773.7±230.9	0.013
MA (mm)	30.3±15.4	50%	17.3±16.9	0.007
		Dose 1	34.6±15.1	0.278
		Dose 2	42.4±12.7	0.013

Bolded cells indicate statistical significance. M±SD – Mean±standard deviation, MA – Maximum amplitude

natural and artificial colloids impair haemostasis more than crystalloids.^[9,10] The impairment of coagulation when using different dilutions of PPF5% was evaluated as a primary endpoint in this study. The coagulopathy comprised of decreased clot strength (MA). With a greater degree of dilution (still to be identified), the reduction in clot firmness (MA) was accompanied by impaired initiation of coagulation (R time), indicating a deficiency of factors needed for thrombin generation. Again, the definitive dilution level is variable from patient to patient and remains unidentified.^[4,11] There are also continued debates and conflicting evidence on the colloid with the worst effect on coagulation, which colloid to use, as well as its dose and indications.^[12]

In terms of coagulation screening tests, previous studies found that traditional coagulation screening tests (PT, APTT, platelet count and fibrinogen levels) were of limited value in acute haemorrhage and did not correlate well with bleeding outcomes,^[13,14] and in-line with such propositions, the current study did not use any of these haemostatic tests. We focused on global coagulation, fibrinogen level and function changes, and we observed that *in vitro* 30% dilution (equivalent to 1.5 litre PPF5% for an average 70 kg adult male) had minimal coagulation and blood chemistry effects (enhanced coagulation [shortened R time] in the functional fibrinogen curve and small insignificant decrease in clot strength). Such initial enhanced coagulation was also observed in another study because of unknown cause/s; however, this observation was found in *in vitro* studies in the absence of clinical *in vivo* changes as consumption coagulopathy.^[15,16] The 50% dilution (equivalent to 2.5 litre PPF5% for an average 70 kg adult male) had greater coagulation consequences (although clot initiation and strength impairments were still within normal acceptable values [$r 7.1 \pm 1.7$ min and MA 58.0 ± 4.9 mm in males^[17]]); and the 70% dilution (equivalent to dilution with 3.5 litre of PPF) failed the coagulation in terms of initiation and

clot strength. These findings are important, as we tried to identify the PPF transfusion limit to reach haemoglobin <8 and double R time or whichever occurred first when diluting blood of normal humans with haemoglobin >14 g/dl [Figure 4]. We did not analyse factor XIII or its role in dilution, as its effect on final clot strength was reported to be minimal even at 60% dilution.^[18,19]

Acidosis in bleeding trauma patients impairs coagulation by depleting fibrinogen and platelets and inhibiting coagulation kinetics,^[20] an effect not corrected by bicarbonate pH neutralization. In terms of the secondary endpoints of the current study (acidosis and blood chemistry changes after PPF5% transfusion), we observed that 70% dilution induced acidosis (pH <7.3, SID <20) while the 30% and 50% dilutions (equivalent to transfusion of 1.5–2 litre of PPF5% in 70 kg normal adult) were not associated with severe acidosis or SID impairment.^[20] Our findings, despite being *in vitro* and not considering the role of the kidney and buffer mechanisms of the body, suggest that transfusing large amounts of PPF5% might be safe, although further clinical studies are required to determine the maximum amounts that may be used for resuscitation.

As for the related issue of chloride content, fluids used for hydration and resuscitation have supra-physiological concentrations of chloride that induce/exacerbate hyperchloremia and metabolic acidosis and might cause renal vasoconstriction and decreased glomerular filtration rate. Using a chloride-restrictive strategy in a tertiary intensive care unit (ICU) was associated with a significant decrease in the incidence of acute kidney injury and use of renal replacement therapy.^[21] In the present study, the chloride content and the albumin itself are anions that increased significantly as observed in the resulting acidosis that was acceptable at *in vitro* dilutions up to 50%. However, *in vitro* experiments do not reflect accurately the *in vivo* effects as the role of fluid redistribution, buffering and kidney effects are not accounted for.

Although the maximum safe PPF5% amount that can be used remains to be identified, our findings agree with a meta-analysis of randomised trials, involving survival after human albumin administration found no effect of albumin on mortality. Therefore, and any such effect may be small.^[22] Additionally, no significant effect of albumin on mortality was observed in trials

of hypovolemia in patients, who received human albumin, undergoing surgery for trauma.^[23]

In connection with albumin administration, a randomised controlled trial (RCT) demonstrated that human albumin administration did not affect the blood loss as compared to infusion of lactated ringer's solution, and human albumin use did not affect the need for blood transfusion, the incidence of post-operative complications or in-hospital stay.^[13] The dilution level and amount of human albumin were not determined in this trial, but the authors observed that albumin decreased coagulation competence during major surgery and the blood loss was related to MA in TEG rather than to plasma coagulation variables.^[13] Other authors found that the need for blood transfusion increased by about 30% among patients who received HES 130/0.4 and lactated ringers (LR) solution compared to albumin.^[10,24] Still, other research also reported that maximal clot firmness lower in the albumin group compared to LR group when assessed post-operatively, and others reported that only 15% dilution did not impair coagulation *in vitro* while 20% produced minimal changes on TEG.^[23-25]

In terms of fibrinogen and platelets, in agreement with our findings, previous investigations have shown that increasing the fibrinogen concentration improves dilution-dependent impairment of clot formation by colloids.^[26] We observed that a dose equivalent to 5–7.5 gm fibrinogen for average adult promoted clot formation and corrected the 50% dilution induced coagulopathy as evidenced by our prolonged R time and short MA. Likewise, we did not assess platelet number and function because MA remained >40 mm even with extreme dilution to 70% while fibrinogen level and function assay demonstrated that MA was significantly low in 70% dilution but not the 30% or 50% dilutions suggesting that the clot strength decrease at 70% dilution was mainly because of low fibrinogen and not platelets. In agreement with our findings, significantly decreased fibrinogen concentration was the earliest event occurring with the dilution of blood while critical levels of other coagulation factors (prothrombin, FV, FVII, FVIII and platelets) were reached later in the course of massive dilution of blood (60%).^[27] *In vitro* fibrinogen substitution was dependent on the fibrinogen dose and the type of solution used to dilute the blood samples.^[22] Others also found that fibrinogen failed to completely reverse coagulation variables in the blood samples diluted with 6% HES 130/0.4 or the combination of LR and 6% HES

130/0.^[24] Furthermore, synthetic colloids interfere with photometric fibrinogen assays, where samples diluted with colloids exhibited larger fibrinogen values than the samples diluted with saline.^[19]

This study has limitations. Our findings should not be extrapolated directly into clinical practice as there were no endothelium-counteracting effects to haemostasis (providing tissue factor, thrombin inhibitors and receptors for protein C activation). In addition, no fluid redistribution, buffering or electrolyte homeostasis occurred *in vitro*. The strengths of this study include the creation of a platform for further clinical investigations to clearly determine the *in vivo* impact of high dilutions of human PPF and large dose fibrinogen on blood coagulation activity, speed, cascade and competence, as well as the accompanying haemostatic viscoelastic and clot changes and blood chemistry. In other words, the question becomes, how much PPF5% could be used safely without negative effects of coagulation and acid-base balance to prevent premature use of blood and blood products?

CONCLUSION

In vitro PPF5% to replace blood loss up to 30% dilution did not have significant coagulation and blood chemistry effects *in vitro*. A 50% dilution induced minor coagulopathy while extreme dilutions (70%) caused major coagulopathy and acidosis. A low-dose fibrinogen equivalent to 5 gm for an average 70 kg adults improved clot initiation, speed and strength when blood was diluted *in vitro* to 50% in PPF5%.

Declaration of patient consent

The authors certify that they have obtained all appropriate consent forms. In the form, the participant(s) has/have given his/her/their consent for his/her/their images and other clinical information to be reported in the journal. The patients understand that their names and initials will not be published, and due efforts will be made to conceal their identity, but anonymity cannot be guaranteed.

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Conflicts of interest

There are no conflicts of interest.

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