Research Article

Disordered hemostasis associated with severely depressed fibrinolysis demonstrated using a simultaneous thrombin and plasmin generation assay during L-asparaginase induction therapy in pediatric acute lymphoblastic leukemia.

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Abbreviations:

ALL, acute lymphoblastic leukemia
AT, antithrombin
BCP, B-cell precursor
BFM, Berlin-Frankfurt-Münster
Dana, danaparoid sodium
EP, endogenous potential
EZR, Easy R
Fbg, fibrinogen
FDP, fibrin-fibrinogen degradation products
FFP, fresh frozen plasma
IT, intra-thecal therapy
JACLS, Japan Association of childhood Leukemia Study
L-Asp, L-asparaginase
LMWH, low-molecular-weight heparin
LT, lag time
MPAL, mixed phenotype acute leukemia
NS, not significant
PAI-1, plasminogen activator inhibitor
PARKAA, Prophylactic Antithrombin Replacement in Kids with Acute Lymphoblastic Leukemia Treated with Asparaginase
Peak, peak levels
P-EP, endogenous plasmin potential
Ph, Philadelphia chromosome-positive
PIC, plasmin-α2 plasmin inhibitor complex
PL, phospholipid vesicles
Plt, platelet
PNP, pooled normal plasma
P-Peak, plasmin peak height
RV, reference value
T/P-GA, thrombin and plasmin generation assay
TAT, thrombin-antithrombin complex
T-EP, endogenous thrombin potential
TF, tissue factor

tPA, tissue-type plasminogen activator
ttPeak, time to peak
y, years old

Abstract

Background: L-asparaginase (L-Asp)-associated thromboembolisms are serious complications in pediatrics patients with acute lymphoblastic leukemia (ALL), especially at ≥10.0 years old, but the pathogenesis remains to be clarified. **Procedure:** We have conducted a multi-center, prospective study of 72 ALL patients aged 1.0 to 15.2 years treated with either a Berlin-Frankfurt-Münster 95-ALL oriented regimen or Japan Association of Childhood-Leukemia-Study-ALL-02 protocol. We divided into each of treatment protocol and investigated the dynamic changes in coagulation and fibrinolysis using simultaneous thrombin and plasmin generation assay. Patients' plasma samples were collected at the pre-phase (T0), intermittent-phase (T1) and post-phase of L-Asp therapy (T2), and post-induction phase (T3). Measurements of endogenous thrombin potential (T-EP) and plasmin peak height (P-Peak) were compared to normal plasma. **Results:** None of the cases developed thromboembolisms. Median ratios of T-EP and P-Peak to control in the JACLS group were 1.06 and 0.87 (T0), 1.04 and 0.71 (T1), 1.02 and 0.69 (T2) and 1.20 and 0.92 (T3), respectively, whilst those in the BFM group were 1.06 and 1.00 (T0), 1.04 and 0.64 (T1), 1.16 and 0.58 (T2) and 1.16 and 0.85 (T3), respectively. In particular, P-Peak ratios were depressed at T1 and T2 compared to T0 in the BFM group ($p<0.01$). Moreover, P-Peak ratios in patients ≥10.0 years old were lower at T1 in the BFM group ($p=0.02$). **Conclusions:** The results demonstrated that hemostatic dynamics appeared to shift to a hyper-coagulable state with marked hypo-fibrinolysis associated with L-Asp therapy, especially in patients ≥10.0 years old of the BFM regimen.

Introduction

Thromboembolism remains a serious complication of acute lymphoblastic leukemia (ALL) chemotherapy. The incidence of venous thromboembolism, including symptomatic and asymptomatic cases, has been reported to range between 1.1 and 36.7%.[1-3] L-asparaginase (L-Asp) is a key drug for ALL treatment, although cessation of chemotherapy is sometimes necessary due to critical side effects including anaphylaxis, pancreatitis and thromboembolism. L-Asp is known to depress the hepatic synthesis of pro-coagulant, anti-coagulant and fibrinolytic factors, but the detailed pathogenesis of the disordered hemostasis in these circumstances is complex, and remains to be clarified.[4-7] Clinical management of thrombosis-related complications may include antithrombin (AT) concentrate supplementation, fresh frozen plasma (FFP) transfusion and administration of low-molecular-weight heparin (LMWH) or danaparoid sodium (Dana). At present, however, it is difficult to predict whether the coagulation abnormalities predispose to hemorrhage or thrombosis, and the appropriative supportive therapy may not be readily apparent.

Conventional laboratory tests of blood coagulation have been designed mainly to investigate isolated mechanisms of hemostasis, and do not provide adequate data for the assessment of simultaneous coagulation and fibrinolytic potential. In this context, therefore, we focused on the dynamic hemostatic balance between coagulation and fibrinolysis likely to be associated with the clinical thromboembolisms mediated by induction therapy in pediatric ALL. Recently, a novel assay designed to assess the interplay between coagulation and fibrinolysis with the simultaneous measurement of thrombin and plasmin generation (T/P-GA) has been established and utilized in several hemostatic disorder reports.[8-13] In the present study, we have extended these investigations to assess the balance of coagulation and fibrinolytic potential using T/P-GA during induction chemotherapy in 72 children newly diagnosed with ALL.

Materials and Methods

The study was approved by the Medical Research Ethics Committee of Nara Medical University (No.902), Kobe Children's Hospital (No.26-43), Osaka Women's and Children's Hospital (No.744), and Kyoto Prefectural University of Medicine (No. ERB-C-674). Blood samples were obtained after informed consent following local ethical guidelines.

Enrolled patients - The study population included consecutive newly diagnosed ALL patients enrolled from August 2014 to March 2018 at four core hospitals for pediatric hematology and oncology in Japan. All patients received induction chemotherapy following the guidelines of either the Japan Association of childhood Leukemia Study (JACLS) ALL-02 protocol, consisting

of a total of six doses of *E.coli* L-Asp (Leunase®, Kyowa Hakko Kirin Co., Ltd, Tokyo, Japan) 6000 U/m^2 (JACLS group, Day 15, 17, 19, 22, 24 and 26), or the Berlin-Frankfurt-Münster (BFM) 95 oriented protocol consisting of a total of eight doses of *E.coli* L-Asp (Leunase®) 5000 U/m^2 (BFM group, Day 12, 15, 18, 21, 24, 27, 30 and 33) (Figure 1).[14,15] Thromboprophylaxis including FFP transfusion, usually without cryoprecipitate because of uncommon product in Japan, for low fibrinogen (Fbg) levels (≤50 mg/dL), AT supplementation for low AT levels (≤70% of control) and administration of Dana for anticoagulation was included in these protocols if required ultimately based on the decision of each clinician. The treatment protocols and supportive therapies for each patient were non-randomized and selected according to the treatment strategy of each institution. No specific intervention rules were imposed on local medical practice.

Plasma sampling - Pooled normal plasma (PNP) was prepared from 20 normal non-age-matched adult healthy individuals. Blood samples were collected in plastic tubes containing 3.2% sodium citrate at a 9:1 ratio. Platelet-poor plasma was obtained after centrifugation of citrated whole blood for 15 min at 1,500×*g*. All plasma samples were stored at -80°C, and thawed at 37°C immediately prior to assay. Patients' plasmas were collected at the following time-points: T0; pre-phase of L-Asp (*i.e.* before the first administration of L-Asp, Day 15 of JACLS and Day 8 of BFM, respectively), T1; intermittent phase of L-Asp (*i.e.* just after the third administration of L-Asp in the JACLS group, and just after the fourth administration of L-Asp in the BFM group, Day 22 of both JACLS and BFM), T2; post-phase of L-Asp (*i.e.* after the end of L-Asp therapy, Day 29 of JACLS and Day 36 of BFM, respectively), and T3; post-induction phase, approximately one week after T2 (Day 36 of JACLS and Day 43 of BFM, respectively) (Figure 1).

Simultaneous thrombin and plasmin generation assay (T/P-GA) - The T/P-GA technique has been recently described by our research group.[8-13] Briefly, blood coagulation and fibrinolytic reactions were initiated by the addition of a mixture of optimized final concentrations of tissue factor (TF; 1 pM), phospholipid vesicles (PL; 4 μM) and tissue-type plasminogen activator (tPA; 3.2 nM). Thrombin and plasmin generation were monitored simultaneously using individual fluorescent substrates in separate 96-well microtiter plates. Standard curves were established using purified α-thrombin and plasmin. Data analyses were performed using Excel software. The first derivatives (velocity) of thrombin and plasmin generation were utilized to define the parameters, lag time (LT), endogenous potential (EP), peak levels (Peak), and time to peak (ttPeak) (Figure 2A). Endogenous thrombin potential (T-EP) and the plasmin peak height (P-Peak) parameters were chosen as analytical indices for coagulation and fibrinolytic activity,

respectively. Our previous data indicated that the measurements of endogenous plasmin potential (P-EP) were unsuitable for assessing fibrinolytic activity at lower P-Peak levels after hematopoietic stem cell transplantation in pediatric acute leukemia and during induction therapy for pediatric solid tumors.[9,10] Plasmin generation was delayed in these circumstances, and plasmin generation curves were unreliable at intersections on the time axes (*i.e.*, horizontal axes).[9] Following the several recent reports for T/P-GA,[9-13] ratios of T-EP and P-Peak to PNP (as control normal plasma) were calculated, therefore, to estimate the balance of coagulation and fibrinolytic potential in patients' plasmas. A ratio >1.0 was defined as high coagulation or fibrinolytic potential relative to normal.

Measurement of other coagulation and fibrinolytic markers and platelet counts - Fbg, fibrin-fibrinogen degradation products (FDP), AT and total plasminogen activator inhibitor 1 (PAI-1) antigen were measured in an automated blood coagulation analyzer using the respective commercial kits (Coagpia®Fbg, Nanopia®P-FDP; Testteam®S AT-III; Nanopia®PAI-1; Sekisui Medical, Tokyo, Japan). In addition, thrombin-AT complex (TAT) and plasmin-α2 plasmin inhibitor complex (PIC) were measured using an automated immunoassay system (HISCL®-2000*i* analyzer, Sysmex, Kobe, Japan) with the respective commercial kits (HISCL™ TAT Assay Kit and HISCL™ PIC Assay Kit). Platelet (Plt) counts were measured in an automated blood cell analyzer at the same time-points. Total PAI-1 was measured only at T0 and T2, whilst the other measurements were obtained at all four time points. The reference value of total PAI-1 in normal individuals (n=19) in our laboratory was 14.0±4.5 ng/mL (mean±SD).

Data analyses - All statistical analyses were performed using EZR (Easy R) statistical software, which is a graphical user interface for defining R (The R Foundation for Statistical Computing, Vienna, Austria).[16] More precisely, EZR is a modified version of the R commander designed to add statistical functions for frequently used biostatistics. For the purposes of our statistical analyses, patient variables were grouped in the following manner: Time points of T-EP ratios and P-Peak ratios, T0 vs. T1 vs. T2 vs. T3; treatment protocol, BFM vs. JACLS; onset-age category, ≥10.0 years old (y) vs. 1.0-9.9 y; FFP transfusion, non-FFP vs. FFP; AT supplement, non-AT vs. AT; thromboprophylaxis with Dana, non-Dana vs. Dana. Both parameters obtained from T/P-GA and other conventionally hemostatic laboratory data were analyzed by being divided into the JACLS and BFM groups. Baseline patient demographics, disease characteristics, laboratory data, clinical course results and thromboprophylaxis were compared using Fisher's exact test for categorical data. Significant differences for continuous data were determined by the Mann-Whitney *U*-test between two groups and the Kruskal-Wallis rank sum

test among 4 time points, respectively. *P*-values <0.05 were considered as statistically significant.

Results

Patients' characteristics - Seventy-two pediatric patients were enrolled (Table 1). Seventeen cases (23.6%) were treated according to JACLS ALL-02 protocol (B-cell precursor (BCP)-ALL; n=13, T-ALL; n=2, Philadelphia chromosome-positive ALL (Ph-ALL); n=1, Mixed phenotype acute leukemia (MPAL); n=1). The other 55 cases (76.4%) were treated according to BFM 95 oriented protocol (BCP-ALL; n=47, T-ALL; n=7, MPAL; n=1). Median age among all patients at onset was 5.5 y (range, 1.0-15.2 y) and was similar in the JACLS group and the BFM 95 group (median [range]: 4.3 y [1.2-14.2] and 5.5 y [1.0-15.2]; p=0.31, respectively). In addition, 21 cases (29.2%) were categorized in the ≥10.0 year-group and 51 cases (70.8%) in the 1.0-9.9 year-group (median [range]; 12.6 y [10.0-15.2] and 4.3 y [1.0-9.5], respectively). The percentages of patients in each onset-age group were similar in the JACLS and BFM categories (p=0.76). There was no evidence of the past history of thrombosis, and none of the patients had any thromboembolic and other L-Asp-associated adverse events during induction therapy or failed to survive this intensive first-line treatment. Induction was unsuccessful in two cases (2.8%) but there were no differences in immediate outcome between each protocol (p=0.41). The percentages of patients treated with AT supplementation were not significantly different between the JACLS and BFM protocols (p=0.21), although the number of patients treated with FFP transfusions was significantly greater in the JACLS group than in the BFM group (p<0.01). Thromboprophylaxis with Dana was chosen for the BFM group alone (n=8, 11.1% of all patients). Hence, comparisons between the JACLS and BFM groups were not informative in this category.

Dynamic changes in thrombin and plasmin generation during the period of induction therapy - Coagulation and fibrinolytic responses were assessed using simultaneous T/P-GA during induction therapy in all 72 children with newly diagnosed ALL and divided into each protocol group. Figure 2B shows the time-dependent changes in thrombin and plasmin generation waveforms obtained in a representative case (UPN 1). Almost all of the other cases in both groups showed similar and characteristic patterns. Increased T-EP was observed at T3, whilst markedly low levels of P-Peak were evident during T1 to T2 (Table 2). The specific thrombin and plasmin generation data provided by this assay, however, are likely to be governed by base-line levels of a number of hemostasis-related components in individual plasma samples. Comparisons of these results alone might not be appropriate, therefore, and we further assessed the relationship between thrombin and plasmin generation in individual cases using ratios of

T-EP and P-Peak in patients' plasmas to those in control plasma.

These analyses demonstrated that the T-EP ratios were increased at T3 ($p<0.01$) and P-Peak ratios were not significantly depressed at T1 ($p=1.00$) and T2 ($p=1.00$) compared to T0 in the JACLS group, whilst the T-EP ratios were increased at T2 to T3 and P-Peak ratios were depressed at T1 and T2 compared to T0 in the BFM group (median T-EP ratio/P-Peak ratio: (A) JACLS group; 1.06/0.87 at T0, 1.04/0.71 at T1, 1.02/0.69 at T2 and 1.20/0.92 at T3, (B) BFM group; 1.06/1.00 at T0, 1.04/0.64 at T1, 1.16/0.58 at T2 and 1.16/0.85 at T3, respectively) (Figure 2C showed the box plot of the BFM group). The increased T-EP ratios indicated enhanced coagulation potential at the latter phase of induction therapy, whilst the decreased P-Peak ratios suggested depressed fibrinolytic potential during the L-Asp phase in the BFM group (no significant depression of fibrinolysis during L-Asp treatment phase compared to pre-treatment phase in the JACLS group: T1; $p=1.00$ and T2; $p=1.00$ compared to T0, respectively). The data indicated, therefore, that dynamic changes in the overall hemostatic balance reflected a relatively coagulation-predominant state at the late stages of induction therapy (mild hyper-coagulation and severe hypo-fibrinolytic potential) especially in the BFM group.

Comparisons of the different treatment protocols demonstrated that T-EP ratios were more elevated and P-Peak ratios were significantly less at T2 in the BFM group compared to the JACLS group (median T-EP ratio and P-Peak ratio in JACLS and BFM: T-EP ratio; $p=0.02$ and P-Peak ratio; $p=0.02$, respectively) (Table 2). These findings were in keeping with a greater tendency towards hyper-coagulation and hypo-fibrinolytic in the BFM-treated patients. In addition, P-Peak ratios were lower at T0 in the JACLS group compared to the BFM group, suggesting more depressed fibrinolytic potential just before starting L-Asp treatment in the JACLS cohort (median P-Peak ratio in JACLS and BFM; $p=0.02$). The results suggested that the different treatment protocols used in these pediatric patients with ALL might have contributed to the extent of the disordered hemostatic balance between coagulation and fibrinolysis.

In regards to the age of onset, T-EP ratios were significantly lower at T1 and T2 in the ≥10.0 y group (n=4) compared to the 1.0-9.9 y group (n=13) and no statistical difference of P-Peak ratios in the JACLS group. These results showed no thrombogenetic tendency during L-Asp treatment phase of the JACLS protocol (Table 3). However, ≥10.0 y group in this cohort was very small population. Whilst, in the BFM group, analyses of the age of onset showed that P-Peak ratios were significantly lower at T1 in the ≥10.0 y group (n=17) compared to the 1.0-9.9

y group (n=38). These results identified a more pronounced hypo-fibrinolytic potential during L-Asp treatment of the BFM oriented protocol in patients ≥10.0 years of age (Table 3). T-EP ratios were not significantly different in the BFM group. This evidence was in keeping with the possibility that depressed fibrinolysis during L-Asp therapy in ALL patients ≥10.0 years of age might contribute to an increased thrombotic tendency in these older children in the BFM protocol.

We further examined the balance between coagulation and fibrinolysis in patients with or without thromboprophylaxis. Analyses of comprehensive hemostatic changes of both non-FFP vs. FFP and non-AT vs. AT identified no statistical differences of T-EP ratios and P-Peak ratios at T0 to T3 in the JACLS and BFM group (Table S1). In the small number of patients treated prophylactically with Dana (n=8), which was used only in the BFM group, T-EP ratios were significantly lower at T1 compared to the non-Dana group (n=47) (Table 3). These limited data indicated that Dana therapy suppressed coagulation potential, and might have helped to offset the imbalance between coagulation and fibrinolysis during the L-Asp induction phase of the BFM protocol in our pediatric patients.

Changes in specific components of coagulation and fibrinolytic mechanisms - The potential effects of the commonly accepted conventional components of coagulation and fibrinolytic mechanisms, including Fbg, FDP, AT, TAT, PIC and Plt counts were also examined. The time-related changes in these parameters during the induction phase of the JACLS and BFM protcols are shown in Table 2. Fbg levels were significantly lower from T0 to T2 compared to T3 in the JACLS group ($p<0.01$), whilst lower at T1 and T2 compared to T0 and T3 in the BFM group ($p<0.01$). AT levels were reduced from T1 to T3 compared to T0 ($p<0.01$ for each protocol group). FDP levels were significantly lower at T2 in the BFM group ($p=0.02$) although they remained within reference value at all time points. Measurements of TAT, however, did not show any significant changes during induction phase.

PIC levels were significantly decreased at T0 to T2 in the JACLS group and at T1 and T2 in the BFM group ($p<0.01$ for each protocol group), although the median PIC remained within the reference value. Median total PAI-1 at both T0 and T2 was elevated above the reference value and was significantly increased during induction treatment in the BFM group ($p=1.00$ for JACLS and $p<0.01$ for BFM, respectively). The age of onset did not appear to significantly affect total PAI-1 during induction therapy (data not shown).

Plt counts were initially low, and increased significantly towards the latter of induction therapy ($p<0.01$ for each protocol group).

In contrast to the results of simultaneous T/P-GA, therefore, the changes in conventional components of coagulation and fibrinolysis, except for total PAI-1, did not reflect a predominance of either coagulation or fibrinolytic mechanisms during induction therapy, although the increased total PAI-1 tended to support the presence of a hypo-fibrinolytic state.

Discussion

Enhanced thrombin generation during induction therapy in ALL has been identified in several reports,[1,17-20] but previous studies have not focused on the balance between coagulation and fibrinolysis. The present study was devised to assess the global changes in mechanisms of hemostasis during induction therapy in pediatric ALL, especially after the start of L-Asp therapy. Our investigations were based on a novel T/P-GA designed to investigate the interplay between coagulation and fibrinolysis, dividing into two cohorts based on the treatment protocol of either JACLS or BFM. Although none of the participants developed any thrombotic or bleeding events, the T/P-GA data demonstrated that a significant decrease of P-Peak and a mildly elevated T-EP relative to normal were associated with L-Asp therapy especially in the BFM group. The conventional components demonstrated that Fbg and AT levels were significantly reduced in keeping with the low P-Peak ratios observed in the T/P-GA, whilst the FDP, TAT (except for JACLS group) and PIC levels each remained within the respective reference value. Total PAI-1 levels were higher compared to its reference value, however, consistent with suppressed fibrinolytic potential. The markedly reduced P-Peak ratios pointed especially to a depression of fibrinolytic function that could exacerbate the thromboembolic risk. These disturbances seemed more likely due to decreased levels of circulating fibrinolytic factors mediated by the effects of L-Asp on hepatic synthesis, rather than a consumptive decrease in these components. Corticosteroids may enhance PAI-1 expression from endothelial cells,[21] and combination therapy with L-Asp and corticosteroids might further promote a thrombotic tendency. Differences in the T/P-GA that correlate with thrombotic or bleeding outcomes would be of great interest, hence, further investigations among the cases complicated with thrombotic or bleeding events during induction phase (and early consolidation phase) for ALL are conducted.

Our findings suggest that thromboprophylaxis may modulate hypercoagulability during L-Asp induction therapy. In these circumstances, replacement of hemostatic components using AT treatment or FFP transfusion might be insufficient for complete prevention of thrombotic-related events. In contrast, the promising results of thromboprophylaxis with Dana in the BFM group

showed significant reduced T-EP ratios during L-Asp treatment phase (T1). However, patients with Dana-prophylaxis were small population in the BFM group (n=8), hence, we should assess the anti-coagulant effect of Dana during the ALL induction therapy in more large population with BFM protocol in future. Nevertheless, our promising findings highlighted the possibility that thromboprophylaxis could be beneficial at the initiation of L-Asp induction therapy. The optimum length of time to continue this type of treatment remains a difficult clinical decision, however. Improvements in the overall balance between coagulation and fibrinolysis appeared not to be completely adequate at the end of induction therapy (T3). Further studies are required, therefore, to examine the hemostatic balance during consolidation therapy after induction therapy.

Our data were derived using a plasma-based global assay in the absence of platelets. Satisfactory Plt counts were largely observed at the later stages of induction (T2 and T3), and these tended to coincide with an improvement in the relationship between T-EP and P-Peak ratios. Other studies have shown that Plt counts and P-selectin levels increase at the later phases of induction,[5] and it may be that restoration of platelet function plays an important role in these overall mechanisms. Further studies are required to examine the interplay between plasma-derived components and Plt by using whole blood global clotting assays, *e.g.* rotational thromboelastometry (ROTEM®).[22]

Furthermore, the most appropriate type of thrombo-prophylactic agent for a L-Asp-associated thromboembolism remains to be established. In our study, FFP transfusion did not appear to significantly improve the balance between coagulation and fibrinolytic potential during the induction therapy. The efficacy of FFP for thromboprophylaxis in this setting is controversial.[23-25] FFP contains free asparagine and may replace the plasma asparagine pool during L-Asp therapy.[26] Hence, FFP may impair the anti-leukemic effects of L-Asp, and may be not suitable for thromboprophylaxis. In addition, the PARKAA trial (Prophylactic AT Replacement in Kids with ALL Treated with L-Asp), which was the first prospective randomized trial in pediatric ALL to determine the efficacy and safety of AT supplementation,[27] failed to demonstrate any significant benefits of AT thromboprophylaxis because the PARKAA trial was stopped early due to poor enrollment and was not expected therefore to be able to show statistical significance for the study question. Our findings also demonstrated that AT supplementation appeared unlikely to significantly correct the hemostatic imbalance. In contrast, AT thromboprophylaxis has been reported to be effective in adult ALL.[28] Alternatively, the recent THROMBOTECT trial (a randomized study comparing LMWH, AT and unfractionated heparin for thromboprophylaxis during induction therapy of ALL in children and adolescents) suggested

that maintaining AT activity at 80% or higher throughout the induction phase could significantly protect patients from thrombosis.[29] Although the THROMBOTECT trial reported that enoxaparin (LMWH) and activity-adapted AT substitution were equally effective, a lower incidence of thrombotic events was shown in children aged over 6 years old. In this context, several reports have also recently demonstrated that an onset-age of ≥10.0 years is a risk factor of L-Asp associated thromboembolism.[29-31] In our study, the T/P-GA technique indicated that the P-Peak ratios at T1 in the ≥10.0 y group of BFM were significantly more decreased than in the younger age-group, whilst T-EP ratios at T1 and T2 in the ≥10.0 y group of JACLS were significantly reduced. However, the statistical results regarding onset-age in the JACLS group might be misinterpreted because the ≥10.0 y group of JACLS was very small population (n=4). The findings in the BFM cohort indicated that the thrombotic risk in patient aged ≥10.0 years might reflect a greater depression of overall fibrinolytic potential than that in younger patients during treatment with L-Asp. In this study, however, PNP was derived from 20 healthy adult individuals and we could not calculate any ratios with age-matched PNP. Moreover, thromboprophylaxis was not randomized and each our cohort showed heterogeneous population according to the doses, frequency, timing of intervention and modality of thromboprophylaxis.

Some contrasting results were evident between patients treated using the JACLS protocol and those managed with the BFM protocol. In particular, P-Peak ratios in the JACLS group at T0 were more significantly decreased compared to those in the BFM group, whilst there were no significant differences in total PAI-1 between the groups at the same time-point. A major difference between these treatment regimens is the choice corticosteroid administered just before T0 (BFM, prednisolone; JACLS, dexamethasone). The difference of steroids on thrombotic effect study had been already reported that prednisolone appeared to be more pro-thrombotic effect rather than dexamethasone in BFM protocol.[32] However, anti-leukemic agents had already been administered at T0 of JACLS, whilst only corticosteroids (and intra-thecal therapy (IT)) had been administered at T0 of BFM, hence, these differences of protocol construction might contribute any effect for the imbalance of comprehensive hemostatic condition. In addition, the number of patients treated with FFP in the JACLS cohort was greater than that in the BFM group (Table 1), and this could account for the differences in the global hemostasis parameters recorded in these individuals. Therefore, we should investigate the changes of comprehensive hemostatic potentials during induction therapy for ALL in each of treatment protocol, but not together with some protocol series.

In conclusion, our findings suggest that T/P-GA could reflect the balance between coagulation and fibrinolysis more effectively than conventional components and that there is a

hyper-coagulable and hypo-fibrinolytic state during induction therapy with L-Asp that could explain the reported association with clinical thrombosis, particularly in children over age 10 years. The effect of pharmacologic thromboprophylaxis in these assays raises intriguing questions about its potential benefit in pediatric patients during induction therapy for ALL. Hence, we should conduct a randomized clinical trial for each uniform thromboprophylaxis using T/P-GA with age-matched PNP to establish optimal anti-thrombotic treatment for onset-age during induction and early consolidation phase in major unified treatment protocol, *e.g.* BFM regimen, in future.

Conflict of Interest

All authors have no direct and indirect conflicts of interest.

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Figure Legends

Figure 1 Treatment schedule and time points of plasma collection - *Panel* **A** illustrates the induction procedure in the JACLS ALL-02 protocol, consisting of a total of 6 doses of L-Asp 6000 U/m^2 (L). Cyclophosphamide (E) was administered to high-risk and extremely high-risk patients. *Panel* **B** shows the induction therapy in the BFM 95 protocol, consisting of a total of 8 doses of L-Asp 5000 U/m^2 (L). Dexamethasone (DEX) was administered to patients with T-ALL, whilst the other patients received prednisolone (PSL) alone as a corticosteroid. The total period of intrathecal injection (IT) was increased in patients with central nervous system involvement. Patients' plasmas were collected at the following time-points: T0=before L-Asp treatment, T1=intermittent L-Asp phase, T2=post L-Asp phase, and T3=post-induction phase. Abbreviations: V, vincristine; D, daunorubicin.

Figure 2 Simultaneous T/P-GA: a representative waveform and the parameters derived during induction therapy in pediatric ALL - (*Panel* **A**) Simultaneous T/P-GA; Mixtures of TF, tPA, and PL (f.c. 1.0 pM, 3.2 nM and 4.0 μM) were added to normal plasma as described in Methods. Representative curves of thrombin generation (**a**) and plasmin generation (**b**) are illustrated. Parameters calculated were: LT, lag-time; Peak, peak thrombin or peak plasmin; ttPeak, time to peak; EP, endogenous potential of thrombin or plasmin. *Panel* **B** shows the curve changes in thrombin and plasmin generation using T/P-GA in a representative case (UPN 1, BFM group) at the indicated times (T0, T1, T2 and T3). These curves demonstrated markedly low levels of P-Peak during T1 to T2 (**b**). *Panel* **C** shows the ratios of T-EP and P-Peak, based on the parameters in the BFM group showed in Table 2. The median values are depicted within the boxes. The boxes end at the 25th and 75th percentiles, and the whiskers extend to the furthest points that are not outliers. Outliers are depicted as open dots. The upper and lower dotted lines show the 95% confidential interval of parameters from normal controls. The ratios of T-EP and P-Peak of patients' plasmas to those of control normal plasmas were calculated. There were significant differences in both ratios between time points (T-EP ratios/P-Peak raitos, p=2.2×10^{-5}/3.7×10^{-15} in the BFM group using the Kruskal-Wallis rank sum test). *P*-values were adjusted using the Bonferroni method (*, p<0.05 and **, p<0.01, respectively)

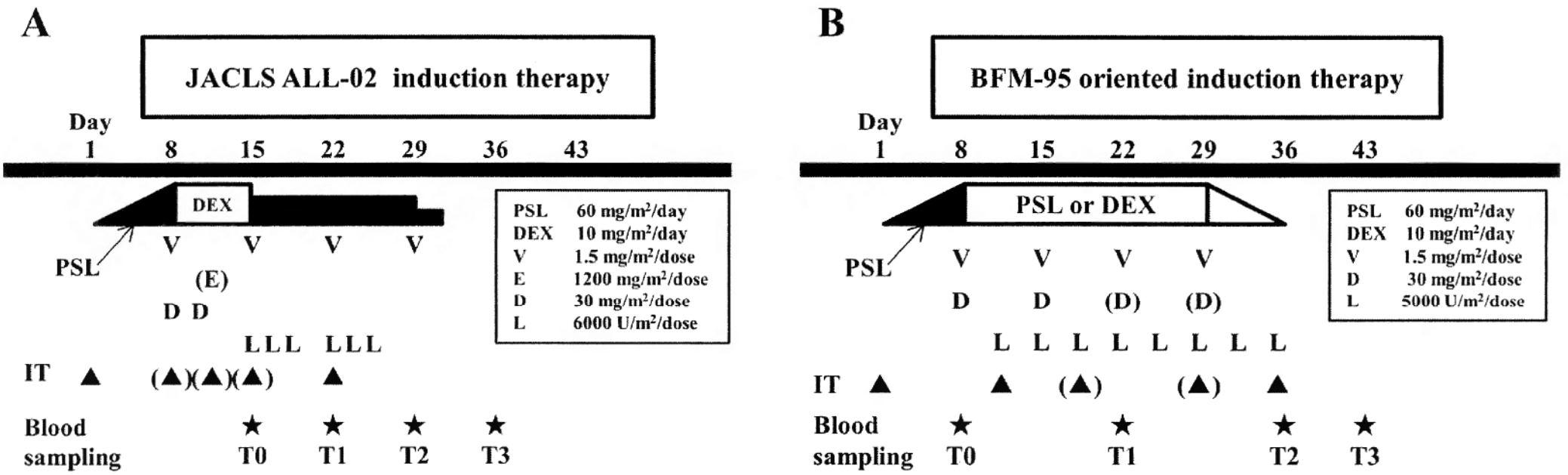
A
JACLS ALL-02 induction therapy
Day
1 8 15 22 29 36 43
DEX
PSL
V V V V
(E)
D D
L L L L L L
IT ▲ (▲)(▲)(▲) ▲
Blood sampling ★ ★ ★ ★
T0 T1 T2 T3
PSL 60 mg/m²/day
DEX 10 mg/m²/day
V 1.5 mg/m²/dose
E 1200 mg/m²/dose
D 30 mg/m²/dose
L 6000 U/m²/dose
B
BFM-95 oriented induction therapy
Day
1 8 15 22 29 36 43
PSL or DEX
PSL
V V V V
D D (D) (D)
L L L L L L L L
IT ▲ ▲ (▲) (▲) ▲
Blood sampling ★ ★ ★ ★
T0 T1 T2 T3
PSL 60 mg/m²/day
DEX 10 mg/m²/day
V 1.5 mg/m²/dose
D 30 mg/m²/dose
L 5000 U/m²/dose

Figure 1

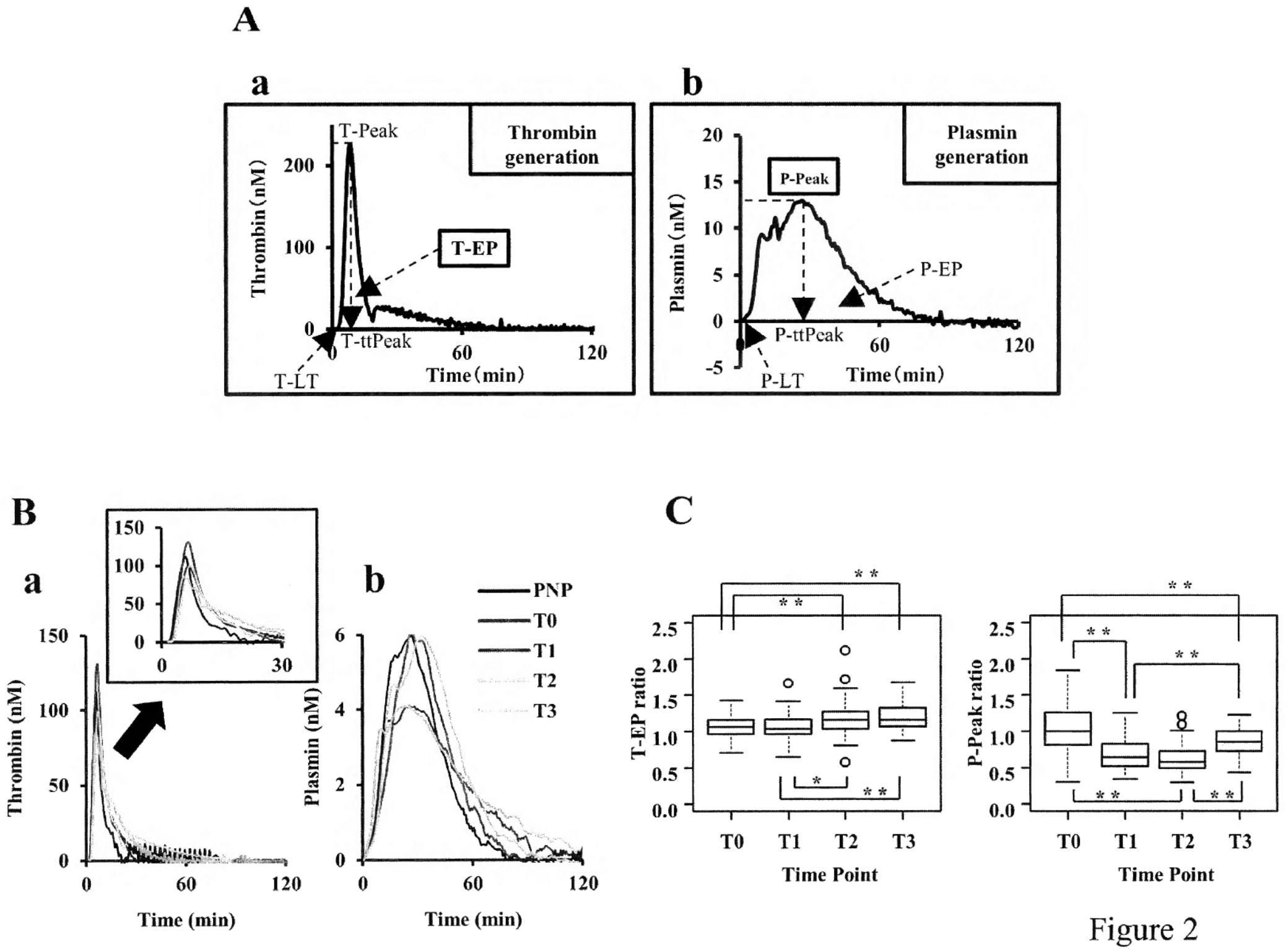
A
a
Thrombin generation
T-Peak
T-EP
T-ttPeak
T-LT
Thrombin (nM)
Time (min)
b
Plasmin generation
P-Peak
P-EP
P-ttPeak
P-LT
Plasmin (nM)
Time (min)
B
a
b
PNP
T0
T1
T2
T3
Thrombin (nM)
Plasmin (nM)
Time (min)
C
T-EP ratio
P-Peak ratio
Time Point

Figure 2

TABLE 1 Characteristic of pediatric ALL patients enrolled

Factors	Category	Total patients (n=72)	JACLS group (n=17)	BFM group (n=55)	*p*-value
Age (years old), median [min-max]		5.5 [1.0-15.2]	4.3 [1.2-14.2]	5.5 [1.0-15.2]	NS
Onset age, n (%)	≥10.0 y	21 (29)	4 (24)	17 (31)	NS
	1.0-9.9 y	51 (71)	13 (76)	38 (69)	
Sex, n (%)	Male	43 (60)	13 (76)	30 (55)	NS
	Female	29 (40)	4 (24)	25 (45)	
Diagnosis, n (%)	BCP-ALL	60 (83)	13 (76)	47 (85)	NS
	T-ALL	9 (13)	2 (12)	7 (13)	
	Ph-ALL	1 (1)	1 (6)	0 (0)	
	MPAL	2 (3)	1 (6)	1 (2)	
Past history of thrombosis, n (%)	No	72 (100)	17 (100)	55 (100)	–
Thromboembolism, n (%)	No	72 (100)	17 (100)	55 (100)	–
Induction death, n (%)	No	72 (100)	17 (100)	55 (100)	–
Induction failure, n (%)	Yes	2 (3)	1 (6)	1 (2)	NS
	No	70 (97)	16 (94)	54 (98)	
AT supplement, n (%)	Yes	52 (72)	10 (59)	42 (76)	NS
	No	20 (28)	7 (41)	13 (24)	
FFP transfusion, n (%)	Yes	13 (18)	12 (71)	1 (2)	*<0.01
	No	59 (82)	5 (29)	54 (98)	
Infusion of danaparoid sodium, n (%)	Yes	8 (11)	0 (0)	8 (15)	NS
	No	64 (89)	17 (100)	47 (85)	

**P*-values <0.05 were considered as statistically significant using Fisher's exact test between JACLS group and BFM group.

Abbreviation: NS, not significant.

TABLE 2 Changes in T/P-GA and laboratory data at indicated time points during induction phase among the each treatment protocol

(A) JACLS group

Parameters	Time points				
	T0	T1	T2	T3	PNP
T-EP (nM×min)	1,131 [803-1,321]	1,055 [885-1,412]	1,099 [972-1,423]	1,277 [1,057-1,581]	1,102 [885-1,299]
T-EP ratio	1.06 [0.68-1.21]	1.04 [0.77-1.30]	1.02 [0.84-1.28]	1.20 [1.00-1.41]	
P-Peak (nM)	4.80 [2.24-6.82]	3.99 [2.57-5.85]	4.20 [3.32-6.33]	5.39 [3.44-7.37]	5.51 [4.59-7.15]
P-Peak ratio	0.87 [0.41-1.22]	0.71 [0.40-1.12]	0.69 [0.58-1.16]	0.92 [0.63-1.31]	
Fbg (mg/dL) (RV, 200-400)	134 [86-243]	104 [76-242]	97 [59-181]	299 [120-579]	
FDP (μg/mL) (RV, <5.0)	2.2 [1.0-5.4]	2.1 [0.6-3.6]	1.8 [0.8-3.6]	2.4 [1.3-5.1]	
AT (%) (RV, 80-130)	131 [106-149]	79 [63-118]	80 [61-102]	95 [80-120]	
total PAI-1 (ng/mL) (RV, 9.5-18.5)	26.3 [7.3-83.2]	–	27.0 [10.3-50.9]	–	
TAT (ng/mL) (RV, <4.0)	4.7 [0.7-28.4]	4.4 [0.8-22.2]	4.4 [1.6-60.0]	3.7 [2.0-27.4]	
PIC (μg/mL) (RV, <0.8)	0.2 [0.2-1.5]	0.2 [0.2-1.4]	0.2 [0.2-0.3]	0.6 [0.2-11.6]	
Plt ($x10^3/\mu L$) (RV, 158-348)	67 [18-222]	143 [40-318]	217 [112-430]	262 [51-424]	

(B) BFM group

Parameters	Time points				PNP
	T0	T1	T2	T3	
T-EP (nM×min)	1,134 [779-1,526]	1,140 [735-1,810]	1,272 [644-1,873]	1,281 [994-1,775]	1,102 [885-1,299]
T-EP ratio	1.06 [0.70-1.44]	1.04 [0.65-1.66]	1.16 [0.56-2.12]	1.16 [0.88-1.67]	
P-Peak (nM)	5.47 [1.54-10.53]	3.59 [1.86-7.49]	3.39 [1.68-6.94]	4.75 [2.32-7.50]	5.51 [4.59-7.15]
P-Peak ratio	1.00 [0.30-1.83]	0.64 [0.34-1.25]	0.58 [0.29-1.21]	0.85 [0.44-1.23]	
Fbg (mg/dL) (RV, 200-400)	172 [60-307]	98 [54-293]	97 [49-297]	316 [78-780]	
FDP (μg/mL) (RV, <5.0)	3.5 [1.3-11.1]	2.5 [1.2-69.1]	2.5 [1.2-6.1]	3.3 [1.8-4.9]	
AT (%) (RV, 80-130)	148 [99-150]	83 [62-145]	81 [41-145]	107 [70-150]	
total PAI-1 (ng/mL) (RV, 9.5-18.5)	23.8 [7.3-210.0]	–	40.6 [7.3-140.0]	–	
TAT (ng/mL) (RV, <4.0)	3.6 [0.4-39.4]	3.1 [1.0-11.5]	2.7 [1.1-71]	2.7 [1.1-18.1]	
PIC (μg/mL) (RV, <0.8)	0.4 [0.1-6.1]	0.3 [0.0-1.4]	0.3 [0.0-0.6]	0.4 [0.1-1.9]	
Plt ($x10^3$/μL) (RV, 158-348)	80 [18-422]	88 [11-536]	240 [31-584]	147 [16-481]	

All data show the median values and [min-max]. Patients' plasmas were collected at the following points as described in Materials and Methods: T0=before L-Asp treatment, T1=intermittent L-Asp phase, T2=post L-Asp phase, and T3=post-induction phase. Abbreviation: RV, reference value; PNP, pooled normal plasma.

TABLE 3 The ratios on T/P-GA parameters at time point in the age of onset among the each treatment protocol and for the thromboprophylaxis with danaparoid sodium in the BFM group

(A) JACLS group

	Time points							
	T0		T1		T2		T3	
Parameters	≥10.0 y	1.0-9.9 y	≥10.0 y	1.0-9.9 y	≥10.0 y	1.0-9.9 y	≥10.0 y	1.0-9.9 y
T-EP ratio	1.04 [0.68-1.21]	1.06 [0.73-1.20]	0.86 [0.77-0.92]	1.08 [0.87-1.30]	0.93 [0.84-0.99]	1.08 [0.95-1.28]	1.13 [1.05-1.35]	1.21 [1.00-1.41]
	NS		*p=0.01		*p=0.01		NS	
P-Peak ratio	0.83 [0.50-1.22]	0.87 [0.41-1.13]	0.61 [0.40-0.79]	0.76 [0.56-1.12]	0.64 [0.58-0.90]	0.75 [0.62-1.16]	0.91 [0.71-1.16]	0.92 [0.63-1.31]
	NS		NS		NS		NS	

(B) BFM group

	Time points							
	T0		T1		T2		T3	
Parameters	≥10.0 y	1.0-9.9 y	≥10.0 y	1.0-9.9 y	≥10.0 y	1.0-9.9 y	≥10.0 y	1.0-9.9 y
T-EP ratio	1.03 [0.77-1.30]	1.06 [0.70-1.44]	1.04 [0.65-1.19]	1.03 [0.83-1.66]	1.16 [0.57-1.71]	1.17 [0.81-2.12]	1.32 [0.88-1.67]	1.14 [0.92-1.57]
	NS		NS		NS		NS	
P-Peak ratio	0.97 [0.30-1.83]	1.03 [0.57-1.64]	0.57 [0.34-0.90]	0.68 [0.34-1.25]	0.56 [0.40-0.81]	0.65 [0.29-1.21]	0.91 [0.44-1.23]	0.85 [0.50-1.18]
	NS		*p=0.02		NS		NS	
Thrombo-prophylaxis	non-Dana	Dana	non-Dana	Dana	non-Dana	Dana	non-Dana	Dana

T-EP ratio	1.06 [0.77-1.44]	0.97 [0.70-1.25]	1.06 [0.65-1.66]	0.97 [0.83-1.14]	1.18 [0.57-2.12]	1.09 [0.86-1.27]	1.18 [0.88-1.67]	1.13 [0.91-1.42]
	NS		*p=0.048		NS		NS	
P-Peak ratio	0.95 [0.30-1.83]	1.04 [0.81-1.64]	0.65 [0.34-1.25]	0.60 [0.46-0.93]	0.63 [0.36-1.21]	0.55 [0.29-0.88]	0.86 [0.44-1.23]	0.82 [0.45-1.00]
	NS		NS		NS		NS	

The data show the median value and [min-max]. *P-values <0.05 were considered as statistically significant using the Mann-Whitney U-test. Patients' plasmas were collected at the following points: T0=before L-Asp treatment, T1=intermittent L-Asp phase, T2=post L-Asp phase, and T3=post-induction phase. Abbreviation: Dana, danaparoid; NS, not significant.

SUPPLEMENTAL TABLE S1 The ratios on T/P-GA parameters at time point for the thromboprophylaxis with FFP/AT supplement between each treatment protocol

(A) FFP transfusion

		Time points							
		T0		T1		T2		T3	
Protocol	Parameters	non-FFP	FFP	non-FFP	FFP	non-FFP	FFP	non-FFP	FFP
JACLS	T-EP ratio	1.06 [0.73-1.20]	1.06 [0.68-1.21]	1.06 [0.91-1.10]	0.99 [0.77-1.30]	1.11 [1.00-1.26]	1.01 [0.84-1.28]	1.22 [1.00-1.41]	1.18 [1.04-1.41]
		NS		NS		NS		NS	
	P-Peak ratio	0.67 [0.41-1.06]	0.92 [0.50-1.22]	0.59 [0.56-0.91]	0.76 [0.40-1.12]	0.66 [0.63-0.76]	0.77 [0.58-1.16]	0.91 [0.63-1.21]	0.93 [0.71-1.31]
		NS		NS		NS		NS	

(B) AT supplement

		Time points							
		T0		T1		T2		T3	
Protocol	Parameters	non-AT	AT	non-AT	AT	non-AT	AT	non-AT	AT
JACLS	T-EP ratio	1.07 [0.84-1.20]	1.05 [0.68-1.21]	1.06 [0.87-1.30]	0.97 [0.77-1.25]	1.08 [0.96-1.28]	0.99 [0.84-1.26]	1.22 [1.04-1.41]	1.15 [1.00-1.41]
		NS		NS		NS		NS	
	P-Peak ratio	0.96 [0.41-1.07]	0.75 [0.50-1.22]	0.77 [0.58-1.12]	0.70 [0.40-1.01]	0.76 [0.62-1.03]	0.66 [0.58-1.16]	0.91 [0.63-1.31]	0.93 [0.71-1.16]
		NS		NS		NS		NS	
BFM	T-EP ratio	1.07 [0.94-1.44]	1.05 [0.70-1.30]	1.03 [0.87-1.42]	1.04 [0.65-1.66]	1.19 [0.81-1.57]	1.12 [0.57-2.12]	1.27 [0.96-1.57]	1.16 [0.88-1.67]

	NS		NS		NS		NS	
P-Peak ratio	1.06 [0.57-1.83]	1.00 [0.30-1.64]	0.79 [0.46-1.12]	0.62 [0.34-1.25]	0.64 [0.38-1.21]	0.58 [0.29-1.09]	1.00 [0.50-1.14]	0.85 [0.44-1.23]
	NS		NS		NS		NS	

The category of FFP selected only JACLS protocol treatment showing in this table because only one patient used FFP in BFM group. The data show the median value and [min-max]. *P-values <0.05 were considered as statistically significant using the Mann-Whitney U-test. Patients' plasmas were collected at the following points: T0=before L-Asp treatment, T1=intermittent L-Asp phase, T2=post L-Asp phase, and T3=post-induction phase. Abbreviations: Dana, danaparoid; NS, not significant.