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Anticoagulant effects of protein C, protein S, and antithrombin levels on the protein C pathway in young children

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Response to Reviewers:	Editor-in-Chief

Prof. Dr. Takaori-Kondo
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We responded to all comments of the reviewers. According to the reviewers’ comments, we revised the text. Responses to the reviewers’ comments are presented below. We have prepared a revised version that marked all changes in red color. We thank the reviewers for the efforts. We hope that our revised version of this review paper would be accepted in this journal.

Sincerely yours,
Kenichi Ogiwara

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Response to the Reviewer 1 comment
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Additional Information:	
Question	**Response**
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Response to the Reviewer 2 comment

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Anticoagulant effects of protein C, protein S, and antithrombin levels on the protein C pathway in young children

Type of manuscript: Original article

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Abstract

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Key words; developmental hemostasis, protein C, protein S, antithrombin, thrombin generation

1. INTRODUCTION

The human hemostatic mechanism consists of primary hemostasis, secondary hemostasis (coagulation), and fibrinolysis. In growing or developing children, hemostasis and thrombosis are controlled by different mechanism(s) from those in adults, referred to as "developmental hemostasis" [1-5]. Because of their immature liver function, the plasma levels of most coagulation factors in newborns are approximately half those in adults [5]. Although vitamin K (VK) deficiency influences both pro- and anticoagulant activities, a recent protocol for the oral administration of VK contributes to reducing the risk of critical bleeding [6]. Depending on the specific parameter, adult values are usually reached between a few months of age and up to 16 years [5].

The protein C (PC) pathway is important in the pathogenesis and pathophysiology of neonatal infections and thrombosis [7] and implicated in many thrombogenic conditions, including congenital thrombophilia [8]. In Japan, where PC concentrates have not yet been approved, activated PC (APC) concentrate products are indicated for the acute stage of thrombosis but are expensive and not readily available because of the rarity of the disease. Therefore, fresh frozen plasma (FFP) is often used for initial treatment. However, only few studies have evaluated the efficacy of FFP on anticoagulant activity.

In addition to PC and protein S (PS), factor V (FV) is also involved in the PC pathway as a cofactor for PC/PS function. Moreover, conditions with increased thrombin generation (TG) such as high factor VIII (FVIII) levels, prothrombin abnormalities, FV abnormalities, and conditions that show APC resistance are also involved in deteriorated PC pathway function [9]. ThromboPath®, ProC Global®, and other assays have been used to evaluate the overall PC pathway function [9-11]. In young children, levels of these factors (including PC, PS, antithrombin [AT], prothrombin, and FV but not FVIII) are physiologically decreased, which may directly affect TG or the PC pathway function that regulates TG. Nevertheless, only few reports have used these assays in newborns and infants [12].

We hypothesized that the PC pathway function in younger children is important but differs from that in adults. Therefore, we aimed to investigate the characteristics of PC pathway function in newborns, infants, and toddlers using the ThromboPath® assay to comprehensively evaluate PC pathway function and further evaluate the effects of spiked AT,

PC, and FFP *in vitro*.

2. MATERIALS and METHODS

2.1 Ethics

This study was approved by the Nara Medical University Medical Research Ethics Committee (Nos. 2503 and 8072). Residual blood samples were used after obtaining informed consent and approval from the patients or their parents in accordance with the university's ethical guidelines.

2.2. Reagents

Plasma-derived PC (Haematologic Technologies Inc., Essex Junction, VT, USA), PC- or PS-deficient plasma (Affinity Biologicals, ON, Canada), HemosIL™ ThromboPath® kit (Instrumentation Laboratory, Bedford, MA, USA), Thromborel®S (Sysmex Corporation, Kobe, Japan), Hemoclot™ Protein S (Hyphen BioMed, Neuville-sur-Oise, France), Test Team®S ATIII and Test Team®S PC (Sekisui Medical, Tokyo, Japan) were obtained from the indicated vendors.

2.3. Blood samples

Plasma samples were obtained from 35 newborns, 42 infants, and 35 toddlers with no hemostatic abnormalities and 20 healthy adults with no history of medication affecting hemostatic function, 4 adult patients with liver disease, and 6 adult patients taking warfarin, all who consented to participate in the study. Adult PC- or PS-deficient plasmas (n=2, each) were commercially purchased. The samples from newborns and infants were 3.2% sodium-citrated plasma remaining after performing hemostatic screening tests on admission or preoperative evaluation. Plasma samples were also collected from healthy adults. Platelet-poor plasma was obtained by centrifuging citrated whole blood for 15 min at 2,300×g. All plasma samples were stored at -80 °C and thawed at 37 °C immediately before performing the assays.

2.4. Laboratory tests

Plasma prothrombin time (PT) and AT activity were measured as normal hemostatic screening when the hemostatic test was requested. The measurements were performed using a Blood Coagulation Automated Analyzer CP3000™ (Sekisui Medical). PC, PS, and AT

activities, which were not tested in the hemostatic screening, were measured using the CP3000™ simultaneously with ThromboPath® measurements during the lysis of frozen residual plasma.

2.5. Assay for assessing global PC/PS pathway function

The total potential of PC/PS anticoagulant pathway function was evaluated using the Hemos IL™ ThromboPath® kit, which utilizes snake venom extract (protac) to fully activate PC contained in plasma. Subsequently, APC/PS degrades activated FV (FVa) and FVIIIa in phospholipids and reduces tissue factor-triggered TG. The PC/PS-induced anticoagulant mechanism is potentiated in the presence of cofactors such as FV. Hence, the ratio (percentage of protac-induced coagulation inhibition, PiCi%) of the amounts of TG without (**ODB**) and with (**ODA**) protac provides an overall measurement of PC/PS-related functional activity and is clinically used to comprehensively assess PC/PS pathway function in patients with thrombotic tendencies. Briefly, the optical density was measured at 405 nm on an ACL-TOP™CTS300 analyzer (Instrumentation Laboratory, IL Japan, Tokyo, Japan) after the addition of thrombin-specific chromogenic substrate (S-2796; Z-D-Arg-Ser-Arg-pNA) with or without the addition of protac. The results are expressed as PiCi%, as recommended by the manufacturer, and were determined using the ratio (ODB – ODA)/ODB x 100.

2.6. Statistical analysis

All data were summarized as means with standard deviation (SD). Correlation and regression analyses were conducted for each age group to investigate their relationships between PiCi% and other factors (PT- international normalized ratio [PT-INR], PC, PS, and AT). Additionally, to assess the changes in the relationship between coagulation parameters with age, the correlation coefficients between each parameter were estimated. The degree of correlation was classified based on the |r| values as strong (>0.7), moderate (>0.5), or weak (>0.3). To examine the differences in ODB, ODA and PiCi% between newborns with and without AT spikes, a paired *t*-test was conducted as a post-hoc analysis. The two-sided significance level was set to 0.05.

3. RESULTS

3.1. Anticoagulant potentials according to ThromboPath® in the healthy subgroup by age

The ODB and ODA were obtained by measuring the amounts of TG produced without and

with protac, respectively, in plasma samples from the healthy group according to age using ThromboPath®. PiCi% was calculated as described in the Methods section (**Table 1**). The ODB and ODA in healthy adults (n=20) were 801.8±63.6 and 105.0±29.6 mOD/min, respectively. TG was suppressed by 78.6–92.7% with the addition of protac, and the PiCi% was 86.9±3.4%. The ODB in 35 newborns was 525.0±73.8 mOD/min, approximately two-thirds of that in adults, whereas the ODA was 300.5±51.4 mOD/min, approximately three-fold higher than the TG in adults. The PiCi% was 42.1±9.9%, approximately half that in adults. In the 42 infants, the ODB was 719.6±95.8 mOD/min, an approximately 1.4-fold increase in TG relative to newborns and 90% of that in adults, indicating significant changes in procoagulant potentials. The ODA was 210.5±62.2 mOD/min, approximately two-fold higher than that in adults, resulting in a lower PiCi% than that in adults (69.8±11.0%). In the 35 toddlers, the ODB and ODA were 785.2±53.2 and 133.3±33.8 mOD/min, corresponding to approximately 98% and 127% of the levels in adults, respectively, resulting in a PiCi% of 82.9±4.4% which was comparable to that in adults.

Figure 1 shows the parameters for all healthy samples in order of age in months. The results of this assay showed that procoagulant potentials (ODB) were low in newborns and dynamically increased in infants, especially within the first 6 months of life, and were close to adult levels in toddlers. In contrast, residual TG after the activation of PC pathway (ODA) was three-fold higher in newborns than in adults, gradually reduced during infancy, and was close to the levels of adults in healthy toddlers. Therefore, anticoagulant potentials evaluated with PiCi% were very low in newborns (32.2–52.0%, ±1SD), markedly increased in infants (especially within the first 6 months) but remained low (58.8–80.8%), and approached adult levels (83.5–90.3%) in toddlers (78.5–87.3%).

3.2. Difference in PiCi% between pathological and healthy plasma samples

PiCi% is reduced not only in healthy children but also in adult patients with liver disease or taking anticoagulants such as warfarin [13,14]. The low PiCi% in such patients in the present study suggested qualitative differences in pathological samples compared with in healthy samples (**Table 1**). **Figure 1B** shows a two-dimensional map of ODB (without protac) and ODA (with protac). All samples from patients with liver disease overlapped with healthy samples. Furthermore, plasma samples from patients taking warfarin (older individuals with underlying cardiac diseases such as atrial fibrillation) or PC or PS deficiency suggested different positions in the two-dimensional maps compared with healthy samples. In other

words, among samples with lower PiCi% (less than the cutoff value of 80%), procoagulant potentials (ODB) differed from those of healthy children, even in similar PiCi% ranges (very low ODB in the warfarin group or very high ODB and ODA in the PC- or PS-deficient groups).

3.3. Relationships of plasma levels of PT-INR, PC, PS, and AT with PiCi%

To clarify the correlation of critical plasma components with ThromboPath® parameters, we measured the levels of PT-INR, PC, and PS, as well as AT activity in the plasma. The results are summarized in **Table 1**. Because of the limited sample volume, the number of measurable samples was restricted in several tests (shown in brackets in Table 1). Multiple regression analysis was difficult to perform because of the small sample size; therefore, the correlation coefficients for each parameter were analyzed in a round-robin manner.

Results of the total sample are shown in **Supplemental Data**. All analyzed pairs showed a significant correlation ($p<0.0001$) with |r| values. Among the PT-INR, PC, PS, and AT activities, PS showed the highest |r| value with PiCi% (r=0.86), followed by PC (r=0.85). The highest |r| value with ODB and ODA was observed in PC (r=0.85 and -0.77, respectively). The distributions of ODB, ODA, and the resulting PiCi% values changed dramatically with age; thus, the same analyses were repeated for all four age groups. We observed no significant correlations between PiCi% and PT-INR in toddlers (r=-0.14) or adults (r=-0.05); however, a weak negative correlation was observed in newborns (r=-0.41, $p<0.05$), and a moderate negative correlation was observed in infants (r=-0.50, $p<0.001$) (**Figure 2A**). No significant correlation was observed between PiCi% and PC activity in newborns (r=0.28) or adults (r=0.05); however, a strong positive correlation was observed in infants (r=0.73, $p<0.001$), and a moderate positive correlation was found in toddlers (r=0.55, $p<0.001$) (**Figure 2B**). No significant correlation was observed between PICI% and PS in adults (r=0.48, $p>0.05$) or infants (r=0.34, $p>0.05$); however, a moderately significant positive correlation was found (r=0.66, $p<0.01$) (**Figure 2C**). No significant correlation was observed between PiCi% and AT activity in adults (r=-0.36), toddlers (r=0.38), or infants (r=0.30); however, a weak positive correlation was observed in newborns (r=0.38, $p<0.05$) (**Figure 2D**).

We summarized the results of the correlations of ODB, ODA, and PiCi% with PT-INR, PC, PS, and AT in the four age groups in **Figure 3** and **Supplemental Data**. The characteristics for each age group were as follows:

(i) Newborns: Procoagulant potential (ODB) was strongly correlated with AT, PC, PS, and PT-INR. PiCi% was moderately correlated with PS and weakly correlated with AT or PT-INR but not with PC. PT-INR was negatively correlated with PC, PS, and AT, whereas AT was positively correlated with PC and PS, suggesting that both procoagulant and anticoagulant potentials in newborns were low, probably because of immature liver function.
(ii) Infants: ODB was moderately correlated with PC and PT-INR and weakly correlated with AT but not with PS. ODA was moderately correlated with PC, resulting in a strong correlation between PiCi% and PC. PT-INR was weakly correlated with PC, AT, and PiCi%.
(iii) Toddlers: ODB was strongly correlated with PC but not with PT-INR or AT. ODA was weakly correlated with PC, resulting in a moderate correlation between PiCi% and PC. PS was not evaluated in this age group because of the lack of plasma samples.
(iv) Adults: ODB was moderately correlated with PC and AT but not with PT-INR, suggesting that PT-INR was plotted within a narrow range (1.01±0.06 in Table 1) owing to mature procoagulant potentials in adults (probably similar in toddlers). ODA was moderately correlated with AT in this age group. PiCi% was not correlated with any factor, probably because PiCi% was plotted within a narrow range in healthy adults.

3.4. Effect of spiked AT on ODB, ODA, and PiCi% in healthy newborn plasma

As ThromboPath® was an indicator of total PC pathway function, PC and PS were likely to be strongly correlated with this assay. The involvement of PT-INR was also convincing, as PiCi% is based on endogenous TG potentials [9]. Therefore, the weak but significant correlations between AT and PiCi% in newborns and infants were unexpected. Therefore, we performed additional experiments to confirm the effects of AT alone on the plasma of healthy newborns. ThromboPath® was performed on the plasma from newborns (n=9) in the presence or absence of added commercial AT (Neuert®; final concentration, 100%). Spiked AT slightly reduced TG in ODB ($p<0.05$) but did not change ODA or PiCi% (**Table 2**), suggesting that the correlation between AT and PiCi% occurred owing to confounding factors (PC and PS). Newborns with low AT levels also had low PC and PS levels, and the latter was associated with low PiCi% in this age group.

3.5. Impact of spiked PC or FFP on PiCi% in PC-deficient plasma

ThromboPath® was performed with commercially available severe PC-deficient plasma spiked with PC (0–150%) to estimate the effect of PC concentrates, which are clinically used for the treatment of severe congenital PC deficiency, on PiCi% (**Figure 4A**). TG was

suppressed by the addition of protac (ODA) as the amount of spiked PC increased. The PiCi% was approximately 80% at 50% of the spiked PC and reached a plateau at 70% of the PC where the PiCi% exceeded the cutoff value for adults (>80%).

We also examined the effects of additional spiked FFP at various PC concentrations (**Figure 4B**). Plasma (150 mL) with PC activity (0–150%) was mixed with FFP (50 mL) to simulate the clinical setting of FFP transfusion at 15 mL/kg. The expected increase in PC activity was approximately 25%. Spiking FFP into PC-deficient plasma (PC=0%) improved the PiCi% level (57.5%; **Figure 4B**), which corresponded to a 10–25% increase in PC activity (48.8–66.3%; **Figure 4A**). The effect of spiked FFP on PiCi% was no longer observed at PC concentrations >50%.

4. DISCUSSION

Many pediatric textbooks indicate that children cannot be considered miniature adults; rather, children are in the process of growth and development. This description also applies to hemostasis and thrombosis: pediatric and adult hemostatic balances differ. Although the PC pathway has been comprehensively evaluated in adults [9-11], the evaluation in developing young children has been limited to individual factors such as PC and PS [1-5]. The strength of our results lies in demonstration of the evolution of the overall function of the PC pathway with age.

The results of the present study demonstrated that PiCi%, a key parameter of the overall PC pathway function, changed dramatically from the neonatal stage to infancy (**Figure 1A**). In newborns, the most important factor that correlated with PiCi% was PS and not PC; in infants, it was PC but not PS (**Figures 2,3**). This finding does not indicate that PC is unimportant in newborns; rather, under conditions of low PC values (29.8±15.6%) in healthy newborns, PS values may be in the rate-limiting phase. Our findings also indicated that in infants, in whom the values approached the adult level but showed large variability (68.2±23.6%), PC values may be in the rate-limiting phase. Many studies have investigated age-related changes in each component of coagulation/anticoagulation factors. In general, levels of most coagulation factors in newborns are approximately half those in adults, with most reaching adult levels by 6 months, whereas others do not reach those levels until adolescence. Likewise, PC and PS levels are low at birth (usually <50% in adults) and in the first weeks of life, approaching

adult levels by 6–12 months of life. Low PS levels may be partially counterweighed by a higher proportion of free PS because the levels of its carrier protein, the C4b-binding protein, are also reduced (or may even be undetectable) in newborns [5]. AT levels are relatively low at birth and in the first weeks of life but gradually increase thereafter, approaching adult levels by 3–6 months of life.

Plotting of the PC pathway function on the two axes of ODB and ODA, as well as PiCi%, showed a continuum of change with age among the healthy subgroups, whereas some groups showed a different distribution from the normal range owing to pathological conditions or anticoagulant therapy (**Figure 1B**). The four cases in the group of (older) adults with chronic hepatitis or liver dysfunction had PiCi% of 68.1±13.4%, close to that in the infant group (69.8±11.0%); the 2D-plots also overlapped. In contrast, in the six adults on warfarin, the PiCi% was 63.5±12.9%, similar to the values in healthy infants or adults with liver disease. However, the ODB and ODA values differed in the 2D map. In these plasma samples, VK deficiency caused a decrease in TG (low ODB) owing to a decrease in VK-dependent coagulation factors.

The warfarin-treated group demonstrated lower ODA (with higher PiCi%) compared with healthy newborns with comparable ODB values, although the lower PC and PS were probably equivalent. Although the reason for this difference is unclear, the influence of other factors that are VK-independent coagulation factors involved in PC pathway function (such as FV) has been speculated. Adult ~~cases~~ plasmas of ~~congenital~~ PC or PS deficiency also showed a different distribution compared with healthy newborns, with equally low PiCi%. The ODB values remained as high as those of healthy toddlers or adults, whereas the ODA values were extremely high owing to very low PC or PS values, resulting in a different distribution. Although the present study did not include samples from newborns and infants with congenital PC or PS deficiencies, discriminating the pathology of abnormal PC pathway function by using age-group references in ThromboPath® is possible.

Although we examined the relative contributions of PT-INR, PC, PS, and AT to the total data of all age groups in terms of PiCi%, this analysis was inappropriate because PiCi%, the outcome of the regression analysis, varied greatly with age. Therefore, we performed a comparison using a correlation matrix, which allowed us to identify the characteristics of each age group (**Figure 3, Supplemental Data**). It is difficult to explain the involvement of

AT in PC pathway function because AT inhibits thrombin and FXa in the presence of heparin. However, the overall analysis revealed a strong correlation with PiCi% and a weak but significant correlation in newborns. To assess the validity of these findings, we added exogenous AT to newborn plasma. However, the PiCi% did not change significantly (**Table 2**), suggesting that the correlation between AT and PiCi% may have been mediated by confounding factors. AT showed a strong correlation with PC and PS, suggesting that the effects of PC and PS variation can be assessed. Although the *in vitro* addition of PC or PS to the samples would have allowed the assessment of PiCi% improvement, we were unable to do so due to insufficient sample volume. Instead, we observed a concentration-dependent improvement in PiCi% when PC was added to commercial PC-deficient plasma (**Figure 4A**). However, newborn plasma with the same PC concentration range (<25%), showing large changes in PiCi%, did not correlate with PC concentration. The effect of PS concentration on PiCi% was not assessed in the present study but has been reported previously [9], including by our group (data not shown).

We also evaluated the effects of *in vitro* addition of FFP equivalent to 15 mL/kg (**Figure 4B**). The effect of FFP on PiCi% was reasonable, although each clotting factor was expected to increase by approximately 25% with FFP. These results were obtained using commercially available PC-deficient plasma, which differs from the composition of newborn plasma in neonatal fulminant purpura, to which the actual administration is expected. However, if adult FFP, which is equivalent to 25% of the plasma volume, is administered to newborns, additive or synergistic effects of PC and other relevant factors can be expected.

The results of this study have clinical implications. ThromboPath® is a highly sensitive assay for PC, PS, FV, lupus anticoagulant, high FVIII levels, and changes in PT abnormality related to PC pathway function and had shown great promise for application in screening tests for thrombophilia. However, the clinical significance of screening tests remains controversial [15]. Moreover, screening tests for asymptomatic patients are not currently recommended because thrombosis does not always develop in groups or families with abnormal levels; hence, the results may cause anxiety, and the tests are not cost-effective [15]. Thrombosis is more likely to develop not when laboratory values are out of the normal range but rather in combination with exacerbating factors such as vessel wall condition, genetic factors, and environmental factors. In the present study, we attempted to identify not only PiCi% but also the relationships with and without protac. However, the pathological samples demonstrated

variation by age group (liver disease) and according to warfarin use and PC/PS deficiency). Therefore, the potential clinical applications of our findings include the following:

(i) A new index that considers the balance between coagulability and anti-coagulability, in addition to PT-INR, for patients taking warfarin and comparison with those on direct oral anticoagulants (DOACs).

(ii) A new index of VK efficacy in newborns.

(iii) Assessment of the association with thrombotic risk in the context of a similar single test value for thrombophilia (PC, PS, AT, and FV_{Leiden}).

(iv) The potential for understanding the pathophysiology of liver disease by assessing whether the coagulation/anticoagulation balance is physiological (outside adult reference values but within the age group reference distribution) or pathological.

This development was initially anticipated; however, ThromboPath® was discontinued. We hope that this assay, which is easy to use and reproducible, will be relaunched. The development of similar methods is also expected, and we are currently developing similar methods [11,16].

This study had some limitations, including the lack of ThromboPath® availability and the small sample sizes, especially the number of PS-deficient samples. Additional limitations included the blood collection volume, quality of the blood samples used, and lack of comparison with other coagulation tests (such as activated partial thromboplastin time, fibrinogen, D-dimer, and FV).

In conclusion, the assay for evaluating total TG in plasma with and without protac enabled the identification of the characteristics of the PC pathway function in younger children by age group. In addition to the evaluation of individual factors, a fully automated method with a small sample volume (150 μL), such as the assay used in our study, is useful for the analysis of developmental hemostasis in younger children.

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Hospital) for helpful statistical advice. We would like to thank Honyaku Center Inc. for English language editing.

Authorships

Contribution

TN carried out the experiments, analyzed the data, created the figure, wrote the paper; **KO** designed the study, analyzed the data, created the figures, wrote the paper, and approved the final version for publication; **HT** supported clinically**; YT** and **TN** supported clinically and supervised this research, **KN** designed the study, interpreted the data, wrote the paper, and edited the manuscript.

Conflict of interests

The authors declare that they have no conflicts of interest.

Data availability: The full data that support the findings of this study are available on request to the corresponding author.

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FIGURE LEGENDS

Figure 1. **Thrombin generation without and with protac and resulting PiCi% by ThromboPath® for healthy plasma samples by age group and pathological patient samples.**

(*Panel* **A**) The thrombin generation values in the absence (ODB; *blue bars*) and presence (ODA; *red bars*) of protac in each healthy plasmas by age group (newborn, infant, toddler, and adult) were measured at 405 nm after the addition of S-2796 using ThromboPath® as described in Methods. The results were expressed as percentage of protac-induced coagulation inhibition (PiCi%; *closed dots*) that were determined by using the (ODB – ODA)/ODB x 100.

(*Panel* **B**) The thrombin generation values without and with protac in healthy plasmas by age group (*panel* A) and pathological plasmas (liver dysfunction, warfarin-treated, and PC or PS-deficiency) were plotted as a 2-demensional map. The straight lines indicate the 10-90% PiCi% positions.

Figure 2. Relationship of plasma levels of anticoagulation parameters on PiCi% by age group

Plasma levels of PT-INR (*panel* **A**), PC (*panel* **B**), PS (*panel* **C**), and AT (*panel* **D**) activity were measured and were compared to PiCi% by age group. The analyzed pairs were calculated as the correlation with variable |r| values. The symbol used are as follows, newborn; *blue circles*, infant; *red circles*, toddler; *green circles*, adult; *black circles.*

Figure 3. Relationship of correlations of ODB, ODA, and PiCi% to PT-INR, PC, PS, and AT, by age group

The |r| values were shown as thickness of the lines. The degree of correlation was classified based on the |r| values: strong (>0.7), moderate (>0.5), and weak (>0.3). Significant correlations ($p<0.05$) were shown as solid lines and no significant correlations as dotted lines. Positive and negative correlations were shown as black and gray lines, respectively.

Figure 4. Effect of spiked purified PC or FFP on PiCi% in PC-deficient plasma

The thrombin generation values in the absence (ODB; *blue bars*) and presence (ODA; *red bars*) of protac in commercially available severe PC-deficient plasma spiked with purified PC (0-150%) (*panel* **A**) and with FFP (*panel* **B**) were measured using ThromboPath® as described in Methods. The results were expressed as PiCi%) *closed dots*) that were determined by using the (ODB – ODA)/ODB x 100.

Table 1

Table 1. Summarized results of PiCi% and anticoagulant factors in healthy groups by age or patient groups.

PiCi%: Protac-induced coagulation inhibition%, PT-INR: prothrombin time-international ratio, PC: protein C, PS: protein S, AT: antithrombin, Ac: activity, N/A: not applicable, ODB: thrombin generation without protac, ODA: thrombin generation with protac.

Data were shown as mean±SD.

	Newborns	Infants	Toddlers	Adults	Liver dysfunction	Warfarin-treated	PC or PS deficiency
Number	35	42	35	20	4	6	4
Age (month)	0.16±0.25	5.3±3.3	25.2±10.3	353±66	851±121	1,055±96	N/A
ThromboPath®				(*mOD/min*)			
ODB; protac (－)	525.0±73.8	719.6±95.8	785.2±53.2	801.8±63.6	705.3±106.5	406.5±61.5	796.6±21.1
ODA; protac (+)	300.5±51.4	210.5±62.2	133.3±33.8	105.0±29.6	223.7±92.6	143.2±32.7	648.7±89.2
PiCi% (%)	42.1±9.9	69.8±11.0	82.9±4.4	86.9±3.4	68.1±13.4	63.5±12.9	18.6±10.1
Labo data							
PT-INR	1.25±0.23 (n=29)	1.07±0.13	1.00±0.09	1.01±0.06	N/A	N/A	N/A
PC:Ac (%)	29.8±15.6 (n=25)	68.2±23.6 (n=41)	86.1±19.2	113.9±13.4	N/A	N/A	N/A
PS:Ac (%)	32.9±20.0 (n=14)	77.3±17.2 (n=16)	N/A	96.9±14.0 (n=10)	N/A	N/A	N/A
AT:Ac (%)	49.0±19.6 (n=29)	93.4±25.3 (n=35)	122.4±23.2 (n=16)	104.0±11.8	N/A	N/A	N/A

Table 2

Table 2. PiCi% on the spike of exogenous AT in newborn plasma (ThromboPath®)

PiCi%: Protac-induced coagulation inhibition%, AT: antithrombin, n.s.: not significance.

ODB: thrombin generation without protac, ODA: thrombin generation with protac

A paired *t*-test was conducted as a post-hoc analysis. The significance level is set at 0.05 (two-sided). Data were shown as mean±SD.

	No spike of AT	Spike of AT	p value
	mOD/min		
ODB; protac (−)	506.6±79.0	475.2±90.3	<0.05
ODA; protac (+)	288.6±70.0	272.5±55.8	n.s.
PiCi% (%)	40.9±19.0	40.2±17.4	n.s.

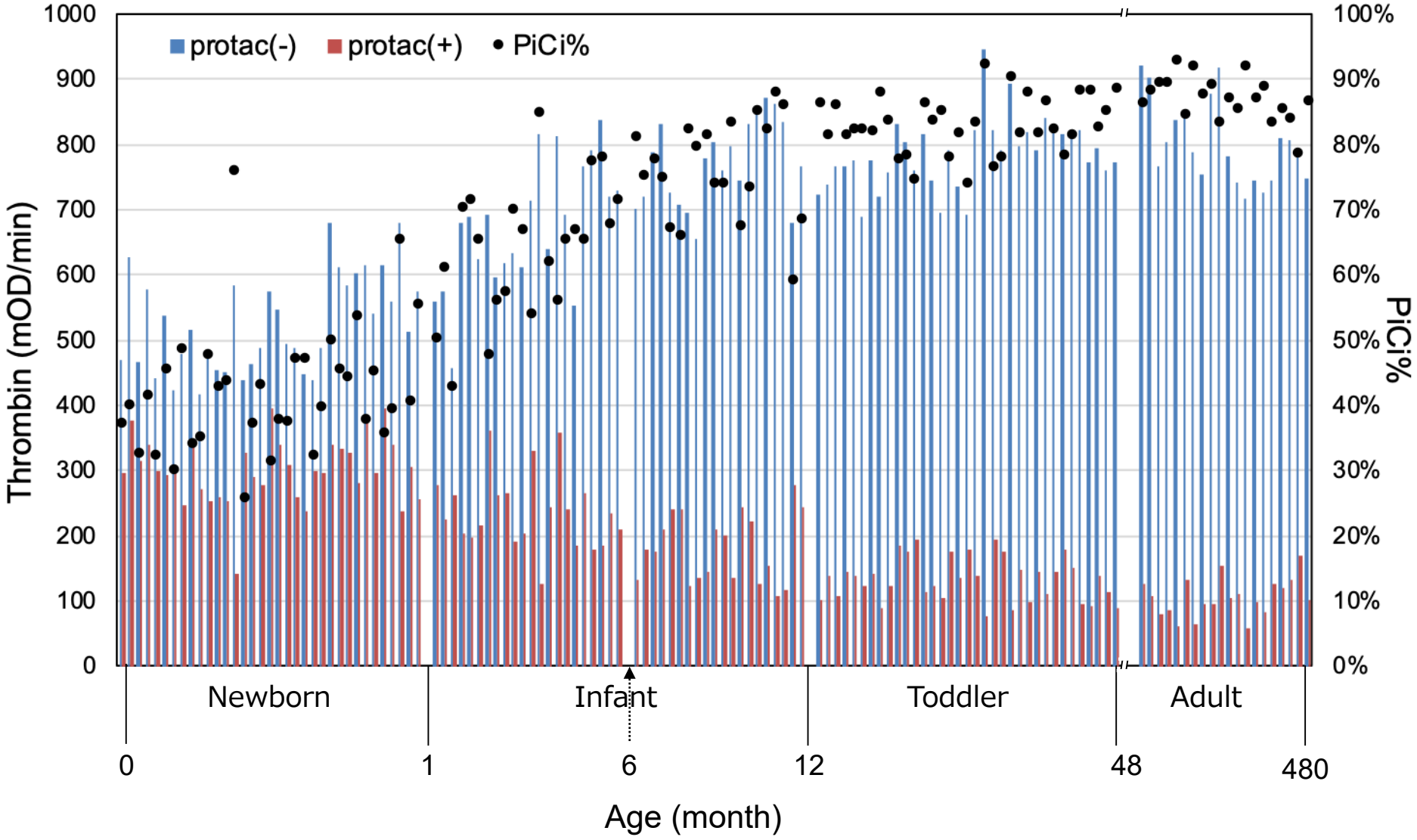
protac(-)
protac(+)
PiCi%
Thrombin (mOD/min)
PiCi%
1000
900
800
700
600
500
400
300
200
100
0
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Newborn
Infant
Toddler
Adult
0
1
6
12
48
480
Age (month)

Figure 1A

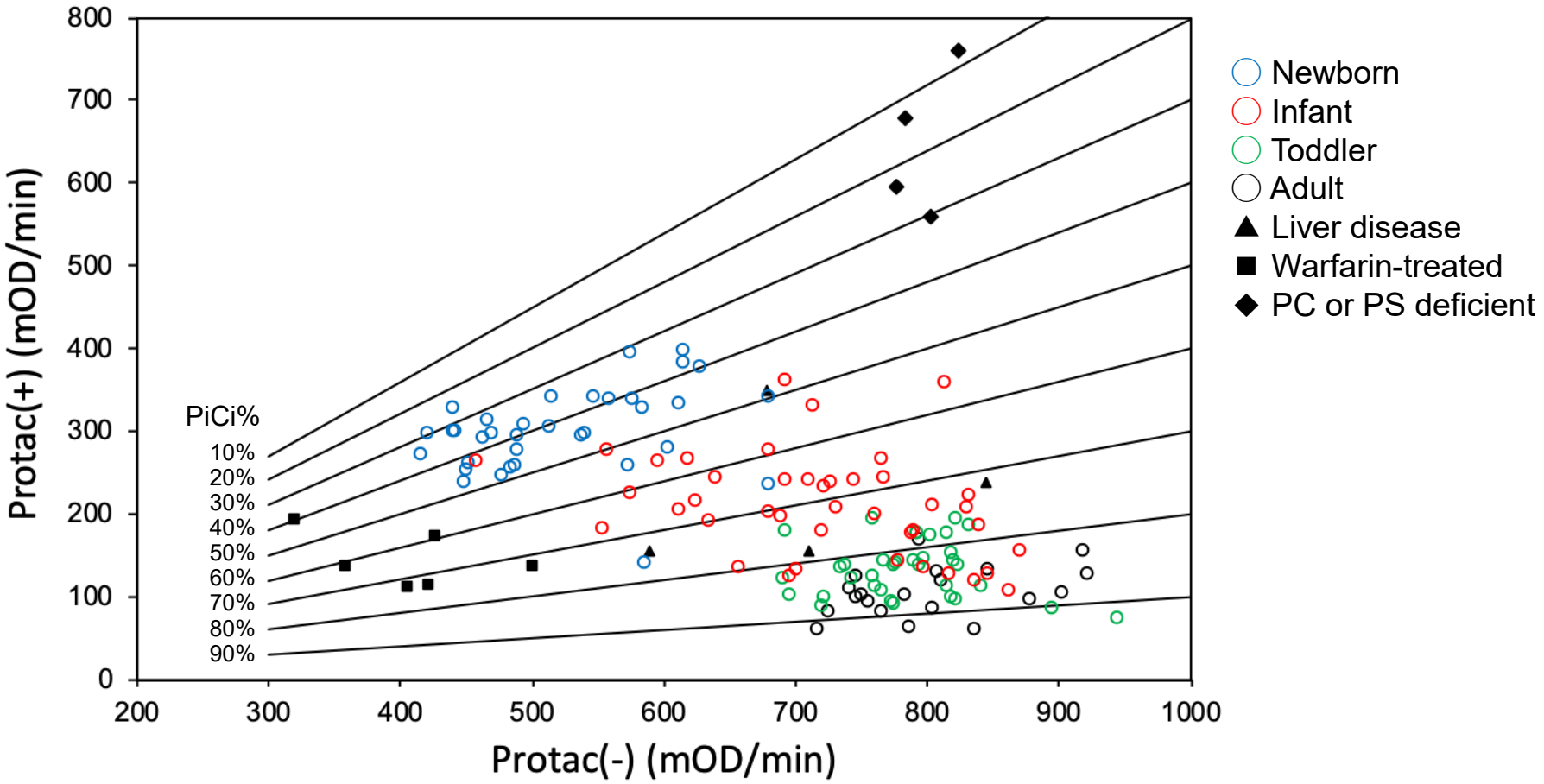
Protac(+) (mOD/min)
Protac(-) (mOD/min)
0
100
200
300
400
500
600
700
800
200
300
400
500
600
700
800
900
1000
PiCi%
10%
20%
30%
40%
50%
60%
70%
80%
90%
Newborn
Infant
Toddler
Adult
Liver disease
Warfarin-treated
PC or PS deficient

Figure 1B

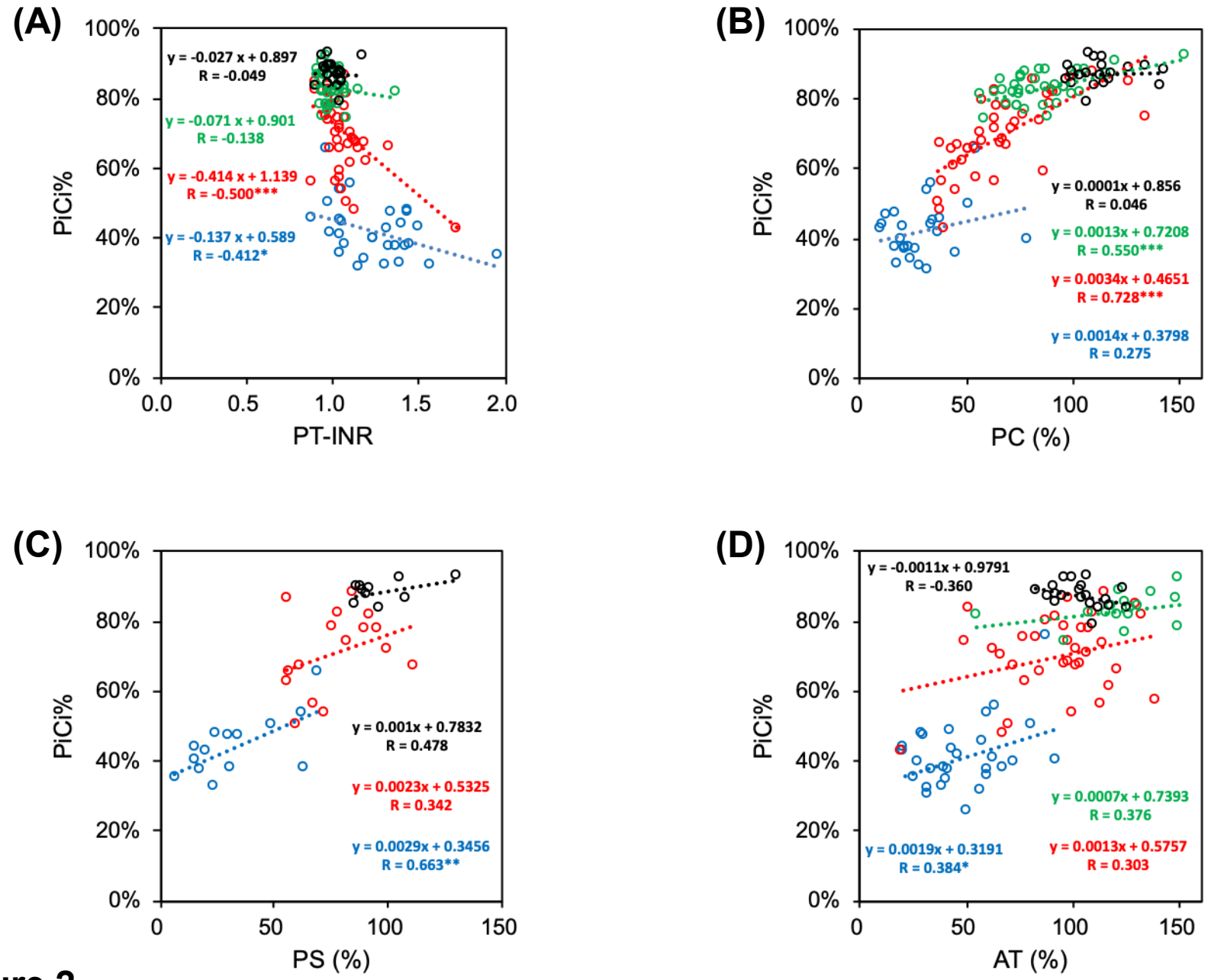
(A)
y = -0.027 x + 0.897
R = -0.049
y = -0.071 x + 0.901
R = -0.138
y = -0.414 x + 1.139
R = -0.500***
y = -0.137 x + 0.589
R = -0.412*
PiCi%
PT-INR
(B)
y = 0.0001x + 0.856
R = 0.046
y = 0.0013x + 0.7208
R = 0.550***
y = 0.0034x + 0.4651
R = 0.728***
y = 0.0014x + 0.3798
R = 0.275
PiCi%
PC (%)
(C)
y = 0.001x + 0.7832
R = 0.478
y = 0.0023x + 0.5325
R = 0.342
y = 0.0029x + 0.3456
R = 0.663**
PiCi%
PS (%)
(D)
y = -0.0011x + 0.9791
R = -0.360
y = 0.0007x + 0.7393
R = 0.376
y = 0.0019x + 0.3191
R = 0.384*
y = 0.0013x + 0.5757
R = 0.303
PiCi%
AT (%)

Figure 2

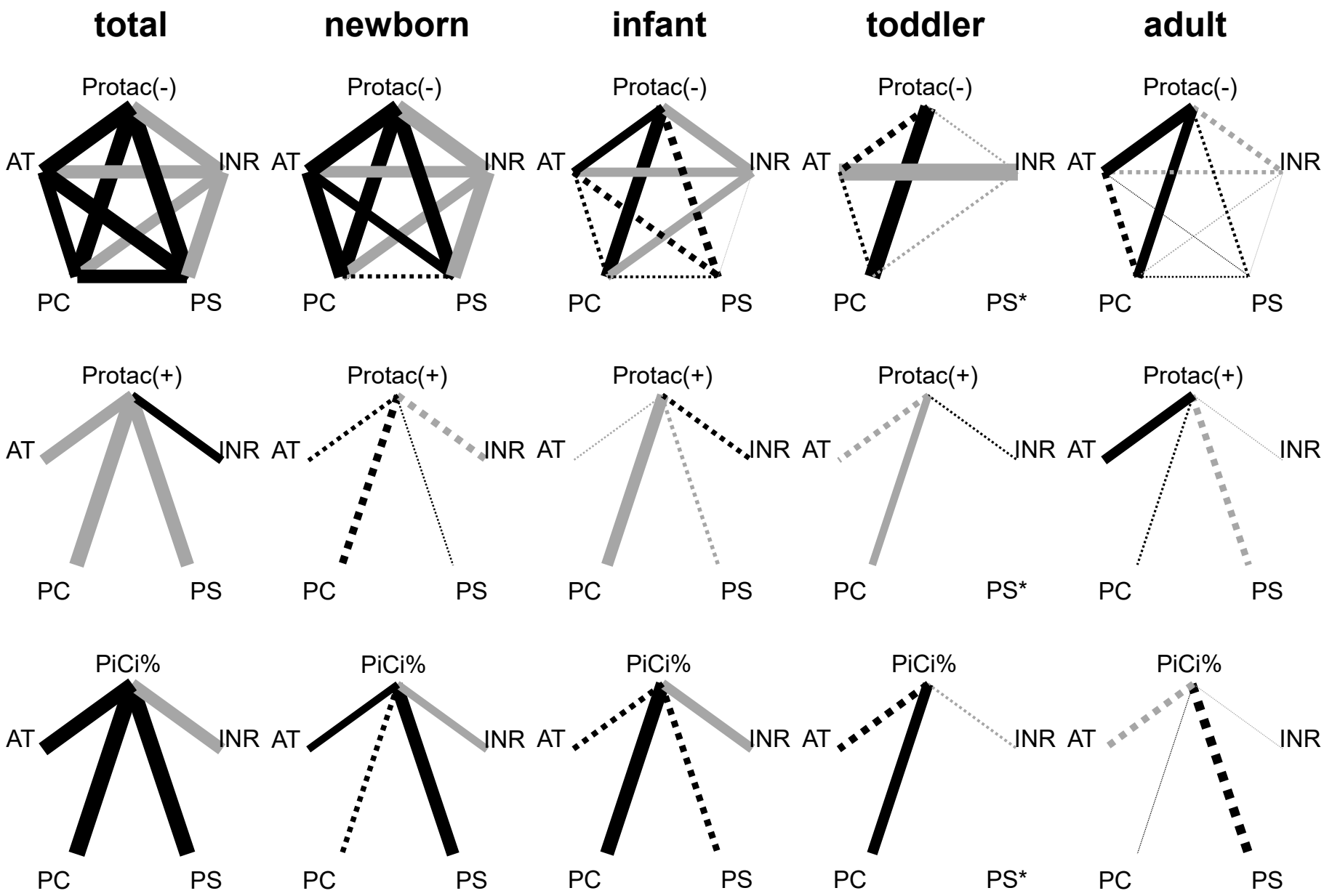
total
newborn
infant
toddler
adult
Protac(-)
Protac(+)
PiCi%
AT
INR
PC
PS
PS*
|r|=0.3
|r|=0.5
|r|=0.7

Figure 3

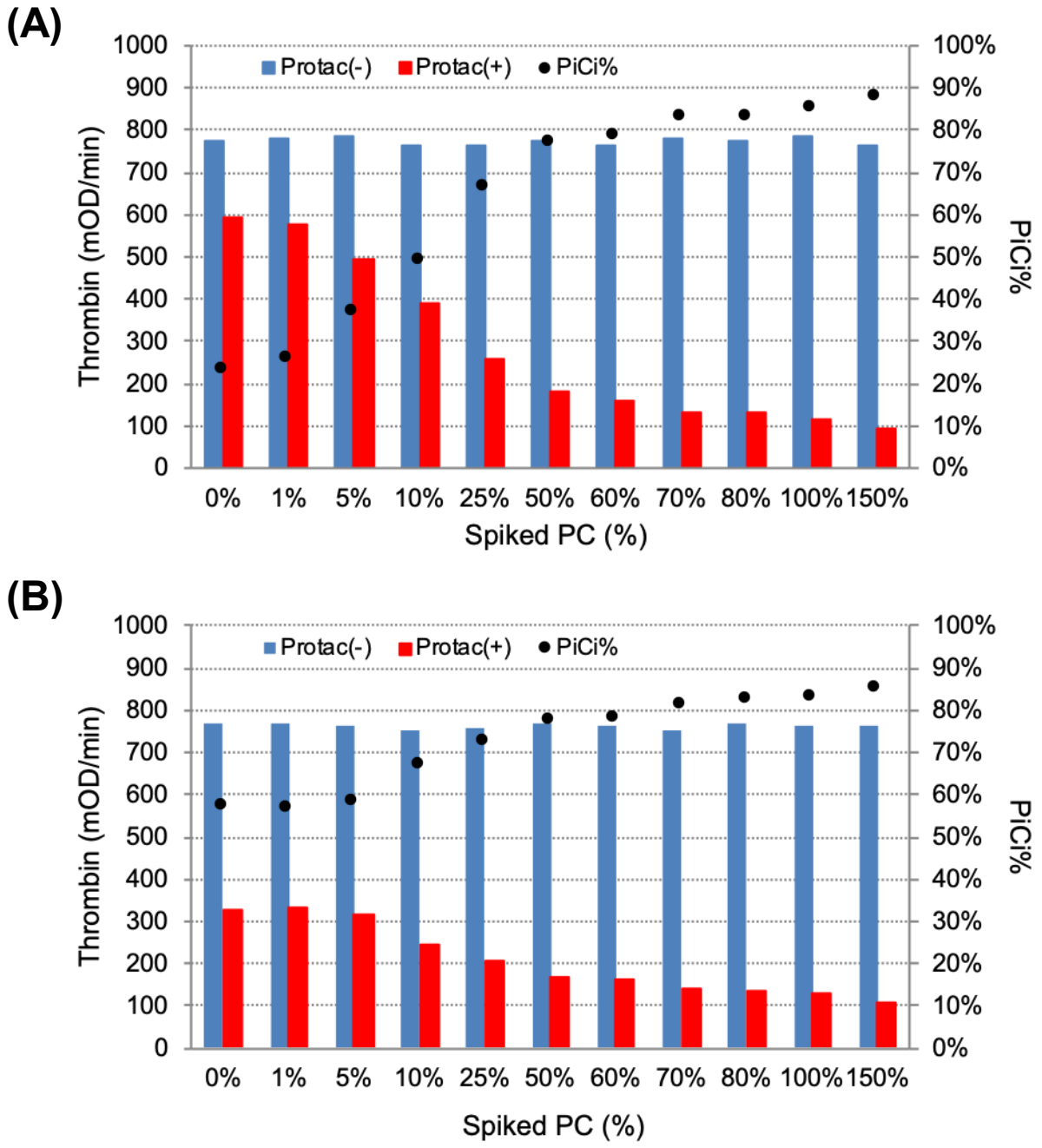
(A)
Protac(-)
Protac(+)
PiCi%
Thrombin (mOD/min)
PiCi%
0
100
200
300
400
500
600
700
800
900
1000
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
0%
1%
5%
10%
25%
50%
60%
70%
80%
100%
150%
Spiked PC (%)
(B)
Protac(-)
Protac(+)
PiCi%
Thrombin (mOD/min)
PiCi%
0
100
200
300
400
500
600
700
800
900
1000
0%
10%
20%
30%
40%
50%
60%
70%
80%
90%
100%
0%
1%
5%
10%
25%
50%
60%
70%
80%
100%
150%
Spiked PC (%)

Figure 4

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Supplementary Material

231011_Supplemental_data.xlsx

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[12, October, 2023]

Akifumi Takaori-Kondo
Editor-in-Chief
International Journal of Hematology

Dear Editor:

I wish to submit an Original Article for publication in the *International Journal of Hematology*, titled "Anticoagulant effects of protein C, protein S, and antithrombin levels on the protein C pathway in young children". The paper was coauthored by Takashi Nakagawa, Hitoshi Tonegawa, Yukihiro Takahashi, Toshiya Nishikubo, and Keiji Nogami.

This study evaluated the contribution of protein C (PC) pathway function during early childhood by administering protac (a PC activator) and measuring changes in plasma thrombin generation (TG). The results showed that the percentage of protac-induced coagulation inhibition (PiCi%) increased dynamically during infancy and was correlated with protein S (PS) levels in newborns and PC levels in young children. In PC-deficient plasma, adding approximately 25% PC or fresh frozen plasma equivalent to 15 mL/kg (approximately 25% PC) improved the PiCi%. Finally, the assay for evaluating total plasma TG with and without protac enabled the identification of the characteristics of the PC pathway function in younger children by age group. We believe that our study makes a significant contribution to the literature because although the PC pathway has been comprehensively evaluated in adults, the evaluation in developing young children has been limited to individual factors such as PC and PS. Our findings demonstrated the evolution of the overall function of the PC pathway with age.

This manuscript has not been published or presented elsewhere in part or in entirety and is not under consideration by another journal. All study participants provided informed consent, and the study design was approved by the appropriate ethics review board. All authors have read and understood your journal's policies, and we believe that neither the manuscript nor the study violates any of these. There are no conflicts of interest to declare.

Thank you for your consideration. I look forward to hearing from you.

Sincerely,

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