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ORIGINAL ARTICLE

# Age dependency of coagulation parameters during childhood and puberty

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**Summary.** *Background:* Use of age-adjusted reference values is crucial for correct diagnosis and management of thrombotic and hemorrhagic disease in children. They vary with utilized reagents and analyzers. *Objectives:* We established reference values with the Sysmex CA-1500 System and in parallel with the Behring BCS System using reagents from Siemens Healthcare Diagnostics Products GmbH. *Methods:* After informed consent, blood samples were obtained from 218 healthy children and 52 healthy adults, grouped as 1–6 months ($n = 29$), 7–12 months ($n = 25$), 1–5 years ($n = 57$), 6–10 years ($n = 57$), 11–18 years ($n = 50$) and > 19 years ($n = 52$). *Results:* Most coagulation parameters demonstrate good comparability between analyzers with the exception of PT and APTT. Single coagulation factors fibrinogen, factor (F) II, FIX, FXI and XII were significantly decreased in the youngest children; the strongest age dependency was found for coagulation inhibitors Protein C and S, both significantly decreased in infancy and young childhood. We confirmed that high levels of von Willebrand factor are found in the youngest children without increased levels of FVIII followed by decreased von Willebrand levels in the subsequent age group. In children with blood group O a less distinct increase in time was found, compared with individuals with one of the other blood groups. *Conclusions:* The correlation between the CA-1500 and the BCS system was remarkable. Differences were most pronounced between children < 12 months and older children and adults, confirming the phenomenon of developmental hemostasis. The rationale for age-related changes in the hemostatic system remains unraveled. Our results underline the need for age-specific reference ranges.

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## Introduction

Since the concept of developmental hemostasis was introduced by Maureen Andrew in 1987, several studies have confirmed the age-related numerical changes in pediatric hemostasis [1,2]. Monagle *et al*. [3] underlined the need for age-related reference ranges to be determined for each reagent-analyzer combination. Their results provided useful data for laboratories using the same Diagnostica Stago analyzer-reagent combination as used in their study. Indeed, they too confirmed the concepts of developmental hemostasis as launched by Andrew. With the development of novel reagents, methodologies and instruments, new reference values are continuously necessary. Currently, tests are underway that require smaller volumes for accurate monitoring of coagulation proteins. Consequently in the future, very young children could be tested without major ethical dilemmas in order to obtain normal reference ranges. However, the venapuncture itself and extraction of a sufficient amount of blood remains a challenge in this age group. In addition, it must be taken into account that in the very young clinical symptoms such as polycythemia and asphyxia will influence results [4]. In conclusion, pediatric reference ranges are not easy to generate and may be difficult to interpret for physicians not familiar with the concept of developmental hemostasis.

Ongoing discussions regarding the ethical difficulties of collection of blood samples from otherwise healthy children remain obstructive in the designing of studies on normal pediatric coagulation protein levels. All clinicians underline the importance of both age and reagent-analyzer appropriate reference values as a normal level according to one list may be misclassified as pathological in another reference list, with possible serious consequences. We establish reference intervals for a large number of coagulation parameters with a Sysmex CA-1500 System (Sysmex Corporation, Kobe, Japan) and in parallel with the Behring BCS System (Siemens Healthcare Diagnostics Products GmbH, Marburg, Germany) using reagents from Siemens Healthcare Diagnostics Products GmbH.

## Methods

After informed consent, blood samples were obtained from healthy subjects. For comparison purposes age groups were classified as in earlier studies and grouped as follows: (i) 0–6 months, (ii) 7–12 months, (iii) 1–5 years, (iv) 6–10 years, (v) 11–18 years and (vi) > 19 years and < 50 years (adults). The study was performed in accordance with the Erasmus Medical Centre - Sophia Children's Hospital Ethics Committee. Written informed consent was obtained from the adult controls and from parents and/or guardians of the children and from the children older than 12 years.

The inclusion criteria were: healthy children and adults without previous thromboembolic or hemorrhagic events or any other coagulation disturbances, with no anticoagulant therapy and no other interfering disease; a birth weight of > 3000 g and delivery at full-term for the group 0–6 months old; children undergoing a minor surgical procedure needing intravenous access for anesthesia; and an available written informed consent.

### *Blood sampling*

At least 14-mL and maximally 20-mL citrated blood samples were obtained from the children with 18–24 G catheters. From the adults and older children citrated blood samples were obtained by peripheral venapuncture from the antecubital vein employing only a light tourniquet to avoid stasis. Standard blood collection tubes containing 0.5 mL of 3.2% (0.105 M) trisodium citrate were used. Immediately after sampling, the blood was centrifuged twice (15 min at 2500 × *g* and 5 min at 10 000 × *g*, room temperature) and frozen at < −70 °C within 2 h of blood withdrawal. All blood samples were thawed within 10 months and measured within 4 h after thawing (except for Protein S and D-dimer assays, which were performed within 2 h after thawing).

### *Analyzers*

Samples were analyzed on two different analyzers, the Behring Coagulation System (BCS®) from Siemens Healthcare Diagnostics Products GmbH (Marburg, Germany) and the Sysmex® CA-1500 System from Sysmex Corporation (Kobe, Japan). Both systems are fully automated analyzers for clotting, chromogenic and immunologic assays. The BCS System is a high-throughput centrifugal analyzer with a wide range of assay applications and the CA-1500 System is a compact medium size analyzer. The investigated coagulation parameters and methods are listed in Table 2. All reagents were from Siemens Healthcare Diagnostics Products GmbH and used according to the instructions given by the manufacturer, except for minor deviations regarding sample storage for some assays.

### *Statistics*

Statistical analysis and the determination of percentiles were performed using SAS-Software Version 9.1 (SAS Institute Inc., Cary, NC, USA). The results are presented as median, mean and the central 90% interval (5th to 95th percentile) of the different age groups. Statistical differences between age groups and methods were investigated using the *t*-test with $P < 0.05$ considered statistically significant. For statistical analysis, values above or below the reported range were defined as Y = 0.1 (or 0.01 for D-dimer) ± limit of the reporting range. If results for percentiles were equal to Y, percentiles were expressed as less than or greater than the limit of the reporting range. ABO blood group-dependent differences (O vs. non-O) were calculated for FVIII and von Willebrand factor using the *t*-test.

## Results

Samples were obtained from 218 healthy children and 52 adults. Results are reported for six different age groups: (i) 1–6 months ($n = 29$), (ii) 7–12 months ($n = 25$), (iii) 1–5 years ($n = 57$), (iv) 6–10 years ($n = 57$), (v) 11–18 years ($n = 50$) and (vi) > 19 years ($n = 52$); demographics are shown in Table 1. Children and adults involved had different ethnic backgrounds, reflecting the Dutch population. As the youngest recruited child was 1 month old, the first group will be denoted as the 1–6 months group. In this group seven children are in the range of 1–3 months old, three children are on formula feeding only, three children are on breastfeeding combined with formula feeding, and only one child is receiving breastfeeding only. In the Netherlands it is recommended that children on breastfeeding only receive a supplementation of 150 μg vit K daily until the age of 3 months. Infant formula contains as standard about 40 μg vit K/100 g.

The employed reagents for the BCS and Sysmex CA-1500 are given in Table 2. Tables 3–7 summarize the results for different age groups by showing the median, mean and the 90% central interval for each parameter and method. For some samples, the amount was insufficient to perform all methods; the affected age groups and methods are indicated in table footnotes.

All global assays (Table 3) decreased during childhood, with the highest changes in the APTT. The analysis revealed two extreme outliers, which were eventually excluded from final analysis. The first outlier was a member of the 1–6 months group, who for the APTT demonstrated results of 154 s with Pathromtin SL and 22 s with Actin FS. Unspecified pediatric lupus anticoagulants were suspected because Pathromtin SL is more sensitive in detecting these antibodies than Actin FS; the APTT result obtained with Pathromtin SL was thus excluded. The second outlier was a member of the 6–10 years group with extremely low results for all single coagulation factors and immeasurable clotting times for PT and APTT on the BCS. Pre-analytic clotting was suspected and all results of this sample were also excluded from the final analysis.

The differences between global assays are minimal in the age groups 1–5 years, 6–10 years and 11–18 years and are not likely to be clinically relevant. For all global assays, results in the first group (1–6 months) showed a high variability leading

**Table 1** Demographic data

| | 1–6 months | 7–12 months | 1–5 years | 6–10 years | 11–18 years | > 19 years < 50 years |
|---|---|---|---|---|---|---|
| No. per group | 29 | 25 | 57 | 57 | 50 | 52 |
| M/F | 14/15 | 19/6 | 35/22 | 30/27 | 24/26 | 27/27 |
| Minor surgery | | | | | | |
| ENT | 2 | 2 | 29 | 26 | 24 | – |
| Surgery | 7 | 4 | 4 | 4 | 2 | – |
| Urology | 1 | 9 | 4 | 5 | 2 | – |
| Plastic surgery | 11 | 7 | 5 | 6 | 4 | – |
| Orthopedic surgery | 7 | – | 2 | 4 | 11 | 2 |
| Oral surgery | – | – | 1 | 4 | 5 | – |
| Ophthalmology | – | – | – | 1 | – | – |
| Others | 1 | 3 | 12 | 7 | 2 | 50 |
| Blood group | | | | | | |
| Non-O | 14 | 12 | 27 | 27 | 33 | 30 |
| O | 15 | 10 | 29 | 28 | 17 | 22 |
| Not known | – | 3 | 1 | 2 | – | – |

ENT, otolaryngologist; M, male; F, female.

**Table 2** Employed reagents for BCS and Sysmex® CA-1500

| Parameter | BCS | CA-1500 |
|---|---|---|
| Prothrombin time (PT) | Thromborel® S | Dade® Innovin® |
| Activated partial thromboplastin time (APTT) | Pathromtin SL | Dade® Actin® FS Activated PTT Reagent |
| Fibrinogen | Multifibren U | Dade® Thrombin Reagent |
| Thrombin time | n.d. | Thromboclotin |
| Batroxobin time | n.d. | Batroxobin Reagent |
| Coagulation factors VIII, IX, XI and XII | Coagulation factor deficient Plasmas and Pathromtin SL | Coagulation factor deficient Plasmas and Dade® Actin® FS Activated PTT Reagent |
| Coagulation factors II, V, VII and X | Coagulation factor deficient Plasmas and Thromborel® S | Coagulation factor deficient Plasmas and Dade® Innovin® |
| Coagulation factor XIII | Berichrom® FXIII | n.d. |
| Antithrombin (AT) | INNOVANCE® Antithrombin *(FXa-based method)* | Berichrom® Antithrombin III (A) *(FIIa-based method)* |
| Protein S (PS) | Protein S Ac | Protein S Ac |
| Protein (PC) | Protein C Reagenz *(clotting method)* | Berichrom® Protein C *(chromogenic method)* |
| von Willebrand factor Antigen (VWF:Ag) | VWF Ag | VWF Ag |
| von Willebrand factor Ristocetin cofactor activity (VWF:RCo) | BC von Willebrand reagent | n.d. |
| D-dimer | INNOVANCE® D-Dimer | INNOVANCE® D-Dimer |
| Plasminogen | Berichrom® Plasminogen | Berichrom® Plasminogen |
| α2-Antiplasmin | Berichrom® α2-Antiplasmin | Berichrom® α2-Antiplasmin |

to wide ranges for the central 90% interval. The values were approximately twice as high as similar values in adults (Fig. 1). Results for PT and APTT are statistically significantly different between methods for all age groups.

When single coagulation factors were evaluated (Table 4), age dependency was most distinct in FIX (Fig. 1). Coagulation factors VIII, X, XI and XII demonstrated considerably lower 5% percentiles in young children (< 1 year) as compared with older children and adolescents. Fibrinogen and coagulation factors II, IX, X, XI and XII demonstrate good comparability between methods performed with the BCS and CA-1500 (exemplarily shown for FIX and FXII in Fig. 1). Values for FV and FVII are higher with Thromborel S (BCS) compared with Dade Innovin (CA-1500) and values for FVIII are higher with Pathromtin SL (CA-1500) compared with Actin FS (BCS). Significant differences between methods are only found in late childhood, adolescence and adulthood. Statistical differences between methods are not detected in the first year of life (except for FV), which might be due to high variability and/or low number of reference individuals in these groups.

All investigated natural coagulation inhibitors are significantly higher in adults compared with children (Table 5). The inhibitors demonstrate high comparability between methods; differences between methods were insignificant in most age groups (Fig. 2). Significant differences between methods in the 1–5 years group (antithrombin) and in the 6–10 years group (protein S) are small and do not exceed 5% for the mean and for the 5% percentile.

**Table 3** Median, mean and central 90% interval for global assays. The first row shows the median/mean with results *t*-test between methods and age groups. The second row shows the boundaries including 90% of the central population

| Assay | Method | 1–6 months $n = 29^{\dagger}$ (14M/15F) | 7–12 months $n = 25^{\ddagger}$ (19M/6F) | 1–5 years $n = 57$ (35M/22F) | 6–10 years $n = 56$ (29M/27F) | 11–18 years $n = 50^{\S}$ (24M/26F) | > 19 years $n = 52$ (27F/25M) |
|---|---|---|---|---|---|---|---|
| PT (sec) | Thromborel S | 12.5/12.8#* | 12.2/12.4#* | 12.1/12.2#* | 12.6/12.6#* | 12.8/12.6#* | 11.7/11.8* |
| | **BCS** | **11.2–15.5** | **11.4–13.5** | **11.2–13.4** | **11.5–14.0** | **11.4–13.8** | **10.7–12.9** |
| | Innovin | 10.7/10.7 * | 10.6/10.6* | 10.6/10.6 * | 10.9/10.9#* | 10.8/10.9#* | 10.5/10.6* |
| | **CA-1500** | **10.0–12.7** | **9.5–12.8** | **10.0–11.4** | **10.2–11.6** | **10.1–11.9** | **9.7–11.4** |
| PT (%) | Thromborel S | 92/89#* | 95/93#* | 97/96#* | 91/91#* | 89/91#* | 101/101* |
| | **BCS** | **64–108** | **81–105** | **81–108** | **76–104** | **78–105** | **88–116** |
| | Innovin | 103/104* | 106/106* | 106/106* | 100/100#* | 101/100* | 108/108* |
| | **CA-1500** | **72–122** | **71–128** | **89–121** | **86–116** | **81–118** | **89–129** |
| APTT (sec) | Pathromtin SL | 41/42#* | 39/39#* | 36/37#* | 37/37#* | 35/36#* | 34/34* |
| | **BCS** | **33–56** | **32–49** | **31–44** | **31–44** | **30–43** | **27–40** |
| | Actin FS | 29/29#* | 28/28#* | 27/27#* | 28/28#* | 27/27#* | 25/25* |
| | **CA-1500** | **21–33** | **24–33** | **24–30** | **25–32** | **25–30** | **22–28** |
| TT (sec) | Thromboclotin | 19.2/20.0# | 18.0/18.0 | 17.0/17.2 | 17.5/17.4 | 17.4/17.8 | 17.4/17.5 |
| | **CA-1500** | **16.2–24.9** | **15.4–21.1** | **15.3–19.7** | **14.5–19.9** | **15.2–24.0** | **15.5–20.5** |
| BT (sec) | Batroxobin | 21.0/21.4# | 20.2/20.5 | 20.2/20.3 | 20.2/20.2 | 19.8/19.9 | 20.1/20.1 |
| | Reagent CA-1500 | 19.7–25.0 | 19.1–24.0 | 18.8–22.7 | 19.1–21.5 | 18.8–21.5 | 18.7–22.4 |

M, male; F, female; PT, prothrombin time; sec, seconds; BCS, Behring Coagulation System; CA-1500, Sysmex CA-1500 Analyzer; APTT, activated partial thromboplastin time; TT, thrombin time; BT, batroxobin time. #Indicates statistically significant difference between child groups and adults for the Student's *t*-test. *Indicates statistically significant difference between devices for the Student's *t*-test. $^{\dagger}n = 28$ for APTT with the BCS; $^{\ddagger}n = 24$ for PT, TT and BT with the CA-1500; $^{\S}n = 49$ for batroxobin time (one sample was excluded because of an extremely outlying result of 13.3 sec).

D-dimer, plasminogen and alpha-2 antiplasmin data are shown in Table 6. Significantly higher levels of D-dimer are demonstrated in the two youngest age groups. Only data on the CA-1500 are shown for plasminogen and alpha-2 antiplasmin because the volume was not sufficient to perform additional measurements on the BCS. D-dimer levels remain increased throughout the first year of life compared with adults.

Table 7 shows the values of VWF antigen and activity (RCO) levels further stratified by age group and AB/A/B (non-O) vs. O blood group. The differences in VWF antigen levels were consistent for both devices. VWF antigen and activity levels were higher in the youngest age group compared with older children independently of blood group. Antigen and activity levels in the non-O blood groups reached a nadir at about 12 months and then gradually increased towards adulthood. We noticed a significant difference in the activity and antigen levels in children aged 1–5 years and adulthood with blood group O compared with non-O (Fig. 3). The levels of VWF antigen and activity were always 10–20% lower in O vs. non-O children and adults. Differences between blood group non-O vs. O demonstrated the same trend for FVIII.

## Discussion

The concept of developmental hemostasis was introduced in the late 1980s by Dr Maureen Andrew [5] and is currently widely accepted. The understanding of physiological age-dependent changes in the coagulation system is crucial to an accurate diagnosis in the case of problems of thrombosis or bleeding, especially in the very young child. In general, young children have decreased physiological levels of coagulation proteins such as factors II, VII, IX, X, XI and XII, and low levels of proteins involved in fibrinolysis (plasminogen and tissue plasminogen activator) and natural coagulation inhibitors (such as antithrombin and protein C and S). However, there are no data to support an increased risk of thrombosis or bleeding during infancy; in fact it is stated that neonates and children are protected against thrombotic and bleeding complications when compared with adults [3].

Recent studies have provided reference ranges that delineate age-dependent characteristics of global coagulation assays and coagulation factors and inhibitors. However, these may vary considerably with the use of different reagents and analyzers [6].

The concept of developmental hemostasis is also confirmed in our study, using Siemens analyzers and reagents. Both the CA 1500 and the BCS were tested. Both analyzers use photo-optical clot detection methods; however, they differ in size and speed. As in earlier studies by others, we collected blood samples from healthy children and adults in 3.2% citrate test tubes and stored the samples at $< -70$°C prior to thawing for analysis.

### *APTT and related single coagulation factors (factors VIII, IX, XI and XII)*

The prolonged APTT values in the youngest group may be explained by markedly decreased concentrations of the vitamin K-dependent factors such as FIX. The prolonged APTT levels may also be due to lowered levels of contact activators such as FXI and FXII or to contact factors that were not measured during this study (e.g. prekallikrein and high-molecular-weight

**Table 4** Median, mean and central 90% interval for single coagulation factors. The first row shows the median/mean with results *t*-test between methods and age groups. The second row shows the boundaries including 90% of the central population

| Assay | Method | 1–6 months $n = 29^{\dagger}$ (14M/15F) | 7–12 months $n = 25^{\ddagger}$ 19M/6F | 1–5 years $n = 57^{\S}$ 35M/22F | 6–10 years $n = 56^{\P}$ 29M/27F | 11–18 years $n = 50^{**}$ 24M/26F | > 19 years $n = 52^{\dagger\dagger}$ 27F/25M |
|---|---|---|---|---|---|---|---|
| Fbg (g/L) | Multifibren U | 2.2/2.3# | 2.3/2.6# | 2.5/2.7* | 2.3/2.6# | 2.3/2.5# | 2.9/3.0 |
| | **BCS** | **1.5–3.8** | **1.8–4.8** | **1.9–3.9** | **2.0–3.9** | **1.9–3.7** | **2.1–4.2** |
| | Dade Thrombin | 1.9/2.0# | 2.2/2.3# | 2.4/2.5#* | 2.3/2.4# | 2.3/2.4# | 2.7/2.8 |
| | **CA-1500** | **1.3–3.3** | **1.6–4.0** | **1.7–3.5** | **1.8–3.6** | **1.8–3.3** | **2.0–3.9** |
| FII (%) | Thromborel S | 93/91# | 98/99# | 104/105# | 99/99# | 96/99# | 117/119 |
| | **BCS** | **66–112** | **83–132** | **85–126** | **78–121** | **78–132** | **96–147** |
| | Innovin | 86/86# | 95/97# | 102/103# | 97/98# | 92/94# | 114/116 |
| | **CA-1500** | **60–109** | **77–134** | **81–126** | **77–116** | **70–120** | **93–151** |
| FV (%) | Thromborel S | 114/114 | 114/117* | 108/111 | 97/99#* | 95/99#* | 112/113* |
| | **BCS** | **82–145** | **97–148** | **85–153** | **80–123** | **76–132** | **84–149** |
| | Innovin | 118/110# | 102/102#* | 102/104# | 93/92* | 87/87* | 89/91* |
| | **CA-1500** | **56–148** | **66–141** | **68–143** | **62–127** | **55–119** | **57–128** |
| FVII (%) | Thromborel S | 98/97# | 96/98# | 99/99#* | 96/98#* | 100/101#* | 105/108 |
| | **BCS** | **54–126** | **74–131** | **81–117** | **79–119** | **75–130** | **86–142** |
| | Innovin | 93/91# | 89/88 | 84/85#* | 86/87#* | 86/86#* | 101/101 |
| | **CA-1500** | **38–129** | **41–148** | **61–111** | **61–127** | **55–115** | **67–146** |
| FVIII (%) | Pathromtin SL | 90/96# | 95/100# | 109/109#* | 100/101#* | 109/108#* | 123/123* |
| | **BCS** | **58–144** | **59– > 152** | **76–143** | **68–137** | **70–148** | **87– > 152** |
| | Actin FS | 108/107# | 116/119# | 124/125#* | 118/119#* | 118/122#* | 133/140* |
| | **CA-1500** | **67–141** | **70–213** | **83–170** | **75–163** | **80–166** | **96–216** |
| FIX (%) | Pathromtin SL | 53/57# | 64/68# | 77/78# | 78/80# | 84/85# | 102/104* |
| | **BCS** | 41–87 | **42–109** | **58–99** | **57–106** | **60–117** | **78–139** |
| | Actin FS | 57/57# | 71/72# | 78/78# | 77/80# | 87/89# | 110/116* |
| | **CA-1500** | **44–78** | **46–114** | **63–97** | **60–108** | **72–116** | **87–174** |
| FX (%) | Thromborel S | 90/90# | 100/99# | 104/104# | 95/95# | 88/94#* | 112/115 |
| | **BCS** | **66–132** | **74–124** | **84–129** | **74–120** | **73–128** | **90–149** |
| | Innovin | 88/87# | 97/99# | 101/100# | 92/92# | 84/86#* | 110/114 |
| | **CA-1500** | **55–120** | **67–146** | **75–124** | **69–118** | **66–117** | **78–159** |
| FXI (%) | Pathromtin SL | 83/80# | 86/88# | 100/100 | 95/96# | 88/91# | 104/104* |
| | **BCS** | **54–101** | **65–125** | **72–134** | **75–127** | **72–122** | **77–130** |
| | Actin FS | 85/82# | 88/91# | 104/104# | 99/100# | 93/95# | 115/113* |
| | **CA-1500** | **57–105** | **64–129** | **74–134** | **78–131** | **78–122** | **83–158** |
| FXII (%) | Pathromtin SL | 75/72# | 88/81# | 95/92# | 96/90# | 96/89# | 102/101 |
| | BCS | **29–112** | **35–113** | **44–127** | **41–122** | **44–116** | **52–140** |
| | Actin FS | 76/74# | 82/82# | 88/87# | 92/88# | 92/88# | 106/108 |
| | **CA-1500** | **28–116** | **31–126** | **36–122** | **37–123** | **43–122** | **53–165** |
| FXIII (%) | Berichrom FXIII | 96/99# | 97/97# | 99/100# | 104/103# | 99/97# | 116/115 |
| | BCS | **63–152** | **42–128** | **71–139** | **76–133** | **64–133** | **68– > 156** |

M, male; F, female; PT, prothrombin time; sec, seconds; BCS, Behring Coagulation System; CA-1500, Sysmex CA-1500 Analyzer; Fbg, fibrinogen; F, coagulation factor. #Indicates statistically significant difference between child groups and adults for the Student's *t*-test. *Indicates statistically significant difference between devices for the Student's *t*-test. $^{\dagger}n = 28$ for FVII, FVIII, FIX, FX with the BCS and $n = 27$ for FXIII; $^{\ddagger}n = 24$ for Fbg, FII, FV, FVII, FX and FIX with the CA-1500, $n = 23$ for FXI with the BCS and $n = 18$ for FXIII; $^{\S}n = 53$ for FXI with the BCS and $n = 50$ for FXIII; $^{\P}n = 55$ for FII with the CA-1500, $n = 53$ for FXI with BCS and $n = 51$ for FXIII; $^{**}n = 48$ for FXI with BCS and FXIII; $^{\dagger\dagger}n = 51$ for FXI with BCS and $n = 49$ for FXIII.

kininogen) [7]. The age dependency regarding the 95th percentiles was more pronounced for Pathromtin SL (BCS) compared with Actin FS, which may be due to the higher sensitivity for lupus anticoagulants of Pathromtin SL when compared with Actin FS. Actin FS is a highly sensitive reagent for reduced levels of factors VIII, IX and XI [8]. Therefore, the prolongation of APTT in childhood detected by Actin FS most likely displays the reduced levels of related single coagulation factors VIII, IX, XI and XII. Besides reduced levels of intrinsic factors, the values measured by Pathromtin SL may partially be explained by the higher incidence of transient anticoagulants found in pediatric patients [9–11]. Klarmann *et al*. [12]used the same method to measure APTT (Pathromtin SL on the BCS); in summary, the results are very comparable, except for the children < 1 year, who presented with longer clotting times in our study. This difference may be due to differences in the study population; Klarmann *et al*. excluded all individuals with C-reactive protein values beyond the age-specific reference ranges, whereas our study only excluded children with clinically apparent infections. The number of individuals with infection-related lupus anticoagulants might thus be higher in our population compared with the population of Klarmann *et al*.

**Table 5** Median, mean and central 90% interval for coagulation inhibitors. The first row shows the median/mean with results *t*-test between methods and age groups. The second row shows the boundaries including 90% of the central population

| Assay | Method | 1–6 months $n = 29^{\dagger}$ 14M/15F | 7–12 months $n = 25^{\ddagger}$ 19M/6F | 1–5 years $n = 57$ 35M/22F | 6–10 years $n = 56$ 29M/27F | 11–18 years $n = 50$ 24M/26F | > 19 years $n = 52$ 27F/25M |
|---|---|---|---|---|---|---|---|
| AT (%) | INNOVANCE AT, **BCS** | 105/104# | 110/109# | 110/109#* | 108/107# | 104/104# | 116/115 |
| | | **81–126** | **90–132** | **93–128** | **92–122** | **90–119** | **97–133** |
| | Berichrom AT | 106/103# | 110/108# | 113/113* | 110/109# | 105/106# | 113/114 |
| | **CA-1500** | **78–129** | **88–132** | **97–129** | **97–122** | **93–122** | **98–131** |
| PS (%) | Protein S Ac | 78/79# | 81/80# | 85/83# | 84/84#* | 82/86# | 101/105* |
| | **BCS** | **60–103** | **61–95** | **65–99** | **63–97** | **69–119** | **83– > 130** |
| | Protein S Ac | 84/83# | 85/82# | 85/87# | 87/89#* | 90/90# | 116/114* |
| | **CA-1500** | **59–99** | **59–110** | **60–115** | **63–116** | **62–126** | **86– > 130** |
| PC (%) | Protein C Reagent, **BCS** | 70/71# | 83/85# | 97/97# | 98/97# | 100/103# | 120/118 |
| | Berichrom | **41–115** | **60–117** | **63–133** | **62–134** | **71–144** | **78–148** |
| | Protein C | 66/67# | 76/78# | 88/92# | 90/92# | 93/96# | 114/115 |
| | **CA-1500** | **43–102** | **59–103** | **71–125** | **75–120** | **70–131** | **83–153** |

M, male; F, female; PT, prothrombin time; sec, seconds; BCS, Behring Coagulation System; CA-1500, Sysmex CA-1500 Analyzer; AT, Antithrombin. #Indicates statistically significant difference between child groups and adults for the Student's *t*-test. *Indicates statistically significant difference between devices for the Student's *t*-test. $^{\dagger}n = 28$ for PS with CA-1500 and $n = 27$ for PC with BCS; $^{\ddagger}n = 24$ for AT with CA-1500.

**Table 6** Median, mean and central 90% interval for D-dimer, α2-antiplasmin and plasminogen. The first row shows the median/mean with results *t*-test between methods and age groups. The second row shows the boundaries including 90% of the central population

| Assay | Method | 1–6 months $n = 29^{*}$ 14M/15F | 7–12 months $n = 25^{\dagger}$ 19M/6F | 1–5 years $n = 57$ 35M/22F | 6–10 years $n = 56$ 29M/27F | 11–18 years $n = 50$ 24M/26F | > 19 years $n = 52$ 27F/25M |
|---|---|---|---|---|---|---|---|
| D-dimer (mg/L FEU) | INNOVANCE D-dimer, BCS | 0.28/0.50# | 0.25/0.46# | 0.19/0.25 | 0.19/0.24 | < 0.17/0.28 | < 0.17/0.21 |
| | | < 0.17–2.81 | < 0.17–3.32 | < 0.17–0.64 | < 0.17–0.49 | < 0.17–0.99 | < 0.17–0.44 |
| | INNOVANCE D-dimer, CA-1500 | 0.33/0.39# | 0.27/0.34# | 0.20/0.27 | 0.20/0.26 | < 0.19/0.27 | < 0.19/0.23 |
| | | < 0.19–3.49 | < 0.19–10.9 | < 0.19–0.65 | < 0.19–0.52 | < 0.19–0.75 | < 0.19–0.48 |
| α2-antiplasmin (%) | Berichrom | 122/121 | 123/125# | 128/128# | 119/121 | 114/113# | 118/119 |
| | α2-antiplasmin, CA-1500 | 103–139 | 100–151 | 107–145 | 103–140 | 97–126 | 103–133 |
| Plasminogen (%) | Berichrom | 81/79# | 93/94# | 104/106# | 99/99# | 95/99# | 112/117 |
| | Plasminogen, CA-1500 | 56–102 | 66–115 | 84–130 | 75–126 | 83–128 | 92–150 |

M, male; F, female; PT, prothrombin time; sec, seconds; BCS, Behring Coagulation System; CA-1500, Sysmex CA-1500 Analyzer; FEU, fibrinogen equivalent units. #Indicates statistically significant difference between child groups and adults for the Student's *t*-test. Differences between devices for D-dimer were statistically not significant for the Student's *t*-test. $^{*}n = 28$ for D-dimer with CA-1500; $^{\dagger}n = 24$ for α2-antiplasmin and plasminogen.

### *PT and related single coagulation factors (II, V, VII and X)*

We did not find clinically relevant changes for the PT in this cohort of children. Most changes occur in the first weeks after birth and our youngest child was 1 month of age. By the age of approximately 6 months factors II, VII and X were still approximately 10–20% reduced compared with adult values. We confirm the data of Andrew *et al.* and Monagle *et al.*, [1,3], which state that normal adult levels are achieved at 6 months of age. Detecting reference ranges for newborns is much more difficult, as was demonstrated by Monagle *et al.* [3] in a recent study: of 827 mothers approached, only 159 consented to participate.

### *Thrombin time and Batroxobin time*

Our study is the first to analyse developmental changes for thrombin time (TT) and batroxobin time. We found a slight increase of both clotting times in the group of children aged 1–6 months, which may be due to low fibrinogen values in this group.

### *Fibrinogen*

Like others we found increasing levels of fibrinogen with age [3,9]. Fibrinogen is the final substrate to form an insoluble clot but also an acute phase reactant. Fibrinogen levels may be raised in relation to a variety of physiological variables (such as age) but also to inflammatory conditions; even the season of the year has been found to affect fibrinogen measurements (i.e. higher in wintertime) [13]. Additionally, an individual polymorphism of the beta fibrinogen gene promoter (G-455A) has been associated with increased levels of fibrinogen in humans [14].

Both the BCS and the Sysmex 1500 analyzers measure functional fibrinogen according to the Clauss method. This is a

**Table 7** Median and central 90% interval for von Willebrand factor and coagulation factor VIII

| Method | Blood group# | 1 to 6 months | 7–12 months | 1–5 years | 6–10 years | 11–18 years | > 19 years |
|---|---|---|---|---|---|---|---|
| VWF Ag | All (*n*) | 106 (27) | 82 (25) | 86 (57) | 91 (56) | 93 (50) | 111 (50) |
| BCS (%) | | 58–206 | 53–153 | 52–140 | 58–145 | 57–147 | 65–182 |
| | AB/A/B (*n*) | 106 (13) | 80 (12) | 97* (27) | 100 (27) | 98 (30) | 118* (30) |
| | | 77–215 | 64–155 | 66–141 | 59–150 | 52–142 | 65–196 |
| | O (*n*) | 102 (14) | 86 (10) | 71* (29) | 80 (27) | 90 (17) | 99* (20) |
| | | 56–192 | 50–122 | 45–152 | 45–144 | 61–152 | 59–161 |
| VWF Ag | All (*n*) | 109 (28) | 96 (25) | 90 (57) | 94 (56) | 99 (50) | 112 (52) |
| CA-1500 (%) | | 63–223 | 60–158 | 60–140 | 60–142 | 60–159 | 72–188 |
| | AB/A/B (*n*) | 110 (14) | 87 (12) | 101*( 27) | 98 (27) | 102 (30) | 119* (30) |
| | | 76–243 | 67–163 | 71–140 | 63–153 | 56–160 | 72–199 |
| | O (*n*) | 104 (14) | 100 (10) | 77* (29) | 86 (27) | 98 (17) | 103* (22) |
| | | 61–192 | 59–141 | 50–158 | 46–141 | 63–165 | 62–162 |
| VWF: RCO | All (*n*) | 98 (27) | 73 (25) | 74 (57) | 77 (56) | 85 (50) | 93 (50) |
| BCS (%) | | 56–> 150 | 51–> 150 | 51–128 | 46–138 | 51–147 | 56–>150 |
| | AB/A/B (*n*) | 94 (13) | 68 (12) | 82* (27) | 83 (27) | 90 (30) | 106* (30) |
| | | 62–>150 | 56–>150 | 57–138 | 47–>150 | 43–147 | 61–>150 |
| | O (*n*) | 103 (14) | 88 (10) | 66* (29) | 71 (27) | 84 (17) | 82* (20) |
| | | 55–>150 | 52–114 | 41–122 | 38–127 | 51–> 150 | 50–116 |
| FVIII | AB/A/B (*n*) | 98 (13) | 114* (12) | 121* (27) | 105* (27) | 110 (30) | 131* (30) |
| BCS (%) | | 72–>152 | 77–>152 | 89–148 | 71–138 | 69–>152 | 83–>152 |
| | O (*n*) | 86 (15) | 87* (10) | 100* (29) | 90* (27) | 103 (17) | 116* (22) |
| | | 50–130 | 59–115 | 65–132 | 52–143 | 70–134 | 86–145 |
| FVIII | AB/A/B (*n*) | 113 (14) | 129* (12) | 128* (27) | 122 (27) | 124 (30) | 142* (30) |
| CA-1500 (%) | | 82–142 | 93–232 | 102–171 | 84–172 | 79–190 | 92–221 |
| | O (*n*) | 104 (15) | 100* (10) | 115* (29) | 107 (27) | 116 (17) | 127* (22) |
| | | 67–134 | 69–129 | 76–158 | 59–172 | 93–147 | 96–160 |

BCS, Behring Coagulation System; CA-1500, Sysmex CA-1500 Analyzer. *Indicates statistically significant difference between non-O and O blood groups for the Student's *t*-test. #Blood group is not known for three individuals from the 7–12 months group, for one individual from the 1–5 years group, for two individuals from the 6–10 years group and for three individuals from the 11–18 years group. Values for coagulation FVIII for all individuals are given in Table 4.

photometric assay with stimulation with thrombin in excess. Differences between analyzers are low and not likely to be of clinical relevance [15].

### *Coagulation inhibitors*

Compared with adults, children have decreased physiologic levels of proteins involved in inhibition of hemostasis (antithrombin, protein C and protein S). Proteins C and S are vitamin K-dependent coagulation factors and like FIX show lowest levels in the youngest age groups. Like others we confirm that protein C and S levels remain 10–20% reduced throughout childhood [3,5]. Measurements demonstrate equivalent trends between the two devices used.

Antithrombin levels reach adult levels by the age of 7–12 months. Antithrombin is regularly used to point to possible alternate functions of coagulation proteins during fetal life [6,16]. Antithrombin also plays other relevant roles outside the hemostatic system, such as a strong anti-angiogenic [17] and anti-inflammatory [18] role. A difference in the balance of isoforms of antithrombin between the proteins with a role in angiogenesis and with an antithrombotic role might be the reason for low neonatal levels of antithrombin. As angiogenesis becomes less important in the neonate the antithrombotic role takes over, leading to adult levels in infancy [19]. As differences in molecular weight suggest differential post-translational modifications of the components of fibrinogen with age [20], Teruel *et al.* [16] suggest that significant changes at the transcriptional level may also contribute to age-related changes in the coagulation system. They studied the role of microRNAs (miRNAs), functioning as regulators of gene expression at the post-transcriptional level, in the hemostatic system of neonatal and adult mice. The levels of miRNAs targeting antithrombin inversely correlated with antithrombin mRNA, suggesting that miRNAs may be potential modulators of the hemostatic system after birth. Future research on miRNAs might help us to understand the mechanisms and rationale for the marked age-related changes in developmental hemostasis.

### *von Willebrand factor*

Our findings are comparable to those of Klarmann *et al.* [12], with higher levels of VWF antigen and activity in the youngest groups, decreasing to a nadir at about 1 year of age, to finally increase to adult levels. This last trend was less distinct in individuals with blood group O. Especially the VWF activity levels in this group demonstrated only a slight increase in time from a median of 66% to 82% in adults, whereas adults with blood group non-O reached a median level of 106%. The concentration and activity of VWF are influenced by several factors, including blood group, inflammation and proteolysis by 'A Disintegrin and Metalloprotease with ThromboSpondin

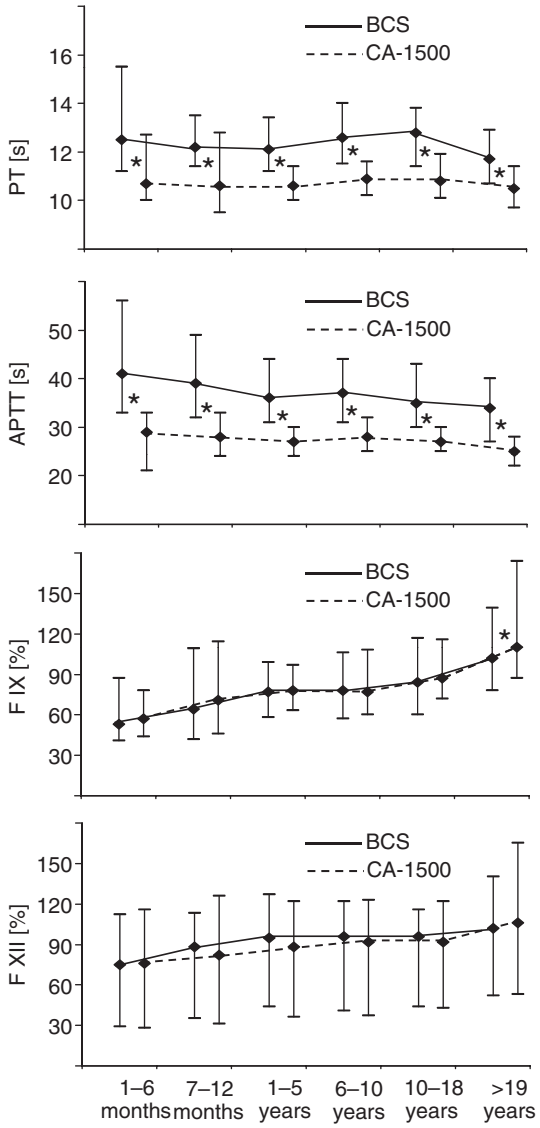


**Fig. 1.** Comparison of PT, APTT, factor IX and factor XII data with BCS and CA-1500. *Significant differences between methods for age groups ($P < 0.05$).

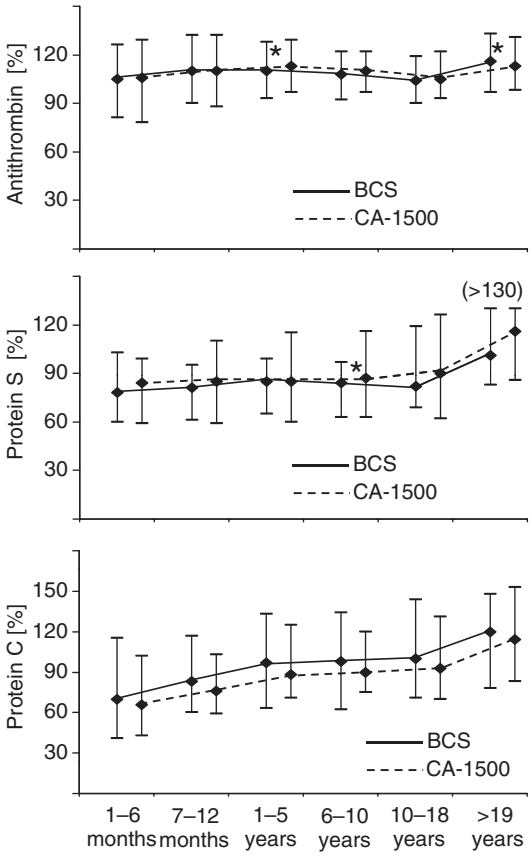


**Fig. 2.** Comparison of antithrombin, protein C and protein S data with BCS and CA-1500. *Significant differences between methods for age groups ($P < 0.05$).

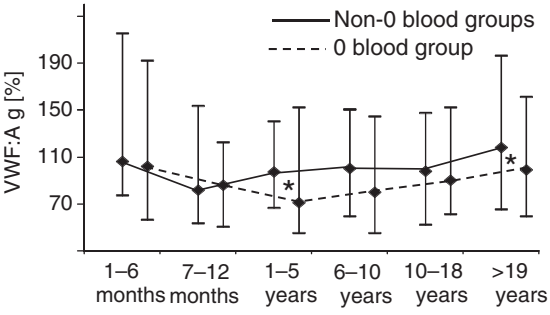


**Fig. 3.** Comparison of von Willebrand factor in blood groups non-O and O with BCS. *Significant differences between age groups ($P < 0.05$).

motif' (ADAMTS13). ADAMTS13 is a recently discovered metalloprotease that specifically cleaves VWF multimers. The fragments are less active in platelet aggregation compared with the uncleaved VWF multimers [21].

Some mechanisms have been proposed through which blood group affects VWF levels, such as the clearance of VWF, which differs in the various blood groups owing to the varying carbohydrate structure of plasma glycoproteins [22]. Furthermore, gene loci were suggested that have a quantitative effect on plasma levels of VWF; however, the distant location of the gene for VWF on chromosome 1 compared with the gene for ABO blood group on chromosome 9 does not point to a direct relation. Otherwise it has been found that VWF of blood group O is more susceptible to the proteolytic activity of ADAMTS13 compared with VWF of non-O blood groups [23,24]. It is well known that compared with adults the red blood cells of neonates carry fewer A and B antigens and are thus less complex structures. If the amount of A, B and H antigens of VWF is comparably low, the lack of blood group differences in VWF levels in early childhood may be related to low ABH antigen expression on VWF. At approximately 18 months the normal adult status in ABH-bearing structures of red blood cells is reached. This is in line with our observation that statistically significant differences between O and non-O blood groups were not found in young children aged < 12 months. Feys *et al.* [25]found that both ADAMTS13 activity and antigen levels were lower in neonates than in adult controls,

which may contribute to higher VWF levels found in our youngest children. It is remarkable that the high VWF factor levels in the youngest children are not accompanied by increased levels of FVIII even though it is well known that VWF increases the half-life of FVIII. Andrew *et al.* and Klarmann *et al.* [2,12] made this same observation. Probably this could be related to the fact that no blood group components are present on the FVIII molecule. We only included adults until the age of 50 as it is well known that VWF antigen levels increase with age [26].

### *D-dimer, plasminogen and alpha2-antiplasmin*

The lower ends of the measuring range for D-dimers are 0.17 mg/L on the BCS and 0.19 mg/L on the CA-1500. D-dimer was found to be higher in the first year of life. However, it should be noted that the cut-off level for the exclusion of thromboembolism is the relevant biological decision point for D-dimer and not the percentiles of the reference intervals. Plasma concentrations of alpha2-antiplasmin and plasminogen are decreased to 50% and 80% at birth, respectively [2]. As reported by Andrew *et al.* [2] we found that plasminogen levels increase to adult levels by approximately 6 months of age. During fibrin formation plasminogen binds to fibrin and is incorporated into the consolidated thrombus. Tissue plasminogen-activator will also bind to fibrin, where it converts plasminogen to plasmin, which in turn will degrade fibrin. The major inhibitor of plasmin is alpha2-antiplasmin. As reported earlier, we did not demonstrate differences between infant and adult levels of alpha2-antiplasmin. Alpha2-antiplasmin levels reach adult levels within the first week of life [1]. The fibrinolytic system therefore seems activated at birth. Neonatal plasminogen itself is qualitatively impaired with slow activation kinetics by its major activator protein, tissue plasminogen-activator (t-PA). One study found that five times the amount of t-PA was required in the newborn to achieve similar activation of plasminogen to plasmin to that seen in adults [27]. Conversely, plasminogen activator inhibitor (PAI), a primary inhibitor of fibrinolysis, exhibits normal to elevated values at birth. When combined, these differences lead to an overall decrease in plasmin generation and fibrinolytic activity in neonates only [28]. However, this was not investigated in this study.

In summary, pediatric reference data were established for a wide range of coagulation parameters. Our study is the first of such studies to compare two different methods (analyzers and reagents) in the same study population. The correlation between these methods is remarkable for coagulation inhibitors and von Willebrand factor. Most groups do not demonstrate significant differences between methods even though methods do not only differ regarding the used analyzer but also differ between reagents (e.g. method for AT is FXa based on BCS or FIIa based on CA-1500). Single coagulation factors also showed a good comparability between methods (except for FV). The largest differences between methods were found for the PT and APTT; these differences were not more pronounced in children compared with adults for the PT. APTT is the only parameter for which age dependency seems to be different between methods. The first group demonstrates a considerably higher 95% percentile (140% of adult value) with Pathromtin SL on the BCS compared with the 95% percentile with Actin FS (120% of adult value). All other age-dependent parameters (e.g. protein C, FIX, FXII) demonstrated equivalent trends between methods; the percentage differences between child groups and adults for the biological decision points/medians were nearly equal between methods. It may be tempting to use our findings to justify the expression of pediatric reference intervals as ratios of adult values (except for the APTT). This would tremendously help laboratories who do not have the resources to establish their own reference intervals, as is recommended by most manufactures. However, our data are far too limited to support such a procedure as this study was performed with one study population using reagents and analyzers from one single company. Differences between methods from other companies are likely and large multicenter studies need to either confirm or reject the approach of expressing pediatric reference values as a ratio of adult values. Results in the youngest group show that inter-individual variability is highest in young children. Differences were most pronounced between children aged $< 12$ months and older children and adults, confirming the phenomenon of developmental hemostasis. Our results underline the need for age-specific reference ranges. However, age-related physiological changes of the coagulation system are most pronounced in the fetal and neonatal periods. Therefore, in neonates and infants multiple reference samples are required to define the normal range of coagulation proteins for age more precisely.

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## Disclosure of Conflict of Interests

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