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Promising Biomarkers for the Prediction of Catheter-related Venous Thromboembolism in Children: An emphasis on prevention and personalized care

THESIS

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by

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LIST OF ABBREVIATIONS

HA-VTE = Hospital-Acquired Venous Thromboembolism

CVC = Central Venous Catheter

MP = Microparticles

FVIII = Factor VIII activity

TG = Thrombin Generation

PPV = Positive Predictive Value

IBD = Inflammatory Bowel Disease

CF = Cystic Fibrosis

pOR = pooled odds ratio

γ'fib = γ' fibrinogen isoform

sPS = soluble P-selectin

IL-6 = Interleukin 6

TNF-α = Tissue necrosis factor alpha

IL-1β = Interleukin 1 beta

IL-8 = Interleukin 8

CPMP = Circulating Pro-coagulant Microparticles

TGA = Thrombin Generation Assays

CAT = Calibrated Automated Thrombogram

SNP = Single nucleotide polymorphisms

ECMO = extracorporeal membrane oxygenation

CRF = Case Report Form

SBS = Short Bowel Syndrome

TPN = Total Parental Nutrition

PPP = Platelet Poor Plasma

PL = Phospholipids

ttPeak = Time to Peak

Peak = Peak Thrombin Generation

ETP = Endogenous Thrombin Potential

VI = velocity index

XaCT = Xa clotting time

WBC = White Blood Cell Count

ANC = Absolute Neutrophil Count

CRP = C-reactive protein

PICCs = Peripherally Inserted Central Catheters

ROC = Receiver Operating Characteristic

AUC = Area Under the ROC Curve

NPV = Negative Predictive Value

+LR = Positive Likelihood Ratio

-LR = Negative Likelihood Ratio

CHAT = Children's Hospital Acquired Thrombosis

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ABSTRACT OF THE THESIS

Promising Biomarkers for the Prediction of Catheter-related Venous Thromboembolism in Children: An emphasis on prevention and personalized care

by

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Master of Science in Biomedical and Translational Science

University of California, Irvine, 2018

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Background: Pediatric hospital-acquired venous thromboembolism (HA-VTE) has increased over the past ten years, with central venous catheters (CVC) being the strongest risk factor. Current tools are not sufficient to predict VTE risk at this time. The utility of biomarkers in predicting CVC-related VTE has been minimally explored.

Aims: Determine the utility of microparticles (MPs), factor VIII activity (FVIII) and thrombin generation (TG) in prospectively predicting VTE occurrence in hospitalized children with CVCs.

Methods: In this cohort pilot study, 42 hospitalized acutely ill children needing CVC placement (1 month to 21 years) and 42 age-matched healthy controls were enrolled. Venous samples were collected prior to or within 24 hours of CVC placement and processed using a strict protocol to minimize pre-analytical variables. MPs were measured using factor Xa initiated clot-based assay. FVIII was

measured using a one-stage clot-based assay. TG was measured using the calibrated automated thrombogram.

Results: There were three CVC-related VTE events (7%) in our cohort. Xa clotting time (XaCT) ratio was significantly lower, while FVIII, peak thrombin (peak), estimated thrombin potential (ETP) and velocity index (VI) were significantly higher in patients with CVC-related VTE, as compared to healthy controls and patients without CVC-related VTE. Sensitivity/specificity analysis revealed optimal cutoff values for XaCT ratio (0.75), FVIII (370), ETP (1680), Peak (315) and VI (130), with AUC values of all biomarker ROC curves >0.9.

Conclusion: MPs, FVIII and TG can potentially predict pediatric CVC-related VTE in a prospective fashion. Further studies are needed to explore if stratification according to VTE risk will guide preventative efforts in this patient population.

Key Words: Venous thromboembolism, Central venous catheter, Biomarkers, Children, Prediction, Personalized medicine

CHAPTER 1: INTRODUCTION

In an era of flourishing with scientific discovery, there has been tremendous progress in understanding the biological and pathophysiological basis underlying disease development and progression. This is especially true in the field of pediatric hematology oncology and, in particular, in pediatric hemostasis and thrombosis. With this understanding, there has been a stronger emphasis in incorporating this knowledge into tools that allow improved accuracy in disease prediction, thus ensuring timely prevention efforts are directed towards at-risk patient populations. Even though the majority of these efforts have been directed at developing molecular approaches[1], evaluation of biological markers may result in more precise prediction in specific risk groups, such as prediction of hospital-acquired venous thromboembolism (HA-VTE) occurrence in children. Improving our predictive ability will help guide clinical decision-making and provide patients with more information to make an informed decision in their care.

Pediatric HA-VTE has increased over the past ten years[2], with an associated increase in both VTE-associated complications and treatment-related adverse events. Specifically, these adverse events are related to subsequent anticoagulation, the complications of organ damage due to embolization of clots to the lung[3] and the occurrence of post-thrombotic syndrome[4]. This increase has resulted in significant preventable morbidity and mortality with potential of adverse long-term outcome into adulthood. In addition, children with VTE have a four-fold increase in the length of admission compared to children without VTE[2], and VTE occurrence has been associated with a two-fold increase in admission cost in adults[5]. The presence of a central venous catheter (CVC) has been shown to be the

strongest risk factor in children[6], while other associated risk factors, such as pediatric malignancies and inflammatory conditions have also been strongly associated with VTE[7].

The majority of children requiring inpatient admission have pathological risk factors associated with VTE occurrence[7]. Predicting and preventing pediatric VTE will impact a wide variety of patients, while limiting complications associated with CVC placement in children. However, thrombosis is a multi-factorial process that involves interaction between vascular, immune and hemostatic components. To optimize the accuracy of VTE predictive models, a comprehensive approach addressing the multiple aspects of thrombus pathogenesis is required. Identification of dependable, rapid and cost effective biomarkers for the prediction of VTE in children is essential in guiding anti-coagulation prophylaxis, thus minimizing VTE risk among hospitalized children.

Current tools including clinical risk-prediction scoring systems as well as genetic risk factors lack the needed sensitivity and specificity to predict initial and recurrent VTE events in pediatric patients[8]. Furthermore, to date, biomarkers have not been sufficiently evaluated in pediatric VTE. Developing a translational and practical approach in identifying at-risk children who may benefit from more aggressive VTE prophylactic strategies while minimizing its potential adverse events will result in improved long-term outcome of children with VTE. Given the rising incidence of pediatric VTE[9] and its associated financial burden, the ability to use biomarkers to stratify patients according to VTE occurrence risk, may provide a vital tool to guide preventative efforts while minimizing unnecessary expense and toxicities of such treatments to low risk patients.

The purpose of this research is to evaluate the clinical feasibility and potential predictive value of microparticles (MPs), factor VIII activity (FVIII) and thrombin generation (TG), in the setting of hospitalized children with CVCs. The first aim of this thesis is to evaluate the positive predictive value (PPV) of each of these biomarkers, as compared to current clinical risk-prediction tools, in predicting CVC-VTE. We hypothesize that elevation in biomarkers will differentiate between patients with and without CVC-VTE, resulting in a higher PPV when compared to current clinical risk-prediction tools. The second aim of this thesis is to determine the relationship of these biomarkers in hospitalized children needing CVC placement and children with acute VTE, as compared to age-matched healthy controls. We hypothesize that elevation of all biomarkers will be detected in cases of both study arms, as compared to age-matched healthy controls.

This thesis is organized into four additional chapters. Chapter 2 provides a comprehensive literature review outlining clinical VTE risk factors, the current state of clinical VTE risk prediction tools and detailed description of current evidence supporting several potential biomarker candidates. Chapter 3 describes the study design, clinical collection tools and laboratory methods used to perform the study. Chapter 4 details the study results, from a clinical and laboratory prospective, with special emphasis on epidemiological analysis and risk prediction evaluation. Chapter 5 will include a discussion of our findings, including the strengths and limitation of this thesis, in addition to discussing several areas of future research.

CHAPTER 2: BACKGROUND

1. Pediatric VTE and associated risk factors

The incidence of pediatric HA-VTE has increased by 70% to an annual incidence of 34 – 58/10,000 admissions[2], with an associated increase in both VTE-associated complications and treatment-related adverse events. This increase has resulted in significant preventable morbidity and mortality with potential of adverse long-term outcome into adulthood. There are many known clinical risk factors for the development of VTE, including venous stasis, endothelial injury, inflammation and thrombophilia – inherited and acquired[10]. Specifically, inflammation (acute or chronic) has been established as a risk factor for thrombosis, as shown in inflammatory bowel disease (IBD)[11] and cystic fibrosis (CF)[12].

The presence of a CVC is the strongest risk factor to development of VTE in children[6]. CVCs have gained greater importance as a risk factor for HA-VTE due to their increasing use for venous access in the pediatric population. The incidence of CVC-VTE in a retrospective single center study was 3.2% (with 0.3 events per 1000 catheter-days)[13]. Recent preliminary data indicate that the true incidence of all CVC-VTE is slightly higher at 5.7%, with a large majority related to non-tunneled CVC at 7.5%[14]. Even though the majority of CVC-VTE occurs within the first 7 days[15], some can occur at a maximum of 44 days after insertion[16].

2. Clinical risk-prediction models of pediatric VTE

Several attempts have been made to utilize VTE-related risk factors in an evidence-based risk assessment tool to help predict HA-VTE. Specifically, there are two pediatric clinical risk prediction models that have been developed through logistical regression analyses of any hospitalized cases, with age-matched controls. Sharathkumar et al. derived and validated a six-risk-factor weighted scoring tool with 70% sensitivity and 80% specificity for risk score ≥ 3. However, their calculated post-test probability was only 2.84%, which is attributed to the low prevalence of pediatric hospital-acquired VTE (0.34 – 0.71/10,000 hospital admission)[17]. Branchford et al. reported similar results showing a post-test probability of 3.6% when children had the following 3 risk factors: mechanical ventilation, systemic infection and hospitalization for ≥ 5 days[18].

Recently, Arlikar et al. developed a new risk assessment scoring system based on presence of CVC, length of stay and infection that had a PPV of 8.8% in non-cardiac patients admitted to the intensive care unit[19]. Furthermore, Mahajerin et al. performed a systematic review and meta-analysis on pediatric HA-VTE and identified several limitations that exist in the majority of these studies including lack of standardized definition of specific risk factors (e.g. admission to intensive care, immobilization), inclusion of prolonged hospitalization in the risk assessment tool reflecting its retrospective characteristic and the relatively low odds ratios provided by many of the clinical risk factors. He also evaluated the pooled odds ratio (pOR) for several known clinical risk factors such as admission to ICU (pOR 2.14 [1.97-2.32]) and presence of any CVC (pOR 2.12 [2-2.25])[8].

Even though there was great heterogeneity between studies evaluated and the majority of studies were not case-control in design, the current literature sheds light on the limitation of the data supporting the isolated use of clinical risk factors in predicting HA-VTE in children. It is also important to note that even though the risk for major bleeding with prophylactic anti-coagulation seems to be minimal in children receiving prophylactic anticoagulation[20], the overall reported bleeding incidence in a large retrospective cohort study that included adolescents was 1.1%[21]. As a result, we need more robust risk models to overcome the potential for bleeding complications and other adverse effects, associated with pharmacological prophylaxis.

3. Evaluation of biomarker candidates

Thrombosis is a multi-factorial process that involves interaction between vascular, immune and hemostatic components[22-24]. Unfortunately, this heterogeneity complicates any attempt to utilize a single biomarker for the prediction of VTE occurrence. This heterogeneity is easily observed in clinical practice, where, even though the majority of children requiring inpatient admission have several pathological VTE risk factors, only a minority of children develop VTE. After a comprehensive evaluation of the literature for candidate biomarkers, we examined the feasibility of several novel biomarkers as representatives of each major component of thrombus pathogenesis. Candidate biomarkers are microparticles, factor VIII activity, thrombin generation, γ' fibrinogen isoform (γ'fib), soluble P-selectin (sPS) and inflammatory cytokines such as Interleukin 6 (IL-6), tissue necrosis factor-alpha (TNF-α), Interleukin 1beta (IL-1β) and Interleukin 8 (IL-8). A

summary of biomarker, name of respective assay used and what each assay is measuring is present in Appendix A.

3.1 Microparticles

Microparticles are microvesicles released from the membranes of many cell types, with the vast majority being derived from activated platelet and endothelium. MPs are released upon activation or death of these cells and have antigens specific to their cell of origin[25]. Mechanistically, they serve as a source of phosphatidylserine, which acts as a procoagulant platform for clotting complex assembly and action. Even though MPs are traditionally measured via flow cytometry, recent literature has supported the utilization of functional assays and clot-based assays in the evaluation of pro-coagulant MPs[26,27,28]. Multiple studies have shown the association of elevated MPs with acute VTE in a variety of adult patient populations, with established VTE risk factors[29,30,31,32]. In a study by Deutschman et al., circulating pro-coagulant microparticles (CPMP) were found to be elevated in children with IBD, an established clinical risk factor for VTE occurrence[33]. As a result, MPs may serve as an important biomarker to evaluate in patients with VTE, especially CVC-VTE.

3.2 Thrombin Generation

Indirect markers of thrombin generation, such as Prothrombin fragment 1+2 and Thromin-antithrombin complex, have been evaluated in multiple clinical settings as markers of thrombin activation[34, 35]. In relation to VTE, Prothrombin fragment 1 + 2 was found to predict recurrence of VTE once off anti-coagulants in adults only but this has not

been studied in children[36]. Older thrombin generation assays (TGA) have been assessed for potential correlation to VTE risk but showed no correlation except when thrombomodulin was added[37]. The same technique without thrombomodulin was correlated with children with IBD with and without active flare but none of these subjects had evidence of active thrombus[38]. A recent review of the use of TGA in adults as it relates to the occurrence of unprovoked and recurrent VTE illustrated the current lack of standardization of these assays, their variable sensitivity and their unclear correlation with clinical outcomes[39]. However, utilizing the Calibrated Automated Thrombogram (CAT) in hospitalized adults at high risk for VTE, Espitia et al. showed promising results that support using this method in as a risk assessment tool, with sensitivity and specificity of 83%[40]. This promising tool has not been evaluated in hospitalized children at this time.

3.3 Factor VIII activity and novel genetic markers

Factor VIII activity has been established as an important risk factor for VTE occurrence[41]. However, due to its classification as an acute phase reactant, it has lacked the specificity needed to become a clinically relevant tool. Despite this limitation, the role of FVIII was evaluated in the intensive care setting as a potential predictor of VTE occurrence in sick children. Even though FVIII's sensitivity was > 90%, its specificity was < 50% due to the low cut-off threshold (i.e. 100%) chosen by the study group, thus resulting in limited success and clinical applicability[42]. On the contrary, a much stronger relationship was found in adult patients with cancer and CVC-VTE, where a FVIII > 260% resulted in a hazard ratio of 3 after adjusting for confounders[43].

Finally, with the maturation of next generation sequencing and advance molecular testing, there has been an explosion of potential genetic markers of VTE risk[44]. Recently, there are several new genetic markers that emerged as being associated with an elevated thrombotic risk[45]. Specifically, two promising single nucleotide polymorphisms (SNP) are the lead SNP at the TSPAN15 locus (rs78707713) and the lead SNP at the SLC44A2 locus (rs2288904)[46]. Other potential genes with promising rare variants include SMAP1, B3GAT2 and RIMS1[47]. These are especially important as potential biomarker since they have not yet been evaluated in children.

3.4 Miscellaneous biomarkers

Fibrinogen is an essential glycoprotein involved in the final stages of the clotting cascade and clot formation. γ'fib is a unique form of fibrinogen that seems to have an anti-thrombotic role. Specifically, it acts as a poor platelet aggregator/activator and results in thrombin inhibition due to its high affinity to thrombin[48]. It was shown in adult studies that γ'fib and the γ'fib/total fibrinogen ratio was lower in adults with VTE compared to controls[49]. They also examined the genetic variation in the fibrinogen gene among these patients and discovered a strong association between risk of thrombosis and the presence fibrinogen gamma haplotype 2 (FGG H2) SNP 10034C>T (rs2066865)[50]. In addition, even though previous literature has shown the relationship between low expression of this isoform and increased thrombotic risk, this has been challenged recently in the adult VTE literature[51]. As a result, the exact relationship with thrombosis is not clear and it has not been evaluated in children, regardless of thrombosis presence.

P-selectin is a cell adhesion molecule present on activated platelets and activated endothelial cells. Platelet P-selectin, measurement via flow cytometry, is elevated in pediatric patients with congenital heart disease[52, 53]. sPS measured via ELISA does not allow identification of its source, but it has still been shown to be significantly elevated in adults with proven VTE[54]. In addition, sPS was a potential biomarker for prediction of VTE in adult cancer patients in a review article by Pabinger et al.[55]. In children with ALL, sPS was elevated at diagnosis and increased with therapy[34]. However, the role of sPS as a predictive biomarker in the occurrence of VTE in children has not been explored to date.

IL-6 is a pro-inflammatory cytokine secreted by immune and vascular smooth muscle cells in response to trauma or infection, where its specific function depends on the location in which it is secreted. In an adult systematic review published in 2005, there was a 2-6x increase in risk of DVT associated with elevation of plasma levels of IL-6[56]. In addition, adults with residual thrombus and elevated D-dimers also had elevated IL-6 levels compared to ones with resolution of thrombotic findings[57]. In a prospective study of children with ALL, IL-6 was elevated at baseline in the two cases with thrombotic event[34]. In addition, IL-6 was evaluated in children with VTE, with or without infection, and confirmed a higher level of IL-6 in the infection-related VTE. However, the reason for this elevation was not clear due to the co-presence of infection in this population[58]. As a result, there is insufficient literature evaluating the impact of Il-6 in children with VTE. In addition, other inflammatory cytokines such as TNF-a, IL-1β and IL-8 have been implicated in the risk assessment of patients with VTE[59].

4. Selection of study biomarker

Combined with the heterogeneity of VTE risk factors in hospitalized children, the relative low prevalence of pediatric VTE has lead to the poor performance of current clinical risk-prediction scoring systems in predicting HA-VTE[7]. Furthermore, most of these tools utilize VTE prediction factors that are commonly determined after the patient's admission, such as length of hospitalization and mechanical ventilation. As a result, they depend on knowledge of the patient's clinical course, which is usually not available for the clinician faced with the dilemma of VTE prophylaxis. In contrast, biomarkers, measured at the time of line placement, may predict VTE occurrence prospectively, regardless of their clinical variability.

The utility of biomarkers in predicting VTE in adults has been evaluated in different settings[52,40,26,41], but has been minimally explored in children. Having an objective laboratory evaluation, with assays that utilize a small sample size and have a rapid turn around time, will be of great value in identifying a small subset of pediatric patients at the highest risk of developing pathologic thrombosis, thus potentially benefiting from pharmacological prophylaxis. This approach will overcome the enormous clinical variation and utilize the current knowledge of thrombus pathogenesis to potentially resolve the debate among hematologists on the role of pharmacologic prophylaxis in children at risk of VTE occurrence. After a comprehensive evaluation of the literature for candidate biomarkers, we evaluated the clinical feasibility and potential predictive value of MPs, FVIII and TG, in the setting of hospitalized children with CVCs.

CHAPTER 3: MATERIALS AND METHODS

1. Study design

This study is a prospective cohort pilot study that is composed of three arms: 1) children that are having a CVC placed for various medical indication during a current hospitalization, 2) children that developed an acute VTE at or during their admission, and 3) Healthy age-matched controls, recruited from patients undergoing anesthesia or elective surgery, such as circumcision and umbilical hernia repairs, without a history of current infection or chronic disorder. As a proof of concept, we evaluated the acute VTE cohort to establish the utility of our biomarkers in patients with an established active thrombus. We collected serial samples from the non-control arms to establish the trajectory of our biomarkers over time.

2. Patients selection and clinical data collection

Institutional review board approval to conduct this pilot study and informed consent was obtained prior to enrollment. Patients included in this study were hospitalized patients, ages 1 month to ≤ 21 years, whose corrected gestational age was at least 44 weeks, and who had an ultrasound-proven acute VTE or who had a CVC placement during the current admission. Exclusion criteria to avoid any potential of disrupting the endovascular environment were: undergoing extracorporeal membrane oxygenation (ECMO) within past 6 weeks, cardiac catheterization procedures within the past week, and dialysis or regular plasmapheresis within the past 6 weeks. Consecutive eligible patients were recruited during work hours, as long as inclusion and exclusion criterias were satisfied and selection bias was avoided.

Using the case report form (CRF) outlined in Appendix B, we collected extensive clinical information from the electronic medical records to account for the variety of potential risk factors associated with VTE occurrence. All data points were transferred in the REDCap online data collection tool, which was formatted based on the original CRF. Data collected included demographic data (i.e. age, gender, ethnicity), age category (i.e. ≤ 1 year, > 1 year to 5 years, 6 years to < 12 years and ≥ 12 years), transfusion history within three months of enrollment, surgical history within one year of enrollment and cardiovascular history. Significant immobility, defined by a state in which the child has a limitation in independent, purposeful physical movement of the body or of one or more extremities for > 48 hours[60], was recorded.

Other non-hematologic acute risk factors, such as oncologic history, infectious history, and presence of burns were recorded. A subjective evaluation of the presence of inflammation was performed, noting if the inflammation was chronic (i.e. persistent for > or = 3 months), acute (i.e. present for < 3 month) or acute-on-chronic. Non-hematologic chronic risk factors were evaluated and included: IBD, short bowel syndrome (SBS), nephrotic syndrome, autoimmune disorders, arrhythmias, CF, obesity, mechanical vascular obstruction syndromes (ex. May Thurner syndrome) and medications associated with VTE (such as steroids, oral contraceptives and L-Asparaginase). We also evaluated chronicity and number of non-hematologic chronic medical conditions for each patient, regardless of the state of the condition. Hematologic risk factors associated with VTE and their details were recorded and included: factor V Leiden, Prothrombin mutations, Homocysteine

elevation, protein deficiencies such as protein C, protein S and anti-thrombin 3 deficiency, lipoprotein(a) elevation and the presence of anti-phospholipid antibody syndrome, polycythemia or sickle cell disease states.

Hospital course details were also abstracted, such as hospital location at initial admission, need for transfer to an intensive care setting, intubation, need for vaso-active medication, surgical interventions, presence of total parental nutrition (TPN), duration of admission and/or intensive care stay and mortality. Clinical laboratory values, such as complete blood counts (CBC), albumin, coagulation testing (PT/INR, aPTT, fibrinogen and D-dimers) and inflammatory markers (CRP and ESR) were recorded as long as they were performed within 48 hours of sample collection. CVC-related characteristics were collected on patients with CVC, such as tunneled vs. non-tunneled catheter, the specific catheter type, location of catheter placement including limb and sidedness, caliber of vessel of insertion, internal length of catheter, number of lumens, appropriate location of catheter tip and any associated complications. For patients who had an acute VTE, thrombus characteristics (e.g. location, radiologic characteristics, clinical presentation) and anticoagulation therapy (e.g. medication, length of therapy, adverse events) were recorded.

3. Blood collection and plasma preparation

Venous samples were collected within 24 hours of enrollment for both study arms and serial samples (at 7 days post-enrollment and at removal of line or resolution of acute VTE) were collected when possible. Samples were collected via venipuncture or through the CVC by experienced personnel into vacutainers (BD Bioscience, Franklin Lakes, New

Jersey, USA) containing 3.2% buffered sodium citrate in a 9:1 ratio. The investigator directly observed sample collection and blood was gently mixed immediately after collection. Sample was transported manually to the lab without agitation. All blood samples were processed within 2 hours of collection. To prepare platelet poor plasma (PPP), samples were centrifuged at room temperature for 15 minutes at 2500 g with slow acceleration and no brake. The supernatant plasma was carefully removed and repeat centrifugation of the supernatant was done at room temperature for 15 minutes at 2500 g with slow acceleration and no brake. The resultant supernatant plasma was aliquoted in 250 µL aliquots and stored at -80°C for future analysis. This processing procedure was determined to minimize any pre-analytical bias based on recent literature[61, 62]. In addition, sample-related factors, such as timing of collection in relation to CVC insertion, presence of anti-coagulant or anti-platelet agents and evidence of hemolysis on visual inspection, were assessed to account for potential pre-analytic confounders.

4. Thrombin Generation

Thrombin generation was measured using the CAT (Thrombinoscope BV, Stago, Maastricht, The Netherlands). The assay was performed according to manufacturer instructions, which is thoroughly described in the literature.[63, 64] All samples were tested in triplicate and TG was determined under 2 experimental conditions (final plasma concentrations): PPP Reagent low (Stago; 1 pM TF and 4 mmol/L phospholipids [PL]) and PPP Reagent (Stago; 5 pM TF and 4 mmol/L PL). PPP (80 µL) sample was added to each well. To correct for inner filter effects and substrate consumption, each TG measurement was calibrated against the fluorescence curve obtained in the same plasma with a fixed

amount of thrombin-a2-macroglobulin complex (Thrombin Calibrator, Stago, Maastricht, The Netherlands). Each reagent (20 µL) was added to one set of wells and thrombin calibrator (20 µL) was added to the other set of wells. The plate was warmed to 37c and Fluca-Kit Reagent (20 µL) was automatically added to each well to re-calcify the sample and start the reaction. Fluorescence was read at fixed time intervals over a 1-hour period in an Ascent Reader (Thermolabsystems, Thermo Fisher Scientific, Waltham, MA USA), and TG curves were calculated with the Thrombinoscope software (Thrombinoscope BV). Five parameters were derived from the thrombin generation curves: lag time, time to peak (ttPeak), peak thrombin generation (peak), endogenous thrombin potential (ETP; area under the curve) and velocity index (VI), the latter calculated as follows: Peak/[(ttPeak) – (lag time)]).

5. Microparticles Assays

The functional aspects of MPs were evaluated via two methods. The first assay is the Zymuphen CPMP-Activity ELISA (DiaPharma, West Chester, OH), which measures the phospholipid-dependent functional potential for thrombin production. This assay was performed according to the manufacturer's protocol as outlined in the literature[65,28]. PPP samples and kit controls were diluted 1:20 in sample diluent (containing calcium and factor IIa and factor Xa inhibitors) and applied to microplate wells coated with streptavidin and biotinylated annexin V. Following incubation at 37°C for 60 minutes to allow binding of CPMPs to annexin V, the wells were washed five times followed by addition of calcium, bovine factor Xa/factor Va (100 µL) and purified human prothrombin (50 µL). As a result, pro-thrombinase complex assembled on phospholipid surface of the CPMP allowing the

cleavage of prothrombin to thrombin. A chromogenic thrombin substrate (50 µL) was added, and the reaction was stopped with 2% citric acid. Absorbance was measured at 405 nm. CPMP concentrations (nM [PS] equivalent) were calculated from a calibration curve obtained from serially diluted calibrator provided by the manufacturer.

The second functional MP assay is the STA-Procoag-PPL assay (Diagnostica Stago, Parsippany, New Jersey, USA), which was performed according to the manufacturer's protocol[66-68]. This assay measures phospholipid-dependent clotting time, which is dependent on the phospholipid content of the sample as its only source. A shortened clotting time indicates increase pro-coagulant activity due to higher phospholipid content. Two kit controls, with a known clotting time, were analyzed to check the reproducibility of the assay. Using an automated Stago platform (STA Compact, Diagnostica Stago, Parsippany, New Jersey, USA), PPP sample (25 µL) is mixed with phospholipid depleted human plasma (25 µL) in a cuvette and pre-warmed for 2 minutes at 37°C. Coagulation was triggered by adding 100 µL of pre-warmed factor Xa/calcium reagent (containing 0.01 U/mL bovine factor Xa in a buffered calcium solution), followed by measurement of the clotting time in seconds (i.e. Xa clotting time – XaCT). To obtain a reference clotting time, 20 plasma samples form 20 healthy individuals, that were confirmed to have normal PT/INR/aPTT, were measured and the mean value was used to calculate a XaCT ratio.

6. Factor VIII activity

Factor VIII activity was measured using a one-stage clot-based assay (Diagnostica Stago, Parsippany, New Jersey, USA) according to the manufacturer's protocol. This assay

measures factor VIII-dependent clotting, which is dependent on the factor VIII content of the sample as its only source. Using an automated Stago platform (STA Compact, Diagnostica Stago, Parsippany, New Jersey, USA), a calibration curve was obtained from serially diluted calibrator provided by the manufacturer. Two kit controls, with a known clotting time, were analyzed to check the reproducibility of the assay. PPP sample is diluted at 1:40 ratio and 50 µL of the diluted sample is mixed with factor VIII deficient plasma (50 µL) and phospholipid aPTT reagent (50 µL, containing cephalin and phospholipid) in a cuvette and pre-warmed for 2 minutes at 37°C. Coagulation was triggered by adding 50 µL of calcium chloride followed by measurement of the clotting time in seconds. FVIII was extrapolated from the calibration curve obtained from the serially diluted calibrator.

7. Statistical analysis

Pre-study statistical assessment was performed using control results (4.3 nmol/L PS eq [R: 1.3 – 14.5]) of CPMP levels from previous studies[33, 69, 70] as the primary measure for sample size calculation for this pilot study. To show a difference that approximates one standard of deviation from the available data (i.e. > 7.5 nmol/L PS eq), with 90% power and $p < 0.05$, we calculate a minimum sample size of 25 patients would be needed in each group. To minimize the risk of a type II error and account for any possible confounders due to heterogeneity of the target population, we aimed to recruit 50 subjects per group with a 1:1 age-matched control ratio. At the conclusion of the study period, 42 patients were enrolled on each arm of the study with appropriate number of matched healthy controls.

Data were analyzed using SPSS v23 and graphs were created using Excel v14.

Analysis of each of the variables collected was done through descriptive statistics. Categorical variables (nominal/ordinal) were evaluated by the frequencies of each occurrence, while continuous variables were evaluated for normality and if deemed not normally distributed, the median and interquartile range (IQR) was calculated. Missing data were noted for each variable and cases with missing values were not analyzed. The relationship between the variables and the outcomes was assessed by Fisher's exact test or the independent T-test, depending on the type of independent variable in question. A p-values < 0.05 was considered significant. In addition, sensitivity/specificity analysis was performed to evaluate for appropriate biomarker cutoffs to guide future studies.

CHAPTER 4: RESULTS

1. Demographic and sample-related data

Forty-two patients were enrolled on each arm of the study and forty-two healthy controls were recruited. There was no significant difference between the three groups in terms of gender, median age or age group (i.e. ≤ 1 year, > 1 year – 5 years, 6 years – < 12 years, ≥ 12 years) – (see Table 1). A second sample was collected in 21 patients (50%) at a median interval from enrollment of 12 days (IQR=29). A third sample was collected in 5 patients (12%) at a median interval from enrollment of 43 days (IQR=136). The 1st sample was collected prior to CVC insertion in 17 of 42 (41%) patients and between 12 and 24 hours post insertion in 25 of 42 (59%) patients.

Table 1. Demographic data

	Healthy Controls[1] (n = 42)	**CVC Group[1] (n = 42)**	**VTE Group[1] (n = 42)**
Age – Median (IQR) – in years	9 (10.3)	10 (11.3)	12.2 (13.4)
Age Cat 1 (≤ 1 yr) – n (%)	4 (8)	6 (15)	6 (14)
Age Cat 2 (> 1 yr – 5 yr) – n (%)	13 (32)	9 (21)	7 (17)
Age Cat 3 (6 yr – < 12 yr) – n (%)	10 (24)	9 (21)	8 (19)
Age Cat 4 (≥ 12 yr) – n (%)	15 (36)	18 (43)	21 (50)
Male	23 (55)	22 (52)	21 (50)

[1] Number of cases = 42, unless otherwise specified in the table.

When evaluating potential for contamination of samples with anti-coagulants or anti-platelet agents, 21% of 1st samples (n=9/42), 62% of 2nd samples (n=13/21) and 40%

of 3rd samples (n=2/5) were exposed. However, the majority of exposures were due to heparin flushes, with confounding avoided with appropriate wasting technique to avoid contamination. None of the samples had any visible evidence of hemolysis. Thirteen patients (31%) had transfusion exposure within 3 months of enrollment. In addition, the median number of transfusion products was 5 (IQR=6) and the median interval of last transfusion from enrollment was 6 days (IQR=6).

2. VTE-related clinical risk factors

The majority of patients receiving a CVC had chronic medical conditions – (n=27, 64%), with 44% (n=12/27) having a single condition, 22% (n=6/27) having 2 conditions, 26% (n=7/27) having 3 conditions and 8% (n=2/27) having more than 3 conditions. In addition, 43% (18/42) had a surgical procedures in the year prior to enrollment. Past cardiac history was present in 17% (n=7/42) of patients. Inflammation was present in the majority of patients (n=36/42, 86%), with the majority having acute onset without any evidence of chronic inflammation at baseline (n=30/36, 83%). Infectious causes attributed for most patients with acute inflammation, as it was the most common non-hematological acute risk factor (n=32/42, 76%). Most infections were bacterial (n=30/32, 94%) with more than 75% having microbiological evidence. The majority of these patients needed CVC to complete anti-microbial treatment with an excellent response rate. Due to the predominance of non-tunneled CVC in our cohort, there were a small number of patients with an oncological diagnosis (n=7/42, 17%), none of whom developed a CVC-related VTE.

Unlike non-hematological acute risk factors, which were present in the majority of patients (n=38/42, 91%), only 33% (n=14/33) of patients had non-hematological chronic risk factors for VTE. IBD was present in 3 patients, with two patients having significantly uncontrolled disease with multiple recent admissions and CVC placements but no VTE. Three patients had CF with an acute exacerbation, with two patients having 3-4 admissions in the previous year and multiple previous CVC (range 2 – 8) with no history of CVC-VTE. Even though 14% of patients had some components of thrombophilia testing, none had any identifiable laboratory risk factors. One patient had sickle cell disease, which was controlled on hydroxyurea. There were no patients with positive family history of VTE. Three patients had positive past medical history of VTE due to a single previous episode, one of whom was treated with aspirin for secondary prophylaxis. None of these patients developed a CVC-related VTE. A summary of the VTE-related clinical risk factors is outlined (see Table 2).

Table 2: Clinical risk factors for VTE

	CVC Group[1] (n = 42)
Presence of past cardiac history – n (%)	7 (17)
Transfusion exposure within 3 month of enrollment – n (%)	13 (31)
Number of transfusion products – Median (IQR)	5 (6)
Days from last transfusion to enrollment – Median (IQR)	6 (6)
Acute risk factors – n (%)[2]	38 (91)

Inflammatory state – n (%)	36 (86)
Acute inflammation – n (%)	30/36 (83)
Acute on Chronic inflammation – n (%)	4/36 (11)
Chronic inflammation – n (%)	2/36 (6)
Significant Immobility – n (%)	6 (14)
Acute infection / sepsis – n (%)	32 (76)
Types of infections[3]	
Viral – n (%)	1/32 (3)
Bacterial – n (%)	30/32 (94)
Fungal – n (%)	4/32 (13)
Confirmed via microbiology – n (%)	22/32 (76)
Oncological diagnosis – n (%)	7 (17)
Lymphoma/Leukemia – n (%)	4/7 (57)
Solid tumor – n (%)	3/7 (43)
Chronic risk factors – n (%)[4]	14 (33)
Inflammatory bowel disease – n (%)	3 (7)
Rheumatologic disease – n (%)	1 (2)
Cystic Fibrosis – n (%)[5]	3 (7)
Obesity – n (%)[6]	7 (17)
Medications associated with VTE – n (%)[7]	6 (14)
Presence of chronic medical problems – n (%)	27 (64)
1 chronic medical problem – n (%)	12/27 (44)

2 chronic medical problems – n (%)	6/27 (22)
3 chronic medical problems – n (%)	7/27 (26)
> 3 chronic medical problems – n (%)	2/27 (8)
Hematological risk factors – n (%)[8]	4 (10)
Thrombophilia testing done – n (%)	6 (14)
Sickle cell disease – n (%)[9]	1 (2)
PMH of VTE – n (%)	3 (7)

[1] Number of cases = 42, unless otherwise specified in the table. [2] There were no patients with burns. [3] There were no parasitic infections. [4] No patients had short bowel syndrome (SBS), nephrotic syndrome, cardiac arrhythmias or anatomical obstruction. [5] JIA, RF+, controlled disease, not on steroids, Dx since 30 months prior to enrollment. [6] All Obese patients had BMI > 95% for age and had no recorded co-morbidities (HTN, DM or pre-DM or lipid pathology). [7] All pro-coagulant medications were steroids (no OCPs, no L-Asparaginase). [8] No patients had polycythemia. [9] Patient had sickle cell disease controlled on hydroxyurea and thrombocytosis (on aspirin for primary prophylaxis).

3. Admission characteristics

On Admission, 62% (n=26/42) of CVC patients were admitted to the regular pediatric floor, with 19% (n=5/26) of these patients needing transfer to an intensive care during the same admission. The accumulative intensive care admission in our cohort was 50% (21/42), with a median intensive care admission length of 3 days (range=2 – 27). Intubation occurred in 43% (n=9/21) and need for vaso-active medication was present in 24% (5/21). Interestingly, surgical intervention during admission was present in all patients with CVC-VTE, even though not statically significant (p=0.06). In our cohort, TPN was used in 31% (13/42) of patients, for a median duration of 23 days (IQR=12). The mean white blood cell count (WBC) around the 1st sample was elevated at 11 x 103/μL (SD=6), with an absolute neutrophil count (ANC) of 7146/μL (SD=5060). Fibrinogen (n=17) was

elevated at 428 mg/dl (SD=164) and the C-reactive protein (CRP) level (n=30) was 126 mg/L (SD=146). Surgical intervention was needed in 41% (n=17/42). The mean admission duration was 30 days (SD=14), with overall mortality rate of 7% (n=3/42). However, none of the fatalities were associated with the CVC or a VTE event. Summary of the admission characteristics is outlined (see Table 3).

Table 3: Admission characteristics[5]

	CVC Group[1] (n = 42)
Admission length – Mean (SD) – in days	30 (14)
Admission to general pediatric floor – n (%)	26 (62)
Transfer to PICU during admission – n (%)	5/26 (19)
Admission to PICU/CVICU – n (%)[2]	16 (38)
Total patient admitted to PICU during admission – n (%)	21 (50)
Need for intubation – n (%)	9/21 (43)
Need for vaso-active medication – n (%)	5/21 (24)
PICU admission length – Median (range) – in days	3 (2 – 27)
TPN need during admission – n (%)	13 (31)
Duration on TPN – Mean (SD) – in days	23 (12)
Surgery needed during admission – n (%)	17 (41)
Mortality during that admission – n (%)[3]	3 (7)

[1] Number of cases = 42, unless otherwise specified in the table. [2] Only two admissions to CVICU, the remainder were to the PICU. [3] None of the mortalities were associated with CVC or VTE. [5] Relevant lab results around first sample: The mean white blood cell count (WBC)

was elevated at 11 x 103/μL (SD=6), Fibrinogen (n=17) was elevated at 428 mg/dl (SD=164) and the C-reactive protein (CRP) level (n=30) was 126 mg/L (SD=146).

4. CVC-related characteristics

For patients with CVC, median interval between admission and CVC placement was 3 days (IQR=8). The majority of the CVCs (n=38/42, 91%) were non-tunneled peripherally inserted central catheters (PICCs), with the remaining 4 CVCs being tunneled (three Broviac and one Port-a-Cath). Ninety-five percent of the CVC were inserted in the upper extremity (40/42), with 64% (27/42) placed on the right side. There were no documented complications during insertion but the CVC was not in appropriate radiological position in 5% (2/42) of patients, with no apparent complications. Fifty-eight percent of patients (n=13/40) required more than one attempt to reach appropriate location, with 38% (n=15/40) needing a total of two attempts and 20% (n=8/40) needing a total of three attempts. Patients with a history of previous CVC placement accounted for 29% of the cohort (n=12/43), with the majority having 1-2 previous CVCs. The median duration of these lines were 0.75 months (range=0.5 – 14) and only one patient had a previously documented CVC-VTE (1/12) but did not develop a second event during our study.

The indication for CVC placement was prolonged antibiotic administration in 33%, need for TPN in 12%, poor vascular access with need for frequent labs in 2% and the remaining cases had multiple indications. The median duration of CVC was 15 days (range=3 – 332) and most were removed after completion of primary use (86%), with the exception of one CVC removed due to infection, one CVC removed due to VTE occurrence and four CVCs (10%) removed due to malfunction. Two of the CVC malfunction cases were

diagnosed with CVC-related VTE (50%), occurring at an average duration of 11 days post-insertion (range=5 – 19). CVC patency was maintained via continuous intravenous infusion in 71% of patients (n=30/42), while the remainder received intermittent heparin flushes (n=12/42). Three patients developed CVC-VTE, which occurred at a median time of 7 days from CVC placement (range=5 – 39). A summary of CVC-related characteristics is outlined (see Table 4).

Table 4: CVC-related Data[1]	
	CVC Group[2] (n = 42)
History of previous CVC placement – n (%)[3]	12 (29)
Number of attempts at insertion[4]	
One attempt – n (%)	17/40 (42)
Two attempts – n (%)	15/40 (38)
Three or more attempts – n (%)	8/40 (20)
Location of CVC placement	
Insertion in upper extremity – n (%)	40 (95)
Insertion in right-side limb – n (%)	27 (64)
Days from admission and CVC insertion – Median (IQR)	3 (8)
Duration of CVC – Median (range) – in days	15 (3 – 332)
Caliber of vessel of CVC (n = 36) - Mean (SD) – in cm	0.33 (0.09)
Internal length (n = 36) – Mean (SD) – in cm	29.9 (9.7)
Number of lumen	

Single lumen – n (%)	25/38 (66)
Double lumen – n (%)	11/38 (29)
Triple lumen – n (%)	2/38 (5)
Size of CVC	
3/3.5 Fr – n (%)[5]	15/36 (42)
4 Fr – n (%)	9/36 (25)
5 Fr – n (%)	6/36 (17)
6 Fr – n (%)	4/36 (11)
7 Fr – n (%)	2/36 (5)
Indications for removal of CVC	
Completion of primary indication – n (%)	36 (86)
CVC-associated infection – n (%)	1 (2)
CVC malfunction – n (%)	4 (10)
CVC-associated VTE – n (%)	1 (2)
CVC-related complications	
VTE – n (%)	3 (7)
Interval between CVC insertion & VTE Diagnosis – Median (range) – in days	7 (5 – 39)

[1] 4 CVCs were tunneled (three Broviac and one Port-a-Cath). The indication for CVC placement was prolonged antibiotic administration (33%), need for TPN (12%), poor vascular access with need for frequent labs (2%) and the remaining cases had multiple indications. [2] Number of cases = 42, unless otherwise specified in the table. [3] The majority having 1-2 previous CVCs with median duration of previous lines of 0.75 months (range=0.5 – 14). One patient had a previously documented CVC-related VTE (1/12) but did not develop a second event during our study. [4] There were no documented complications during insertion but CVC was radiologically mal-positioned in 5% (2/42) of patients, with no apparent complications. [5] Only 1 patient had a 3.5 Fr size.

5. Patients with CVC-VTE

When comparing the patients with CVC, with and without CVC-VTE, there was no observed significant difference in age, gender, acute risk factors, chronic risk factors and thrombophilic risk factors. However, all subjects with CVC-VTE were admitted for an acute infection to an intensive care setting, had a surgical intervention during their admission and received an upper extremity PICC. Two out three CVC-related VTE events occurred within 7 days from CVC placement. As shown in previous studies, certain admission characteristics, such as length of stay and intensive care admission, were significant on univariate analysis (see Table 5). In addition, the only clinical laboratory values that showed significant difference were the ANC and the CRP, both reflecting the hyper-inflammatory state of patients who develop CVC-related VTE. Interestingly, none of the CVC-related characteristics (e.g. location or lumen size) showed any significant difference between patients with and without CVC-VTE, except for presence of CVC malfunction (p=0.02). The clinical details of the three patients that developed CVC-related VTE are summarized (see Table 6).

Table 5. Clinical factors in children with CVC, with and without CVC-related VTE

	CVC-related VTE Present (n = 3)	**CVC-related VTE Absent (n = 39)**	**p-Value**
Age – Mean (SD) – in years	11.7	9.4	0.54
Male, No. (%)	1	21	0.60
VTE-related risk factors[1]			
Presence of past cardiac history – n	1	6	0.43

Inflammatory state – n	3	33	1.00
Significant Immobility – n	1	5	0.38
Acute infection – n	3	29	1.00
Chronic medical conditions – n	1	26	0.29
Admission characteristics			
Length of stay – Mean (SD) – in days	52 (49)	18 (15)	**0.004**
Admission to PICU/CVICU – n	3	13	**0.049**
ICU stay during admission (n = 21) – n	3	18	0.23
Need for intubation (n=9) – n	1	8	1.00
Need for vaso-active meds (n=5) – n	1	4	0.45
ICU admission length – Mean (SD) – days	37 (58)	9 (9)	**0.04**
TPN need during admission (n=13) – n	2	11	0.22
Duration on TPN – Mean (SD) – in days	49 (31)	17 (10)	**0.009**
Surgery needed during admission – n	3	14	0.06
Clinical laboratory evaluation			
ANC within 48 hours from 1st sample – Mean (SD) – /µL (n = 41)	13,800 (2300)	6600 (5000)	**0.016**
CRP within 48 hours from 1st sample – Mean	377 (169)	99 (116)	**0.001**

(SD) – /μL (n = 41)			
CVC-related characteristics			
Previous CVC – n	1	11	1.00
Interval between admission & CVC insertion – Median (IQR) – in days	4 (4)	6 (16)	0.83
Duration of CVC – Mean (SD) – days	30 (22)	36 (68)	0.88
Left-sided insertion – n	2	13	0.29
Caliber of vessel of CVC –Mean (SD) – in cm (n = 36)	0.37 (0.07)	0.33 (0.09)	0.41
Internal length– Mean (SD) – in cm (n = 36)	34 (8.5)	29.5 (9.9)	0.46
Multiple lumen CVC (n = 40) – n	2	12	0.28
Higher caliber CVC – > 4 Fr (n = 36) – n	2	11	0.54
> 1 attempt at placement (n = 40) – n	3	20	0.25
Intra-catheter thrombosis prevention via continuous IV infusion – n	2	28	1.00
CVC malfunction – n	2	2	**0.02**

ANC – Absolute neutrophil count, CRP – C-reactive protein, [1]None of CVC-VTE occurred in patients with IBD, SBS, Nephrotic syndrome, rheumatoid disease, CF, Obesity, Mechanical obstruction or taking VTE-related medication. None of CVC-VTE occurred in patients with + thrombophilia testing or FH of VTE.

Table 6: Clinical details of children that developed a CVC-related VTE[1]			
	Pre-CVC clinical history	**Hospital course**	**Details of CVC & associate VTE**
*Patient #1***	• 15 years old male with no PSH/PMH or personal/FH of VTE. • Presented with mental status change, 2-weeks of headache.	• Diagnosed with epidural empyema due to complicated sinusitis • PICU for 5 days post craniotomy and abscess evacuation. • Discharged day 7, re-admitted for wound infection, treated with a wound washout and revision. • Re-admitted for PICC-line cellulitis	• PICC inserted during first admission, after initial neurosurgery. • Redness and induration around PICC site for 2 days prior to 3rd admission. • PICC-associated RUE distal occlusive DVT on US doppler • VTE occurred 39 days post-insertion. • No anti-coagulation was started due to mild symptoms and CVC removal, clot resolved after 2 weeks.
Patient #2#	• 3.5 years old female with complex congenital heart disease, status post multiple cardiac surgical corrections. • Single ventricle physiology, heterotaxy asplenia. • No personal/FH of VTE. • On ASA primary prophylaxis for bidirectional Glenn shunt.	• Admitted to CVICU with acute abdomen and signs of sepsis. • Bowel obstruction with perforation, peritonitis • Intubated, and on pressure support. • Multiple surgical interventions during admission, on TPN for 71 days. • Candidemia, on prolonged broad anti-microbial therapy. • Hospitalized 104 days and discharged home	• PICC on admission, removed after 5 days, non-functional, no line study • Day 14, phlebitis at site of previous PICC, US Doppler revealed a LUE chronic non-occlusive proximal DVT • No anti-coagulation was initiated due to risk of bleeding but ASA prophylaxis was continued. • There was no progression of thrombus on serial imaging • Patient developed two unrelated CVC-related thrombi during a future admission.
Patient	• 17 years old	• PICU admit due to	• PICC inserted on

#3$	female, obesity, cholelithiasis, cholecystectomy 4 days PTA. • Presented with acute pancreatitis. • No personal/FH of VTE.	pancreatitis with third spacing. • Imaging revealed dilation of common bile duct and pseudocyst formation. • Transferred to the floor after 2 days. • Bacteremia treated with anti-microbials for 2 weeks. • Stenting of the bile duct, laparoscopic drainage of the pseudocyst. • Hospitalized 44 days, discharged with no sequel.	admission and was on TPN for 27 days. • Pain, redness, palpable cord at PICC site 7 days after insertion. • LUE superficial non-occlusive thrombus on US Doppler. • Due to mild symptoms and risk of hemorrhagic pancreatitis, anti-coagulation was not initiated. • Symptoms worsened, CVC partially non-functional. Repeat imaging revealed progression to deep veins. • Anti-coagulation with complete resolution. • Prophylaxis enoxaparin after resolution until discharge. • CVC was removed at discharge after a total duration of 41 days.

CVC – Central venous catheter, VTE – Venous thromboembolic event, PSH – Past surgical history, PMH – Past medical history, FH – Family history, CSF – Cerebrospinal fluid, PICU – Pediatric intensive care unit, PICC – Peripherally inserted central catheter, RUE – right upper extremity, US – Ultrasound, DVT – deep vein thrombosis, ASA - Aspirin, CVICU – Cardiovascular intensive care unit, TPN – Total parenteral nutrition, LUE – left upper extremity, PTA – prior to admission. [1] None of these patients were on prophylactic anticoagulation during the duration of CVC presence and none had thrombophilia testing after the VTE diagnosis. [*] VTE Risk factors present at diagnosis were the presence of central venous catheter and an acute infection. [#] Risk factors present at diagnosis were history of single ventricle congenital heart disease, the presence of CVC and an acute infection. [$] Risk factors present at diagnosis were obesity, pancreatitis with acute infection and presence of CVC

6. Biomarker univariate evaluation

In our cohort, biomarker values did not differ significantly among age categories, gender or ethnic groups, except for Low Reagent ETP, which demonstrated an increase with age (p=0.006)[71]. When biomarker values of non-contaminated 1st samples from the acute VTE arm (n=16) were compared with control samples, all values were significantly higher in the VTE group (p<0.05). The same relationship was demonstrated when biomarker values of all 1st samples from the CVC arm (n=42) were compared with control samples (p<0.05). However, contrary to prediction, CPMP concentrations were higher in healthy controls as compared to the other two groups. The data comparing biomarker values for healthy controls and CVC patients are summarized in Table 7. There was strong correlation between all TGA parameters when comparing regular TF vs. low TF reagent (r > 0.7, p<0.05). Correlation analysis of the two CPMP assays was also significant (r = -0.659, p<0.05).

Table 7. Biomarker data – Across study arms

	Healthy Controls (n = 42)	**CVC Group – 1st Sample (n = 42)**	**p-Value**
[CPMP] – (nM [PS] equivalent) – Mean (SD)	15 (9)	10 (8)	0.020
Absolute PPL-dependent Clot time (Sec) – Mean (SD)	61.1 (6.3)	50 (12)	<0.0001
PPL-dependent Clot time ratio – Mean (SD)	1.12 (0.12)	0.9 (0.2)	<0.0001
Factor VIII activity (%) – Mean (SD)	115 (37)	281 (138)	<0.0001
Thrombin Generation (TG) via calibrated automated thrombogram (CAT) – Regular TF reagent			

Lag time (min) – Mean (SD)	3.67 (0.69)	4.03 (1.26)	0.23
Endogenous thrombin potential – ETP (nM min) – Mean (SD)	1136 (180)	1442 (383)	0.001
Peak thrombin generation – Peak (nM) – Mean (SD)	175 (38)	292 (112)	< 0.0001
Time to Peak – ttPeak (min) – Mean (SD)	7.44 (1.17)	6.72 (1.61)	0.067
Velocity Index – VI (nM/min) – Mean (SD)	49 (16)	123 (65)	< 0.0001
Thrombin Generation (TG) via calibrated automated thrombogram (CAT) – Low TF reagent			
Lag time (min) – Mean (SD)	5.98 (1.49)	8.09 (2.28)	< 0.0001
Endogenous thrombin potential – ETP (nM min) – Mean (SD)	946 (211)	1322 (420)	< 0.0001
Peak thrombin generation – Peak (nM) – Mean (SD)	136 (42)	226 (113)	< 0.0001
Time to Peak – ttPeak (min) – Mean (SD)	10.06 (1.78)	11.43 (2.63)	0.009
Velocity Index – VI (nM/min) – Mean (SD)	36 (16)	83 (59)	< 0.0001

CPMP: Circulating pro-coagulant microparticles. PS: Phosphatidyl Serine, PPL: Phospholipid, TF: tissue factor.

To account for any potential confounding effect in the CVC arm, biomarker values were analyzed in relation to anti-coagulant/anti-platelet exposure, timing of 1st sample collection and transfusion exposure. There was no statistical difference between biomarker values of patients with and without anti-coagulant/anti-platelet exposure, and with and without transfusion exposure. In addition, there was no statistical difference between biomarker values of patients with pre- and post-insertion collection within the first 24 hours. Furthermore, paired analysis of serial samples, performed using the paired sample t-

test, revealed no significant difference between samples in terms of all measured biomarkers.

First sample biomarker measurements in patients with CVC-VTE were compared with patients who did not develop CVC-related VTE (Table 8). The Xa clotting time assay showed measurable difference between the two groups, both as an absolute value and as a ratio (p=0.04 for both). In contrast, the CPMP-Activity assay did not show a significant difference between patients with and without CVC-related VTE (p=0.27). This is due to the lack of sensitivity of this assay, resulting in its inability to differentiate between affected patients with VTE from those without VTE, within a clinical group with high risk of VTE[69,30]. However, the Xa clotting time assay has demonstrated high clinical utility potential through its ability to produce significant results, while minimizing the time and expertise needed to run the assay.

Table 8. Biomarker measurements of 1st sample in children with CVC, with and without CVC-related VTE

	CVC-related VTE Present (n = 3)	**CVC-related VTE Absent (n = 39)**	**p-Value**
[CPMP] – (nM [PS] equivalent) – Mean (SD)	15 (8)	10 (8)	0.27
Absolute PPL-dependent Clot time (Sec) – Mean (SD)	36.9 (3.9)	51.4 (11.5)	0.04
PPL-dependent Clot time ratio – Mean (SD)	0.68 (0.07)	0.95 (0.21)	0.04
Factor VIII activity (%) – Mean (SD)	461 (120)	267 (130)	0.02
Thrombin Generation via calibrated automated thrombogram – Regular TF			

reagent			
Lag time (min) – Mean (SD)	3.67 (0.34)	4.05 (1.30)	0.62
Endogenous thrombin potential – ETP (nM min) – Mean (SD)	1960 (515)	1401 (347)	0.01
Peak thrombin generation – Peak (nM) – Mean (SD)	492 (102)	276 (98)	0.001
Time to Peak – ttPeak (min) – Mean (SD)	5.70 (0.42)	6.80 (1.64)	0.26
Velocity Index – VI (nM/min) – Mean (SD)	241 (36)	114 (57)	< 0.0001
Thrombin Generation via calibrated automated thrombogram – Low TF reagent			
Lag time (min) – Mean (SD)	7.26 (1.06)	8.16 (2.34)	0.52
Endogenous thrombin potential – ETP (nM min) – Mean (SD)	1828 (485)	1282 (394)	0.03
Peak thrombin generation – Peak (nM) – Mean (SD)	418 (89)	211 (101)	0.001
Time to Peak – ttPeak (min) – Mean (SD)	9.56 (1.28)	11.58 (2.66)	0.20
Velocity Index – VI (nM/min) – Mean (SD)	182 (28)	75 (53)	0.001

CVC – Central venous catheter, VTE – Venous thromboembolic event, CPMP: Circulating pro-coagulant microparticles. PS: Phosphatidyl Serine, PPL: Phospholipid, TF: tissue factor

Factor VIII activity showed an approximate elevation of 200% in individuals who developed VTE, compared to those who did not develop VTE (p=0.02). Similarly, peak thrombin generation, ETP and the velocity index were significantly higher in patients with CVC-related VTE as compared to those who did not develop VTE, using both 1 pM TF and 5 pM TF concentration reagents. The significant difference between the TG curves is illustrated in Figure 1, which shows a two to four-fold increase in both ETP and peak values when compared to both healthy controls and to CVC patient without VTE**.** As described previously[40], TGA using 1 pM TF concentration reagent demonstrated a larger difference

between the two groups. In addition to its significant ability to differentiate between at risk patients, TGA has high clinical potential due to its ability to run samples in batches and relatively small sample size requirement. Even though the assay requires specialized laboratory expertise, with recent standardization efforts[72], TGA can be performed in specific regional centers, thus ensuring the needed quality controls and sufficient patient load necessary to ensure the sustainability of the assay in clinical practice.

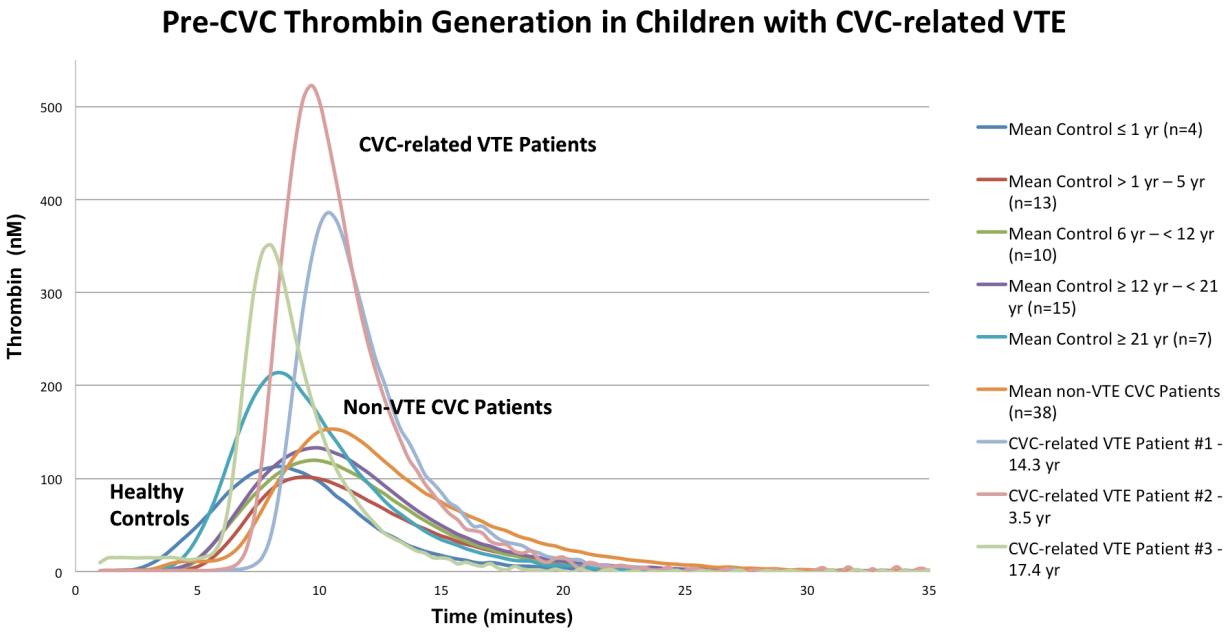

Figure 1

7. Biomarker sensitivity/specificity analysis

A sensitivity and specificity analysis was conducted involving the following biomarker parameters: Xa clotting time ratio, FVIII, ETP, Peak, VI and the clinically obtained CRP. Receiver operating characteristic (ROC) curves were generated for each of the parameters in relation to CVC-related occurrence (see Figure 2.a-f). TGA parameters,

determined using 1 pM TF concentration reagent, were selected as the optimal reagent for differentiating at-risk patients. The cutoff point for each ROC curve was determined by locating the point on the curve with the minimum distance from top left corner (i.e. point (0,1)). To ensure that we obtain the optimal cutoff point, we conducted additional analyses evaluating cutoff points for each of the biomarker parameters, utilizing cutoff points that are 2 SD from the mean of the control group values and 1 SD from the mean of CVC non-VTE group values.

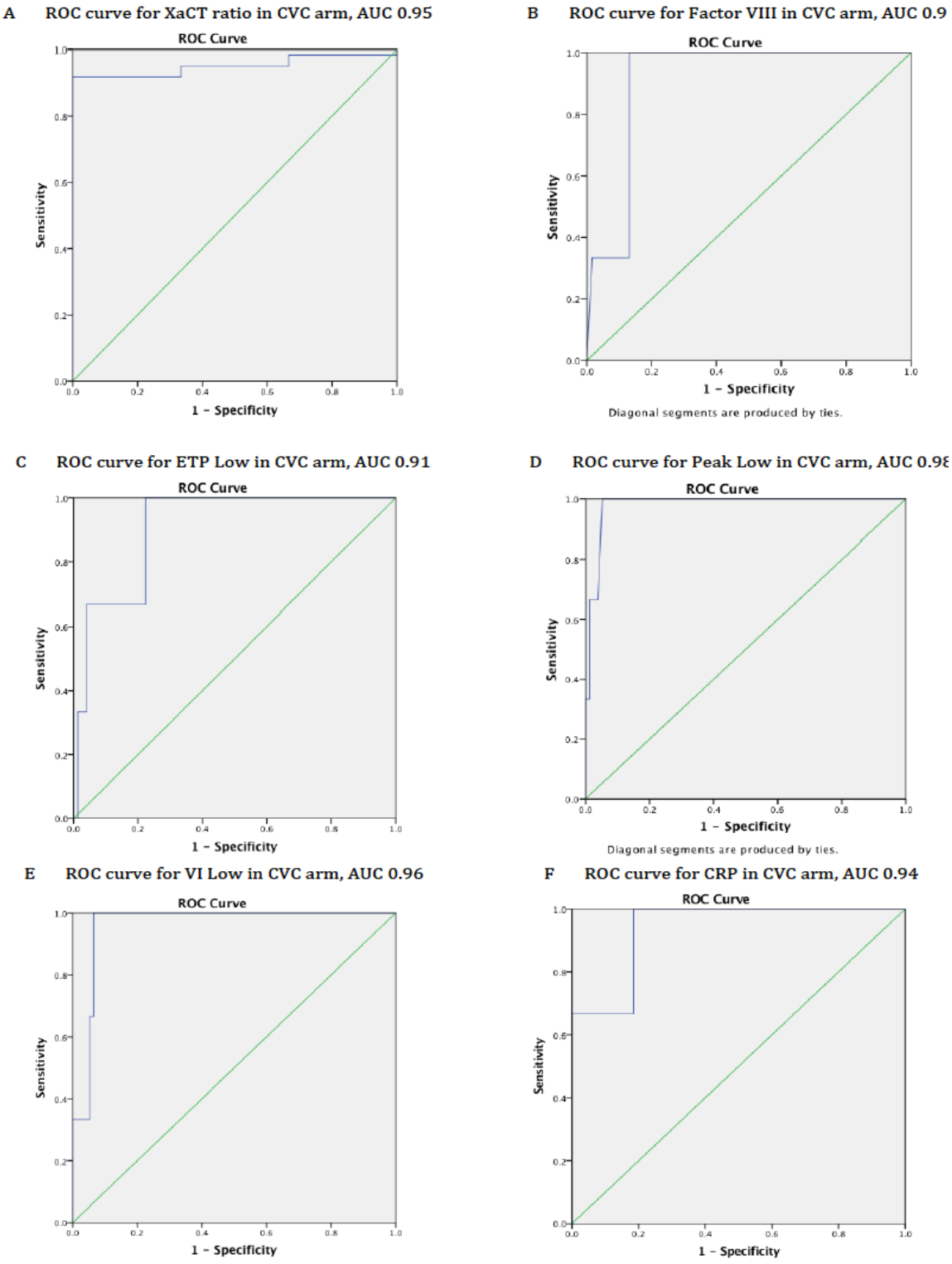

Figure 2. ROC curves showing biomarker function in the CVC arm with AUC values. A, for XaCT ratio; B, for Factor VIII activity; C, for ETP Low; D, for Peak Low; E, for VI Low; F, for CRP.

For each cutoff value, we determined sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), positive likelihood ratio (+LR) and negative likelihood ratio (-LR), as outlined in Table 9. Selection of optimal cutoff point for each biomarker value depended on optimization of PPV and +LR, without compromising sensitivity and specificity characteristics. All biomarker values had excellent AUC values, consistently above 0.9. The PPVs for each of the biomarkers were 27-40%, with overall high sensitivity and specificity except for a slightly lower sensitivity for ETP. The final cut-off values for each proposed biomarker value is summarized in Table 10.

Table 9: Sensitivity and specificity analysis result of Biomarker cutoff values

	Cutoff Value	Sen. (%)	Spec. (%)	PPV (%)	NPV (%)	+ LR	-LR
XaCT ratio							
ROC cutoff	**0.75**	100	87	38	100	7.69	0
2SD of Control cutoff	0.9	100	51	14	100	2.04	0
1SD of CVC non-VTE cutoff	0.8	100	74	23	100	3.85	0
FVIII (%)							
ROC cutoff	**370**	100	80	27	100	5.00	0
2SD of Control cutoff	190	100	36	11	100	1.56	0
1SD of CVC non-VTE cutoff	400	33	80	20	94	1.65	0.84
ETP Low (nM min)							
ROC cutoff	1350	100	55	15	100	2.22	0
2SD of Control cutoff	1370	67	58	16	96	1.60	0.57
1SD of CVC non-VTE cutoff	**1680**	67	92	40	97	8.38	0.36

Peak Low (nM)							
ROC cutoff	350	67	92	40	100	8.38	0.36
2SD of Control cutoff	220	100	58	16	100	2.38	0
1SD of CVC non-VTE cutoff	**315**	100	84	33	100	6.25	0
VI Low (nM/min)							
ROC cutoff	155	100	87	38	97	7.69	0
2SD of Control cutoff	70	100	63	18	100	2.70	0
1SD of CVC non-VTE cutoff	**130**	100	84	33	100	6.25	0
CRP (/µL) – ROC cutoff	**175**	100	82	38	100	5.56	0

XaCT: Xa clotting time, Sen,: Sensitivity, Spec.: Specificity, PPV: Positive predictive Value, NPV: Negative predictive value, LR: Likelihood ratio, ROC: Receiver operator curve, SD: Standard Deviation, CVC – Central venous catheter, VTE – Venous thromboembolic event, FVIII: Factor VIII activity, ETP: Endogenous thrombin potential, VI: Velocity index, CRP: C-reactive protein.

Table 10: Final Biomarker cutoff values

	Final Cutoff Value	Sen. (%)	Spec. (%)	PPV (%)	+ LR
XaCT ratio	0.75	100	87	38	7.69
FVIII (%)	370	100	80	27	5.00
ETP Low (nM min)	1680	67	92	40	8.38
Peak Low (nM)	315	100	84	33	6.25
VI Low (nM/min)	130	100	84	33	6.25
CRP (/µL)	175	100	82	38	5.56

XaCT: Xa clotting time, Sen,: Sensitivity, Spec.: Specificity, PPV: Positive predictive Value, LR: Likelihood ratio, FVIII: Factor VIII activity, ETP: Endogenous thrombin potential, VI: Velocity index, CRP: C-reactive protein.

CHAPTER 5: DISCUSSION

1. Current VTE risk prediction and biomarker application

The incidence of pediatric VTE and its associated acute and long-term complications are rising, necessitating accurate risk prediction to improve prevention efforts through appropriate pharmacological prophylaxis. Our study evaluated the utility of biomarkers in prospectively predicting VTE occurrence in relation to known clinical risk factors. In our study, microparticles, factor VIII activity and thrombin generation were evaluated for the prediction of VTE occurrence in this population. With the exception of CPMP, these biomarkers were significantly different in patients with an acute VTE event and in patients needing inpatient CVC placement, in comparison to healthy controls. This study demonstrates a hypothesis generating approach that constitutes the first step towards the identification of valid, reliable, feasible and rapid biomarkers to help identify and prevent VTE in children.

Due to the heterogeneity of the at-risk patient population and the variability of clinical course and outcomes, VTE risk prediction must be studied prospectively. Several attempts have been made to utilize VTE-related clinical risk factors in an evidence-based risk assessment tool to help predict HA-VTE, which was comprehensively reviewed by Mahajerin et al.[8] Significant clinical risk factors common to these studies were length of stay, intensive care admission and evidence of systemic infection.[17,18,73,19] However, these factors alone are not able to prospectively predict VTE occurrence and had variable sensitivity/specificity with PPV ranging from 2.84% to 12.5%. Slightly higher PPVs were

produced when these risk assessment tools were applied to our population (10% to 15%), which may be much improved if combined with concomitant biomarker evaluation.

Previous studies to utilize MPs [66,33] and TGA [74,75] in the pediatric setting were not able to show causality due to the lack of VTE occurrence in their patient population, but the potential utility of VTE biomarkers in adults has been more extensively investigated. Bucciarelli et al. demonstrated in a case-control study a linear dose-response effect between VTE risk and total MP measured >3 months post-VTE occurrence (adjusted OR=2.2).[29] A similar relationship was demonstrated in adults with thrombophilia-associated VTE (adjusted OR=2.72)[30] and in adults with cancer (adjusted OR=3.72),[76] with a sensitivity, specificity and PPV in cancer patients of 80%, 78% and 25%, respectively. Several adult studies demonstrated the association of elevated TGA parameters with the occurrence of a first VTE event (OR=1.7)[77] and with recurrence of unprovoked VTE (ORs=1.6-4.6).[78,79,80] Similarly, FVIII has shown prospective predictive potential in a subset of adults, both in association with CVC[81] and without CVC (adjusted OR=3),[43] with FVIII levels > 200%. None of these studies evaluated the value of combining clinical prediction tools and biomarkers but several compared the two approaches in the evaluation of at-risk adults.[40,76,43]

2. Impact of study results on Biomarker VTE research

In our study, the Xa clotting time ratio, factor VIII activity, and TGA measured within 24 hours of CVC placement were significantly different in children with CVC who developed

VTE, in comparison to those who did not develop VTE. A parallel relationship was demonstrated for CRP measurement within 48 hours of initial sample collection, reflecting the pro-inflammatory state of these patients and illustrating the important role of inflammation in thrombosis. Furthermore, sensitivity/specificity analysis for all significant biomarkers demonstrated excellent ROC curves with AUCs > 0.9.

In practice, our candidate biomarker assays use a small sample size, have a rapid turnaround time and, with the exception of TGA, require minimal technician time and effort. In addition, these assays can be performed on frozen plasma, allowing patching of samples, lowering cost, and providing the infrastructure for standardized procedures and practical workflows. As a result, they can be considered as excellent candidates for future large multi-institutional studies in combination with known clinical risk factors, to validate a clinical-biomarker risk prediction tool for VTE occurrence in children. One such study is the Children's Hospital Acquired Thrombosis (CHAT) Registry, a multi-institutional database for prospective identification of independent risk factors for hospital acquired pediatric VTE.[82] If used in clinical practice, a validated clinical-biomarker risk prediction tool could lead to stratification of VTE prophylaxis strategies, which will prevent overuse of pharmacological prophylaxis and prevent the occurrence of CVC-related VTE, similar to those that occurred in our patient cohort.

Since our patient cohort received PICCs as their CVC, we cannot comment on the utility of our biomarkers in non-PICC CVCs. However, since our biomarkers reflect the biological pro-coagulant state of the patient, irrespective of the patient condition or type of

CVC placed, the results further support more research into the utility of these biomarkers in patients with any CVC type. By combining biomarker-based risk stratification with clinical risk assessment tools, we will improve the PPV and thus target the population at the highest risk for VTE development. Furthermore, this may improve the effectiveness of our prophylactic strategies and potentially tailor interventions to the patient's individual needs.

3. Limitations and proposed solutions

There are several limitations to our study that will be addressed and we will provide several proposed solutions to overcome these sources of potential bias in future studies. One major limitation is the small number of cases that developed CVC-related VTE within our CVC study arm. Even though this was an expected result due to the pilot nature of our study and the rarity of CVC-related VTE in the pediatric population, the results still showed a significant difference, similar to several adult studies evaluating patients with VTE occurrence that were previously cited in this thesis. Furthermore, our results correlated with the event rate of CVC-related VTE in the pediatric literature (3 – 8%).[2,14] To address this inherit limitation in future studies, there are several proposals for future studies: 1) design a phase 2 validation study with appropriate power to confirm our findings, 2) recruit multiple institutions as part of future studies, with potential incorporation into future CHAT projects, 3) consider utilizing appropriately timed ultrasound surveillance programs on selected patients as part of study protocol.

As mentioned previously, the prevalence of HA-VTE in the literature is estimated at 0.71%, while overall prevalence of all CVC-related VTE is estimated at 5.7%. Applying specific clinical risk-assessment tools from the literature has improved PPV to a mean value of 5% (range 2.84 – 8.8%). The proposed phase 2 validation study design is a nested case-control design, with cases defined as subjects who developed CVC-related VTE and controls defined as subjects who do not develop a VTE from the same cohort, age-matched at a 3:1 ratio. Using data from our pilot study, we performed a power calculation to allow us to show an improvement in PPV to 33%, with 80% power and p-value < 0.05. Our calculation resulted in a sample size of cases of 27 subjects, with age-matched controls with CVC without CVC-related VTE of 81 subjects. Given the annual event rate of non-tunneled CVC-related VTE is 7.5%, the total number of subjects needed for recruitment is 360 patients. Even though we plan to recruit consecutive patients to avoid missing cases due to the known inherit low prevalence of events, we may consider implementing random selection as a way to avoid any selection bias. If recruitment of additional institutions is successful, this proposal is feasible for completion over a 3-year period.

To further overcome the inherently low prevalence of CVC-related VTE, we may also consider inclusion of asymptomatic CVC-related VTE that are detected through implementation of ultrasound surveillance, limited to clinically-determined high-risk children with CVC. There is active debate among the pediatric hematology community about the significance of asymptomatic CVC-related VTE, especially in relation to long-term complications, such as post-thrombotic syndrome, and potentially limiting future central access for patients with specific chronic diseases. These sub-clinical events may result in

the formation of collaterals, with eventual increase risk of venous insufficiency and/or future thrombotic complication. In this phase 1 study, we only considered VTE if it was symptomatic, thus explaining the much lower prevalence. However, a US surveillance study had a 25% event rate for line-related thrombus[83], regardless of symptoms, with a quoted event rate of 15 – 25% in the published literature. In addition, screening ultrasound at Day 7 post-placement detected the majority of the CVC-related clots[84]. As a result, a single complete US Doppler of the limb utilized for the CVC-insertion performed at Day 7 post-placement or prior to removal (whichever comes first) may result in detection of clinically significant CVC-related thrombus without introducing additional unwanted bias.

Another limitation that we faced during the conduct of this study involved the inability to consistently collecting all proposed serial samples specified in our initial protocol. This occurred due to two main reasons: 1) lack of communication between medical and research staff to allow for appropriately timed sample collection, 2) difficulty in co-ordination between timing of sample collection and availability of research staff, mainly due to work-related responsibilities and limited number of research staff. To overcome these obstacles, education was provided to staff that are involved with enrolled subjects, visual reminders were implemented and additional clinical research staff were recruited, supported by local grant funding. This allowed us to obtain serial samples on approximately 50-60% of subjects. Interestingly, paired analyses of serial samples did not show an overall decrease in biomarker values, which may reflect a baseline pro-coagulant state. However, this finding suggests the need for a longer time interval between samples to further study the time course of biological risk factors, such as inflammation, which was

beyond the scope of this study. This finding will aid in determining optimal timing of follow-up samples in future long-term, larger prospective studies.

Due to the known higher prevalence of HA-VTE in neonates,[7, 9, 85] the exclusion of this important population from our study constitutes another important limitation. However, because of its pilot nature, we decided to evaluate this first in non-neonates to establish the utility of these biomarkers prior to involving such a fragile population. After obtaining appropriate preliminary results, we obtained IRB approval to expand our inclusion criteria to include neonates that are at least 2 kg and 30 weeks corrected gestational age. We also implemented strict sample volume restrictions that were conservative and simple to follow. Even though we were not able to recruit any neonatal subjects due to limitation of time and resources, this unique population should be included in future validation studies as evaluation of the impact of developmental hemostasis is essential in the development of CVC-related VTE in this high-risk population.

4. An approach to advancing personalized care

Current scientific knowledge continues to rapidly evolve, with disproportionate impact on current medical care provided directly to patients. This is due in part to stringent expectations applied to clinical and translational research, limiting delivery of novel approaches to the patient. Even though it is essential to have methodologically sound approaches to conducting research, these obstacles hinder the enthusiasm of young researchers and may result in a disserve to our patients. A prime example of the impact of overcoming such hurdles and providing cautious utilization of a promising research tool to

patient care is the utilization of rapid whole genome sequencing in the care of pediatric patients at Rady Children's Hospital San Diego. I have witnessed first hand the impact of this novel and personalized approach on the quality and timeliness of care to our patients. When this approach is conducted in a careful manner, through appropriate acknowledgment of any limitation and utilization of data as a component of the complete clinical picture, it results in outstanding, cutting-edge and personalized preventative patient care.

As outlined in this thesis, I have provided sufficient evidence of the utility and feasibility of biomarkers in the prediction of CVC-related VTE. I believe that this evidence is strong enough, despite the limitations outlined above, to support the utility of these biomarkers as appropriate adjuncts to the clinician, as an additional decision-making tool, in the prevention effort of HA-VTE. By utilizing these laboratory tools in the care of the patient, we allow for timely delivery of personalized medicine, in additional to potentially avoiding long-term complications and providing cost-effective care. To have sufficient statistical power to establish these benefits, we must continue to evaluate the impact of these novel tools as part of quality improvement initiatives. Most importantly, this can only be achieved through appropriate clinician education, emphasizing the current limitation of these tools, and continual re-assessment of their utility in a real-life setting, which may be best achieved through a Bayesian approach.[86, 87] Finally, utilizing these tools to stratify patients with the highest risk will allow for evaluation of the efficacy of several prophylactic strategies through future randomized phase 3 multi-center studies.

5. Future research to advance personalized care in Hemostasis

As an effort to investigate the utility of our proposed biomarkers in patients with inherited bleeding disorder, we are completing a multi-center cohort study to evaluate the utility of TGA and MPs in the prediction of the clinical phenotype of patients with non-severe hemophilia A and B and rare bleeding disorders. Mild and moderate forms of hemophilia A and B (factor levels ≥ 1%) account for approximately 50% of subjects with hemophilia. Unlike severe hemophilia, the moderate to mild forms of this disorder, make up a more heterogeneous group in regards to bleeding phenotype[88,89]. There have been several attempts to correlate clinical bleeding with specific Factor VIII or Factor IX gene variants but these have been limited by small sample size and the lack of a standardized non-molecular biomarker[90,91]. More importantly, the correlation between factor levels and clinical phenotype in this population has been inconsistent[92-94]. Similar poor correlation exists in von Willebrand disease[95,96] and other rare bleeding disorders (deficiency in factors I, II, V, VII, X, XI and XIII)[97,98,99].

This gap in knowledge negatively impacts clinical decision making for management of these disorders. Since continuous prophylactic therapy at a young age is the gold standard for prevention of joint disease in severe hemophilia, prophylaxis in non-severe hemophilia may improve outcomes through strict prevention of all bleeding events[100]. Prediction of phenotype may help guide utilization of prophylaxis in this under-researched population, as there are no guidelines for prescription of prophylaxis in subjects with mild and moderate hemophilia. Specifically, TGA will provide a global assessment to help guide

clinicians in their risk-benefit assessment prior to starting prophylaxis for subjects with a more severe phenotype.

At this time, we have enrolled 91 subjects, with the majority having mild and moderate. We utilized validated bleeding assessment tools that have been studied extensively in the inherited bleeding disorders literature with overall significant correlation[101,102,103,104,105,106,107] and establishment of normal ranges in normal controls[108]. These questionnaires are self-administered and age-appropriate, further supporting their utility in research as appropriate patient-centered tools. Furthermore, we included standardized self-administered chronic pain assessment tools[109,110], which will evaluated patient-centered assessment of chronic pain intensity and its impact on their day-to-day activities. Preliminary data show significant negative correlation between ETP, peak and VI values, in relation to bleeding scores, indicating higher biomarker values in patients with less bleeding. As hypothesized, the same relationship was not demonstrated when FVIII, FIX and VW factor were evaluated at the same time. However, contrary to our initial hypothesis, higher XaCT values correlated with lower bleeding scores, a finding that may need further investigation in the future.

6. Conclusion

Developing novel approaches to bridge the gap between scientific discovery and clinical implementation continues to be of high priority in our scientific community. Implementation of these approaches in pediatric hemostasis thrombosis requires appropriate scientific evaluation and continual quality assessment. Utilizing biomarkers to predict patient-centered outcomes and prevent complications is an important aspect of

personalized care in pediatric hemostasis thrombosis. Specifically, the ability to use these biomarkers, in combination with clinical risk prediction tools, to stratify patients according to their VTE risk has great clinical, safety and financial benefits. Our study helped provide preliminary data by *prospectively* measuring specific biomarker assays, thus showing the potential for predicting CVC-related VTE in a heterogeneous patient population, without knowledge of the patient's future clinical course. Our results support the potential utility of microparticles-dependent clotting time, factor VIII activity and thrombin generation as biomarkers to differentiate children with CVCs that may develop a CVC-VTE as a HA-VTE. Further research is needed to validate these results prospectively in a larger cohort with appropriate controls and to expand the utility of biomarkers in the field, with emphasis on evaluating the utility of combining these biomarkers with established clinical risk factors.

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Appendix A

Summary of Biomarker testing and its cost.		
Biomarker	**Name of Assay**	**Component of VTE formation being measured**
CPMP	ZYMUPHEN MP-Activity kit	Measures pro-coagulant phospholipid-based activity
PPL-XaCT	STA-PROCOAG-PPL	Measures pro-coagulant phospholipid-based activity
γ' fibrinogen/Total fibrinogen ratio	γ' fibrinogen Sandwich ELISA	Measures γ' fibrinogen since lower endogenous levels decreases its anti-thrombotic role as a poor platelet aggregator and activator
Lead SNP at FGH2 locus	5' nuclease/Taqman assay	Measure presence mutation in γ' fibrinogen gene that results in its decrease production relative to other isoforms of fibrinogen
Soluble P-selectin	Human souble P-Selectin/CD62P Sandwich ELISA Kit	Measures degree of platelet activation
IL-6, IL-1β, IL-8 and TNF-α	Multiplex inflammatory marker assay	Measures degree of different aspects of inflammation
Thrombin generation (lag time, peak and ETP)	Calibrated Automated Thrombogram	Measures degree of thrombin generation potential
Lead SNP at TSPAN15 locus	5' nuclease/Taqman assay	Unclear but maybe involved in WBC attachment to endothelium
Lead SNP at SLC44A2 locus	5' nuclease/Taqman assay	Unclear but maybe involved in WBC & endothelial activation

Appendix B

Case Report Form

Demographics:

- Age (yr): _____
- Gender (M = 1, F = 0): _____
- Ethnicity (white = 0, Black = 1, Hispanic = 2, Asian = 3, Other = 4): _____
- Date of admission (M/D/Y): _____
- Lag of line placement from 1st sample (Duration from admission – days): _____

Past History:

- Transfusions? Y / N
  - How many in last 3 month: _____
    - # of PRBC: _____
    - # of Plt: _____
    - # of FFP: _____
    - # of Cryo: _____
  - Lag & type(s) of last transfusion from 1st sample (days): _____
  - Details (include any transfusions during study): _____

- ECMO? Y / N
  - Lag of ECMO from 1st sample (days): _____
  - Duration of ECMO (days): _____
  - Details (include indication, any complications especially ischemia, number of times circuit needed to be switched): _____ _____

- Dialysis? Y / N
  - Lag of Dialysis from 1st sample (days): _____
  - # of episodes in 6 weeks prior to 1st sample: _____
  - # of episodes per week: _____
  - Duration of Dialysis (days): _____
    Details (include type of dialysis, indication): _____

- Plasmapheresis? Y/N
  - Lag of Plasmapheresis from 1st sample (days):
  - # of episodes in 6 weeks prior to 1st sample: _____
  - # of episodes per week: _____
  - Duration of Plasmapheresis (days): _____

- Details (include type of replacement, indication):
  _____

- Past Surgical History (in past yr)? Y/N
  Details (Indication, specific name and presence of foreign material):
  _____
  _____

- Past Cardiac History (lifetime)? Y/N
  Details (Indication, specific name, age at surgery and presence of foreign material):
  _____
  _____

- Non-hematological acute VTE risk factors? Y/N
  - Significant limited mobility? Y/N
    - Major spinal, lower extremity or pelvic surgery *(Impact = 2)*? Y/N
    - Spinal cord injury - congenital, traumatic or acquired *(Impact = 2)*? Y/N
    - Trauma to lower extremities with/without casting *(Impact = 2)*? Y/N
    - Details: _____

  - Acute infection / Sepsis? Y/N
    - Type of infection: Viral / Bacterial / Fungal / Parasitic
    - Mode of confirmation: Clinical only / Microbiological
    - Treatment: Supportive / Anti-microbial
    - Duration of treatment (include duration of IV antimicrobial): _____
    - Time to significant response to treatment (ex. Becoming afebrile, returning close to baseline): _____
    - Details: _____

  - Malignancy? Y/N
    - Type of Malignancy: Leukemia / Lymphoma / Solid (Specific Dx _____)
    - Leukocytosis present at VTE? Y/N (If yes, what's the highest WBC _____)
    - Treatment around VTE: Chemo / radiation / Both
    - Details (Lag from diagnosis, phase of treatment, recent surgical resection, treatment details):
      _____

  - Burns needing treatment? Y/N
    - Percentage of body effected: _____
    - Details: _____

- Non-hematological chronic factors:
  - Inflammatory bowel disease? Y/N
    - Type of IBD: Crohns Disease / Ulcerative Colitis
    - Controlled disease (via symptom assessment)? Y/N

- CVL present? Y/N
- ESR closest to admission: _____
- Details (symptoms, severity, medication acute/chronic control, treatment plan, TPN): _____

- Short bowel syndrome? Y/N
  - Cause of SBS: _____
  - Duration of SBS: _____
  - TPN-dependent? Y/N
  - Lag from placement of last CVL if VTE present (months): _____
  - Number of CVL placed in lifetime: _____
    Details (cause of SBS, growing well, type of CVL, other medical problems):
    _____

- Nephrotic syndrome? Y/N
  - Duration of symptoms: _____
  - Steroid use? Y/N
  - Need for dialysis? Y/N
  - Albumin level closest to admission: _____
  - Fibrinogen level closest to admission: _____
  - Details (diagnosis, biopsy?, response to steroids, duration until remission, Lipid issue): _____

- Autoimmune disease? Y/N
  - Type of autoimmune disease: _____
  - Which auto-antibodies: _____
  - Complications/Uncontrolled? Y/N
  - Steroid use? Y/N
  - Lag from diagnosis if VTE present (months): _____
  - Details (complications, treatment details, duration until remission, degree of severity, degree of control):
    _____
- Cardiac arrhythmias? Y/N
  Details (type, treatment with medication and/or surgical, CHD present, anti-coag): _____

- Cystic Fibrosis? Y/N
  - Severity (subjective & via lung function): _____
  - Number of admission in last year: _____
  - CVL present? Y/N
  - Lag from placement of last CVL if VTE present (days): _____
  - Details (complications, treatment, type CVL, duration of admission & Abx treatment):
    _____
    _____

- Obesity? Y/N
  - BMI: _____
  - Abnormal Lipid profile? Y/N
  - Hypertension? Y/N
  - DM or Pre-DM? Y/N
  - Details (co-morbidities such as HTN, DM, HyperLipid, relevant FH, complications):
    _____
    _____

- Mechanical obstruction (ex. May-Thurner syndrome, Paget Schroetter, tumor related)? Y/N

- Significant Past Medical History not mentioned above? Y/N
  Details (Diagnosis, treatment, persistent or resolved, controlled:
  _____
  _____

- Medication used associated with VTE? Y/N
  - Hormonal contraception? Y/N
  - Glucocorticoids? Y/N

- Hematological factors? Y / N
  - Inherited Thrombophilia? Y / N / UT
    - Factor V Leiden mutation? Y / N / UT
      - Details (Hetero vs. Homo): _____
    - Prothrombin mutation? Y / N / UT
      - Details (Hetero vs. Homo): _____
    - Elevated homocysteine levels? Y / N / UT
      - Details (any syndromes, severity, random vs. fasting): _____
    - MTHFR? Y / N / UT
      - Details (type of mutation, hetero vs. homo, homocysteine done?):
        _____
    - Anti-thrombin 3 deficiency? Y / N / UT
      - AT3 Level (closest to 1st sample): _____
      - Details (AT3 trends if done multiple times, any supplementation, sources of loss present): _____
    - Protein C deficiency? Y / N / UT
      - Protein C Activity (closest to1st sample): _____
      - Protein C Antigen (closest to 1st sample): _____
      - Details (PC trends if done multiple times, Coumadin use, Liver function, recent transfusion, severity): _____
    - Protein S deficiency? Y / N / UT
      - Protein S Activity (closest to1st sample): _____

- Protein S Total Antigen (closest to 1st sample): _____
- Protein S Free Antigen (closest to 1st sample): _____
- Details (PS trends if done multiple times, Coumadin use, Liver function, recent transfusion, severity): _____

➢ Elevated Factor VIII? Y / N / UT
- Factor VIII Level (closest to 1st sample): _____
- Course: Transient / Persistent
- Details (Factor VIII trends if done multiple times): _____

➢ Elevated Lipoprotein(a)? Y / N / UT
- Lipoprotein(a) (closest to 1st sample): _____
- Details (other lipid problems): _____

- Anti-phospholipid antibody testing? Y / N / UT
  - ➢ Meets syndrome criteria? Y / N
    - Lag from diagnosis if VTE present (months): _____
  - ➢ Presence of other autoimmune disease? Y / N
  - ➢ Anti-cardiolipin +? Y / N
  - ➢ Anti-Beta2Glycoprotein1 +? Y / N
  - ➢ Lupus anti-coagulant +? Y / N
  - ➢ Details (age at diagnosis of APAS, other autoimmune disease, trends in APA treatment):

    _____

- Sickle cell disease? Y / N
  - ➢ History of serious complications (recurrent ACS or strokes): Y/N
  - ➢ Hydroxyurea: Y/N
  - ➢ Chronic transfusions: Y/N
  - ➢ Details (genotype, complications, frequency of admission):

    _____

    _____

- Polycythemia? Y / N
  - ➢ Hb level: _____
  - ➢ Hct level: _____
  - ➢ Details (age, cardiac disease, transfusions, treatment, course):

    _____

- Past medical history of VTE or stroke? Y / N
  - ➢ Number of previous thrombotic events: _____
  - ➢ If VTE present, is this a recurrence of same location? Y / N
  - ➢ If VTE present, Lag from last VTE/Stroke/MI (month): _____
  - ➢ Was subject on any anti-coagulant treatment/prevention at recurrence? Y / N
  - ➢ Details (Type of previous VTE/Stroke/MI, age of occurrence of these events, any additional reversible risk factors, treatment modalities [including

duration, dosing, lab goal and overall compliance], prevention modalities, any long term complications including bleeding side effects):

_____
_____

Family history of Thrombosis? Y / N

Include type, location, age, treatment [acute & chronic], complications for each relative & each event → preferably in 1st & 2nd degree relative:

- FH of VTE (DVT or PE):

  _____
- FH of stroke < 50 yr:

  _____
- FH of MI < 50 yr: _____
- FH of > or = 3 miscarriages: _____

Current admission:

- Location at initial admission: _____
- If PICU/CVICU, duration admission to the unit: _____
- Intubation? Y / N
- Use of vaso-active medication to maintain blood pressure? Y / N
- All Vascular access (PIV, CVL, art line, etc …) during study periods and duration of each:

  _____
- TPN needed and for how long? _____
- Diagnosis at admission (may mentioned presenting symptoms such as fever):

  _____
- Surgical procedures during admission:

  _____
- Overall pertinent clinical factors in course and treatment:

  _____

VTE-related Data (if applicable):

- S & S at present: _____
- Duration of symptoms prior to Dx (days): _____
- Pre-diagnoses established VTE risk factors (clinical & laboratory):

  _____
- Dehydration? Y / N
- Type of imaging used to confirm Dx & other images (dopplers & serial images – dates):

  _____
- Location (include vessels & superficial vs. deep if limb):

  _____
- Occlusive or non-occlusive at Dx ? O / Non-O
- Date of Dx & duration from admission and/or CVL placement:

  _____

- Treatment plan:
  - If no anti-coagulation, why (contraindication, mild S & S, line removed)?
    _____
  - If anti-coagulated, with which medication (including bridging)?
    _____
  - How long until therapeutic for each medication?
    _____
  - Adverse events (especially bleeding)?
    _____
  - Treatment outcome (total duration, Complete resolution?, Evidence for chronic clot and after how long, presence of collaterals, date of stopping treatment)
    _____
  - Use of secondary prevention? Y / N
  - If secondary prevention, mention persistent risk factors, type of treatment and duration: _____

- Any complication related to VTE (ex. post-thrombotic syndrome, PE if DVT)?
  _____

CVL insertion-related Data (if applicable):

- Number, location, duration and age of previous CVL:
  _____
- Hx of CVL-related VTE & details as above:
  _____
- Location of insertion: _____
- Number of attempt of insertion and their locations: _____
- Size of needle used: _____
- Vessel(s) where CVL is located: _____
- Caliber of vessel where CVL is located (if available): _____
- Type and size of CVL: _____
- Brand of CVL: _____
- Number of lumens: _____
- Length of CVL inside patient: _____
- Location of tip on X-ray: _____

- Any complications during insertion (ex. bleeding, pneumothorax)?
  _____
- Is prophylaxis used at time of sample(s) (1^{st}, 2^{nd}, 3^{rd})?
  _____
- If prophylaxis used, mode of delivery, dose, overall frequency and time from sample draw:
  _____
- Is VTE consult done & was SCD used/recommended?
  _____

- Was the CVL non-functional at any time? If yes, mention day post-insertion and intervention taken (ex. tPA)?

  _____

- Any line-associated infections? Details:

  _____

- Did a line-associated VTE occur? (If yes, documents details as per VTE data collection outlines above): Y / N

Laboratory evaluation:

- CBC closest to 1st, 2nd, 3rd samples (# days: prior is – & post is +):

  _____

  - WBC count/ANC/ALC): _____
  - Hemoglobin/MCV: _____
  - Platelet count (include trend): _____

- CMP closest to 1st, 2nd, 3rd samples (# days: prior is – & post is +):

  _____

  - BUN/Creat: _____
  - ALT/AST: _____
  - Albumin (include trend): _____

- DIC closest to 1st, 2nd, 3rd samples (# days: prior is – & post is +):

  _____

  - PT/INR (include trend if on Coumadin): _____
  - PTT (include trend if on UFH/other): _____
  - Fibrinogen (include trend): _____
  - D-dimers (include trend): _____

- CRP closest to 1st, 2nd, 3rd samples (# days: prior is – & post is +) & include trends:

  _____

- ESR closest to 1st, 2nd, 3rd samples (# days: prior is – & post is +) & include trends:

  _____

- Anti-Xa closest to 1st, 2nd, 3rd samples (# days: prior is – & post is +) & include trends:

  _____