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Global coagulation assays – Proposed reference intervals for healthy controls

Reference intervals for global coagulation assays

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Dear Editors,

Global coagulation assays (GCA) have been proposed as better surrogate measures of the haemostatic profile compared to traditional coagulation studies[1]. Traditional coagulation assays such as prothrombin time (PT) and activated partial thromboplastin time (APTT) measure the time to the start of clot formation, representing less than 5% of total clot formation[1]. Furthermore, they were developed to measure anticoagulants, with their role in assessing bleeding and thrombotic risk is unclear. GCA, on the other hand, measure the end products of the coagulation cascade such as thrombin and fibrin, and provide a more comprehensive assessment of the haemostatic profile. These assays have been shown to be potentially more useful in assessing for bleeding and thrombotic disorders[1]. One of the challenges of any new assay is understanding what is normal, the variations seen with standard epidemiology factors of age and gender, and developing a reference range for these parameters. We have previously reported significant age and gender differences in thromboelastography (TEG®) and calibrated automated thrombogram (CAT®) parameters in the normal population[2,3]. Since our previous report, we have expanded our cohort of healthy controls and here, we report the reference intervals for TEG, CAT and a fibrin generation assay, overall haemostatic potential (OHP).

Healthy volunteers were recruited and written informed consent obtained. Volunteers with history of thrombosis, active cardiovascular risk factors on treatment, such as hypertension, diabetes and hyperlipidaemia, as well as those on hormonal therapies, anticoagulation or antiplatelet agents were excluded. All volunteers also underwent testing with full blood count, renal and liver function and coagulation studies and were excluded if the results were abnormal. Samples were collected via venepuncture from the cubital fossa using 21G vacutainer or butterfly collection set. Whole blood collected in citrated tube was used for immediate testing with TEG® within 4 hours and additional samples were double-spun at 2500g to obtain platelet-poor plasma (PPP). The PPP was immediately stored at -80°C and tested at a later time for CAT and OHP. The study was approved by the Austin and Northern Health Human Research Ethics Committees (H2013/04977 and P5/13).

Thromboelastography measures the changes in the viscoelastic properties of whole blood during fibrin clot formation, propagation and dissolution and was performed using TEG® 5000

(Haemonetics, USA) as per manufacturer's guidelines. 1000µL citrated blood was mixed with 40µL kaolin. 340µL of this mix was added to a cup preheated to 37°C, containing 20µL of 0.2M Calcium chloride. The cup oscillates around a suspended pin. As the fibrin clot forms, the torque on the wire is recorded. CAT® (Diagnostica Stago, France) determines the rate and extent of thrombin formed over 60 minutes after a tissue factor stimulus. 80µL of PPP was added to either 20µL of PPP reagent or 20µL of thrombin calibrator. The final mixture has a concentration of 5pM tissue factor; 4µM phospholipids. Coagulation is triggered with the addition of calcium chloride in a buffer with fluorogenic substrate. OHP is derived from a fibrin aggregation curve formed from repeated spectrophotometric measurements measured on the FLUOstar Optima (BMG Labtech) at 405nM. 75µL of thawed PPP was added to wells with 75µL of buffer containing either (i) thrombin (0.006IU/mL) to generate the overall coagulation potential (OCP) or (ii) thrombin and tissue plasminogen activator (tPA) (600ng/mL) to generate the OHP. Figure 1 illustrates the key parameters as measured using TEG®, CAT® and OHP.

Statistical analysis: Reference intervals were calculated using the non-parametric approach based on the recommendations of the International Federation of Clinical Chemistry and Laboratory Medicine[4]. Screening for outlier exclusion was undertaken using the Reed test[5] or the Tukey test[6]. Sex-dependent differences were explored using a Mann-Whitney U test. Age-dependent differences were assessed using analysis of variance[7]. Where differences were found for a given analyte, sex partitioned reference intervals were calculated. As described in the Clinical and Laboratory Standards Institute (CLSI) Guidelines C28-A3[4], the non-parametric rank method was used to calculate the reference interval for partitions with a sample size ≥120 participants. For analytes with partitions containing <120 participants, the robust method of Horn and Pesce was used to calculate the reference interval[4]. The data are presented as 2.5-97.5th percentile ranges (two-sided) with 90th percentile confidence intervals. The MedCalc software was used for statistical analysis.

A total of 153 healthy volunteers, aged 18 to 80 years (mean age 42±17 years, 98 females), were prospectively recruited. Table1 shows gender and age-specific reference intervals for TEG®, CAT® and OHP. For TEG®, the manufacturer's reference intervals were R-time, 2-8 min; K-time, 1-3 min; α-angle, 55-78^{o}; maximum amplitude (MA), 51-69 mm; LY30 0-8%. Age did not appear to influence TEG® parameters while gender did. Figure 2 shows the age and gender differences in some of the key GCA parameters. Compared to the manufacturer's reference intervals, 25 (20.3%) of healthy controls were outside of the reference interval for R-time, 19 (15.4%) for K-time, 47 (38.2%) for α-angle, 18 (14.6%) for MA and 4 (3.2%) for LY30. Only 58 (47.2%) controls had all parameters within the manufacturer's reference intervals. This

discordance is consistent to Scarpelini et al which reported a specificity of 81% for the manufacturer's "normal "values[8]. Of note, the manufacturer's reference interval was established using small studies of volunteers or hospitalised patients, which may limit its generalisability.

To the best of our knowledge, there are no manufacturer's reference interval for CAT. Previously published reference intervals for CAT either did not delineate age and gender or used a different tissue factor concentration[9,10]. The results from the Gutenberg Health study involving over 1000 healthy controls and included results derived from 5pM tissue factor, reported a median of 2.7 (interquartile range (IQR) 2.3, 3.0) minutes for lag time, a mean of 1322±196 for endogenous thrombin potential (ETP) and 236±52 for peak height for males, and 2.4 minutes (IQR 2.3, 2.7) for lag time, 1318±212 nM.min for ETP and 259±53 nM for peak height for females[10]. Interestingly, the ETP was not significantly different between genders in the Gutenberg Health Study ($p=0.71$) while the female controls in our study was more hypercoagulable than the males (1391±263 vs 1236±219 nM.min, $p<0.001$). Both studies reported higher peak height in females (our study 236±68 vs 190±54 nM, $p<0.001$) even after adjusting for age[3,10]. While the lag time was longer in the male controls in the Gutenberg Health Study ($p<0.001$)[10], the difference was not significant in our study (3.0 (2.7, 3.7) vs 3.3 (3.0, 3.7) min, $p=0.097$). No age group specific ranges were reported in the Gutenberg Health Study[10].

There are no published reference intervals for the OHP assay and previous studies have utilised different reagent concentrations[11]. In our study, we found significant age and gender differences in the OHP parameters. Females appear more hypercoagulable than males (Overall coagulation potential, OCP 38.0±8.9 units vs 20.9±9.5 units ($p<0.001$); OHP 7.9±3.1 vs 5.9±3.4 units ($p<0.001$); and overall fibrinolytic potential, OFP 79.3±6.2 vs 81.5±6.6 % ($p=0.020$). The older controls also demonstrated higher OCP (39.9±9.1 vs 32.6±9.0 units, $p<0.001$) and OHP (9.0±3.6 vs 6.1±2.6 units, $p<0.001$) values and reduced OFP (77.4±7.6 vs 81.7±4.8 %, $p<0.001$).

The proposed reference intervals derived from this study represents an adult population within Australia's metropolitan area with diverse ethnicity background (56% Caucasians, 35% South Asians and 9% South Asians). Importantly, and unique to many normal control studies, our volunteers have been specifically screened for pre-existing cardiovascular and prothrombotic risk factors. This study confirms the gender differences seen in some of the parameters, which are not quantified by the currently published TEG® reference intervals or other studies involving CAT® using this specific study protocol. Statistically significant age-dependent differences were found for lag time, OCP, OHP and OFP. However, combined with the

uncertainty in the partitioned reference intervals and for standardisation, an arbitrary 50 years old cut-off was considered appropriate for these analytes. In addition, we note there is some differences across the different ethnic group, particularly with the South Asian group – the MA and ETP are significantly elevated in this group compared to the rest of the group but we note that there are only 13 subjects in this group which limits how meaningful and applicable this finding may be.

To the best of our knowledge, this is also a first-in-kind study looking at reference intervals in the OHP assay, a fibrin generation assay. Given the discordance of our results to reference intervals provided by the TEG® manufacturer, many otherwise well healthy individuals in our population would have been identified as "abnormal" supporting the recommendations by the manufacturer to establish each institution's local ranges. The clinical implications of these differences were not explored in this study but given that TEG® is a point-of-care device used to support transfusion in bleeding patients, it is important to raise awareness of these discrepancies to avoid potentially unnecessarily transfusion. We acknowledge that our locally determined reference intervals, however, may not be directly transferable in laboratories serving different populations or using different study protocol and trigger reagents. Laboratories should embark on establishing local reference intervals where possible, however, our reference intervals may be useful for transference checking[12].

In conclusion, this letter reports reference intervals for three commonly used global coagulation assays, TEG®, CAT® and OHP assays based on 153 healthy volunteers of varying age, gender and ethnic background. It is important to note that the applicability and generalisability of these reference intervals are subject to the study protocol and local population characteristics. Nevertheless, our reported reference intervals will add to the literature and understanding of global coagulation assays.

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Table and figure legends

Table 1 Proposed reference intervals for the global coagulation assay parameters

Figure 1 shows the traces of the global coagulation assays. Fig 1A represents the TEG® trace which includes parameters such as R-time (minutes) = reaction time, time from start of test to initial clot formation; K-time (minutes) = time taken to achieve a certain level of clot strength; α-angle (^{0}) = rate of clot formation; maximum amplitude (MA, mm) = strength of fibrin clot; LY30 (%) = percentage of decrease in amplitude at 30 minutes after MA (degree of fibrinolysis). Fig 2B represents the CAT curve which includes parameters such as lag time (min) = time required to reach one-sixth of the peak height; peak height (nM); endogenous thrombin potential (ETP, nM.min) = area under the curve or amount of thrombin formation; velocity index (nM/min) = maximum slope of the initial part of the curve. Fig 1C illustrates the

OHP trace which includes overall coagulation potential (OCP, units) derived from the addition of thrombin only; overall haemostatic potential (OHP, units) derived from the addition of thrombin and tissue plasminogen activator, and overall fibrinolytic potential (OFP, %) which is calculated using ((OCP-OHP/OCP) x 100%).

Figure 2 Boxplots showing age and gender differences in the key parameters for TEG®, CAT® and OHP. (The box component indicates the 25th and 75th percentiles (interquartile range) with the median indicated by the line within the box. The whiskers indicate the upper and lower adjacent values and extreme values outside these adjacent values are indicated by dots.)

Table 1 Proposed reference intervals for the global coagulation assay parameters

	Manufacturers' reference intervals	Age group (years)	Male			Female		
			Reference intervals	Lower limit 95% CI	Upper limit 95% CI	**Reference intervals**	Lower limit 95% CI	Upper limit 95% CI
Number of volunteers			**55**			**97**		
APTT	25.0-38.0	20-<80	**22.1-36.6**	20.8-23.5	35.0-38.1	**21.0-34.2**	20.0-22.0	33.0-35.3
PT (sec)	11.0-17.0	20-<80	**10.3-15.3**	9.8-10.8	14.8-15.7	**9.3-11.9**	9.1-9.6	11.7-12.2
Thromboelastography (TEG®) (n=123)								
Number of volunteers			**43**			**75**		
R time (min)	2.0-8.0	20-<80	**2.3-11.0**	2.6-3.8	12.1-17.4	**2.9-9.3**	2.4-3.5	8.7-9.8
K time (min)	1.0-3.0		**1.3-3.8**	1.0-1.5	3.5-4.0	**0.7-3.2**	0.4-0.9	2.9-3.5
a-angle (deg)	55.0-78.0		**36.1-75.2**	31.5-41.2	69.8-79.3	**36.5-83.3**	31.9-41.7	79.5-86.3
Maximum amplitude (mm)	51.0-69.0		**46.7-69.2**	44.0-49.7	66.9-71.6	**49.2-74.2**	47.1-51.5	71.6-76.6
LY 30 (%)	0.0-8.0		**0.0-4.9**	0	3.6-6.1	**0.0-8.6**	0	6.7-16.7
Calibrated automated thrombogram (CAT) (n=153)								
Number of volunteers			**55**			**98**		
Lag time (min)	Not available	20-<50	**1.8-4.2***				1.7-2.0	4.0-4.4
		50-<80	**1.7-5.3***				1.3-2.2	4.8-5.7
ETP (nM.min)		20-<80	**789-1678**	708-878	1580-1766	**861-1912**	787-932	1833-1985

Velocity Index (nM/min)			**16.2-147**	13.1-20.8	120.8-177.7	**24.6-215.6**	20.7-30.0	184.7-248.6
Thrombin Peak (nM)			**80-299**	62-100	278-318	**101-373**	82-121	355-391
Overall haemostatic potential assay (OHP) (n=144)								
Number of volunteers			**33**			**54**		
OCP (units)	Not available	20-<50	**9.0-43.2**	4.8-14.1	37.3-47.6	**18.0-51.9**	14.1-21.9	47.4-55.9
OHP (units)			**1.7-11.2**	1.3-2.1	8.6-14.2	**3.1-13.0**	2.8-3.6	11.4-14.8
OFP (%)			**75.3-92.3**	72.7-77.9	90.0-94.7	**73.6-90.1**	71.8-75.5	88.3-91.8
Number of volunteers			**18**			**39**		
OCP (units)		50->80	**20.2-55.7**	14.4-25.5	49.7-60.2	**21.2-61.2**	16.8-27.0	56.4-65.3
OHP (units)			**3.5-13.9**	2.9-4.4	10.6-16.5	**4.0-20.9**	3.3-5.2	17.4-24.8
OFP (%)			**73.1-88.2**	70.6-75.6	85.3-90.3	**64.1-92.3**	59.9-68.9	87.5-96.5

* Asterisk indicates common reference interval for both male and female; CI = confidence interval; APTT = activated partial thromboplastin time; PT = prothrombin time; ETP = endogenous thrombin potential; OCP = overall coagulation potential; OHP = overall haemostatic potential; OFP = overall fibrinolytic potential