The implementation of a high-throughput sequencing test for diagnosing inherited bleeding, thrombotic, and platelet disorders: lessons learned from an international study.

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**Objective:** Inherited bleeding, thrombotic, and platelet disorders (BPD) are heterogeneous and a molecular analysis for patients with a BPD is often unavailable. The ThromboGenomics is a platform that provides a DNA-based test to diagnose patients suspected of having an inherited BPD.

**Methods:** A high-throughput sequencing platform was designed targeting 63 genes relevant for BPDs. Sequencing data were analyzed in combination with an analysis approach based on using the Human Phenotype Ontology (HPO) terms for coding phenotype. 300 samples from 13 different countries were enrolled in this study from i) patients with known pathogenic variants (known-group, n=159), ii) patients with phenotypes that strongly indicate a particular disorder (suspected-group, n=61), iii) patients having clinical bleeding or thrombotic problems but normal laboratory coagulation and platelet function tests (uncertain-group, n=76) and iv) 4 samples from unaffected relatives.

**Results:** All previously determined variants in the know-group were detected (empirical sensitivity 100%). In 56 out of 61 cases from the suspected-group pathogenic (n=29) or likely-pathogenic (n=28) variants that fully or partially explain the disease phenotype were identified (sensitivity 91.8%). In the uncertain-group, genetic defects were found only in 8 out of 76 cases corresponding to a sensitivity of 10.5%. Interestingly, negative Sanger sequencing results were overturned in 3 cases in the suspected- and uncertain-group, respectively, demonstrating that this platform can outperform Sanger sequencing in terms of sensitivity.

**Conclusion:** These results indicate that the ThromboGenomics platform has an excellent sensitivity to detect known causal variants in known BPD genes. However, the low rate of identified genetic variants in patients with uncertain etiology demonstrates the need for further research into the molecular etiology of uncharacterized BPDs. The BRIDGE-BPD is a research platform which will help identifying novel variants not in the known Tier 1 genes.
Diagnosing heparin-induced thrombocytopenia (HIT) in 30 minutes: A prospective evaluation of a rapid diagnostic work-up.

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**Laboratory**
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**Objective:** We assessed 3 immunoassays (IAs) detecting anti-PF4/heparin antibodies for their ability to predict the result of the functional gold standard Heparin Induced Platelet Aggregation Test (HIPA). Our aim was to prospectively evaluate a rapid algorithm able to confirm or exclude HIT within 30 minutes.

**Methods:** We conducted a retrospective (05.2014 – 08.2015; n=220) and a prospective (09.2015 – 08.2016; n=234) evaluation study in patients with clinical HIT suspicion. Plasma samples were analyzed by 3 IAs (ELISA Zymutest HIA monostrip IgG, AcuStar HIT IgG and ID-H/PF4-PaGIA). Employing ROC analysis we compared areas under the curve (AUC) and determined cut-offs with 100% negative (NPV) and positive (PPV) predictive values for a positive HIPA.

**Results:** HIT diagnosis could be proven by positive HIPA in 10% (22 patients) in the retrospective and in 9.4% (22 patients) in the prospective study. AcuStar showed an AUC of 0.98 both in the retrospective (p=0.02 compared to Zymutest; p=0.59 to PaGIA) and in the prospective study (p=0.06 to Zymutest; p=0.52 to PaGla). A 100% PPV was observed with a result >1.37 U/ml (identifying 18/22 of HIT cases) and >0.77 U/ml (20/22 of HIT cases), respectively. A 100% NPV was observed with a result ≤0.12 U/ml (excluding 18/24 of HIPA-neg cases) and ≤0.18U/ml (excluding 200/212 of non-HIT cases). PaGIA showed AUC of 0.99 and 0.97. In both studies a titer ≥16 had a 100% PPV (identifying 28/44 of HIT cases) and a titer ≤1 had a 100% NPV (excluding 210/244 of non-HIT cases). In the prospective study, applying the cut-off values <0.12 U/ml = HIT-neg and >1.00 U/ml = HIT-pos, AcuStar excluded HIT in 192/234 (82%) and diagnosed it in 18/234 (8%) patients, recognizing 18/22 of HIT cases. 24 out of 234 results (10%) were in the AcuStar “grey zone” (0.12 – 1.00). Among them, 12 were correctly solved by PaGIA (HIT excluded by titer ≤1) while 12 (5% of the total) remained unclear until HIPA was performed. There were no false positive or negative results.

**Conclusion:** The sequential application of two rapid immunoassays (AcuStar HIT IgG and ID-H/PF4-PaGIA) with in-house cut-off values with 100% NPV and PPV enables a reliable and conclusive diagnostic work-up for 95% of patients with clinical suspicion of HIT, with a laboratory turn-around-time of 30 minutes. We are now undertaking a prospective validation of this rapid diagnostic algorithm.
Multianalyte determination of NOAC using LC-MS/MS and comparison with functional coagulation assays

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**Objective:** New oral anticoagulants, also called NOAC, are direct inhibitors of coagulation factors which meant improvement in anticoagulation therapy. Currently, three NOAC are used in anticoagulation therapy: dabigatran, direct thrombin inhibitor and rivaroxaban and apixaban, direct factor Xa inhibitors. Even though routine monitoring of NOAC is not needed, there are certain situations in which it is necessary to know the plasma level of anticoagulants, eventually their coagulation effect.

Monitoring of NOACs can be addressed by measurement of anticoagulant activities, measurements of trombin production or by quantification of drug levels. Compared to functional tests such as trombin generation for all NOACs, calibrated chromogenic anti-Xa assays for rivaroxaban and apixaban or a calibrated diluted thrombin time assay for dabigatran, liquid chromatography-coupled tandem mass spectrometry (LC-MS/MS) remains the gold standard in quantitative drug analysis.

**Methods:** This study was conducted on a set of blood samples from 116 patients with therapy by NOACs. Our LC-MS/MS method enables simultaneous determination of apixaban, dabigatran and rivaroxaban. The aim of our study was comparison of this method with functional tests for determination of NOACs as aPTT, dTT assay, PT, anti-Xa activity assay and TGA assay.

**Results:** The results of dabigatran levels showed statistically significant relationship between LC-MS/MS and aPTT (r = 0.82; p < 0.001), dTT (Hyphen: r = 0.817, p < 0.001, HemosIL: r = 0.819, p < 0.001) and one TGA parameter - tLag (r = 0.44, p = 0.0045). Correlation of remaining TGA parameters (Peak, AUC) was not significant as their initial values show high variability.

The results of rivaroxaban shows better relationship between LC-MS/MS and anti-Xa assay (r=0.7025) than TGA. TGA assay did not show statistically significant relationship of its parameters (tLag, tPeak, Peak, AUC) and rivaroxaban concentration in plasma.

**Conclusion:** The results of all methods for dabigatran determination were compared using HPLC-MS as reference. dTT HemosIL showed better results for low concentrations when compared to HPLC-MS than dTT Hyphen as HemosIL uses non-linear calibration curve and therefore gives more accurate results.

The determination of rivaroxaban and apixaban by Anti-Xa assay shows better results than TGA.

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Fibrin clot formation properties in patients on treatment with oral anticoagulants correlate with drug concentration and intensity of anticoagulation

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Objective: Vitamin-K-antagonists (VKA) and direct oral anticoagulants (DOAC) are treatment options for stroke prevention in atrial fibrillation (AF), but there is no global coagulation assay that equally quantifies their anticoagulant effects. Therefore, we investigated the in-vitro properties of fibrin clot formation in AF patients on oral anticoagulation and analyzed the correlation with the INR in patients on VKA and drug concentration in patients on DOACs, respectively.

Methods: Patients with AF on treatment with anticoagulation were recruited into a prospective registry and blood samples were taken in trough or peak levels. DOAC plasma concentrations were measured on a liquid chromatography tandem mass spectrometry platform. Fibrin clot turbidity was read on a photometer after addition of tissue factor (2 pmol/L), phospholipids (4 μmol/L), and CaCl₂ (20 mmol/L) to citrate plasma. Clot formation lag-phase corresponds to the time to clot formation onset, maximum rate describes the maximum increase in clot formation over time, and peak clot turbidity corresponds to the maximum density of the clot.

Results: Out of 349 patients, 163 patients (46.7%) were receiving VKA with a median INR of 2.0 (25th to 75th percentile 1.8–2.4), 31 patients (8.9%) were on dabigatran with a median plasma concentration of 140 ng/ml (63.3–290), 82 (23.5%) on rivaroxaban with a median concentration of 111.0 ng/ml (33.6–204.75), 22 (6.3%) on apixaban with a median concentration of 179.5 ng/ml (102.4–268.0 ng/ml), and 26 control AF patients were not anticoagulated.

DOAC plasma concentrations as well as INR levels in VKA patients correlated positively and strongly with fibrin clot formation lag-phase (r=0.63 p<0.001 and 0.61 p<0.001, respectively DOAC and VKA), negatively and moderately with the maximum rate of clot formation (r= -0.481 p<0.001 and r=-0.32 p<0.001), and negatively, weakly with peak clot turbidity (r= -0.33 p<0.001 and r=-0.15 p=0.06). DOAC samples in trough levels had significantly longer lag-phases (Mann-Whitney-U p<0.001) and lower clot formation rates (p=0.04), compared to patients in target INR range on VKA treatment (table).

Conclusion: Fibrin clot properties correlate to both DOAC plasma concentrations and INR levels in VKA treatment, thus equally reflecting their anticoagulant effects. The fibrin clot formation assay may allow an in-vitro comparison between the anticoagulant effects of DOACs and VKA.
Spatial fibrin clot formation is less affected by clotting factor deficiency than thrombin generation

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Objective: Thrombodynamics (TD) and Calibrated Automated Thrombography (CAT) are two methods frequently used in basic and translational research. Both assays differ from standard coagulation methods because they detect continuing enzymatic action after onset of clot formation, thus providing better insight into subtle changes of the haemostatic balance. In contrast to CAT, TD allows to differentiate between ‘initiation phase’ and ‘propagation phase’ as coagulation is activated by immobilized tissue factor (TF). This study aimed to assess the sensitivity of TD to deficiency of several clotting factors in comparison with CAT.

Methods: TD assays were performed with varying concentrations of (F) II, V, VII, VIII, IX, X and XI, using respective factor-deficient plasma samples in fractions of 0 to 100%. TD parameters (lag time, initial speed, average speed, clot size) were compared with CAT parameters [lag time, endogenous thrombin potential (ETP), peak, and time-to-peak (ttPeak)].

Results: Lag time, initial speed, average speed, and clot size determined by TD exhibited a reliable dependency on concentrations of all tested clotting factors. Although substantially reduced, clot development and propagation were still detected at 0% FVIII, FIX or FXI. Clot propagation declined exponentially at FII concentrations below 4% as well as at FV and FX concentrations below 1%. FVIII levels below 10% resulted in thrombin generation traces too low for evaluation (ETP not calculable), while TD parameters were less affected. TD parameters exhibited lower sensitivity but higher consistency than CAT parameters in detecting coagulation factor deficiencies.

Conclusion: The TD assay shows that reduced but persistent clot formation occurs even at complete deficiency of FVII, FVIII, FIX, or FXI. This can be explained by the compensatory effects of extrinsic and intrinsic components perpetuating clot formation even in conditions where thrombin generation is largely abrogated. Very small amounts of thrombin (>4%) were required to maintain stable clot propagation when tissue factor was immobilized on a surface. TD parameters reflect in vivo conditions of hemophiliacs exhibiting a significantly higher risk of bleeding at FVIII levels <5% better than CAT parameters due to higher sensitivity towards very low FVIII levels.
Catching microparticles - a methodical comparison

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**Objective:** The field of microparticle (MP) research is constantly expanding. In 2000 there were 155 publications about MPs, in 2015 pubmed lists 1174. The increasing number of research groups calls for standardization of MP isolation, to ensure comparability and transparency of results. In 2013 Lacroix R. et al published a protocol for standardization of sample preparation for flow cytometric analysis of microparticles (J Thromb Haemost 10.1111/jth.12207). We prepared our samples according to this protocol. For further isolation and accumulation of microparticles there is no common consensus. The majority of work groups uses high-speed- or ultra-centrifugation.

In this study we compare two different methods for MP-accumulation:

a) high-speed centrifugation of samples at 10.000g for 90 minutes (2009_Muralidharan-Chari);

b) capture with Annexin-V-coated magnetic beads (Gieseler et al. Cell Biol Int. 2013).

**Methods:** We analyzed patient derived effusion obtained from the University Hospital Schleswig-Holstein (UKSH, Germany).

For depletion of larger cell debris we centrifuged our samples twice at 2.500g for 15 minutes and discarded the pellet, thus obtaining low speed centrifuged samples ("lsEV"). Afterwards we divided our samples, one part was centrifuged again at 10.000g for 90 minutes obtaining high-speed EVs ("hsEV"), the other part was processed according to our previously published capture method wheer annexin-V coupled magnetic beads are used, obtaining captured EVs ("capEV"). Afterwards, we characterized the samples by high-resolution cytoflow as well as various ELISA based assays, and tested their cellular effects such as the activation of the PAR2/ G protein/ ERK signaling pathway and the induction of tumor cell migration.

**Results:** Soluble factors such as growth factors or cytokines, which might interfere in functional assays, are depleted by both assays. As compared to the ISTH protocol, both methods resulted in a depletion of small EVs such as exosomes. capEVs had higher tenase activity and they were more potent inducers of tumor cell migration than hsEV.

**Conclusion:** EV-capture by anexin-V coupled magnetic beads selects phosphatidylserine (PS) positive EVs, whereas high speed centrifugation includes all EVs, therefore the combination of the methods might be useful to determine different subtypes of EVs with different cellular effects.