POSSIBILITIES FOR LABORATORY EXAMINATIONS OF CONGENITAL AND ACQUIRED THROMBOPHILIA

Ph.D. thesis

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ABSTRACT

Thrombophilia can be defined as an increased, persistent tendency to hypercoagulability and venous thrombosis. The laboratory examination of thrombophilia is a costly and time-consuming process, as each defect should be separately investigated such as deficiencies of antithrombin (AT), protein C (PC), protein S (PS), FV Leiden mutation, prothrombin gene mutation. A single laboratory test for detecting multifactorial thrombophilia would be useful. It would be desirable both for patients and doctors to have a reliable global coagulation test that can differentiate patients who have or do not have hereditary thrombophilia. The Coagulation Inhibitor Potential (CIP) is a promising new global test.

In the first part of the study, our aim was to adapt the manual application of the original CIP method to an optical coagulation analyser, to examine reproducibility and reliability of the CIP method and to measure the samples of the healthy subjects and the patients with hereditary thrombophilia. According to our results, CIP was found to be a suitable and reliable test to differentiate between the healthy subjects and the patients with thrombophilia. It could be possible that only those patients need to be further tested with special thrombophilia tests who have positive CIP results. By automation the CIP test would be easier, faster and more precise allowing the performance of simultaneous analyses of samples. The possible random errors of the manual laboratory activity could also be eliminated.

In the second part of the study, we examined blood coagulation tests (prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), fibrinogen and D-dimer) of samples from uncomplicated pregnancies at gestational weeks 16, 26 and 36. In addition the CIP method was used on this samples. Our results confirm that pregnancy is associated with a progressively increasing hypercoagulable state and risk factors for venous thromboembolism. We established specific reference intervals and cut-off values for these conventional routine blood coagulation parameters for gestational weeks 16, 26 and 36. Our results could support appropriate clinical decision making.
INTRODUCTION

Haemostasis is the consequence of balanced interactions of cellular and molecular components responsible for the maintenance of an intact circulation. Dysfunction of cells or proteins participating in the haemostasis system may result in thrombophilia or a haemorrhagic diathesis.

The formation of blood clots within the cardiovascular system results in a thrombus. The clinical disease is called thrombosis. When the thrombus or part of the thrombus splits and gets stuck in a farther part of the blood vessels, it is called embolisation. Depending on the location of its development it can be arterial or venous thrombosis.

Deep vein thrombosis (DVT) and pulmonary embolism (PE) represent different manifestations of the same clinical entity, which is referred to as venous thromboembolism (VTE). VTE has a relatively high prevalence in Western populations. The incidence rate of a first VTE is 1-2 events per 1000 patient per years, and VTE also has a considerable fatality-rate (3-25%). VTE recurs frequently, about 30% of patients develop recurrence within the following 10 years.

Venous thromboembolism is now recognized as a complex, multifactorial disease, involving both environmental exposures (e.g. clinical risk factors) and both genetic and environmental interactions. In order to improve survival, avoid recurrence and prevent complications, the occurrence of VTE must be reduced. To reduce VTE incidence, persons at risk for VTE must first be identified. Thrombophilia (the tendency to develop thrombosis) can be inherited, acquired, or both.

Hereditary thrombophilia is a genetically determined predisposition primarily for VTE diseases. Hypercoagulability state, even without the known, acquired risk factors, in itself can generate VTE diseases. The homozygous mutations, and combinations of genetic deficiencies could also lead to thromboembolic diseases. Environmental risk factors are also often present, which may contribute to the manifestation of thromboembolic events. Hereditary thrombophilia should be supposed to be the cause of VTE if it occurs in young age (under 45 years), if the thrombosis is in unusual localisation (not lower limbs), or in case of recurrent thromboses in the family.
Hereditary thrombophilia risk factors for VTE: AT-, PC-, PS deficiencies, FV Leiden mutation (FV:Q506), prothrombin gene mutation (FII G20210A), certain types of dysfibrinogenemia and elevated FVIII.

The most important acquired risk factors for VTE: malignant diseases, heart failure, nephrosis syndrome, paroxysmal nocturnal haemoglobinuria, varicose veins, antiphospholipid syndrome, surgeries, immobilisation, hyperhomocysteinaemia (of vitamin deficiency), pregnancy, puerperium, oral contraceptive therapy, hyperviscosity syndromes: myeloproliferative neoplasms (i.e. polycythaemia vera, essential thrombocythaemia) and lymphoproliferative diseases (i.e. multiple myeloma, Waldenström’s macroglobulinaemia), trauma, obesity, increasing age, previous thromboembolic event.
AIMS

I. Thrombophilia examination with Coagulation Inhibitor Potential (CIP) method

1. Adapting the original, manual CIP method to an optical coagulation analyser.
2. Examining the test performance characteristics of the automated CIP method.
3. Evaluating the CIP test with commercially available AT-, PC- and PS deficient plasma samples.
4. Measuring the samples from healthy subjects with the CIP method.
5. Collecting samples from patients with a previous VTE, or with a family member having a previous VTE as a consequence of a specific form of inherited thrombophilia. These samples were collected at the thrombophilia outpatient clinic at the 1st Department of Internal Medicine, University of Pécs.
   Examining these samples from patients with inherited thrombophilia with the CIP method and conventional specialized blood coagulation laboratory tests.
6. Comparing the results of the conventional blood coagulation laboratory tests with the results of the CIP method of the same samples from the patients with hereditary thrombophilia.
7. Evaluating the application of the CIP method.

II. Blood coagulation examination throughout uncomplicated pregnancy

1. Collecting samples from the same pregnant women at the 16th, 26th and 36th gestational weeks. Examining the routine blood coagulation tests: PT, APTT, TT, fibrinogen and D-dimer at each of these three gestational weeks.
2. Establishing gestational age specific reference intervals and cut-off values for these conventional routine blood coagulation parameters.
3. Comparing gestational age specific reference intervals with the non-pregnant, normal reference intervals for PT, APTT, fibrinogen and D-dimer.
4. Examining the samples from pregnant women at the 16th, 26th and 36th gestational weeks with the CIP method.
THROMBOPHILIA EXAMINATION WITH COAGULATION INHIBITOR POTENTIAL (CIP) METHOD

I. Introduction

The diagnosis of hereditary thrombophilia is a costly and time-consuming process, since each defect should be separately investigated. A single laboratory test for detecting multifactorial thrombophilia would be useful. It would be desirable both for patients and doctors to have a reliable global coagulation test that can differentiate patients who have or do not have hereditary thrombophilia. The Coagulation Inhibitor Potential (CIP) is a promising new global test.

Currently, no routine laboratory method exists to examine the global hypo-, or hypercoagulability state of blood. The Coagulation Inhibitor Potential (CIP) assay is a promising new global test, sensitive for most of the hereditary thrombophilias, developed for manual methodology by Abildgaard et al. We aimed to adapt the original method to an optical coagulation analyser. By automation the CIP test would be easier, faster and more precise allowing the performance of simultaneous analyses of samples. The possible random errors of the manual laboratory activity could also be eliminated.

The CIP method detects the aggregation of fibrin after the addition of tissue factor comparing two aliquots of the same sample in the absence and in the presence of Protac and pentasaccharide. Protac and pentasaccharide are added to plasma in order to enhance inhibition of coagulation by accelerating the activation of PC and the effect of AT. Protac activates PC, which then inactivates FVa and FVIIIa leading to enhanced inhibition of coagulation. In case of FV Leiden mutation (the cleavage site of FVa by the activated PC is affected by the mutation) the rate of FVa inactivation by the activated PC is slower and consequently thrombin formation carries on for a longer time and thus a hypercoagulation state develops. The pentasaccharide accelerates the AT effect, thus inhibition of blood coagulation factors (FIIa, FXa, FXIa, FXIIa, plasmin, kallikrein) regulated by AT develops faster and earlier.
II. Methods
II.1. Patients

Plasma samples were collected from 319 persons (126 healthy subjects and 193 patients with thrombophilia) after taking their informed oral and written consent. The study was approved by the local research ethics committee (approval No.6544).

The patients attended our thrombophilia outpatient clinic at the 1st Department of Internal Medicine, University of Pécs. The patients either had a previous VTE, or a family member with a previous VTE as a consequence of a specific form of inherited thrombophilia. The samples were collected after at least 3 months following VTE. The patients on oral anticoagulant therapy were treated with LMWH instead for at least 2 weeks prior to sampling.

The specific type of thrombophilia was determined by standard methods. AT-, PC-, PS activities and APC ratio were measured with an ACL blood coagulation analyser using IL reagents or kits according to the manufacturer’s instructions (Instrumentation Laboratory, Milano, Italy). We selected the samples whose AT-, PC- and PS activities were below cut off in repeated tests. FV Leiden mutation analysis was performed according to Zöller and Dahlbäck, FII G2021A mutation analysis according to Danneberg and co-workers publication.

II.2. Preparation of blood samples

Peripheral venous blood was drawn into Vacutainer tubes (Becton Dickinson) containing 1/10 volume of 0.129 M sodium citrate. The blood was centrifuged at 2000 x g at room temperature for 20 min to obtain platelet poor plasma (PPP). Plasma was frozen in aliquots and stored at −70 °C until the tests were performed (based on Clinical and Laboratory Standards Institute (CLSI) H21-A5 document).

II.3. The principle of the Coagulation Inhibitor Potential (CIP) method

The CIP assay includes two reactions: run A and run B. In the CIP assay, citrated plasma was activated by tissue factor and CaCl₂. Fibrin polymerization changed the opacity, which was recorded at 405 nm for 20 min. The AUC was calculated (run A). In run B, inhibition of activation was potentiated by adding pentasaccharide and Protac, which activated AT and
protein C, respectively. The decrease in the coagulant activity was more marked in normal plasma than in thrombophilic plasma. The inhibition was calculated as the reduction of the AUC observed in run B (with the activation of coagulation inhibition), relative to the result obtained in run A (without activation): \[(A-B)/A\] x 100, expressed in units (U). A value of 100 Units is defined as complete inhibition.

II.4. Automation of the CIP method

The CIP assay was performed using an ACL 9000 optical blood coagulation analyser (Instrumentation Laboratory, Milano, Italy) instead of the previous manual method.

First, the new reagents were defined, followed by the steps of the reaction and the volumes of reagents (µl), and finally the wavelength (nm) and time (s) of the measurement.

An aliquot of 100 µl citrated plasma was used in both run A and run B. In run A, only buffer (85 µl) was added. In run B, Protac (22 µl) and buffer (35 µl) were added 5 min prior to, and Arixtra (28 µl) immediately prior to recalcification, making their final concentrations 0.11 IU/ml and 1.4 µg/ml, respectively. The reactions were started by adding a mixture of thromboplastin (Innovin 1/9000, 0.67 pmol/l) and CaCl2 (17 mmol/l) in a volume of 15 µl, thus obtaining a total volume of 200 µl. Buffer was 66.0 mmol/l Tris and 130 mmol/l NaCl, pH 7.4.

The absorbance was measured at 405 nm with the ACL 9000 at 37 ºC every second for 1000 seconds. By plotting the absorbance against time, a curve was obtained. The AUC was calculated as the sum of the measured absorbance data. The data were evaluated by Microsoft Office Excel. The results are expressed in units (U) using \[(A-B)/A\] x 100 formula.

II.5. Statistical analysis

The results are reported as median values and interquartile ranges. The distribution of CIP values was examined by Kolmogorov-Smirnov Test. Kruskal-Wallis Test with Dunnett post hoc was used to examine the statistical differences between the thrombophilia subgroups and the healthy controls. To obtain the optimal cut-off value, ROC (Receiver Operating Characteristic) curve was analysed. All analyses were made with SPSS 24.0 (IBM Corporation NY, USA). The p-values < 0.05 were considered to be of statistical significance.
III. Results

III.1. Reproducibility of the CIP assay, within-run and day-to-day variability

Reproducibility of the CIP method was examined by measuring the within-run (intra-assay variability) and the day-to-day (inter-assay variability) precision. One healthy control and one thrombophilic patient (FVL hom) were selected and measured eighteen times within one day (within-run precision) and once a day for eighteen days (day-to-day precision). The CV<10% was considered to be appropriate, thus the reproducibility of the CIP method is acceptable.

III.2. Evaluation of the reliability of the CIP method with AT-, PC- and PS deficient plasmas

Commercially available AT-, PC- and PS deficient plasmas were used for the evaluation of reliability of the CIP method. The normal plasmas were diluted with deficient plasmas to gain samples with 50%, 55%, 60%, 65%, 70% and 75% AT-, PC- and PS activities, and subsequently the CIP method was performed in each dilution. The CIP values were 32.0 U for the 50% AT deficient plasma, 50.0 U for 50% PC deficient plasma and 42.0 U for 50% PS deficient plasma. The CIP values were above 90.0 U for plasma with 75% AT-, 70% PC- and 65% PS activity. These activity values exceed the lower limit of the AT-, PC- and PS reference intervals in our laboratory, respectively.

III.3. Patient characteristics

Plasma samples were collected from 319 persons, 126 healthy subjects and 193 patients with thrombophilia AT deficiency (n=12), PC deficiency (n=14), PS deficiency (n=4), FVL hom mutation (n=9), FVL het mutation (n=115), FII het mutation (n=8) and combined thrombophilia conditions (n=29) were examined. The patients in the combined thrombophilia group had at least two hereditary deficiencies which were combinations of inhibitor deficiencies (AT-, PC-, PS deficiency) and/or genetic abnormalities (FV Leiden-, FII mutation).

The types of thrombophilia examined were divided into two groups according to the clinical severity. 36% of the patients were high risk thrombophilia (AT 6%, PC 7%, PS 3%, FVL hom 5%, combined deficiencies 15%; within the combined group the subgroup of FVL
het + FII het comprises 4%, with subgroup was analysed separated in the result section). 64% of the patients were low risk thrombophilia (FVL het 60%, FII het 4%).

III.4. Evaluation of the CIP results of healthy subjects and thrombophilia patients

The distribution of the CIP results of healthy controls and thrombophilia patients was assessed with Kolmogorov-Smirnov Test. The distribution of data was significantly different from normal distribution (p<0.001), therefore the Kruskal-Wallis Test with Dunnett post hoc was used for the comparison of the control and thrombophilia groups. Significantly lower median CIP values were found for AT-, PC-, PS deficiencies, FVL hom, FVL het, combined thrombophilia (without the subgroup of FVL het and FII het) (p<0.01) compared to controls. The separation of the FVL het + FII het subgroup from the other combined thrombophilia (with at least one type of high risk thrombophilia) was necessary, because the CIP values of this subgroup approached the value of the controls. It happened despite the significant difference between CIP values of FVL het + FII het (p<0.01) and the controls. There was no significant difference between the FII het (p=0.669) thrombophilia group and the controls. In each high risk group the median CIP values were significantly lower than in the control group. Median CIP values were 96.7 U for controls, 25.0 U for high risk thrombophilia groups (including 38.5 U for AT-, 34.0 U for PC-, 13.5 U for PS deficiency, 16.0 U for FVL hom, 20.5 U for combined thrombophilia (except subgroup of FVL het + FII het) and 80.0 U for FVL het + FII het). This latter median value (80.0 U for FVL het + FII het) was relatively high, but was still considerably below the median value of controls (96.7 U). The median value was 74.0 U for the low risk thrombophilia group (including 72.0 U for FVL het and 95.0 U for FII het).

III.5. Sensitivity and specificity of the CIP method

Receiver operating characteristic (ROC) curves showed sensitivity and specificity of the CIP test within the different subgroups, with different cut-off values. The best performance of the test was achieved at the cut-off value of 90.0 U (area: 0.981) with 96% sensitivity and 92% specificity in the high risk thrombophilia group estimated by ROC analysis (SE=0.011; CI: 0.961-1.000). If all thrombophilia types or all except FII het were examined, sensitivity was lower (72%, 75% respectively) at a cut-off value of 90.0 U.
IV. Discussion

The CIP assay is considered to be a promising global thrombophilia screening test. This assay was performed originally manually; a pilot study suggested that the CIP assay was sensitive to high risk thrombophilia. In the present study we adapted the CIP assay to ACL 9000 optical blood coagulation analyser and measured the CIP results of known thrombophilia patients and healthy controls. We studied the analytical performance, cut-off value and usefulness of the test in the diagnosis of hereditary thrombophilias.

According to our results, CIP was found to be appropriate and reliable for the detection of AT-, PC- and PS deficiencies, homozygous FV Leiden mutation and also for combined deficiencies, except some cases of subgroup of FVL het + FII het. The CIP method proved to be insensitive for FII G20210A and showed great heterogeneity concerning the heterozygous FV Leiden mutation. Since CIP is intended to be a thrombophilia screening test, high sensitivity was required. The best performance of the test was found to be at a cut-off value of 90.0 U for high risk thrombophilias with a sensitivity of 96%, which indicated the usefulness of the test for screening severe hereditary thrombophilias.

Based on our investigations the normal result of the CIP test (>90.0 U) excluded hereditary thrombophilias except the lowest risk heterozygous FV Leiden- and heterozygous FII G20210A mutations. Therefore, in cases when the CIP results are within the normal range, high risk thrombophilias could be ruled out but FII G20210A- and FV Leiden mutation tests need to be performed additionally to reliably cover all thrombophilia types. These three tests, CIP and the two mutation analyses (FV Leiden and FII G20210A) therefore should be done in all thrombophilia examinations, although heterozygous FV Leiden- and FII G20210A mutations are low risk mutations, which do not increase recurrence rate to an extent that influences treatment, if not combined with another deficiency. Tests for AT-, PC- and PS deficiencies should be and are required to be performed only if the CIP value is below 90.0 U.

We compared the cut-off value of the automated CIP assay to the manual method described by Andresen et al. The cut-off value was 90.0 U in our method and 32.9 U in the manual method at the best performance of the tests, that is at the best balance between specificity and high sensitivity, concerning high risk thrombophilia types. In case of our method, there was a smaller overlap between normal controls and high risk thrombophilia samples than in the manual method. The difference may have been due to the different
measurement methods, the source and concentration of reagents and instruments, moreover to
the different number of cases examined.

The automated method may be more applicable to screen high risk thrombophilia types.

The CIP assay may have a higher sensitivity for hereditary high risk thrombophilias
than other global assays. The Calibrated Automated Thrombogram (CAT) assay, also known
as the Endogenous Thrombin Potential assay (ETP), presents abnormal results in hereditary
AT deficiency and homozygous FII G20210A mutation. Currently, the CAT assay has
different variants, with different sensitivity to coagulopathies due to difficulties of
international standardisation. Because of the high inter-laboratory variability and the lack of
consistent results, the CAT is not yet recommended for the detection of thrombophilias in
clinical practice. Another global assay, the ProC Global assay (PCG) showed only
deficiencies of the protein C system.

The prevalence of the different types of hereditary thrombophilias in our study
corresponded to that in other Western nations according to literary data, except heterozygous
FV Leiden mutation which represented 60% of all hereditary thrombophilias in our patient
population. This mutation in Hungarians was found to be more frequent than in many other
Western nations, resulting in one of the highest prevalence in middle Europe. These results
contradict the presumed distribution along a South to North gradient and demonstrate a more
complex pattern. The explanation for such an overrepresentation of the mutation in
Hungarians may be the nation’s Northern origin. On the other hand there is no difference in
the prevalence of high risk thrombophilias.

The low risk mutations are frequent in Western populations but apparently absent in
Asian and African populations. In contrast, the high risk mutations are rare in Western
populations but apparently more frequent in Asian populations. Therefore the CIP assay is
even more feasible for routine thrombophilia screening in the Asian populations, because the
two genetic tests (FV Leiden and FII G20210A) may not need to be performed.

Based on our results, the CIP method could be applied for those patient populations
whose thrombophilia examination would otherwise be time-consuming and expensive.
BLOOD COAGULATION EXAMINATION THROUGHOUT UNCOMPLICATED PREGNANCY

I. Introduction

Haemostatic complications often occur during pregnancy, delivery and puerperium leading to high maternal and fetal morbidity or mortality. Pregnancy and puerperium are well-established risk factors for VTE, with an incidence 4–50 times higher compared to non-pregnant women especially in the third trimester of pregnancy and in the period of puerperium. Pregnancy is normally associated with significant changes in all aspects of the Virchow's classical triad: venous stasis, endothelial damage and a tendency to enhanced coagulation resulting in a shift of the hemostatic balance towards hypercoagulability. This is one of the protective mechanisms involved in avoiding serious bleeding complications at delivery. Elevated markers of coagulation and fibrinolytic system activation indicate increased thrombin generation and increased fibrinolysis following fibrinogen-fibrin conversion throughout pregnancy. Szécsi at al. found that the level of coagulation factors II, V, X, XI, XII and antithrombin, protein C, APTT and PT remained essentially unchanged during pregnancy, delivery and postpartum and were within non-pregnant reference intervals. However, levels of coagulation factors VII, VIII, IX, fibrinogen and D-dimer increased markedly. J. Liu et al. found that PT, APTT and TT decrease significantly, but fibrinogen level rizes with the gestational age, which refer to a hypercoagulable state. Protein S activity decreased substantially, while free protein S decreased slightly and total protein S was stable. These changes are considered physiological for maintaining placental function during pregnancy and preventing significant blood-loss at delivery, but may predispose to thrombosis and placental vascular complications at the same time. In most cases these changes exceed the biological variability, as determined by Westgard by the measurements of non-pregnant healthy individuals’ various laboratory parameters. The CV₁ (within-subject biological variation) of PT is 4.0% and the CVg (between-subject biological variation) is 6.8%. The CV₁ of APTT is 2.7% and the CVg is 8.6%. The CV₁ of fibrinogen, a blood coagulation parameter and a positive acute phase protein, is 10.7%. The CVg of fibrinogen is 15.8%. Whether the fibrinogen increase during pregnancy is due to an acute phase reaction or to pregnancy related changes, can be examined if determined together with a classical positive acute phase protein,
e.g. C-Reactive Protein (CRP). The biological variability of CRP is also high: the CV₁ of CRP is 42.2% and the CVg is 76.3%. The CV₁ and CVg of D-dimer are also remarkable: 23.3% and 26.5%, respectively. The TT is missing from this database (http://www.westgard.com/biodatabase1.htm).

Most reference values established by laboratories are based on samples obtained from non-pregnant women. Clinical symptoms of VTE can be misleading in pregnant woman. Trimester specific reference intervals for D-dimer and fibrinogen level may help in the differential diagnosis of VTE. Without knowing these intervals, the elevated level of D-dimer and fibrinogen may falsely strengthen the suspicion of VTE with low probability pre-test Wells scores, for deep vein thrombosis as well as for pulmonary embolism.

Some gestational age-specific reference intervals have been reported. These studies used different analytical methods and/or include a mixture of complicated and uncomplicated pregnancies in their cohort.

In the present study, based on 83 pregnancies, we report gestational age-specific reference intervals for the following coagulation tests: PI, APTT, TT, fibrinogen and D-dimer. Additionally, we also examined these samples with the CIP method, which was presented in the previous section.

II. Methods
II.1. Patients

128 pregnant women in the 16th week of their pregnancy were recruited for the study to have a blood test in our outpatient laboratory. 40 of them did not reappear for taking blood at week 26 and/or 36. Three women with twin pregnancy and 2 having hereditary thrombophilia were excluded from the study. Altogether blood samples of 83 pregnant women were collected at the 16th, 26th and 36th gestational weeks. These three weeks were chosen according to the most widely used obstetrical protocol for blood withdrawal in Hungary. The study was approved by the local research ethics committee (approval No. 3192/2008), and all women gave informed oral and written consents.
II.2. Blood sampling and reagents

Peripheral venous blood was drawn in Vacutainer tubes (Becton Dickinson) containing 1/10 volume of 0.129 M sodium citrate. The blood was centrifuged at 2000 × g at room temperature for 20 min to obtain platelet poor plasma (PPP). Plasma was frozen in aliquots and stored at - 70 °C until the tests were performed (based on Clinical and Laboratory Standards Institute (CLSI) H21-A5 document).

II.3. Statistical analysis

The results are reported either as mean or as median values depending on the type of distribution, and interquartile range. The data were analysed using the Paired-Samples T Test, Kolmogorov-Smirnov Test, Mann–Whitney Test and Wilcoxon Signed-Rank Test. P levels <0.05 were considered to be of statistical significance. SPSS 15.0 (IBM Corporation NY, USA) was used for calculation of all these significance tests. Reference intervals (2.5th and 97.5th percentiles with 90% confidence intervals) were calculated for the measured parameters at gestational weeks 16, 26, 36 using the non-parametric bootstrap method with RefVal software version 4.11 according to the recommendations of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). The conventional normal approximation of the 95% reference intervals was also calculated for comparison as mean±2 SD.

III. Results

III.1. Patient characteristics

From 128 pregnant women 83 were analysed at gestational weeks 16, 26 and 36. The 83 pregnant women had a mean age of 28.9±4.3. Of the pregnant women, 55 had no pathological signs or symptoms („normal or uncomplicated pregnancies”) and 28 had at least one of the following pathological signs or complications („not normal pregnancies”): 1. proteinuria (n=1) (without preeclampsia), 2. oedema in the lower limbs (n=7), 3. hypertension (n=2) (without preeclampsia), 4. varicose veins (n=2), 5. thrombophlebitis (n=1), 6. gestational diabetes (n=13) (balance could be maintained by diet), 7. autoimmune disease (n=1; Raynaud's disease), 8. in vitro fertilisation (IVF) (n=3), 9. habitual abortion during previous
pregnancies (n=2). Between „normal” and „not normal” pregnancy groups there was no significant difference altogether (p>0.05) with Mann–Whitney Test concerning PT, APTT, TT, fibrinogen, D-dimer and CRP levels, the individual variations between these parameters, at gestational weeks 16, 26 and 36, respectively. Henceforward the data of all 83 pregnant women were evaluated together.

III.2. Distribution of examined parameters

The distribution of PT, APTT, TT, fibrinogen, D-dimer, CIP and CRP values were assessed at all three gestational weeks with Kolmogorov-Smirnov Test. The distribution of individual APTT, TT and fibrinogen was not significantly different from normal at all three examined gestational weeks, therefore these results were compared by Paired-Samples T Test. As PT, D-dimer, CIP and CRP results showed right-skewed distribution (p<0.05) at all gestational weeks, Wilcoxon Signed-Rank Test was used for the comparison of these results or the parameters derived from those.

III.3. Changes of PT, APTT and TT during pregnancy

PT (INR) progressively decreased throughout the pregnancy. There were significant differences between the parameters of weeks 16 and 26 (p=0.043), weeks 26 and 36 (p<0.001) and weeks 16 and 36 (p<0.001). PT was expressed in INR (International Normalised Ratio), because INR is the standardized, derived form of PT and many laboratories report the PT results as INR. We analyzed the statistical results for INR. Because of the right-skewed distribution of INR data the non-parametric bootstrap method was used, and the reference intervals for INR were assigned as the range between the 2.5th and 97.5th percentiles.

The gestational age-specific reference intervals for INR became the following:
16th week: 0.80-1.12, 26th week: 0.77-1.18 and 36th week: 0.74-1.02.

In our laboratory the reference interval of healthy non-pregnant patients for INR is 0.90-1.15.

The APTT and TT decreased throughout the pregnancy. In case of APTT, there were significant differences between the values of weeks 16 and 26 (p<0.001), weeks 26 and 36 (p<0.001) and weeks 16 and 36 (p<0.001). In case of TT, significant differences were found
between the values of weeks 16 and 26 (p<0.001), weeks 26 and 36 (p=0.008) and weeks 16 and 36 (p<0.001). We calculated gestational age-specific reference intervals for APTT and TT, as mean±2 SD, since the distribution of APTT and TT was close to normal at all examined gestational weeks. By using the non-parametric bootstrap method, reference intervals for APTT and TT were assigned as the range between the 2.5th and 97.5th percentiles.

_The gestational age-specific reference intervals for APTT became the following:_
16th week: 27.5-38.8 sec, 26th week: 26.3-35.7 sec and 36th week: 24.7-32.7 sec.

In our laboratory the reference interval of healthy non-pregnant patients for APTT is 25.0-37.0 sec.

_The gestational age-specific reference intervals for TT became the following:_
16th week: 12.5-15.6 sec, 26th week: 12.1-14.9 sec and 36th week: 11.8-15.5 sec.

In our laboratory the reference interval of healthy non-pregnant patients for TT is 11.0-17.0 sec.

### III.4. Changes of fibrinogen and CRP during pregnancy

Fibrinogen levels progressively increased throughout the pregnancy. There were significant differences between the parameters of weeks 16 and 26 (p<0.001), weeks 26 and 36 (p<0.001) and weeks 16 and 36 (p<0.001). Moreover at all three gestational weeks, starting with week 16, fibrinogen mean levels were higher than the upper limit of the normal non-pregnant reference interval in our laboratory. We calculated gestational age-specific reference intervals for fibrinogen, as mean±2 SD, since the distribution of fibrinogen was close to normal at all examined gestational weeks. By using the non-parametric bootstrap method, reference intervals for fibrinogen were assigned as the range between the 2.5th and 97.5th percentiles.

_The gestational age-specific reference intervals for fibrinogen became the following:_
16th week: 3.28-6.59 g/L, 26th week: 3.34-6.80 g/L and 36th week: 4.30-8.11 g/L.

In our laboratory the reference interval of healthy non-pregnant patients for fibrinogen is 2.00-4.00 g/L.

In case of the CRP levels statistically significant decrease could be observed between the 16th and the 26th weeks (p=0.003) and between the 16th and the 36th gestational weeks (p=0.001), although the median CRP concentrations stayed below the normal non-pregnant
conventional threshold during the whole course of pregnancy. Accordingly, compared to non-pregnant healthy individuals CRP levels did not change significantly during pregnancy. CRP and fibrinogen are positive acute phase proteins, thus we examined their association. The regression analysis showed that there was a weak, positive linear correlation between CRP and fibrinogen levels (p<0.001, r=0.265).

III.5. Changes of D-dimer during pregnancy

D-dimer concentrations also gradually increased from the week 16, through the weeks 26 and 36 with statistically significant differences between the gestational weeks (p<0.001). While at the 16th week median D-dimer level of pregnant women remained below the normal non-pregnant conventional cut-off point, the median levels at the 26th and 36th gestational weeks exceeded that limit. The D-dimer levels of 42% of all examined pregnant women were above the normal non-pregnant reference cut-off throughout pregnancy and at the 36th gestational week 98% of pregnant women displayed D-dimer levels above the upper limit of the normal non-pregnant reference cut-off point. We calculated gestational age-specific cut-off points for D-dimer. The measured median values of D-dimer were defined as gestational age-specific thresholds because of the right-skewed distribution of these parameters.

*The gestational age-specific cut-off values for D-dimer became the following:* 16th week < 224 ng/mL, 26th week < 309 ng/mL and 36th week < 541.

In our laboratory the cut-off value of healthy non-pregnant patients for **D-dimer** was below 250 ng/mL.

III.6. Changes of CIP during pregnancy

The CIP values decreased throughout the pregnancy. There were significant differences between the parameters of weeks 16 and 26 (p=0.016), weeks 26 and 36 (p<0.005) and weeks 16 and 36 (p<0.001). We calculated gestational age-specific cut-off points for CIP. The measured median values of CIP were defined as gestational age-specific thresholds because of the right-skewed distribution of these parameters.

*The gestational age-specific cut-off points for CIP became the following:* 16th week: 42 U, 26th week: 36 U and 36th week: 32 U.
Based on our previous work, the cut-off value of **CIP** is above 90 U for the normal non-pregnant patients.

**IV. Discussion**

In the present study, we examined the progressive changes of PT, APTT, TT, fibrinogen, D-dimer, CIP and CRP at gestational weeks 16, 26 and 36 in pregnant women.

The determination of PT, APTT and TT may help in cases of blood coagulation disorders and factor deficiencies. The shortened coagulation times reflect hypercoagulability state both in the extrinsic and intrinsic pathways.

D-dimer, a blood coagulation and fibrinolysis parameter, is widely used in clinical decisions to rule out thromboembolism due to its high negative predictive value in case of low probability pre-test Wells scores. Fibrinogen, a blood coagulation parameter and a positive acute phase protein is helpful in diagnosing DIC or hyperfibrinolysis. The CIP results confirm the presence of a hypercoagulable state throughout pregnancy.

However, the conventional cut-off points and reference ranges for these parameteres are mostly determined in non-pregnant healthy people, which can not be applied for pregnant women.

We found that coagulation times (PT, APTT and TT) and CIP progressively decreased, fibrinogen and D-dimer progressively increased from the 16th to the 36th weeks, while CRP remained stable throughout the duration of all pregnancies. Compared to the healthy non-pregnant conventional levels, the median of PT remained within the normal reference range at the 16th gestational week, but declined at the 26th and 36th weeks. APTT and TT stayed in their respective reference ranges at all three gestational weeks, but were continuously reducing throughout pregnancy. Fibrinogen and D-dimer concentrations were increasingly higher at each examined gestational week, D-dimer levels stayed below the cut-off point in the 16th gestational week but exceeded it at the 26th and 36th, while CRP concentrations were always below the threshold during pregnancy. At the 16th gestational week 42%, at the 26th gestational week 66% and at the 36th gestational week 98% of pregnant women displayed D-dimer concentrations higher than the non-pregnant cut-off point. The D-dimer levels increased more steeply than fibrinogen. The CIP values were below the cut-off points at all three gestational weeks representing a hypercoagulable state.
Increased thrombin generation and higher fibrinogen production may lead to more intense fibrinogen-fibrin conversion and thus to the increased fibrinolysis and elevated level of fibrin degradation products (FDP). More intense fibrinogen-fibrin conversion may cause a hyperproduction of fibrinogen. The two factors – increased production and conversion – may occur simultaneously leading to hypercoagulability without any clinical manifestations in most cases.

Although both fibrinogen and CRP are positive acute phase proteins but it was only fibrinogen that progressively increased, while CRP remained below the non-pregnant cut-off point, i.e. the elevation of fibrinogen levels was not due to an acute phase reaction. On the contrary in complicated pregnancies, particularly in preeclampsia there is a large CRP increase. The median CRP levels did not exceed the non-pregnant threshold in pregnancies, values measured at the 16th gestational week were significantly higher than at the subsequent times of measurements. It remains to be explored if this change in CRP level is connected to the normal course of pregnancy.

The tendency towards blood coagulation is considered to be activated in pregnancy by several authors. The calculated reference ranges differ between the studies and examined populations. Szecsi et al. found similarly shortened reference intervals for PT and APTT, higher reference values for all gestational ages for D-dimer, and lower ranges for fibrinogen, although this difference may be due to the different reagents used. In the study of Kovac et al., who used the same reagents as we did, the reference ranges are similarly comparable with our results. J. Liu et al. found that PT, APTT and TT decrease significantly, but fibrinogen level rises with the gestational age, which refer to a hypercoagulable state. Abbassi-Ghanavati et al. suggest reference intervals for pregnant women at all three gestational weeks. These reference intervals are available on the internet (http://www.perinatology.com), including different parameters of haematology, haemostaseology and biochemistry, too.

These differences suggest that laboratories should establish their own reference intervals and cut-off points also in pregnancy in order to help the clinician's work, although data from such studies can be exemplary in many clinical situations.
SUMMARY OF NEW RESULTS

In our present work, we investigated congenital and acquired thrombophilia with conventional and special laboratory methods.

New results achieved by Coagulation Inhibitor Potential method:

1. First, we adapted the manual application of the original CIP method to an optical coagulation analyser. According to our results, CIP was found to be an appropriate, reliable and reproducible method.
2. According to our results, CIP was found to be appropriate and reliable for the detection of commercially available AT-, PC- and PS deficiencies plasmas.
3. Comparing controls to thrombophilic plasmas, we found that the CIP method was suitable to differentiate between high risk thrombophilia patients and controls without hereditary thrombophilia.
4. We determined the optimal cut-off value of the automated CIP method for the detection of AT-, PC-, PS deficiencies, APC resistance and combined deficiencies.

New results achieved by examining coagulation parameters during pregnancy:

1. We determined reference ranges and cut-off values of different gestational ages in normal pregnancies for coagulation parameters: PT, APTT, TT, fibrinogen and D-dimer.
2. We found that coagulation times (PT, APTT and TT) progressively decreased, fibrinogen and D-dimer progressively increased throughout the duration of pregnancies.
3. Our observations about the examined coagulation parameters such as PT, APTT, TT, fibrinogen and D-dimer supported the development of a hypercoagulable state throughout uncomplicated pregnancy.
4. The CIP values were below the cut-off point at all three examined gestational weeks.
5. The CIP method was suitable for detecting hypercoagulation state under pregnancy.
ARTICLES RELATED TO THE THESIS

Papers:


Total impakt faktor: 3,546

Published abstracts:

Barbara Réger, Hajna Losonczy, Ágnes Nagy, Emese Kátai, Ágnes Péterfalvi, Nelli Farkas, Attila Miseta, Tóth Orsolya. Introduction of an automated global coagulation assay, the coagulation inhibitor potential method (CIP). CCLM 2018; Volume 56, (Issue 9) eA128


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