Evaluation of prolonged surface activated coagulation time

Master degree thesis, 30 ECTS

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Abstract
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Evaluation of prolonged surface activated coagulation time

by Amalie Jesting

Background: Blood coagulation is an essential defense mechanism to prevent bleeding. Disorders in the coagulation system can be severe and blood tests measuring the blood’s ability to coagulate are important. Activated partial thromboplastin time (APTT) is a blood test that measures blood coagulation time. An abnormal prolonged APTT can both be associated with a bleeding tendency or a risk of thrombosis. Additional blood tests are needed to discover the cause of a prolonged APTT. One potential test is the APTT mixing study, which can separate samples with and without inhibitors.

The aim of this project is to investigate how the cause of a prolonged APTT is evaluated today and to examine if it is possible to indicate the cause of a prolonged APTT using the APTT mixing study performed on routine samples. The goal is to be able to indicate the cause of a prolonged APTT immediately when it is first discovered. This will save time and help guide the physicians in their work with the patient.

Methods: Retrospective data is used to examine how the cause of a prolonged APTT is evaluated today. Samples with known cause of prolonged APTT are used to establish a cut-off value for the APTT mixing study to indicate the cause of a prolonged APTT. The cut-off values are then tested using routine APTT samples. Pre-analytical variables relevant to APTT are also investigated.

Results: Today, specialized departments request most special coagulation blood tests. The APTT mixing study can separate samples with and without inhibitors with 90% specificity and sensitivity using index of circulating anticoagulants cut-off value of 16.0. In regard to pre-analytical variables, the centrifugation force affects the plasma platelet count but not APTT and sample storage has an affect on APTT.

Conclusion: The APTT mixing study can be implemented as an additional test to indicate the cause of a prolonged APTT on routine samples.

Keywords: Blood test, Coagulation, APTT, Pre-analytical variables, APTT mixing study, decision tree.
This Master's thesis has been defended on August 21, 2018 at the Faculty of Health and Society, Malmö University.

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Preface

A thesis submitted in fulfilment of the requirements for the degree of Master programme in Biomedical Surface Science at Malmö University

This Master’s thesis was conducted as a part of the Master’s program in Biomedical Surface Science at Malmö University. All experiments were carried out at Rigshospitalet at the Department of Clinical Chemistry, Copenhagen in collaboration with Metropolitan University College, Copenhagen. The Master’s thesis was carried out in the period of January 2018 to August 2018.

Sebastian Björklund, Malmö University was the internal supervisor on this master’s thesis project. External supervisors were Jens Peter Gøtze, Rigshospitalet and Søren Frank Jørgensen, Metropolitan University College.

I thank my supervisors Sebastian, Jens Peter and Søren for their help and guidance throughout the project. A special thanks, goes to Jens Peter for giving me the opportunity to conduct this project at the Department of Clinical Chemistry, Rigshospitalet and for letting me be a part of his research group. In this regard, I would also like to thank Søren for agreeing to supervise this project and to share his ideas with me.

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## Abbreviations

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<td>APTT</td>
<td>Activated Partial Thromboplastin Time</td>
</tr>
<tr>
<td>HK</td>
<td>High molecular weight Kininogen</td>
</tr>
<tr>
<td>ICA</td>
<td>Index of Circulating Anticoagulants</td>
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<tr>
<td>INR</td>
<td>International Normalized Ratio</td>
</tr>
<tr>
<td>ISTH</td>
<td>International Society on Thrombosis and Haemostasis</td>
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<tr>
<td>LAC</td>
<td>Lupus Anticoagulant</td>
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<tr>
<td>PK</td>
<td>Prekallekrein</td>
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<td>PLT</td>
<td>Platelets</td>
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<td>PT</td>
<td>Prothrombin Time</td>
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<td>TF</td>
<td>Tissue Factor</td>
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Chapter 1

Introduction

1.1 Background

Blood coagulation is an important defense mechanism to stop bleeding and involves several different factors. Disorders in the coagulation system may result in bleeding or thrombosis. It is therefore important to be able to measure the blood’s ability to coagulate using laboratory tests [1, 2].

Many physicians and clinical departments are exposed to a diagnostic dilemma when receiving an abnormal result of a blood test measuring the blood coagulation time [2]. One commonly used measure of the blood coagulation is a blood test referred to as activated partial thromboplastin time (APTT). APTT is used for preoperative screening to indicate bleeding risk, as well as to monitor anticoagulant heparin therapy [2–5]. The reliability of preoperative screening with APTT is widely discussed since an abnormal APTT \(^1\) does not always indicate an increased risk of bleeding [2–5].

Abnormal prolongation of a patient’s APTT can be caused by several factors with different consequences for the patients [2]. To identify the cause of a prolonged APTT additional blood tests are needed. At Rigshospitalet, Copenhagen, APTT results are delivered electronically to the physicians without explanatory comments. It is now up to the involved physicians to decide which additional blood tests the laboratory should perform. Knowledge about these special blood tests is not something every physician has, and specialized clinical departments are typically involved [2]. In a preoperative setting, an unexpected abnormal APTT may result in increased cost and delayed surgery as well as emotional stress for patients and their families [3].

If the laboratory performs additional tests immediately when an abnormal prolonged APTT is measured, it might give sufficient information to add an explanatory comment to the APTT result. An explanatory comment could help guide the physicians in the further investigation of the cause of the prolonged APTT.

The main purpose of this project is to investigate the use of a test known as the APTT mixing study to indicate the cause of an abnormal APTT. The APTT mixing study is a test known to distinguish between possible causes of a prolonged APTT [2, 6]. The goal is to implement the mixing study as an extra test that automatically is performed when a sample with prolonged APTT is measured. The hypothesis is that the APTT mixing study provides sufficient information for the laboratory to connect an explanatory comment to the

\(^1\)At the Department of Clinical Chemistry, Rigshospitalet, a normal APTT range from 25-35 s. In this project a prolonged APTT is classified as >45 s.
APTT result, thereby providing a clear guide to the physicians suggesting which blood tests to order next.

1.2 Aim

The aim of this project is to investigate how the cause of a prolonged APTT is evaluated today and to examine if it is possible to indicate the cause of a prolonged APTT using the APTT mixing study performed on routine samples.

1.3 Theory

This section includes an introduction to blood coagulation in vivo and in vitro followed by a description of APTT, the APTT mixing study and the factors known to cause a prolonged APTT.

1.3.1 Introduction to blood coagulation

When damage occurs at the vascular endothelium, a hemostatic response is initiated. First, platelets aggregate forming a primary hemostatic plug at the site of injury. Then, blood coagulation is initiated resulting in a clot of fibrin polymers that reinforces the platelet plug [7, 8].

Blood coagulation is a series of reactions involving cell surfaces and activation of coagulation factors, which end by the formation of fibrin polymers. Coagulation factors are assigned roman numbers in the order they where discovered while the suffix “a” indicate when a coagulation factor is activated [8]. In vivo coagulation occurs in three distinct but overlapping phases: initiation, amplification and propagation, which involve a multitude of reaction steps where different complexes are formed.

In the initiation phase tissue factor-bearing cells, that are normally located outside the vasculature, play a key role. Upon vessel injury, the tissue factor-bearing cells come in contact with the blood resulting in factor VII activation by tissue factor. The activated factor VIIa then forms a complex with tissue factor on the surface of the tissue factor-bearing cell (Figure 1.1) [7]. This complex activates factor IX and factor X (Figure 1.1a and b). The prothrombinase complex is then formed by factor Xa and factor Va on the tissue factor-bearing cell. This prothrombinase complex is then able to activate a small amount of prothrombin (II) to thrombin (IIa) (Figure 1.1c). Among other possible activation routs, factor V can be activated by factor Xa. [7].

During the amplification phase, the activated thrombin (IIa) from the initiation phase is involved in several processes including activation of factor V, VIII and XI (Figure 1.2). The activated thrombin also activates platelets located at the site of injury thereby priming the platelets for the propagation phase [7].

In the propagation phase, platelet-bound factor Xla activates factor IX to IXa (Figure 1.3a). This factor IXa, in combination with factor IXa from the initiation phase, binds to factor VIIIa forming a tenase complex on the platelet surface (Figure 1.3b). The tenase complex is now able to activate factor X to factor Xa, which forms a prothrombinase complex with platelet-bound factor Va (Figure 1.3b and c). As in the initiation phase, prothrombin (II) is transformed to thrombin (IIa) by the prothrombinase complex (Figure 1.3c) [7]. Factor Xa cannot travel from the tissue factor-bearing cells to the platelets, as is the case for factor
Figure 1.1: Initiation phase. A complex composed by tissue factor (TF) and factor VIIa is formed on the tissue factor-bearing cell. This complex now activates factor IX (a) and factor X (b). Factor Xa binds to membrane-bound factor Va forming a prothrombinase complex (c). The prothrombinase complex activates a small amount of prothrombin (II) to thrombin (IIa). Factor IXa produced in the initiation phase play a role in the propagation phase.

Figure 1.2: Amplification phase. Factor V, VIII and XI are activated by thrombin (IIa) during the amplification phase.

IXa. Therefore is the platelet-associated factor X activation essential for the formation of an efficient fibrin clot [7].

Figure 1.3: Propagation phase. Factor IX is activated by platelet bound factor XIa (a). The tenase complex composed by factor IXa and VIIIa activate Factor X (b). Factor Xa form the prothrombinase complex with factor Va, activating prothrombin (II) to thrombin (IIa) (c).

A common way to display blood coagulation is the cascade model, which in vitro shows how blood coagulation can be activated via either the intrinsic (Figure 1.4a) or the extrinsic pathway (Figure 1.4b). Both pathways end up in the common pathway (Figure 1.4c) with fibrin as the end product. The intrinsic and common pathways can be measured with APTT while the prothrombin test (PT) is a measure of the extrinsic and common pathways [2, 7, 8]. Calcium ions (Ca^{2+}) are involved in several steps of coagulation (Figure 1.4) [8].
1.3.2 APTT and APTT mixing study

During an APTT test, blood coagulation is initiated through activation of factor XII and measured as the time until formation of a fibrin clot. This makes APTT a measure of the intrinsic and common pathway of the coagulation cascade.

The blood sample used for APTT is collected in a tube containing liquid trisodium citrate that traps calcium ions from the blood, preventing coagulation. The sample is centrifuged, according to local standards, and inserted into automated equipment. Here plasma is incubated at 37°C with a solution containing an activator (e.g. silica particles) and artificial phospholipids. The activator initiates blood coagulation by surface activation of factor XII [9]. The phospholipids simulate the surface of activated platelets (Figure 1.3, 1.4).

Without available calcium ions, the coagulation cannot proceed (Figure 1.4). Therefore a second solution containing calcium ions is added to the sample which starts coagulation. When calcium ions are added coagulation can run to the end forming a fibrin clot. Clot formation is measured as an abrupt increase in light absorbance. The result of APTT is the time, in seconds, from calcium ions are added to the abrupt increase in light absorbance is measured (Figure 1.5).

The APTT mixing study is used to indicate whether a prolonged APTT is caused by a coagulation factor deficiency or the presence of an inhibitor. This is done by measuring APTT on a mixture of patient plasma and normal plasma. There are two possible outcomes of an APTT mixing study. 1) The APTT result is corrected due to the supply of coagulation factors from the normal plasma. This indicates that the patient plasma has reduced concentrations of one or more coagulation factors. 2) The result is not corrected and still prolonged, which indicates that there is an inhibitor in the blood (Figure 1.6) [2].
Figure 1.5: Absorbance measured throughout an APTT test. An abrupt increase in light absorbance indicates clot formation (marked with vertical line). The APTT result is the time in seconds from calcium ions are added to clot formation is measured.

Figure 1.6: Overview of potential causes of a prolonged APTT. The schema indicates how the mixing study can be used to distinguish between factor deficiency and inhibitors in a blood sample.

Reduced concentrations of one or more coagulation factors of the intrinsic or common pathway will result in a prolonged coagulation time measured as APTT. Patients with the bleeding disorders hemophilia A, B and C lack factor VIII, IX or XI respectively. Patients with deficiencies of factor XII, high molecular weight kininogen (HK) and prekallekrein (PK) have no bleeding tendencies despite a prolonged APTT [2, 7]. This is explained by the activation of factor XI by thrombin (IIa) that occurs In vivo during the amplification phase (Figure 1.2) [2, 7]. Coagulation factors are produced in the liver which is why liver disease may result in coagulation factor deficiencies that will prolong both APTT and PT [2].

Certain inhibitors can also influence the in vitro coagulation time resulting in a prolongation of APTT. There are three types of inhibitors affecting APTT: 1) Anticoagulant drugs, 2) specific coagulation factor inhibitors that bind to coagulation factors and 3) non-specific inhibitors, which target phospholipids [2].

There are several types of anticoagulant drugs that work in different ways. Some, e.g. Rivaroxaban and Dabigatran are direct coagulation inhibitors, affecting factor Xa or thrombin (IIa) [10]. Others work by affecting the production of coagulation factors in the liver e.g. vitamin K antagonists such as Warfarin [2, 11]. The anticoagulant drug Heparin works by
forming a complex with anti-thrombin that inactivates several coagulation factors including factor Xa and thrombin (IIa) [12].

Specific coagulation factor inhibitors are antibodies directed against e.g. factor VIII. These specific antibodies can be produced as an immune response to factor replacement therapy given to hemophilia patients [13, 14].

Lupus anticoagulant (LAC) is a non-specific inhibitor known to prolong APTT. LAC is a type of antibody directed against protein-phospholipid complexes (Figure 1.7). LAC prolongs APTT by binding phospholipid surfaces, thereby blocking e.g. the prothrombinase complex. Even though LAC prolongs coagulation time measured with APTT, LAC is not associated with a bleeding tendency. Instead, the presence of LAC in plasma is associated with a higher risk of thrombosis and pregnancy morbidity [10]. Since LAC affects APTT by binding to phospholipids, the amount of phospholipid in the APTT reagent controls how sensitive an APTT reagent is to LAC [6]. LAC can also bind to platelets left in a plasma sample after centrifugation, which is why it is recommended to measure LAC in platelet poor plasma [6].

Figure 1.7: The effect of lupus anticoagulant (LAC) on APTT measurements. LAC binds to phospholipids in the APTT reagents, thereby hindering the prothrombinase complex from binding to the phospholipid surfaces. This results in a prolonged coagulation time measured as APTT.

1.4 Project plan

This project is divided into three parts. First, the current approach to evaluating a prolonged APTT is investigated through a retrospective data study. Secondly, the mixing study and its ability to differentiate between samples with and without inhibitors is investigated. The third part focuses on how pre-analytical variables relevant to coagulation assays affect APTT.

The Subcommittee on Lupus Anticoagulant/Antiphospholipid Antibody of the Scientific and Standardisation Committee of the International Society on Thrombosis and Haemostasis (ISTH) has described guidelines for LAC detection in the article "Update of the guidelines for lupus anticoagulant detection" from 2009 [6]. These guidelines are used as basis in regard to sample handling, setup and interpretation of the APTT mixing study.

In the following sections, the individual objectives and the experiential approaches of the three project parts are described. This includes a brief introduction to the theories behind the experimental approaches and statistical analyses used in each part of the project. The following chapters of this report will follow the same structure as in this section.

1.4.1 Part 1: Retrospective study

The retrospective data study is performed to clarify how the cause of an unexpected prolonged APTT is identified today. This is essential to understand how the APTT mixing
study can improve the evaluation of an unexpected prolonged APTT. The focus is to investigate the time it takes from an abnormal APTT is measured to the cause is known as well as which blood tests and clinical departments are involved in the process. To do this, retrospective data from the laboratory database (LABKA) including patients with prolonged APTT is analyzed.

1.4.2 Part 2: APTT mixing study

The central goal of this project is to investigate the ability of the mixing study to differentiate between samples with and without inhibitors. To do this a simple machine learning approach is used. This approach is described by Richardson et al. in the article “Clinical chemistry in higher dimensions: machine-learning and enhanced prediction from routine clinical chemistry data” from 2016 [15]. In machine learning, algorithms, e.g., in the form of decision trees, are used to determine splits in data that best divide the data set into homogenous groups [15]. Another central concept of machine learning is the “train-test” splitting of data. The idea is to use part of a data set to train a machine learning algorithm and the other part of the data set to test the efficiency of the algorithm [15].

In this project, samples with known causes of prolonged APTT are used as training data to establish a decision tree algorithm to separate samples with and without an inhibitor. Since the result of the mixing study is the only variable introduced in this project, a simple decision tree algorithm with one cut-off value is sufficient to divide the samples. The established cut-off value is then tested using a test data set composed by a group of routine samples with prolonged APTT. The three steps of Part 2 are displayed in Figure 1.8.

![Figure 1.8: Overview Part 2. The schema shows the three steps included in Part 2.](image)

There are different ways to interpret the output of the mixing study. One is direct use of the APTT mixing study measurement in seconds. The other is to calculate the index of circulating anticoagulants (ICA) as shown in Equation 1.1 [6, 16]. This ICA calculation includes both the APTT of the patient plasma and the APTT of the normal plasma used to mix with the patient plasma. A high ICA value indicates that an inhibitor is causing the prolonged APTT while coagulation factor deficiencies will result in a low ICA value.

\[
ICA = \frac{\text{APTT of mixture} - \text{APTT of normal plasma}}{\text{APTT of patient plasma}} \times 100 \quad (1.1)
\]

To investigate which of the two interpretation methods gives the best possible split between samples with and without inhibitors, receiver operating characteristic (ROC) curves are used. ROC curves show the sensitivity of all possible cut-off values against specificity of the same cut-off values [17]. The sensitivity and the specificity of a diagnostic test define the accuracy of the test. In this case, the test sensitivity is defined as the probability that a sample containing an inhibitor will be correctly classified. Specificity is, on the other hand, the probability that samples without an inhibitor will be correctly classified. Changing a
cut-off value of a test will change the accuracy of the test where sensitivity and specificity are inversely related [17].

In this project, sensitivity and specificity is weighted equally when determining the best cut-off value. This cut-off value is found using the R package rpart [18]. To calculate the sensitivity and specificity achieved with a specific cut-off, the results are classified as true or false and negative or positive according to Table 1.1.

Table 1.1: Classification of mixing study results. How to classify the outcome of the mixing study as true negative, false negative, false positive or true positive.

<table>
<thead>
<tr>
<th></th>
<th>No inhibitor</th>
<th>Inhibitor</th>
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<tr>
<td>Corrected</td>
<td>True negative</td>
<td>False negative</td>
</tr>
<tr>
<td>Not corrected</td>
<td>False positive</td>
<td>True positive</td>
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Sensitivity and specificity are calculated from Eqs. 1.2 and 1.3, respectively: [19].

\[
\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \tag{1.2}
\]

\[
\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}} \tag{1.3}
\]

The established cut-off value is tested using routine samples with prolonged APTT. Routine samples are used because they represent the type of samples the mixing study will be applied on in the future. The samples, used to establish the cut-off, are handled differently from routine samples, which is why testing the cut-off on routine samples is essential. Due to ethical restrictions it is not possible to find out why the routine samples have prolonged APTT. To test the cut-off value routine samples with known cause of prolonged APTT is needed.

As an extra control of the established cut-off value, routine samples with normal APTT are also included in the project.

1.4.3 Part 3: Pre-analytical variables

The goal of investigating pre-analytical variables is to understand how these affect APTT and thereby the APTT mixing study. This is essential knowledge before implementing the APTT mixing study to indicate the cause of prolonged APTT. Pre-analytical variables are factors that can influence a sample prior to the actual sample analysis. In this project, two pre-analytical variables that are known to influence coagulation assays, centrifugation and storage, are investigated. Method comparison studies are used to analyze the effect of the pre-analytical variables.

Centrifugation

Sample centrifugation is done to achieve separation of the blood cells and the plasma. The coagulation factors will stay in the plasma in the top part of the sample tube, while the cells will gather in the bottom of the tube. The amount of platelets left in the plasma after
centrifugation is related to the centrifugation gravity (g) force as well as the centrifugation time [20].

Platelets left in plasma supply phospholipids that might influence the effect of non-specific inhibitors on the APTT measurements. This is why ISTH recommend the use of platelet poor plasma with platelet count of less than $10 \times 10^9$/L for LAC detection [6].

Different approaches on how to efficiently achieve platelet poor plasma is described and discussed in literature. ISTH recommend that samples are double centrifuged first at 2000 g for 15 minutes then at $\geq 2500$ g for 10 minutes [6]. Another article state that platelet poor plasma can be generated by centrifugation by 1500 g for at least 15 minutes [20].

At the Department of Clinical Chemistry at Rigshospitalet, the samples for LAC detection is double centrifuged in a similar manner as recommend by ISTH. Samples for routine APTT is on the other hand single centrifuged at 2000 g for 10 minutes or at 3500 g for 10 or 15 minutes.

To understand the effect of centrifugation, the plasma platelet count obtained through two different centrifugation forces are investigated as well as the influence of the platelet count on APTT measurements.

Aside from affecting LAC detection, can platelets in the plasma sample also cause neutralization of heparin, which might result in false low heparin measurements [12].

Storage

Sample storage can be defined as storage before and after centrifugation. The focus of this project is storage after centrifugation since all routine APTT samples are centrifuged within two hours after sample collection, which is well below ISTH’s maximum of four hours [6].

How storage affects APTT is investigated to know whether the samples can be stored before performing the mixing study or not. Understanding the effects of long storage is important as many hospitals have a common practice for storing samples for special coagulation tests, such as LAC detection and coagulation factor activity, before analysis [20].

In this project, effect of storage time and temperature on APTT is examined and discussed.

Method comparison studies

The comparisons performed for centrifugation and storage studies are analysed regarding correlation and agreement using linear regression and Bland-Altman plots. Linear regression can be used to investigate the correlation between paired measurements. A Bland-Altman plot is a type of difference plot showing the difference between two paired measurements and is used to show the agreement between the measurements [21].

1.5 Scope

The main focus of this project is on the use of the APTT mixing study to indicate the cause of prolonged APTT on routine samples. It is well known that the different reagent kits used for measuring APTT vary in composition affecting e.g. sensitivity to LAC [6, 13, 16]. In this project, a LAC sensitive APTT reagent (APTT-SP) is used as this is the reagent used for routine APTT measurement at the Department of Clinical Chemistry at Rigshospitalet. The difference in reagent composition and its importance for the performance of APTT is an interesting topic that is outside the scope of this thesis work. The results produced in this project (Part 2-3) can only be applied when using the reagent APTT-SP.
Chapter 2

Materials and Methods

In this section, the materials and methods used in this project are described. First, the retrospective study in Part 1 is described. Then the materials used in both Part 2 and Part 3 are listed with a part describing the different samples used and how they are pre-analytically treated. The method section describes how the practical parts of this project are executed.

2.1 Retrospective study (Part 1)

Data from the laboratory database LABKA is used. The data contains information about blood samples collected at the outpatient clinic from 208 patients between 16 and 50 years of age. A time period of three years is investigated (from January 2015 to December 2017). Data includes patients with at least one APTT measurement higher than 45 s analyzed during this period.

All coagulation related blood tests performed at Rigshospitalet are included in the data set. Besides these, blood tests informing about liver diseases and pregnancy are included since these conditions can affect blood coagulation. The data also show information about the patient age, gender and which clinical departments requested the blood tests. A list of blood tests included in the data set can be found in Appendix A. The data is anonymous and it is not possible to identify any of the patients from the data.

Using Microsoft Excel and RStudio, each patient case is reviewed and the following factors are noted:

- What is the cause of the prolonged APTT
- Types of coagulation tests performed (if any)
- Is the patient pregnant or has a liver disease
- If factor deficiency present, does it seem to be a known or unknown condition
- If the presence of LAC is analyzed what is the result e.g. LAC negative, LAC positive, anticoagulant drug or another result
- If special coagulation tests are performed, which clinical department have requested the tests
2.2 Materials

Equipment

- Automated Coagulation Laboratory, ACL TOP 750 CTS
- Sysmex XN 9000
- Centrifuges, Hettich

Reagents

- APTT: Haemosil® APTT-SP (liquid) – 0020006300 (Instrumentation Laboratory, USA)
  - Silica particles, synthetic phospholipid, buffer and conservatives
  - Calcium Chloride 0.025 mol/L
- Mixing study: Normal plasma (see section 2.3.1 page 13)
- Platelet count: Sysmex reagents

Quality control material

- Quality control material for APTT from Medirox
  - Normal (NKP, GHI162)
  - Abnormal (OKP, GHI167B)
- Quality control material for platelet count, Sysmex XN-Check
  - Level 1 (QC-81481101)
  - Level 2 (QC-81481102)
  - Level 3 (QC-81481103)

Statistical software

- RStudio with the following packages
  - rpart
  - ggplot2
  - pROC
- Microsoft Excel

2.2.1 Blood samples

In this section, the different types of blood samples are listed. The pre-analytical sample handling is performed according to local guidelines and described in this section.
Chapter 2. Materials and Methods

Blood samples used for Part 2 - mixing study

- Samples measured for the presence of LAC with prolonged APTT (>45s) and normal International Normalized Ratio (INR)\(^1\) (<1.3)
  - 29 LAC positive samples
  - 6 LAC negative samples
  - 31 samples with anticoagulant drugs
- 36 factor VIII deficient samples with prolonged APTT (>45s) unknown INR
- Routine samples with normal INR (<1.3)
  - 121 prolonged APTT (>45s)
  - 9 normal APTT (25 – 35s)

Blood samples used for Part 3 – pre-analytical variables

- 65 x 3 Routine samples with varying APTT, three samples/patient (figure 2.1 page 14)

Conditions common for all samples used

All blood samples used in this project are analyzed and collected at The Department of Clinical Chemistry, Rigshospitalet. Some of the samples are sent to the department from other hospitals. The samples are collected from a stock of previously analyzed blood samples and therefore no informed consents from the patients are needed.

The blood samples used in this project are collected in vacuum sample tubes from VAC-UETTE Greiner Bio-One containing anticoagulant liquid trisodium citrate (3.2%) in a 1 + 9 ratio. The sample volume is controlled by visual inspection.

All frozen plasma samples are thawed in a water bath at 37\(^\circ\)C for about 5 minutes and mixed thoroughly prior to analysis.

For samples used in part 2 (mixing study), samples with an INR of >1.3 are excluded. This is done to avoid samples not relevant to this project e.g. samples from patients treated with vitamin K antagonists or with a liver disease. The INR is not known for all the samples and it is possible that some of the samples included in the study have an INR >1.3.

Samples measured for the presence of LAC – used in Part 2

For the investigation of the presence of LAC three blood samples are collected from each patient. The samples are kept on ice and centrifuged at 3500 g for 15 minutes at 4\(^\circ\)C within 1 hour after sample collection. Then the top 2/3 of the plasma is transferred to new tubes using single-use plastic pipettes and centrifugation is repeated. After the second centrifugation the top 2/3 of the plasma is transferred to 3 tubes and stored at -20\(^\circ\)C. Double centrifugation is performed to secure platelet poor plasma. Most of the LAC samples used for the mixing study (project part 2) have not been thawed prior to this project.

\(^1\)INR is calculated from the PT test
Chapter 2. Materials and Methods

Factor VIII deficient samples – used in Part 2

Samples ordered for the measurement of factor VIII are kept on ice before centrifugation at 3500 g for 15 minutes. The centrifugation is performed within one hour after sample collection. The plasma is transferred to a sample tube using single-use plastic pipettes and stored at -20°C. The samples used in this project have been thawed and frozen once prior to this project.

Routine samples – used in Parts 2 and 3

Blood samples for routine APTT analyses at the hospital are delivered to the laboratory within two hours from sample collection. The majority of the routine samples are centrifuged at 2000 g for 10 minutes. Because more than one centrifuge is used for the routine samples, some of the routine samples are centrifuged at 3500 g for 15 minutes. After routine analysis, the samples are stored at room temperature and/or 4°C. Plasma from the routine samples used for the mixing study (project part 2) are transferred to new tubes and stored at -20°C. Before the samples are frozen they are stored for a maximum of four hours, in the primary sample tube, at room temperature and/or 4°C.

2.3 Methods

2.3.1 Preparation of pooled normal plasma (Part 2)

Pooled normal plasma is prepared according to the guidelines from ISTH [6] and the local procedure used at the department. Blood from nine healthy volunteers from the department is used. None of the volunteers were taking anticoagulant drugs or were pregnant at the time of blood collection. The samples are double centrifuged in the same manner as LAC samples (Section 2.2.1). The pooled normal plasma is stored at -80°C in aliquots until use.

2.3.2 APTT and APTT mixing study (Part 2)

Routine samples are after centrifugation inserted directly to the ACL TOP equipment, in their primary sample tube, using a sample rack that holds 10 samples. A needle pierces the lid of the tube and extracts a specific amount of plasma. The plasma is transferred to a cuvette and the APTT measurement is performed as described section 1.3.2 page 4. Clot formation is measured at 671 nm.

For the mixing study, 150 µl patient plasma is manually mixed with 150 µl pooled normal plasma in 2 ml sample cups. The sample cups fit into sample racks that are inserted into the ACL TOP equipment and the APTT measurement is performed automatically as described in Section 1.3.2. Besides the APTT of the mixture of patient plasma and pooled normal plasma, a new APTT measurement is performed on the undiluted patient sample.

For every new aliquot of pooled normal plasma used, APTT of the pooled normal plasma is measured. The average APTT of the aliquots are used to calculate ICA (Eq. 1.1 at page 7).

The APTT reagent is mixed thoroughly before use. The accuracy of APTT is controlled at least four times daily using quality control material with known values.
2.3.3 Pre-analytical variables (Part 3)

Samples from 65 patients are used to investigate the influence of storage on APTT and centrifugation on APTT and platelet count. Three samples from each patient drawn at the same time are used for comparisons. To investigate the effect of centrifugation and storage on APTT the samples are pre-analytically treated differently. An overview of the samples and how they are treated is displayed in Figure 2.1.

![Diagram](image)

**Figure 2.1**: Overview of Part 3. Three samples are drawn from 65 patients. One handled in the routine way (2.2.1), one centrifuged at 2000 g and one for 3000 g (10 minutes). The majority of the routine samples are centrifuged at 2000 g for 10 minutes but some might be centrifuged differently. After centrifugation, APTT and plasma platelet count is measured. Then the routine sample and the sample centrifuged at 2000 g are stored for two hours at room temperature and 4°C respectively before APTT is repeated.

One of the samples for the centrifugation and storage study is the routine sample, which is treated in the regular manner described above (Section 2.2.1). The two additional samples are either centrifuged at 2000 g or 3000 g for 10 minutes. After the first centrifugation, APTT and the platelet count is measured for all samples. Then the routine sample and the sample centrifuged at 2000 g are stored for two hours at room temperature and 4°C respectively. After two hours the APTT measurements are repeated.

2.3.4 Platelet measurement (Part 3)

The platelets are counted using a method based on impedance and flow cytometry performed using the Sysmex XN 9000 equipment. The method can count platelets down to $3 \times 10^9$/L and is controlled daily using quality control material with known values.
Chapter 3

Results

In this section, the results obtained in this study is presented starting with the results of the retrospective study in Part 1. Next, the results of the APTT mixing study (Part 2) with the establishment and test of the cut-off value is shown. The last part of this section presents the results of the study of the pre-analytical variables performed in Part 3.

3.1 Part 1: Retrospective study

The retrospective data set contained a total of 208 patient cases all with at least one APTT higher than 45 s measured in the period. Reviewing the patient cases lead to the following observations.

Factor VIII and IX activity are measured for 50 and 15 patients respectively with 5 patients having both their factor VIII and IX activity measured. For the patients with low factor VIII and IX activity the prolonged APTT can be explained by hemophilia A or B. From the data set, it is not possible to determine if the factor deficiencies are known conditions.

The presence of LAC is investigated in 53 patient cases where 32 are found to be LAC positive. A LAC positive test will be associated with a prolonged APTT measurement.

For about 10 patients cases the blood tests indicate a liver disease and about 19 patient cases have blood tests indicating pregnancy. For some of the pregnant patients, tests for LAC are also measured. Liver disease might be the cause of the prolonged APTT.

All but 2, patients where special coagulation tests such as factor VIII and IX activity or presence of LAC is measured are at some point connected to a clinical department specialized in hematology and bleeding disorders.

3.2 Part 2: APTT mixing study

In this section, the results obtained in Part 2 concerning the ability of the mixing study to differentiate between samples with and without inhibitors are shown. First, the two different ways to interpret the outcome of the mixing study is shown. The following section displays the ROC curves and the obtained test accuracy of the different cut-off values found. At last the cut-off values are tested using routine samples with prolonged and normal APTT.
3.2.1 Interpretation of APTT mixing study

The four types of samples with a known cause of prolonged APTT (anticoagulant drug, LAC positive, LAC negative and factor VIII deficiency) are shown in Figure 3.1, using different colors and forms. Interpretation of the results by direct use of the APTT mixing study in seconds is shown in Figure 3a and the calculated ICA value is shown in Figure 3.1b.

![Figure 3.1: APTT of patient plasma against APTT mixing study (a) and ICA (b). Color and forms indicating the different types of known causes.](image)

Figure 3.2 displays the same measurements as Figure 3.1. The only difference is that samples containing a drug and the LAC positive samples are grouped as samples with an inhibitor. The samples without inhibitors represent LAC negative samples and factor deficient samples. It is not known if the LAC negative samples are from patients with factor deficiencies.

![Figure 3.2: APTT of patient plasma against APTT of APTT mixing study (a) and ICA (b). Red dots contain an inhibitor, blue dots does not contain an inhibitor.](image)

**Pooled Normal plasma**

The pooled normal plasma used in the APTT mixing study have a platelet count of less than $3 \times 10^9/L$. The APTT of the pooled normal plasma is measured 16 times and have a mean APTT of 29.3 s with a standard deviation of 0.4 s.
3.2.2 ROC curve and best cut-off value

To investigate the two methods to interpret the mixing study results, ROC curves are shown in Figure 3.3. The ROC curves reveal that ICA gives higher sensitivity and specificity than direct use of the mixing study result.

![ROC curve of ICA and mixing study](image)

Figure 3.3: ROC curve of the APTT mixing study results. Orange line: ROC curve by directly use of the APTT mixing study. The blue line: ROC curve of the calculated ICA value. Best cut-offs are marked with grey circles.

The ICA cut-off value that gives the highest possible specificity and sensitivity, from the samples measured in this project, is 16.0. This cut-off induces both specificity and sensitivity of 90%.

If instead the APTT mixing study is used directly, the best possible cut-off value is 36.9 s resulting in a specificity of 67% and a sensitivity of 93%.

How the ICA cut-off value of 16.0 separates the samples is shown in Figure 3.4. Samples above the cut-off line are classified as containing an inhibitor while samples lower than the cut-off is classified as not containing an inhibitor. All four types of samples have between one and three samples that are falsely classified as with or without an inhibitor by the cut-off value of 16.0.

3.2.3 Test of cut-off value

In Figure 3.5, the ICA cut-off value of 16.0 is used to indicate the causes of the prolonged APTT of routine samples. According to this cut-off, 66 out of 119 samples contain an inhibitor and 53 out of 119 have no inhibitor. Since it is not known why the samples have prolonged APTT it is not possible to say to which extent the cut-off value has correctly divided the samples according to the presence of inhibitors.

3.2.4 Normal controls

Samples with normal APTT are shown as a control for the APTT mixing study in Figure 3.6. Some of the normal samples have negative ICA values because the APTT of the undiluted plasma is lower than the APTT of the normal plasma. None of the samples with normal APTT have ICA values higher than the cut-off.
Figure 3.4: Division of known samples. How the ICA cut-off value of 16.0 (blue line) divides the known samples. There are both positive and negative predictions of all four types of causes.

Figure 3.5: Test of cut-off value. Mixing study (ICA) of routine samples with prolonged APTT and how the ICA cut-off value of 16.0 classifies the samples according to the presence of inhibitors. 66 out of 119 samples are classified as containing an inhibitor and 53 out of 119 as not containing an inhibitor.

Figure 3.6: Mixing study (ICA) of routine samples with normal APTT. All samples have ICA values lower than 16.0.
3.3 Part 3: Pre-analytical variables

In this section, the results obtained in project part 3 concerning pre-analytical variables as shown. First the results regarding the effect of centrifugation force are presented. Then the part investigating how storage affects APTT is discussed. Finally, the effect of one freeze-thaw cycle on the APTT is presented.

3.3.1 Centrifugation and platelet count

The platelet counts for samples centrifuged at 2000 g for 10 minutes ranged from 8 - 93 x 10⁹/L. Four out of 64 samples (6%) have a platelet count below 10 x 10⁹/L (Figure 3.7). The samples centrifuged at 3000 g for 10 minutes have platelet counts ranging from 2 - 34 x 10⁹/L where 43 out of 64 samples (67%) have a platelet count lower than 10 x 10⁹/L (Figure 3.7). One outlier is removed justified by the fact that the platelet count for 3000 g (82 x 10⁹/L) is much higher than the platelet count for 2000 g (46 x 10⁹/L). This is likely to be a measurement error.

Figure 3.7: Plasma platelet count. Graph of platelet counts in plasma samples centrifuged at 2000 g and 3000 g. The red line indicating the 10 x 10⁹/L guideline [6]. 6% and 67% of the samples centrifuged at 2000 g and 3000 g respectively, have platelet counts less than 10 x 10⁹/L.

How the centrifugation, and thereby platelet count, affects the routine APTT measurements is shown in Figure 3.8. Linear regression (Figure 3.8a) shows a high correlation with R² of 0.994 and a regression equation of y = 1.03x -0.95. A good agreement between APTT of the two centrifugation methods is shown in a Bland-Altman plot (Figure 3.8b). All samples are within the 95% limit of agreement of -1.75 – 2.31 s and the mean difference (bias) is 0.28 s.

3.3.2 Storage

The effect on APTT of storage at 4°C for two hours is shown in Figure 3.9. The linear regression (3.9a) shows a good correlation with R² of 0.984 and a regression equation of y = 0.908x + 2.8. A 95% limit of agreement of -3.03 - 3.92 s is shown in the Bland-Altman plot (3.9b). This shows a relatively good agreement between APTT measurements before and after storage at two hours at 4°C. One point is above the upper 95% limit of agreement.

How storage at room temperature for two hours affects APTT measurements is shown in Figure 3.10. A high correlation can be seen from the linear regression with R² of 0.822 and
Chapter 3. Results

Figure 3.8: Centrifugation. Linear regression (a) and Bland-Altman plot (b) APTT in samples centrifuged at 2000 g and 3000 g. No systematic difference between the centrifugation methods is shown in the Bland-Altman plot. All the differences and the identity line (red line) are within 95% limit of agreement -1.75 – 2.31 s (black lines). The average difference (bias) of the results is 0.28 s (marked with blue line).

Figure 3.9: Storage at 4°C. Linear regression (a) and Bland-Altman plot (b) of APTT in samples stored two hours in a fridge compared to the original APTT measurement at time 0. The linear regression shows a good correlation between the measurements. No systematic difference caused by the storage is shown in the Bland-Altman plot. The average difference (bias) is 0.44 (marked with blue line). All but one point are within 95% limit of agreement -3.03 - 3.92 s (marked with black dotted lines). The identity lines for both plots are marked with red lines.

A regression equation of \( y = 1.02x + 0.31 \). The Bland-Altman plot, on the other hand, reveals a systematic difference between the APTT before and after 2 hours storage at room temperature. A relatively wide 95% limit of agreement of -11.21 - 9.38 s indicates that the storage at room temperature affects the sample and the measurement of APTT. Samples with APTT in the higher range have lower APTT after storage. One point falls under the lower 95% limit of agreement.

The APTT of the routine samples used to test the cut-off value in Part 2 is measured both before and after storage. The samples are first stored at room temperature and/or in a fridge for a maximum of four hours before the plasma is transferred to new tubes and the samples are frozen at -20°C. How this storage process, which includes one freeze-thaw cycle, affects the samples and the APTT measurements is displayed in Figure 3.11. Both the samples
Chapter 3. Results

Figure 3.10: Storage at room temperature. Linear regression (a) and Bland-Altman plot (b) of APTT in samples stored two hours at room temperature and the original APTT measurement at time 0. The linear regression shows a good correlation between the measurements. A systematic difference caused by the storage is shown in the Bland-Altman plot. The average difference (bias) is -0.91 s (blue line). All but one point are within 95% limit of agreement -11.21 - 9.38 s (black lines). The identity lines for both plots are marked with red lines.

with normal (25 - 35 s) and prolonged (>45 s) APTT (at time 0) are included. Two samples with APTT higher than the measurement range of APTT (>240 s) are removed.

Figure 3.11: Storage freeze-thaw. Linear regression (a) and Bland-Altman plot (b) storage of routine samples used for the mixing study (part 2). The APTT at time 0 is compared to the APTT measurement repeated after storage. The linear regression shows low correlation between the measurements. The 95% limit of agreement is -38.92 - 27.62 (black lines) and a bias of -5.65 (blue line) is found. The identity lines for both plots are marked with red lines.

Both the linear regression (3.11a) and the Bland-Altman plot (3.11b) reveal that the storage has an effect on the APTT measurements. The samples with normal APTT (25 - 35 s) seem less affected than the samples with prolonged APTT (>45 s). For the samples with prolonged APTT, there is generally low agreement with a wide spread to both sides of the identity line. A group of samples with APTT >75 s have lower APTT after storage, where some of these fall below the lower 95% limit of agreement of -38.92 s.
Chapter 4

Discussion

In this section, the results obtained throughout this project are discussed. First, the retrospective study (Part 1) are discussed including the findings of two other retrospective studies from the literature. Secondly, the results of the APTT mixing (Part 2) is discussed. The methods to evaluate the results of the APTT mixing study and to establish a cut-off value is discussed and compared to cut-off values found in other studies. The last part concerns the study of the pre-analytical variables relevant to APTT.

4.1 Part 1: Retrospective study

The main objective of the retrospective study is to investigate how an unexpected prolonged APTT is evaluated today. The retrospective study indicates that the majority off specialized coagulation tests performed at Rigshospitalet are requested by clinical departments specialized in hematology and bleeding disorders. This indicates that patients with unexpected prolonged APTT need to go through these specialized clinical departments in order to find out if their prolonged APTT is associated with a bleeding tendency or potentially a risk of thrombosis.

More detailed knowledge about the patient’s history is needed to evaluate the cause of the prolonged APTT for all patient cases in the data set. The prolonged APTT in some of the patients cases does seem to be caused by coagulation factor deficiencies or LAC.

A retrospective study of 8069 patients undergoing surgery performed by Tagariello et al. investigates the incidence of prolonged APTT and how this affects surgery [3]. They found that 463 samples had mildly prolonged APTT ratio $>1.20$ and 240 had prolonged APTT ratio $>1.3$. Vitamin K antagonists caused 201 of these prolonged APTT samples. LAC was detected in 7 patients, four had multiple factor deficiencies due to liver disease, three had factor XII deficiency, two had known mild hemophilia A and one had mild combined factor XI/XII reduction. The remaining 22 were for some reason not investigated. Since surgery was performed safely in all patients, they argue that pre-operative blood testing is unnecessary and can be replaced by collecting a thorough patient history combined with physical examinations [3].

Chng et al. investigated the causes of prolonged APTT in an acute care general hospital in Singapore [22]. They found that 94 of 177 samples with prolonged APTT (normal PT and thrombin time (TT)) were due to LAC. Deficiencies of factor VIII, XI and XII were found in three, six and nine samples respectively. They conclude that a prolonged APTT in an

\[^{1}\text{APTT ratio to normal plasma}\]
acute hospital setting is usually not related to a bleeding tendency. They suggest using an algorithm to investigate the cause of a prolonged APTT where the first step is the APTT mixing study [22].

The approach and the studied populations differ between the two studies which might explain the difference in the number of samples with LAC and anticoagulant drugs found. Both studies described here find that a prolonged APTT is seldom caused by coagulation factor deficiencies associated with increased bleeding risk. They do, however, not agree about whether to perform the APTT or not. To perform APTT or not is a discussion about on one hand to avoid unnecessary blood tests and on the other hand to be able to rule out a risk of unnoticed bleeding tendency. The APTT alone cannot tell if a patient has a bleeding disorder but in corporation with the mixing study it can be a useful screening tool to prevent potentially dangerous situations involving bleeding patients.

Factor deficiencies and LAC is both found to be the likely cause of some of the prolonged APTTs in the retrospective study performed in this project. It is therefore relevant to investigate how the evaluation of an unexpected prolonged APTT can be improved.

4.2 Part 2: APTT mixing study

4.2.1 Interpretation of APTT mixing study, ROC curve and best cut-off value

When comparing the two methods to interpret mixing study results it can be seen that a better split between samples with and without inhibitors can be achieved by calculating the ICA value. This can be concluded from the graphs in Figures 3.1 and 3.2 as well as the ROC curves in Figure 3.3. When defining the best cut-off value as the value that gives the highest possible sensitivity and specificity simultaneously an ICA cut-off value of 16.0 is found in this study. This cut-off separates the known samples with both a 90% sensitivity and specificity. If instead the result of the APTT mixing study is used directly the best cut-off would be 36.9 s. The cut-off value of 36.9 splits the known samples with a slightly higher sensitivity of 93% while achieving a lower specificity of 67% compared to the ICA cut-off.

Interpretation of the APTT mixing study result is also investigated in a study by Kumano et al. [16]. In this study, they evaluate the use of ICA and the direct use of the mixing study result for different APTT reagents including APTT-SP. They calculate cut-off values as the 99th and 95th percentile of 41 LAC-negative samples with prolonged APTT and of 41 samples from healthy donors with normal coagulation tests. They compare the cut-off values to 22 LAC positive samples and calculate the specificity and sensitivity for each cut-off value. Using the reagent APTT-SP, and the 95th percentile, they get cut-off values of 14.8 for samples from healthy donors and 14.4 for LAC negative samples. From their data, these cut-off values result in sensitivities of 86% and 91% for healthy samples and LAC negative samples respectively and specificity of 100% for both sample types [16].

Kumano et al. calculate the cut-off values as the 99th and 95th percentile because ISTH recommends establishing a cut-off value by performing the APTT mixing study on plasma from healthy donors and taking the cut-off as the value above the 99th percentile of the distribution. As an alternative method, ISTH recommend calculating ICA [6]. Taking the cut-off as the value above the 99th percentile of the distribution is a different approach to establish a cut-off value than the machine learning approach used in this project. A cut-off value established by the 99th percentile of healthy donors will often have a tendency to secure high specificity but not necessarily a high sensitivity since it can increase the amount of false negative predictions.
Figure 3.4 shows how the ICA cut-off value of 16.0 separates the four types of know samples used in this project. Some of the samples without inhibitors are falsely classified as containing an inhibitor and vice versa.

Deciding on a cut-off value will always be a trade-off between sensitivity and specificity. The question is if false positive or false negative predictions are associated with the greater risk for the patients. According to ISTH false positive LAC predictions should be avoided due to the risk of long and unnecessary oral anticoagulant treatment [6]. Implementing the APTT mixing study as an extra test to indicate the cause of a prolonged APTT is meant to be a tool to ease the evaluation of an unexpected prolonged APTT and not alone lead to decisions about treatment e.g. with anticoagulant drugs. When the APTT mixing study indicates the pretense of an inhibitor, a new blood test for analyzing the presence of LAC should be requested. If the APTT mixing study instead indicates that no inhibitor causes the unexpected prolonged APTT the next step will be to request samples for coagulation factor activity. When patients are on anticoagulant treatment there is no need for additional blood tests since the anticoagulant drugs will cause a prolonged APTT. LAC testing should in general not be performed on patients receiving anticoagulant treatment [6, 10].

4.2.2 Test of cut-off value

When applying the "train-test" method it is normal to use the majority of a data set to train the algorithm and the rest of the data set to test the algorithm. This is convenient when working with a large data set. In this project, the data set used to train the algorithm (establish a cut-off value) is composed of 102 samples with known causes of prolonged APTT. Since a larger dataset is needed before it can function as both training and test data sets, a different approach is used in this project.

The established cut-off value is instead tested using routine samples with prolonged APTT. Another reason to tests the cut-off using routine samples is that these samples represent the type of samples the mixing study will be applied on in the future. This way the cut-off value is tests using the correct pre-analytical conditions.

Figure 3.5 shows how the ICA cut-off value of 16.0 classifies the routine samples with prolonged APTT. Some of the APTT samples analyzed at the department come from in-house patients in anticoagulant heparin treatment. It is therefore expected that some of the samples shown in Figure 3.5 are inhibited by heparin. As the cause of the prolonged APTT is not known for the routine samples, a new study using routine samples with known causes of prolonged APTT has to be conducted.

The samples with normal APTT shown in Figure 3.6 indicate that the ICA cut-off value of 16.0 does not falsely classify normal samples as containing an inhibitor.

To summarize, the results of Part 2 show that the APTT mixing study can be used to indicate if a prolonged APTT is caused by an inhibitor or not. The cut-off that gives the highest possible sensitivity and specificity is found by calculating the ICA value and is for the samples used in this project found to be 16.0. Routine APTT samples with a known cause of prolonged APTT is necessary to validate the cut-off value.

4.3 Part 3: Pre-analytical variables

4.3.1 Centrifugation and platelet count

The plasma platelet counts achieved by centrifugation at 2000 g and 3000 g is shown in Figure 3.7. Neither centrifugation for 2000 g nor 3000 g (10 minutes) secures platelet-poor
plasma with platelet count below the ISTH guideline of $10 \times 10^9$ /L for all samples [6]. The samples centrifuged at 3000 g are closer to fulfill the guidelines with 67% of the samples having a platelet count lower than $10 \times 10^9$/L. Only four out of 64 samples (6%) of the samples centrifuged at 2000 g have a platelet count below $10 \times 10^9$/L. Since the majority of the APTT routine samples are centrifuged at 2000 g for 10 minutes, these results show that the routine samples do not meet the ISTH guideline according to platelet count. The ISTH guidelines are prepared for the purpose of LAC detection and do not refer to the appropriate platelet count for other coagulation tests [6].

Influence of the different centrifugation forces on routine APTT is shown in Figure 3.8. The linear regression shows a good correlation between the APTT measurements of the centrifugation methods with a $R^2$ of 0.994. The 95% limit of agreement of -1.75 – 2.31 and bias of 0.28 from the Bland-Altman plot show good agreement between APTT for the two centrifugation forces. These results indicate that it makes no difference for routine APTT samples whether the sample is centrifuged at 2000g or 3000g for 10 minutes. It thereby also suggests that platelet count does not influence routine APTT measurements. It has been shown that routine coagulation tests, including APTT, are not affected by platelet counts up to $200 \times 10^9$/L when measured on fresh samples [20, 23].

Though it does not seem to be crucial to achieve platelet poor plasma for routine APTT it might still be important when measuring LAC or heparin [6, 12, 20, 23].

The samples used for the centrifugation study in this project come from unknown patients. It is therefore not possible to say whether any of the samples contain LAC or heparin. Though no difference is found between APTT of samples centrifuged at 2000 g and 3000 g in Figure 3.8 only a few samples have prolonged APTT (>45 s). It is thereby likely that the majority of the samples used in this study do not contain inhibitors such as drugs or LAC. It can, therefore, be speculated that the platelet count will have more influence on the APTT measurements of samples with APTT > 45 s, than seen in Figure 3.8.

When using an APTT mixing study to indicate the cause of a prolonged APTT it is crucial that e.g. LAC or heparin is not affected by platelets in the samples. A platelet count higher than $10 \times 10^9$/L might, therefore, be a problem and routine centrifugation at 2000g for 10 minutes will not be sufficient. One possible way to avoid this problem is to repeat the centrifugation at 3500g for 15 minutes before performing the APTT mixing study.

The number of platelets left in the plasma after centrifugation can also be important when samples are frozen before they are analyzed. This is due to lysis of any remaining platelets during a freeze-thaw cycle releasing phospholipids to the plasma [20].

Platelet-poor plasma is achieved for the pooled normal plasma produced and used in this project. This shows that double centrifugation does result in a platelet count below $10 \times 10^9$/L and that the pooled normal plasma in this project is not a source of interfering phospholipids.

4.3.2 Storage

The Bland-Altman plot, of the difference between APTT at time 0 and after two hours at room temperature, show a wide 95% limit of agreement (-11.21 - 9.38s). The differences are both positive and negative for the samples with APTT less than 50s. The samples with higher APTT (>50s) all have a lower APTT measurement after two hours at room temperature than at time 0. These samples may contain heparin since it is known that heparin can be neutralized in a sample. It has been shown that heparin can be underestimated after storage for two hours [12]. The same study concluded that room temperature is the best for storage of Heparin samples [12]. Another possible explanation to the lower APTT after storage can be lysed platelets in the sample affecting LAC detection. The wide 95% limit
of agreement in the Bland-Altman plot implies that APTT is not stable when stored two hours at room temperature.

The samples stored at 4°C for two hours show a better agreement with the measurements at time 0, with 95% limit of agreement of -3.03 - 3.92s. The differences are evenly distributed for all levels of APTT. The results of the storage study imply that APTT is stable after two hours at 4°C.

According to local guidelines, APTT is stable at 4°C up to four hours after centrifugation. Samples are continuously stored in the fridge during the day but can sometimes be left at room temperature for some time before being stored. The routine APTT samples used in this project are collected maximum four hours after the first APTT measurement. It was not possible to control for how long they were stored at room temperature.

The low correlation ($R^2 = 0.765$) and agreement (95% limit of agreement is -38.92 - 27.62) for APTT measured before and after storage shown in Figure 3.11 might be due to a combination of several factors. First of all, the time and temperature at which the samples are stored before they are frozen might affect the APTT. Secondly, the freeze-thaw cycle can affect the samples. From Figure 3.11 is can be seen that samples with normal APTT (25-35 s) are hardly affected by storage opposite samples with prolonged APTT (>45 s) that are highly affected by storage. These results show the importance of performing the APTT mixing study on fresh samples instead of samples that have been stored for later analysis.

In summary, centrifugation force does influence the plasma platelet count but does not seem to influence APTT of fresh routine samples. Achieving platelet poor plasma might still be beneficial when measuring samples containing LAC or heparin and when samples are frozen prior to analysis.

APTT seem to be more affected when samples are stored at room temperature compared to storage at 4°C for two hours. Storage of the routine samples used to test the cut-off value in Part 2 (Figure 3.11) has affected the APTT measurements. To avoid errors due to sample storage, the APTT mixing study should be performed on fresh samples.
Chapter 5

Conclusion

Based on this project, it is clear that it is possible to use the APTT mixing study to indicate the cause of a prolonged APTT. From the samples used in this project, the best separation of samples with and without inhibitors is achieved by using an ICA cut-off value of 16.0. This cut-off results in a sensitivity and specificity of 90%. To further test the validity of the cut-off, fresh routine samples with a known cause of prolonged APTT should be used.

It can be seen from the retrospective data that special coagulation testing is generally controlled by clinical departments specialized in hematology and bleeding disorders. Implementing the APTT mixing study can be a way to guide physicians with other specialists when discovering an unexpected prolonged APTT.

The study of pre-analytical variables and their effect on APTT measurements shows the importance of including pre-analytical considerations when working with blood tests.

A way to secure a low amount of platelets in plasma for the APTT mixing study can be to repeat the centrifugation before analysis. But more importantly, the APTT and the APTT mixing study should be performed on fresh plasma samples and not stored for later analysis.

There is an immense potential for reducing costs and improve treatment quality and time by introducing the APTT mixing study as an extra test to indicate the cause of a prolonged APTT. The immediate next step should be to validate the established cut-off value using fresh routine samples with a known cause of prolonged APTT.
Appendix A

Appendix A: List of blood tests included in the retrospective data set

- P-Antithrombin; arb.stofk. AT3ENZ
- Antithrombin; (imm.; IS 93/768);P AT3AG
- P-Apixaban: massek. APIXA
- Cardiolipin-antistof(IgG);P CARDIGG
- Cardiolipin-antistof(IgM);arb.stofk.;P CARDIGM
- P-Dabigatran: massek. DABI
- P-Fibrin D-dimer; arb.stofk. (mg/l FEU) DIMER
- P-Fibrinogen FIB
- P-Heparin, lav molmasse HEPARIN
- P-Koagulation, overfladeindtroduceret (APTT); tid APTT
- P-Koagulationsfaktor II (koagulation); arb.stofk. KF2
- P-Koagulationsfaktor II+VII+X(koagulation); arb. stofk. KF2710
- Koagulationsfaktor II+VII+X [INR];P INR
- P-Koagulationsfaktor II-antistof; arb.stofk. KF2AB
- P-Koagulationsfaktor VIII(koagulation); arb. stofk. KF8
- P-Koagulationsfaktor VIII-antistof; arb. stofk. KF8AB
- Koagulationsfaktor V (koag.);P KF5
- Koagulationsfaktor V-Ab (koag.);P KF5AB
- Koagulationsfaktor VII; (koag.; IS 09/172);P KF7
- Koagulationsfaktor VII-Ab (koag.);P KF7AB
- Koagulationsfaktor IX; (koag.; IS 09/172).;P KF9
• Koagulationsfaktor IX-Ab (koag.); P KF9AB
• Koagulationsfaktor X; (koag.; IS 09/172); P KF10
• Koagulationsfaktor X-Ab (koag.); P KF10AB
• Koagulationsfaktor XI (koag.); P KF11
• Koagulationsfaktor XI-Ab (koag.); P KF11AB
• Koagulationsfaktor XII (koag.); P KF12
• Koagulationsfaktor XII-Ab (koag.); P KF12AB
• Koagulationsfaktor-antistof; (overfl.induc.koag.; 0 1); P KFAB
• Koagulationsfaktor XIII (enz.); P KF13
• Lupus antikoagulans; P LUPUS
• Lupus antikoagulans, konfirmatorisk; P LUPUSKO
• P-Protein C (enz.; Coamatic); arb.stofk. PROTE
• P-Protein S (frit) PROTSFR1
• P-Rivaroxaban; massek. RIVA
• P-von Willebrand-faktor (antigen); arb.stofk. VWFK
• P-von Willebrand-faktor (Ristocetin Cofaktor aktivitet); arb.stofk. (kIU/l) VWFRIST
• P-Choriongonadotropin + beta [HCG] HCG+BETA
• P-Choriongonadotropin beta-kæde; arb. stofk. hcgbeta
• P-Pregnancy-associated plasma protein A; arb stofk. (IU/L) PAPPAK
• B-Thrombocytter; antalk. (PLT) THROM
• P-Creatininium; stofk. CREA
• P-Alanintransaminase ALAT
• ADAMTS13 protein; P ADAM13
• ADAMTS13-antistof; P ADAM13AB
Bibliography


