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Rapid, hand-held paper diagnostic for measuring Fibrinogen Concentration in blood

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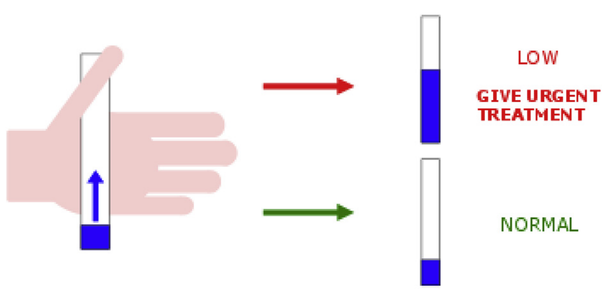
HIGHLIGHTS

- We have developed a new hand-held, paper-based blood diagnostic which can measure fibrinogen concentrations.
- Testing is completed within 1 minute and needs only 3 μL of plasma – making it ideal for diagnosing hypofibrinogenemia.
- The test can withstand acidosis, alcohol use, warfarin use, severe haemolysis and severe hypertriglyceridemia.
- The test can also withstand a wide range of temperatures and humidities making it suitable for outdoor use.
- The test has the potential to revolutionise point of care testing and save two million lives each year.

GRAPHICAL ABSTRACT



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ABSTRACT

Critical bleeding causes over 2 million deaths a year. Early hypofibrinogenemia is a strong predictor of mortality in critically bleeding patients. The early replenishment of fibrinogen can significantly improve outcomes. However, over replenishment can also be dangerous. Furthermore, there is no rapid, cheap, hand-held diagnostic that can aid critically bleeding patients in fibrinogen replacement therapy. In this study, we have developed a hand-held paper diagnostic that measures plasma fibrinogen concentrations. The diagnostic has the potential to be used as a point of care device both inside and outside of hospital settings. It can vastly reduce the time to treatment for fibrinogen replacement therapy. The diagnostic is a two-step process. First, thrombin and plasma are added onto horizontially-orientated paper strips where the fibrinogen is converted into fibrin, drastically increasing the plasma's hydrophobicity. Second, an aqueous blue dye is pipetted onto the strips and allowed to wick through the fibrin. The distance the blue dye wicks through the strip correlates precisely to the fibrinogen concentration. The diagnostic can provide results within a minute. It can distinguish low fibrinogen concentrations (ie. <2 g/L) from normal fibrinogen concentrations. It shows remarkable reproducibility between healthy individuals. It is unaffected by common blood conditions such as acidosis, blood alcohol, severe hypertriglyceridemia, severe

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Coagulopathy
Fibrinogen concentration

haemolysis and warfarin administration. Finally, it is unaffected by humidity and can withstand cold temperatures.

1. Introduction

This study aims to develop a rapid hand-held paper diagnostic for measuring fibrinogen concentrations in plasma. The diagnostic can revolutionise the treatment of heavily bleeding patients. It can vastly reduce the time to treatment before fibrinogen replacement therapy. Furthermore, it can be used as a point of care device in a multitude of locations - not only in hospital emergency rooms, but also in ambulances or outdoors at the site of injury.

Fibrinogen is the effector clotting protein in blood. It is normally present in the plasma at concentrations between 2 and 4 g/L [1,2]. When bleeding is induced, fibrinogen is polymerized into fibrin at the site of blood vessel breakage [3,4]. It then forms the backbone to clots to stop bleeding [5–9]. Patients with hypofibrinogenemia are at a significantly greater risk of mortality due to critical bleeding [10,11]. The early depletion of fibrinogen can not only cause weaker clots to develop, but also increase the clot's susceptibility to degradation [12,13].

Critical bleeding can occur anywhere in the world. Outside clinics, trauma-induced major haemorrhage is the leading cause of death for people aged 18–39 - contributing two million deaths worldwide a year [14–20]. Inside clinics, critical bleeding can arise as a result of childbirth (75% of all cases), cardiac surgery, liver transplant and post-operative complications [21–25]. The incidence of hypofibrinogenemia in critical bleeding is common. In 2017, McQuilten et al. identified that 21.2% of major trauma patients had fibrinogen levels below 2 g/L [26]. Fibrinogen is one of the first proteins to be depleted in critically bleeding patients [27]. Hence, the early replenishment of fibrinogen levels can significantly improve outcomes [28–30].

When hypofibrinogenemia is detected in critically bleeding patients, fibrinogen replacement therapy (FRT) is used. European clinical guidelines state that FRT should be implemented when plasma fibrinogen concentrations fall below 2 g/L [31]. However, FRT needs to be handled carefully to prevent unnecessary fibrinogen supplementation. Over-supplementation can lead to thrombotic episodes, induce iatrogenic effects and waste expensive resources [32,33].

Traditionally, fibrinogen replacement therapy (FRT) was provided indirectly through fixed volumetric transfusions [34]. This consisted of fixed volumetric ratios of donor red blood cells, platelets, cryoprecipitate and fresh frozen plasma (FFP) being transfused into critically bleeding patients. Since these components had all the blood products needed for clotting, there was no need for a diagnostic step to determine the causes of bleeding. However, fixed volumetric transfusions have been scrutinized because: 1) The time required to thaw and transfuse both cryoprecipitate and FFP is over an hour [35]. 2) The contents of the cryoprecipitate and FFP are unstandardized [36,37]. 3) The transfusion of cryoprecipitate or FFP can cause dangerous side effects [20,36,38–40]. 4) Even though the FFP and cryoprecipitate contains fibrinogen, the transfused red blood cells and platelets dilute the patient's fibrinogen to be below the threshold levels for clotting [41–43].

Recently, the availability of lyophilized clotting factors has allowed major haemorrhage protocols to be carried out more safely. Therefore, FRT has been successfully implemented by using fibrinogen concentrate (FC). Rahe-Meyer et al. 2011 reported that FC only needs a supplementation time of 5 min [20]. However, a means of diagnosing hypofibrinogenemia must be executed to prevent the inappropriate supplementation of FC.

The main clinical assays currently used to detect hypofibrinogenemia are unreliable. The Clauss assays are the most common standard laboratory technique for quantifying fibrinogen concentration. They do so by taking a plasma sample, adding thrombin and measuring the time taken for fibrin formation to occur. Longer times correlate to lower fibrinogen concentrations [44]. Likewise, viscoelastic haemostatic assays have risen in popularity in the last twenty years. This is due to their ability to quantify numerous clotting factors in whole blood per assay. Viscoelastic haemostatic assays measure fibrinogen through the change in viscoelasticity when fibrin is formed [45]. However, both assays require well controlled, sensitive and expensive instruments with long assaying times and have discrepancies in accuracy [46].

Therefore, a rapid, cheap, portable and hand-held plasma fibrinogen concentration diagnostic is needed to greatly reduce the time to treatment needed for fibrinogen replacement therapy.

In our previous study, we have developed a paper test based on the principles of vertical wicking [47]. First, plasma is added onto paper strips pre-wetted with thrombin. Second, the test is placed vertically into an infinite reservoir of blue dye. The fibrinogen concentration can be measured by how far the blue dye wicks up the strip over a set amount of time. This is because when the fibrinogen in plasma reacts with the thrombin to form fibrin, it increases the hydrophobicity of the strip. This in turn decreases the blue dye wicking rate. The higher the fibrinogen concentration, the stronger the strip hydrophobicity and hence the lower the blue dye wicking height. Therefore, the tests of patients with hypofibrinogenemia will produce further wicking than healthy people.

However, the vertical wicking test has limitations that confines it to research laboratories. First, the test-setup is not easy-to-use. The infinite reservoir must rest on a stable horizontal surface and the paper strips need to be placed stationary in it for accurate reading. This is not feasible in outdoor scenarios. Second, the test is limited by gravity. As the strip must be held vertically, the rate at which the blue dye moves up the strip is slower than what it would be in a flatter orientation. Therefore, we aim to modify this test so that it is better suited as a rapid, hand-held point of care device. We intend to achieve this by not-only substituting the infinite reservoir with pipetting droplets of blue dye on the strip, but also by orientating the strips horizontally instead of vertically.

In this paper, we develop a rapid and sensitive paper-based test that can be hand-held horizontally for point of care use. We investigate the effect that different human plasma samples have on the test - to conclude wherever the test varies between healthy individuals. We compare its performance to a hospital-grade Clauss assay. Finally, we investigate the effect of common blood abnormalities and weather conditions. This includes the effects of acidosis, alcohol, heparin, warfarin, haemolysis and dietary lipids as well as surrounding temperatures and humidities. It is our objective to show the proof of concept of a point of care paper fibrinogen diagnostic.

2. Materials and methodology

2.1. Materials

Tissue paper of 30 GSM and 0.16 mm was used to create the paper strip cutouts. They were produced in the Kimberly Clark Experimental Forming Unit (EFU), Neenah (WI), USA. Tissues consist of eucalyptus fibres formed with a 3 layer headbox. 10 kg/T Kymene, a PAE wet strength agent, was added to each layer. Tissues were moulded and thoroughly air-dried. Two 1×1 mm^2 square steel rods were cut into 35 mm lengths to hold the paper strips horizontally. J Burrows Magnets were used to attach the paper strip cut-outs to the steel bar. A $7.5 \times 12.6 \times 26.7$ cm^3 Falcon tube holder was used to hold the metal bars in place and keep paper strip cut-out suspended over the tabletop. A schematic of the setup is shown Supplementary Figure 1.

Brilliant Blue FCF (from Queen Blue Food Colouring) was used to make the blue dye (for wicking). Ponceau 4R (from Queen Red Food Colouring) was used to make the red dye (for colouring the thrombin solution).

3 lots of citrated plasma from healthy samples (with fibrinogen concentrations of 2.8 g/L, 3.2 g/L and 3.3 g/L) were sourced from R2 diagnostics. Each plasma sample was pooled from the platelet-poor plasma of at least fifteen different donors. Thrombin (T8885), unfractionated heparin (H5515), warfarin (PHR1435), triglycerides (17810), hydrochloric acid solution (150696), sodium chloride (S7653) and the haemoglobin assay kit (MAK115) was sourced from Sigma Aldrich. Absolute ethanol (AJA214) was sourced from Ajax Finechem. Ethylenediaminetetraacetic acid (EDTA) anticoagulated whole blood was sourced from the Australian Red Cross. Soy wax (NatureWax® C3 Container Blend) was sourced from Candle Supply.

MilliQ water was used to dilute the dyes to the required concentrations and reconstitute vials of citrated plasma and thrombin.

"Tubes PCR Strip Tubes 0.2 ml Thin Wall & Flat Caps" from Axygen were used as the tubes for mixing. They are referred to as PCR tubes throughout the paper.

An iPhone 8 with the app ProMovie was used to record wicking. A Yellowstone 60″ Tripod was used to hold the iPhone in place during recording. A Manfrotto Universal Smartphone Clamp was used to attach the iPhone to the tripod.

3. Methodology

3.1. Paper strip cut-out preparation

Paper strip cut-outs were prepared as such on CorelDRAW X6 with the dimensions shown in Supplementary Figure 2. Paper was cut on a Laser Cutter (60 Watt Epilog Helix) using the cut-outs prepared on CorelDRAW X6. The print settings used were 100% speed, 5% Power and 500 Hz Frequency. A Petri dish filled with soy wax was melted at 150 °C on a Corning hot plate (#6795-420D). Once molten, 10 μL of molten wax was applied across the bottom of each strip below the dye pad. This ensured that the blue dye only travelled in one direction.

3.2. Plasma sample preparation

Vials of citrated plasma were fully reconstituted with 1 mL of distilled water each. This plasma is referred to as normal or healthy plasma throughout the paper. Then, 50 μL aliquots of normal plasma were pipetted into Eppendorf Tubes and snap-frozen in liquid nitrogen. They were later stored at −86 °C until used.

Defibrinogenated plasma was prepared through heat precipitation. Normal plasma was transferred to Eppendorf Tubes and heated in a water bath (Thermo Scientific Precision GP20) at 56–58 °C for 30 min - to selectively precipitate the fibrinogen. Afterwards, the Eppendorf Tubes were centrifuged at 12500 rpm using a 11124-H rotor for 10 min in a Sigma 2-16P centrifuge. The supernatant - which contained no fibrinogen - was collected, aliquoted 50 μL each into Eppendorf Tubes, snap frozen in liquid nitrogen and stored at −86 °C until used.

When both normal and defibrinogenated plasma samples were thawed, they had to be re-mixed using a pipette - to ensure their contents were uniformly distributed. Plasma samples of different fibrinogen concentrations were prepared by mixing normal plasma and defibrinogenated plasma to the appropriate ratios.

3.3. Thrombin solution preparation

50 μL of Queen Red Food Colouring was diluted in 450 μL MilliQ Water. One vial of thrombin was reconstituted with 300 μL of the solution to make vials of 30 NIH U/mL Thrombin. The vials were then aliquoted 50 μL each into Eppendorf Tubes, snap-frozen in liquid nitrogen and stored at −86 °C until used. (NB: Any food colouring can be used. We used red colouring as it contrasted with the blue dye used in the test and allowed its microfluidics to be observed more easily).

When these thrombin solution samples were unfrozen and thawed, they had to be re-mixed using a pipette - to ensure their contents were uniformly distributed.

3.4. Main test

Experiments were performed in a temperature and humidity controlled room at 23 °C and 50% relative humidity.

0.5 μL of Thrombin Solution was pipetted into the bottom of a PCR tube. Using a pipette, 3 μL of plasma sample was quickly mixed with the thrombin solution until the red colouring was homogenous. It was then applied across the 0 mm marking of the paper strip. The mixed plasma had to be evenly applied across the width of the strip to prevent gaps forming in the fibrin network. This was achieved by quickly pipetting the plasma dropwise onto the left-hand side, right-hand side and centre of the strip – thus forming a zone of hydrophobicity. An example of how mixed plasma was applied to the paper strip is shown in Video 1.

After 30 s, a 30 μL droplet of 5% Queen Blue Food Colouring was applied from the bottom of the dye pad. It was important to ensure that the shear force of the blue dye did not destroy the fibrin network that was formed. Therefore, the blue dye wicking through the network was controlled in two ways. 1) The blue dye was added slowly and continuously over a period of 10 s. 2) The blue dye was added from the bottom of the dye pad (to provide enough room for the droplet to sink in). The wicking length was measured 30 s after the blue dye began to be added to the strip. Fig. 1 illustrates this procedure.

3.5. Wicking length measurement

The midpoint for the wicking front was used to measure the wicking length. The iPhone was held directly over the paper strips and ProMovie (downloaded from the App Store) was used to film the main test. ProMovie was used because it could manually focus on the paper strip markings for analysis (see Supplementary Figure 3). To measure wicking length, snapshots of each strip in the recorded videos were taken 30 s after blue dye was first applied. They were then analysed on ImageJ to determine their wicking length - as shown in Supplementary Figure 4.

3.6. Testing of different substances

Heparin, warfarin, alcohol and lipids were all tested as they are all common substances found in plasma that may have impacted on the test. Solutions of 14 U/mL heparin, 60 mg/L warfarin, 4 g/dL ethanol and 25,000 mg/dL triglycerides were all prepared by

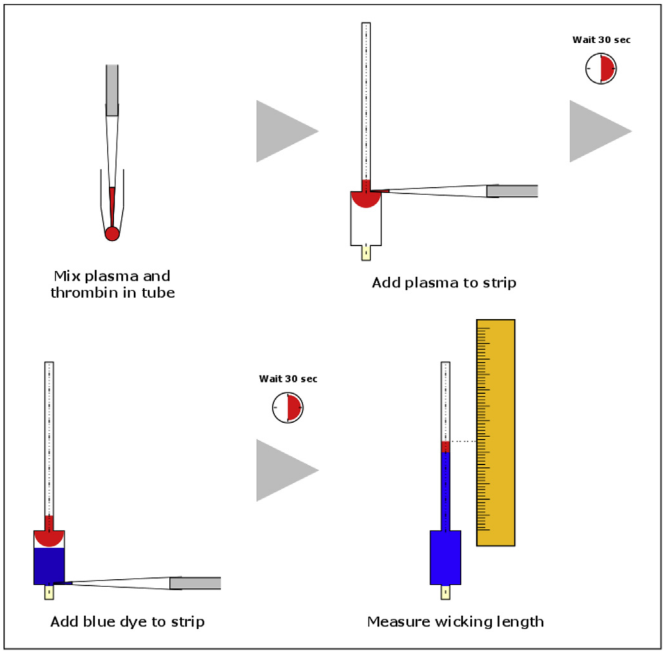

Fig. 1. Schematic illustration of the main test procedure.

dissolving or diluting in MilliQ water.

Each substance was diluted 1:20 times in either normal or defibrinogenated plasma. This created plasma samples with physiologically significant concentrations of each substance. These samples were then tested.

3.7. Testing of acidosis

A pH Meter (Thermo Fisher) was used to record the pH of 2 mL of normal plasma. This was performed in a 15 mL Falcon tube - because it could allow the plasma to reach a high enough level for pH measurement. The pH was measured to be 7.6. 500 μL of normal plasma was initially collected and kept aside.

1 M hydrochloric acid was added carefully to drop the pH of the remaining plasma to 7.3 and another 500 μL was collected. This process was repeated twice more with the remaining plasma further dropped to 7.1 and 6.9 and collected each at 500 μL. The final concentration of hydrochloric acid in the pH 6.9 sample was calculated. Then, the equivalent molar concentration of sodium chloride was added to 250 μL of normal plasma with 1 M sodium chloride. This created a control sample that determined whether any observed effects were driven by decreased pH or increased salt concentration.

Half the volume of each sample was heated at 56–58 °C for 30 min to also prepare defibrinogenated samples at different pHs. These samples were then tested.

3.8. Testing of haemolysis

1 mL of whole blood was pipetted into an Eppendorf tube. It was then centrifuged at 12500 rpm using a 11124-H rotor for 10 min in a Sigma 2-16P centrifuge. The plasma was carefully discarded using a pipette - leaving behind the red blood cells (RBCs). The RBCs were lysed by snap-freezing them in liquid nitrogen and re-thawing them at room temperature three times. This created haemoglobin concentrate. To confirm that the cells had successfully lysed, a 1 μL sample of haemoglobin concentrate was viewed under a Nikon H600L microscope and verified by the lack of cells present.

The concentration of the haemoglobin concentrate was quantified by using the haemoglobin assay kit and following the technical bulletin instructions. The assay was performed on a Tecan Infinite M Nano microplate reader. The haemoglobin concentrate was measured to be 36 g/dL.

1.8 g/dL haemoglobin plasma samples were created by diluting the haemoglobin concentrate 1:20 times in normal and defibrinogenated plasma. Furthermore, a 0.9 g/dL haemoglobin plasma sample was created by diluting the haemoglobin concentrate 1:40 times in defibrinogenated plasma. These samples were then tested.

3.9. Testing of humidity

A dessication chamber (Dry Keeper Sanplatec Corp C–3B) was used and filled with three bottles of silica gel contained in petri dishes to drop the relative humidity from 50% to 30%. The test as per normal was carried out inside the chamber. The door was opened when adding reagents onto the strip but was closed immediately to prevent the humidity from rising.

3.10. Testing of temperature

An incubator (Carun Diurnal Incubator Model 6042) was used to change the temperature to 6 °C, 23 °C, 36 °C and 44 °C. The test setup and all reagents were placed inside the incubator and allowed to equilibrate with the set temperature for 10 min. Afterwards, the test as per normal was carried out inside the chamber. The door was opened when adding reagents onto the strip but was closed immediately to prevent the temperature from returning to room temperature.

3.11. Clauss assay

A Sta-R Analyser - with commercial reagents from Diagnostica Stago (STA® Fibrinogen 5) - was used. This was then compared to the paper test.

4. Results

Paper strips were prepared as shown in Supplementary Figure 2. The dye pad design is deciphered in Supplementary Figure 8. Fig. 2 illustrates the concept behind the test. The videos that Fig. 2 sources from are found in Video 2 (0 g/L fibrinogen) and Video 3 (3.2 g/L fibrinogen).

Plasma and thrombin (dyed red) were quickly mixed with a pipette in a PCR tube until the mixture became homogenously red. The mixture was then quickly pipetted across the 0 mm marking and allowed to react for 30 s. This formed a zone of hydrophobicity. Accurate plasma volumes were confirmed by measuring the equilibrium stain size on paper (Supplementary Figure 13) [48].

A droplet of blue dye was then deposited on the dye pad. The wicking length on paper was measured 30 s after the blue dye was first applied. This was chosen as the most suitable wicking time for this study (Supplementary Figure 10 and 11). It was important that the blue dye passed the fibrin network at a controlled velocity to prevent it from causing damage. This was achieved by: 1) adding it slowly over a period of 10 s, and 2) adding it from the bottom of the strip (to ensure that the droplet had sunk into the paper before it reached the fibrin network).

The further the wicking front migrates to, the lower the plasma fibrinogen concentration. The mechanism is predominantly hydrophobicity-driven. The conversion of fibrinogen into fibrin increases the hydrophobicity of the paper. Therefore, when the blue dye reaches this hydrophobic zone, its contact angle with the paper increases significantly and wicks at a much slower rate. This

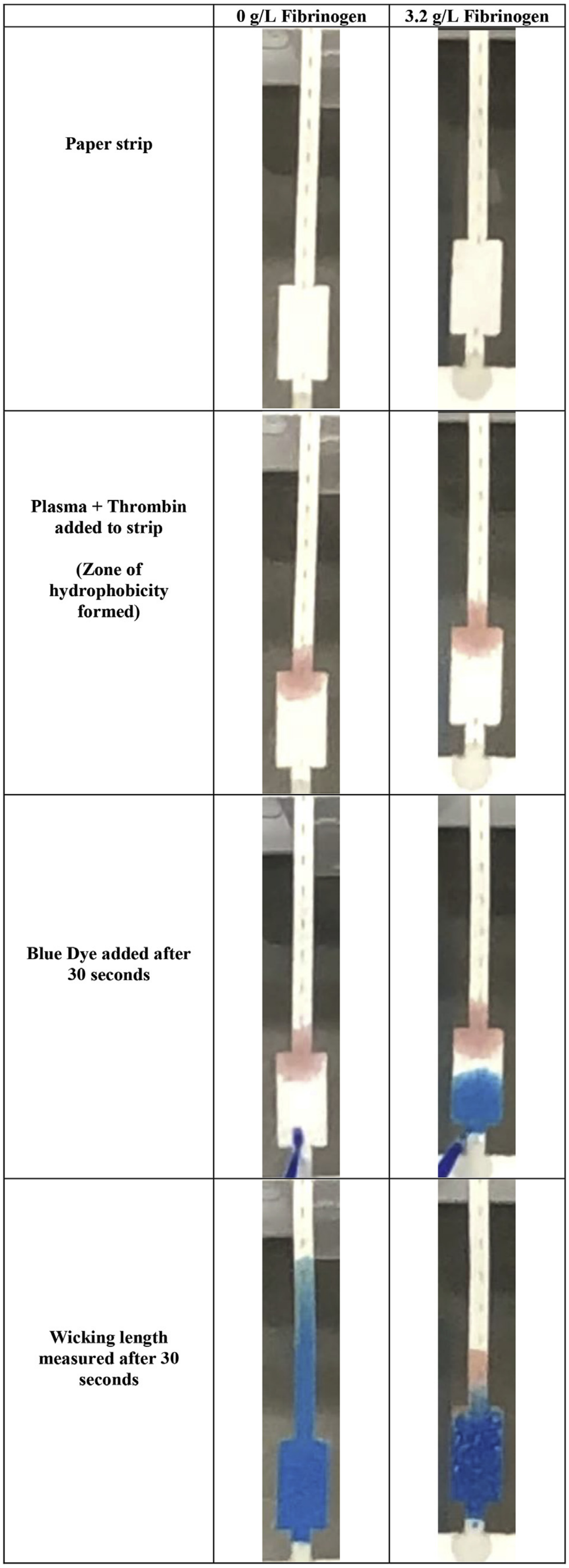

Fig. 2. Summary of wicking test for plasma samples without fibrinogen (0 g/L) and with fibrinogen (3.2 g/L).

decreases the wicking length after 30 s.

The amount of fibrin present on paper dictates the extent of hydrophobicity (in this hydrophobic zone), which controls the wicking lengths reached by the dye droplet. There is a clear and reproducible correlation between the length that the droplet wicks along each paper strip and the plasma fibrinogen concentration. The discovery forms the concept of a hand-held diagnostic for measuring plasma fibrinogen concentrations in blood.

The test was diagnosed using three different healthy plasma samples. Each sample of plasma was pooled from at least fifteen different donors and citrated. The test's sensitivity was compared to an automated Clauss assay (Stago STA® Fibrinogen 5). Finally, the effects of haemolysis, acidosis, alcohol, heparin, warfarin and dietary lipids as well as surrounding temperatures and humidities were also compared.

4.1. Comparison between plasma samples

Different plasma samples were experimented with to verify whether the test varies between healthy individuals. Results are shown in Figs. 3 and 4.

Plasmas from two different samples were artificially made to different fibrinogen concentrations by diluting with defibrinogenated plasma. The wicking length on paper was then measured. This experiment was performed in triplicates and the average and standard deviation are reported in Fig. 3. Each trendline was created from the normal and defibrinogenated plasmas of a single sample. For example, the Plasma 1 trendline featured the dilution of plasma sample 1 with defibrinogenated plasma created from plasma sample 1. There are two observations of interest. First, a non-linear relationship is observed for both curves. Fibrinogen concentrations below 2 g/L displayed optimal sensitivity, as denoted by the strong gradients of the curves. Second, no variability is observed between the curves. The sample the plasma is sourced from does not impact wicking length. The use of defibrinogenated plasma as an appropriate dilutant is shown in Supplementary Figs 7 and 14.

Plasma from three different samples was tested without dilution. Each sample was tested up to twenty times and the normal range (solid block) and total range (line bars) are reported in Fig. 4.

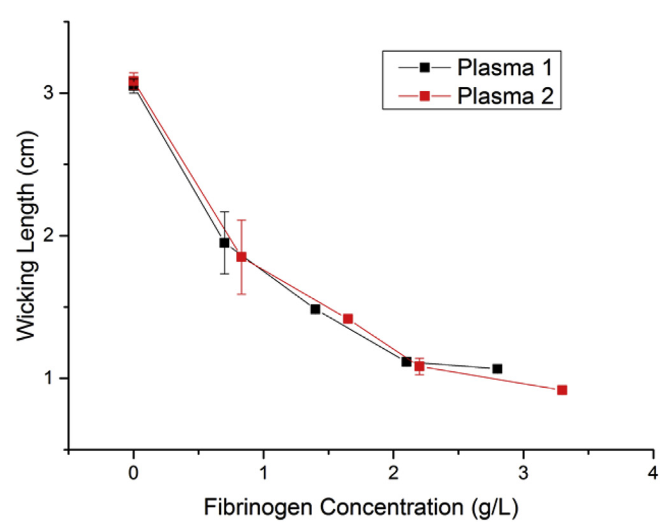

Fig. 3. Testing of plasma samples with different fibrinogen concentrations. Plasma from samples 1 and 2 contained 2.8 g/L and 3.3 g/L fibrinogen respectively before they were diluted with defibrinogenated plasma to different fibrinogen concentrations. Each data-point was tested three times and the mean and standard deviations are reported.

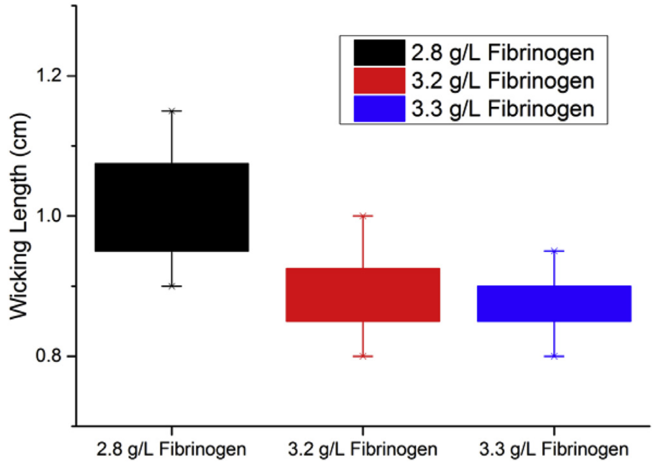

Fig. 4. Testing of normal plasma from three samples. Citrated plasma from samples 1, 2 and 3 contained 2.8 g/L, 3.2 g/L and 3.3 g/L fibrinogen respectively. Each plasma was tested twenty times. The normal range (ie. mean ± standard deviation) is denoted by the solid block, the total range (ie. range of entire set of data) is denoted by the line bars.

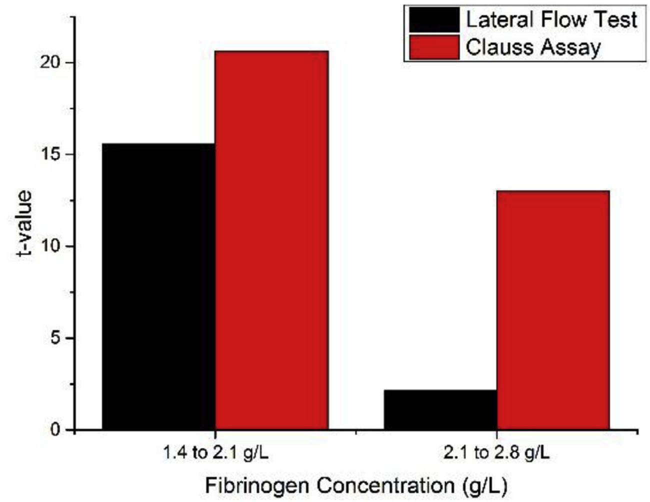

Fig. 5. Analysis of variance between the horizontal wicking assay and the automated Clauss assay (Stago STA® Fibrinogen 5). Defibrinogenated plasma from sample 1 was made to different fibrinogen concentrations and tested on both diagnostics. Samples (1.4, 2.1 and 2.8 g/L) were tested on each diagnostic three times. The means and standard deviations were taken from each diagnostic and used to calculate the t-values across a range of fibrinogen concentrations.

Two observations are present. First, there is a clear relationship between plasma fibrinogen concentration and wicking length. Plasma samples of higher fibrinogen concentrations wick less. Second, even non-optimal fibrinogen concentrations (ie. above 2 g/L) still produced a clear difference in wicking. This is demonstrated by the non-overlapping normal ranges between the 2.8 g/L fibrinogen sample and the other two samples. Therefore, plasma samples in the optimal fibrinogen concentration range (ie. below 2 g/L) are unlikely to overlap at all.

4.2. Comparison to Clauss assay

The sensitivity of the paper test was compared to a hospital grade automated Clauss assay (Stago STA® Fibrinogen 5). Plasma from sample 1 (fibrinogen concentration: 2.8 g/L) was diluted to 1.4 g/L and 2.1 g/L fibrinogen with defibrinogenated plasma. Each sample was tested in triplicate on both the paper test and the Stago STA® Fibrinogen. An analysis of variance was then performed to quantify the ability of each assay to distinguish between the three concentrations.

The t-value was used as the metric for the Analysis of Variance. It quantifies how well both devices could distinguish a 1.4 g/L (low) fibrinogen sample from a 2.1 g/L (borderline-normal) fibrinogen sample as well as a 2.1 g/L sample from a 2.8 g/L (normal) sample. The higher the t-value, the greater the sensitivity between two fibrinogen concentrations. Results are shown in Fig. 5. The Clauss assay displays greater sensitivity in samples across all fibrinogen concentrations. An example showing how the t-value is calculated is provided in Supplementary Figure 6.

4.3. Patient condition: effect of chemical substances in plasma

Patients requiring the measurement of blood fibrinogen concentration might be on anticoagulants, have drunken alcohol, or suffer from high cholesterol. These conditions should not affect the test. Extreme plasma concentrations of heparin, warfarin, alcohol and dietary fats were therefore all tested individually to observe whether they impact on wicking distance. These substances were added to defibrinogenated plasma to determine whether they influenced the blue dye migration rate. These additives were also added to normal plasma to investigate if they interfere with the fibrin formation process and subsequent wicking impedance. Each substance was tested five times in both defibrinogenated and normal plasma and the mean and standard deviations are reported in Fig. 6.

Two observations are noted: 1) No substance alone affects the blue dye migration rate (as shown in the top graph). It was hypothesised that triglycerides would at least impair the wicking of defibrinogenated plasma due to its hydrophobic properties. However, this is not the case. 2) Heparin is the only substance to diminish the level of fibrin formation and cause further migration of blue dye than expected (as shown in the bottom graph). This is due to its ability to inhibit already activated thrombin. Heparin can activate the plasma protein anti-thrombin, which in turn can inhibit thrombin. This can drop the thrombin activity and subsequentially the amount of fibrin formed. Although both warfarin and alcohol can act as anti-coagulants, they cannot do so by inhibiting active thrombin. For example, warfarin exerts its effects by inhibiting the vitamin K dependent synthesis of coagulation factors in the liver (including thrombin, factor VII, IX and X).

4.4. Effect of acidosis

Acidosis is commonly present in heavily bleeding patients. It results from the build-up of lactic acid due to insufficient blood flow to the tissues. Acidosis is fatal as it impairs coagulation when the blood pH drops below 7. The pH of normal plasma was decreased by mixing it with small volumes of concentrated hydrochloric acid and subsequentially measuring the pH with a pH meter. The initial pH of the normal plasma was 7.6. The effect of acidosis was observed by dropping the pH of the plasma to three key levels: 7.3 (moderate acidosis), 7.1 (critical acidosis), 6.9 (fatal acidosis). Each pH was tested five times and the mean and standard deviations are reported in Fig. 7.

The effect of acidosis is insignificant. At moderate acidosis levels, no effect is observed. However, at extreme levels a small decrease in wicking length is noted. To validate whether this small decrease was due to the drop in pH or increase in chloride ion concentration, sodium chloride was also added to pH 7.6 plasma. No difference is shown between the pH 7.6 or pH 7.6 + NaCl plasmas, verifying that

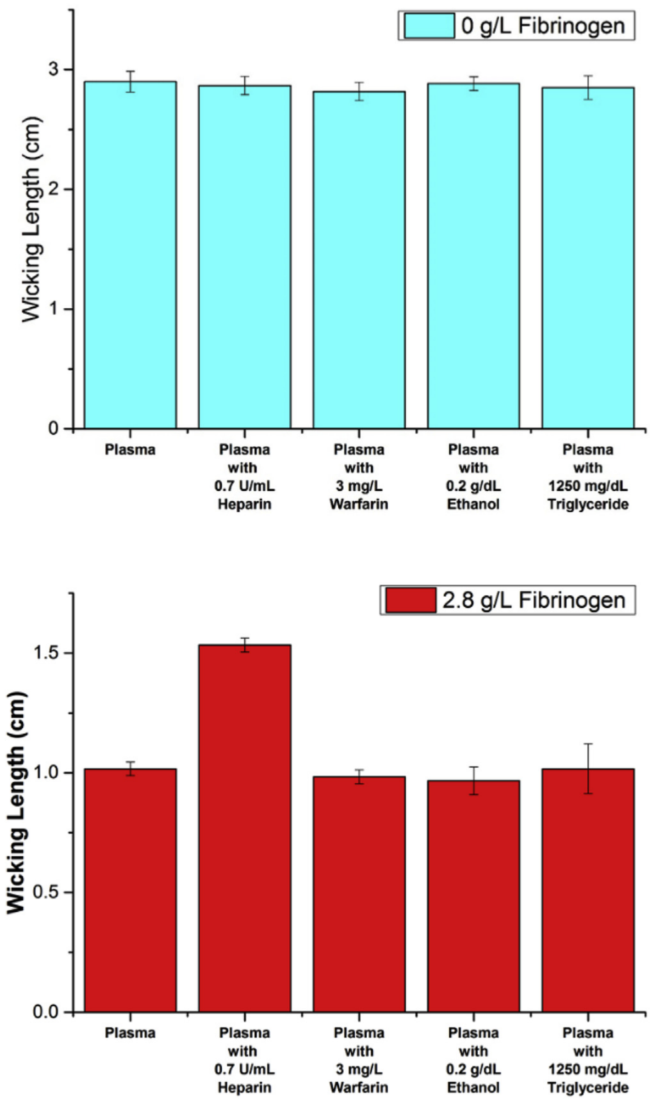

Fig. 6. Testing of different substances on defibrinogenated plasma (top) and normal plasma (bottom). Each substance was tested five times in both plasmas. The means and standard deviations are reported accordingly.

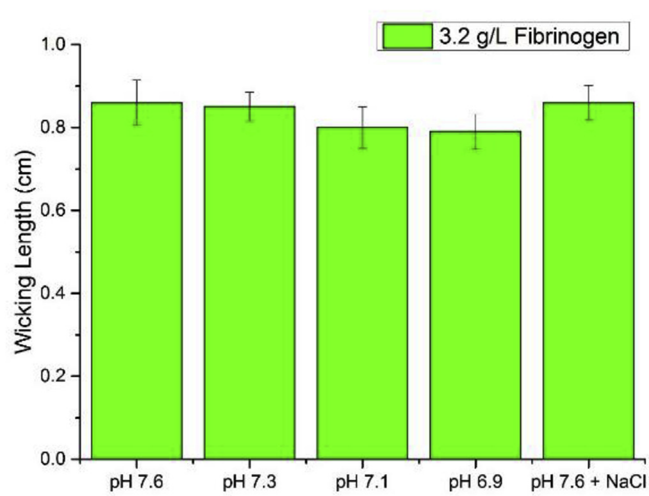

Fig. 7. The effect of acidosis on normal plasma was tested five times. The means and standard deviations are reported accordingly.

the effect is solely pH driven.

The effect of acidosis and increase in osmolarity was also observed in defibrinogenated plasma (result not shown). No effect is observed by decreasing the pH or increasing the sodium chloride concentration.

4.5. *Effect of haemolysis*

The destruction of red blood cells commonly occur in bleeding disorders such as disseminated intravascular coagulation. The resulting effect is the release of haemoglobin into the plasma. Plasma haemoglobin concentrations above 0.5 g/dL are classified as severely haemolysed.

The release of haemoglobin can increase the viscosity of the plasma. Therefore, the effect on the blue dye migration rate was observed. Haemoglobin concentrate was created by lysing red blood cells. Its haemoglobin concentration was then quantified. Normal and defibrinogenated plasmas of different haemoglobin concentrations were made by mixing different quantities of haemoglobin concentrate into the plasma samples. Each concentration was tested four times and the mean and standard deviations are reported in Fig. 8.

The effect of haemolysis is negligible. Fibrin formation in normal plasma is unaffected by haemoglobin concentration. Furthermore, the blue dye migration rate is largely unaffected by haemoglobin as well. A small, but insignificant decrease in wicking length is noted at extreme haemoglobin concentrations (ie. 1.8 g/dL) in defibrinogenated plasma.

4.6. *Effect of temperature and humidity*

Because critical bleeding can occur anywhere in the world, the test was experimented at different humidities (Fig. 9) and temperatures (Fig. 10). This is because humidity and temperature affect the mechanical and structural properties of paper.

Experiments at 50% relative humidity were performed in a temperature and humidity controlled room. A desiccator cabinet filled with silica gel was used to drop the relative humidity to 30%. Defibrinogenated plasma was used to test the direct effect humidity

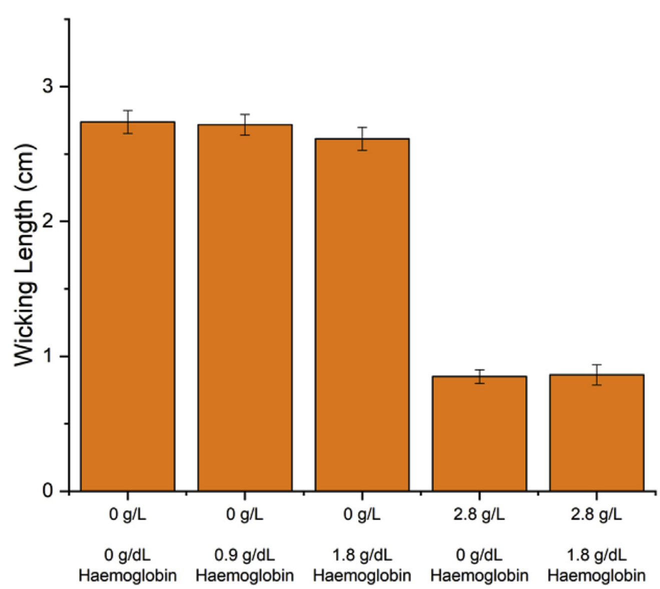

Fig. 8. The effect of haemolysis on defibrinogenated (0 g/L) plasma and normal (2.8 g/L) plasma was tested four times. The means and standard deviations are reported accordingly.

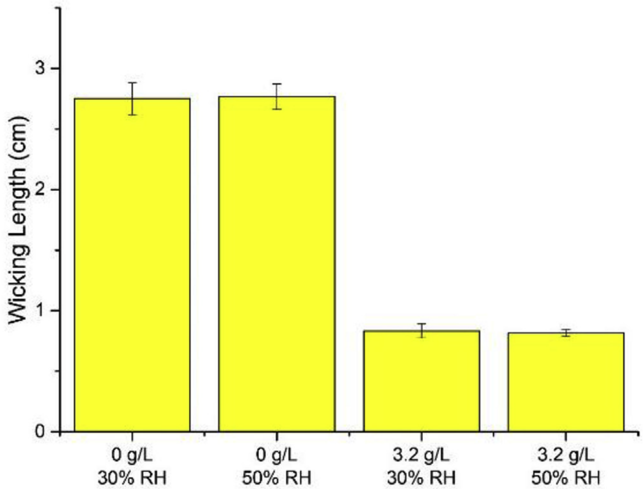

Fig. 9. The effect of humidity on defibrinogenated (0 g/L) plasma and normal (3.2 g/L) plasma was tested four times. The temperature was kept constant at 23 °C. The means and standard deviations are reported accordingly.

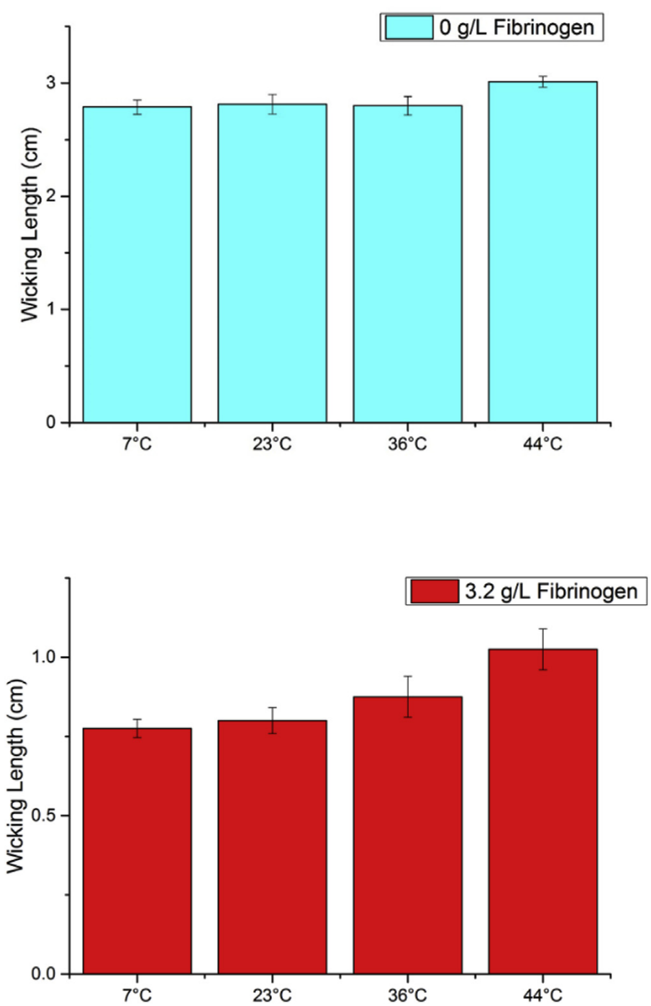

Fig. 10. The effect of temperature on defibrinogenated plasma (top) and normal plasma (bottom) was tested four times. The means and standard deviations are reported accordingly. The relative humidities for each temperature condition are summarized in the Appendix.

had on the blue dye migration rate due to drying. Normal plasma was used to test the effect humidity had on fibrin formation. Each condition was tested in quadruplets in both defibrinogenated and normal plasma and the mean and standard deviations are reported in Fig. 9.

No difference in wicking length for either the defibrinogenated or the normal plasma is observed (Fig. 9).

Experiments at different temperatures were performed using a temperature incubator. Four temperatures were tested: cold (7 °C), temperate (23 °C), hot (36 °C), and extremely hot (44 °C). Defibrinogenated plasma was used to test the direct effect temperature had on the blue dye migration rate due to viscosity changes. Normal plasma was used to test the effect temperature had on fibrin formation. Each condition was tested in quadruplets in both defibrinogenated and normal plasma and the mean and standard deviations are reported in Fig. 10.

Two observations are noted. 1) The blue dye wicking rate is unaffected by cold to hot temperatures, but slightly increases at extremely hot temperatures. 2) The fibrin formation is unaffected by cold to moderate temperatures, but is affected by hotter temperatures.

5. Discussion

5.1. Impact

In this study, we have developed the first ever paper based hand-held test for diagnosing hypofibrinogenemia. Clear results can be obtained in only a minute. Therefore, it can strongly reduce the time to treatment before fibrinogen replacement therapy. As discussed in our first study [47], the test predominantly works on the basis of hydrophobicity. When plasma and thrombin react with each other on paper, they convert fibrinogen into fibrin. This creates a zone of hydrophobicity. When the droplet of blue dye is applied onto the strip, the zone of hydrophobicity retards its capabilities to wick through the paper strip [47]. The amount of fibrinogen present dictates the magnitude of the hydrophobicity. Therefore, the plasma fibrinogen concentration can control the rate at which the blue dye can wick down the strip. This is modelled through the Lucas-Washburn equation which states:

$$L = \sqrt{\frac{\gamma r t \cos\theta}{2\eta}} \tag{1}$$

where L = length, γ = surface tension, r = pore radius of paper, η = blue dye viscosity, t = wicking time and θ = contact angle between blue dye and paper. The zone of hydrophobicity increases the contact angle between the wicking front of the blue dye and the paper surface. Therefore, it decreases the length that the blue dye wicking front can travel through the strip. This means that the plasma fibrinogen concentration can dictate the length of the wicking front as demonstrated in Fig. 2.

This study has achieved two objectives over the previous study [47]. The first is by successfully replacing the infinite reservoir in the vertical wicking test for a step where a droplet of blue dye can be applied by hand. The second is by creating a horizontal setup that can distinguish physiologically-relevant plasma fibrinogen concentrations from each other and accurately measure them. Furthermore, a blue dye wicking time of only 30 s is required to provide clear results. This is significantly better than the 7 min wicking time previously needed for the vertical wicking test [47]. This means that the new test can be engineered into a portable hand-held device and has the potential to save more than two million lives worldwide every year [14–20].

Although the plasmas tested were artificially diluted, the paper diagnostic shows optimal sensitivity below 2 g/L fibrinogen, the range of clinical interest (Fig. 3). The results are very reproducible. These trends are consistent with those observed with the vertical wicking test [47]. This new methodology can rapidly identify whether a bleeding patient has a blood fibrinogen concentration below 2 g/L or not. It can also distinguish cases of mild hypofibrinogenemia (1.5–2 g/L) from severe hypofibrinogenemia (<0.5 g/L).

This new test is unaffected by the source of plasma. The trendlines generated in Fig. 3 were created from the normal and defibrinogenated plasmas of two different citrated plasma samples. They show clear similarity to each other. Furthermore, undiluted plasma from all three healthy plasma samples were tested twenty times each (Fig. 4). The plasmas containing 3.2 g/L and 3.3 g/L fibrinogen do not show noticeable separation. On the other-hand, the plasma containing 2.8 g/L fibrinogen wicks further than the other two similarly concentrated plasmas. This means that the wicking length is controlled only by the plasma fibrinogen concentration in healthy individuals.

5.2. Performance

The paper test was compared to a hospital-grade automated Clauss assay (Stago STA® Fibrinogen 5). Even though the paper test has a lower sensitivity than the hospital grade Clauss assay (as shown in Fig. 5), it still possesses five important advantages over other hospital grade assays that make it particularly suitable for emergency situations.

First, the test can be transported easily to a patient. This makes it suitable as a point of care device. Whereas, other hospital grade assays require bulky and heavy table top systems dedicated to a laboratory environment. Second, the paper test clearly works within the physiological range of plasma fibrinogen. In contrast, other hospital grade assays including Clauss assays require up to 1:30 times dilutions to achieve clear results [49]. As the paper test does not require pre-dilutions, it saves a considerable amount of time, complexity and reduces human errors. Third, the paper test can be performed immediately at room temperature. However, other hospital grade assays require that the blood or plasma sample be pre-warmed at 37 °C for 4–6 min before the assay starts [50]. Given the urgent situation of critical bleeding, these pre-incubation times are disturbing. Fourth, hospital grade assays require complicated endpoint determination techniques to identify when clot formation has occurred [51]. The endpoint refers to the point of time when fibrin formation has completed. For example, the Stago STA® Fibrinogen 5 detects when fibrin formation has been completed by monitoring the motion of metal ball bearings. A metal ball bearing is added to the plasma sample once the assay has started and is allowed to move around with the aid of magnets. The formation of fibrin impedes the movement of the ball bearing. The moment the ball bearing stops moving is when fibrin formation is completed. However, this requires complicated apparatus such as ball bearing dispensers, applied magnetic fields and detectors to achieve this. However, as the paper test measures the fibrinogen concentration after 30 s of wicking, it takes out the requirement for endpoint determination altogether. Finally, the machine requires a computer screen to display fibrinogen concentration readings. On the contrary, the paper test is interpreted directly-unassisted to the naked eye.

Given that clinics utilise the same fibrinogen replacement therapy procedures once they find a patient has below 2 g/L plasma fibrinogen, further sensitivity below those values is not as important. Therefore, this test is better suited for diagnosing hypofibrinogenemia than typical hospital grade assays.

5.3. Adaptability

Since the paper test could successfully measure plasma fibrinogen concentrations in healthy individuals, we also quantified the effect that important physiological and pharmaceutical conditions can have on the test. We looked at those most likely to cause deviations away from the results expected with healthy and defibrinogenated plasmas. This included anticoagulant drugs, alcohol, dietary lipids, acidosis and haemolysis (Figs. 6, 7 and 8). Furthermore, because the test is suitable as an outdoor point of care device, we also tested the effects of different temperatures and humidities (Figs. 9 and 10). Only conditions known to alter thrombin activity affect the test. Heparin exerts the most potent effect. However, extreme heat does negatively affect the test also.

Anticoagulant drugs such as heparin and warfarin are used during and after cardiac surgery to prevent acute coronary syndrome. Warfarin is preferred over heparin due to its ability to be administered orally and its longer half-life. However, warfarin takes longer to exert its effect [52]. Therefore, heparin and warfarin are both initially used for the first five days to prevent anticoagulation [53]. Once anticoagulation has stabilized, then heparin therapy is discontinued and patients can continue taking warfarin.

Heparin is clinically used as either unfractionated heparin or low molecular weight heparin. Because unfractionated heparin has a much greater inhibitory effect on thrombin, it was used in this study [54]. Furthermore, the use of unfractionated heparin also indicates how other pathophysiological thrombin inhibitors - such as fibrin/fibrinogen degradation products - or other thrombin inhibiting anticoagulants - such as direct oral anticoagulants - might behave [55]. Clinical target therapeutic concentrations of heparin in plasma are as high as 0.7 U/mL [56]. Therefore, we added heparin in healthy plasma to that concentration and quantified its effect. The effect of heparin is significant (Fig. 6 - bottom graph). When compared to a healthy plasma sample of 2.8 g/L fibrinogen, the heparin causes an extra 5 mm of further wicking. From Fig. 3, this correlates with a reading of roughly 1.5 g/L fibrinogen. The inhibitory mechanism of heparin on thrombin is well documented. Heparin binds to the enzyme anti-thrombin and activates it. This in turn inactivates thrombin [57]. Given that the amount of thrombin used in the paper test was not in huge excess, the inhibitory effect is not surprising. The inhibitory effects on thrombin (of heparin or other inhibitors) can be fixed through several means. The simplest solution is by using higher thrombin concentrations for sufficient competition.

Likewise, we added 3 mg/L warfarin to healthy plasma as it is the upper threshold of clinical target therapeutic concentrations. However, no effect is observed (Fig. 6). This is expected as warfarin does not form inhibitory complexes with thrombin like heparin does. Instead, its mode of action is to prevent the vitamin K dependent liver synthesis of coagulation factor precursors into their active forms, including thrombin [58]. Given that the thrombin adsorbed on the paper strip is already active, warfarin cannot exert any effect on it.

High blood alcohol concentrations are problematic in situations such as drink-driving traffic accidents. This represents a significant cause of trauma. In 2017, Mitra et al. reported that 16% of trauma patients involved in traffic accidents on Australian roads had blood alcohol concentrations above 0.05 g/dL [59]. Ethanol is known to impair the initial clot formation time but has no reported effect on thrombin [60]. We tested plasma mixed with four times the Australian legal driving limit of 0.05 g/dL blood alcohol concentration. However, we observed no effect compared to healthy plasma (Fig. 6). This means that the test is unaffected by alcohol.

In our first manuscript we concluded that the mechanism behind the test is largely hydrophobicity driven. Therefore, we wanted to assess wherever blood lipids exerted their own effect on blue dye wicking. This is because blood lipids have hydrophobic components that may impede wicking if concentrations get too high [61]. Triglycerides make up 50% of all plasma lipids in the healthy population. Normal baseline triglyceride concentrations do not exceed 150 mg/dL. However, in cases of very severe hypertriglyceridemia, they can exceed 1000 mg/dL [62]. When we add 1250 mg/dL of triglycerides to defibrinogenated plasma, we do not observe any changes in results (Fig. 6). This indicates that plasma lipids do not impact on wicking.

Acidosis is another complication in heavily bleeding patients. It is caused by the build-up of lactic acid from anaerobic metabolism due to decreased blood flow and oxygen delivery to tissues [63]. Healthy blood pH is tightly controlled between 7.35 and 7.45. However, when blood pH drops below 7.35, then the effects of coagulopathy worsen [64]. Because the healthy plasma samples used were citrated, their pH levels were recorded to be 7.6. By adding hydrochloric acid to healthy plasma, we dropped its pH from 7.6, to 7.3 (mild acidosis), 7.1 (critical acidosis) and 6.9 (fatal acidosis). When the pH is dropped to 7.3, no difference in wicking is observed when compared to a pH of 7.6 (Fig. 7). This means that the test can adapt to blood pH levels between those two values. However, when the pH is dropped to fatal levels, a small decrease in wicking length - averaging roughly 0.5 mm - occurs. To verify wherever this effect is due to the drop in pH or the increase in chloride ions, sodium chloride was also added to healthy plasma. The added sodium chloride does not impede further wicking, thus confirming that the slight decrease in wicking is pH driven. Although the effect is insignificant, it is likely because thrombin works optimally at a pH of 6.5 [65]. Hence, low blood pH can enhance thrombin activity and further impair wicking.

Microangiopathic haemolytic anaemia is a common symptom in bleeding disorders such as disseminated intravascular coagulation. Vessel rupture and fibrin deposition is thought to cause the formation of abnormal flow channels and hence the mechanical destruction of red blood cells (RBCs). This results in RBCs releasing their cytosolic contents into the plasma (including haemoglobin) [66]. As haemoglobin is a large and globular protein, it was hypothesised that increasing the haemoglobin concentration in plasma would also increase its viscosity. Hence, it may have perturbed blue dye wicking. Severe haemolysis is diagnosed when plasma haemoglobin concentrations rise above 0.5 g/dL [67]. When defibrinogenated plasma samples are made to 0.9 g/dL haemoglobin, no impediment in wicking is observed (Fig. 8). It is only when the plasma haemoglobin concentration is made to 1.8 g/dL - a concentration extremely rarely reached [67] - that an insignificant wicking perturbation is observed. Furthermore, the addition of haemoglobin does not affect the wicking of normal plasma - proving that it has no impact on fibrin formation. Therefore, the test is unaffected by clinically relevant levels of haemolysis.

Traumatic injury can occur in different geographical environments around the world. These environments can all have different weather conditions. Therefore, we measured the effect of temperature and humidity on the test. We originally hypothesised that dropping the humidity would hasten drying and hence retard wicking. However, Fig. 9 shows that no effect is observed when the humidity is dropped from 50% to 30%. Likewise, we also hypothesised changing temperature would exert two different effects. One was that increasing temperature would decrease blue dye viscosity and cause further wicking. The other was that either increasing or decreasing the temperature to extreme values would decrease thrombin activity and cause further wicking. Fig. 10 (top graph), shows that the blue dye wicking rate on its own is insensitive to temperature change. Furthermore, Fig. 10 (bottom graph) demonstrates that fibrin formation is unaffected at low to moderate temperatures. However, at high temperatures, fibrin formation is perturbed and further wicking occurs. This can lead to fibrinogen readings being falsely low. A solution is to use a correction factor based on the ambient temperature the test is performed at. Alternatively, a means to protect the test from extreme heat can also be used. Otherwise, the test is suitable in a wide range of different weather conditions and locations around the world.

5.4. Limitations

The main limitation of the paper test is the distribution of plasma and thrombin evenly through the paper strip. When the fibrinogen in plasma reacts with thrombin on paper, it forms a fibrin network that perturbs the wicking of the blue dye. However, when the fibrinogen and thrombin are not perfectly distributed on paper, it creates areas of unreacted plasma. Because these areas are more hydrophilic than the surrounding fibrin network, the blue dye travels through them preferentially and wicks further than expected. This is demonstrated in Supplementary Figure 5. Initially, we intended to add the thrombin onto paper first and then add the plasma on top of the thrombin afterwards. However, the plasma would frequently push the thrombin out of its way instead of mixing with it. This created results that were difficult to reproduce. Therefore, a means to keep the thrombin in place is needed for the test. However, our solution to this issue for this study was to premix the plasma with thrombin before applying to paper.

The pre-mixing caused extra limitations in our study.

First, the concentrations of thrombin we used had to be low (30 U/mL) to provide enough time to mix with the plasma and apply to the paper before it could clot. We tried using concentrations of 600 U/mL (similar to that of a Clauss assay) [44]. However, it causes the plasma to clot immediately in the pipette tip before it could be applied to paper. Additionally, the low thrombin concentrations used in the test may be the reason why the test did not work with therapeutic unfractionated heparin concentrations. By finding a way to remove the pre-mixing step, we may be able to react the plasma uniformly with higher thrombin concentrations on paper.

Second, the mixed plasma has to be applied evenly across the width of the strip. If the mixed plasma is added to one section of the strip, it creates a gap surrounding the fibrin network for the blue dye to easily go through. Hence, the blue dye wicks further than expected as shown in Supplementary Figure 5. In our methodology, we fixed this issue by pipetting approximately one third of the mixed plasma on the left-hand side, one-third on the right-hand side and the other third in the centre of the strip. An example of this is shown in Video 1. Because pipetting technique varies from individual to individual, a means of distributing the plasma evenly across the strip is needed for the test.

The resulting fibrin network formed on the strip is fragile and susceptible to damage from excessive shear stresses. Therefore, the velocity of the blue dye has to be controlled as it wicks through the network. In this study, we achieved this through three mechanisms. 1) By expanding the width of the dye pad (see Supplementary Figures 8 and 9). 2) By adding the blue dye from the bottom of the dye pad (to provide settling distance between the dye and the fibrin network). 3) By adding the blue dye slowly over a period of 10 s. Performing the test with this technique does not create outliers.

Our study was also limited by the choice of plasma reagent. We worked with platelet-poor citrated plasma of healthy donors. This was because no extra substances were added into the plasma samples that could affect wicking rates or thrombin activity. Hence, we can claim that the results observed in Fig. 4 are due only to fibrinogen concentration. However, as these samples were derived from healthy donors with minimal modifications, the fibrinogen concentrations were all around 3 g/L. Therefore, they had to be artificially diluted in defibrinogenated plasma to reach the desired fibrinogen concentrations for testing. The use of defibrinogenated plasma as an appropriate dilution medium is validated in Supplementary Figs 7 and 14. These results support the hypothesis that the trend observed in Fig. 3 would be similar if we used unmodified plasma samples of different concentrations instead.

We did not have access to samples from patients already containing the substances tested in Fig. 6. Therefore, we added those substances into normal and defibrinogenated plasmas instead. Whilst most of these substances exert a direct effect in the plasma, warfarin does not. Instead, warfarin inhibits the synthesis of coagulation factors in the liver. This normally takes up to five days to have an effect. Whilst warfarin is shown not to impact the test, further work is needed to determine whether the plasma of a patient on warfarin for five days produces the same effect. In this study, we used thrombin concentrations similar to a thrombin time assay (ie, 30 U/mL). As it is known that any depletion of endogenous thrombin (due to the effects of warfarin) does not impact a thrombin time assay, we assume this diagnostic will not be impacted by it either.

Finally, the effects of dysfibrinogenemia were not considered in this study. We assumed that if a patient suffered from dysfibrinogenemia and their fibrinogen could not react with the thrombin in our test, then it could not react with the thrombin in their plasma either. Hence, they would need fibrinogen replacement therapy anyway. Whilst there are some types of dysfibrinogenemia that cause thrombosis, Korte et al. 2017 found that there was no definitive evidence to suggest that fibrinogen replacement therapy increases the risk of thrombosis in these patients [68]. Therefore, we prioritised the development of a diagnostic that could measure the concentration of clottable fibrinogen as opposed to total fibrinogen.

6. Conclusion

In this study, we have developed a rapid, hand-held paper-based diagnostic for measuring fibrinogen concentrations in human plasma. The test can provide a result within a minute. This time is suitable for facilitating fibrinogen replacement therapy. The test has optimal sensitivity for fibrinogen concentrations below 2 g/L - the critical diagnostic concentration - and shows reproducibility between healthy individuals. Whilst the diagnostics does not have the same sensitivity as a hospital-grade automated Clauss assay, it is portable, much faster, requires no plasma pre-treatment, pre-incubation or clot formation detection mechanism and has a simple visual readout. The test can also withstand common blood conditions such as acidosis, blood alcohol, severe hypertriglyceridemia, severe haemolysis and warfarin administration. Finally, the test is unaffected by humidity and can withstand cold temperatures. This makes it suitable as a point of care diagnostic to detect hypofibrinogenemia early - both inside and outside of the hospital setting. Therefore, it can greatly reduce the time to treatment needed for fibrinogen replacement therapy and save more than two million lives a year.

Ethical statement

De-identified human blood samples were provided by the Australian Red Cross Blood Service, obtained with written informed consent in accordance with the recommendations of Blood Service Human Research Ethics Committee (BSHREC). This study was carried out with approval of and in accordance with the recommendations of the Monash University Human Research Ethics Committee (MUHREC).

Author contribution

Marek Bialkower: Lead Author.
Gil Garnier: Corresponding Author.
Clare Manderson: Set-up image analysis.
Heather McLiesh: Troubleshooted problems.
Rico Tabor: Troubleshooted problems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2019.12.046.

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