Assessment of procoagulant activity of microparticles

Ditte Sørensen

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Written by:

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Ditte Sørensen

Main supervisors:
Shona Pedersen
Egon toft

Clinical supervisor:
Søren Risom Kristensen

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Preface

This report was written by group 957, Translational Medicine, 4th semester of the Master's Degree in Medicine with Industrial Specialization, Aalborg University, Denmark. The project was compiled from September 1st 2011 to June 1st 2012. The group has been supervised by Shona Pedersen, Ph.D. at Department of Clinical Biochemistry, Aalborg Hospital, Egon Toft , professor at the Faculty of Medicine, Aalborg University and Søren Risom Kristensen, Professor at Department of Clinical Biochemistry, Aalborg Hospital.

First chapter is an introduction giving the reader an overview of topics relevant to the project. The following chapters include materials and methods, results, discussion, and conclusion related to the experimental part. The structure throughout the report is based on a four-leveled subdivision of the chapters.

The knowledge acquired during the project was primarily from scientific articles of high validity found on databases such as PubMed or through the State and University Library (Statsbiblioteket). Keywords used during the literature search were: [illegible], microvesicles, hypercoagulability, tissue factor, prothrombinase complex, cancer, haemostasis and thrombosis.

Abstract

Studies are on going to identify microparticles (MP) populations and develop methods to quantify these, as MP can serve as diagnostic and prognostic biomarkers of disease. However, the potential for MP as biomarkers has been limited by inadequate validation and standardization of sample preparation and handling, reagents and assays. The commercial available Zymuphen Microparticle Activity assay and the Zymuphen MP-TF assay claim to quantify phosphatidylserine positive MPs and tissue factor bearing MPs, respectively, present in plasma. In order to move towards standardized handling of the assays the centrifugation recommended by the manufacturer was compared to the one most prominent in the literature and the one used at the department, to see if the one suggested in literature could be preferred. Plasma from healthy men and women were studied. All three centrifugations gave platelet free plasma, however, the amount of phosphatidylserine positive MPs registered by the assay differed within samples from the same person, suggesting that there might be some platelet residues due to centrifugation. Additionally to difference as a consequence of centrifugation, both assays showed a difference when analysing fresh and frozen plasma. Our preliminary data suggest that both assays are able to discriminate between healthy individuals and cancer patients.

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1. Introduction

Thrombosis is a multifactorial disease

Thromboembolism is a multifactorial disease and risk factor include events such as malignancy, extensive surgery, trauma, burns, myocardial infarction, use of oral contraceptive, and immobilisation (Line, 2001). Thrombosis occurs when the balance in haemostasis is shifted towards procoagulant factors. It is associated with high morbidity and mortality and is the leading course of death in the western societies. Additionally, the prophylaxis, diagnosis, and treatment consume a significant amount of the national health care budget. The risk of thromboembolism increases by age; and the rate of thrombosis is approximately 5 per 10,000 per year across age in the Western Europe and the United States (Fowkes, 2003; White, 2003). The venous thromboembolism (VTE) includes deep vein thrombosis (DVT), and pulmonary embolism. Thus, identifying at-risk persons and reducing the incidence of venous thrombosis would be of great importance for the morbidity, cost of care and most importantly the mortality. Several biomarkers have been proposed for the purpose of identifying at-risk persons (Rak, 2010; Sud, 2009).

Microparticles

One such biomarker could be microparticles (MPs) as MPs are present in low numbers in the blood of healthy individuals and are increased in various diseases (Burnier, 2009). MPs are fragments shed from the cell membrane of most stimulated or apoptotic cells. After having been considered cell debris for the last 40 years, there is growing evidence that MPs are physiologically important. Increased production of MPs has been associated to various physiological and pathophysiological conditions such as cell adhesion, apoptosis, immune response, vascular function, vascular remodelling and angiogenesis, haemostasis and thrombosis, cardiovascular diseases, cancer, infections, and normal and pathological pregnancy (Burnier, 2009; Freyssinet, 2003).

Evidence indicating that MPs contribute to thrombus formation and the development of VTE is accumulating (Hugel, 2005). In this connection, it could be highly useful if MPs could serve as a prognostic marker. However, results are conflicting regarding the usability. On one side, highly elevated MP levels were measured shortly after an acute VTE event in cancer patients indicating that MPs might serve as a marker (Chirinos, 2005; Tesselaar, 2007). However, MP levels were not elevated 3 months after a VTE event in a high-risk population of non-cancer patients with a history of recurrent VTE (Ay, 2009). Meaning it might be difficult to predict the occurrence of VTE from the MPs status, if MPs are only elevated around the time of the VTE. It has however been demonstrated that MPs are guided towards a developing thrombus and this accumulation of MPs supports thrombus growth (Furie, 2008; Thaler, 2011).

MPs - a pool of bioactivity

The structure of MPs and the proposed interaction with the coagulation system will be described in the following, as this knowledge is important when assessing the potential of MPs as a biomarker.

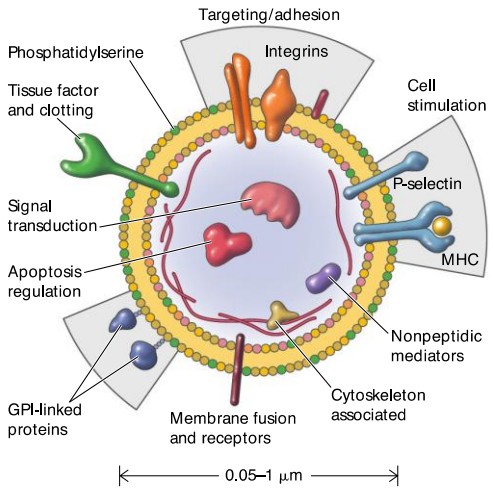


Figure 1 Cellular circulating microparticle. Membrane microparticles are shed from the membrane of stimulated cells. They consist of different components, including phosphatidylserine, GPI-linked proteins, p-selectin, and intergrins. The size ranges from 0.05-1 µm (Hugel, 2005)

MPs have a diameter ranging from 0.05 to 1 µm (Figure 1) and are rich in phosphatidylserine (PS). Normally, the particles serve as important signalling structures between cells (Théry, 2009). MPs have the characteristics of the cells they are derived from and enclose components such as enzymes, transcription factors and mRNA originating from their parent cell (Morel, 2011). They mediate cell-to-cell communication by transferring these components from the parental cell to the target cell (Mause, 2010). By this mechanism MPs interact with adjacent and remote cells. Additionally, MPs may bind and fuse with the plasma membrane of the target cell or be engulfed by the target cell (Mause, 2010).

MPs are defined by their size and the antigens from the parental cell located on their surface. The parental cell may mainly be platelets, but also erythrocytes, granulocytes, monocytes, lymphocytes and endothelial cell derived MPs have been observed (Zwaal, 1992)

MP formation

The mechanism that control MP formation *in vivo* has yet not been established. There is however some knowledge on MP formation *in vitro*, which originates from experiments performed on isolated or cultured cells (Burnier, 2009). The top of Figure 2 illustrates the

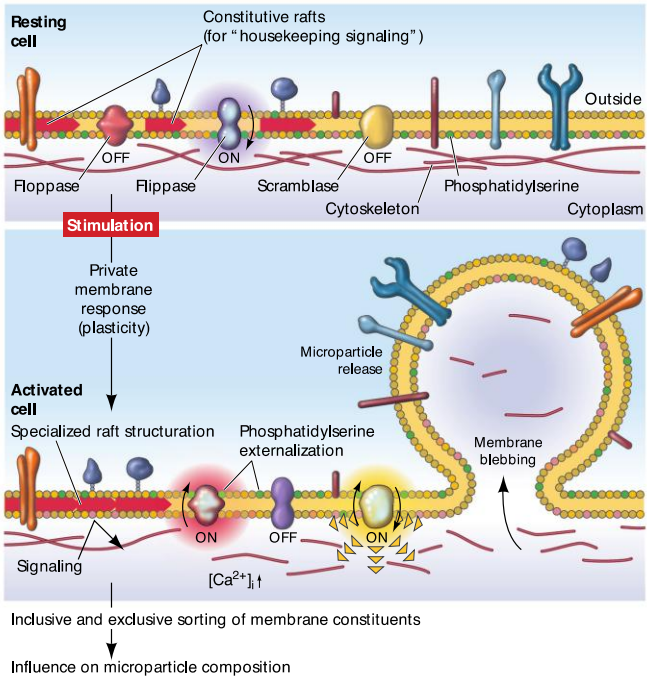


Figure 2 The mechanism of MP formation. The top of the picture shows the resting cell membrane. The membrane is characterised by a controlled distribution of proteins and lipids between the two leaflets. When the cell is stimulated the membrane units are redistributed, leading to externalisation of phosphatidylserine and the formation of a microparticle. (Hugel et al., 2005).

resting state of a cell membrane. Here, the membrane is asymmetric regarding the lipid composition. PS and phosphatidylethanolamine (PE) are present in the inner leaflet of the cell membrane and phosphatidylcholine and sphingomyelin are located in the outer leaflet. This asymmetric distribution of the lipid bilayer is maintained by three enzymes: flippase, floppase and scramblase. Flippase specifically transfers the PS and PE from the outer to the inner leaflet and floppase transports phospholipids from the inside to the outside of the cell membrane. Lastly, the scramblase promotes bidirectional redistribution across the lipid bilayer and is inactive when cells are in resting state (Bevers, 1999).

The formation of MPs during cell activation, apoptosis, or senescence is thought initiated by a significant increase of Ca^{2+} released by the endoplasmic reticulum of the cell that release the MP. See Figure 2. The Ca^{2+} inactivates the flippase and activates floppase and scramblase, leading to loss of phospholipid asymmetry. Moreover, the Ca^{2+} increase activates the enzymes calpain and gelsolin, which hydrolyses actin-binding proteins. Such hydrolysis leads to a decrease in the association between actin and membrane glycoproteins, as well as cleavage of actin capping proteins (Fox, 1990; Wiedmer, 1990). This results in membrane budding and shedding of MPs enriched with PE and PS on the surface. The distribution and the amount of

PE and PS vary depending on the cell that releases the MP and the way of activation. The MPs contain cytoplasmic proteins and display hijacked components and bioactive lipids implicated in a variety of fundamental processes on their surface, see Figure 1 (Hugel, 2005).

Cellular sources of MPs

Different cell types release different types of MPs as mentioned and the three major sources of MPs are thought to be platelets, monocytes and endothelial cells. See Figure 3. The level of MPs in blood depends on the balance between the rate of release and the rate of clearance (Burnier, 2009). The mechanisms of clearance of MPs in vivo have not been described. It has been suggested that clearance might occur by phagocytosis in the same manner as apopototic bodies or MPs might be destroyed by the phospholipases (Flaumenhaft, 2006).

Platelet derived MPs

Several studies have found that platelets are the major source of PS positive MPs and that they represent 70-90% of all circulating MPs (Owens, 2010). The platelet derived MPs display the surface antigen CD41, which is a platelet membrane protein, see Figure 3. Platelet derived MPs can be produced by various stimuli, including platelet agonist, calcium ionophore, complement-binding proteins or high shear stress (Burnier, 2009). Platelet derived MPs are generated under high shear stress in atherosclerotic arteries, but platelet derived MPs are not generated under normal shear stress (Holme, 1997).

Monocyte derived MPs

Monocyte derived MPs are thought to be the source of MPs bearing tissue factor (MP-TFs). A recent study showed that MPs expressing TF also express the monocyte marker CD14. Furthermore, another study showed that MPs positive for both TF and CD14 are increased in a human endotoximia model (Aras, 2004). Lastly, monocyte derived MP-TF are also elevated in sickle cell disease (Shet, 2003). In summary, these studies indicate that monocytes are likely to be the major source of MP-TF in healthy and diseased individuals.

Endothelial cell derived MPs

Endothelial cells express TF when they are stimulated with agonists such as cytokines and LPS. However, it is not certain whether endothelial cells express TF *in vivo*. The *in vivo* studies have been ambiguous because when TF was positively stained in endothelial cells, also other markers such as the leukocyte marker PSGL-1 were stained. Therefore it is possible that the positive staining of TF came from monocyte derived MP-TFs and not endothelial cell derived MP-TFs. This makes it difficult to determine the cellular origin of the MPs (Owens, 2010). In patients with crisis of sickle cell disease endothelial cell derived MPs expressed both TF and the endothelial cadherin CD144 (Shet, 2003). See Figure 3. These findings indicate that endothelial cells might release MP-TFs in certain circumstances.

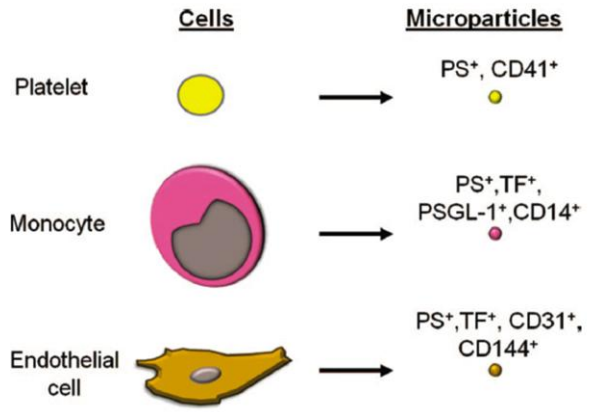


Figure 3 Surface markers of microparticles (MPs) released by platelets, monocytes and endothelial cells. (Owens III & Mackman, 2010)

MPs and haemostasis

MPs positive of PS provide a catalytic surface for the assembly of blood coagulation factors, thereby promoting the coagulation cascade and thrombin generation (Morel, 2006). The surface of PS positive MPs has 50- to 100-fold increased procoagulant ability than the same surface area of an activated platelet (Sinauridze, 2007). The importance of MPs in haemostasis is demonstrated in haemophilia where MP levels are higher than normal and can be additionally elevated in case of acute bleeding. Here MPs provide the ability to mobilize the haemostatic system without the threat of thrombotic complications since there is insufficient amount of thrombin formed (Freyssinet, 2003). Haemostasis is the hosts defence system aimed against bleeding and at preserving the integrity of the circulatory system in mammals. Haemostasis is a highly complex and tightly regulated process involving blood cells, soluble plasma proteins, and the vessel wall. In normal physiological conditions the blood flows within the vessels and the endothelium serves as a wall that separates blood cells and vessel wall proteins involved in blood coagulation. Haemostasis is triggered after injury of the vessel wall endothelium, which allows exposure of blood to the extravascular tissue, giving rise to primary haemostasis, which is the formation of a platelet plug that is the first to occlude the vascular lesion, and secondary haemostasis that is blood coagulation via the coagulation cascade. The primary and secondary haemostasis happens simultaneously. While the clot is formed the coagulation cascade forms fibrin that strengthens the clot. Anticoagulant mechanisms ensure careful control of coagulation and under normal circumstances these mechanisms ensures a balance between anticoagulant and procoagulant factors. Disturbances of this balance due to genetic or acquired factors may result in bleeding or thrombotic disorders (Dahlbäck, 2000; Furie, 2008). Haemostatic clots are attached to the vessel wall and do not greatly impair blood flow in the vessel. Thrombotic clots however, result in impairment of blood flow and sometimes even total occlusion of the vessel, leading to hypoxia of the tissue (Owens, 2010).

Prothrombinase-and tenase-complexes

The coagulation cascade has three coagulation complexes. The two will be described in the following. TF and FVIIa form the initiating or extrinsic tenase complex (Eilertsen, 2004). The importance of the TF:FVIIa complex is demonstrated in mice that either lack TF or FVII, as they cannot survive (Mackman, 2005). The extrinsic tenase complex converts FIX and FX to their active forms, FIXa and FXa. The enzyme FXa is the one part of the next complex. FXa and its cofactor FVa, form the prothrombinase complex. This complex activates prothrombin to thrombin, in combination with Ca^{2+}(Mann, 1993).

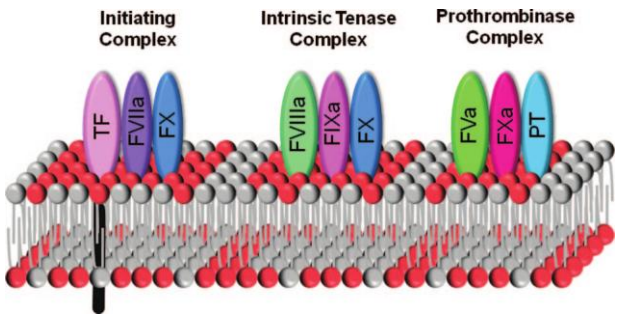


Figure 4 The coagulation complexes on a negatively charges PS membrane. The coagulation complexes TF:FVIIa, FVIIIa:FIXa and FV:FXa assemble on a membrane surface. The red dots indicates PS.

Both the prothrombinase- and tenase-complex is dependent on a negatively charged surface in order to assemble. It was previously believed that only platelets provided this surface, but animal studies in the Par4-null mouse have now demonstrated that fibrin generation is normal in these mice, although these mice' platelets can not be activated by thrombin (Vandendries, 2007). This finding suggests that something else than activated platelets provide the surface on which the prothrombinase and tenase complexes assemble. PS positive MPs have been suggested to be the providers of this surface as they can be observed as smaller versions of activated platelets and express receptors for both collagen and von Willebrand factor (VWF) (Owens, 2010). See Figure 4.

Blood borne tissue factor

Tissue factor is a 263 amino acids long protein. It consists of a hydrophilic extracellular domain of 219 residues, a hydrophobic transmembrane segment of 23 residues and a cytoplasmic tail of 21 residues. Four cysteine residues in the extracellular domain form two intermolecular disulphide bonds that are important for the interaction with FVII/FVIIa. (Eilertsen, 2004). The dogma has been that TF was only present on the surface of nonvascular cells and TF was thought absent in circulating blood. The findings of Giesen et al in 1999 forced a diversion from this dogma, as blood-borne tissue factor was discovered. They identified circulating and thrombi-associated TF-bearing cells. This raises the idea that MPs might be the source of blood-borne TF (Giesen, 1999). The relative contribution from vessel wall and blood-borne TF to a developing thrombus has not been established. However, it is known that larger vessels contain more TF than smaller vessels. The ratio of MP-TF to vessel

wall TF is 1000:1 in healthy individuals and mice (Butenas, 2005; Day, 2005). Suggesting that MP-TFs are more likely to play a role to haemostasis in small vessels were the abundance of vessel wall TF is low (Burnier, 2009).

In addition to MP-TF and vessel wall TF, a soluble alternative spliced form of TF (asTF) have been discovered. asTF contains most of the extracellular domain of TF but lacks a transmembrane domain and terminates with a special peptide sequence. (Bogdanov et al., 2003) Earlier a soluble form called truncated TF (tTF) has been reported. tTF consist of the extracellular domain of TF (Morrissey, 1993; Paborskysb, 1991). It has not been established whether blood-borne TF circulates as asTF, tTF, MP-TF or as all types at the same time (Steffel, 2006). The vessel wall keeps TF in nonvascular cells from FVII, but the idea of blood-borne TF requires other hypothesis of how MP-TF is kept from activating coagulation. Possibly, the concentration of TF is sufficiently low, under the threshold of activation of blood coagulation, until TF is concentrated at the site of vessel injury, and thereby preventing random coagulation and keeping coagulation nearby the clot. Random coagulation might be avoided because the concentration of MP-TF is not sufficient to overcome inhibition by TFPI. This should be further investigated. When TF is present on MPs it is either in a latent ("encrypted") form or in an active ("decrypted") form. The active form is the only one showing procoagulant activity (Bach, 1990). The mechanisms that turn TF from active to the latent form may involve dimerization, lipid reorganisation or cellular secretion of TF-rich granules (Burnier et al., 2009). It has not been established to what extent asTF, tTF and MP-TF contributes to cloth growth.

Procoagulant activity of MPs

MPs can harbour active TF from the parent cell and become MP-TFs (Lechner, 2008). It is suggested that there are different levels of procoagulant activity of the MPs, dependent on the amount and placement of PS and the presence of TF. MPs containing both PS and TF have the highest level of procoagulant activity. See Figure 5. The PS is important for the expression of the full procoagulant potential of the MP-TF and for the decryption process of TF (Key, 2010).

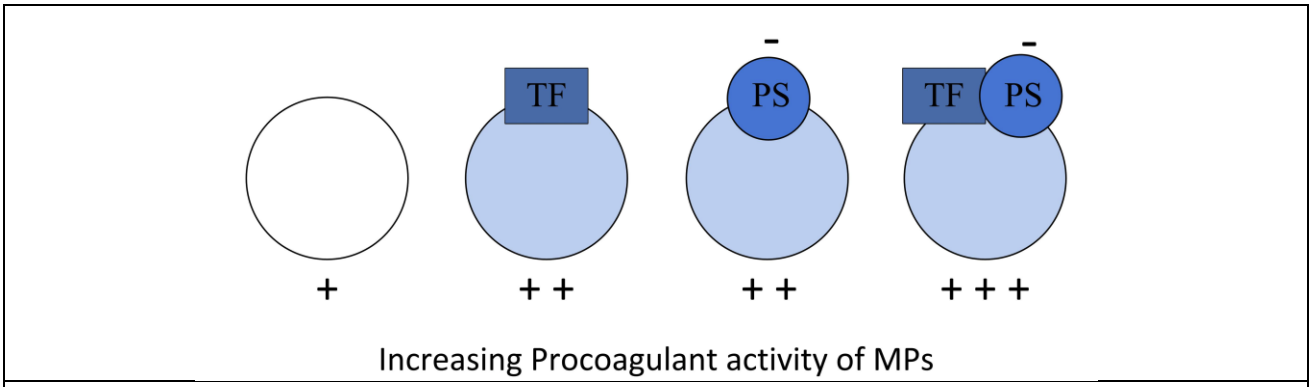


Figure 5 The increasing procoagulant activity of microparticles (MPs). The parental cell is decisive to whether the MP is bearing tissue factor (TF). To left the MP that maintains phosphatidylserine (PS) on the inside and does not express TF would have very little procoagulant activity, the procaugulant activity increases to the right and the MP showing both PS and TF is the most procoagulant. The negative bars over PS (–) indicate that the surface is negatively charged. (Key, 2010), figure by Dr. Nigel Mackman.

Analytical methods for MP measurements

A better understanding of the mechanisms that control the PS exposure process will make it possible to develop new pharmacological agents to reduce thrombotic risk by controlling the amount of PS available at the cell surface and the rate of membrane vesiculation (Weiss, 1997). MPs are of particular interest for the monitoring of the efficiency of therapeutic treatments, but before MPs can be used as biomarkers and for monitoring they need to be characterised to a greater extend (Freyssinet, 2003). The evidence that exist at present comes from a wide variety of methods used to measure, quantify, and phenotype MPs. These data are not supportive of each other and there is a lack of consensus. Hence, there is a critical need for standardisation in order to reliably compare the results from different studies (Furie, 2006).

Preanalytical variables

At current both the preanalytical and the analytical methods to detect MPs vary widely. The varying factors of the preanalytical step are blood collection, plasma isolation, storage, and MP isolation (Thaler, 2011). During blood collection it is important to avoid tourniquet and use needle and collection system that apply minimal stress to the blood drawn. The high pressure of a tourniquet and the high shear on blood, when it is collected through small needles into tubes with vacuum, might cause haemolysis and the formation of erythrocyte MPs (Lacroix, 2012). It is important to choose a anticoagulant that limit platelet activation inside the tube, as the activation of platelets will release MPs and the sample will not represent the in vivo status. The most common used anticoagulant is citrate, which chelates Ca^{2+} (Lippi, 2006). After having collected the blood, the plasma must be isolated and the platelets must be removed by centrifugation. In order to get platelet free plasma a two-step procedure is often used, where the first spin is by 1,500G for 15 minutes followed by a high-speed centrifugation at 13,000 for 2 min (Freyssinet, 2005; Robert, 2009). However, the high-speed centrifugation might be too vigorous and deplete the sample of some MPs (Yuana, 2011). Therefore, Lacroix and colleagues suggested a double centrifugation that might be more fit for routine laboratories. Centrifuging the sample twice by 2,500G for 15 minutes produces platelet free plasma (Lacroix, 2012). Ideally, the plasma should be analysed fresh in order to resemble in vivo conditions, but this is mostly not possible for analyse of multiple samples. Therefore, it is important to use the same freeze and thaw procedures as both freezing and thawing affect residual platelets, that might unintentionally be present in the sample, to release MPs (Yuana, 2011). Lastly, some methods require isolation of MPs. When analysing isolated MPs there is less interference of plasma proteins and the concentration of MPs is higher. However, the direct measurement of plasma is to be preferred as the MP isolation might disturb the morphology and decrease the number of MPs (Yuana, 2011).

Analyses of MPs

Many different methods are used in the effort of analysing MPs including flow cytometry, functional assays on isolated MPs and capture based assays among others. There are advantages and disadvantages of all methods. Collectively, the recently applied methods are

all promising in giving an accurate measurement of MPs, but these methods do not provide any information on the functional properties of MPs. Additionally, the methods are mostly not suitable for routine laboratories as they require much labour and only few samples can be analysed at a time (Yuana, 2011).

Flow cytometry

Flow cytometry appears to be the most preferred method for analysis of MPs (Ahn, 2004).The method can measure directly on plasma and it can be used for assessment of cellular origin and enumerating of MPs (Shet, 2008). The enumeration of MPs can either be done based on the forward and sideward light scatter, which represent the size and granularity, or by using size calibration beads. However, the use of classical flow cytometers for MP analyses must be done with caution and with thought of the technical limitations that it entails. In flow cytometry the MPs appear close to the electronic noise, together with cellular debris, because of their small size, and it can therefore be difficult to identify them. Additionally, light scatter might not be the correct way of counting MPs. The laser beam of common flow cytometers excites at 488nm and MPs vary in size between 50-1000nm, but particle size cannot be measured when the incident light is in the same range as the particle Furie, 2006).

Capture assays

In solid phase capture assays the wells of a microtiter plate are coated with a probe, antibody or Annexin V that specifically binds MPs. These assays have made it possible to analyse large sample numbers at a time, presumed that the samples are frozen at stored until time of analysis. The advantages of functional assays include their simplicity and the use of well-defined reagents. The assays are semi-quantitative regarding MP concentration, but cannot give information on size and total number of MPs (Owens, 2010; Shet, 2008).

MP activity assay

Aupix and colleagues was the first to report of an assay with annexin V coated wells for the quantification of the procoagulant activity of MPs based on the presence of PS and the prothrombinase activity leading to conversion of thrombin (Aupeix et al., 1997). A commercial assay called Zymuphen MP-activity assay (Hyphen Biomed) is available. See Figure 6. The assay is assumedly based on the method developed by Aupix et al in 1997. However, the insert does not state that (Appendix M). The assay uses biotinylated annexin V bound to streptavidin-coated plates. Diluted plasma samples supplemented with calcium, FXa and thrombin inhibitors are added to the wells. When this is incubated, the phospholipids of MPs bind to the annexin V. Following a washing step, FXa and FVa are added together with prothrombin. The prothrombinase complex will, in the presence of Ca^{2+}, assemble on the surface of the captured MPs as they project PS. The amount of active FXa:FVa complex will convert prothrombin to thrombin. Then a thrombin specific substrate is added, and cleaved by thrombin, releasing the chromophore p-nitroaniline (pNA). pNA emits a yellow colour, which absorbance is recorded at 405nm. The absorbance is directly proportional to the amount of phospholipids in the sample. The calibration curve is made from a washed and lysed platelet concentrate (Appendix M). This is different from the calibration curve used by

Aupix et al, which contains 33% PS and 67% phosphatidylcholine (mol/mol) described by Pigeault and colleagues. Pigeault 1994. The concentration of MPs in the Zymuphen MP activity assay is established respectively to an internal standard and it is expressed in nanomolar (nM) PS equivalent. The controls CI and CII are prepared with human plasma. The insert does not reveal if there is something else in the controls (Appendix M).

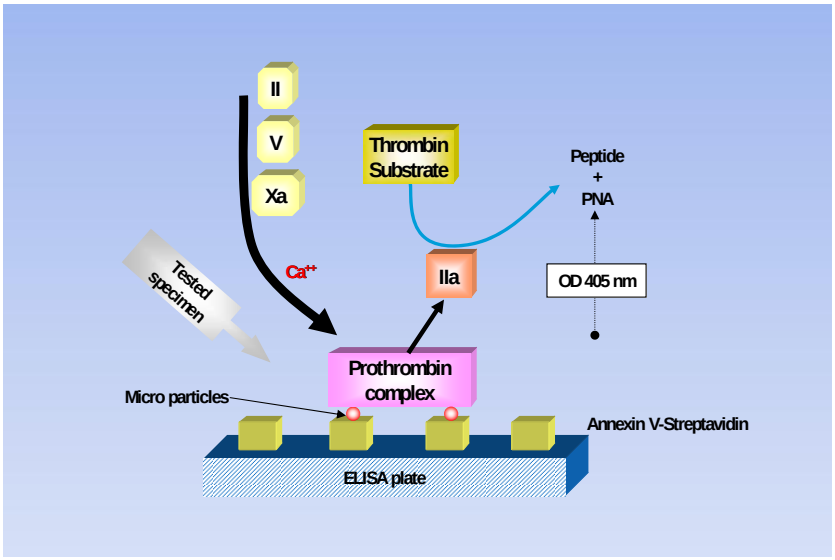


Figure 6 The MP activity assay principle.

MP TF assay

Aras and colleagues reported of an assay measuring the TF activity of MPs captured by monoclonal antibodies directed at human fibroblast surface protein 1B10. This antibody captures a variety of cells, including monocytes. Here the activity is measured as TF dependent FXa generation (Aras et al., 2004). Another study captured MP-TFs using a biotinylated anti-TF antibody. The TF activity of the captured MP-TFs were analysed by adding FVIIa and FX and measuring the amount of activated FX (FXa) (Bakouboula, 2008). When using this assay, it must be performed both in the presence and in the absence of an anti-human TF antibody to distinguish TF-dependent and TF-independent FXa generation. There is a commercial assay available that measures the activity of tissue factor-bearing MPs by FXa generation. This is called Zymuphen MP-TF assay (Hyphen Biomed). See Figure 7a and Figure 7b for a graphichal presentation of the principle of the assay. The activity assay is prepared by adding assay enhancer, undiluted plasma samples, calibrators, and controls to the wells of a microplate. MP TFs are captured by a murine monoclonal (MoAb) directed against the extracellular domain of TF, that does not inhibit TF activity. Following overnight incubation and a washing step, FVIIa and FX are added to the microplate wells. The extrinsic tenase complex consists of TF, FVII, and Ca^{2+} as an activating ion. Following incubation the TF-FVIIa complex forms. The inactive protease FX is converted to the active protease FXa by the complex in presence of Ca^{2+}. FXa generation is dependent on the presence of TF and anionic

phospholipids on the MP's surface with TF being a limiting factor. An FXa-specific substrate is added, reacts with FXa, release pNA, which absorbance is recorded at 405 nm. The absorbance is directly proportional to the amount of MP TF present in the sample. The calibration curve is prepared with recombinant full length human TF (1-263) and synthetic liposomes. The concentration is established against an internal standard. And the concentration of MP-TF is expressed as TF antigen equivalents. The insert does not state what the controls are made of (Appendix N).

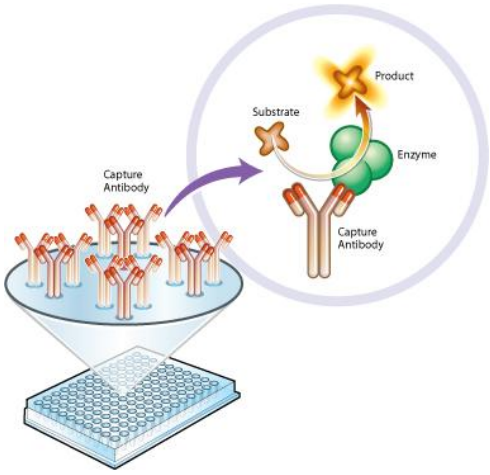


Figure 7a **The MP-TF assay principle.**

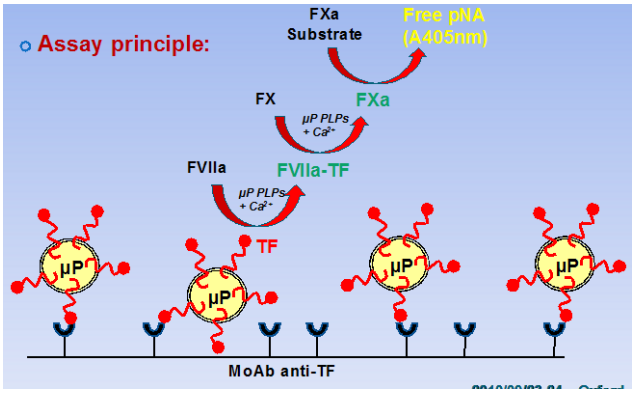


Figure 7b **The MP-TF assay principle schematically.**

2. Aim of the study

The aim of the study is to evaluate two novel commercially available ELISA assay's ability to detect the procoagulant activity of circulating microparticles in plasma.

The factors that will be evaluated are:

- Calibration
- Intra- and inter-assay variability
- The difference of using fresh and frozen samples
- The effect of different centrifugation procedures
- Ability to discriminate between healthy and cancer samples

3. Method and materials

Sample collection

Blood samples were taken from the antecubital vein, with light tourniquet, through a 21-gauge butterfly needle into 9 ml or 3.5 ml vacutubes containing 3.2% (0.109 mol) sodium citrate (Vacuette®, Greiner Bio-One). The first few millilitres were collected in a separate tube and discarded to avoid the potential artefact generated by the contact activation. Within two hours of blood collection the cells were removed by the respective centrifugation needed.

Centrifugation

The collected blood samples were centrifuged by tree different centrifugation methods. 1) The departmental routine centrifugation method, spinning 3220 g for 20 min at room temperature (RT). 2) A two-step centrifugation, recommended by the manufacturer of the applied assays, spinning first 15 min at 1500 g at RT, and a second spin for 2 min at 13000 g at RT. 3) A double centrifugation spinning twice for 15 min at 2500 g at RT. All centrifugations were performed on an eppendorf centrifuge 5810R (Eppendorf AG, 22331 Hamburg, Germany), except second spin in method 2, where an eppendorf centrifuge 5224 (Eppendorf AG, 22331 Hamburg, Germany) was used.

In method 2 and 3 the supernatant was transferred after the first centrifugation step into two 1.5 ml polypropylene microtubes (Gemü FlipTube®) and a 15 ml polypropylene Greiner centrifuge tube (Sigma Aldrich), respectively. Aspiration from both the 3.5 ml and the 9ml tubes was stopped 1cm above the buffy coat. After the second spin the supernatant was collected, leaving 100 µl of the pellet. The plasma was homogenized by rotation and split into 600 µl aliquots. Schematic over the centrifugations are shown in Appendix B.

Sample storage and handling

Platelet count was performed on the centrifuged supernatant plasma by a Sysmex KX-21N haematology analyser (Sysmex America Inc, Illinois, USA). The plasma was either analysed within 4 hours or immediately frozen and stored at -80 °C. Generally analysis was performed on frozen samples centrifuged by the double centrifugation, when nothing else is mentioned. Frozen samples were thawed in a water bath at 37 °C for 3 minutes.

Calcium ionophore stimulated plasma sample

Buffy coats collected the previous day and kept on ice are filled in tubes and centrifuged at 480 g, for 6 min at RT. Plasma was collected, pooled and treated with Ionomycin (Sigma-Aldrich, cat # IO 634), dissolved in ethanol, 10 µmol/l for 10 to 30 min, at 37 °C. This was then

centrifuged at 1850 g for 10 min at RT. Supernatant was collected in a new tube and centrifuged at 3000 g for 15 min at RT. The supernatant was transferred to a second new container and centrifuged at 3000 g for 5 min at RT. Plasma was aliquoted, snap-frozen in dry-ice and kept at 80 °C.

Solid-phase capture assays

The plasma samples were analysed by three different solid-phase capture assays from Hyphen Biomed (Nueville-Sur-Oise, France); the MP activity assay and the MP-TF assay. The assays were measured spectrophotometrically by a Spectramax M5 multimode microplate reader (Molecular devices, Sunnyvale, USA).

3.5.1 MP activity assay

The MP procoagulant activity was determined using the kit Zymuphen MP-activity from Hyphen Biomed (ref 521096, lot 110601H PK1). The manufacturers protocol was followed, see Appendix M. The MP activity assay was performed on plasma samples centrifuged by the three different centrifugation methods, both pre and post freezing, and on the calcium ionophore stimulated plasma sample.

3.5.2 MP-TF assay

The activity of TF associated with MPs was determined using the kit Zymuphen MP-TF from Hyphen Biomed (ref 521196, lot 111214F, lot 110315A). The manufacturers protocol was followed, see Appendix N. The MP TF assay was performed on plasma samples centrifuged by the three different centrifugation methods, both pre and post freezing, and on the calcium ionophore stimulated plasma sample

4. Results

Detection of platelets

In the supernatant plasma no platelets could be detected when using a Sysmex KX-21N haematology analyser (Sysmex America inc.), regardless of the centrifugation used.

MP activity assays

During the project nine assays were performed. The first six assays were performed on an entire 96-well microtiter plate (8 wells x 12 strips) and the last three assays were run a half plate (8 wells x 6 strips). See Appendix K for all data from MP activity. The amount of active microparticles was measured as equivalents of phospholipids (MP as nM PS), as it is the phospholipids that provide the negative surface which catalyse the prothrombinase complex. The concentration of MP as nM PS is determined with regards to a calibration curve, adhering to Lambert-Beer's law. See Appendix A

Calibration curves

A series of calibrator dilutions and two control values were run with every assay. Seven of the calibration curves were positioned in the same range, see Figure 8. The calibrator curve from MP 2 and MP 4 were very low and the controls did not fall within the respective acceptance ranges. As all of the assays were performed in the same laboratory with the same equipment, it is not likely that the two dropouts were due to instrumental changes but rather a wrong preparation of the calibration dilutions. Therefore the assays were excluded from the data analysis.

The calibration curves from MP 1-4 and MP 6 were prepared with six calibrator dilutions as the assay recommends. The curve from MP 5 was made from ten calibrator dilutions in order to closer determine the course of linearity for the calibrator and finally the curves from MP 7-9 was made from 5 dilutions to save space on the plate for samples. When using these calibration curves two curves out of seven were able to deliver both control values within their acceptance range. The assays where the calibration curves fitted to the controls were MP 8 and MP 9. It is observed in Figure 8 that the calibration curves bend off at the concentration 1,5/1,45 nM PS. In order to produce a linear calibration curve without this bend off, the highest concentration (3,0 or 2,9 nM PS) was removed and alternative curves were made. When using these alternative curves both control values fit in the remaining assays MP 1, MP 3, MP 5, MP 6 and MP 7. See the alternative calibration curves in Appendix E.

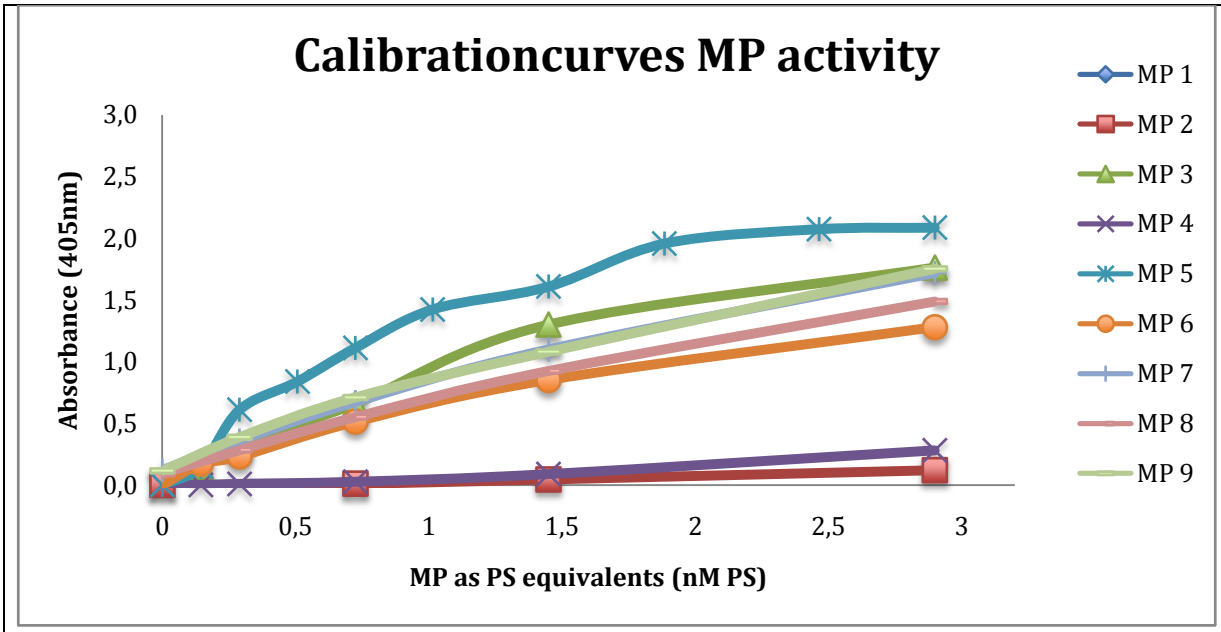


Figure 8 Calibration curves from 9 assays (MP1-9). The curves are prepared according to the insert of the assay. Calibraton curves MP 1-4 and MP 6 is made from six calibrator dilutions, MP 5 is made from ten and MP 7-9 is made from five calibrator dilutions. There is linearity in the curves from zero to 1,45/1,5 in concentration. After 1,45/1,5 the curve bends off.

Dynamic range

In order to determine the dynamic range of the assay a MP rich sample was analysed. Stimulating platelet rich buffy coats with a calcium ionophore produced a plasma sample rich in MPs. The concentration of MPs in the sample was not known but it was expected to be very high, so the sample was diluted heavily. The sample was assayed undiluted and diluted in two serial dilutions; a four-fold dilution ranging from 1/4 - 1/256 and 10 fold dilution ranging from 1/10-1/100000. With the exception that the dilution 1/100 was not analysed and a dilution of 1/500000 was added. Additional to these serial dilutions the sample (dilutions) was diluted as the assay prescribes (1/20, sample or dilution/buffer). See Appendix G for additional data on the dilutions.

The undiluted MP rich sample had a strong absorbance at 2,58; this exceeds the application of lambert beers law. The dilution 1/4 had a lower absorbance of 1,73. It was however not only 25% of the undiluted which had an absorbance of 94,3, it was 67%. This pattern was the same throughout the dilutions; samples are continuously lower in absorbance as the concentration lowers, however the relationship is not linear to the undiluted sample. Data are presented in Figure 9.

When the undiluted sample is set at 100% there is no linearity between the dilutions, see Figure 9. The goodness of fit for the trend line is 0,77804. When the 1/4 dilution (absorbance 1,73) is set to be 100% of the sample and the 1/256 dilution (absorbance 0,04) is the lowest included point, there is a good linearity, with a goodness of fit of 0,91229. When the lowest point is 1/256 and the 1/10 dilution (absorbance 1,17) or the 1/16 dilution (absorbance

0,74) is set to be 100% the linearity is excellent, 0,99992 and 1 respectively. This indicates that the limit of linearity, which is the upper limit of the dynamic range, is somewhere between the 1/4 dilution and 1/10 dilution. Meaning for this assay that the highest readable absorbance is between 1,73 and 1,17. These absorbances correspond to a MP concentration of 63,4 and 42,8 nM PS, respectively.

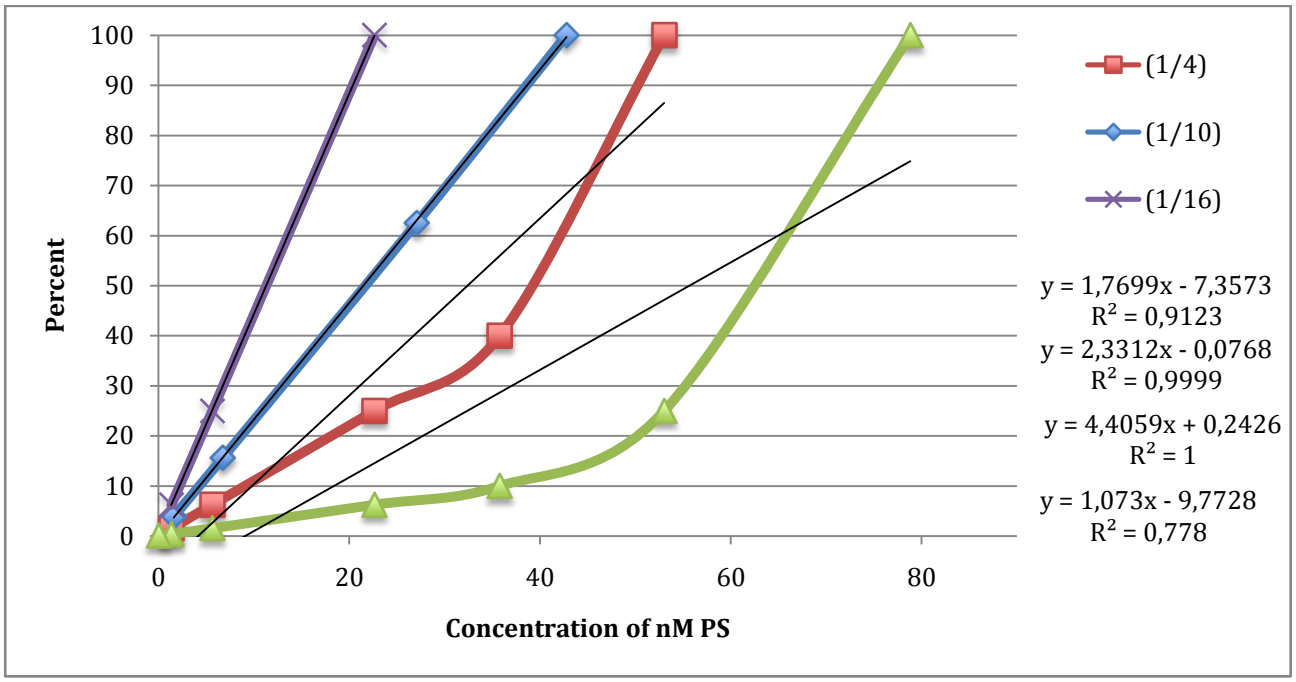
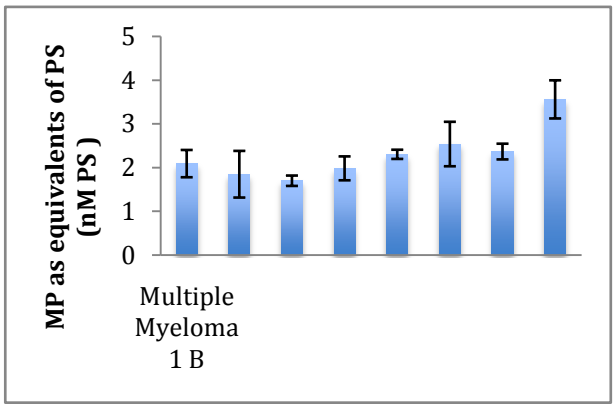


Figure 9 The linear relationship between dilutions and the value set to be 100% of undiluted MP rich sample. The linear regressions and the goodness of fit is showed in the same order as the curves: (1/4), (1/10), (1/16) and (undiluted).

The noise of the assay was 0,109 in absorbance. When the noise is subtracted from the readings the dilution 1/1000 is the highest dilution with an absorbance. This is 0,0021, giving a concentration of 0,04 nM PS. For the rest of the strongly diluted samples, the value is zero. However, this dilution 1/1000 is not linear with neither the 1/4 dilution nor the 1/10 dilution, so the concentration of 0,04 nM PS is the lower limit of detection. The lower limit of quantification and the lower limit of dynamic range is somewhere between the 1/1000 dilution and the 1/256 dilution. In absorbance this is 0,0021 and 0,04, giving concentrations of 0,4 and 1,6 nM PS.

Intra-assay variability

In order to test the assays ability to re-find the same value a sample from one multiple myeloma patient was analysed. The sample was loaded in eight rows of three wells, which gives 24 readings of the same sample. Additional to the data presented a setup with fresh healthy, frozen healthy, and frozen cancer samples was prepared in order to test reproducibility for different kinds of samples. However this assay was excluded as mentioned above.



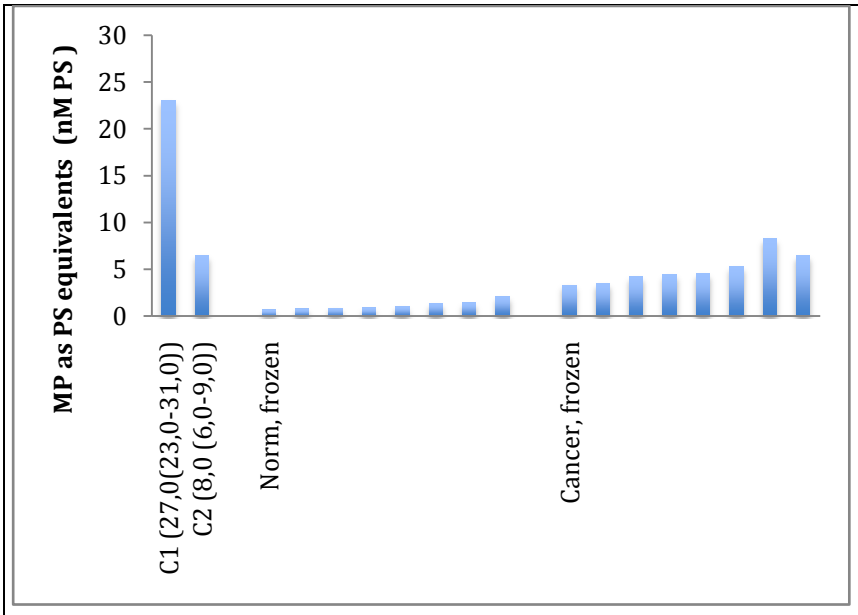
Number	24
Min	1,4
Max	4
Std	0,62
Mean	2,3
CV% (Std/Mean)	27

Figure 10 Intra assay variation of one sample. Eight rows of three wells are loaded with the same sample, giving 24 readings. Error bars indicating standard deviation of the triplet.

Table 1 Specifications on the readings presented in Figure 10.

The intra-assay variability was measured on plasma samples from one healthy subject and one cancer patient. We observe a top to bottom drift when analysing the same sample within different columns in the microtiter plate, giving increasingly higher concentrations of MP. See Figure 12 for the placing of the samples in the microtiter plate. Reagents were dispensed within 10 minutes and with equal time intervals according to the manufacturer's directions. The CV% is 40 for the healthy sample and 33 for the cancer sample Figure 11b.

After the observation of this drift on the plate, the duplets or triplets of samples were no longer placed adjacent to each other. The samples, controls and calibrator dilutions were placed randomly with one duplet (or triplet) in the left side of the plate and on in the right side of the plate.



Healthy, frozen	
Repeats	8
Min	0,7
Max	2
Std	0,46
Mean	1,1
CV% (Std/Mean)	40
Cancer, frozen	
Repeats	8
Min	3,3
Max	8
Std	1,66
Mean	5,0
CV% (Std/Mean)	33

Figure 11 Top to bottom drift on the plate, represented as left to right drift in these columns.

Table 2 Specifications for readings presented in Figure 11.

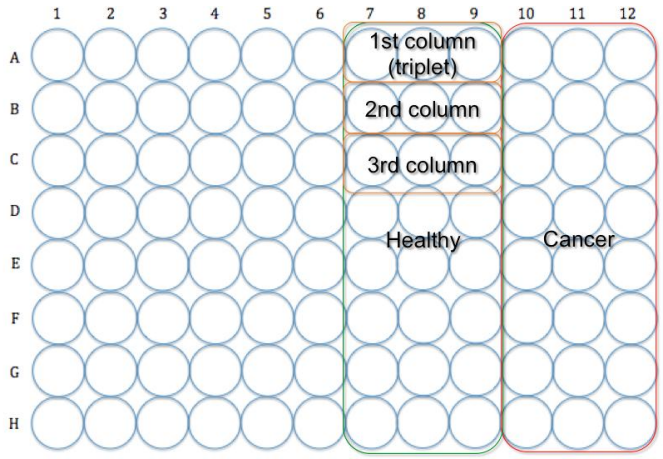


Figure 12 **The placing of the samples on the microtiter plate. The top row of the microtiter plate was loaded with samples that is presented in the first column of plasma samples in Figure 6a, the second row was loaded with samples that is presented in the second column of plasma samples in Figure 6a and so forward.**

Inter-assay variability

The mean value of the noise from 8 assays was 0.1015 and in the last assay the noise was 0,444. The total mean from 9 assays was 0,1395.

Two identical setups were run five days apart in order to determine the inter-assay variability. Plasma from seven healthy individuals and two patients was collected in 3,5 ml citrate tubes and centrifuged by double centrifugation. Additionally two samples from the patients were centrifuged by two-step centrifugation. Pt 1 was a coronary artery bypass graft patient and Pt 2 had atrial fibrillation. The difference in % between MP 7 and MP 8 was lowest in the control samples. In the healthy samples and the patient samples the difference between MP 7 and MP 8 was ranging from 9,8% to 50,9%. As seen in Figure 13 the error bars are not overlapping in MP 7 and MP 8. See Appendix I for additional data. The CV of the controls was 8%, the CV of the normal samples was 36% and the CV of the coronary patients was 19%.

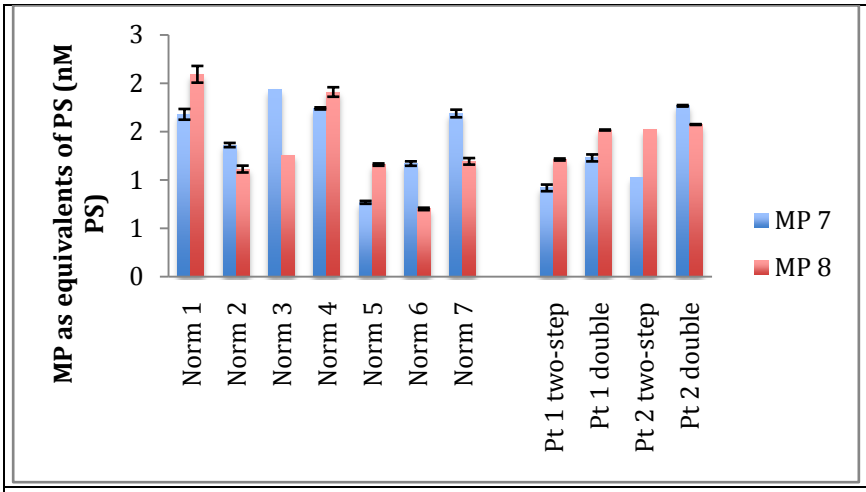


Figure 13 **Illustrating MP 7 and MP 8. Error bars represent the standard deviation of the duplet.**

Centrifugation

Plasma from three healthy volunteers (1, 2, 3) was centrifuged according to three protocols called single, double, and two-step. The data is presented in Figure 14. The samples were run in duplets and the error bars represent these. The single centrifugation gives the highest concentration for all three samples. The two-step centrifugation is lower than the single centrifugation and the double centrifugation has the lowest concentration of them all.

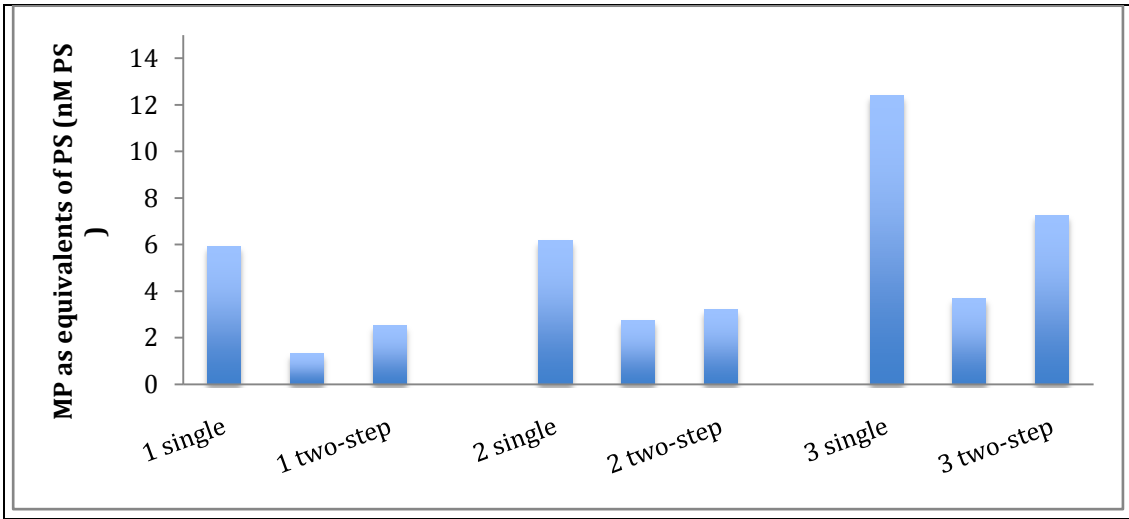


Figure 14 Illustrates the effect of centrifugation on the analysis. Samples from three persons, centrifuged by single, double and two-step centrifugation.

The samples used in this assay were provided by other on-going studies in the Department of Clinical Biochemistry, Aalborg Hospital. Therefore three of the samples are centrifuged different from the centrifugations described in the section Materials and methods. Samples were gathered from three different multiple myeloma patients (1, 2, 3). The samples had been centrifuged according to procedure A, B and C. A = 2000g, 15min, RT. B = 2 x (2500g, 15 min, RT). C = 2500g, 25min, RT + 2500g, 15min, RT. The two different samples centrifuged by C procedure were highest in concentration. The third sample was centrifuged by A and B. Procedure B (which is the double centrifugation) had the lowest concentrations. These samples were also analysed in an ELISA assay that measures the concentration of full length tissue factor antigen. The results can be seen in Appendix J.

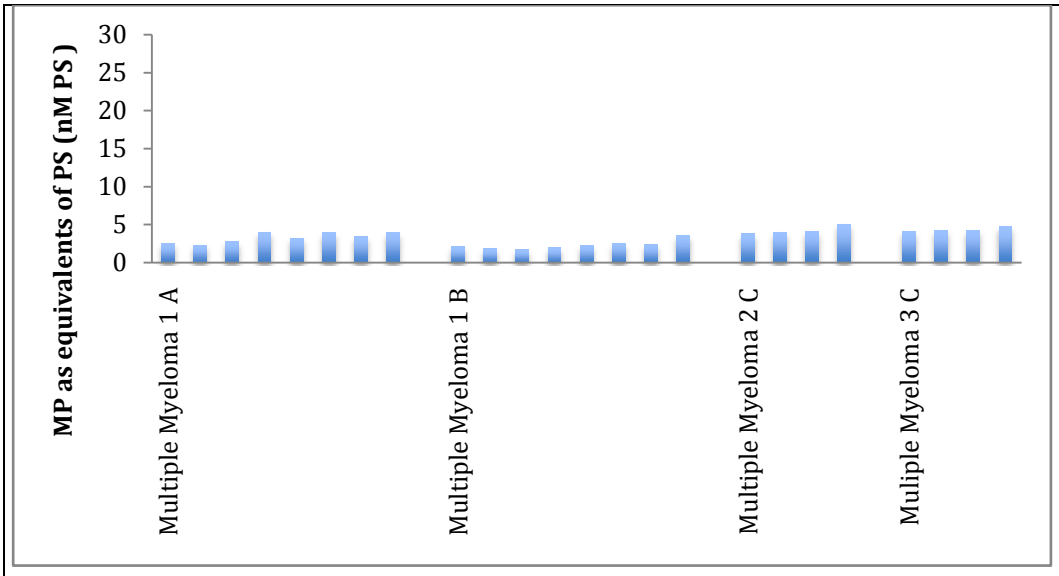


Figure 15 Impact of different centrifugation procedures.. Samples from three different multiple myeloma (MM) patients (1,2,3). Three centrifugation methods from the different studies (A, B, C). A = 2000g, 15min, RT B = 2 x (2500g, 15 min, RT). C = 2500g, 25min, RT + 2500g, 15min, RT. The first eight columns are readings of MM1A, the next eight columns are readings of MM1B, the next four columns are readings of MM2C and the last four columns are readings of MM3C.

Fresh and frozen samples

The effect of using frozen samples compared to fresh was analysed by having two setups with the same samples. The frozen samples had been in -80°C for seven days. In the single centrifugation the frozen samples are higher than the fresh samples. The two-step and double centrifugation showed little change in concentration. The fresh samples are higher in concentration than the frozen samples. The fresh single centrifuged sample is 47% different from the frozen single centrifuged sample, the fresh double centrifuged is 59% different from the frozen double centrifuged and the two-step centrifuged fresh sample is 67% different from the frozen two-step sample.

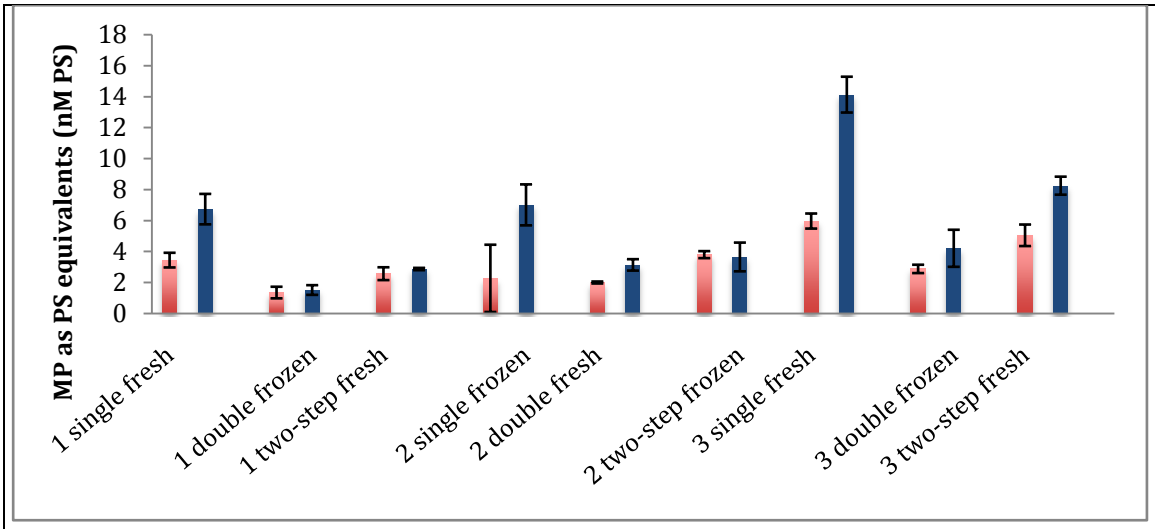


Figure 16 **The effect of using frozen samples, shown for three centrifugations.**

Healthy and cancer samples

In order to use the assay clinically it must be able to differentiate between healthy and disease states. A box plot of 27 readings from one healthy volunteer and 18 readings from one stomach cancer patient is seen in Figure 17. There is a two-fold difference between the mean of the healthy sample and the mean of the cancer sample. There is a no difference observed between two cardiac patients and seven healthy in Figure 13.

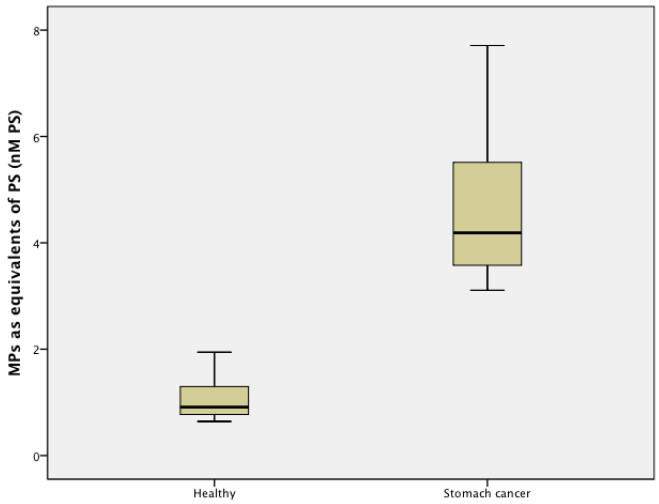


Figure 17 **The difference of 27 readings from one healthy volunteer and 18 readings from one stomach cancer patient.**

MP-TF assays

During the project 7 assays were performed. The first four assays were performed on an entire 96well plate (8 wells x 12 strips) and for the last three, a half plate was used (8 wells x 6 strips). The amount of MPs that carries active TF (MP-TF) was measured as equivalents of TF (pg/ml). The concentration of MP-TF as pg/ml was determined with regards to a calibration curve, adhering to Lambert Beer law (see Appendix A). If the absorbance from a sample is lower than the blank value, which represents noise, the sample is set to 0.

Calibration curves

A series of calibrator dilutions and two control values were run with every assay. The calibration curves from all seven assays are in the same range and all calibration curves were linear. For the MP TF 2 the two control values did not fall within their acceptance range, therefore this assay was excluded from the data analysis. Calibration curves can be seen in Appendix F.

Dynamic range

The MP rich sample was assayed in the same dilutions as described in the section MP activity, Dynamic range. The dilutions are shown in Appendix G. For this MP TF assay the control values were within their acceptance range. When the noise was removed from the readings all of the assayed samples were zero.

Intra-assay variability

Each of the two controls provided with the MP-TF assay were positioned in 5 rows and 3 wells per row, which gives 15 readings of each control. The CV% was 12 for the high control and 7 for the low control. The triplet readings had a low standard deviation, illustrated by the error bars on Figure 18.

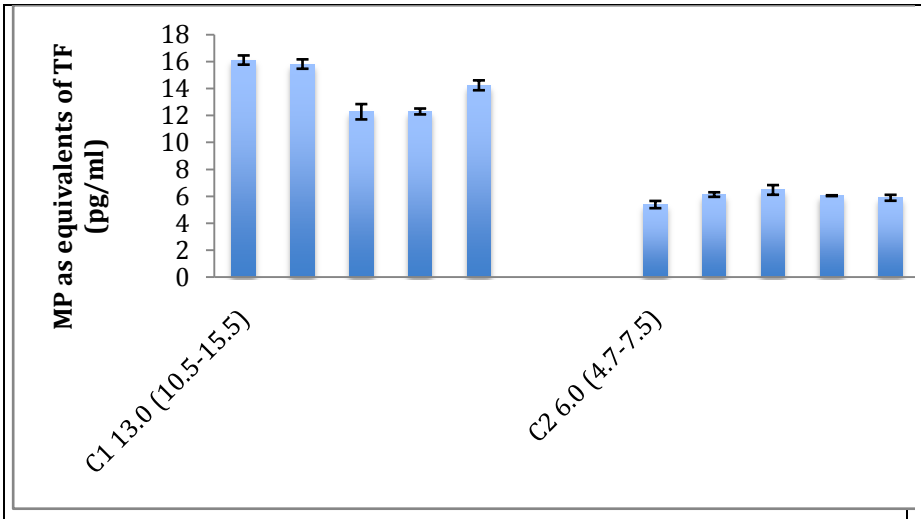


Figure 18 **Reproducibility of controls**

C1	
Number	15
Min	11,58
Max	16,35
Std	1,74
Mean	14,08
CV (Std/Mean)	12%
C2	
Number	15
Min	5,02
Max	6,82
Std	0,42
Mean	5,92
CV (Std/Mean)	7%

Table 3 **Specifications for Figure 18.**

To see the reproducibility of healthy plasma, 1 sample was positioned in 9 rows of 3 wells per row, which gives 27 readings of the sample. The error bars in Figure 19 show the standard deviation for each of the triplets. The CV of the 27 readings is 58%. Also 1 cancer sample was assayed to see the reproducibility of a pathological sample. The cancer sample was placed in 6 rows of 3 wells, which gives 18 readings of the sample. The CV% of the 18 readings is 32%.

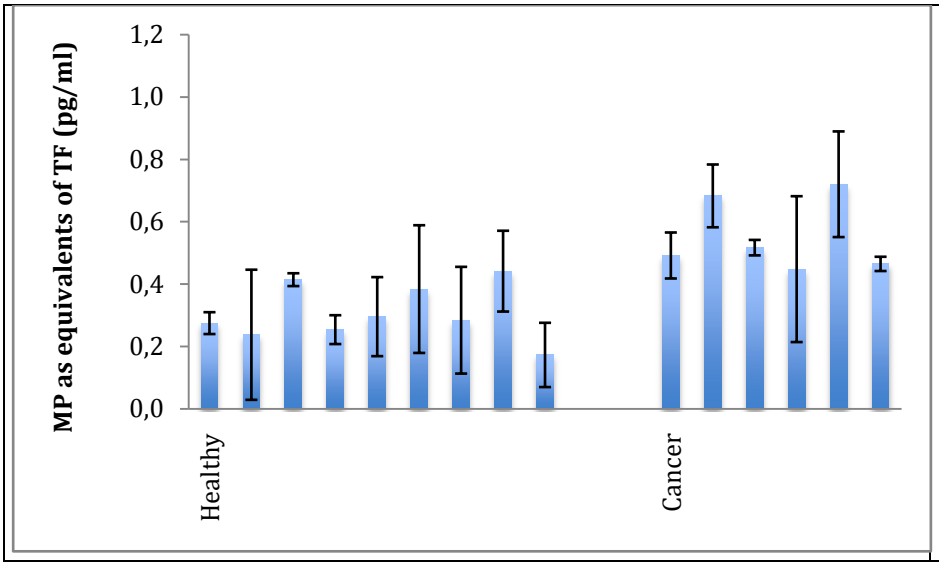


Figure 19 Reproducibility of one healthy sample and one cancer sample

Healthy	
Number	27
Min	0,002
Max	0,522
Std	0,140
Mean	0,241
CV (Std/Mean)	58%
Cancer	
Number	18
Min	0,147
Max	0,830
Std	0,158
Mean	0,494
CV (Std/Mean)	32%

Table XXX Specifications for Figure 19.

Inter-assay variability

The mean value of noise in the 7 assays was 0,2032. To test inter-assay variability a 96 well plate was divided in two and the two halves were run five days apart, with aliquots from the same samples. Control values were within their acceptance range for both assays, see Figure 20. There was a signal from all 11 samples in MP TF 5, but in the MP TF 6 the signal from Norm1, 3, 6 and 7 and Pt. 1 double and Pt. 2 two-step were below the noise. These values are set to be 0. It is therefore only possible to evaluate the inter-assay variability on the control values, see Figure 21.

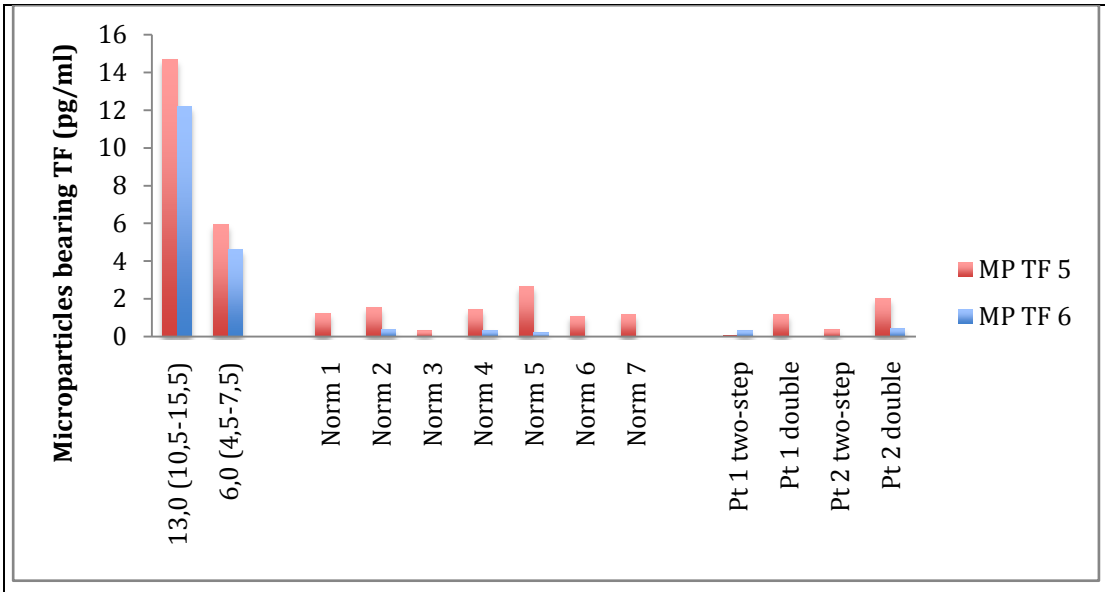


Figure 20 **The inter-assay variability of MP TF 5 and 6.**

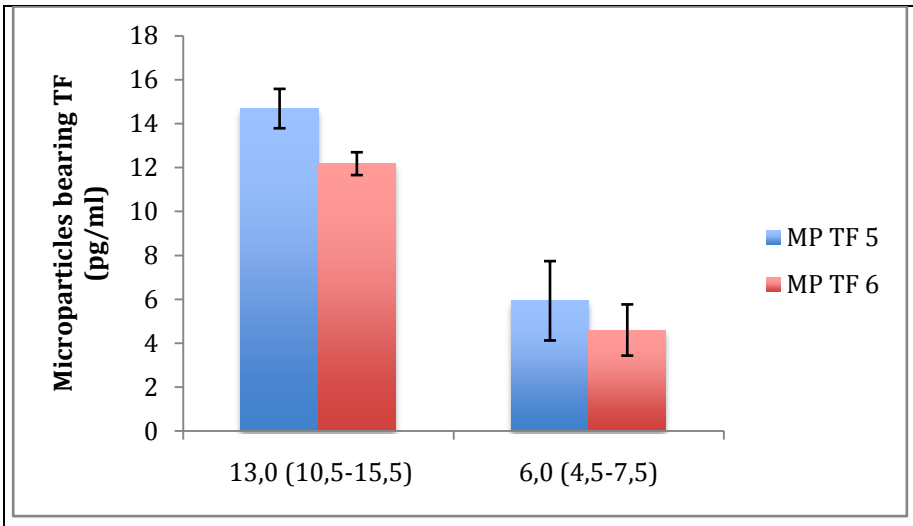


Figure 21 **Control values from MP TF 5 and 6.**

The controls were run in triplets and the error bars represent the standard deviation of the triplets. The high control from MP TF 6 is 17% different from the high control in MP TF 5. The low control in MP TF 6 is 22% different from the low control in MP TF 5.

Centrifugation

Plasma from three healthy volunteers (1, 2, 3) was centrifuged according to the three different protocols called single, double and two-step, see Figure 22. The samples were run in duplets and the error bars in Figure 22 represents these. The mean of the samples from person 2 are in general highest regardless of the centrifugation. Samples from person 1 and 3 are in the same range, however errors bars of the samples from person 3 are overlapping with person 2.

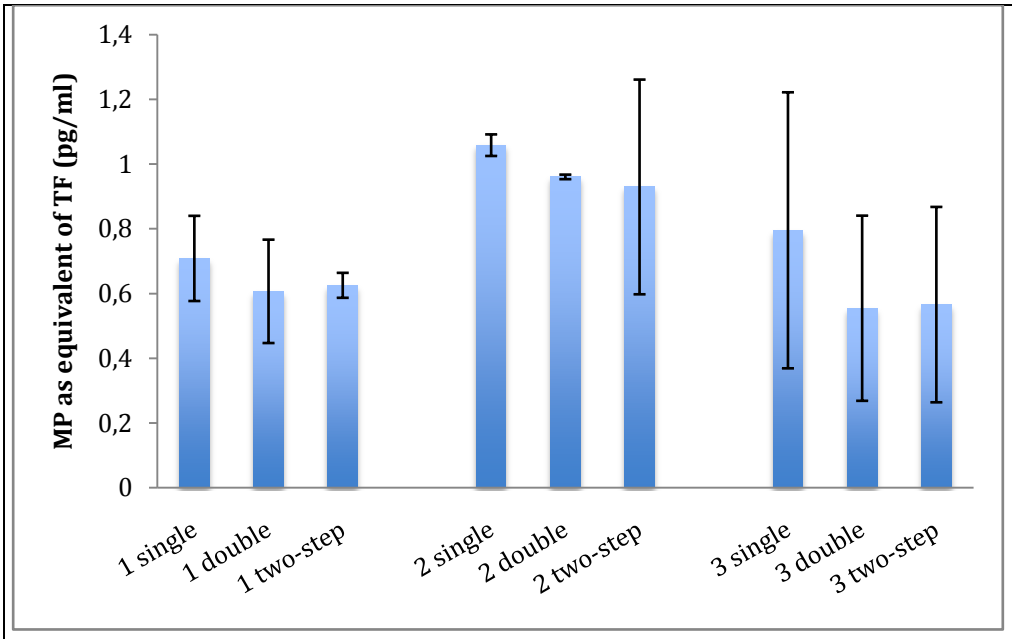


Figure 22 **The effect of centrifugation on the MP TF assay.**

Fresh and frozen samples

The majority of data on the difference between fresh and frozen samples were excluded from the analysis because an assay was excluded, as the controls were not within the acceptance ranges. The plasma from 1 person was analysed both fresh and frozen and centrifuged by the three centrifugations; single, double and two-step. Regardless of centrifugation, there is a higher concentration of TF in the fresh samples, than in the frozen samples.

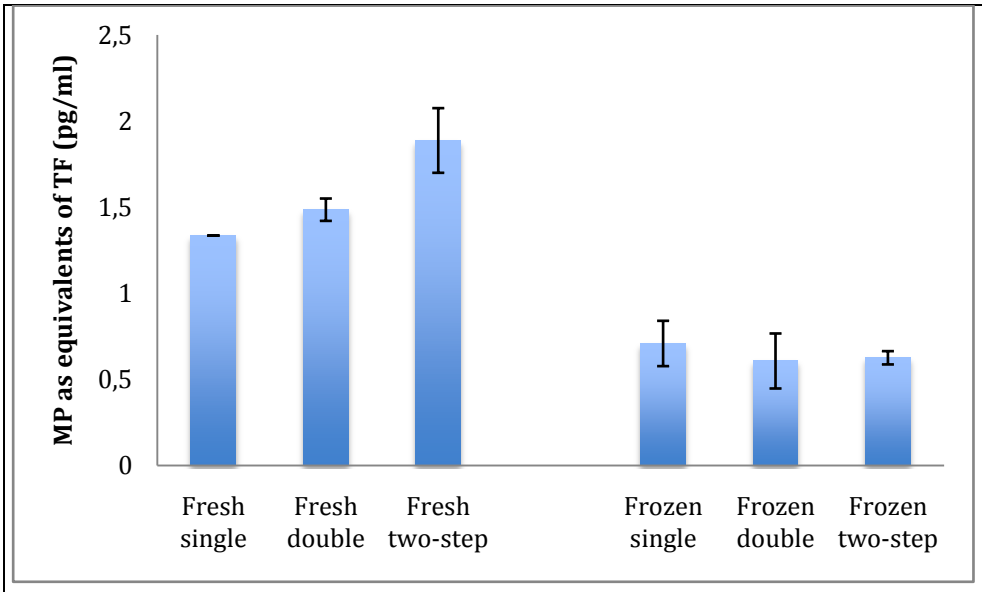


Figure 23 **The difference of fresh and frozen samples in the MP TF assay. Samples from one person, centrifuged by three procedures (single, double and two-step) for a fresh and a frozen sample.**

Healthy and cancer samples

The box plot of a sample from one healthy person compared to one cancer sample shows that the cancer sample is higher in concentration than the healthy. Error bars are overlapping in Figure 24.

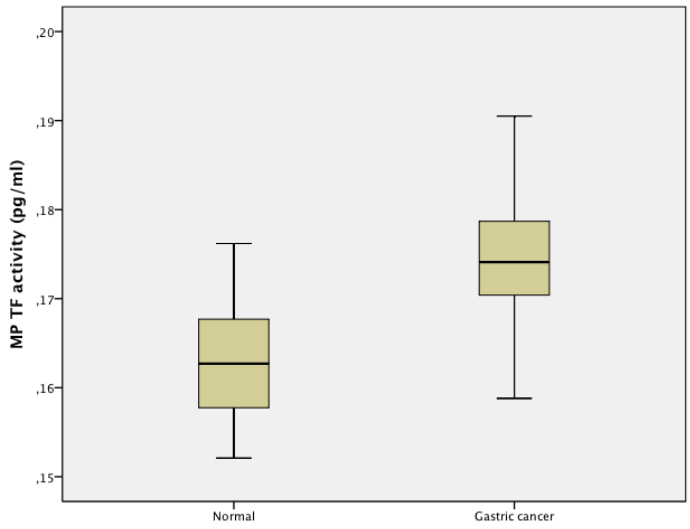


Figure 24 The difference between one healthy sample and one cancer sample.

5. Discussion

MP activity assay

Calibration curve

Seven out of nine calibration curves were not able to place the controls within their acceptance ranges. At the same time it was observed that the calibration curves bend off at 1,5/1,45 nM PS. Therefor alternative calibration curves without the highest concentration of the calibrator was calculated. These curves placed the controls within their acceptance range. When buying a commercial assay the idea is that one follows the insert from the kit and if there is any reason why one should deviate from these procedures, alternatives should be suggested in the kit. The insert states that if the controls are out of their ranges, then the assay should be re-run. This is problematic as the assays are very expensive. Furthermore, a re-run requires additional sample. This is a problem if the amount of available sample is limited. No laboratory would accept to reject 7 assays for every 9 performed.

An analytical issue is that the calculation of the MP concentration in plasma is based on comparison with a calibrator that does not have the same characteristics as plasma MPs. The calibrator is made of washed and lysed platelets and the samples are diluted plasma. The calibrator might behave differently than the MP plasma samples therefore comparison is somewhat far-fetched.

Dynamic range

The MP rich sample was analysed in different dilutions and this showed an excellent linearity between the absorbances 0,04 and 1,17. The lowest detectable absorbance was 0,0021, when the noise was removed. This gives a concentration of 0,04 nM PS. This is in accordance with the detection threshold of ≤0,05 nM stated in the insert. The sample used for this setup was artificially made very MP rich. When stimulating buffy coats with calcium ionophore the produced particles are platelet derived MPs (Burnier, 2009). This homogene collection of MPs does not resemble the collection of MPs that are found in plasma from either healthy or diseased individuals. But the sample served as a verification of the assays ability to find linearity in a series of dilutions. In order to test the linearity of samples that resembles ones from healthy, the control values could be used. The controls are made with human plasma and the manufacturer establishes the concentration. A series of dilutions of the high control would give a more natural picture of the linearity that can be expected when analysing plasma samples.

Intra- and inter-assay variability

The insert informs that the intra-assay CV is 3-8%. We found that for healthy plasma the CV is 40%, for cancer plasma the CV was 33% and 27% for multiple myeloma plasma. There is no

correlation between our findings and the one informed. These high CV%'s of the healthy and cancer sample might be due to the drift observed on the plate. However, the multiple myeloma sample was assayed in another setup where there were no obvious drift and the CV was still as high as 27% for this sample.
The inter-assay CV is 5-10% according to the insert. The CV of the controls was 8%, the CV of the normal samples was 36% and the CV of the coronary patients was 19%. The CV% of the controls is within the 5-10% stated; the plasma samples are far from the suggested range.

Centrifugation

There is observed a clear effect when different centrifugation methods are used. The double centrifugation gives the lowest concentration of nM PS. The single centrifugation is highest for all three samples. This is suspected to be due do platelet residues, although all samples were tested free of platelets on a Sysmex KX-21N haematology analyser. However, the sensitivity of the instrument is not high enough to detect fewer than 10 platelets in the sample. So even though the platelet count was zero there might have been some platelets present in the sample. These platelets would release MPs during freezing of the sample, giving a higher concentration than in vivo.
The findings of great variance of concentrations between different centrifugations of the same sample emphasis the need of using the same centrifugation procedure across laboratories and studies.

Fresh and frozen samples

The effect of freezing samples before analyses was very dependent on the centrifugation method used. Again the single centrifugation showed a large difference between fresh and frozen samples. This is suspected to platelets residues as explained above.

Healthy and cancer

The insert states that normal plasma has a concentration of or below 5 nM PS and that pathological samples are expected to be two fold that of normal, e.g. 10 nM PS. There is more than a two- fold difference between the means of 27 readings of one healthy sample and 18 readings of one stomach cancer sample. It is of course speculative to extract too much from the readings of two samples. The difference observed could very well be a coincidence. But it suggests that the assay has the ability to discriminate healthy and cancer samples.

MP-TF assay

To account for the specificity of the monoclonal antibody (MoAb) used, the manufacturer states in the insert: "MP-TF reactivity is inhibited by an anti-TF polyclonal antibody spiked into the sample". The MoAb used in the assay targets an epitope of the extracellular domain. Amino acid 1-219 is the extracellular domain of full length TF. The insert states that: "The MoAb does not react with truncated TF (1-219) nor synthetic liposomes". It is not explained how the MoAb differentiates between MP-TF and truncated TF. Additionally it was not

possible to find the term “truncated” TF in the scientific database available through Pubmed. So the company was asked to define truncated TF, in order to understand if the extracellular domain of truncated TF is different from the extracellular domain of full length TF. Unfortunately the company have not replied. It was possible to find two sources outside of pubmed, that uses the term truncated TF. One of the sources defines truncated TF as the extracellular domain of full length TF (Paborskysb, 1991). This leaves the idea that some of the MoAbs of the assay might be occupied by an eventual presence of a soluble antigen, such as truncated TF or the soluble asTF. The possible interference of soluble antigens may lead to underestimation of the MP levels.

Calibration curves

All of the calibration curves were fine and linear for this assay. This is probably owed to that the calibration curve is made of recombinant TF and liposomes, so the substrate is specifically designed for this assay.

Intra- and inter-assay variability

The intra- and inter-assay CV is not provided in the insert as it was for MP activity. The observed intra-assay CV for the high control was 12% and for the low control it was 7%. The CV for healthy samples was 58% and cancer sample 32%. As observed in the MP activity assay the reproducibility is highest for the controls and much lower for the plasma samples.
The inter-assay CV can only be established on the analyses of the high and low control. The CV of the high control is 28% and for the low it is 5%.

Centrifugation

It does not appear that the centrifugation method is of great importance in this assay. The three differently centrifuged samples from three persons are all positioned in the same range. The measured activity of this assay is not directly reliant on the presence of PS, so it is not the possibility of residual platelets. This suggests that samples used for this assay should not be sensitive to freezing.

Fresh frozen samples

There is a difference between the fresh and frozen samples. The fresh samples are higher in TF concentration than the frozen ones. This is contrary to the assumption made when looking barely at the centrifugation data for this assay. These data suggest that of samples are frozen for this analysis, it is important that all the analysed samples are frozen. Furthermore, it suggests that the measured activity of frozen samples might be lower than the actual in vivo activity.

Healthy and cancer samples

There is a difference between the healthy and the cancer sample. The cancer sample is higher than the healthy. However, both values are very low which is contrary to the assumption that MP-TFs might be present in high numbers in cancer cells. Actually the finding is in agreement with the finding of a study that found healthy individuals to have very low levels of MP-TF

avtivity (0.21±0.11 pg/ml) (Khorana, 2008). Another study showed that the procoagulant activity of MP-TF is at the same level in cancer patients without VTE as in normal healthy volunteers. However, cancer patients with VTE had significantly higher mean MP TF activity (1.7±3.8pg/ml, $p<0.05$) compared with cancer patients without VTE (0.5±0.5 pg/ml, $p<0.05$). The findings were measured within 24 hours of diagnosis of the VTE. (Manly, 2010). If MP TF activity should be used as a marker for the risk of VTE we need to know if the raised procoagulant activity is present all the time or only in the time interval surrounding the occurrence of the VTE. Some interesting questions would be: Is it possible to detect raised procoagulant activity before the first VTE? Is it possible to detect raised activity after the first occurrence and before a recurrence of VTE?

Solid phase assays in general

The drawback of a method often depends on the quality of reagents and the monoclonal antibody used. When a commercial assay is bought there is no internal quality measurements of the reagents and antibody at the purchaser, they rely on the manufacturer. If the assays were to be used routinely an in-house quality procedure had to be developed.

When analyzing MPs by solid phase assay, the advantage is that MPs are quantified and the procoagulant activity is measured at one time. However, one study states that MPs cannot always be detected by the binding to annexin V. This study in patients with sickle cell disease showed that all platelet derived MPs are positive of PS, but not all endothelial cell and monocyte derived MPs are positive of PS (Shet, 2003). So when using the assay the MP concentration might be calculated too low. The question is then if this is a good reflection on how the in vivo procoagulant activity is. If there is no PS present and no PS measured, then there might not be an increased procoagulant activity in vivo despite of a raised concentration of microparticles. If this is true, then the assays are still relevant for clinical use.

6. Conclusion

- The inter-assay reproducibility was good for controls and poor for plasma samples in **MP activity.**
- **MP TF** accurately determines the supplied control values, with a CV% of 12 and 7, respectively.
- The **MP activity** for frozen samples was higher than that observed for fresh samples as if freezing induces increased procoagulant activity.
- For the **MP TF** assay it appears as if the freezing of samples interferes with the assay's ability to detect MP bearing TF.
- Centrifugation plays a role in the **MP activity** and **MP TF** assays; where double centrifugation produced the lowest activity in healthy individuals in both assays.
- Our preliminary data suggest that both assays are able to discriminate between healthy individuals and cancer patients.

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