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UNIVERSITY OF CALIFORNIA, SAN DIEGO

**DETECTION OF PROTEASES IN WHOLE BLOOD AND OTHER**

**BIOLOGICAL FLUIDS IN DIFFERENT DISEASES**

A Dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Bioengineering

by

Augusta E. Modestino

Committee in charge:

      Professor Michael J. Heller, Chair
      Professor Antonio De Maio
      Professor David A. Gough
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      Professor Geert W. Schmid-Schönbein
      Professor Dorothy D. Sears

2014

The dissertation of Augusta E. Modestino is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

University of California, San Diego

2014

# DEDICATION

I dedicate all my work to my family, friends, and of course to Venezuela. This is all because and for you. Gracias.

# EPIGRAPH

"Tiene que haber una mejor forma de hacer esto"

Vanessa Coll

"We are very lucky to be here"

Dr. Michael J. Heller

"When you have a good set of results, the laboratory becomes the most interesting

place in the world"

Dr. Geert Schmid-Schönbein

"La cultura es el ejercicio profundo de la identidad."

Julio Cortazar

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SAO          Splanchnic Arterial Occlusion

T2DM        Type 2 Diabetes Mellitus

MMP        Metalloproteases

CLL         Chronic Lymphocytic Leukemia

POC         Point of Care

MOF        Multi Organ Failure

ELISA       Enzyme-Linked Immunosorbent Assay

ECM        Extracellular Matrix

PBS         Phosphate Buffered Saline

TBE         Tris Borate EDTA

RFU         Random Units of Fluorescence

EDTA       Ethylenediaminetetraacetic acid

IR           Insulin Receptor

# ACKNOWLEDGMENTS

I would like to acknowledge all the support that I have received throughout this years from my friends and family, especially from my parents, and my brother. It would have been simply impossible to do any of this work without their constant help.

I would like to acknowledge Dr. Schmid-Schönbein, not only for his constant support in my work, but it is him that introduce me on my first week in San Diego, to the *Autodigestion Hypothesis,* and how he transmitted how important it is for us, as scientists, to act as detectives, in order to understand the mechanisms behind all diseases. It has been a great honor, and truly a pleasure to work with him all this years, and I certainly look forward to many more.

I would like to acknowledge the chair of my committee. Dr. Michael J. Heller, who has been an incredible mentor, and has supported all my efforts since the very first day. It has been a tremendous honor working with him. It is because of Dr. Heller, that I have understood the importance of translating our work into the real life, a lesson that is of incredible importance, not only for this work, but for the rest of my life.

For the rest of my committee members: Dr. Erik Kristler, Dr. David Gough, Dr. Dorothy Sears, and Antonio De Maio, thank you very much in your input, and mentorship throughout my research.

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Altshuler AE., Richter MD., Modestino AE., Penn AH., Heller MJ., Schmid-Schönbein GW. Removal of luminal content protects the small intestine during hemorrhagic shock but is not sufficient to prevent lung injury. *Physiological Reports,* 2013, 1(5): e00109.

Levine JM., Cohen ND., Heller, MJ., Fajt, VJ., Levine, GW., Kerwin, SC., Trivedi, AA., Fandel, TM., Werb, Z., Modestino, AE., Noble, LJ. Efficacy of a Metalloproteinase Inhibitor in Spinal Cord Injured Dogs. *PLoS One*, *2014*, 9(5), e96408.

## ABSTRACT

Heller, MJ., Modestino, AM., Yu, J., Bhide, M. Lab-on-a-Chip device for rapid detection of Thrombin and other Protease activities directly in whole blood samples. InternationalConference of Biosensors, and Bioeletectronics. Las Vegas, 2012.

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ABSTRACT OF THE DISSERTATION


**DETECTION OF PROTEASES IN WHOLE BLOOD AND OTHER**

**BIOLOGICAL FLUIDS IN DIFFERENT DISEASES**


by


Augusta E. Modestino

Doctor of Philosophy in Bioengineering

University of California, San Diego, 2014

Professor Michael J. Heller, Chair

Proteases are enzymes that cleave proteins by hydrolyzing their peptide bonds. Proteases have been shown to be elevated in many diseases including shock, diabetes, several types of cancers, and coagulation disorders. Thus the ability to measure protease activity directly in whole blood, and other complex samples will allow for better diagnostics, better understanding of the disease progression, and the development of better therapeutics. Current techniques used to measure protease activity required considerable amounts of sample preparation, which is 1) time-consuming 2) costly and 3) alters the sample, making the reads less accurate. In this dissertation we have further developed a novel assay, which allows rapid measurements of different proteases activities directly in whole blood, and other complex samples, requiring no sample

preparation. The technology used by the charge-changing fluorescent substrates assays consists of a simple electrophoretic format.

In the past proteases have not been fully studied in many conditions, because sample preparation lead to un-accurate measurements of the proteases in most cases. In this dissertation we present the measurement of protease activity in the lymph fluid of rats, after physiological shock. An elevation of the metalloproteases-2, and -9, trypsin, and chymotrypsin activity after shock was observed. We also were able to measure the protease activity in whole blood samples of type 2 diabetics during a meal; where we found an elevation of metalloproteases -2, and -9, and elastase activity. Metalloproteases-2, and -9 activities were measured in whole untreated blood samples of CLL patients, where they were elevated, and it was observed that this activity came from B-cells. Because of the simple format of our assay, we have design and tested a prototype for a point of care system, which detected thrombin activity in whole untreated blood in only 27-mintues.

We have then demonstrated, that our charge-changing fluorescent substrate assay can be used with real complex clinical samples and no sample preparation for the detection of protease activities to: 1) gain a better understanding of the disease progression, 2) develop better diagnostics, and 3) develop new therapeutics for the different diseases, which will ultimately help patients managing their condition.

# CHAPTER 1

# INTRODUCTION

## 1.1. Motivation for Detection of Proteases

Proteases are a family of enzymes that catalyze the hydrolysis of specific peptide bonds of proteins, degrading them [1]. There are well over a thousand proteases in the body, and they play an important role in the life and death of organisms, for example: they are central in the digestion of food, the coagulation cascade, angiogenesis and apoptosis [2]. When the protease activity is not properly regulated, they are linked to the initiation and progression of many diseases and disorders, which include: physiological shock [3-8], diabetes [9-12], cancers [12-23], coagulation disorders (e.g. thrombosis, hemophilia) [24-27], among others. It is for this reason, that the detection of proteases could serve as an important tool for identification of new biomarkers, which would lead to better diagnostics, monitoring, and ultimately to the development of new therapeutics in all of these disease; this will ultimately increase the quality of life of the patients.

Protease detection in past decades has been insufficient in measuring in a reliable manner protease activity in a crude sample, such as blood, preventing advancements in the understanding of proteases in disease progression. Current techniques to measure protease activity require significant sample preparation, which not only adds time and cost of the measurement of protease activities, but it also jeopardizes the reliability of the reading. A new technique has been designed and tested by our group, this technique is capable of detecting low concentrations of protease activity in whole blood [28-30]. The subject of this dissertation is the further development of the technique for the detection new proteases, and to utilize it to help elucidate the role of proteases in the progression of the following diseases: physiological shock, type 2 diabetes, chronic lymphocytic leukemia, and coagulation disorders. This will be done in order not only to understand the role of proteases in the initiation, and progression of disorders, but also because this will ultimately lead to the development of better diagnostics, monitoring, and therapeutics.

## 1.2.    Organization of the Thesis

The presentation of this dissertation work has been divided into 7 chapters. In the first chapter, a literature review is presented as evidence of the role of proteases in: physiological shock, diabetes and metabolic disorder, chronic lymphocytic leukemia, and coagulation disorders. These reviews present the motivation behind this dissertation, and serve as evidence on the necessity of understanding the role of the proteases in diseases. The chapter closes with the presentation of the charge changing

fluorescent substrates, and the electrophoretic technique used through-out the dissertation, to measure protease activity in untreated biological samples.

The second chapter shows the detection of proteases in physiological shock in a rat model. In this chapter pancreatic proteases trypsin, and chymotrypsin, and metalloproteases -2 and -9 (MMP-2, and -9) are measured in lymphatic fluid of rats subjected to splanchnic arterial occlusion (SAO). Here we showed evidence that protease activity is present in the lymph fluid after SAO is induced. This provides a piece of evidence for the *Autodigestion Hypothesis.*

The third chapter shows the detection of pancreatic proteases: trypsin, and elastase, and also MMP-2, and -9, in untreated whole blood of a human after a high calorie, fat and sugar meal. The individuals in this study are suffering from type 2 diabetes mellitus (T2DM), or pre-type 2 diabetes mellitus (pre-T2DM). This is an exploratory study to gain understanding of the role of proteases in the initiation and progression of T2DM. It was shown that MMP-2, and -9, and elastase where elevated in T2DM. Moreover, it was shown that after a meal there is significant change in protease activity for T2DM patients. The results suggests that uncontrolled protease activity in the circulation might play a role in the initiation, and progression of diabetes.

The fourth chapter will focus on the detection of metalloproteases activity in chronic lymphocytic leukemia (CLL). The activity is measured in whole untreated blood, as well as plasma, and isolated B-cells. The findings of this study were that there is an elevation in the MMP-2, -9 activity in the blood of CLL patients; and that the

activity most likely came from the B-cells. This suggests that MMP-2, -9 in the B-cells is helping in the angiogenesis, and tumor invasion that leads to metastasis, and the progression of CLL.

The fifth chapter presents the detection of thrombin activity in whole blood. In this chapter the design and testing of a new charge-changing substrate specific to thrombin enzyme is presented. This new fluorescent charge changing peptide substrate was capable of detecting thrombin activity in whole untreated human blood within minutes, and using only a few micro-liters of blood, and most importantly required no sample preparation. We also, present the ability of this assay to differentiate between coagulation patterns of normal patients, and a normal healthy individual on a daily dose of aspirin. This finding, suggests that with further development, this thrombin activity assay could be used to diagnose, and monitor mild coagulation disorders.

The sixth chapter shows the design of a prototype of a point-of-care (POC) system for the detection of thrombin activity directly in whole blood. We present here a proof of principle POC device, capable of detecting thrombin activity directly in whole blood in only 27-minutes. This proof of principle POC prototype device, insinuates that it is possible to develop a POC device for the measurement of protease activity in whole blood. A seventh chapter will present the general conclusions of the findings of all this work, and the suggestions on the future direction for this project.

**1.3.** **Physiological Shock, Proteases and the Autodigestion Hypothesis**

Physiological shock followed by multi-organ failure (MOF) is a life threatening condition, which is responsible for taking the lives of hundreds of thousands of people each year in the U.S. alone. In spite of the fact that this condition is the primary cause of deaths of people under 40 years old in the intensive care units, no treatment other than alleviation of syndromes is available [31-32]. The impairment for the developing of therapeutics for this condition, has been a lack of understanding in the molecular mechanism leading to physiological shock.

Over the past two decades a theory has been proposed to explain the progression of shock to MOF, which ultimately leads to death, this theory is the *Autodigestion Hypothesis.* The hypothesis began its formulation because for decades there has been a clinical observation that pinpointed the intestine as a central player in the initiation, and progression of physiological shock [8]. The intestine has the peculiarity of containing active digestive enzymes (e.g. trypsin, chymotrypsin and elastase), which are discharged from the pancreas in their inactive zymogen form, and are activated by enterokinases in the small intestine. The fully activated enzymes are part of normal digestion, they are proteases that degrade food, and thus necessary for digestion. Protection against autodigestion, by the active proteases in the intestine, is possible through compartmentalization of the proteases inside the lumen of the intestine. The compartmentalization is possible by a layer of epithelium cells, and mucin secreted by goblets cells. During physiological shock it has been observed that there is an elevation in the permeability of the mucosal layer. This elevation in the permeability allows for

**1.4.    Physiological Shock, Proteases and the Autodigestion Hypothesis**

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the enzymes, and the inflammatory products from their digestion to enter the wall of the intestine, followed by further transport into the systemic circulation via the portal venous system, the intestinal lymphatics, and the perinoteneal cavity. Protease activity can then reach peripheral organs, and might be then the cause for MOF and death in shock **(Figure 1-1)**. Multiple studies support this hypothesis [3-8, 33-35], and recently it has been demonstrated that blockage of the pancreatic proteases after shock increases the chances of survival [4]. It is thus hypothesized that an early blockage of the protease activity in the lumen of the intestine of patients will increase their chances of survival. But it is necessary to understand the transport of these proteases in-vivo, and the development of specific inhibitors to the proteases that are unregulated, and present uncontrolled activity [7].

It is thus of great importance to be able to detect rapidly protease activity in different crude physiological samples (e.g. blood, plasma, lymph fluid) not only to gain understanding of the progression of the disease, but design and monitor therapeutics that will block the activity of these proteases during sepsis, preventing the action of proteases, which are probably responsible for the progression of physiological shock to MOF. Here we present the possibility of detecting these proteases in the lymphatic fluid of rats after they are subjected to a physiological shock model, using our charge changing substrates and the electrophoretic technique, as a first attempt to use our assay to understand the progression of physiological shock.
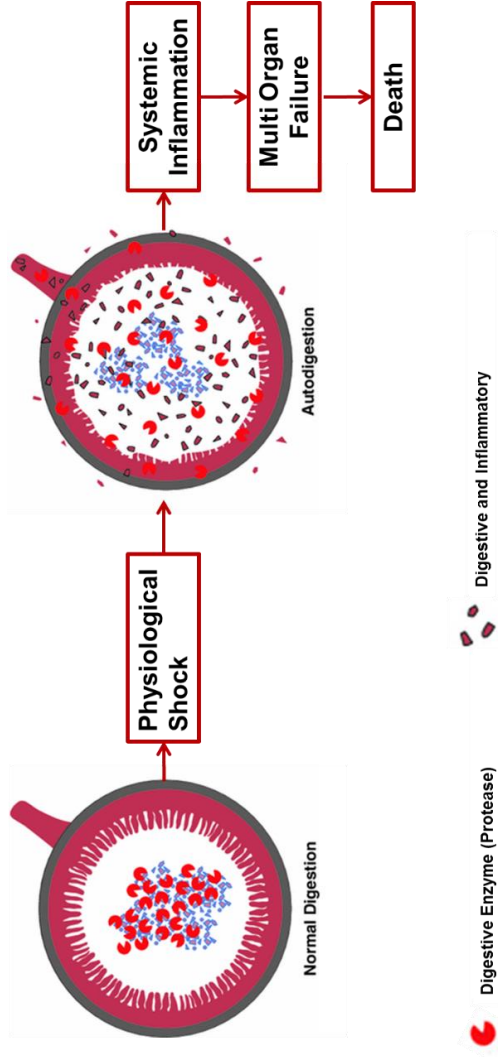
**Figure 1-1 Cross Section of Small Intestine during Normal Digestion, and After Physiological Shock.** In normal digestion digestive enzymes are compartmentalized in the lumen to digest food particles. Under shock conditions the intestine permeability increases, and allows the escape of digestive enzymes into the wall of the intestine (Autodigestion). Digestive enzymes and inflammatory fragments they generate escape into portal veins, into intestinal lymphatics and into the peritoneal space, reaching peripheral organs, and are responsible for MOF and death.

### 1.5.    Type 2 Diabetes Mellitus and Proteases and the Autodigestion Hypothesis

Type 2 diabetes mellitus (T2DM) is a disease associated with obesity, which develops when chronic over-nutrition, and genetic susceptibility align to cause insulin resistance [36]. Insulin normally controls glucose homeostasis, by binding to the insulin receptor, and stimulating the glucose up-take by the cells, and by suppressing the release of stored lipids from adipose tissue, in T2DM insulin is incapable of binding to the insulin receptor, and the uptake of glucose by the cells is impaired, raising the glucose levels in the circulation [37] **(Figure 1-2)**. Because of the staggering growth of obesity, diabetes has become a global epidemic, the proper prevention, and management of the disease will only be possible with a full understanding of the mechanism leading to T2DM.

A new mechanism to explain the development of insulin resistance has been proposed, which states that the resistance is caused by uncontrolled proteolytic activity of serine, cysteine or metalloproteases. These proteases are capable of cleaving the extracellular portion of the insulin receptor, causing an attenuation in the tolerance of glucose [38-40] **(Figure 1-3)**. The source of the uncontrolled protease activity remains to be investigated, but it has been proposed, that a less severe form of inflammation than shock, can cause a less severe form of increase permeability of the intestine, allowing digestive enzymes to escape from the lumen of the intestine, and as in shock enter the circulation. It is expected that these proteases will be less concentrated in the circulation in T2DM, than in physiological shock, but that they contribute with the chronic inflammation condition presented in patients suffering from T2DM.

Gaining an understanding of the activity of proteases in whole blood will allow us not only to understand the molecular mechanism leading to T2DM, and thus help not only to design better diagnostic, and disease management techniques, but it will help in the prevention of the T2DM global epidemic. Here charge changing peptide substrates for: trypsin, elastase, and MMP-2, and -9, and the electrophoretic technique were used to measure the activity of proteases directly in untreated whole human blood before, and after a high calorie, fat, and sugar meal; in order to help elucidate the complicated relation between protease activity and T2DM progression.
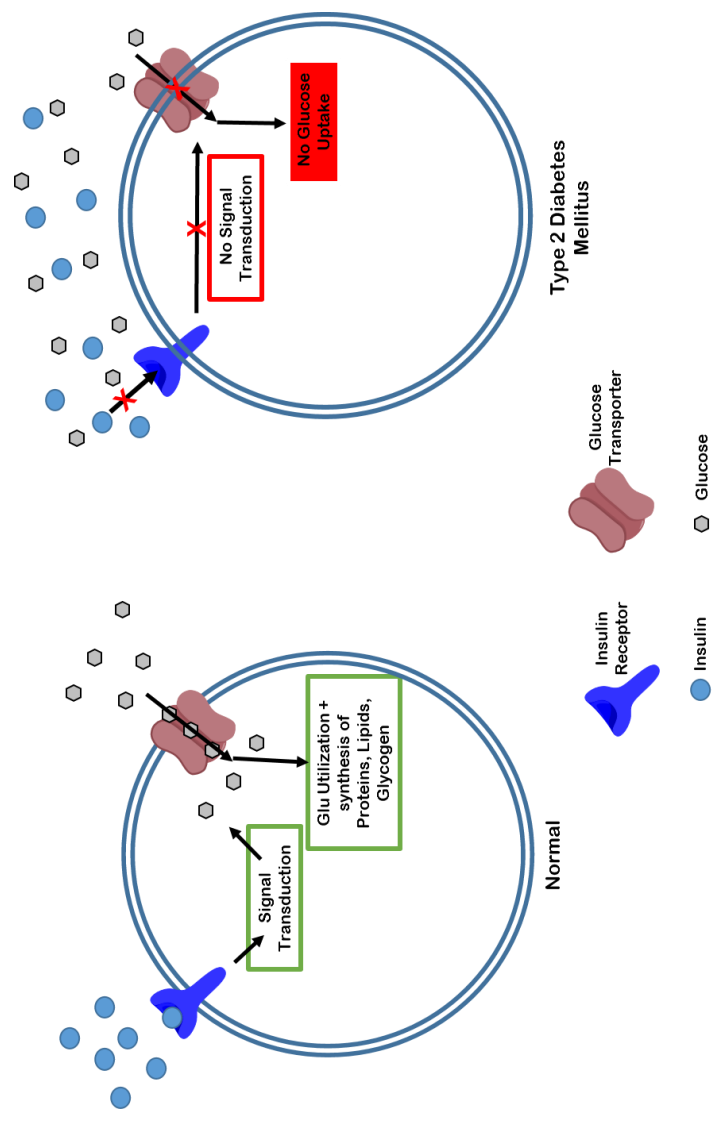
**Figure 1-2 Simplified Diagram of Glucose Uptake by the Cells under Normal and T2DM conditions.** Insulin normally binds to the insulin receptor, stimulating the glucose up-take by the cells. In T2DM insulin is incapable of binding to the insulin receptor, and the uptake of glucose by the cells is prevented, raising the glucose concentration in the blood.
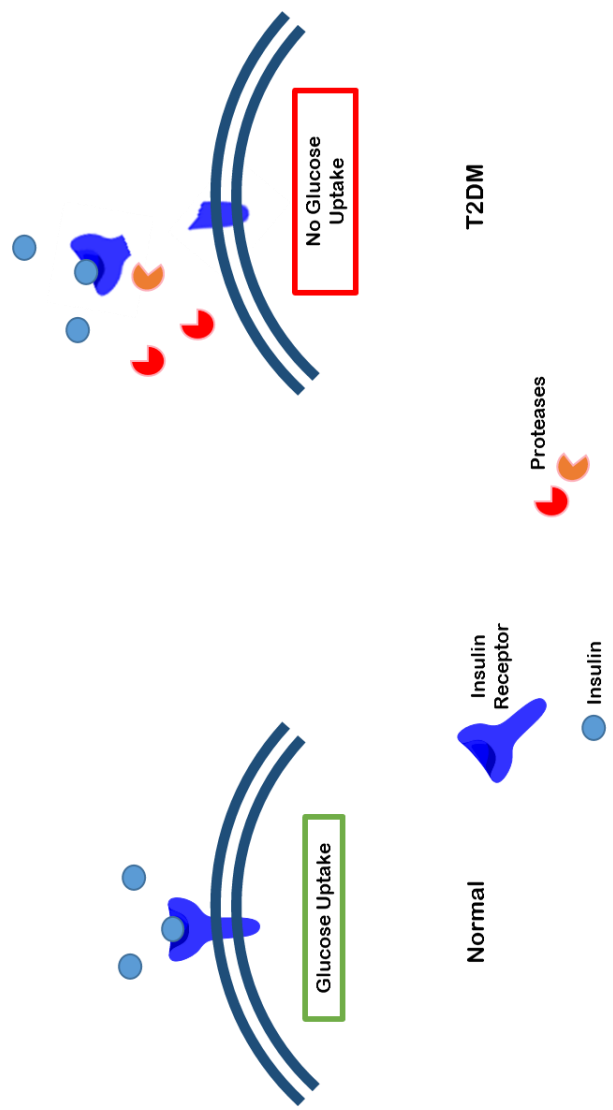
**Figure 1-3 Proposed Mechanism for T2DM.** Uncontrolled protease activity in the extra-cellular matrix, cleaves the insulin receptor, preventing the uptake of glucose by the cells.

## 1.6.    Chronic Lymphocytic Leukemia and Proteases

Study of proteases, especially metalloproteases, has been of importance in understanding the progression of multiple cancers, since they are all characterized by an increased in angiogenesis, tumor progression, and metastasis [41]. Metalloproteases (MMPs) are a family of proteases that have the ability of remodeling the extracellular matrix. From the MMPs, it is MMP-2, and -9 are of most interest, since they can cleave the mayor component of the basal layer (Collagen IV), facilitating the invasion of malignant cells into peripheral tissues, and collaborating in the progression of cancer. Chronic lymphocytic leukemia (CLL) is characterized by the accumulation of monoclonal, slow-dividing $CD5^+$ B lymphocytes in the peripheral blood. The progression of CLL is dependent in the infiltration of these B-cells into the bone marrow, and the secondary lymphoid tissue [42-44]. Growth factors, cytokines, among other molecules are responsible for the migration of the malignant cells into other tissues; but it is proteolytic degradation of the vascular basement membrane by the matrix MMPs that allows the migration to occur. Of the MMPs, MMP-2, and -9, are not only the responsible for degrading the principal component of basal layer, but they are also the principal MMPs produced in the B-Cells [43], and thus are of main importance in the initiation and progression of CLL.

Different studies have already associated the uncontrolled activity of MMPs with the progression of CLL [42-45]. Current techniques are labor intensive (e.g. ELISA, zymographies), and require significant sample preparation; making them useful in a research setting, but limited for use in clinics. We have used the charge-changing

substrates in an effort to investigate the activity of MMPs directly in untreated whole human blood of CLL patients. Using our technique we can gain understanding of the disease initiation and progression, and potentially use this technique for an early diagnostic of the CLL, which is a key factor in increasing the survival rate of patients.

## 1.7.    Coagulation Disorders and Thrombin Activity

Thrombin is a serine protease that is the key enzyme in the coagulation cascade. Activation of thrombin in the coagulation cascade permits the conversion of fibrinogen to fibrin, that crosslinks, forming a thrombus (blood clot), preventing blood loss in the case of injury [46]. Thrombin is then the pivotal enzyme, a high concentration of active thrombin will put patients at risk of generating thrombosis, while a low concentration of active thrombin will put patients at risk of bleeding.

From a clinical perspective, unregulated thrombin activity is linked to various clotting disorders [24, 25], cardiovascular diseases [47], several types of cancers [48, 49], sickle cell anemia [50], and inflammatory bowel disease [51], among others. The ability of measuring thrombin *in-vivo* is then of great interests to (1) further elucidate the relationship of thrombin concentrations in diseases, improve (2) diagnoses, (3) monitoring, and (4) management of these diseases.

Current method to assess thrombin levels and coagulation activity measure either the mechanical properties of the blood, or the concentrations of specific factors in the coagulation cascade. These techniques give a global view of the state of the coagulation, but fail to detect the mild bleeding disorders [24]. As an alternative technique thrombin generation tests have been developed; these tests require significant

sample preparation, adding to time, cost and also altering the concentrations of the factors in the coagulation cascade, thus risking to compromise the reliability of the results [24-25, 52-54]. Current development of thrombin assays focus on progressing in the sensitivity of the detection of thrombin activity, but they do not treat the problem of sample preparation, which is what prevents the rapid detection of thrombin activity *in-vivo.*

We created a charge-changing fluorescent peptide substrate that is specific to the thrombin enzyme, and that when combined with fresh untreated blood it is capable of detecting thrombin concentrations in just minutes, with a simple electrophoretic separation, and using only a few micro-liters of sample. The ease of use then gives promises to the development of a point-of-care (POC) system for the detection of thrombin activity, which can help in the diagnosis, monitoring and therapeutics of diseases that deal with unregulated activity of thrombin enzyme. A prototype of the POC system was also designed and tested for the rapid detection of thrombin activity in untreated whole human blood.

## 1.8.    Charge-Changing Protease Assay for Protease Detection with No Sample Preparation

Current techniques for the detection of the activity of proteases are labor intensive and require significant sample preparation. We believe that this is the main reason why proteases have been under-studied all of these years, even with the significant role that proteases might be playing in different diseases and disorders. Our group developed a charge changing substrate electrophoretic assay that allows for the

detection of proteases directly in whole untreated blood in a (1) simple, (2) rapid, (3) sensitive and (4) specific manner [28-30].

The assay consist of charge changing fluorescent peptide substrates, which are designed specifically to the protease that we plan to detect; and an electrophoretic separation. The charge changing substrates are designed to be either negative, or neutral; upon cleavage by the proteases of interests two fragments are produced, a negative one, and a positive one, which is tagged by the fluorescent molecule. An electrophoretic separation in a polyacrylamide gel, allows for separation of the un-cleaved, cleaved fluorescent substrates, and the sample, which contains multiple proteins that are mainly negative in charge (**Figure 1-4Figure 1-5**). The fluorescent activity captured in the gel, is then related to the concentration of protease activity in the sample.
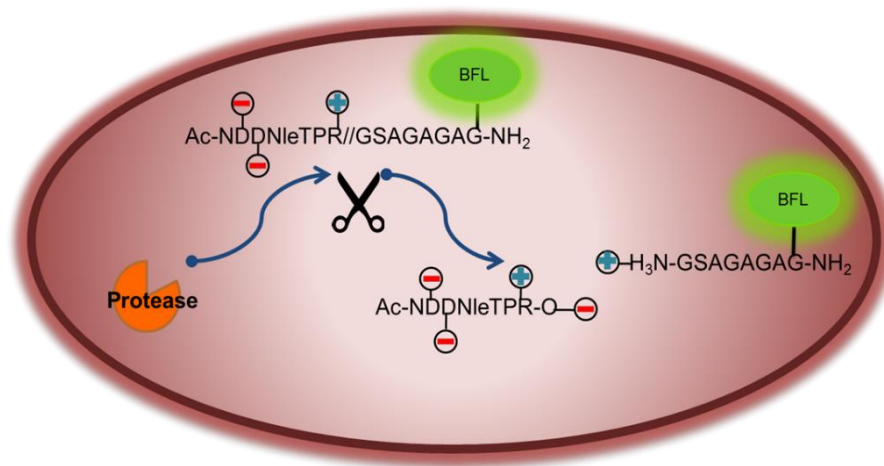
**Figure 1-4  Schematic of Charge Changing Peptide Substrates Whole Blood Protease Assay** Blood sample is combined with the substrate. Cleavage produces two fragments: a negative, and a positive fluorescently tagged.
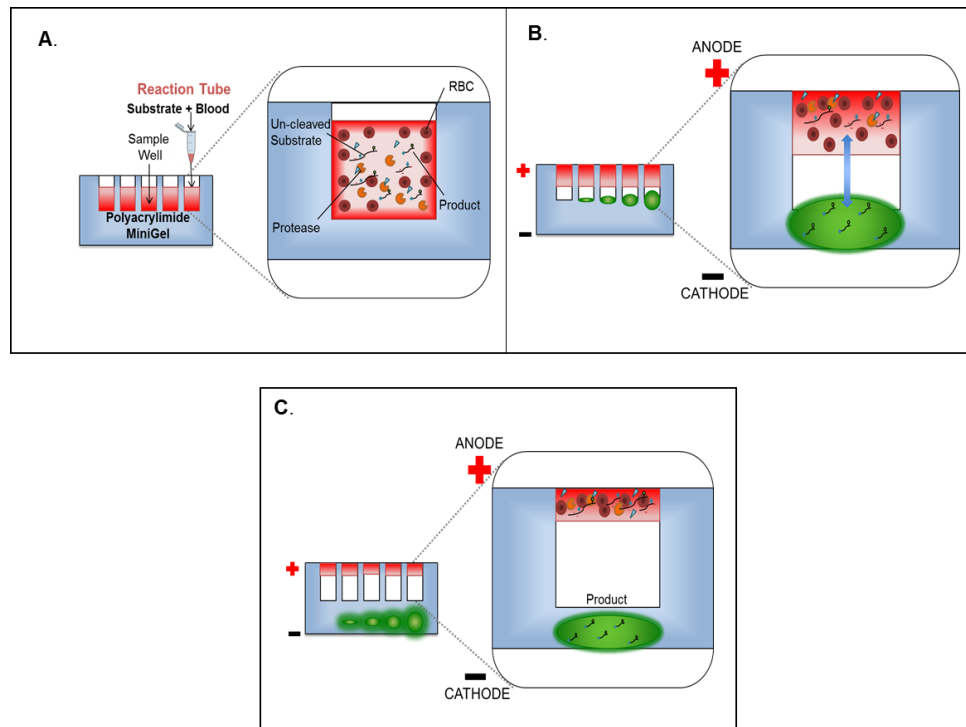
**Figure 1-5 Electrophoretic Separation- Charge Changing Protease Assay (A)** The substrate and blood sample are allowed to react for 30 minutes of time in a reaction tube. **(C)** Samples are loaded onto a polyacrylamide mini-gel, and electrophoresed for 10 minutes at 500V in the polyacrylamide mini-gel. **(D)** Separation of the charged components is observed, fluorescent signal is detected.

# CHAPTER 2 PHYSIOLOGICAL SHOCK-PROTEASE ACTIVITY IN THE LYMPH FLUID

## 2.1. Summary

Physiological shock, which leads to multiple organ dysfunction is placed among the most prevalent causes of death in intensive care units. Advancement in the treatment of this condition has been prevented by a lack of understanding of the molecular mechanism behind the progression of the disease. In this chapter, we present a study that measures the activity of pancreatic proteases: trypsin and chymotrypsin; and metalloproteases-2, and -9, in the lymphatic fluid of rats exposed to shock, in an effort to elucidate the relation of proteases and the progression of physiological shock. An increase in the protease activity after intestinal ischemia in the SAO experimental group was observed, when compared to baseline activity, suggesting that proteases in the

lymph can contribute with the progression of physiological shock that leads to MOF and death.

## 2.2.    Introduction

Physiological shock, and the subsequent multi organ failure (MOF) is placed among the leading causes of death in the intensive care units across the country [32]. Although, the high prevalence of the condition, the advancement in the treatment has been impaired by a lack of understanding of its mechanism of action. In this study, we used a new electrophoretic technique developed by our group [28-30] to evaluate the protease activity in the lymphatic fluids of rats after being subjected to splanchnic arterial occlusion (SAO), in order to gain understanding in the series of events that start in physiological shock, and eventually leads to MOF and death.

The lymphatic system serves as way of transport between the gut and distant organs. In the past it has been shown that inflammatory cytokines originating in the gut use the mesenteric lymphatics as a transport system to reach the circulatory system [55]. Many studies show the ability of mesenteric lymph fluid collected after a period of intestinal IR to recreate many of the symptoms of shock and MOF [56-60]. To this date there has not been a study measuring the activity levels of proteases in the lymphatics. Our group developed an electrophoretic technique that uses fluorescent charge changing peptide substrates, and a simple electrophoretic format, which allows for the detection of proteases in a rapid, sensitive, specific manner, and requires no sample preparation [61-63]. In this experiment we collected the lymph fluid of rats subjected to SAO, and

normal rats (SHAM), before and after intestinal ischemia. The proteases: trypsin, chymotrypsin, and MMP-2, and -9 activities were measured in the lymph fluid samples collected, and it was shown that their activity was elevated, in the SAO rat in the subsequent hours after shock.

## 2.3. Experiment Design

### 2.3.1. Surgical Procedure

Animal protocols were reviewed and approved by the University of California, San Diego Animal Subjects committee. Male Wistar rats weighing between 250 and 350 g (Harlan Labs, Indianapolis, IN) were administered general anesthesia with an intramuscular injection of 75 mg/kg ketamine and 4 mg/kg xylazine. Local anesthesia (1% Lidocaine) was delivered followed by cannulation of the left femoral artery and vein with PE 50 tubing. Catheters were filled with heparinized saline (10 mg/ml) to prevent clotting. After a 3 cm midline laparotomy, the duodenum and intestines were removed and placed on a 1 cm platform to the left of the animal and covered with saline-soaked gauze and saran wrap, exposing the base of the mesentery and the superior mesenteric lymph duct. A 1 cm bridge was placed under the animal to further expose the lymph duct, which was then cleared of fat and surface peritoneum using blunt dissection. Silastic tubing (0.64 mm internal diameter) pre-filled with heparin (10 mg/ml) was drawn through the right abdominal wall using a suture needle and looped under the vena cava using curved tweezers. The superior mesenteric lymph duct was

cannulated with the silastic tubing, and held in place with VetBond® tissue glue (3M, St. Paul, MN).

Once lymph fluid reached the end of the cannula, the end was placed inside of a 2 ml tube preloaded with 5 µl of heparin. Fluid was collected for one hour under the same conditions for every animal. For the splanchnic arterial occlusion (SAO) group (N=5), two micro-clamps were used to occlude the superior mesenteric artery and the celiac artery for the duration of the second hour, while animals in the SHAM group (N=5) remained perfused. After the second hour, the clamps were removed from the SAO animals to begin reperfusion. Lymph fluid was collected continuously for another three hours, and aliquoted every hour. At the end of each hour, samples were centrifuged (1600 g, 4°C, 20 min) to remove cellular debris and excess fat, the resultant supernatant was stored at -80°C. After the five hours of lymph fluid collection, animals were euthanized (120 mg/kg sodium pentobarbital i.v.).

### 2.3.2. Protease Activity Assay: Charge-Changing Substrates

The activity of protease trypsin, chymotrypsin and MMP-2 and -9, were measured in the lymph fluid collected using an electrophoretic technique developed by our group [28-30]. This method uses synthetic peptide fluorescent substrates, which are specifically cleaved by a protease of interest; the substrates are originally negatively charged, and upon cleavage two fragments are produced a negative one, and a fluorescently labeled positive one, this allows for detection in crude samples with no sample preparation. The substrates were synthesized by Aapptec (Louisville, KY), and

had the following sequences: acetyl-N-DGDAGRAGAGK-NH2 for trypsin, acetyl-N-DGDAGYAGLRGAG-NH2 for chymotrypsin, and acetyl-NGDPVGLTAGAGK-NH$_2$ for MMP-2 and MMP-9. The substrates were tagged with a fluorophore Bodipy-FL-SE (Invitrogen, Carlsbad, CA, USA). Stock solutions of the substrates with concentrations of 1.2mg/mL were prepared using 1XPBS (pH 7.8). Lymph fluid samples, were mixed with the substrate, the final concentration of the substrate in the reaction was of 0.6mg/mL. For the negative control 1XPBS (pH 7.8) was combined with the substrate. The reaction was allowed to proceed for 1 hour, after 6μL aliquots of the reaction were loaded onto the different lanes of the Novex 20% Polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) submerged in 0.5XTBE running buffer (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). The samples were electrophoresed at 500 V for 10 minutes; the gel was imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) at excitation and emission wavelengths of 302 nm and 500–580 nm respectively. The gel was scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) loaded with the ImageQuant v5.2 software using the following settings: fluorescence mode, high sensitivity, 100 mm pixel size, 1000 V photomultiplier tube with a 450 nm excitation filter and a 520 nm long pass emission filter. The image was quantified with ImageJ 1.440 and the fluorescent signal is then in function of specific protease activity in each of the samples.

Callibration curves were created by reacting known concentrations of pancreatic bovine trypsin (T9935) and chymotrypsin (C-4139), and recombinant human MMP2 (M9070) and MMP9 (M8945) all obtained from Sigma Aldrich (St. Louis, MO). Known

enzymes concentrations were combined with each of the substrates in 1XPBS (pH 7.8). The final concentrations of each enzyme of the reaction were: 5, 10, 20, 50, 100, 150, 200, 500 and 800nM in 1XPBS (pH 7.8). The negative control was each substrate combined with 1XPBS (pH 7.8). The reaction was allowed to proceed for 1 hour at room temperature. After the reaction time elapsed aliquots of 6μL aliquots of each reaction were loaded into a gel, and they were electrophoresed, and imaged in the same manner as described above.

### 2.3.3. Statistical Analysis

All measurements are presented as mean±standard deviation, with N=5 rats per group. Analysis of variance between groups was computed. Paired t-tests were used to compare protease activity measurements between time points and experimental groups for the protease activity measurements.

### 2.4. Results

### 2.4.1. Lymph Fluid Flow Rate

The lymph fluid was collected via cannulation of the superior mesenteric lymph duct before ischemia, and at three different time intervals after reperfusion: 1, 2 and 3 hours. Severe dehydration was avoided by repeated intravenous booster shots (once every 20-30 minutes) each flushed into the animal with 0.1-0.2 mL of saline. The amount of lymph fluid collected varied between animals, with larger animals tending to produce more lymph flow than the smaller animals, the lymph fluid flow rates are displayed in **Table 2-1**.

Charge changing substrates were used to measure trypsin, chymotrypsin, MMP-2 and -9 activity in lymph. The protease activity values were normalized to the pre-ischemia values, since the objective of the study was to evaluate the relative changes in protease activity after ischemia is induced. In shock animals trypsin activity in the lymph fluid increased after ischemia. The trypsin activity remained elevated in this rat group, through the three-reperfusion time points; the peak trypsin-like activity was recorded 1 hour after reperfusion, with the activity being three-times higher than the normalized pre-ischemia activity levels. The SHAM control rat group showed very little elevation in trypsin activity in the lymph fluid sample collected **(Figure 2-1)**.

A similar trend was observed for the chymotrypsin-like activity. In the SAO experimental rat group the chymotrypsin activity was higher through the three-reperfusion time points. In the first hour of reperfusion chymotrypsin-like activity was two times higher than the normalized pre-ischemic levels, and remained elevated and constant in the second and third hour of reperfusion. For the SHAM control rat group no significant increase in chymotrypsin-like activity was observed **(Figure 2-2)**.

Using known concentrations of exogenous trypsin and chymotrypsin, we obtained a calibration curve for each protease (**Figure 2-3**). The linear segment of each calibration curve allowed us to approximate the active enzyme concentration in each sample using a least squares fit. The mean trypsin and chymotrypsin activity is presented in **Table 2-2** and **Table 2-3** respectively. Note that these values are only approximate, since trypsin and chymotrypsin enzymes do not cleave the substrates at equal rates to that of pure trypsin and chymotrypsin in buffer.

MMP-2 and -9 activity showed similar trends to that of the pancreatic proteases. MMP-2 and -9 activity during the first hour of reperfusion was over three-times than that of the normalized pre-ischemic levels, the enzyme activity kept increasing through the three hours of reperfusion. After the third hour of reperfusion the MMP-2 and -9 activity in the lymph fluid was over five times than the pre-ischemic levels. For the SHAM control group the MMP-2 and -9 activities in the lymph fluid increased with time, after the three-hours the MMP-2 and -9 activity doubled. **(Figure 2-4)**. Using known concentrations of exogenous MMP-2 and -9 calibration a curve for each protease was created **(Figure 2-5)**. The values obtained were not related to the fluorescent values obtained for the study, because at this stage in the design of the assay it is impossible to differentiate whether the fluorescent signal, comes from the activity of either MMP-2 or -9 enzymes.

## 2.5.    Discussion

### A. Protease Activity in the Mesenteric Lymph

The study showed that charge-changing peptide substrates and the electrophoretic technique was successful in detecting the protease activity in the lymph fluid; moreover the technique showed that it was sensitive enough to detect the changes in activity after ischemia is induced in the intestine. To our knowledge other techniques (e.g. casein substrates and zymographies) lack in sensitivity, and specificity, and it is for this reasons that the change in protease activity after ischemia has never been so clearly recorded. The results of this study show clear evidence of increased protease

activity in the mesenteric lymph following intestinal ischemia. For both rat groups, SAO experimental and SHAM control rat group; the lymph fluid was collected for one hour preceding the hour of intestinal ischemia for the SAO group, or the SHAM ischemia for the control group. This sample point was collected as a standard to compare the protease activity in the lymph fluid at later points, after the ischemia was induced. Surprisingly, these samples displayed a significant amount of protease activity, suggesting the existence of a base level unregulated protease activity in the lymph. There was also a large variance between animals, with older animals tending to have higher protease activity in their pre-ischemic values.

The protease activity in the lymph increased throughout reperfusion, for the SAO experimental rat group. In the SHAM control group, the proteases activities showed remained stable throughout the reperfusion time points. The change in proteases activities in the lymph fluid of SAO experimental group is clearly shown in the fluorescent charge-changing peptide substrates assay. These measurements were only possible with this assay, due to its capability of measuring nanomolar concentrations of specific proteases in a crude sample. The use of this new assay, allowed for the first time to record the change in specific proteases activities in the lymph fluid after intestinal ischemia was induced.

For the charge changing protease substrates the pancreatic proteases activities of trypsin and chymotrypsin, in the first hour of reperfusion, were two and three times greater than the normalized pre-ischemic levels, and remained elevated in the second and third hour of reperfusion. Calibration curves, in which, the charge-changing

substrates were reacted with known concentrations of trypsin and chymotrypsin; gave an estimate of the concentration of these enzymes in the lymph. The 77nM change in trypsin concentration is much smaller than the reported 1-40µM trypsin concentration in the intestinal lumen of rats; but is larger than the 10nM concentration that has been reported in rat plasma samples post-shock [61, 64]. This is by no means a definitive evidence of the path that proteases might be taking to travel from the intestine, to the blood; but it does suggest that there is a possibility that the proteases are escaping the lumen of the intestine through the lymphatics, and reaching blood circulation, through which have access to distant organs. Moreover, the concentration of trypsin that we report here may be quite significant, since it is above the threshold for the activation of the protease-activated receptor (PAR-2), which modulates the inflammatory response to proteolytic activity [64].

The charge-changing protease substrates were also able to measure the metalloproteases activities, MMP-2 and -9. It was observed that after the first hour reperfusion the metalloproteases activity had already increased to more than three-times the pre-ischemic protease measurements. The metalloproteases activity continued to increase throughout the reperfusion time points, after the third hour the activity was over five times the pre-ischemic protease measurements. Trypsin increases the inflammatory response after reperfusion, and it also stimulates the rapid conversion of neutrophil released pro-MMP-9 into the lower molecular weight and enzymatically active MMP-9 [65]. Thus it is possible that the increased in activity of the metalloproteases in the

lymph, is a result of the increased in activity of the pancreatic proteases, specifically trypsin, though further experiments will be required to prove this hypothesis.

### A. Gut Barrier Dysfunction and the *Autodigestion Hypothesis*

The increase in protease activity after intestinal ischemia suggest that proteases can be a contributor to the pathogenesis and the activation of the inflammation cascade leading to MOF. But the increase in protease activity after SAO does not provide conclusive evidence for or against the *Autodigestion Hypothesis*. The hypothesis says that proteases in the lumen of the intestine damage the mucosal barrier, and thus the intestine increases its permeability, digestive proteases (trypsin and chymotrypsin) can then escape, and activate an inflammatory response in the organism, which will end up in MOF [3-8, 33-35], **(Figure 2-6)**. Though the lymphatics system serves as a conduit for intestinally absorbed molecules, it is also a collection point for the entire splanchnic bed, which includes the pancreas and liver. So we can't say with certainty that the protease activity increase after splanchnic IR detected in the lymph fluid, is a result of intestinal proteases escaping the lumen [66].

Other studies have showed that trypsin, chymotrypsin, and elastase concentrations are elevated in homogenates of the intestinal wall, mesentery, and peritoneal fluid [38, 61, 67]. The reason we chose to look in lymph was to see if this elevation is related to the MOF inducing effects of postshock lymph. These results may not give evidence for the Autodigestion theory, but there is certainly a basis for implicating the intestine as a likely source of proteases. Further studies, should focus on measuring the protease activity in the blood of rats after SAO, to gain an understanding

if these proteases can then reach also the circulation, as it is hypothesized that they might. It is important to mention that this results were only possible with the use of charge-changing substrates, and their capability of detecting nanomolar concentrations of specific protease activity in a crude sample.

There is a basic level of protease activity (in the nanomolar range) in the mesenteric lymph that significantly increases relative to levels in the SHAM control rat group after splanchnic arterial occlusion. Charge-changing protease substrates for trypsin chymotrypsin and MMP-2 and -9 made possible to detect a difference in the activity of these proteases after SAO. The increased in activity recorded implicates proteases as a possible mediator for the systemic inflammation and subsequent organ failure seen in patients suffering from circulatory shock.

**Table 2-1** Lymph Volume collected during each time point for the shock (SAO) and SHAM shock groups

| Experimental Group | Pre-Ischemia | Ischemia | Reperfusion Hour 1 | Reperfusion Hour 2 | Reperfusion Hour 3 |
|---|---|---|---|---|---|
| SAO (ml) | 0.73±0.20 | 0.14±0.06 | 6.80±0.83 | 7.05±0.62 | 6.90±0.34 |
| SHAM (ml) | 7.66±1.10 | 7.13±0.74 | 6.96±0.69 | 7.14±0.80 | 6.84±0.51 |
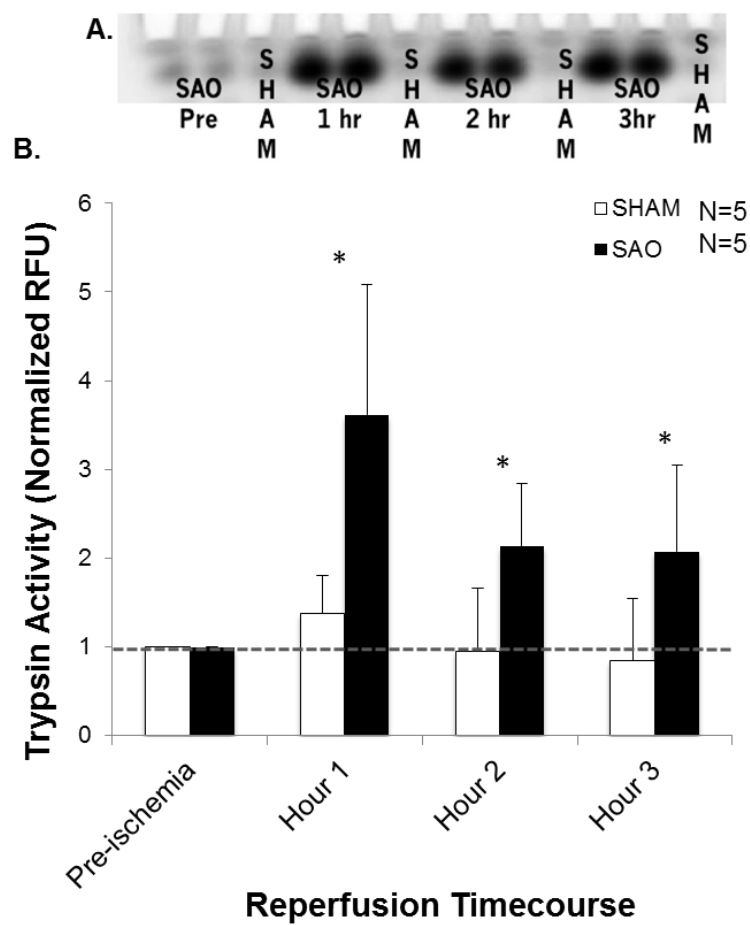
**Figure 2-1 Trypsin Activity in Lymph Fluid (A)** Quantitative gel scan for the electrophoretic pattern generated when reacting the charge-changing fluorescent substrate specific to trypsin with lymph fluid samples **(B)** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between the last three time points were statistically significant, *p<0.05. N=5 for each group.
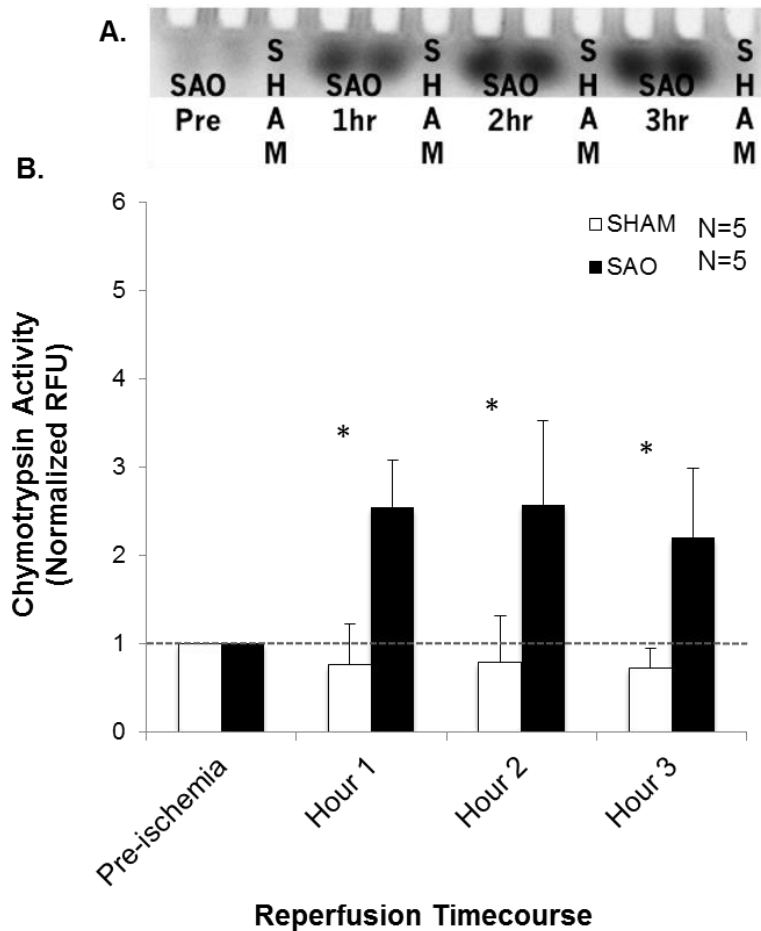
**Figure 2-2 Chymotrypsin Activity in Lymph Fluid** **(A)** Quantitative gel scan for the electrophoretic pattern generated when reacting the charge-changing fluorescent substrate specific to trypsin with lymph fluid samples **(B)** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between the last three time points were statistically significant, *p<0.05. N=5 for each group.
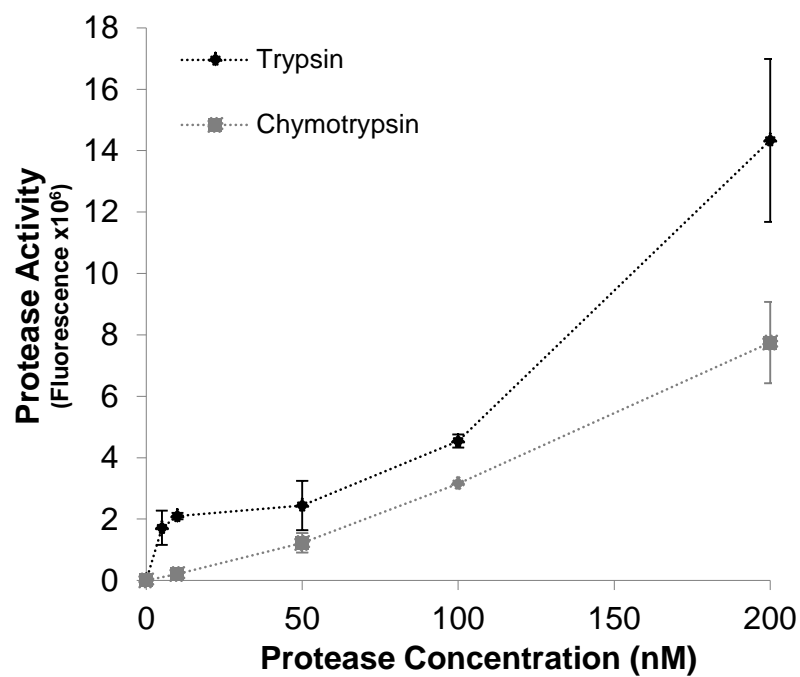
**Figure 2-3 Calibration Curves for Trypsin and Chymotrypsin**. N=3 for each enzyme.

**Table 2-2** Concentration of active trypsin-like enzymes in mesenteric lymph based on a calibration. N=5 for each time point.

| Experimental Group | Pre-Ischemia | Reperfusion Hour 1 | Reperfusion Hour 2 | Reperfusion Hour 3 |
|---|---|---|---|---|
| SAO (nM) | 33.9±12.2 | 110.7±23.6 | 80.2±25.2 | 81.2±26.6 |
| SHAM (nM) | 38.7±9. | 42.8±11.5 | 32.1±10.2 | 27.9±7.9 |

**Table 2-3** Concentration of active chymotrypsin-like enzymes in mesenteric lymph based on a calibration. N=5 for each time point.

| Experimental Group | Pre-Ischemia | Reperfusion Hour 1 | Reperfusion Hour 2 | Reperfusion Hour 3 |
|---|---|---|---|---|
| SAO (nM) | 18.2±1.6 | 31.8±3.8 | 31.3±4.5 | 30.6±5.7 |
| SHAM (nM) | 19.8±0.9 | 16.4±1.7 | 17.0±2.2 | 16.5±0.7 |
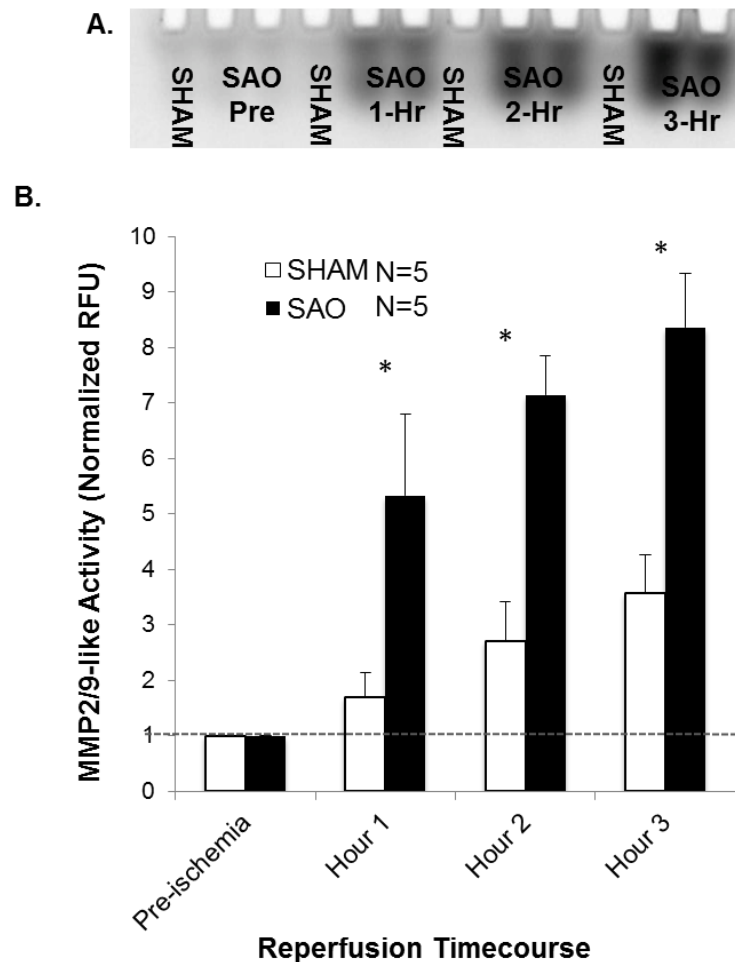
**Figure 2-4 Metalloprotease -2 and -9 Activity in Lymph Fluid (A)** Quantitative gel scan for the electrophoretic pattern generated when reacting the charge-changing fluorescent substrate specific to trypsin with lymph fluid samples **(B)** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between the last three time points were statistically significant, $*p<0.05$. N=5 for each group.
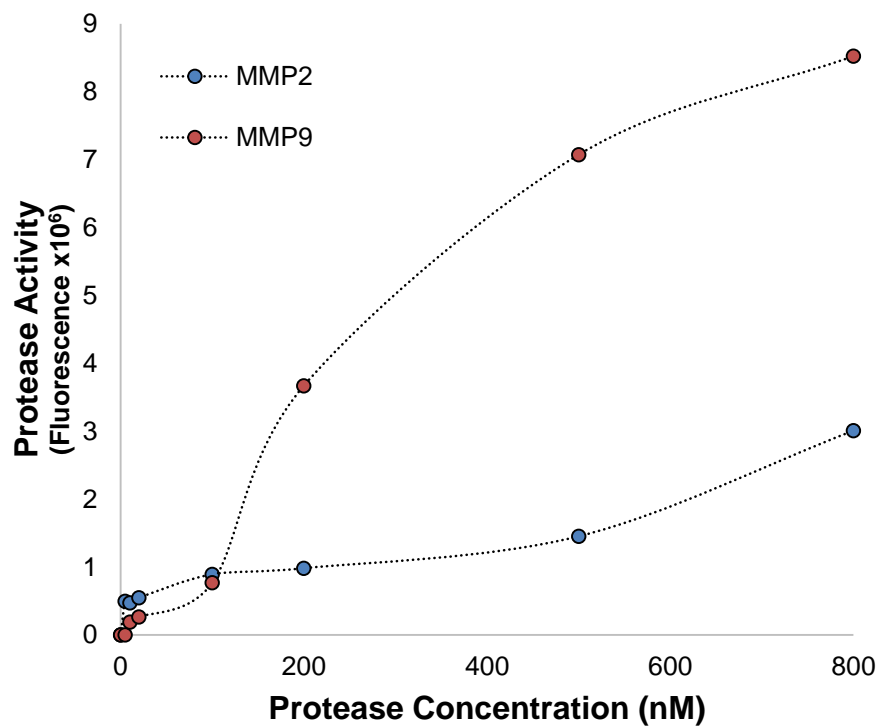
**Figure 2-5 Calibration Curves for MMP-2 and -9**. N=3 for each enzyme.
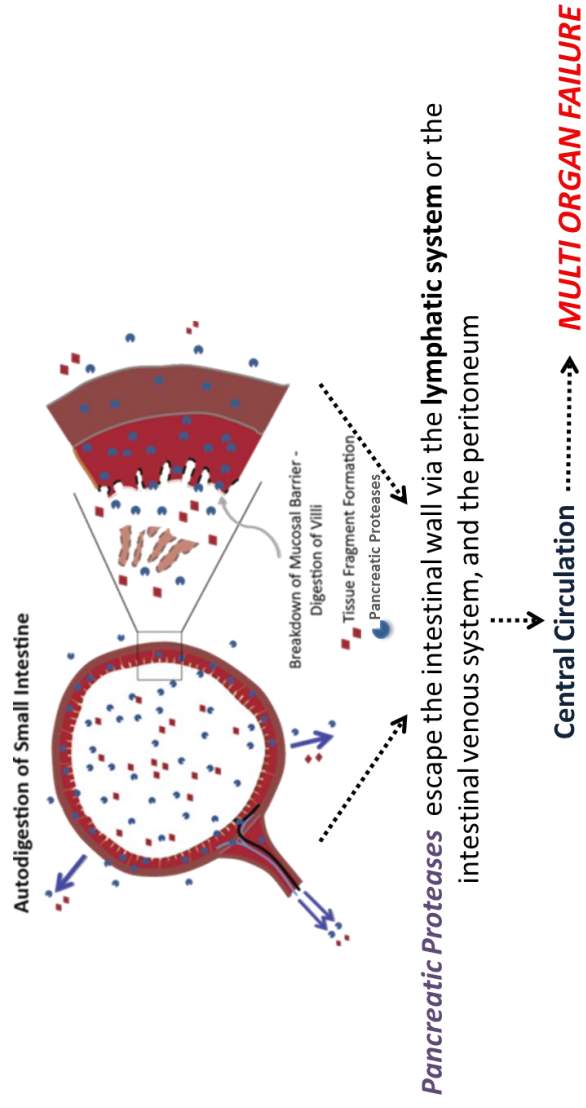
**Figure 2-6 Diagram of a Small Intestine under Acute Ischemia:** *Autodigestion Hypothesis.*

# CHAPTER 3

# PROTEASE ACTIVITY DURING A MEAL

# TOLERANCE TEST IN TYPE 2 DIABETES

# MELLITUS

## 3.1.    Summary

Diabetes mellitus presents a serious burden to the health system, and the projection is that it will continue to increase in incidence. In this chapter we present an exploratory study that aimed to detect protease activity after a high calorie, fat, and sugar meal, in hopes of advancing the understanding of the molecular mechanism leading to the development of type 2 diabetes mellitus. The proteases: MMP-2 and -9, elastase, and trypsin, activities were measured using charge changing substrates. We observed an elevated protease baseline for the diabetics, when compared to the normal group.

Moreover, we were able to observed differences in the change of protease activities throughout the meal.

### 3.2.    Introduction

Type 2 diabetes mellitus (T2DM) is an inflammatory disorder that is characterized by an attenuation in the tolerance of glucose [36, 68-70]. T2DM requires lifelong medical attention and poses a significant burden on the US healthcare system. The incidence of T2DM and insulin resistance has increased dramatically during the past two decades, in 2003 there were 94 million diabetics, and the projection is that this will almost double to 380 million in just 11 years [71]. Although great efforts have been made to understand the underlying molecular mechanism of diabetes, this has not been achieved [38]; thus impairing the development of new more efficient treatments, and prevention strategies for diabetes.

Recent preclinical evidence indicates that in spontaneous hypertensive rats (SHR) there is a correlation between an increase in insulin resistance, and uncontrolled proteolytic activity, and an increase in the insulin receptor (IR) cleavage [38]. Other study has shown that there is an elevation in the soluble IR in plasma samples of T2DM patients [39], and it proposed as a mechanism for the cleavage of the IR, the proteolytic action of a calcium dependent protease [40]. Among the potential causes for the increased in proteolytic activity in the circulation, lies the intestinal permeability. In physiological shock, which is accompanied by a severe form of inflammation, it has been observed that the permeability of the intestine is compromised, allowing for the

proteases, which are normally localized in the lumen of the intestine, to escape through the wall of the intestine into the lymphatics and venous circulation [3-8, 33-35]. T2DM is a less severe, but chronic form of inflammation; it is hypothesized that the intestine might be suffering from a chronic mild increase in permeability, allowing for a low concentration of uncontrolled protease activity in the blood. In this study we investigated if the protease activity in whole blood of T2DM is elevated, as means of providing evidence to this hypothesis. Moreover, we want to see if a meal affects the protease activity in circulation, which will imply that the intestine permeability is also being affected by the meal.

Charge changing protease substrates and the electrophoretic technique developed by our group [28-30], were used to detect the protease activity of: MMP-2 and -9, trypsin, and elastase in untreated whole human blood after a high calorie, fat, and sugar meal. We encountered elevated activity of MMPs, and elastase in the baseline activity levels of these proteases, in the whole untreated blood of T2DM. We also found that the change in protease activity after a meal in T2DM differs from normal subjects. This is the first study, to our knowledge, to investigate protease activity directly in whole human blood of T2DM patients, and to follow their progression of the activity throughout a meal, and it was only possible with the use of the charge changing protease substrates and the electrophoretic technique, which allows for protease activity detection in whole blood, and no sample preparation.

### 3.3. Experimental Design

### 3.3.1. Sample Acquisition

Samples were collected from 4 T2DM, 7 normal, and 1 pre-T2DM patients. The volunteers arrived to the clinic having a minimum of 12 hours of fasting, an intravenous catheter was placed in the antecubital vein of the non-dominant arm. The blood collection was done in collection tubes containing lithium heparin, and EDTA (Becton Dickinson). A first blood sample was collected to serve as a base-line level. A Mc Donald's breakfast meal of total of 1,680 calories was served to the volunteers. The nutritional values of the breakfast are shown in **Table 3-1**. Subsequent blood sampling occurred at time points: 15, 30, 45, 60, 120, 180, 240 and 300 minutes after the meal. A diagram of the experimental design is shown in **Error! Reference source not found.** The blood was stored at 4°C, and was used to measure protease activity within two hours.

Anthropomorphic measurements, and general information on the subjects, diet and exercise data was also collected. Some of this data is shown in **Table 3-2**.

### 3.3.2. Protease Activity in Untreated Whole Blood Measurements: Charge-Changing Substrates

Activity of MMP-2 and -9, trypsin, and elastase were measured in whole blood samples of the volunteers using the charge changing protease substrates, and the electrophoretic method developed by our lab [28-30]. This method uses a synthetic peptide fluorescent substrate, which is specifically cleaved by a protease of interest; the substrates are originally negatively charged, and upon cleavage two fragments are

produced a negative one, and a fluorescently labeled positive one, this allows for detection in whole blood with no sample preparation **(Figure 1-5)**. The substrates were synthesized by Aapptec (Louisville, KY), and had the following sequences: Ac-NGDPVGLTAGAGK-$NH_2$ for MMP-2 and MMP-9; andAc-NDGDAGRAGAGK-$NH_2$ for trypsin, and Ac-DAGSVAGAGK- $NH_2$.The substrates were tagged with a fluorophore Bodipy-FL-SE (Invitrogen, Carlsbad, CA, USA) via the peptide's diaminoethyl-amine group. Stock solutions of the substrates with concentrations of 1.2mg/mL were prepared using 1XPBS (pH 7.8). Whole blood were mixed with the substrate, the final concentration of the substrate in the reaction was of 0.6mg/mL. For the negative control 1XPBS was combined with the substrate. Positive controls consisted of a combination of known concentrations of enzyme: 1000, 100, and 10nM, and the fluorescent substrates. The reaction was allowed to proceed for 30 minutes, after this time 6μL aliquots of the reaction were loaded onto the different lanes of the Novex 20% Polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) submerged in 0.5XTBE running buffer (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). The samples were electrophoresed at 500 V for 12 minutes; the gel was imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) at excitation and emission wavelengths of 302 nm and 500–580 nm respectively. The gel was scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) loaded with the ImageQuant v5.2 software using the following settings: fluorescence mode, high sensitivity, 100 mm pixel size, 1000 V photomultiplier tube with a 450 nm excitation filter and a 520 nm long pass emission filter. The image was then quantified with ImageJ 1.440 and the

fluorescent signal is then in function of specific protease activity in each of the samples.

### 3.3.3. Statistical Analysis

All measurements are presented as mean±standard error, with N=7 for the normal group, and N=4 for the T2DM, and N=2 for pre-T2DM. Analysis of variance between groups was computed. Paired t-tests were used to compare protease activity measurements between time points and experimental groups for the protease activity measurements.

### 3.4.    Results

Protease measurements were made using charge-changing substrates. The total baseline MMP activity for the normal group was lower, than that of the T2DM group. The MMP activity of the T2DM was higher than that of the pre-T2DM group, though the difference was not significant. The next observation is that after the meal for T2DM and pre-T2DM groups, the MMPs activity increased in the first hour, and then, after the second hour decreased. For the normal group the activity remained constant through-out the experiment (**Figure 3-2**, **Figure 3-3**).

The metalloprotease activity was normalized to the first baseline measurement, for each of the groups, and the percentage change of activity was calculated with the formula shown below;

$$\%Protease\ \Delta A = \frac{A\_t_x - At_0}{At_0} * 100 \quad (3.1)$$

Where *%Protease$\Delta A$* is the percentage change of activity, *$A\_t_x$* is the protease activity at time specific time point being measure, in random unit of fluorescence, and *$A\_t_0$* is the protease activity at the baseline. The change in activity of MMPs for T2DM and the normal groups were completely different. During the first hour the MMP-2 and -9 activities for the T2DM had a forty percent increase, while the normal group, it had an increased in the first half hour of less than twenty percent. After the second hour the activity decreased by close to sixty percent for the T2DM group, while it increased by about twenty percent in the normal group. For the pre-T2DM the percentage change of MMP-2 and -9 activity pattern was close to that of the T2DM group. An initial increase in activity in the first hour, peaking at 15 minutes after the meal, with a fifty percent increase in activity. In the second hour the activity decreased, by close to forty percent; at the fourth hour after the meal was taken the MMP activity increased again, and reached a value close to the initial baseline value **(Figure 3-4)**.

For the elastase measurements the total baseline activity for the normal group was lower, than that of the T2DM group. The baseline elastase activity of the pre-T2DM and the T2DM was similar **(Figure 3-5, Figure 3-6)**.

As with the metalloprotease activity, the elastase activity was normalized to the first baseline measurement, for each of the groups, and the percentage change of activity was calculated using *equation 3.1*. The change in activity of elastase for T2DM, the normal groups and the pre-T2DM were different. In the T2DM group the elastase activity decreased in the first forty-five minutes after the meal, reaching a low point at thirty minutes, with an eleven percent activity decrease. At the first hour after the meal

the activity increased by close to twenty percent, after it decreased through the rest of the experiment, reaching almost a sixty percent decrease at the fifth hour after the meal. For the normal group, there was a close to linear decrease in elastase activity, reaching the lowest point at the last time point measured, five hours after the meal, where the percentage decrease was close to forty. The pre-T2DM had an activity decrease during the first three hours after the meal, reaching the lowest point during the second hour after the meal, with a decrease of activity higher than fifty percent. In the last two hours of the experiment the activity increased, to reach a peak during hour four after the meal, with a percentage increase of around ten percent. **(Figure 3-7**).

There was no significant difference between the baseline trypsin activity measurements of the normal, T2DM groups, and the pre-T2DM, though the T2DM and pre-T2DM had slightly higher trypsin activity **(Figure 3-8**, **Figure 3-9**). The trypsin activity was normalized to the first baseline measurement, for each of the groups, and the percentage change of activity was calculated using *equation 3.1*. For the normal group there was a peak in the activity one hour after the meal, with an increase in activity close to thirty percent. After this point the activity decreased, throughout the rest of the experiment, reaching the lowest point at hour five after the meal, with a decrease of close to sixty percent in trypsin activity. The T2DM group, and the pre T2DM had similar trends, with an initial increase in activity 15 minutes after the meal, followed by a decrease one hour after the meal reaching a forty, and ten percent decrease for the T2DM and pre-T2DM respectively. The trypsin activity after this point increased, reaching a high point two hours after the meal, with an increase of close to seventy, and

close to forty percent for the T2DM, and the pre-T2DM respectively. After this point the trypsin activity decreased and remained relatively constant at around twenty percent change through the rest of the experiment, for both groups.

**Table 3-1** Nutritional Information of the McDonald's Breakfast Meal

| Item | Calories | Fat (g) | Protein (g) | Carbohydrates (g) | Sugar (g) | Sodium (mg) |
|---|---|---|---|---|---|---|
| 2 X Sausage Egg Mc Muffin | 900 | 56 | 42 | 60 | 4 | 1720 |
| Hash Brown | 150 | 9 | 1 | 15 | 0 | 310 |
| Medium Orange Juice | 190 | 0 | 3 | 44 | 39 | 0 |
| Medium Hot Chocolate | 440 | 16 | 14 | 61 | 56 | 220 |
| TOTAL | 1680 | 81 | 60 | 180 | 99 | 2250 |
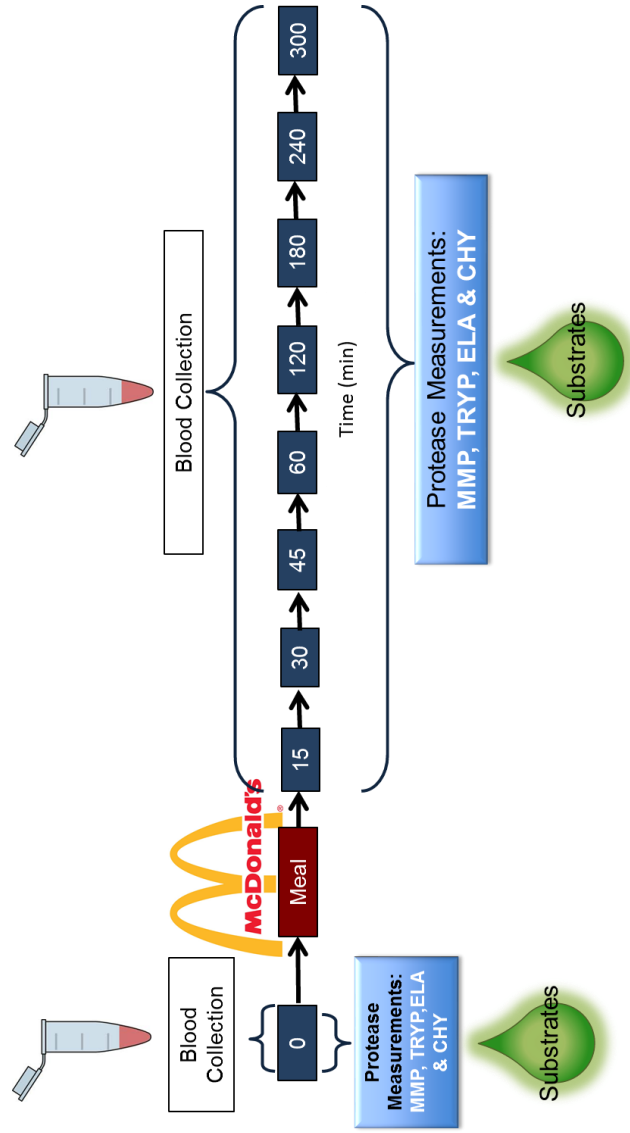
**Figure 3-1 Diagram of the Experimental Set-Up for the Detection of Proteases after a McDonald's Meal.**

**Table 3-2** Anthropometric, and General Information of Subjects

| Subject | Diagnosis | Fasting Time (h) | Height (cm) | Weight (Kg) | Age | 24-hour Food Recall | | | | |
| | | | | | | Calories | %Fat | %Carbs | %Protein | %Alcohol |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | normal | 17 | 183 | 68.3 | 28 | 1886 | 43.3 | 35.5 | 21.1 | 0 |
| 2 | normal | 12 | 183 | 72.1 | 30 | 3222 | 28.5 | 57.2 | 14.3 | 0 |
| 3 | normal | 13 | 157 | 58.2 | 36 | 829 | 19.1 | 36.6 | 44.2 | 0 |
| 4 | normal | 14 | 179.4 | 79.9 | 74 | 2024 | 27.8 | 52 | 20.2 | 0 |
| 5 | normal | 15 | 153.5 | 67.6 | 68 | 788 | 56.1 | 12.2 | 31.8 | 0 |
| 6 | normal | 12 | 170.8 | 85.2 | 55 | 3248 | 52.2 | 30.1 | 17.8 | 0 |
| 7 | normal | 14 | 149.4 | 75 | 58 | 1185 | 46.5 | 33 | 20.5 | 0 |
| 8 | DM | 13 | 180.4 | 127.9 | 43 | 1868 | 34 | 45.9 | 20.1 | 0 |
| 9 | DM | 16 | 178.9 | 92.9 | 79 | 1459 | 24.4 | 49.3 | 17.7 | 8.6 |
| 10 | DM | 14 | 186 | 118 | 38 | 2507 | 46.6 | 39.8 | 13.6 | 0 |
| 12 | pre-DM | 12 | 147.3 | 77 | 68 | 1206 | 40.9 | 40.2 | 18.9 | 0 |
| 14 | DM | 14 | 161.1 | 60 | 40 | 3128 | 58.4 | 25.9 | 15.2 | 0.5 |

**Figure 3-2 MMP-2 and -9- like Activity in Blood through a Meal**. Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between normal, and T2DM is significant where the * is shown. *$p<0.05$. N=7 for normal volunteers, and N=4 for T2DM.
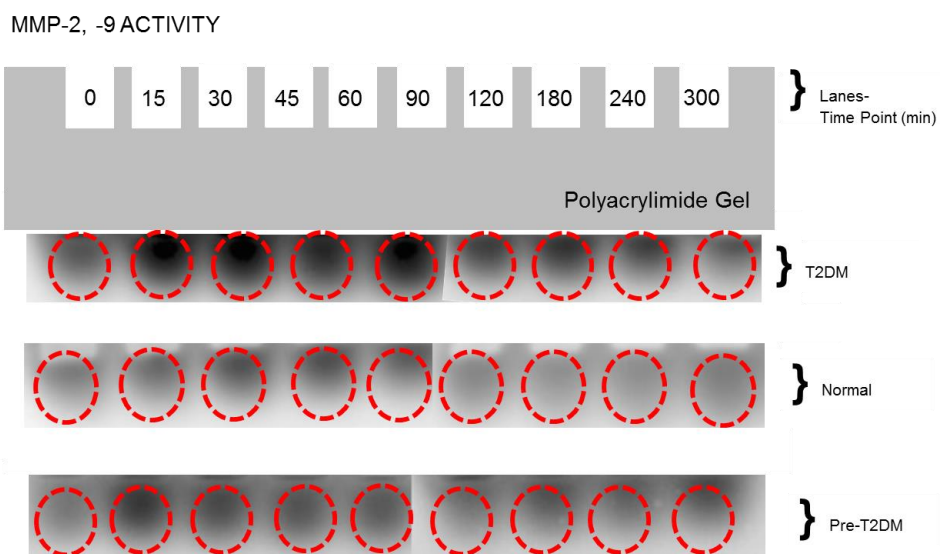
**Figure 3-3 MMP-2, -9- like Activity in Blood Through-out a Meal.** Quantitative gel scan for the electrophoretic pattern generated when reacting the charge-changing fluorescent substrate specific to metalloproteases with blood samples of **(A)** Type 2 diabetics, **(B)** Normal, and **(C)** Pre- diabetics
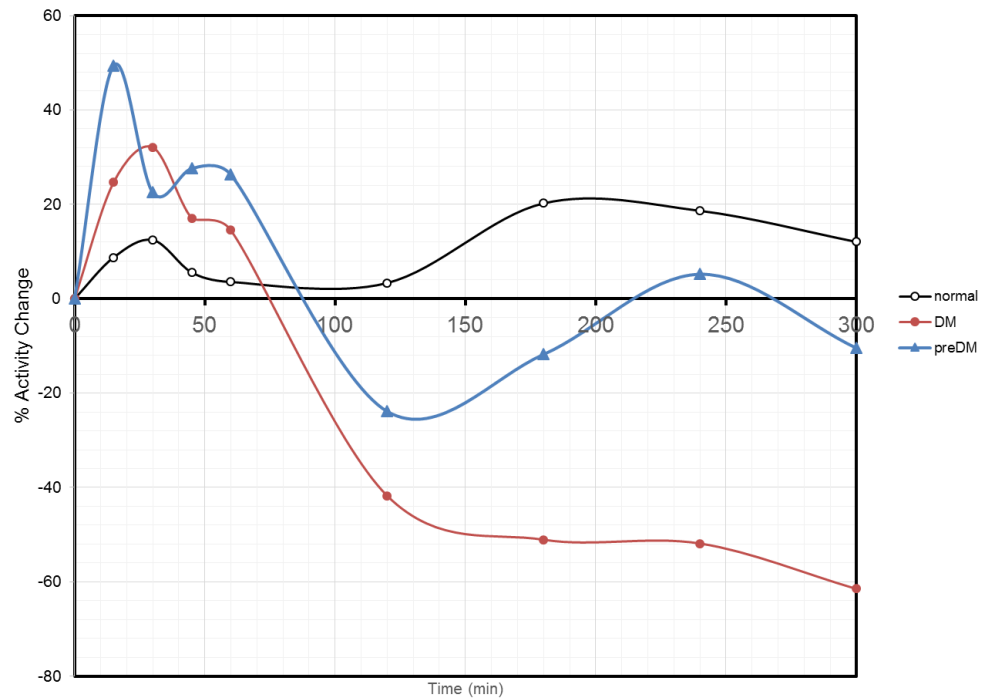
**Figure 3-4 Percentage Activity Change for MMP-2 and -9 Through-out the Meal.**
MMP-2 and -9 activities at the different time points were normalized with respect of the baseline (t=0 minutes) measurement of activity.
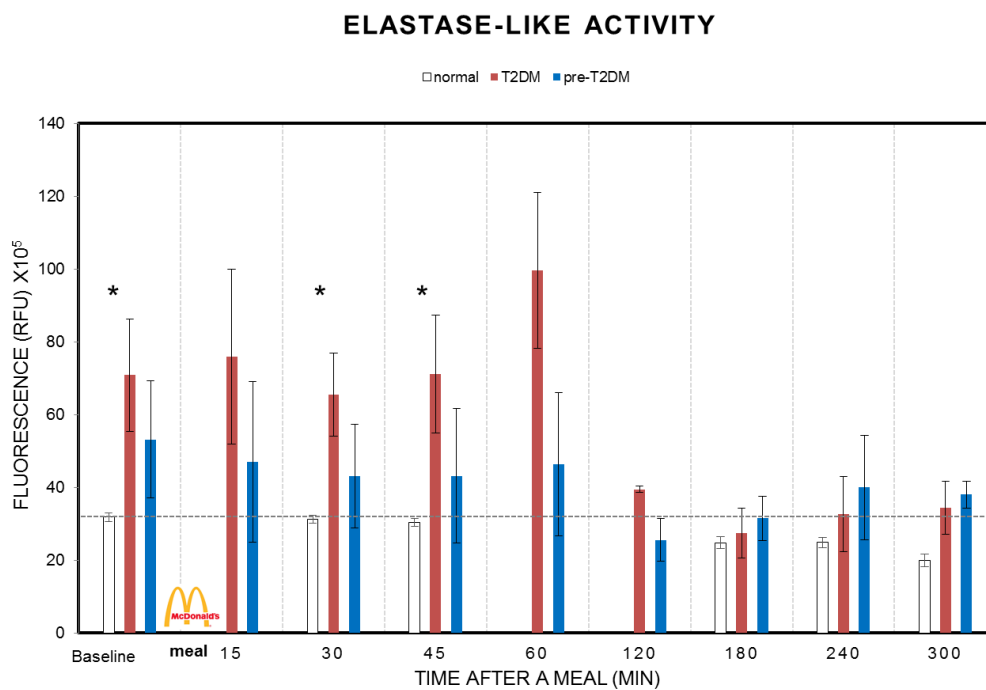
**Figure 3-5 Elastase- like Activity in Blood Through-out a Meal.** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between normal, and T2DM is significant where the * is shown. *$p<0.05$. N=7 for normal volunteers, and N=4 for T2DM.
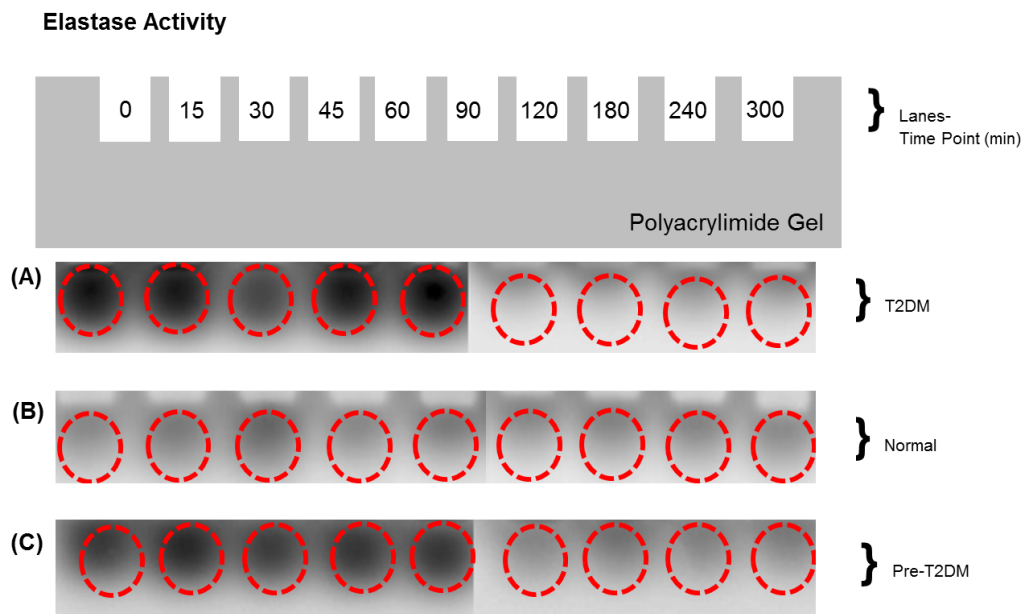
**Figure 3-6 Elastase- like Activity in Blood Through-out a Meal.** Quantitative gel scan for the electrophoretic pattern generated when reacting the charge-changing fluorescent substrate specific to elastase with blood samples of **(A)** Type 2 diabetics, **(B)** Normal, and **(C)** Pre- diabetics
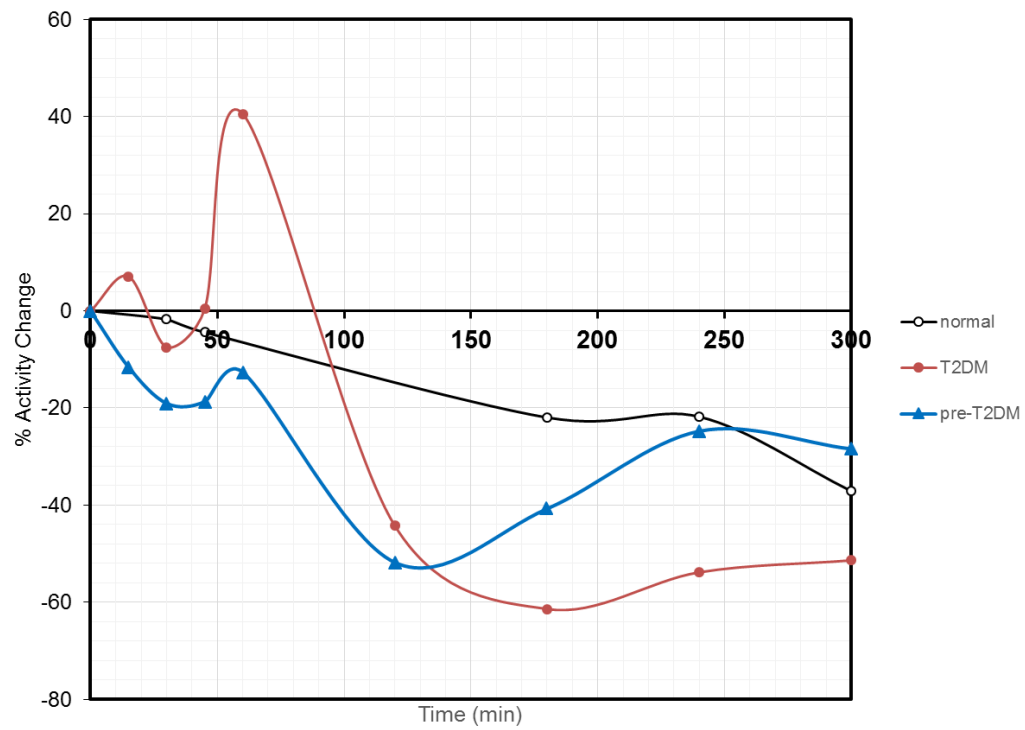
**Figure 3-7 Percentage Activity Change for Elastase Through-out the Meal.** Elastase activities at the different time points were normalized with respect of the baseline (t=0 minutes) measurement of activity.
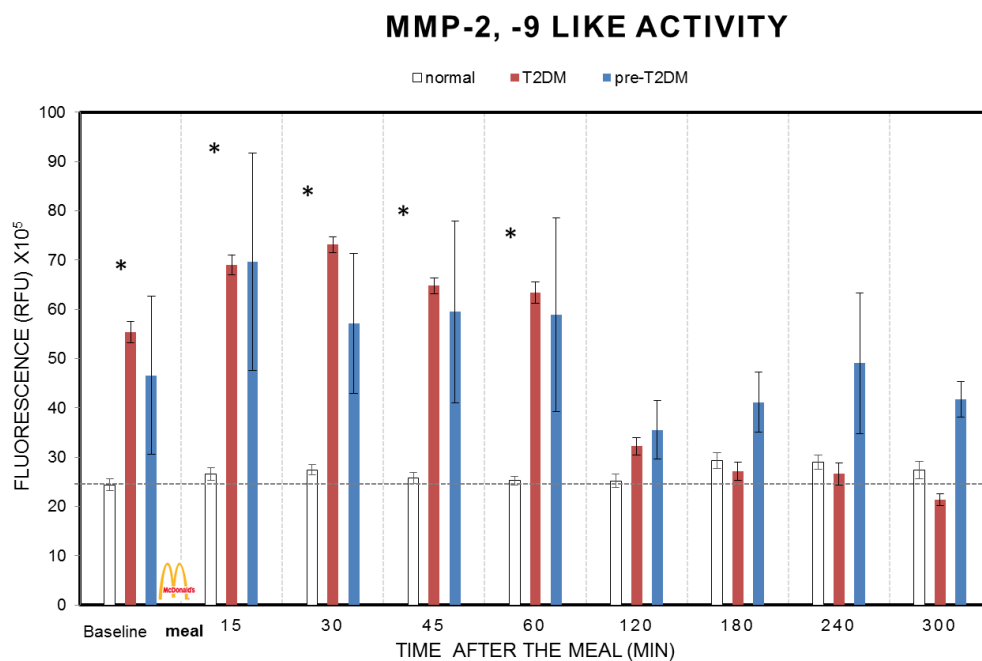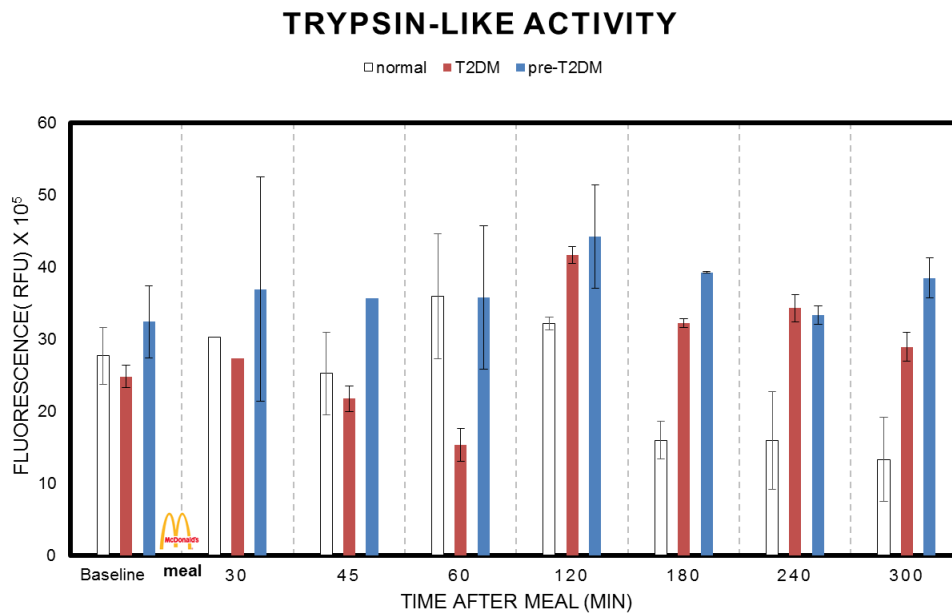
**Figure 3-8 Trypsin- like Activity in Blood through a Meal.** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between normal, and T2DM is significant where the * is shown. *p<0.05. N=7 for normal volunteers, and N=4 for T2DM.
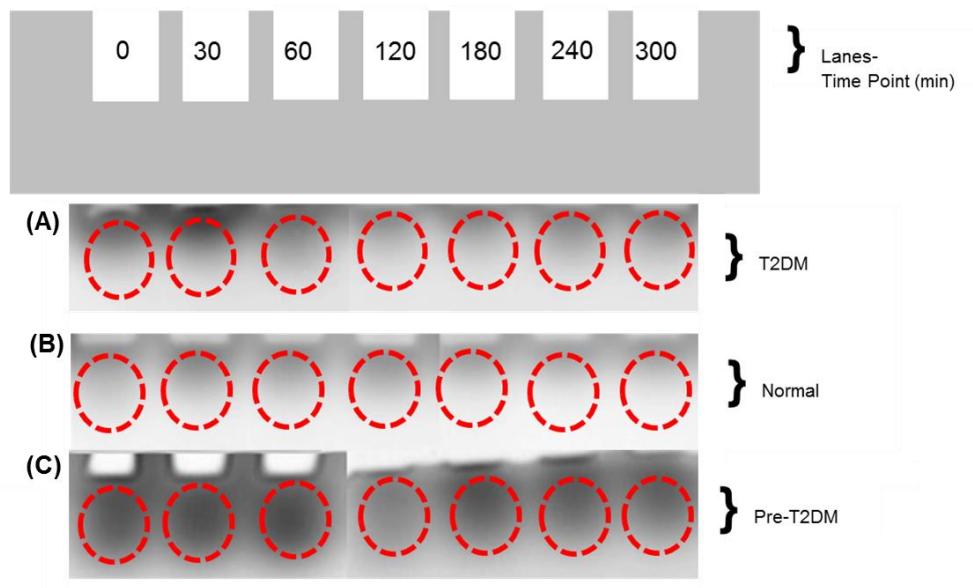
**Figure 3-9 Trypsin- like Activity in Blood Through-out a Meal.** Quantitative gel scan for the electrophoretic pattern generated when reacting the charge-changing fluorescent substrate specific to trypsin with blood samples of **(A)** Type 2 diabetics, **(B)** Normal, and **(C)** Pre- diabetics
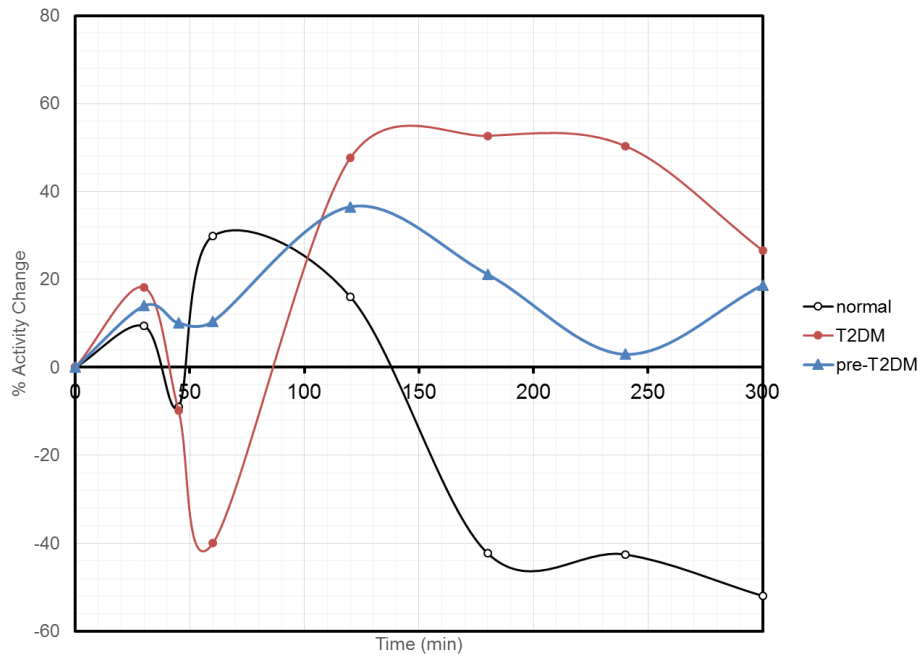
**Figure 3-10 Percentage Activity Change for Trypsin Through-out the Meal.**
Elastase activities at the different time points were normalized with respect of the baseline (t=0 minutes) measurement of activity.

**3.5.    Discussion**

Protease activity has been suspected to be a key factor in the initiation, and progression of T2DM [38-40]. It is for this reason that the activities of trypsin, elastase, and MMP-2, and -9, were measured in whole untreated blood in T2DM patients, after a high calorie, fat, and sugar meal. Most studies done in the past have only looked at the levels of MMP-2, and -9 levels in serum samples of T2DM, and the results of these studies have been conflicting. Some reporting no correlation between plasma levels of MMP-2, and -9 and T2DM [72]; others reporting elevated levels of MMP-2, and -9 in T2DM [10-12]; and finally one reporting lower levels of MMP-2, and -9 in oral glucose tolerance test (OGTT) for T2DM [73].

Our charge changing substrates electrophoretic technique allowed for the detection of protease activity after a high calorie, fat, and sugar meal, directly in untreated whole human blood, giving us for the first time a picture of what happens with the proteases in circulation after we ingest a meal. The first observation was that there is a general elevated protease activity in T2DM, when compared to normal subjects **(Figure 3-2-8).** This elevated protease activity is in line with results obtained in a recent study, where it was shown that spontaneous hypertensive rats (SHR) have increased proteolytic activity in the microcirculation, and that they also present more cleavage of the insulin receptor, and the attenuated glucose tolerance, which is characteristic of T2DM condition [38]. The result also provides more evidence to the observation that there is an increased level of soluble insulin receptor in plasma samples of T2DM [39],

and to the hypothesis that it is the activity of a protease that causes the cleavage of the extracellular portion of the insulin receptor [40].

Of the three proteases studied, only MMP-2, and-9, and elastase **(Figure 3-2-5)** were significantly elevated in the baseline whole blood samples of T2DM patients. MMP-2, and -9 remodel the extracellular matrix, and they have been shown to also be capable of the cleavage of multiple receptors [38, 74, 75]. Elastase is another protease that remodels the extracellular matrix, and it can also cleaves receptors [76]. Thus, although not conclusive, these results do provide a piece of evidence for the hypothesis that the glucose tolerance attenuation in T2DM might be caused by the cleavage of the insulin receptors by uncontrolled protease activity (Error! Reference source not found.). Trypsin baseline activity measurements showed no significant difference between T2DM and normal patients **(Figure 3-8-8)**.

For T2DM patients the percentage change of protease activity, during the five hours after the meal was consumed, was higher than for normal patients **(Figure 3-4Figure 3-7Figure 3-10)**. This implies that T2DM have less control over their circulation protease activity levels after a meal. In blood samples of T2DM we observed an increase of activity for MMP-2 and -9, elastase, and trypsin during the first hour after the meal. The source of this increase in protease activity remains to be investigated, but it is possible that an offense by the high calorie, fat, and sugar meal, to the intestine, increases the permeability of its lumen in T2DM, and thus allow proteases to escape. During severe forms of inflammation such as physiological shock and the subsequent multi organ failure, an increase in the permeability of the lumen intestine has been

observed, which allows for the proteases to escape through the wall of the small intestine, to the circulation, to peripheral organs (*Autodigestion Hypothesis)* [3-8, 55-61] . In less severe forms of inflammation, like T2DM, it is hypothesized that a slight increase in the permeability of the intestine exists, followed by the escape of a low concentration of proteases, that then can enter the circulation [7]. Moreover, the permeability of the intestine is probably constantly changing; it is believed that during the digestion of a meal, the permeability of the lumen of the small intestine increases, allowing for the escape digestive proteases, which could be more important in the case of T2DM, where the permeability of the intestine is hypothesized to already be compromised by chronic inflammation. The results presented are not conclusive, but they do provide a small piece of evidence for this hypothesis, and invite to continue a study on protease progression through a meal, to fully understand the role of proteases in T2DM.

It is observed that during the next four hours after the meal the activity of MMP-2, and-9 and elastase decreased significantly from the baseline levels of T2DM patients; such a significant decrease was not observed in normal patients. A mechanism that explains this remains to be explore, but one possibility is the presence of an inhibitor for these proteases (e.g. TIMP, antitrypsin), in T2DM patients. The trypsin activity reached a low level at the first hour after the meal, but then it increased in T2DM during the remaining hours of the experiment. Further experiments will be needed to understand the physiological consequences of the changes in protease activity. In next experiments it would be of interest to measure the levels of soluble insulin receptor in

circulation, where we would expect to see elevation in T2DM, when compared to normal volunteers; and we would also expect to see an increase of these levels in the subsequent hours after the meal.

A last observation is the differences between the pre-T2DM and the T2DM. Although it was expected that the protease activity base levels of pre-T2DM to be significantly lower than that of T2DM, this was not the case. This might have been due to the fact that only 2 pre-T2DM were sampled, and there is a large variance between the protease activities in people. A larger sample size would be necessary to further our knowledge on protease activity through the progression of T2DM. An interesting observation is that the percentage change in the activity of the proteases for the pre-T2DM was in between that of the normal, and the T2DM groups; suggesting that the change in protease activity during a meal is characteristic in the progression of T2DM.

## 3.6.    Conclusion

In this study we showed for the first time the detection of the activities of: MMP-2 and -9, trypsin, and elastase directly in untreated whole human samples of T2DM, after a high calorie, fat, and sugar meal. We observed elevated elastase and MMP-2, and -9 activities in the blood of T2DM when compared to the blood of normal volunteers. This gives a piece of evidence to the hypothesis that uncontrolled protease activity might be implicated in the cleavage of the insulin receptor that causes an attenuation of glucose tolerance in T2DM. We also observed the differences in the change in activities of proteases in T2DM when compared to normal volunteers, after a meal consumption.

The source of the initial increased in protease activity in T2DM subjects' remains to be investigated, but it is possible that proteases come from the lumen of the small intestine, which might increase its permeability after a heavy meal in T2DM patients. Further experiments will be made to measure the soluble insulin receptor in the plasma sample, as a mean to further elucidate the mechanism behind the progression of T2DM.

# CHAPTER 4

# MMP-2 AND MMP-9 ACTIVITY IN WHOLE UNTREATED BLOOD IN CHRONIC LYMPHOCYTIC LEUKEMIA

## 4.1. Summary

Matrix-metalloproteases (MMP) play an important role in angiogenesis, tumor invasion, and metastasis. In chronic lymphocytic leukemia (CLL), there is an increased in angiogenesis, and more recently it has been shown that there is an increase in the concentration of MMP-9 in early stage CLL patients. The purpose of this study was to measure the activities of MMP-2, and -9 directly in untreated whole blood in CLL patients at different stages. We found that MMP-2, and -9 were elevated in the untreated blood samples of CLL patients. Additionally we measured the MMPs activities in isolated B-cells, and plasma samples, and found that B-cells have a higher MMP activity

than plasma samples. We also measured the trypsin activity in untreated blood samples, and found that normal volunteers, had a higher activity than CLL patients, though the difference was not significant enough. The use of charge changing fluorescent substrates allowed for the rapid detection of proteases activity in whole untreated human blood of CLL patients, this can improve, diagnosis, disease progression understanding, and the development of new therapeutics.

## 4.2. Introduction

Chronic lymphocytic leukemia (CLL) is the most common type of leukemia in adults [7], CLL is characterized by B-cells that growth out of control, and are capable to invade the bone marrow, this migration is possible by the degradation of the basal membrane by the metalloproteases (MMPs) [43-44]. MMPs are a large family of proteolytic enzymes, which are involved in a large range or normal biological processes, such as embryonic development, cell migration, inflammation and tissue repair [42-44]. MMPs are also important in pathogenesis and the tumor progression, angiogenesis and metastasis, because of their abilities to degrade the principal components of the extra-cellular membrane (ECM) [41, 75, 76]. MMP-2 and -9 are the principal, MMPs produced by the B-cells in CLL, and they are the MMPs capable of degrading the major component of the basement membrane (Collagen IV), a necessary process for the migration of malignant cells into new healthy tissue. MMP-2 and -9 also promote malignant cell proliferation and angiogenesis by releasing matrix-bound growth-factors

[42-44]. It is for this reason that we believe that assessing MMP-2 and -9 activity directly in whole blood could serve as a way of assessing the CLL progression.

Previous studies have shown that MMP-9 is secreted by cells from early stage CLL patients. MMP-9 concentration has been shown to be elevated in the serum of early stage CLL patients, thus possibly serving as a new biomarker for early diagnosis of CLL [45]. Also the intracellular MMP-9 concentration correlates with advance stage and poor patient prognosis [43]. These previous studies suggest that MMP-9 is an important mediator in the progression of CLL cancer. Current studies have never been able to detect MMP activity in untreated whole blood, because all of the techniques for measuring proteases (e.g. ELISA, zymographies and Western Blots) require sample preparation. In this chapter we present for the first time the measurement of MMP-2 and -9 activity in untreated whole-human blood. This measurement was made in less than one hour, and required only a few micro-liters of completely untreated blood sample. We were able to observe an elevated MMP-2 and -9 activity in the untreated blood of CLL patients. MMPs were also measured in plasma, and B-cells of CLL patients; the B-cells had a higher activity than the plasma samples. Previous studies have shown that MMPs are in their active form in the B-cells, but that this is not the case in culture cell medium. This observation made us wonder about the mechanism of activation of MMPs. Because trypsin activity can activate MMPs [78], we also measured the activity of trypsin in untreated whole blood. No elevation was observed in the CLL patients, suggesting that the mechanism of activation of MMPs is not cleavage of the pro-MMP form by trypsin. The use of charge-changing fluorescent peptide substrates can be used

to measure the activity of proteases in whole blood of CLL patients, in an easy, rapid, and reliable manner. The findings of this study can serve to deepen the understanding of the progression of CLL, improve diagnostics, and help in the development of new therapeutics.

## 4.3. Materials and Methods

### 4.3.1. Sample Acquisition

Samples were collected from 10 CLL patients and 5 normal healthy volunteers in collection tubes containing lithium heparin (Becton Dickinson). Aliquots of 50uL of the blood were taken away from the blood samples, for the detection of MMP-2 and -9; and trypsin in whole untreated blood. Another aliquot of the sample was used to created plasma, by centrifugation for 10minutes at 1100rpm.

### 4.3.2. B-Cell Isolation

To isolate white blood cells from the blood samples, which contain the B-cells. 5mL of erythrocyte lysis buffer (Buffer EL, Qiagen) were added to 5mL of blood in 15mL conical tubes and vortex for 30sec. The conical tube was placed on ice for 10 minutes, and it was then spun down at 400XG for 10 minutes. The supernatant was disposed, and the same process was repeated three-times until the cell pellet was not tinted red. The cells were then re-suspended in 1mL of freezing medium (Recovery™ Cell Culture Freezing Medium, Gibco®) and store at -80ºC.

### 4.3.3. Protease Activity in Untreated Whole Blood Measurements: Charge-Changing Substrates

Activity of metalloproteases, MMP-2 and -9, and the pancreatic protease trypsin, were measured in untreated whole blood, plasma, and isolated B-cells of CLL, and normal subjects, using charge changing substrates and the electrophoretic method developed by our group [28-30]. This method uses synthetic peptide fluorescent substrates, which are specifically cleaved by a protease of interest; the substrates are originally negatively charged, and upon cleavage two fragments are produced a negative one, and a fluorescently labeled positive one, this allows for detection in whole blood with no sample preparation (**Figure 1-5**). The substrates were synthesized by Aapptec (Louisville, KY), and had the following sequences: Ac-NGDPVGLTAGAGK-NH$_2$ for MMP-2 and -9; and Ac-DAGSVAGAGK- NH$_2$ for trypsin. The substrates were then tagged with a fluorophore Bodipy-FL-SE (Invitrogen, Carlsbad, CA, USA) via the peptide's diaminoethyl-amine group. Stock solutions of the substrates with concentrations of 1.2mg/mL were prepared using 1XPBS (pH 7.8). Whole blood, plasma samples, or isolated B-cells were mixed with the substrate, the final concentration of the substrate in the reaction was of 0.6mg/mL. For the negative control 1XPBS was combined with the substrate. The reaction was allowed to proceed for 1 hour for plasma and isolated B-cells samples; and only 30 minutes for whole blood samples, after this time 6μL aliquots of the reaction were loaded onto the different lanes of the Novex 20% Polyacrylamide gels (Invitrogen, Carlsbad, CA, USA) submerged in 0.5XTBE running buffer (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). The samples

were electrophoresed at 500 V for 12 minutes; the gel was imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) at excitation and emission wavelengths of 302 nm and 500–580 nm respectively. The gel was scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) loaded with the ImageQuant v5.2 software using the following settings: fluorescence mode, high sensitivity, 100 mm pixel size, 1000 V photomultiplier tube with a 450 nm excitation filter and a 520 nm long pass emission filter. The image was then quantified with ImageJ 1.440 and the fluorescent signal is then in function of specific protease activity in each of the samples.

### 4.3.4. Statistical Analysis

All measurements are presented as mean±standard error, with N=10 for the CLL group, and N=5 for the normal group. Analysis of variance between groups was computed. Paired t-tests were used to compare protease activity measurements between time points and experimental groups for the protease activity measurements.

### 4.4. Results

In this study we measure the activity of MMP-2 and -9 in untreated whole blood, isolated B-cells, and plasma samples for 10 CLL patients. It was shown that in general the activity of the MMPs in the whole blood was higher than that of the isolated B-cells, and that this activity was higher than that of the plasma **(Figure 4-1)**. This pattern was also clearly shown in **Figure 4-2**, where we show the average activity, of all CLL patients, from untreated whole-blood, plasma samples, and isolated B-cells.

The MMP-2 and -9 activity was also measured for 5 normal patients. A comparison of the average of the CLL, and normal patients is shown in **Figure 4-3**, where we can observed that the MMPs activity in CLL patients is significantly higher than that of normal patients. Trypsin activity in whole untreated blood was also measured for both groups. It can be observed in **Figure 4-4** that no significant difference was encountered between the activity of trypsin in normal, and CLL groups. Though the activity of trypsin in the normal group was slightly higher.
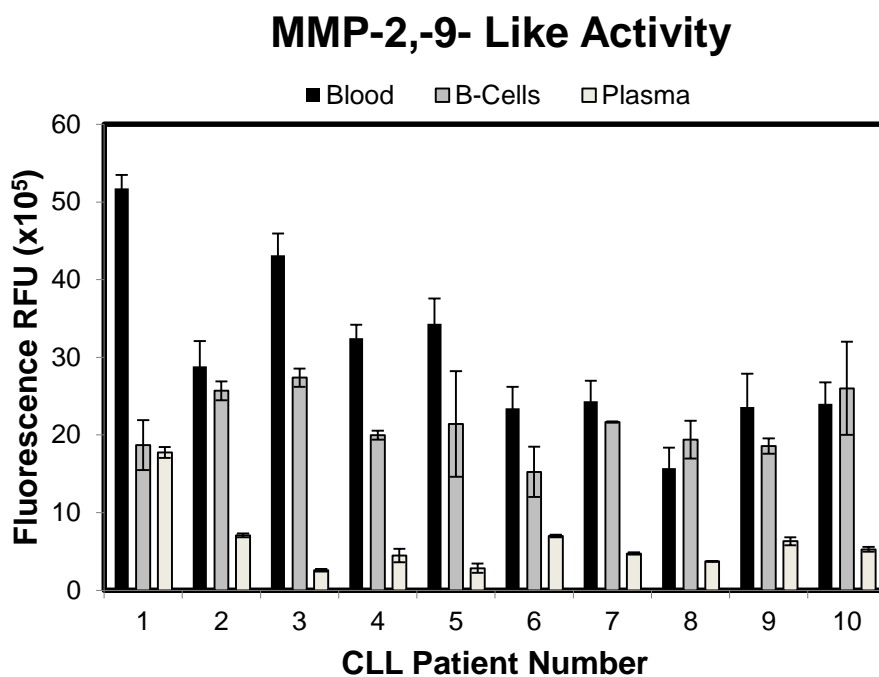
**Figure 4-1 Activity in Whole Blood, Plasma Samples, and B-Cells in CLL Patients** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU).

**Figure 4-2 MMP-2,-9 Average Activity in Whole Blood, Plasma Samples, and B-Cells for 10 CLL Patients** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU).
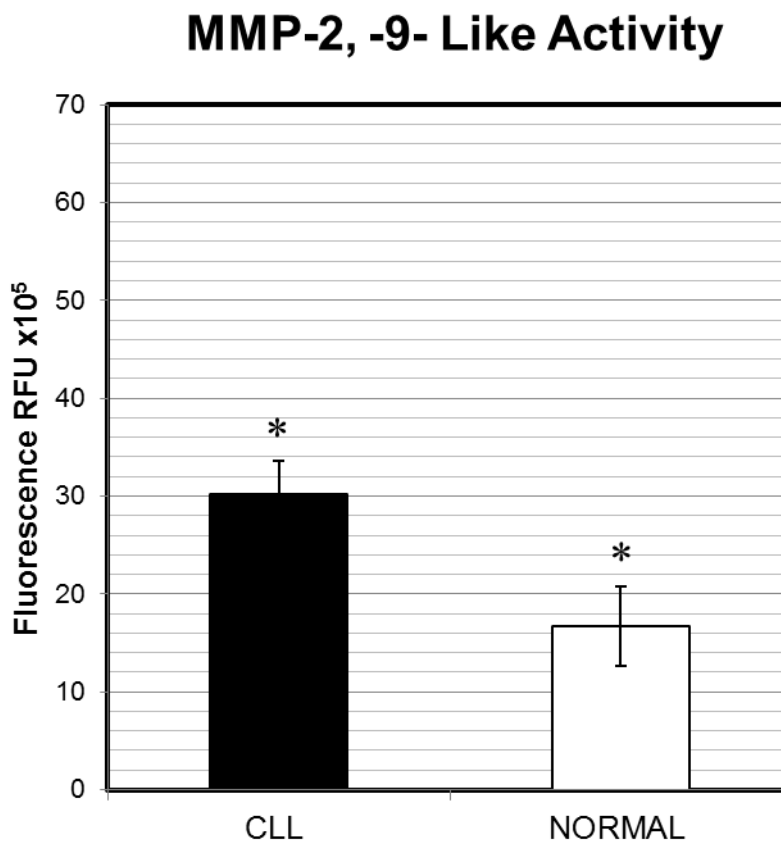
**Figure 4-3 MMP-2,-9 Average Activity in Whole Blood for CLL, and Normal Subjects.** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference in mean activity between CLL, and normal subjects is significant where the * is shown. *$p<0.05$. N=10 CLL volunteers, and N=5 for normal volunteers.
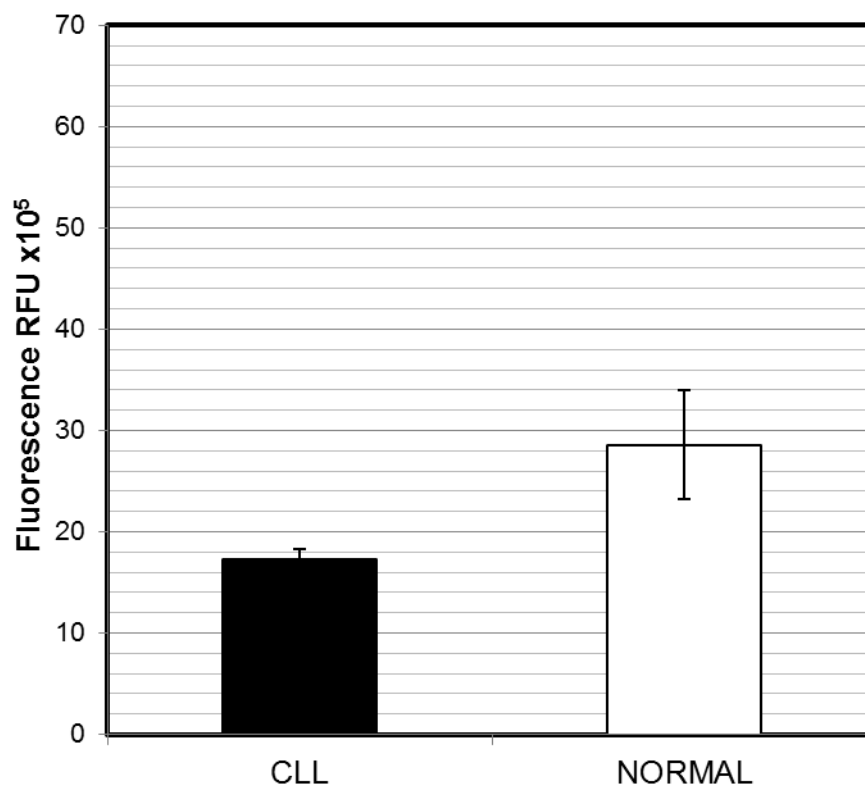
**Figure 4-4 Trypsin Average Activity in Whole Blood for CLL, and Normal Subjects.** Quantification of the gel scan. Fluorescence is reported in millions of relative fluorescence units (RFU). The difference i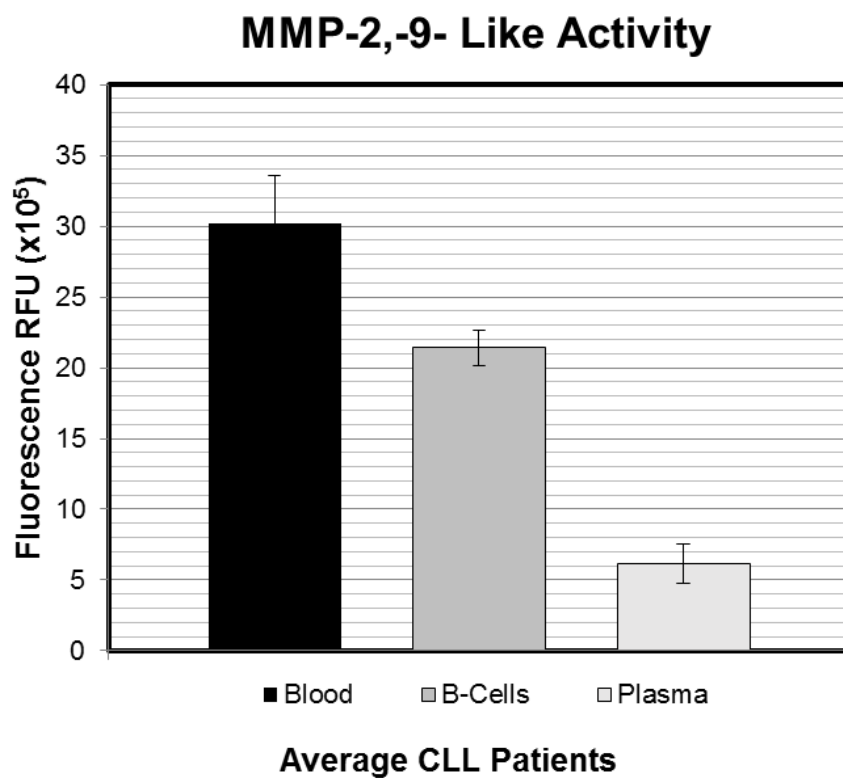n mean activity between CLL, and normal subjects was not significant where the * is shown. *$p > 0.05$. N=10 CLL volunteers, and N=5 for normal volunteers.

**4.5.**     **Discussion**

Metalloproteases have the ability to remodel the extracellular matrix, and for this reason they have since long awoken the interests of researchers, to understand their relation with the progression of different types of cancers [41-42]. MMP-2, and -9 stand out from this family, because their ability to digest collagen IV, the major component of the basal membrane, and thus promoting angiogenesis, tumor invasion, and metastasis. In CLL, like in most cancers, angiogenesis is increased, and thus we thought of interests to determine the MMP-2, 9 activity in CLL untreated blood samples [42-44]. Current techniques to measure the activity of MMPs lack in sensitivity, specificity, are time consuming, and require ample preparation, preventing them from being able to work with untreated whole blood samples, making them not sufficient to be used in the clinic.

Our technique uses a charge changing substrate, which is initially negatively charged; upon cleavage by the protease that is designed for, in this case MMP-2, and 9; two fragments are produced: a negative one, and a fluorescently tagged positive one. Separation of these fragments is possible in a simple electrophoretic format, which also separates the positive product fragment, from the blood proteins that are mainly negatively charged; this eliminates the need for sample preparation **(Figure 1-4)**. Using the charge-changing substrate and the electrophoretic method an increased in the activity levels of MMP-2, and -9 in the untreated blood samples of CLL patients, when we compare them to healthy normal donor, was observed **(Figure 4-3)**.

Another point of importance was to identify the source of MMP activity in the blood. Other studies have shown that the MMP activity comes from intracellular, and extracellular B-cells [43, 79]. We isolated the B-cells, and using our charge-changing MMP assay we measured the MMP-2,-9 activity for the plasma samples, as well than for the B-cells (**Figure 4-1**). We observed that the activity in the B-cells was higher than in the plasma samples. This observation suggests that the activity that was detected in the whole blood samples, came mainly from the B-cells. This is in order with observations made by other studies, and with the idea that the MMP-9 in the cell membrane of B-cells is responsible for degrading the extracellular membrane, and promoting angiogenesis, cell invasion, and tumor suppression [43, 44, 79]. In next studies it will be of importance to measure the activity of the MMP-2, and -9 of the B-cells in normal volunteers, in order to compare with the CLL patients, it is expected that this activity will be lower.

Trypsin is a serine protease that is mainly localized in the intestine, where it plays a role in normal digestion. In past studies it has been shown that trypsin is not completely impermeable to the lumen of the intestine, and that it can reach the circulation under severe inflammatory conditions, such as physiological shock [3-8, 55-61], in this study we wondered if CLL could also lead to leakage of trypsin into the circulation. Trypsin also activates MMP-2 and -9 [78], it is for this reason that trypsin activity was measured in the whole-blood samples of CLL, and normal donors. No significant activity difference was found between the groups, so we believe that the activation of the MMPs in CLL is independent from the activity of trypsin.

**4.6 Conclusion**

In this study we showed for the first time an elevated MMP-2, -9 activity in untreated whole blood samples of CLL patients. This measurement was only possible because of our charge-changing protease assay. We also showed evidence pointing that the source of this activity are B-cells, rather than plasma, suggesting that MMP-2, and -9 help in angiogenesis, by degradation of the extracellular matrix of the B-cells that invade the marrow. Trypsin activity was not elevated in the blood sample of CLL patients, implying that it is not involved in the activation of MMPs in this disease. The ability of measuring MMP in untreated whole blood in CLL patients can help in gaining a better understanding of the disease, developing better diagnostic techniques, and developing better therapeutics that will ultimately increase the quality of life of patients.

# CHAPTER 5

# THROMBIN DETECTION IN WHOLE

# UNTREATED BLOOD

## 5.1.   Summary

This is the pre-peer reviewed version of the following article: Modestino, AM., Tyndall, M., Yu, J., Lefkowitz RB., Schmid-Schönbein GW., Heller MJ. Detection of Thrombin Activity in Whole Untreated Human Blood, *Biotechniques,* Submitted December 2014. Which was submitted for publication.

## 5.2.  Abstract

Present coagulation assays fail to detect hypo- or hypercoagulability states of blood. Though thrombin generation assays can detect these states, they only work in treated blood samples, making them labor intensive, time-consuming and jeopardizing the repeatability of results. We developed a technique for thrombin activity detection in untreated whole human blood assay. The technique uses a thrombin specific charge-changing fluorescent substrate, which was tested for limit-of-detection (LOD) of spiked thrombin in 1XPBS and citrated whole blood, which was 1.97 nM and 6.82 nM respectively. The substrate was tested for the detection of thrombin over 30 minutes in untreated whole blood from healthy volunteers (one aspirin user). Thrombin activity increased over 30 minutes from a baseline level of $2x10^6$ RFU to $1.2 \ x10^{13}$ RFU. The lag time between the blood draw and initial burst of thrombin, was 6 minutes (n=5); and 15 minutes for an aspirin user. Specificity of the substrate was tested by reacting the substrate with heparinized blood and other enzymes resulting in minimal fluorescence signal. A thrombin activity technique was designed and tested in whole human blood, requiring no sample preparation, only minutes, and a only drop of blood.

## 5.3.  Method Summary

We developed a new technique capable of detecting thrombin activity directly in untreated whole human blood. The technique uses a fluorescent charge-changing peptide substrate specific to thrombin, and a simple electrophoretic separation device. The technology has the advantage of requiring no sample preparation, working within

minutes, and using only a drop of blood. The technique has broad applications in research, diagnostics and management of coagulation disorders; as well as a possibility of translation to a POC system.

Thrombin is a serine protease that is the key enzyme in the coagulation cascade. Activation of thrombin in the coagulation cascade permits the conversion of fibrinogen to fibrin, that crosslinks, forming a thrombus (blood clot), preventing blood loss in the case of injury [46]. Thrombin is then the pivotal enzyme, a high concentration of active thrombin will put patients at risk of generating thrombosis, while a low concentration of active thrombin will put patients at risk of bleeding.

From a clinical perspective, unregulated thrombin activity is linked to various clotting disorders [24, 25], cardiovascular diseases [47], several types of cancers [48, 49], sickle cell anemia [50], and inflammatory bowel disease [51], among others. The ability of measuring thrombin in-vivo is then of great interests to *(1)* further elucidate the relationship of thrombin concentrations in diseases, improve *(2)* diagnoses*, (3)* monitoring, and *(4)* management of these diseases.

## 5.4. Introduction

Thrombin is a serine protease, which cleaves fibrinogen, forming a blot-clot and promoting hemostasis [46]. Thrombin irregularities in the blood are present in coagulation disorders [24, 25], disseminated intravascular coagulation in shock [80], cancers [48,49], HIV [81], sickle cell anemia [50], inflammatory bowel syndrome [51], cardiovascular diseases (arterial thrombosis) [24, 25, 47] and pulmonary embolism (venous thrombosis) [24, 25]. Generally, a high thrombin concentration in the blood

will produce less bleeding, but it will increase the risks of developing thrombosis; while a low concentration will decrease the risk for developing of thrombosis, but it will increase bleeding [24, 25]. Therefore, monitoring thrombin concentration and activity is of great importance, since it is an indicator of the coagulability of the blood, and thus could be used as a guide for designing prevention strategies for bleeding or thrombosis.

Coagulation is currently assessed by measuring clotting times, mechanical properties of blood (e.g. tensile strength), or concentrations of specific factors involved in the coagulation cascade. These techniques give information about the general state of coagulation, but they fail to indicate hypercoagulability of blood and are insensitive to detection of mild bleeding disorders [24]. In contrast, TG measurements allow the detection of the hypo- or hypercoagulability state of blood; but they require sample preparation (e.g creation of platelet-poor or rich plasma (PPP or PRP), or dilution of blood sample; and addition of co-factors[24, 25, 52-53] ). Treated samples alter the true composition of the blood, altering the enzymes involved in the coagulation cascade, and thus they fail to determine the coagulability of the blood in a reliable manner.[53].

The ability to rapidly measure thrombin activity directly in untreated whole blood will provide important new *in vitro* diagnostics and bring a powerful tool to clinical researchers to further elucidate the relationship between circulating thrombin activity levels and diseases, as well as allowing investigation of the interactions of thrombin enzyme with other coagulation factors, platelets, and inhibitors. However, to date it has not been possible to measure TG in undiluted untreated blood samples [24]. We have now designed and tested a charge-changing fluorescent peptide substrate

specific for thrombin and develop a technique allowing the measurement of thrombin activity using only a few microliters of untreated whole human blood, requiring no sample preparation. In the study, we also briefly investigated the effects of aspirin on thrombin activity in whole untreated blood; aspirin is known to prolong coagulation by preventing platelet aggregation. The rapid electrophoretic technique was adapted from other work, where charge-changing fluorescent substrates were used to determine protease activities (e.g. chymotrypsin, trypsin, elastase, MMP-2 and MMP-9 in whole blood [28-30]). The charge-changing fluorescent peptide thrombin assay is able to monitor thrombin activity directly in untreated whole blood; this could provide better diagnosis, control and understanding of the coagulability states of blood.

## 5.5. Materials and Methods

### A. Reagents

A thrombin-specific substrate with the following sequence, acetyl-N-DDNleTPRGSAGAG-C-diaminioethyl-NH$_2$, was synthesized by Aapptec (Louisville, KY, USA). The sequence was labeled with the fluorophore Bodipy-FL-SE (Invitrogen, Carlsbad, CA, USA) via the peptide's diaminoethyl-amine group. The labeling was carried out by reacting equal volumes of the peptide substrate (10mg/mL) in 100mM NaHCO$_3$ (pH 8.2), with 10mg/mL of the fluorophore Bodipy-FL-SE in DMSO for 1 hour. The labeled product consists of the following sequence, acetyl-N-DDNleTPRGSAGAG(diamino-ethyl-BodipyFL)-NH$_2$. Human plasma thrombin (2,955 units/mg), calcium-chloride dihydrate (C3881), and ethylenediaminetetraacetic acid (6381-92-6) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Serum, sodium

citrate (0.105M/3.2%) and sodium heparin (30 USP Units) vacuum blood draw tubes were obtained from Becton Dickinson Medical Supplies (Franklin Lakes, NJ, USA). TPCK (N-tosyl-L-phenylalanyl chloromethyl ketone)-treated bovine pancreatic trypsin (T8802); recombinant human MMP-9 (M8945) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Novex pre-cast 1-mm thick 20% Polyacrylamide TBE gels and Novex gel cassettes (for casting custom 1-mm thick gels) were both obtained from Invitrogen. Whole blood samples were obtained from six healthy human donors.

### B. Sample Collection

We collected blood samples from 6 healthy volunteers (institutional review board no. 100556), one of which was on a daily-dose of aspirin (un-related to the study). The blood was collected in sodium citrate tubes, serum, or lithium heparin vacuum tubes (BD Medical Supplies).

### C. LOD of Thrombin activity in 1XPBS and Citrated Whole blood

Different concentrations of thrombin were spiked in 1XPBS (pH 7.8) and citrated whole blood. A stock solution of thrombin (100 units/mL) was prepared in 0.1% BSA (pH 6.5). Nine aliquots of the thrombin-specific substrate were prepared in 1X PBS (pH 7.8) or in the citrated whole human blood, they all were fixed to have a final substrate concentration of 0.45mg/mL, and 56mM of $CaCl_2$. Eight incremental volumes of the thrombin protease were dispensed into respective thrombin-specific substrate aliquots to achieve the following enzyme concentrations: 0.5, 1, 20, 100, 200, 500, and 600 nM. 1X PBS (pH 7.8) was dispensed into the ninth substrate aliquot. After reaction

was allowed for 30 minutes at room temperature, 6μL aliquots of the mixture were loaded onto the different lanes of the Novex Polyacrylamide gels submerged in 0.5X TBE running buffer (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). The samples were electrophoresed at 500 V for 10 min and the gel was imaged using a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA) at excitation and emission wavelengths of 302 nm and 500–580 nm respectively. The gel was also scanned in a Storm 840 workstation (Molecular Dynamics, Sunnyvale, CA, USA) loaded with the ImageQuant v5.2 software using the following settings: fluorescence mode, high sensitivity, 100 mm pixel size, 1000 V photomultiplier tube with a 450 nm excitation filter and a 520 nm long pass emission filter. The image was then digitally analyzed (ImageJ 1.440) and the fluorescent signal intensity was acquired.

### D. Real Time Detection of Thrombin Activity in Untreated Whole Blood

Thrombin activity was measured over time directly in untreated whole blood. **Figure 5-1** summarizes the steps for detection of thrombin in whole untreated human blood. The substrate solution (0.45mg/mL) is combined with the whole untreated human blood (no anticoagulants) *immediately* after the blood is drawn from the volunteers in serum vacuum, and sodium heparin tubes (negative control). The blood sample is allowed to react for a given amount of time, the reaction is then stopped by adding EDTA (0.77mg/mL) at multiple time points over 30 minutes (3 minutes intervals). Aliquots were electrophoresed, visualized, and quantified as described above.

### E. Testing Thrombin-Specific Substrate Against Trypsin and MMP-9

Various concentrations of bovine pancreatic trypsin and recombinant human MMP-9 were prepared as follows: trypsin in 1mM HCl and MMP-9 in TCNB buffer. Solutions of 0.45 mg/mL of the thrombin substrate were prepared in 1X PBS (pH 7.8), with 0.56mM of $CaCl_2$. Fourteen equal aliquots of the thrombin-specific substrate (0.45mg/mL) were prepared in 1X PBS (pH 7.8) for the reaction with each enzyme. Six equal volumes of the trypsin, and of the MMP-9 were dispensed into respective thrombin-specific substrate aliquots to achieve the following enzyme concentrations: 30, 50, 100, 300, 400, 500nM. 1X PBS (pH 7.8) combined with the substrate was used as a negative control. After the reaction was allowed to proceed for 30 minutes at room temperature, 6μL aliquots of the mixture were loaded into the different wells of polyacrylamide gels, electrophoreses was applied and the gels were imaged and quantified as described before.

## 5.6. Results and Discussion

Charge-changing fluorescent peptide substrates allow for the detection of protease activity directly in untreated whole blood, eliminating the need for any sample preparation [28-30]. Current methods used to coagulation lack sensitivity to detect mild bleeding disorders; TG assays have overcome this, but have problems in the repeatability of their results due to differences in sample preparation (e.g. addition of different cofactors, blood dilution, plasma generation). Our new charge-changing fluorescent peptide substrate was designed and tested for the detection of thrombin activity in untreated whole human blood with no sample preparation. The thrombin

specific substrate is shown in **Figure 5-2**: the substrate is cleaved by the enzyme at the peptide bond of Glycine (G) and Argenine (R) residues [1]; upon cleavage, two fragment are generated, one with a net charge of -2 and the other containing the fluorescent label with a net charge of +1. The fluorescently labeled positively charged product can separated from negatively charged uncleaved substrate, as well as from other negatively charged blood components using a simple electrophoretic format (**Figure 5-1**). This separation allows for the technique to be not only able to get rid of the background fluorescent signal of the uncleaved substrate, which has been identified as a problem when measuring proteases using fluorescent substrates [19], but also, it allows for the detection of the thrombin activity in untreated whole blood, which to our knowledge has never been accomplished before. The ability of detecting thrombin in a drop of untreated whole blood, not only brings a powerful research and diagnostic tool, but it also opens the possibility of designing a POC system for the detection of thrombin activity.

To validate our charge-changing fluorescent thrombin substrate, we fist demonstrated its ability to detect different concentrations of spiked thrombin in both, 1X PBS and in citrated whole blood. In citrated blood, the endogenous activity of thrombin is inhibited by the citrate, which acts as a strong chelator of the calcium ions needed as cofactor for the thrombin enzyme. This allowed for measurements of the activity of thrombin enzyme that was spiked in the blood sample, and not the thrombin activity endogenous to the blood. **Figure 5-1** shows the results for the detection of spiked thrombin concentrations in 1X PBS and citrated whole blood. Each well in the

polyacrylamide gels was loaded with a combination of substrate and a thrombin concentration progressing from the highest concentration of thrombin on the left-hand side to the negative control on the right-hand side of the gels **(Figure 5-3**). The negative control for this study was the negatively charged 1XPBS and uncleaved fluorescent peptide substrate, which migrated to the anode (+) in the upper buffer chamber (not shown). Integration of the fluorescent signals in the gels by means of digital analysis shows a non-linear monotonic rise with thrombin concentration in both 1X PBS and citrated human blood sample **(Figure 5-1-C)**. It was also observed that the fluorescence intensity observed for the same concentrations of spiked enzyme in 1X PBS was higher than that observed in citrated blood; this could be due to the presence of citrate in the blood collection tube, which could be inhibiting some of the spiked thrombin.

The LOD of thrombin in 1X PBS and in whole human blood was detected according to IUPAC standards [20], using the following expression: LOD=3 $\sigma_b$ /m, where (m) is the slope of the generated standard curve shown in **Figure 5-1-C, D**, and $\sigma_b$ is the standard deviation of the negative control. The LOD for the spiked thrombin in 1X PBS and citrated whole blood were determined to be 1.97 nM and 6.82 nM, respectively. This is an acceptable detection limit for the detection of thrombin in whole blood with no sample preparation. We believe that the LOD can be further improved by increasing the purity of the substrate, determining the ideal ratio of concentrations of substrate and sample in the reaction, incrementing the reaction time, or using a gel with better resolution (e.g. focusing gel doped with L-glutamic acid).

The charge-changing fluorescent peptide substrate was tested to detect endogenous thrombin activity in untreated whole human blood (no anti-coagulants). This was done by combining the untreated whole blood, with the fluorescent substrate, immediately after venipuncture. The addition of EDTA (a thrombin inhibitor by chelation of calcium) at different time intervals, allowed us to follow the progression of the activation of thrombin over time. We first generated an average thrombin activity curve from all healthy volunteers (n=6) by integrating the fluorescent signal from the gels **(Figure 5-4)**. The thrombin activity curve showed a lag time between the blood drawn and the appearance of fluorescence signal; this lag time corresponds to the time it takes to activate thrombin after the coagulation cascade is triggered. One of the volunteers for this study was a healthy individual on a daily dose of aspirin (unrelated to the study); we observed differences in the detection pattern of the thrombin enzyme over time **(Figure 5-5)**, which helped explain the large error bars obtained in **Figure 5-4**. The main difference observed between individuals not taking aspirin, and the one who is taking it was, a longer lag-time **(Figure 5-5)** for the aspirin taker, in thrombin appearance. This result is in line with aspirin's ability to prevent platelet aggregation by irreversibly acetylating platelet cyclooxygenase [82, 83] and thus prolonging blood clotting time.

When comparing an individual who does not take aspirin, and the one who does we observed that, for the non-aspirin user the lag time for the formation of thrombin was between 3-6 minutes **(Figure 5-6-D, solid circles, red arrow)**. The fluorescent signal for this individual, doubled after 9 minutes of reaction time. For the volunteer on

the daily dose of aspirin, the thrombin the lag time was 15 minutes **(Figure 5-6-D, open squares, red arrow),** and it took over 20 minutes for the fluorescence signal to double. In the latter time points the fluorescence signal corresponding to the thrombin activity is equal for both the individuals. This result implies that aspirin only interferes with the initial velocity of thrombin generation and not the actual capacity of an individual to generate thrombin. The longer lag-time in aspirin users recorded by our assay suggest, that our technique could be used to detect the presence of different anticoagulants in the blood, and thus it has the potential of becoming a monitoring technique for thrombin-related disorders, though other experiments are needed to confirm and develop this claim.

**Figure 5-6** also shows a negative control of whole blood collected in a heparin tube (**Figure 5-6-C),** where no fluorescent signal above background appears. Heparin binds to antithrombin, inducing a conformational change in this molecule, which can then inhibit thrombin activity [84]. The thrombin activity was monitored over time for blood collected in the presence of heparin; after 30 minutes of reaction time, no significant increase in fluorescence signal was observed (**Figure 5-6-C,D, open diamond**). This indicates very low background thrombin activity in the heparinized blood, which is an expected result. The baseline fluorescence signal itself is most likely due to substrate impurities, since there is no increase in the fluorescence intensity over time; thus it is unlikely that other blood proteases are cleaving the thrombin substrate.

Spiking 1X PBS with different proteases and reacting it with the fluorescent substrate further tested the substrate specificity to thrombin. Proteases from two

different groups, were spiked into 1X PBS: trypsin, a pancreatic protease, and MMP-9, a metalloprotease. Minimal increase in fluorescence signal was observed when the proteases were reacted with the thrombin substrate **(Figure 5-7-A)**. It was also shown that, when the same concentration of thrombin enzyme is spiked into 1X PBS the integration of the fluorescence signal obtained is more than five-times greater than that obtained for either one of the proteases **(Figure 5-7-B)**.

This implies that the charge-changing fluorescent peptide substrate is specific to thrombin, and that the background fluorescence signal present is due to impurities in the substrate. The fact that no fluorescence signal, above background, is observed in the heparinized blood sample **(Figure 5-6-B)** and in the samples spiked with other proteases **(Figure 5-7)** supports the hypothesis that thrombin is responsible for the cleavage of the substrate. Thus, the charge-changing fluorescent peptide substrate thrombin assay allows for specific detection of thrombin generation and activity directly in untreated whole blood without any sample preparation, which to the best of our knowledge has not been possible to do until now.

In this study we designed and tested new charge-changing fluorescent peptide substrate with specificity for thrombin. Using this charge-changing substrate we developed a technique for the detection of thrombin activity in untreated whole human blood. The technique can detect thrombin activity directly in untreated whole blood after only minutes and utilizing only a drop of blood. Moreover, we were also able to observe the delaying and inhibiting effect of aspirin on thrombin activity. The major advantages of our thrombin-specific fluorescent substrate thrombin activity assay over current

assays include: (1) no sample preparation, (2) a simple electrophoretic format, (3) requirement of only a very small blood sample volume (5 μL), (4) ability to assess initiation of thrombin activity (coagulation) in whole human blood within minutes, and (5) sufficient sensitivity to detect low concentrations of thrombin in whole blood with considerable options to further improve sensitivity. The ability to use untreated whole blood with no sample preparation opens the possibility to develop a true POC system for rapid thrombin activity coagulation testing for blood disorder diagnostics and monitoring anticoagulant therapies. Our simple system can also be modified to measure other proteases directly in whole blood, as has been done previously for MMPs and pancreatic proteases [28-30]; other substrates could de designed for monitoring other proteases in the coagulation cascade directly in untreated whole blood.
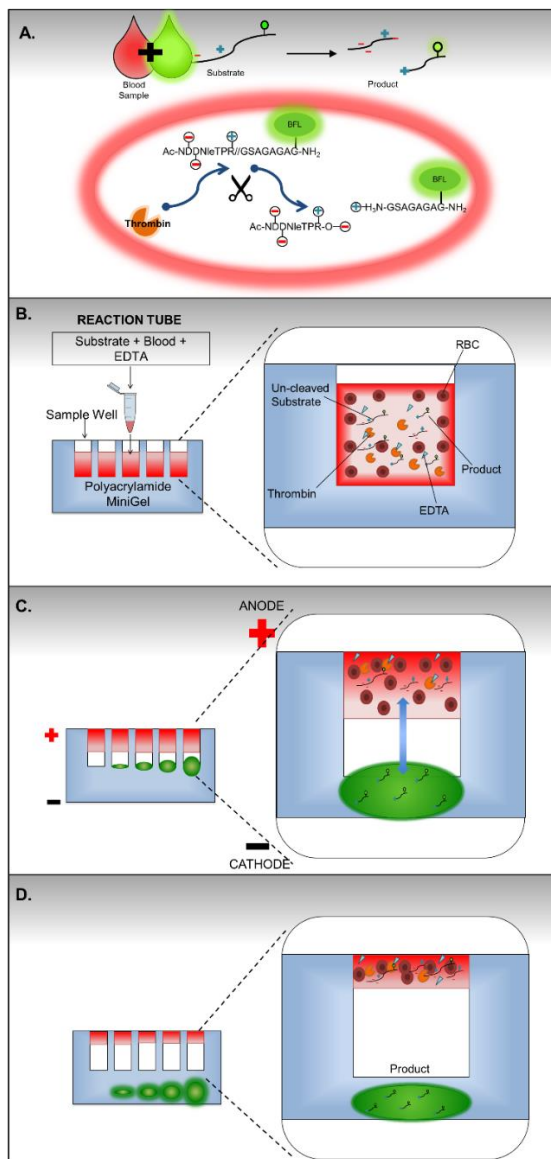
**Figure 5-1Whole-Blood Thrombin Assay. (A)** The charged-changing-fluorescent substrate is added to the untreated-whole-blood. Cleavage of the peptide bond creates two fragments: a positively-charged fluorescent and a negatively-charged one. **(B)** An aliquot is loaded into a polyacrylamide-gel. **(C)** The DC electrophoretic field applied. **(D)** After 10 minutes the cleaved positively-charged fluorescent product fragments are concentrated into the gel.

| Target Protease | Peptide Sequence | Net Charge |
|---|---|---|
| Thrombin | Acetyl-N-D⁻-D⁻ NleTPR⁺ //// GSAGAGAG-**diamino-ethyl-Bodipy-FL** | -1 |
| | **Cleaved Fragments** | |
| N- Terminal | Acetyl-N-D⁻-D⁻ NleTPR⁺-O⁻ | -2 |
| C-Terminal | ⁺H₃N-GSAGAGAG-**diamino-ethyl-Bodipy-FL** | +1 |
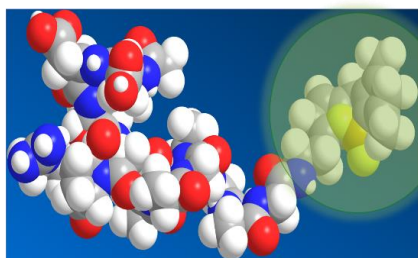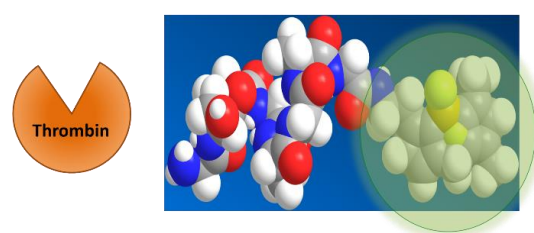


**Figure 5-2 Charge Changing Fluorescent Thrombin Substrate Sequence and CPK-Model.**
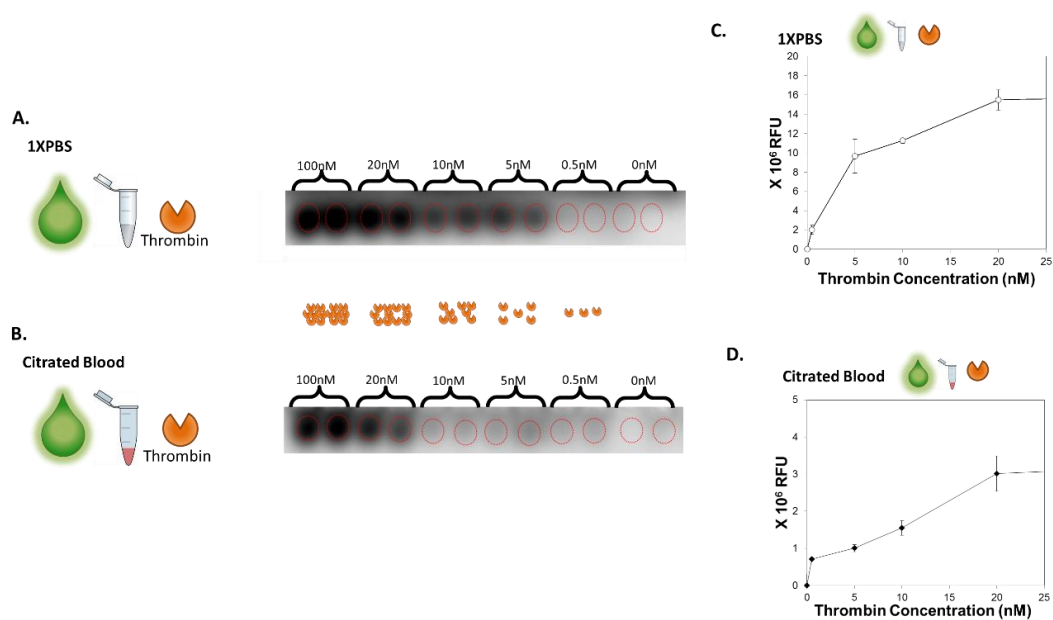
**Figure 5-3. Detection of spiked Thrombin A-D. (A & B)** Quantitative gel scans obtained reacting the substrate, with concentrations of spiked thrombin enzyme in **(A)** 1XPBS, **(B)** citrated-whole- blood. The fluorescent signal is encircled in red. **(C &D)** Show the thrombin activity curves from the gels of A and B. Fluorescence is reported in millions of relative fluorescence units (RFU).
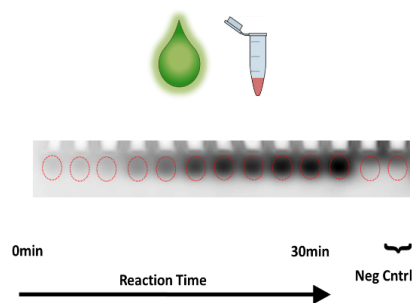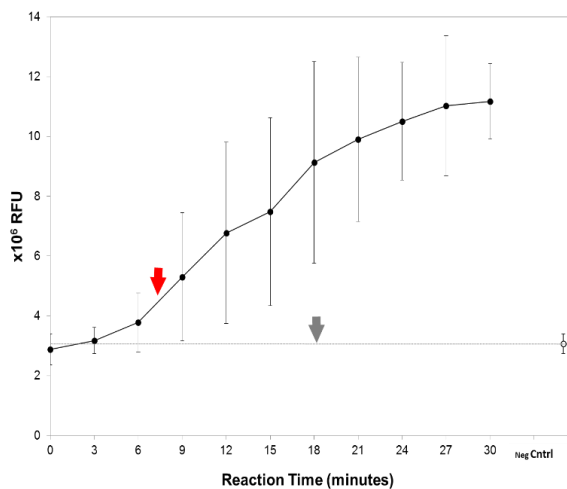
**Figure 5-4 Whole Blood Thrombin Assay. (A)** Quantitative gel scan for untreated whole-blood combined with the substrate. The resulting fluorescent signal is encircled in red. **(B)** Shows a TG curve (n=6, with 3 repeats for each) represented by solid-circles. Open-circle represents, and gray-dotted line represent the negative control (1XPBS). The red-arrow shows the end of the lag-time.
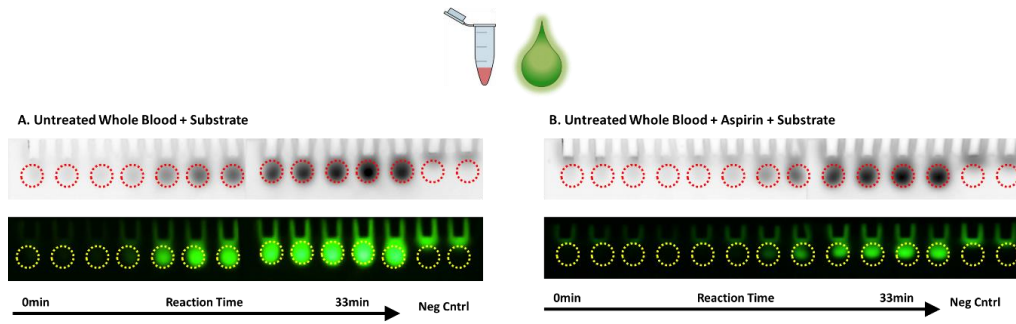
**Figure 5-5 Quantitative gel-scans for thrombin activity over time (A)** whole-blood from non-aspirin user, **(B)** whole-blood from aspirin user
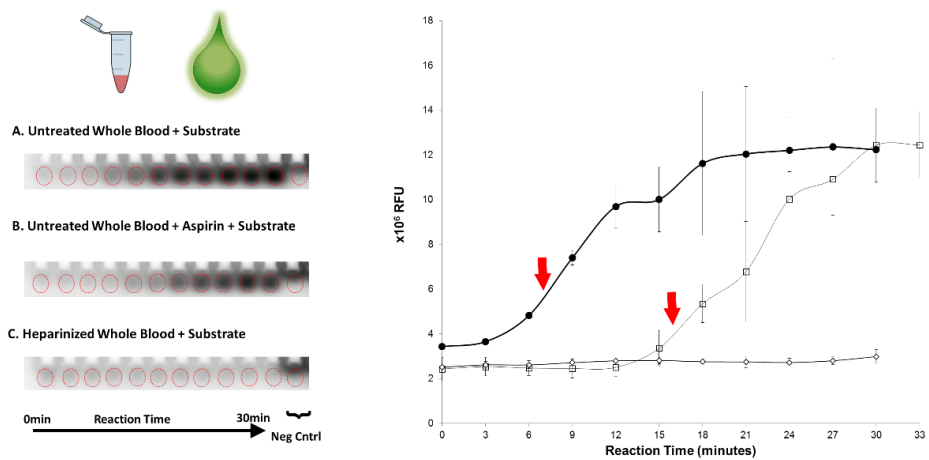
**Figure 5-6 Whole Blood Thrombin Assay Normal and Aspirin User Blood.** Quantitative gel-scans (A-C), and activity curves (D) for **(A)** whole-blood from non-aspirin user, **(B)** whole-blood from aspirin user, and **(C)** heparinized-whole-blood. **(D)** TG curve for A, solid-circles (n=1, 3 repeats), B, open-squares (n=1, 3 repeats), and C, open diamonds(n=3, 3 repeats). The red arrows show the end of the lag-time.
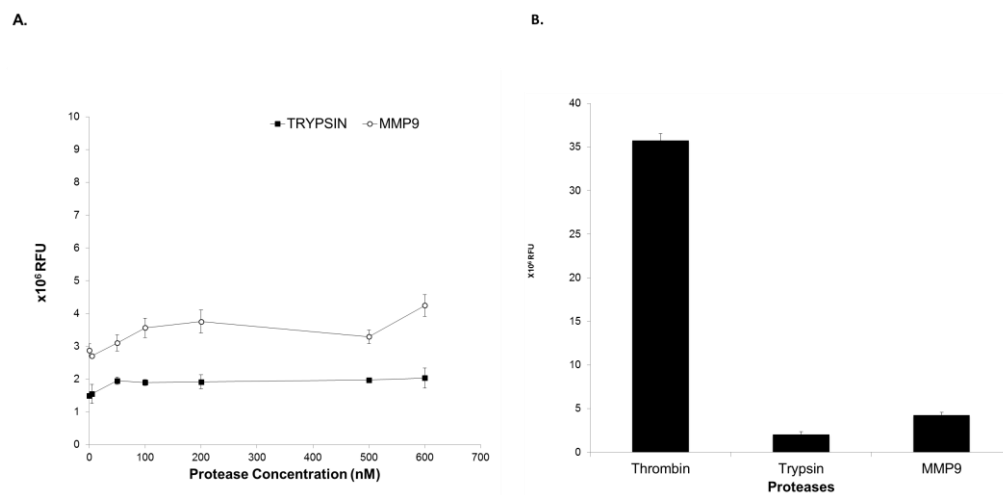
**Figure 5-7 Substrate Specificity. (A)** Fluorescent detection curves for trypsin and MMP-9 combined with thrombin substrate. **(B)** Comparison of the fluorescent signal obtained when reacting the thrombin substrate with 600nM of each of the different enzymes (thrombin, trypsin, MMP-9).

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# CHAPTER 6

# PROTOTYPE FOR A POINT OF CARE

# SYSTEM FOR PROTEASE DETECTION

## 6.1.    Summary

In this chapter we present a prototype for a point-of-care (POC) system for protease detection in whole untreated blood, using the charge-changing substrates, and a simple

## 6.2.    Introduction

As has been discussed through-out this dissertation protease activity plays an important role of the progression of many diseases, here we have discussed: physiological shock, type 2 diabetes mellitus, chronic lymphocytic leukemia, and coagulation disorders; but they are active, and key in the initiation and progression of many more diseases.

The current methods for detection of proteases require significant sample preparation, and this is an impairment in the development of point-of-care (POC) system, that will detect the activity of this proteases in untreated samples. Using charge-changing fluorescent peptide substrates, specific for a protease of interests, we have demonstrated that the detection significant activity of different proteases directly in whole-human blood, in less than 1-hour from sample acquisition, to activity detection. Our ability to work in untreated samples, opens then the possibility for a point-of-care system for detection of protease activity in crude sample that can improve diagnostics, disease management, and help in the development of new therapeutics [28-30].

In this study, we present a first prototype for a POC, which is horizontal channel, with a small gel casted in the middle of the device, for detection. The device is powered by only three 9V batteries. Here we detected thrombin activity in untreated-fresh whole blood, in our prototype POC device. This proof of principle study shows, that it is possible to use charge-changing substrates for the detection of proteases in a battery operated device.

## 6.3. Experimental Design

### 6.3.1. Prototype Device Design

A horizontal channel, with electrodes on each side, and a gel in the middle for capturing of the signal was designed and tested, for the detection of thrombin activity in whole-untreated blood. The horizontal gel format device consisted of an acrylic gasket (1X0.5X0.125cm) bonded onto a silica microscope slide (2.5X

7.5cm) using a medical grade UV adhesive (Dymax 1181, Dymax, Torrington, CT, USA). Leaving a channel in the middle (0.5X3cm) for the blood sample, charge-changing substrate, gel, and buffer (**Figure 6-1**). The gasket was designed in CAD and material was removed using the LaserCAM. The electrode assembly consisted of an acrylic gasket solvent bonded onto two other acrylic plates with dichloromethane, this created two grooves for the placement of two platinum wires. Two holes in opposing corners were cut out from the acrylic gasket and electrode assembly using the LaserCAM. One pin (0.25" long) was installed in each hole for the purpose of aligning the electrodes to the acrylic gasket **Figure 6-1Figure 6-2 Figure 6-3)**

### 6.3.2. Prototype Design Testing

The horizontal gel format device was tested for the detection of thrombin in untreated fresh-whole blood. A 15% polyacrylamide gel, doped with 5% L-Glutamate (Sigma-Aldrich, St. Louis, MO) was casted, and poured into the channel, the recipe for this gel is shown in **Table 6-1**. The gel was cut to form two gels: one in the middle, for the detection of the fluorescent signal; and other to the left-side, which served to separate the sample from the electrodes **(Figure 6-4)**.

Both the sample well, and the buffer well were filled with running buffer 0.5XTBE (44.5 mM Tris-Borate, 1 mM EDTA, pH 8.0). The two platinum wire electrodes were each connected to either the positive or negative terminal. The entire setup was placed in a BioDoc-It M-26 transilluminator (UVP, Upland, CA, USA),

which served as a dark chamber. The camera positioned on top of the chamber facing downwards, this is shown in **Figure 6-5**.

The test sample consisting of: 5uL of untreated blood-sample and 5uL fluorescent thrombin substrate, was then loaded into the sample well. The sample was acquired from a healthy donor, by venous puncture, in serum tubes. The blood was combined with thrombin fluorescent substrate of the following sequence: acetyl-N-DDNleTPRGSAGAG-C-diaminioethyl-NH$_2$, synthesized by Aaptec (Louisville, KY,USA). The substrate was labeled with the fluorophore Bodipy-FL-SE (Invitrogen, Carlsbad, CA, USA) via the peptide's diaminoethyl-amine group.

The electric field in the prototype device was generated using three 9V batteries. The device was turned-on for 30-minutes, and pictures were taken at time 0 and time 25 minutes. The UV-lamp was turned on for the excitation of the cleaved substrate at the times of the pictures, to not exhaust the fluorophores.

## 6.4.    Results and Discussion

The ability of rapidly detect protease activity in whole blood will improve diagnostics, monitoring, therapeutics, and the understanding of the progression of multiple disease and disorders. Here we demonstrated the ability of detecting thrombin activity in an untreated whole blood sample, using a simple proof of principle POC device. We were able to detect a significant fluorescent signal after only 25 minutes of electrophoresis, using 27 V (**Figure 6-6**).

This preliminary results, suggest of the possibility of the detection of different proteases in whole untreated blood, using this prototype POC device. Further development of the device will be required for improvements in the sensitivity, and repeatability of the results. But this was a proof of principle device, which showed the feasibility of moving to a POC system for the detection of protease activities using charge-changing protease substrates.
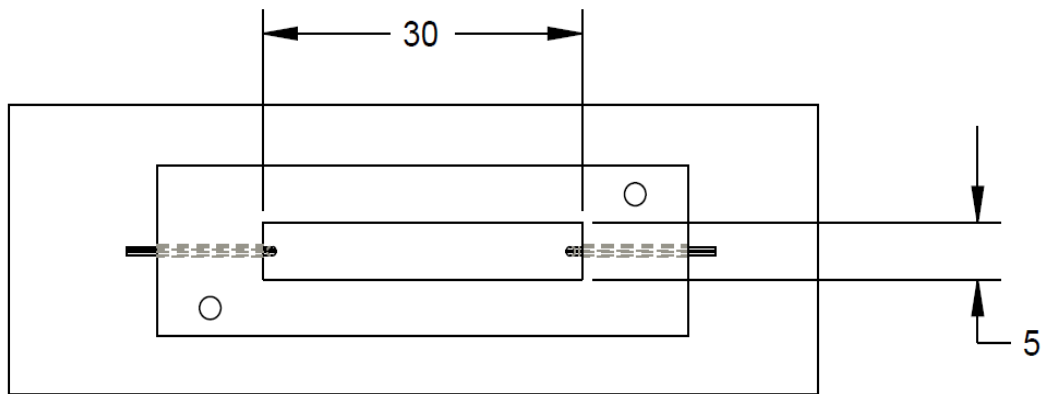
**Figure 6-1 POC Prototype Device Top View** All the dimensions are shown in mm.
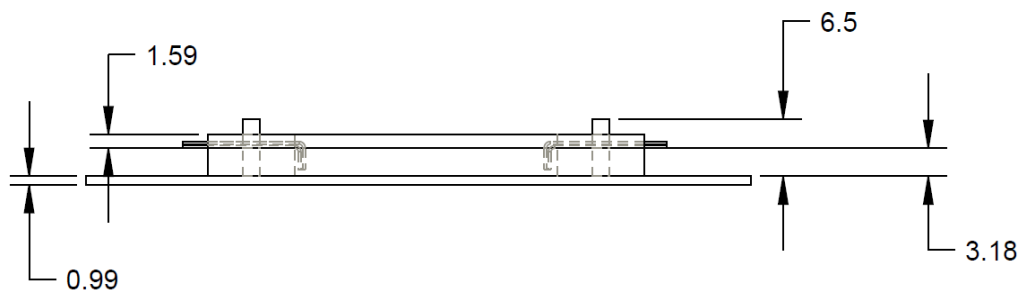


**Figure 6-2 Prototype POC Device Side View** All the dimensions are shown in mm.
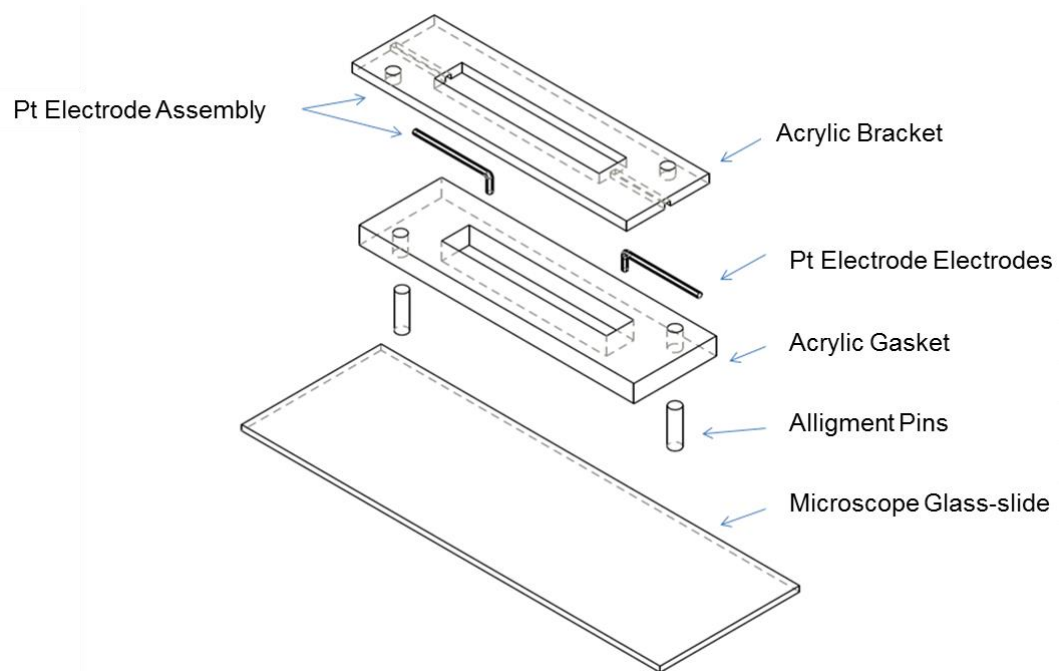
**Figure 6-3 Prototype POC Device Exploded View.** Assembly of the different parts

created the horizontal channel POC device.

**Table 6-1** Components for casting a Gel

| Components | Volume for 15% Doped Gel (uL) |
|---|---|
| Distilled $H_2O$ | 300 |
| 40% Acrylamide (1:29, Acrylamide:bis) | 375 |
| 1.5 M Tris (pH 8.8) | 250 |
| 10% SDS | 10 |
| 10% Ammonium Persulfate | 10 |
| TEMED | 5 |
| Poly-L-Glutamic Acid (5mg in 50uL $H_2O$) | 50 |
| Total Volume | 1000 |
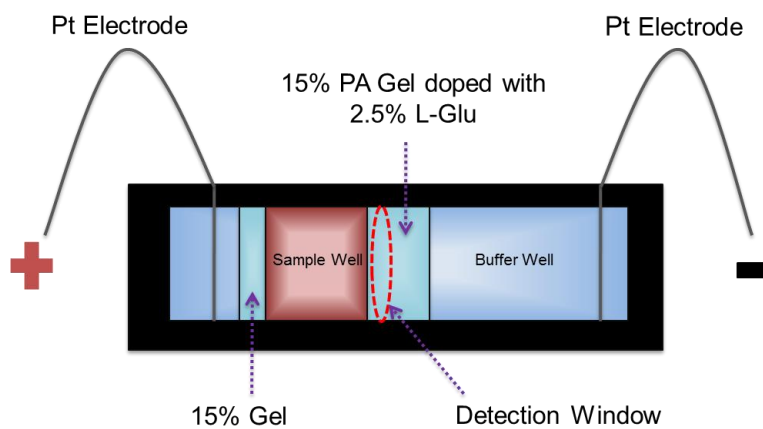
**Figure 6-4 Prototype POC Device General Configuration Top View.**
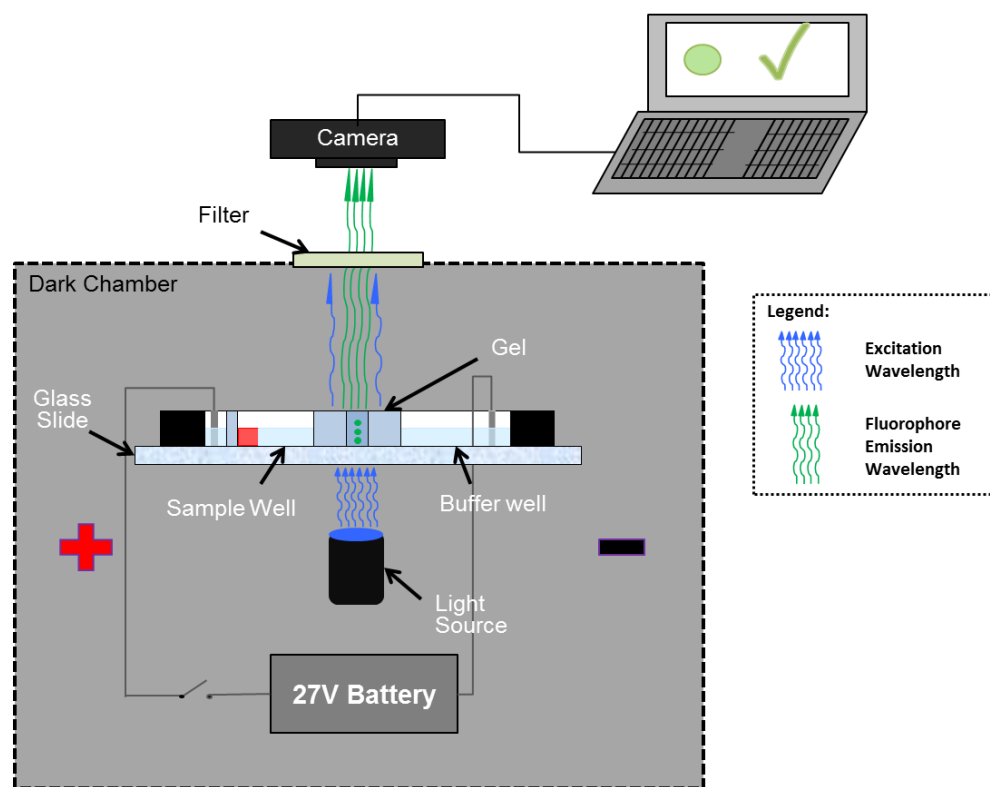
**Figure 6-5 Prototype POC Device System Setup.** The device and battery were placed BioDoc-It transluminator unit. The excitation wavelength was emitted by the light source, which then traveled through the horizontal format device. The filter only allowed the emission wavelength to pass through and the signal was then detected by the camera. Images were then analyzed by the computer.
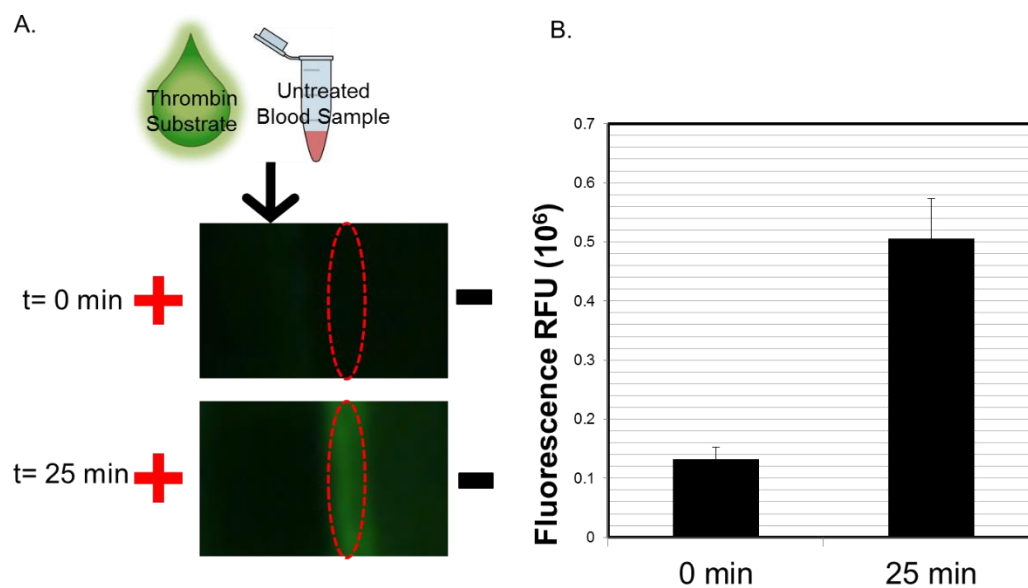
**Figure 6-6   Detection of Thrombin Activity in POC Device**. **(A)** Shows pictures of the detection window, at time 0 of the addition of the sample in the POC Device, and after 25 min of electrophoreses. **(B)** Shows the quantification of this image. Fluorescence is shown in random units of fluorescence (RFU).

# CHAPTER 7

# CONCLUSIONS

## 7.1.  General Conclusions

In Chapter 1, it was shown that the proteases play a pivotal role in the initiation, and progression, of the following diseases: physiological shock, type 2 diabetes, chronic lymphocytic leukemia, and several types of coagulation disorders. Although, they seem to be key factors in the development of these diseases, they have remained under-studied for many decades, possible because of an absence of a technique, allowing for the detection of this family of molecules in untreated biological samples. It was the subject of this dissertation to use the new charge changing fluorescent peptide substrate electrophoretic method to detect protease activity in crude samples in these diseases. Specifically have presented the following findings:

In Chapter 2, the activity of: trypsin, chymotrypsin, and metalloproteases -2, and -9, were detected in lymph fluid of a rat subjected to physiological shock. It was shown

that there was an increase in the activity of proteases following physiological shock; this to our knowledge was the first time that such a clear evidence was presented, and it was only possible due to our charge-changing substrates protease assay. These findings supply a piece of evidence to the *Autodigestion Hypothesis,* which states that the proteases can escape the lumen of intestine after physiological shock and enter the lymphatics, due to an increase permeability of the intestinal wall.

In Chapter 3, the activity of: trypsin, elastase, and metalloproteases -2, and -9, were detected in untreated whole type 2 diabetics, after a high calorie, fat, and sugar meal. We encountered elevated protease activity of elastase, and MMP-2, and -9 in the blood of T2DM. Our technique also allowed for the detection of changes in protease activity after a meal, where we observed more changes in protease activity in T2DM. These findings provide some evidence to the theory that states that the attenuation in glucose tolerance observed in T2DM, come from cleavage in the insulin receptor, by uncontrolled proteolytic activity. Also, the fact that more changes where observed in the protease activity of T2DM after a meal, than in normal, provide some evidence to the idea that the source of the protease activity in the circulation is the small intestine, which is more permeable for patients suffering from T2DM. Our charge changing protease assay then provides means to deepen our understanding of the mechanism of progression of type 2 diabetes.

In Chapter 4, the activity of: metalloproteases -2, and -9, and trypsin were detected in whole untreated blood of CLL patients. We encountered elevated protease activity of elastase, and MMP-2, and -9 in the blood of CLL. We also measured the

activity of MMP-2, and -9 in plasma samples, and B-cells, we found that the activity in the B-cells was higher, than in plasma samples. This finding implies that the activity seen in the untreated whole blood samples, comes from B-cells. Our technique then showed that MMP-2, and -9 can need to be further studied to understand the molecular mechanism behind the progression of CLL.

In Chapter 5, we showed the designed and development of a new charge changing substrate specific to thrombin enzyme. The substrate allowed us for the rapid detection of thrombin activity directly in untreated whole human blood. The assay was also capable of detecting differences in the coagulation patterns of normal patients, and one patient taking a daily dose of aspirin.  To this date, the detection of the activity of thrombin in untreated whole blood samples has not been possible.

In Chapter 6, we used our charge changing thrombin substrate to design a POC prototype system for the detection of thrombin in whole untreated human blood.  Our prototype POC system was capable of detecting thrombin activity in whole untreated human blood within minutes, and the device was powered by only 27V. This prototype device, opens the possibility for the development of POC system for the detection of multiple proteases in whole human blood.

## 7.2.    Further Work

In Chapter 2, we provided evidence of protease activity in the lymph fluid after shock using our charge changing protease substrates. It would be of interests to now, measure the activity in the blood circulation, as well as in peripheral organs, as ways of

understanding the transport of the proteases through the body after physiological shock, and to find how they are involved in multi organ failure. These studies will provide the groundwork to create therapeutics for this condition, that to this date has as the solely treatment the alleviation of symptoms.

In Chapter 3, we showed elevated protease activity in whole blood of patients suffering from T2DM. In order to understand the mechanism of leading to the insulin resistance observed in these patients, it would be necessary to determine if the protease activity is involved in the cleavage of extracellular receptors, in particular the insulin receptor. So it is my suggestion to determine the plasma levels of the soluble insulin receptor in T2DM, before, and after a meal. These findings will provide an insight in the mechanism of this disease that affects a good percentage of the world, and will aid in creating prevention policies, to stop the spread of T2DM.

In Chapter 4, we showed that there is an elevation in the MMP-2, and -9 in the CLL patients. It would be of interests to measure the MMP-2, and -9 levels in the plasma, and B-cells, of normal patients, as a mean of comparison. Moreover, it is a correlation between the stage of CLL, and the MMP-2, and -9 activities level should be made. This will help not only to elucidate the correlation between the protease level, and the progression of the disease; but also it will tell us if these rapid, and simple measurements can be made for an early diagnosis of the disease, that will improve the prognosis of the patient.

In Chapter 5, a new thrombin activity assay in whole untreated blood is shown. Further studies should be made with patients suffering from coagulation disorders, to investigate, whether our simple, rapid, assay can be used for the detection and monitoring of new coagulation techniques. It is also of interests to develop an assay that is capable of measuring the active thrombin in circulation.

In Chapter 6, a prototype for a POC system for the detection of thrombin in whole untreated human blood was presented. It is necessary to further develop this assay in order to extend it to other proteases.

Finally it is suggested that new charge changing peptide substrates, for the detection of other proteases be designed. This will help gaining a better understanding of different diseases and disorders.

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