UC San Diego

UC San Diego Electronic Theses and Dissertations

Title

Charge-Changing Substrates for Degradative Enzyme Activity Detection in Whole Blood

Permalink

https://escholarship.org/uc/item/7h60z2z8

Author

Skowronski, Elaine Alexandra

Publication Date

2017

Peer reviewed|Thesis/dissertation

eScholarship.org Powered by the California Digital Library
University of California

UNIVERSITY OF CALIFORNIA, SAN DIEGO

Charge-Changing Substrates for Degradative Enzyme Activity Detection in Whole Blood

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

in

NanoEngineering

by

Elaine Alexandra Skowronski

Committee in charge:

Professor Michael J. Heller, Chair
Professor Sadik Esener
Professor Paul Mills
Professor Geert Schmid-Schönbein
Professor Joseph Wang

2017

Copyright

Elaine Alexandra Skowronski, 2017

All rights reserved.

The dissertation of Elaine Alexandra Skowronski is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

2017

DEDICATION

This thesis is dedicated to my family, present and past.

EPIGRAPH

"Even in his greatest dedication to science he had never managed to forget that people's goodness and beauty come from what they believe, not from what they know."

–Robert Musil, The Man Without Qualities: Volume 2

TABLE OF CONTENTS

Signature Page iii

Dedication iv

Epigraph v

Table of Contents vi

List of Abbreviations ix

List of Figures xi

List of Tables. xiii

Acknowledgements xiv

Vita xviii

Abstract of the Dissertation xx

Chapter 1 Introduction: Degradative enzymes and their analyses in biological fluids 1
Abstract 2
1.1 Degradative enzymes 3
1.2 Proteases 3
1.2.1 Significance of proteases 3
1.2.2 Structure and classification of proteases 4
1.2.3 Regulation of protease expression and activity 4
1.2.4 Function of proteases 5
1.3 Autodigestion hypothesis: proteases 5
1.4 Analysis of proteases in biological fluids: activity-based assays. 6
1.4.1 Substrate-based protease activity assays 7
1.4.2 Inhibitor-based protease activity assays 7
1.5 Novel assay for protease activity detection 8
1.5.1 Issue of sample preparation 8
1.5.2 Whole blood assay for protease activity detection 9
1.6 Lipases 9
1.6.1 Significance of lipases 9
1.6.2 Structure and classification of lipases 10
1.6.3 Regulation of lipase expression and activity 10
1.6.4 Function of lipases 10
1.7 Autodigestion hypothesis: lipases 11
1.8 Analysis of lipases in biological fluids: activity-based assays 11
1.9 Novel assay for lipase activity detection 11

1.9.1 Issue of sample preparation . . . 12
1.9.2 Whole blood assay for lipase activity detection . . . 12
1.10 Scope of the dissertation . . . 13
References . . . 17

Chapter 2 Pilot Study: Evidence for increased proteolytic activity and proteolytic receptor cleavage after a meal in controls and type 2 diabetics . . . 20
Abstract . . . 21
2.1 Introduction. . . . 22
2.2 Results . . . 24
2.2.1 Study participants . . . 24
2.2.2 Circulating protease activity . . . 25
2.2.3 Inflammatory markers and soluble adhesion molecules . . . 27
2.3 Discussion . . . 28
2.4 Conclusion . . . 33
2.5 Materials and methods . . . 34
2.2.1 Sample acquisition . . . 34
2.2.2 Meal tolerance test study design . . . 34
2.2.3 Metabolic syndrome index (MSI) . . . 34
2.2.4 Protease activity assays . . . 35
2.2.5 Insulin receptor labeling . . . 35
2.2.6 Inflammatory markers and soluble adhesion molecules . . . 36
2.2.7 Statistical analysis . . . 36
Acknowledgements . . . 36
References . . . 48

Chapter 3 Pilot study of circulating proteolytic activity and proteolytic receptor cleavage in schizophrenia and comparison groups . . . 51
Abstract . . . 52
3.1 Introduction . . . 53
3.2 Results . . . 54
3.2.1 Study participants . . . 54
3.2.2 Circulating protease activity . . . 54
3.2.3 Inflammatory markers and soluble adhesion molecules . . . 55
3.3 Discussion . . . 55
3.4 Conclusion . . . 56
3.5 Materials and methods . . . 56
3.5.1 Study population . . . 56
3.5.2 Sample acquisition . . . 57
3.5.3 Protease activity assays . . . 57
3.5.4 Insulin receptor labeling . . . 57
3.5.5 LRP1 receptor labeling . . . 58
3.5.6 Inflammatory markers and soluble adhesion molecules . . . 58
3.5.7 Statistical analysis . . . 59
Acknowledgements . . . 59

References 66

Chapter 4 Associations of circulating proteolytic activity in neonates and length of stay in the neonatal intensive care unit 68
Abstract 69
4.1 Introduction 70
4.2 Results 71
4.2.1 Study participants 71
4.2.2 Circulating protease activity 71
4.3 Discussion 71
4.4 Conclusion 72
4.5 Materials and methods 72
4.5.1 Study design 72
4.5.2 Patients 72
4.5.3 Protease activity assays 73
4.5.4 Length of stay 73
4.5.5 Septic episodes 73
Acknowledgements 73
References 81

LIST OF ABBREVIATIONS

Ac . . . . . acetyl

ADAM . . . . . a disintegrin and metalloprotease

ANCOVA . . . . . analysis of covariance

ANOVA . . . . . analysis of variance

BFL . . . . . Bodipy FL

CHY . . . . . chymotrypsin

CLIA . . . . . clinical laboratory improvement amendments

CuAAC . . . . . copper(I)-catalyzed azide-alkyne cycloaddition

DMSO . . . . . dimethylsulfoxide

EDTA . . . . . ethylenediaminetetraacetic acid

ELA . . . . . elastase

ELISA . . . . . enzyme-linked immunosorbent assay

FRET . . . . . fluorescence resonance energy transfer

IEDDA . . . . . inverse electron demand Diels-Alder

LOD . . . . . limit of detection

MMP . . . . . matrix metalloprotease/metalloproteinase

NICU . . . . . neonatal intensive care unit

OGTT . . . . . oral glucose tolerance test

PAGE . . . . . polyacrylamide gel electrophoresis

PBS . . . . . phosphate buffered saline

PLA1 . . . . . phospholipase a1

PLA2 . . . . . phospholipase a2

POC . . . . . point-of-care

PPMC . . . . . Pearson product-moment correlation

PPMCC . . . . . Pearons product-moment correlation coefficient

pT2DM . . . . . prediabetes

RFU . . . . . relative fluorescence unit

SDS-PAGE . . . . . sodium dodecyl sulfate polyacrylamide gel electrophoresis

SPAAC . . . . . strain-promoted azide-alkyne cycloaddition

T2DM . . . . . type 2 diabetes mellitus

TBE . . . . . tris borate EDTA

THR . . . . . thrombin

TIMP . . . . . tissue inhibitor of metalloproteinases

TRY . . . . . trypsin

LIST OF FIGURES

Figure 1.1. Conventional FRET substrates versus novel charge-changing substrates for protease activity detection 14

Figure 1.2. Sample processing of conventional activity assays 15

Figure 1.3. Whole blood assay for protease activity detection 16

Figure 2.1. Metabolic syndrom index (MSI) calculation flow-chart 38

Figure 2.2. Novel electrophoresis assay for detection of protease activity 39

Figure 2.3. Protease baseline activity 40

Figure 2.4. Postprandial protease activity and glucose levels in circulation 41

Figure 2.5. Insulin receptor density in PBMC incubated with plasma 42

Figure 2.6. Baseline values for inflammation markers and soluble adhesion molecules mean baseline 43

Figure 2.7. Postprandial inflammation marker levels 44

Figure 2.8. Linear correlations of baseline protease activity levels and anthropometric characteristics of participants 45

Figure 2.9. Insulin receptor density changes with reaction time and protease concentration 46

Figure 3.1. Protease activity detection 60

Figure 3.2. Protease activity in circulation 61

Figure 3.3. Mean of protease activity in circulation 62

Figure 3.4. Pearson linear correlations for inflammatory markers and soluble adhesion molecules 63

Figure 3.5. Pearson linear correlation for proteases and inflammatory markers 64

Figure 3.6. Density of cell receptors exposed to proteolytic activity 65

Figure 4.1. Neonate sample acquisition and length of testing 74

Figure 4.2. Gestational age and length of stay in the neonatal intensive care unit 75

Figure 4.3. Time course of circulatory protease activity in neonates 76

Figure 4.4. Activity of pancreatic proteases 77

Figure 4.5. Activity of MMP-2/9 and thrombin 78

Figure 4.6. Proteolytic activity with diagnoses of neonates 79

LIST OF TABLES

Table 2.1. Baseline characteristics of study participants 47

Table 4.1. Peptide substrates for protease activity detection 80

ACKNOWLEDGEMENTS

I owe so much to my advisor, Dr. Michael Heller. He is the reason I applied to graduate school. His unvarnished honesty, passion for real world solutions, and indefatigable support for the people on his team will always inspire me to be a better researcher and a better human being.

My research has been shaped for the better by the counsel and support of my committee members. I appreciate the pragmatic pointers as well as the entertaining stories of Dr. Sadik Esener, Dr. Paul Mills, Dr. Geert Schmid-Schönbein, and Dr. Joe Wang.

The clinical studies that have been the highlight of my research would not have been possible without my collaborators Dr. Augusta Modestino, Dr. Jamie Joseph, and Dr. Shelley Lawrence. Pages and pages would be needed to catalogue all that I owe to Dr. Augusta Modestino, but I will restrict myself to a small but effusive statement—very impressssssed!

There are so many people beyond co-investigators that were critical in making my clinical research possible, including nurses, study coordinators, patients, and volunteers. Little kindnesses can go such a long way in making projects run smoothly.

My mentors off campus have also had a hand in making me a better researcher. I never would have thought I could contribute to research without the confidence that Dr. David Tully and Dr. Donatella Chianelli during my internships at the Genomics Institute of the Novartis Foundation. Dr. Anne Hanneken took me in at The Scripps Research Institute when I was nervous about my research at the University of California, San Diego. The new methods I learned under her made me feel competent again.

My time in the laboratory has been made brighter by the friendships I've made in Dr. Michael Heller's lab group. Dr. Jennifer Marciniak, Dr. Avery Sonnenberg, Dr. Stuart Ibsen, Sareh Manouchehri, and James McCanna helped me cut my teeth on dielectrophoresis and PCR research. Dr. Augusta Modestino taught me the protease activity assays that underlie my research. The helpfulness and cheerfulness of these mentors was further enhanced by the liveliness of the rest of the lab group: Dr. Tsukasa Takahashi, Dr. Youngjun Song, Dr. Sejung Kim, Dr. Taeseok Oh, Dr. Augustine Obirieze, Dr. Jean Lewis, Dr. Daniel Heineck, Yvonne Heaney, and Ben Sarno.

Many offerings were made at the altar of caffeine with my friends on campus as we navigated the coffee dark seas of research together. Bitter coffee was made sweet by the comradery of Dr. Augusta Modestino, Dr. Jennifer Marciniak, Marcela Mendoza, Sohini Manna, Valentin Robert, Ben Dahljelm, and Yvonne Heaney. Both my friends on campus and my friends outside of graduate school, including Katherine Howell, Kristen Ostosh, Keldin Sergheyev, and Crisara Abrams, have improved my time as a researcher by trading stories, going on adventures, taking part in creative diversions, and sharing the joys and sorrows of life.

My life has been made richer by the people outside of science who have taught me so many things. Dr. Gideon Rappaport taught me to respect literature and art, and his meticulous approach to textual analysis will always inspire me to see things clearly. Susie McNamara and Dr. Joshua Feder were instrumental in encouraging me to apply to graduate school and have taught me to see myself and others with clear eyes. I am forever indebted to them.

Saving the best for last, I thank my parents, Papa and Mummie, and my sister, alias TT, for their love and support. My parents gave me my curiosity, my work ethic, and their love. Being blood related to these two amazing people sometimes makes me forget how immensely lucky I am to share my life with such unique, intelligent, and honest people. Not to be outdone, my sister has been my favorite companion since birth. She may have grown out of giving me sloppy baby kisses, but she will never stop being my baby sister, my most trusted confidant, and the person who both knows me best and loves me most despite my flaws. I hope my research and the way I comport myself in life will reflect the values and spread the love I have received with my family.

I also include the following acknowledgments below as required by the University of California, San Diego:

Chapter 2, in part, has been submitted for publication of the material as it may appear in Diabetes Journal, 2017, Augusta E. Modestino, Elaine A. Skowronski, Chris Pruitt, Karen L. Herbst, Geert W. Schmid-Schönbein, Michael J. Heller, Paul J. Mills. "Pilot Study: Evidence for increased proteolytic activity and proteolytic receptor cleavage after a meal in controls and type 2 diabetics." The dissertation author was the secondary investigator and secondary author of the material submitted for publication. The dissertation author ran protease activity assays and receptor cleavage assays, composed the first figure of the material submitted for publication, and added text to this chapter not found in the material submitted for publication.

Chapter 3, in part, is currently being prepared for submission for publication of the material. Jamie Joseph, Elaine A. Skowronski, Augusta E. Modestino, Geert W. Schmid-Schönbein, Michael J. Heller, Colin A. Depp, Dilip V. Jeste. "Pilot study of circulating

proteolytic activity and proteolytic receptor cleavage in schizophrenia and comparison groups." The dissertation author was the secondary investigator and secondary author of the material being prepared for submission for publication. The dissertation author designed and ran protease activity assays and receptor cleavage assays, analyzed data, and composed the text and figures of this chapter.

Chapter 4, in part, is currently being prepared for submission for publication of the material. Elaine A. Skowronski, Michael J. Heller, Shelley M. Lawrence. "Associations of circulating proteolytic activity in neonates and length of stay in the neonatal intensive care unit." The dissertation author is the primary investigator and author of this material.

VITA

2007	Bachelor of Arts in Chemistry, Wellesley College
2014	Master of Science in NanoEngineering, University of California, San Diego
2015	Research Intern, The Scripps Research Institute
2015–2016	Teaching Assistant, Department of NanoEngineering, University of California, San Diego
2016	microMBA, University of California, San Diego
2014–2017	Graduate Student Researcher, Department of NanoEngineering, University of California, San Diego
2016–2017	Lab Safety Manager, Department of NanoEngineering, University of California, San Diego
2017	Doctor of Philosophy in NanoEngineering, University of California, San Diego

PUBLICATIONS

Modestino AE, **Skowronski EA**, Pruitt C, Herbst KL, Schmid-Schönbein GW, Heller MJ, Mills PJ. Pilot Study: Evidence for increased proteolytic activity and proteolytic receptor cleavage after a meal in controls and type 2 diabetics. Diabetes Journal 2017 (***submitted***)

Sonnenberg A, Marciniak JY, **Skowronski EA**, Manouchehri S, Rassenti L, Ghia EM, Widhopf GF, Kipps TJ, Heller MJ. Dielectrophoretic isolation and detection of cancer-related circulating cell-free DNA biomarkers from blood and plasma. Electrophoresis. 2014 Jul 1;35(12-13):1828-36.

Sonnenberg A, Marciniak JY, Rassenti L, Ghia EM, **Skowronski EA**, Manouchehri S, McCanna J, Widhopf GF, Kipps TJ, Heller MJ. Rapid electrokinetic isolation of cancer-related circulating cell-free DNA directly from blood. Clinical chemistry. 2014 Mar 1;60(3):500-9.

PATENTS

Heller MJ, **Skowronski EA**, Modestino AE, Schmid-Schönbein GW, Leiterer C; Regents of The University of California, assignee. Detection, identification, and purification of degradative and non-degradative enzymes in biological samples. US provisional patent application 62/406,781. 2016 Oct 11.

Heller MJ, **Skowronski E**, Song Y, Warner J, Chen S, inventors; Regents of The University of California, assignee. DNA double-write/double binding identity. World patent application WO WO2015017694 A1. 2015 Feb 5.

ABSTRACT OF THE DISSERTATION

Charge-Changing Substrates for Degradative Enzyme Activity Detection in Whole Blood

by

Elaine Alexandra Skowronski

Doctor of Philosophy in NanoEngineering

University of California, San Diego, 2017

Professor Michael J. Heller, Chair

Degradative enzyme activity has been shown to be elevated in many diseases including shock, diabetes, schizophrenia, several types of cancers, and coagulation disorders. The ability to measure degradative enzyme activity in whole blood and other complex samples would be useful for the development of rapid clinical diagnostics, the

elucidation of disease progression, and the design of therapeutics. Current techniques to measure degradative enzyme activity require considerable amounts of time and sample processing steps. In this dissertation, a novel method for rapid detection of degradative enzyme activity, specifically protease activity, in whole blood is applied to several disease states: diabetes, schizophrenia, and premature neonates.

Chapter 1

Introduction: Degradative enzymes and their analyses in biological fluids

Elaine A. Skowronski[a]

[a]*NanoEngineering Department, University of California, San Diego*

Abstract

Degradative enzymes are critical to the physiological homeostasis of a healthy organism. Deregulation of the activity of degradative enzymes is associated with a variety of diseases. Two important classes of degradative enzymes are proteases and lipases. Proteases degrade proteins by cleaving peptide bonds, whereas lipases degrade lipids by cleaving ester bonds. Deregulation of the activity more than the concentration of these enzymes is indicative of a diseased state. Available methods for detecting activity of proteases and lipases require sample processing and are unable to be deployed at the point-of-care. This dissertation reports the use of novel charge-changing substrates for the detection of protease and lipase activity in whole blood without the need for sample preparation.

1.1 Degradative enzymes

Degradative enzymes comprise classes of enzymes responsible for degrading substances endogenous and exogenous to an organism. Degradative enzymes are involved in numerous physiological and pathological processes. This dissertation will focus on two classes of degradative enzymes: proteases and lipases.

1.2 Proteases

Proteases are the efficient nanomachines responsible for the hydrolysis of peptide bonds and therefore the degradation of proteins. Most proteolytic enzymes cleave α-peptide bonds between naturally occurring amino acids [1]. There are some notable exceptions, but these proteolytic enzymes are beyond the scope of this dissertation. According to the Degradome Database, over 500 human proteases exist. Thousands more proteases are found in animals, microorganisms, and plants [26].

1.2.1 Significance of proteases

Proteases are involved in many physiological and pathological processes. Any deregulation of proteolytic enzyme activity may lead to a variety of pathological processes, from minor to catastrophic and from chronic to acute. Deregulation of proteolytic enzyme activity has been shown to be a component of a wide range of diseases, from cancer to chronic inflammatory diseases such as rheumatoid arthritis [17]. The association of their activity with disease and their defined structure have made proteases not only attractive objects of study for researchers trying to elucidate the pathophysiology of various diseases but also attractive targets for pharmaceutical companies.

1.2.2 Structure and classification of proteases

Proteases may be divided into six classes according to their catalytic mechanism: aspartic, glutamic, metallo, cysteine, serine, and threonine proteases. Glutamic proteases have not yet been reported in mammals [24]. For the catalytic process of peptide bond hydrolysis, the aspartic, glutamic, and metalloproteinase enzymes use a water molecule as nucleophile while the others (cysteine, serine, and threonine proteases) use an amino acid containing a nucleophilic side chain according to their respective classification name [16]. Metalloproteinases, as the name implies, require a Zn^{2+} ion to polarize a water molecule to function as a nucleophile to catalyze hydrolysis of the scissile peptide bond of the substrate [31].

1.2.3 Regulation of protease expression and activity

Proteolytic activity is tightly regulated *in vivo* by multiple mechanisms. Organisms control these potent enzymes by regulating gene expression, compartmentalizing enzymes in cellular and/or extracellular space, activating inactive precursors, and inhibiting the active enzyme with endogenous inhibitors. Expression of genes encoding proteases is controlled by growth factors, hormones, cytokines, and physical stress [33]. Proteolytic enzymes are synthesized as inactive precursors (also called pro-enzymes or zymogens) to prevent unregulated protein degradation. Removal of a portion of the inactive precursor by limited proteolysis produces the active enzyme and usually occurs within the subcellular compartment or extracellular space in which the active enzyme exerts its biological function [10]. In general, conversion of inactive precursors to active enzymes is irreversible and so organisms possess inhibitors to deactivate the active enzymes [9].

1.2.4 Function of proteases

One of the most important distinguishing features of a protease is its specificity: where it will cleave a substrate protein or peptide. The cleavage specificity of a protease dictates the peptide and protein substrates it is capable of cleaving efficiently and thus determines its role *in vivo*. Depending on their function in an organism, proteases range from highly particular to promiscuous in their cleavage requirements. For example, thrombin is a coagulation protease that is highly specific in its cleavage preferences, which reflects the importance of maintaining homeostasis between bleeding and clotting [7]. On the other hand, the proteases released by the pancreas are promiscuous in their cleavage preferences, reflecting the need of an organism to be able to digest the diversity of proteins it ingests [34].

1.3 Autodigestion hypothesis: proteases

What prevents auto-digestion of the intestine by digestive enzymes? Pancreatic proteases are secreted into the intestine as active enzymes. These proteases are capable of breaking down almost all proteins as a requirement of digestion. The wall of the intestine does not contain sufficient concentrations of endogenous enzyme inhibitors to prevent auto-digestion. Protection of the intestinal wall from these potent digestive enzymes appears to be provided by the barrier of the mucosal epithelium lining the intestinal wall, which compartmentalizes the proteases in the intestinal lumen where they are restricted to digesting only the contents of the intestine [28, 29].

Under normal physiological conditions, the mucosal layer lining the intestinal wall prevents the escape of pancreatic proteases. However, disruption of the mucosal barrier releases the pancreatic proteases into the intestinal wall and the systemic circulation,

leading to organ failure, blood pressure reduction and mortality within hours [11]. While shock is characterized by this catastrophic release of digestive enzymes, various chronic diseases, e.g. hypertension and diabetes, may also be characterized by release of pancreatic proteases but to a milder degree [30].

The unchecked proteolytic activity that reaches the circulation in chronic diseases is much lower than that in shock, but it is sufficient to produce cell dysfunctions. In the spontaneously hypertensive rat (a genetic model of hypertension) proteolytic cleavage of the insulin receptor ectodomain leads to hyperglycemia and insulin resistance [5]. There is a need for sensitive detection of circulating protease activity and further exploration of proteolytic degradation of cell surface receptors in chronic human diseases such as diabetes.

1.4 Analysis of proteases in biological fluids: activity-based assays

Due to the tight regulation of degradative enzyme activity *in vivo*, measurement of enzyme activity is more meaningful than enzyme concentration for the monitoring of biological or pathological processes. There is a need for simple and robust methods for protease activity detection in biological fluids. Activity of degradative enzymes in biological fluids might be analyzed by two main approaches. The first is based on the use of substrates while the second is based on the use of inhibitors. Substrate-based assays usually require radioactive or fluorescent measurements for monitoring the activity of degradative enzymes while inhibitor-based assays make use of synthetic enzyme inhibitors that target the active site and can be used to enrich or tag active degradative enzymes in an activity dependent manner. Assays that study degradative enzyme activity *in vivo* are

outside the scope of this dissertation. Assays that indirectly study degradative enzyme activity by measuring degradation products are also outside the scope of this dissertation.

1.4.1 Substrate-based activity assays

Radiolabeled substrates used to be used before assays that bypassed the need for radioactive material became available. Radiolabeled substrates were incubated with samples and cleavage products were separated from non-digested proteins by protein precipitation [15]. Zymography is a widely used method for matrix metalloproteinase (MMP) analysis. While zymography is a sensitive technique, it is not quantitative and it cannot distinguish between active and inhibited forms of the enzyme due to the denaturation and refolding of the enzyme during the protocol [36]. Activity-dependent ELISA assays are costly and specificity relies on antibody-enzyme recognition [4]. An easy but not very specific assay format relies on proteolytic cleavage of fluorescent peptide substrates (**Figure 1.1A**). The substrate peptides contain a fluorescent group which is quenched. Proteolytic cleavage of the substrate unquenched the fluorophore [23]. This assay is a very useful tool for monitoring the activity of purified proteases but is inappropriate in biological samples that attenuate the fluorescent signal.

1.4.2 Inhibitor-based activity assays

While the success of synthetic protease inhibitors in clinical applications has been limited, analytical techniques based on derivatives of synthetic protease inhibitors have had success [32]. Activity-based probes (ABP) target active proteases in a family-wide manner, for example in biological fluids, before proceeding to downstream analyses. Downstream analyses are necessary to identify which particular proteases within a targeted family were tagged by the ABP strategy [6].

1.5 Novel assay for protease activity detection

Available protease activity detection methods may be rapid and sensitive but only in buffers or after the sample has been processed into plasma or serum. These available methods are not suitable for the direct measurement of protease activity in whole blood because of the strong autofluorescence, background absorption, and light scattering resulting from the various components of blood.

1.5.1 Issue of sample preparation

Protease activity assays generally overcome the fundamental limitation of blood quenching or obscuring label signals by performing various sample preparation steps, including centrifugation, dilution, and the addition of anticoagulants and other additives. These processing steps alter the concentrations and activities of proteases in blood, reducing the likelihood of the assay reflecting the physiological state of the sampled patient (**Figure 1.2**).

Sample preparation also increases the time, cost, labor requirements and overall complexity of the assay (**Figure 1.2**). The need to draw milliliters of blood to process into plasma precludes these assays from any POC applications. POC tests are generally cheaper, faster, more efficient and provide results for routine analysis in minutes, while tests carried out in central clinical laboratories require many hours or, in some cases, days before a result is available. It is important to eliminate sample preparation and to measure protease activity directly in a whole blood sample to enable timely treatment decisions, especially in time-critical medical situations.

1.5.2 Whole blood assay for protease activity detection

The assays used in this dissertation were developed by Lefkowitz et al. [12–14] and Modestino et al. [21] to bypass sample preparation. The assays are based on the electrophoretic separation of charge-changing peptide substrates from whole blood (**Figure 1.1B**, **Figure 1.3**). Negatively charged or neutral fluorescently-labeled peptide substrates produce positively charged fluorescent products upon cleavage by a target protease after an incubation period in microliters of whole blood. The fluorescent positively charged cleavage product can be separated by application of an electric field from the components of whole blood, the vast majority of which are negatively charged. Thus, this method allows fluorescent signal produced from protease activity to be rapidly resolved from blood and detected.

1.6 Lipases

Lipases are enzymes that degrade fats by hydrolyzing ester bonds. Lipases are interfacial catalysts, rarely acting on substrates in true solution [27].

1.6.1 Significance of lipases

Lipolytic activity results in the release of free fatty acids (FFAs). FFAs are utilized for energy production in the muscle or are re-esterified for storage in the adipose tissue [3]. Hydrolysis of triglycerides (TGs) is essential for energy storage and utilization, whereas variations in TG/FFA levels have a variety of clinical implications that have been extensively reviewed. Deregulation of lipolytic activity is implicated in the invasion of tissues by microorganisms, hyperlipidemia or procoagulant state, and conditions ranging from obesity to cystic fibrosis [25, 35].

1.6.2 Structure and classification of lipases

Lipases can be divided into triacylglycerol lipases and phospholipases. Phospholipase catalyzes the hydrolysis of phospholipids. Phospholipase A catalyzes the hydrolysis of phospholipids to free fatty acid and lysophospholipid; phospholipases A_1 and A_2attack ester bonds in positions 1 and 2 of the phospholipid respectively. Phospholipase B catalyzes the generation of free fatty acid and glycerylphospholipid, and phospholipase C catalyzes the generation of diacylglycerol and a phosphoryl base [2].

The human lipases include the pre-duodenal lingual and gastric lipase and the extra-duodenal pancreatic, hepatic, lipoprotein, and endothelial lipases [22].

1.6.3 Regulation of lipase expression and activity

Whereas numerous exogenous lipase inhibitors have been characterized, mainly representing plant derived components such as saponins, polyphenols, and terpenes, only a limited number of endogenous inhibitors of lipases have been identified [19]. Unlike pancreatic proteases, pancreatic lipases are secreted in their final active form.

1.6.4 Function of lipases

Dietary triglycerides (TGs) contribute a major portion of our daily energy requirement. TGs are digested in the gastrointestinal tract through the action of several lipases including lingual lipase and gastric lipase. However, the majority of dietary TGs are digested in the small intestine by pancreatic lipase (PL) assisted by pancreatic colipase, pancreatic lipase-related protein 2, and carboxyl ester lipase. Lipoprotein lipase (LPL) is the enzyme responsible for controlling plasma TG levels [18].

1.7 Autodigestion hypothesis: lipases

Containment of the digestive process in the lumen of the small intestine requires an intact mucosal barrier. Compromise of this barrier allows leakage of digestive enzymes into the intestinal wall and the circulation, leading to tissue damage termed 'autodigestion.' Lipases generate cytotoxic fragments, e.g. unbound free fatty acids, and the entry of these cytotoxic fragments into the systemic circulation generate multiple cell dysfunctions in other organs [28–30]. More sensitive and specific diagnostic tools to detect lipase activity in patients are required.

1.8 Analysis of lipases in biological fluids: activity-based assays

The available lipase assays have limitations for the determination of lipase activity in biological fluids. The conventional radiometric assay, using ^{3}H- or ^{14}C-labeled trioleoyl glycerol as substrate, requires separation of the released labeled free fatty acids (FFAs), is time-consuming with low throughput, and is limited to research applications due to the environmental concerns associated with the radiolabel [20]. Fluorescence-based assays using coumarin derivatives are attractive because they fluoresce only after conversion to lipolytic products. However, the derivatives are unstable, requiring frequent substrate preparation, and are hydrolyzed nonspecifically by enzymes other than LPL [8]. In addition, these fluorescence-based assays are unable to be performed in whole blood due to the quenching of fluorescent signal by the components of blood. Another commonly used assay, the titrimetric method, is not sufficiently sensitive to be clinically useful [8].

1.9 Novel assay for lipase activity detection

This dissertation reports the development of a novel assay for the detection of lipase activity in whole blood. The simplicity of the assay make it ideal for monitoring of lipase

activity in the clinical setting as well as screening for screening for inhibitors and activators of lipases, thus expediting discovery of drugs of potential clinical value.

1.9.1 Issue of sample preparation

Lipase activity assays generally overcome the fundamental limitation of blood quenching or obscuring label signals by performing various sample preparation steps, including centrifugation, dilution, and the addition of anticoagulants and other additives. These processing steps alter the concentrations and activities of proteases in blood, reducing the likelihood of the assay reflecting the physiological state of the sampled patient (**Figure 1.2**).

Sample preparation also increases the time, cost, labor requirements and overall complexity of the assay (**Figure 1.2**). The need to draw milliliters of blood to process into plasma precludes these assays from any POC applications. POC tests are generally cheaper, faster, more efficient and provide results for routine analysis in minutes, while tests carried out in central clinical laboratories require many hours or, in some cases, days before a result is available. It is important to eliminate sample preparation and to measure protease activity directly in a whole blood sample to enable timely treatment decisions, especially in time-critical medical situations.

1.9.2 Whole blood assay for lipase activity detection

This dissertation reports the development of a novel assay for the detection of lipase activity in whole blood based on charge-changing phospholipid substrates (**Chapter 5**). The fluorescently labeled phospholipid substrates are initially negatively charged and upon cleavage by a target lipase after an incubation period in whole release a positively charge

free fatty acid (FFA) that may be rapidly separated from the negatively charged components of whole blood by the application of an electric field.

1.10 Scope of the dissertation

The overall goal of this dissertation was the design and implementation of innovative techniques for the detection of degradative enzyme activity in complex patient samples. The focus of this dissertation was on *in vitro* instead of *in vivo* methods for enzyme activity measurement as well as on clinical rather than industrial applications of these detection techniques.

Chapter 2 of this dissertation describes the use of protease activity assays based on charge-changing substrates in a pilot study involving diabetic patients, prediabetic patients, and healthy volunteers.

Chapter 3 discusses the use of protease activity assays based on charge-changing substrates in a pilot study involving schizophrenia patients and healthy volunteers.

Chapter 4 describes the use of protease activity assays based on charge-changing substrates in a pilot study involving neonates with various conditions affecting gastrointestinal health and coagulation balance.

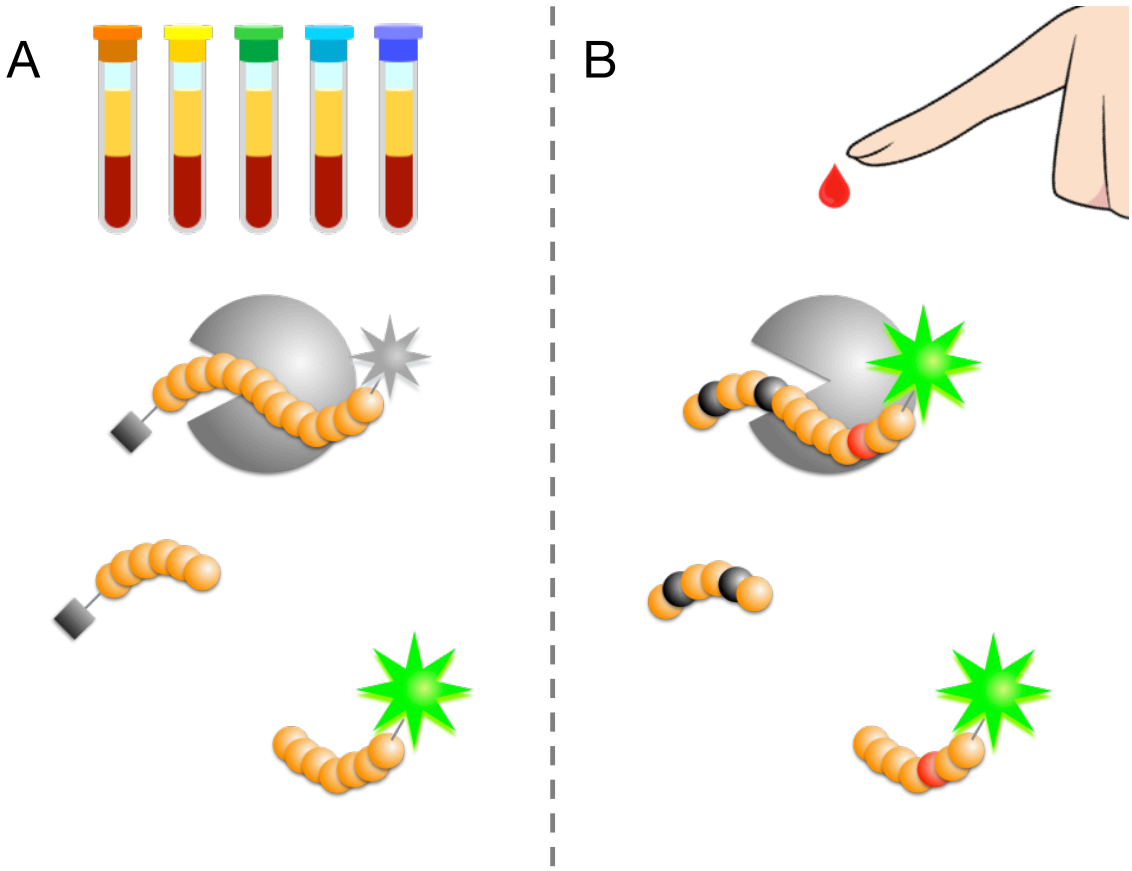


Figure 1.1. Conventional FRET substrates versus novel charge-changing substrates for protease activity detection. (A) FRET substrates for protease activity detection comprise a peptide containing an appropriate cleavage site for the protease of interest, a quencher, and a fluorophore that is quenched until cleavage of the peptide substrate by the protease of interest increases the distance between the quencher and fluorophore. FRET substrates must be run in plasma since whole blood attenuates fluorescence and the need for plasma necessitates milliliters of blood to be drawn, precluding point-of-care applications. **(B)** Charge-changing substrates for protease activity detection comprise a peptide containing an appropriate cleavage site for the protease of interest, charged amino acids or other charged moieties, and a fluorophore or other label. The substrate is initially negatively charged or neutral and upon cleavage produces a positively charged cleavage product with a fluorophore or other label that can be electrophoretically separated from the components of whole blood and detected. Only microliters of whole blood are necessary and so use at the point-of-care is possible.

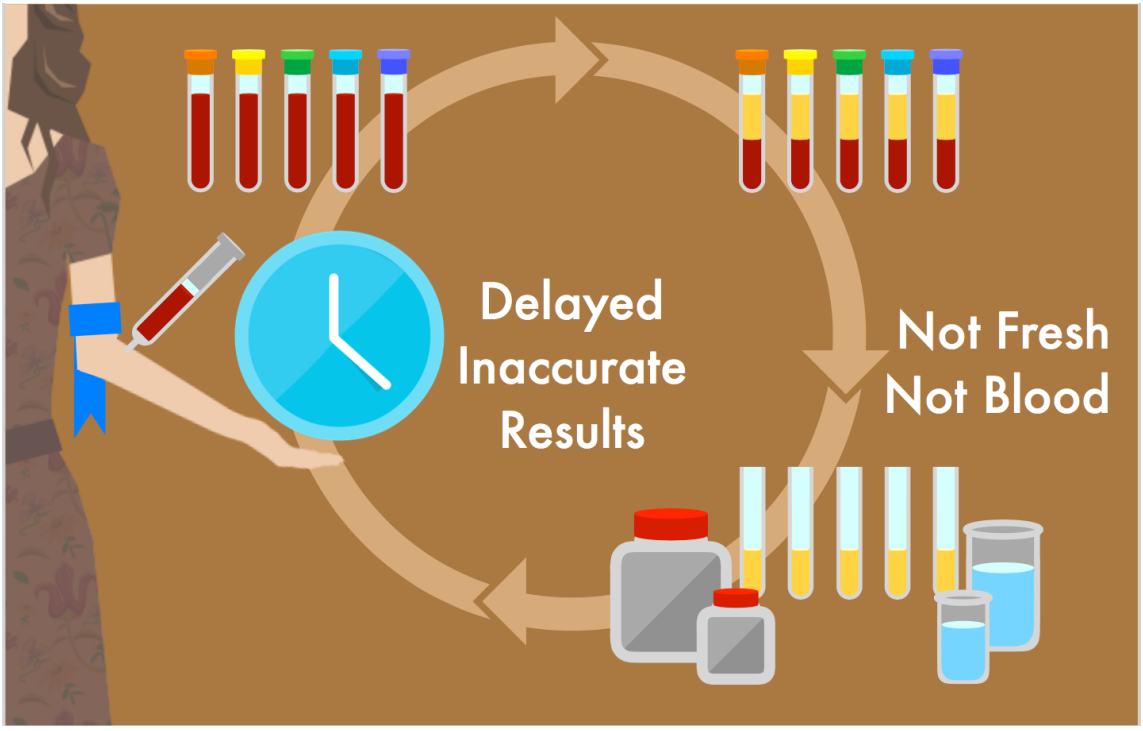


Figure 1.2. Sample processing of conventional activity assays. Conventional assays for the detection of protease or lipase activity are unable to be performed in whole blood due to the components of blood quenching the signal of fluorophores or obscuring other labels. Blood must be centrifuged into plasma, necessitating milliliters of blood to be drawn from a vein, which in turn requires a trained phlebotomist and precludes the development of a point-of-care device for the assay. Blood is not used immediately in assays at the point-of-care and so must be drawn into tubes with anticoagulants before being centrifuged into plasma or allowed to coagulate to produce serum. Plasma or serum may need to be further altered by dilution or additives before use in an assay. These processing steps add time, cost, risk of human error, and manipulation of the sample, frustrating the desire for assays of samples to reflect the true physiological condition of the patient.

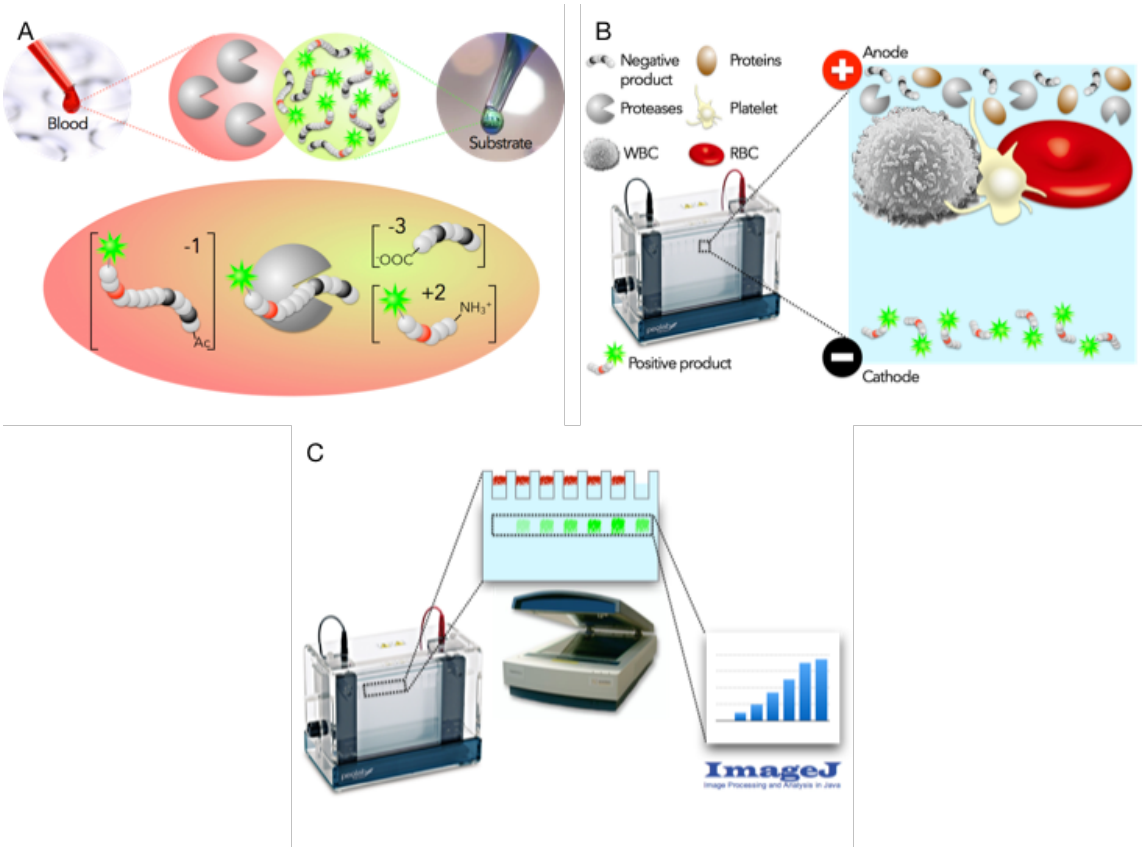


Figure 1.3. Whole blood assay for protease activity detection. (A) Incubation of microliters of whole blood with charge-changing fluorescently labeled peptide substrates designed to be cleaved by the target protease and release a positively charged fluorescently labeled cleavage product. **(B)** Application of an electric field separates the positively charged fluorescently labeled cleavage product from the negatively charged components of blood. Note the reversed polarity of the gel electrophoresis setup. **(C)** Separation by gel electrophoresis of the positively charged fluorescently labeled cleavage product from the negatively charged components of blood concentrates the fluorescent signal for detection. The measured fluorescent signal indicates protease activity.

References

[1] Beynon RJ, Bond JS. Proteolytic enzymes: a practical approach. Oxford: Oxford University Press; 2001.

[2] Borrelli GM, Trono D. Recombinant lipases and phospholipases and their use as biocatalysts for industrial applications. International journal of molecular sciences. 2015 Sep 1;16(9):20774-840.

[3] Carrière F, Renou C, Lopez V, De Caro J, Ferrato F, Lengsfeld H, De Caro A, Laugier R, Verger R. The specific activities of human digestive lipases measured from the in vivo and in vitro lipolysis of test meals. Gastroenterology. 2000 Oct 31;119(4):949-60.

[4] Cheng XC, Fang H, Xu WF. Advances in assays of matrix metalloproteinases (MMPs) and their inhibitors. J Enzyme Inhib Med Chem. 2008 Apr;23(2):154-167. doi: 10.1080/14756360701511292.

[5] DeLano FA, Schmid-Schönbein GW. Proteinase activity and receptor cleavage: mechanism for insulin resistance in the spontaneously hypertensive rat. Hypertension. 2008;52:415–23.

[6] Fonović M, Bogyo M. Activity-based probes as a tool for functional proteomic analysis of proteases. Expert review of proteomics. 2008 Oct 1;5(5):721-30.

[7] Gallwitz M, Enoksson M, Thorpe M, Hellman L. The extended cleavage specificity of human thrombin. PloS one. 2012 Feb 27;7(2):e31756.

[8] Hendrickson HS. Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes. Analytical biochemistry. 1994 May 15;219(1):1-8.

[9] Hibbetts K, Hines B, Williams D. An overview of proteinase inhibitors. Journal of veterinary internal medicine. 1999 Jul 1;13(4):302-8.

[10] Khan AR, James MN. Molecular mechanisms for the conversion of zymogens to active proteolytic enzymes. Protein Science. 1998 Apr 1;7(4):815-36.

[11] Kistler EB, Alsaigh T, Chang M, Schmid-Schönbein GW. Impaired small-bowel barrier integrity in the presence of lumenal pancreatic digestive enzymes leads to circulatory shock. Shock. 2012;38:262–7.

[12] Lefkowitz RB, Marciniak JY, Hu CM, Schmid-Schönbein GW, Heller MJ. An electrophoretic method for the detection of chymotrypsin and trypsin activity directly in whole blood. Electrophoresis. 2010 Jan 1;31(2):403-10.

[13] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. Anal Chem. 2010;82(19):8251-8258. doi: 10.1021/ac101462c.

[14] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for trypsin activity using polyanionic focusing gel electrophoresis. Electrophoresis. 2010 Jul 1;31(14):2442-51.

[15] Lombard C, Saulnier J, Wallach J. Assays of matrix metalloproteinases (MMPs) activities: a review. Biochimie. 2005 Apr 30;87(3):265-72. doi: 10.1016/j.biochi.2005.01.007.

[16] López-Otín C, Bond JS. Proteases: multifunctional enzymes in life and disease. Journal of Biological Chemistry. 2008 Nov 7;283(45):30433-7.

[17] López-Otín C, Matrisian LM. Emerging roles of proteases in tumour suppression. Nature Reviews Cancer. 2007 Oct 1;7(10):800-8.

[18] Lowe ME. The triglyceride lipases of the pancreas. J Lipid Res. 2002 Dec;43:2007-2016. doi: 10.1194/jlr.R200012-JLR200.

[19] Mattijssen F, Alex S, Swarts HJ, Groen AK, van Schothorst EM, Kersten S. Angptl4 serves as an endogenous inhibitor of intestinal lipid digestion. Molecular metabolism. 2014 Apr 30;3(2):135-44.

[20] McCoy MG, Sun GS, Marchadier D, Maugeais C, Glick JM, Rader DJ. Characterization of the lipolytic activity of endothelial lipase. Journal of Lipid Research. 2002 Jun 1;43(6):921-9.

[21] Modestino A, Tyndall M, Yu J, Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Thrombin generation assay in untreated whole human blood. Electrophoresis. 2016 Aug 1;37(15-16):2248-56.

[22] Mukherjee M. Human digestive and metabolic lipases—a brief review. Journal of Molecular Catalysis B: Enzymatic. 2003 Jul 11;22(5):369-76.

[23] Neefjes J, Dantuma NP. Fluorescent probes for proteolysis: tools for drug discovery. Nature Reviews Drug Discovery. 2004 Jan 1;3(1):58-69.

[24] Oda K. New families of carboxyl peptidases: serine-carboxyl peptidases and glutamic peptidases. The Journal of Biochemistry. 2011 Oct 19;151(1):13-25.

[25] Olivecrona G, Olivecrona T. Triglyceride lipases and atherosclerosis. Current opinion in lipidology. 2010 Oct 1;21(5):409-15.

[26] Pérez-Silva JG, Español Y, Velasco G, Quesada V. The Degradome database: expanding roles of mammalian proteases in life and disease. Nucleic acids research. 2015 Nov 8:gkv1201.

[27] Schmid RD, Verger R. Lipases: interfacial enzymes with attractive applications. Angewandte Chemie International Edition. 1998 Jul 3;37(12):1608-33.

[28] Schmid-Schönbein GW. Biomechanical aspects of the auto-digestion theory. Molecular & cellular biomechanics: MCB. 2008 Jun;5(2):83. PMCID: PMC2671552.

[29] Schmid-Schönbein GW, Chang M. The autodigestion hypothesis for shock and multi-organ failure. Annals of biomedical engineering. 2014 Feb 1;42(2):405-14. PMCID: PMC3943906.

[30] Schmid-Schönbein GW, Penn A, Kistler E. The autodigestion hypothesis in shock and multi-organ failure: degrading protease activity. Bol Soc Port Hemorreol Microcirc. 2011 Jul;26(3):6-15. PMCID: PMC4180238.

[31] Tallant C, Marrero A, Gomis-Rüth FX. Matrix metalloproteinases: fold and function of their catalytic domains. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research. 2010 Jan 31;1803(1):20-8.

[32] Turk B. Targeting proteases: successes, failures and future prospects. Nature reviews Drug discovery. 2006 Sep 1;5(9):785-99.

[33] Verbovšek U, Motaln H, Rotter A, Atai NA, Gruden K, Van Noorden CJ, Lah TT. Expression analysis of all protease genes reveals cathepsin K to be overexpressed in glioblastoma. PloS one. 2014 Oct 30;9(10):e111819.

[34] Wang B, Malik R, Nigg EA, Körner R. Evaluation of the low-specificity protease elastase for large-scale phosphoproteome analysis. Analytical chemistry. 2008 Nov 14;80(24):9526-33.

[35] Wang H, Eckel RH. Lipoprotein lipase: from gene to obesity. American Journal of Physiology-Endocrinology and Metabolism. 2009 Aug 1;297(2):E271-88.

[36] Wilkesman J, Kurz L. Protease analysis by zymography: a review on techniques and patents. Recent patents on biotechnology. 2009 Nov 1;3(3):175-84.

Chapter 2

Pilot Study: Evidence for increased proteolytic activity and proteolytic receptor cleavage after a meal in controls and type 2 diabetics

Augusta E. Modestino,[a] Elaine A. Skowronski,[b] Chris Pruitt,[c] Karen L. Herbst,[d] Geert W. Schmid-Schönbein,[a] Michael J. Heller,[a,b] Paul J. Mills[c]

[a]*Bioengineering Department, University of California, San Diego.*

[b]*NanoEngineering Department, University of California, San Diego*

[c]*Department of Family Medicine and Public Health, University of California, San Diego.*

[d]*Department of Medicine, University of Arizona Health Sciences.*

Abstract

Protease activity and intestinal permeability have been identified as contributing to the pathogenesis of insulin resistance in type 2 diabetes mellitus (T2DM). In this study we investigate protease activity and insulin receptor cleavage in T2DM, and the effect of a single high-calorie, - fat, and -sugar meal on them, as a way to elucidate the relationship between over-nutrition, intestinal permeability, protease activity in circulation, and insulin receptor cleavage. We utilize a new method for detection of protease activities directly in fresh whole human blood samples. Pre-meal activity levels of MMP-2/-9- and elastase-like in the blood of T2DM and pre-T2DM patients were significantly elevated compared to normal volunteers ($p<0.01$). Peripheral blood mononuclear cells (PBMC) incubated with plasma from T2DM and pre-T2DM showed a significant lowered density of insulin receptor, when compared to PBMCs incubated with control plasma. No significant difference was found in trypsin-like activity, inflammatory markers and soluble adhesion molecules between control, T2DM, and pre-T2DM. An initial postprandial elevation in protease activity, inflammatory biomarkers, and soluble adhesion molecules was observed in control participants. The insulin receptor density in PBMCs incubated with plasma collected from control participants after the meal, showed a significant reduction when compared to the cells incubated with baseline plasma. The results presented in this study suggests that chronic over nutrition produces chronic elevation of proteases in circulation, which in turn may cleave the insulin receptor, and induce insulin resistivity.

2.1 Introduction

Type 2 diabetes mellitus (T2DM) is an inflammatory disorder characterized by hyperglycemia and insulin resistance [1,2]. T2DM requires long-term medical care, posing a significant burden on the healthcare system. Despite the high prevalence of T2DM, a satisfactory molecular mechanism for the development of insulin resistance has not yet been defined [3]. This incomplete understanding limits the development of more efficient treatment and prevention strategies. In this study we investigate the effect of a meal on circulatory proteolytic activity and on insulin receptor ectodomain density of peripheral mononuclear blood cells (PBMCs) after incubation with plasma collected before and after the meal. The overall aim of this pilot study is to determine if proteases link over-nutrition with the development of insulin resistance in T2DM.

Unchecked protease activity contributes to the development of insulin resistance. In the spontaneous hypertensive rat, proteolytic cleavage of the extracellular domain of the insulin receptor leads to insulin resistance [3]. Enteral blockade of digestive proteases prevents acute insulin resistance after hemorrhagic shock [4]. Other studies have demonstrated elevation of soluble insulin receptor in the plasma of T2DM patients [5], and the proposed mechanism for insulin receptor cleavage is the proteolytic action of a calcium-dependent protease [6].

Increased intestinal permeability may increase proteolytic activity by allowing powerful digestive enzymes to leak out of the intestinal lumen into the systemic circulation. In T2DM, the intestinal barrier has a mild but chronic increase in permeability [7]. Chronic over-consumption of a high-fat, high-sugar diet is the main risk

factor for the development of T2DM; this diet is also believed to increase the permeability of the intestinal mucosa [8]. Thus we hypothesize that T2DM patients have increased proteolytic activity in circulation due to an increased intestinal permeability; this increase in proteolytic activity may trigger insulin resistance by proteolytic cleavage of the insulin receptor.

Despite the link between proteases and insulin resistance, few studies have measured protease activity in the circulation [9]. Previous efforts to study proteases in T2DM have yielded conflicting results and have focused on MMP-2 and MMP-9 levels in plasma. These measurements were carried out under a set of limitations, including the need to isolate blood cells and plasma, low sensitivity, time-consuming steps, and relatively large sample volume.

In this study we used a new electrophoretic technique developed in our laboratory that uses charge-changing fluorescent peptides capable of detecting nanomolar concentrations of active proteases in a single drop of whole blood without the need for sample preparation [10-12]. We measured the activities of the pancreatic proteases, trypsin and elastase, and of the metalloproteases, MMP-2 and -9, in unseparated fresh whole blood of T2DM, p-T2DM and control participants. We examined the effect of a high-calorie, -fat and -sugar meal on protease activity in the circulation to elucidate the relationship between the meal, intestinal permeability, and circulating protease activity. We also measured the insulin receptor density in PBMCs incubated with plasma from T2DM, p-T2DM and control participants collected pre- and post-meal. The overall purpose is to determine if protease activity in the circulation is responsible for clipping

the extracellular domain of the insulin receptor, generating insulin resistance and compromised glucose management. Furthermore, we determined the postprandial levels of serum amyloid-A (SAA), soluble adhesion molecules (sICAM-1 and sVCAM-1) as well as an inflammatory marker, C-reactive Protein (CRP), in T2DM, p-T2DM and control participants over the same time period.

2.2 Results

2.2.1 Study Participants

A total of 34 participants were enrolled and consented in this pilot study, including 14 controls subjects without T2DM, whose measurements served as comparison with the data from participants with T2DM. Four groups were formed: participants with diabetes (T2DM, n=10); with pre-diabetes (p-T2DM, n=10); control (C, n=11); and control that fasted during the whole experiment (Cf, n=3). From the 11 C-group, values for four participants were discarded because their metabolic syndrome index (MSI) was between 3 and 5, and thus were not considered true controls for this study.

No significant differences were found between the baseline values for control and control fasting groups (**Table 2.1**). The body weight was significantly higher in T2DM when compared to control (**Table 2.1**, $p<0.05$,), and to control fasting participants (**Table 2.1**, $p<0.001$), while it was significantly higher for p-T2DM when compared only to Cf (**Table 2.1**, $p<0.05$). The waist circumference and BMI were significantly higher in both T2DM, and p-T2DM when compared to control fasting group (**Table 2.1**, $p<0.05$), but not for the control participants. The fasting glucose levels were significantly higher in T2DM when compared to all other groups (**Table 2.1**, $p<0.001$). The systolic blood

pressure (SBP) was significantly higher in T2DM compared to control (**Table 2.1**, $p<0.05$) and control fasting participants (**Table 2.1**, $p<0.001$). Finally, the MSI was significantly higher for p-T2DM (**Table 2.1**, $p<0.05$) and T2DM (**Table 2.1**, $p<0.001$) compared to control fasting, while only significantly higher for T2DM compared to control participants (**Table 2.1**, $p<0.05$).

2.2.2 Circulating protease activity

Elevated baseline elastase and MMP2/9-like activity in PT2DM and T2DM

We detected elevation in baseline MMP-2/-9-like, and elastase-like activities in T2DM, and p-T2DM, when compared to control (**Figure 2.3A-B**, $p<0.05$), and control fasting participants (**Figure 2.3A-B**, $p<0.001$). While there are differences in the baseline values of the trypsin-like activity between groups, no statistically significant difference between the groups was seen (**Figure 2.3C**).

Calibration curves using the specific fluorescent substrates and spiked proteases were obtained to determine the range of activity observed in each of the groups (**Figure 2.3D-F**). For trypsin the activity detected in circulation ranges between approximately 5.4nM for control fasting and 26.8nM for controls (**Figure 2.3D**); for elastase it ranges from approximately 9.8nM for control fasting, to 37.2nM for T2DM (**Figure 2.3E**); for MMP2/9-activity it ranges from approximately 5.4nM for control fasting to 54.1nM for T2DM (**Figure 2.3F**).

At baseline, for control participants, we detected a strong positive linear correlation between elastase-like activity and age, waist, BMI, and SBP (**Figure 2.8A**, $p<0.05$). MMP-2/-9-like baseline activity had only a strong positive relation with baseline SBP for control participants (**Figure 2.8A**, $p<0.05$). Trypsin-like activity has a strong

positive linear relation with BMI (**Figure 2.8A-B**, $p<0.05$), and moderate positive linear relation with waist circumference (**Figure 2.8A, C**, $p<0.1$).

Initial postprandial increase in circulatory protease activity in control participants

The protease activity in plasma of control participants for MMP-2/9-like, elastase-like, and trypsin-like activities increased significantly already at 15 min after the meal (**Figure 2.4A-C**, $p<0.05$). The T2DM group also showed an increase in elastase-like activity 15 minutes after the meal (**Figure 2.4E**, $p<0.05$). We detected a strong positive correlation between blood glucose during the meal and MMP-2/-9-like activity (**Figure 2.4G**, $p<0.0001$), elastase-like-activity (**Figure 2.4H**, $p<0.0001$), and trypsin-like-activity (**Figure 2.4I**, $p<0.0001$).

Insulin receptor density reduction in PBMC after incubation with plasma

We found that cells incubated with plasma from T2DM, and p-T2DM had significant lower levels of insulin receptor density compared to those incubated with plasma from control participants (**Figure 2.5A**, $p<0.0001$). In addition, there was a significant decrease in insulin receptor density in cells incubated with plasma from all groups compared to cells incubated with 1XPBS (**Figure 2.5A**, $p<0.0001$). Cells incubated with T2DM plasma also showed significant lower levels of insulin receptor density when compared to cells incubated with p-T2DM plasma (**Figure 2.5A**, $p<0.05$).

The insulin receptor density in PBMC incubated with plasma collected at different time points from fasting control participant remained the same throughout the 5-hour observation period (**Figure 2.5B**). In contrast, the insulin receptor density in PBMC incubated plasma collected after a meal from control participants decreased significantly in cells incubated with plasma collected 45minutes after the meal (**Figure 2.5C**, $p<0.01$).

The insulin receptor density in cells of p-T2DM also decreased significantly when they were incubated with plasma collected at 30 and 60minutes after the meal (**Figure 2.5D**, $p<0.01$). For T2DM, although the insulin receptor density decreased in cells incubated with plasma collected 60 minutes after the meal, no statistically significant change was found.

Incubation of PBMC with plasma from control participants 45minutes after the meal caused a decreased in insulin receptor density in PBMC (**Figure 2.9A**), an effect that was larger when the cells were incubated for a longer period of time. We also showed that incubation of PBMC with 5nM of purified trypsin, elastase, and MMP-2 spiked into 1XPBS produced a significant decrease in insulin receptor density on the cell membrane (**Figure 2.9B**).

2.2.3 Inflammatory markers and soluble adhesion molecules

Baseline inflammatory markers and soluble adhesion molecules

Baseline levels of inflammatory markers, and soluble adhesion molecules differed between the groups. The difference in CRP baseline value was only statistically significant between T2DM and control fasting group (**Figure 2.6A**, $p<0.05$); while SAA, sICAM-1, and sVCAM-1 baseline values in p-T2DM and T2DM were significantly higher than in control fasting group (**Figure 2.6A-B**, $p<0.05$).

Initial postprandial increase in circulatory inflammatory markers and soluble adhesion molecules in control participants

Inflammatory markers, and soluble adhesion molecules rose in the circulation of control participants after the meal (**Figure 2.7**). The CRP values increased significantly at times 45 and 60 minutes (**Figure 2.7A**, $p<0.05$), while the SAA values rose significantly

at times 45, 60, and 120 minutes after the meal (**Figure 2.7B**, $p<0.05$). The sICAM-1 values increased significantly at 45 and 120 minutes (**Figure 2.7C**, $p<0.05$), while the sVCAM-1 rose at times 45, 60 and 120 minutes after the meal (**Figure 2.7D**, $p<0.05$). We detected a strong negative correlation between blood glucose and plasma CRP levels (**Figure 2.7I**, $p<0.0001$), SAA (**Figure 2.7J**, $p<0.0001$), sICAM-1 (**Figure 2.7K**, $p<0.0001$), and sVCAM-1 (**Figure 2.7L**, $p<0.0001$).

2.3 Discussion

Digestive enzymes may be driving one mechanism of T2DM development. Chronic over-nutrition is central to the development of T2DM. Intestinal permeability is a key factor in the development of insulin resistance and T2DM [7-8,13]. Inside the lumen of the intestine, powerful pancreatic proteases, including trypsin and elastase, reside in the fully active state and degrade most biomolecules within hours as part of normal digestion. During a meal the concentration of pancreatic proteases increases inside the intestine, accompanied by an increase in intestinal permeability, enabling absorption of important nutrients from the intestine into the bloodstream [14]. We hypothesize that one mechanism leading to glucose sensitivity and T2DM is leakage of digestive proteases from the lumen of the intestine into the peripheral circulation due to chronic over-nutrition. Once these proteases reach the circulation, they can activate other proteases (e.g. metalloproteases) and cleave cellular receptors (e.g. insulin receptor), resulting in reduced insulin sensitivity and compromised glucose management and T2DM.

We detected elevation of baseline values for MMP-2/-9 and elastase activity in the circulation of p-T2DM and T2DM participants. We showed that cells incubated with

plasma from T2DM had a significant decrease in membrane density of the insulin receptor ectodomain. After a meal there is an initial transient elevation of proteases, soluble adhesion molecules, and inflammatory markers in the circulation of control participants, which returns to normal values hours after the meal. We also present evidence of reduced insulin receptor density in cells incubated with plasma from control and p-T2DM after a meal, which links meal consumption with an elevation in protease activity, an increase in inflammatory state, a depletion of insulin receptor, and an increase in blood glucose.

Protease activity in the circulation was measured using our novel electrophoretic assay, which enables protease activity detection in unprocessed whole blood (**Figure 2**). Limited data is available in the literature on plasma levels of proteases in T2DM. Previous efforts to study proteases in T2DM have yielded conflicting results and have focused mainly on MMP-2 and MMP-9 levels in plasma of T2DM. Some reports indicate no correlation between plasma levels of MMP-2 and -9 and T2DM [15]; others show elevated levels of MMP-2 and -9 in T2DM [16-18]; and one reports lower levels of MMP-2 and -9 after an oral glucose tolerance test for T2DM [19]. We believe that these discrepancies are due to the techniques used to measure protease activity and whether they were measured directly in whole blood or after separation of plasma and blood cells. Immunoassays, such as ELISAs, use antibodies that do not distinguish between the active and inactive forms of proteases; moreover, they require treated samples. Manipulation of blood samples introduces the possibility of error and loss of protease activity. The novel electrophoretic assay bypasses these limitations by: 1) detecting protease activity derived

from nanomolar levels of proteases; 2) detecting only active proteases; and 3) working with a small blood sample volume (3.5μL) [10-12].

The baseline elevation of MMP-2/-9 and elastase activity in T2DM (**Figure 2.3A-B**) may be responsible for cleaving the insulin receptor extracellular domain and resulting in the poor blood glucose management of these patients. Proteases, such as elastase, MMP-2 and MMP-9, are capable of not only re-shaping the extracellular matrix [20] and activating other proteases but also cleaving extracellular receptors [21-22]. We show that freshly isolated PBMCs incubated with plasma from T2DM or with purified proteases (MMP-2, elastase, and trypsin) exhibit a significant depletion of their insulin receptor density (**Figure 2.5A** and **2.9**). We cannot rule out that the insulin receptor has been internalized by PBMCs, but the fact that cells incubated with proteases spiked into 1XPBS also showed depletion of the insulin receptor (**Figure 2.9**) suggests that cleavage and not internalization of the insulin receptor is occurring. In addition, other groups have shown an elevation in soluble insulin receptor in T2DM plasma [5-6], and research in animal models suggests that insulin resistance is triggered by proteolytic cleavage of the insulin receptor [3].

A major source of the increase in proteolytic activity in the circulation of T2DM may be the intestine. Chronic over-nutrition is a key driving force in the development of T2DM. We show that a single high-calorie/fat/sugar meal produces a significant initial increase in protease activity after meal consumption in control participants (**Figure 2.4A-C**). The protease activity did not change significantly in control fasting participants (**Figure 2.4A-C**), suggesting that the increase of pancreatic proteases in the circulation is due to digestion. In control participants, the meal also caused an initial increase in soluble

adhesion molecules and inflammatory makers (**Figure 2.7A-D**) and an initial decrease in the insulin receptor density in cells incubated with plasma collected 45 minutes after the meal (**Figure 2.5C**). In contrast to control participants, T2DM had a significant initial increase in only elastase activity after the meal (**Figure 2.4D-F**) and no significant depletion in insulin receptor density (**Figure 2.5E**). T2DM participants are known to have elevated intestinal permeability [7-8] and have been shown by this study to have chronic elevated proteolytic activity in circulation (**Figure 2.3**), which may result in a reduced baseline insulin receptor density, making daily proteolytic activity and insulin receptor density fluctuations less pronounced than in healthy individuals.

The initial elevation in elastase activity observed in T2DM might be caused not only by an increase in pancreatic elastase, which originates in the intestine, but also by neutrophil elastase, which increases in a state of inflammation [23]. In p-T2DM we show a decrease in the insulin receptor density of PBMC incubated with plasma taken 30 and 60 minutes after the meal (**Figure 2.5D**). The measurements of the insulin receptor density were made using plasma from only three of the p-T2DM participants. Even if no significant post-prandial increase in proteolytic activity, inflammatory markers and soluble adhesion molecules was observed in the p-T2DM group (n=10), these three p-T2DM participants showed a significant increase in proteolytic activity after the meal. This increase in postprandial proteolytic activity may be responsible for the decrease in insulin receptor density shown in PBMC and the increase in blood glucose levels (**Figure 2.5D**). The strong positive correlation between protease activity and blood glucose levels (**Figure 2.4G-I**) suggests than an elevation in proteolytic activity is linked to uncontrolled blood glucose levels. In contrast, we find a strong negative correlation

between inflammatory markers and soluble adhesion molecules, implying that these values are a later consequence of the meal and may not be driving forces behind elevated blood glucose in T2DM.

We did not find a significant difference in trypsin activities, inflammation markers, and soluble adhesion molecules in circulation between T2DM and control participants. The relatively high trypsin activity levels found in control participants (**Figure 2.3C**) may be due to their relatively high BMI and waist circumference, which are the only anthropometric characteristics with a positive correlation to trypsin activity values in circulation in controls (**Figure 2.8**). This may help explain why control fasting participants, who on average had the lowest BMI and waist circumference (**Table 2.1**), had the lowest trypsin activity (**Figure 2.3**). In addition, trypsin activity levels were low and close to the detection limit of our electrophoretic assay, making it difficult to identify significant differences in activities between groups. Chronic inflammation is a hallmark of T2DM [2], and studies report elevation of inflammation markers CRP and SAA in T2DM [24-26]. A possible reason for the relatively high levels of CRP and SAA in control participants is their relatively high waist circumference and BMI (**Table 2.1**); high levels of CRP and SAA have been associated with high waist circumference and BMI [27-28]. Soluble adhesion molecules are derived from proteolytic cleavage of the membrane bound adhesion molecule [29]. Though the mechanisms controlling the shedding remain unclear, membrane bound proteases may cleave the extracellular portion of the adhesion molecules, releasing the extracellular fragments into the plasma as “soluble” peptide [29-30]. We expected the higher proteolytic activity found in T2DM to yield higher levels of soluble adhesion molecules. A possible reason for the relatively low

values in soluble adhesion molecules in T2DM may be that chronic elevation of proteolytic activity in circulation leads to further cleaving of the soluble adhesion molecules, degrading their antibody binding sites and preventing detection by the immunological technique used (ELISA).

Future studies will include fasting p-T2DM and T2DM groups and a greater number of participants. A limitation of our novel electrophoretic assay is that although its specificity is high it is not absolute. It would be of interest to measure the insulin receptor in the cells derived from different patients directly, bypassing the need for incubation of cells from a healthy volunteer with patient plasma. Finally, it would of interest to normalize the meal with respect to the BMI of each of the participants as well to test other meals to elucidate the effects of macronutrients on intestinal permeability. The results of this pilot study suggest that blockage of elevated proteolytic activity in circulation may be a therapeutic target for T2DM.

2.4 Conclusion

In conclusion, the results provide new evidence for the hypothesis that the digestive system, and in particular pancreatic proteases, are driving the development of T2DM. Pancreatic proteases, which are normally compartmentalized inside the intestinal lumen, may be reaching the circulation due to an increase in intestinal permeability after a meal. In the systemic circulation pancreatic proteases can degrade biomolecules. In this report we showed that proteases could be responsible for the reduction of the ectodomain of the insulin receptor in cells, impairing glucose control. Many membrane receptors have extracellular domains that may be subject to cleavage by proteases, which could be the reason for the diversity of cell dysfunctions that accompany T2DM.

2.5 Materials and Methods

2.5.1 Sample acquisition

Samples were collected from 10 T2DM, 10 pre-T2DM and 14 normal patients (11 received meal, and 3 fasted). Volunteers participated in the study after giving informed written consent according to the guidelines of the Review Board for Human Research Protections Program at the University of California, San Diego. The volunteers fasted for a minimum of 12 hours. An intravenous catheter was placed in the antecubital vein of the non-dominant arm. Blood was withdrawn into collection tubes containing lithium heparin and EDTA (Becton Dickinson). An initial blood sample was collected to serve as a baseline level. Anthropomorphic measurements, general information on the subjects, as well as diet and exercise data were collected (**Table 2.1**).

2.5.2 Meal tolerance test study design

A McDonald's meal consisting of hot chocolate, orange juice, hash browns, and egg McMuffins with a total of 1,680 calories was served to the volunteers. Blood samples were collected postprandially at 15, 30, 45, 60, 120, 180, 240 and 300 minutes. Blood was drawn in 4mL lithium heparin tubes (60 USP units of lithium heparin, Becton, Dickinson and Company Medical Supplies), stored at 4^{o}C, and used to measure protease activity within two hours. An additional blood sample was drawn into an EDTA tube for plasma generation and measurement of inflammatory markers, soluble adhesion molecules, and glucose concentration.

2.5.3 Metabolic syndrome index (MSI)

In order to define a control population we used an adaptation of the proposed Metabolic Syndrome Index (MSI). This proposed MSI is a five-point scale that assigns

one point to each of the five metabolic syndrome components: waist circumference, blood pressure, triglycerides, high-density lipoprotein (HDL) cholesterol, and fasting blood glucose. Here we adapted the index with the measurements that we had collected for all participants and created a five-point MSI, which assigned one point for each of the following components: waist circumference, BMI, SBP, DBP, and fasting blood glucose. The MSI value is the sum of the scores assigned to each of the four components (**Figure 2.1**). With a score between 3 and 5, four control participants were discarded from the analysis of the study.

2.5.4 Protease activity assays

Activities of MMP-2 and -9, elastase, and trypsin were measured in fresh whole blood samples of volunteers using the charge-changing protease substrates and electrophoretic method developed by our lab [10-12]. This method uses synthetic fluorescent peptides that are specifically cleaved by a protease; the peptides are originally negatively or neutrally charged; upon cleavage by the protease of interest, two fragments are produced, a negative one and a fluorescently labeled positive one, which allows for electrophoretic separation and detection in whole blood with no sample preparation (**Figure 2.2**). This method has been described in detail [10-12].

2.5.5 Insulin receptor labeling

Freshly isolated PBMC cells from a healthy volunteer were incubated with plasma from control fasting, control, p-T2DM and T2DM participants collected at different time points after the meal, or with spiked proteases (elastase, MMP-2, and trypsin). A 50μL PBMC suspension and 150μL plasma solution were incubated for 1 hour at 37^{o}C. The cells were then spread as a blood smear, fixed (10% formalin, neutral

buffered) and labeled with a primary antibody against the extracellular domain of the insulin receptor (sc-710 polyclonal antibody mapping to the N-terminus, Santa Cruz Biotech) followed by the biotin/avidin Vector TMB peroxidase enzyme substrate. The cell images were collected using a bright field microscope (40X and 60X objective) and digitally analyzed (NIH Image J 1.440) to determine light absorption as a measure of the surface density of the insulin receptor extracellular domain.

2.5.6 Inflammatory markers and soluble adhesion molecules

Whole blood was preserved with EDTA. Following centrifugation at 4 ^{o}C, plasma was stored at -80 ^{o}C until assayed. Circulating levels of C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular cell adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were determined by commercial ELISA (MSD, Rockville, MD). Intra- and inter-assay coefficients were <5%.

2.5.7 Statistical analysis

All measurements are presented as mean ± standard error (SEM). Differences in individual variables during the meal tolerance experiment were analyzed by one-way repeated measurements ANOVA significance (F value for differences across the 6 time points) with paired t-tests where a significant difference ($p < 0.05$) was found. Paired t-tests were used for between group comparisons of the variables. Correlations were analyzed using Pearson linear correlation coefficients.

Acknowledgments

Funding was provided by a CTRI Translational Research Pilot Award.

Chapter 2, in part, has been submitted for publication of the material as it may appear in Diabetes Journal, 2017, Augusta E. Modestino, Elaine A. Skowronski, Chris

Pruitt, Karen L. Herbst, Geert W. Schmid-Schönbein, Michael J. Heller, Paul J. Mills. "Pilot Study: Evidence for increased proteolytic activity and proteolytic receptor cleavage after a meal in controls and type 2 diabetics." The dissertation author was the secondary investigator and secondary author of the material submitted for publication. The dissertation author ran protease activity assays and receptor cleavage assays, composed the Figure 2.2 of the material submitted for publication, and added text to this chapter not found in the material submitted for publication.

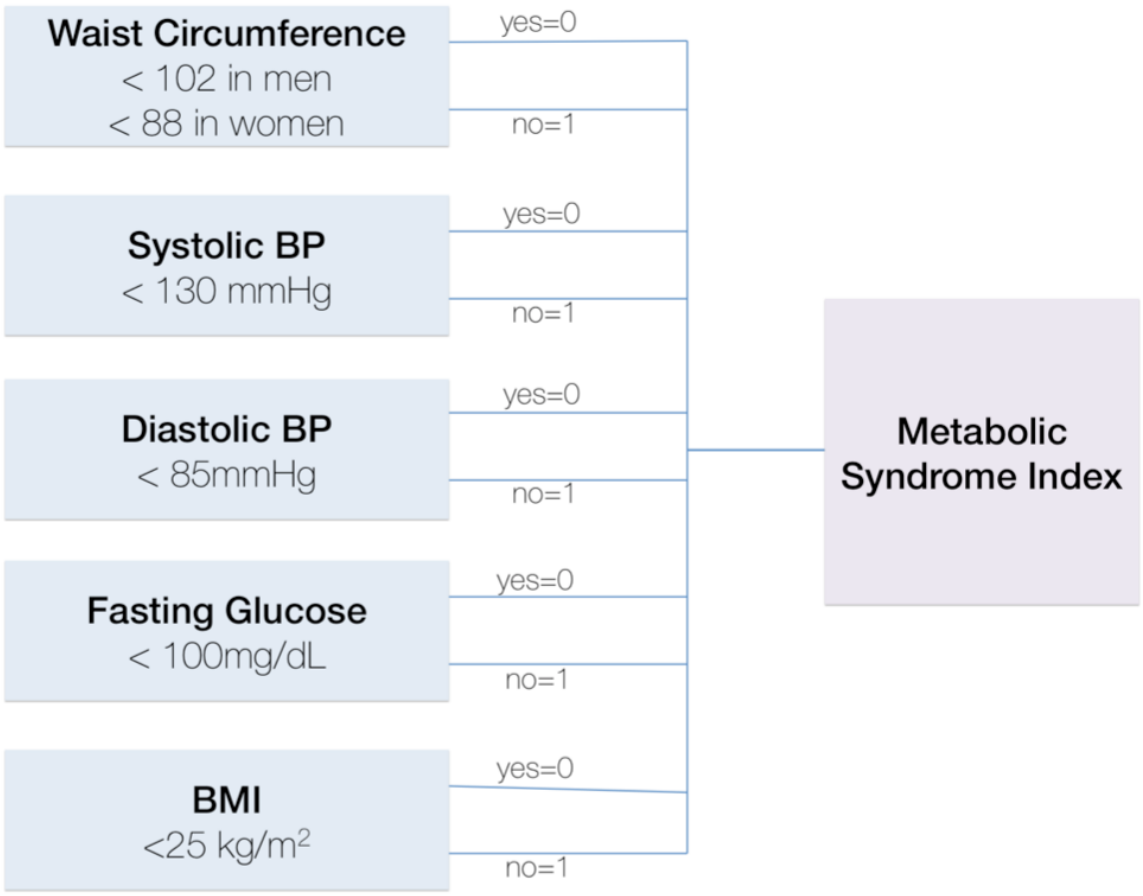


Figure 2.1. Metabolic syndrome index (MSI) calculation flow-chart.

A. Target Protease	Charge-Changing Fluorescent Sequences and Cleavage Site	Charge	
		Substrate	C- Product
Trypsin	Ac-N-DGDAGR // AGAGK(ε-BFL)-NH_2	-1	+1
Elastase	Ac-N-DA//G//SV//A//G//A//GK(ε-BFL)-NH_2	-1	+1
MMP-2/9	Ac-NGDPVGL//TAGAGK(ε-BFL)-NH_2	-1	+1

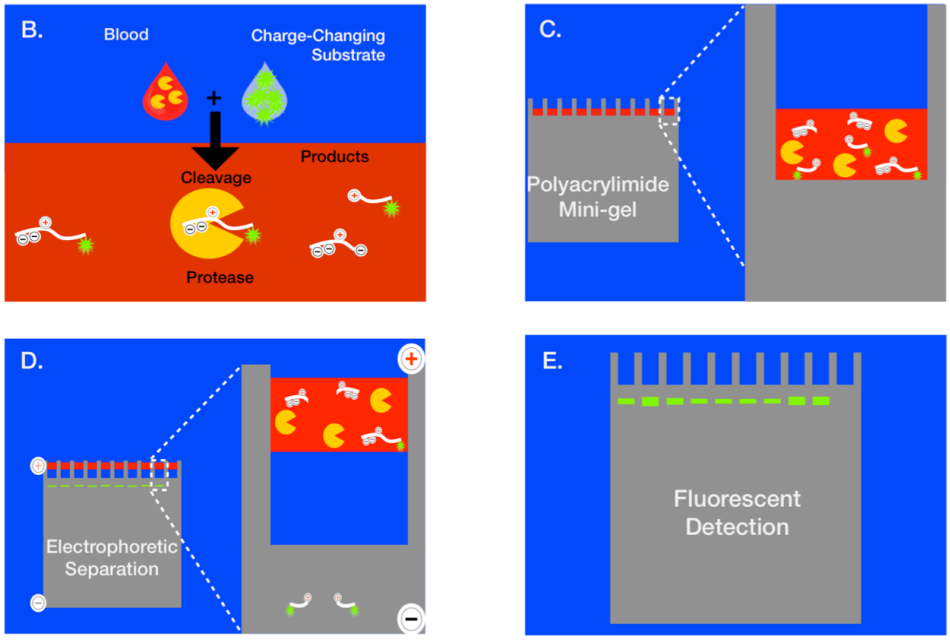


Figure 2.2. Protease activity detection. (A) Charge-changing fluorescently labeled substrate sequences and cleavage sites. **(B-E)** Novel electrophoretic assay for protease activity detection. **(B)** 4µL of whole blood is combined with 4µL of charge-changing substrate designed to be cleaved by a target protease. **(C)** After a 30 min reaction, 6µL aliquots of reaction mixture are loaded into a polyacrylamide mini-gel. **(D)** Gel is run at 500V for 10 minutes **(E)** Detection of fluorescence is carried out in a fluorescence scanner.

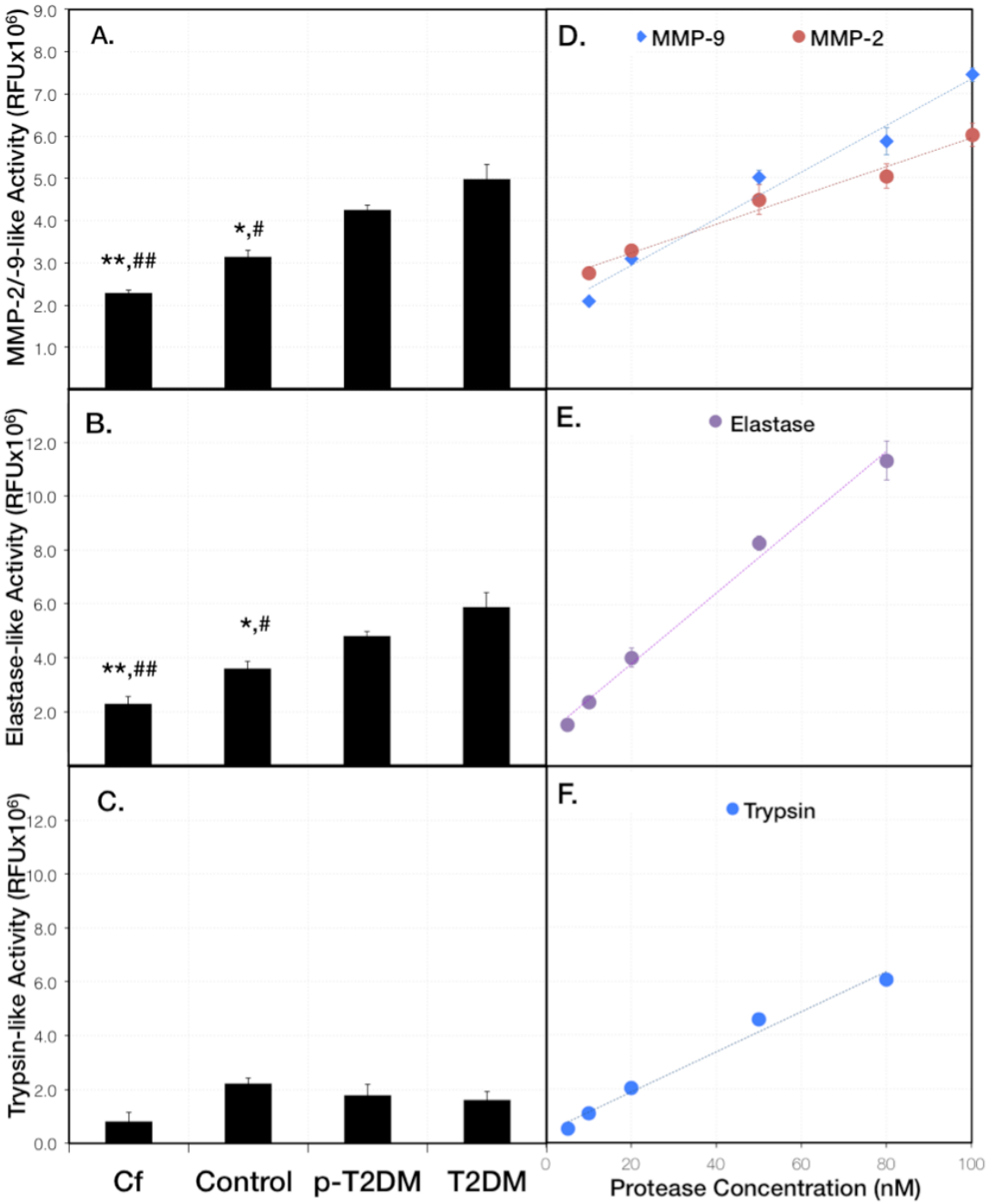


Figure 2.3. Protease baseline activity. (A-C) Mean baseline activity for **(A)** trypsin, **(B)** elastase, and **(C)** MMP-2/9-like activities. Activities were measured after mixing plasma with charge-changing peptide substrates for each protease followed by electrophoresis and fluorescent imaging (see Methods section). **p<0.05, **p<0.001 versus T2DM and #p<0.05, ##p<0.001 versus p-T2DM.* **(D-F)** Calibration curves in 1X PBS spiked with different concentrations of **(D)** trypsin, **(E)** elastase, and **(F)** MMP-2 and MMP-9.

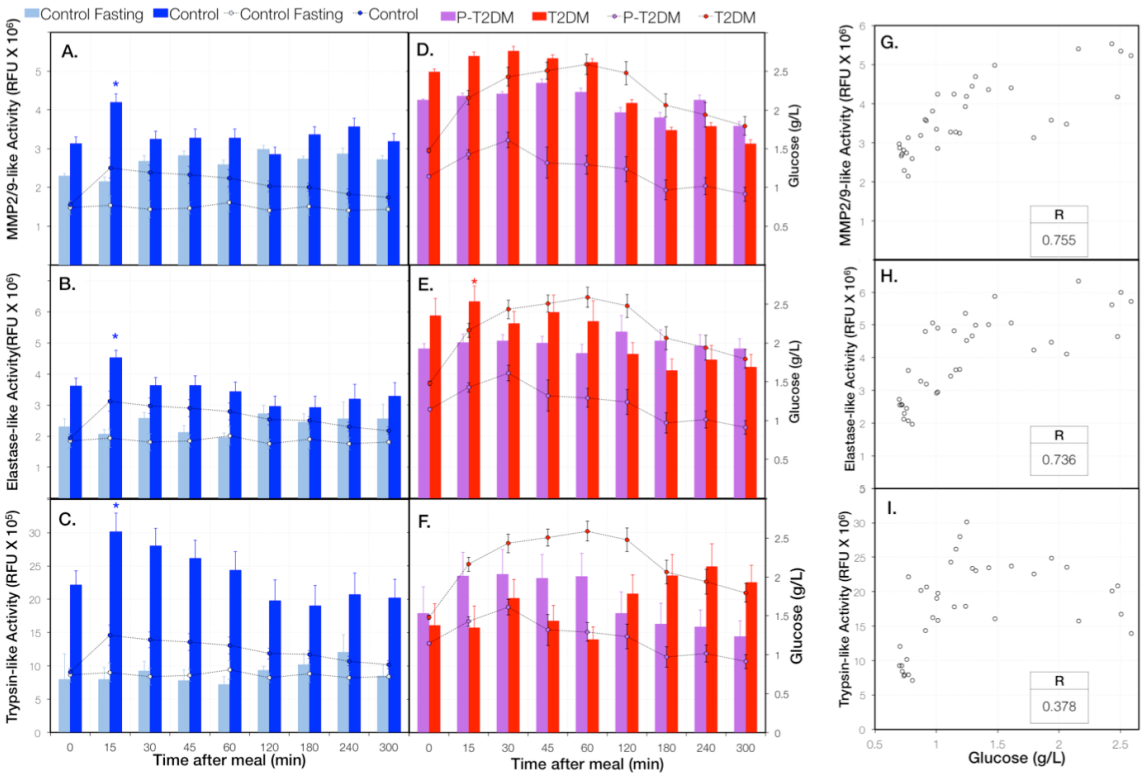


Figure 2.4. Postprandial protease activity and glucose levels in circulation. (A) Level of MMP-2/9-like activity, **(B)** elastase-like activity, and **(C)** trypsin-like activity in control and control fasting participants after a meal. **(D)** Level of MMP-2/9-like activity, **(E)** elastase-like activity, and **(F)** trypsin-like activity in pre-T2DM and T2DM after a meal. Activities were measured after mixing plasma with charge-changing peptide substrates for each protease followed by electrophoresis and fluorescent imaging (see Methods section). **p<0.05 versus time 0 within group.* **(G-I)** Linear correlation of blood glucose levels and **(G)** MMP-2/9-like activity (*p<0.0001*), **(H)** elastase-like activity (*p<0.0001*), and **(I)** trypsin-like activity (*p<0.01*).

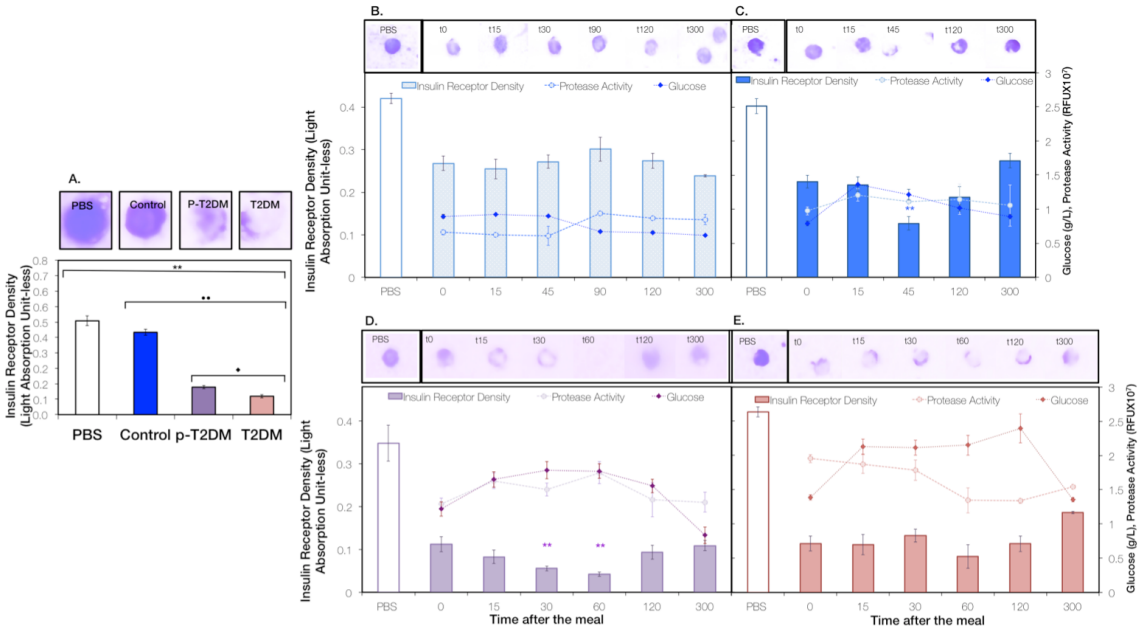


Figure 2.5. Insulin receptor density in PBMC incubated with plasma. (A) Ectodomain insulin receptor density in PBMC incubated with PBS and baseline plasma from control, p-T2DM, and T2DM. ***,••$p<0.01$*. Incubation with 1X PBS versus control, p-T2DM, and T2DM plasma; ••$p<0.01$ for control plasma versus incubation with p-T2DM and T2DM plasma; •$p<0.05$ for incubation with p-T2DM plasma when compared to cells incubated with T2DM plasma. **(B-E)** Density of insulin receptor label after incubation with plasma samples at different times after a meal and with 1X PBS as control. **(B)** Incubation with plasma from control fasting (n=2), **(C)** with plasma from control (n=3), ***$p<0.01$ versus time 0*, **(D)** with plasma from p-T2DM (n=3), ***$p<0.01$ versus time 0*, and **(E)** plasma from T2DM (n=3).

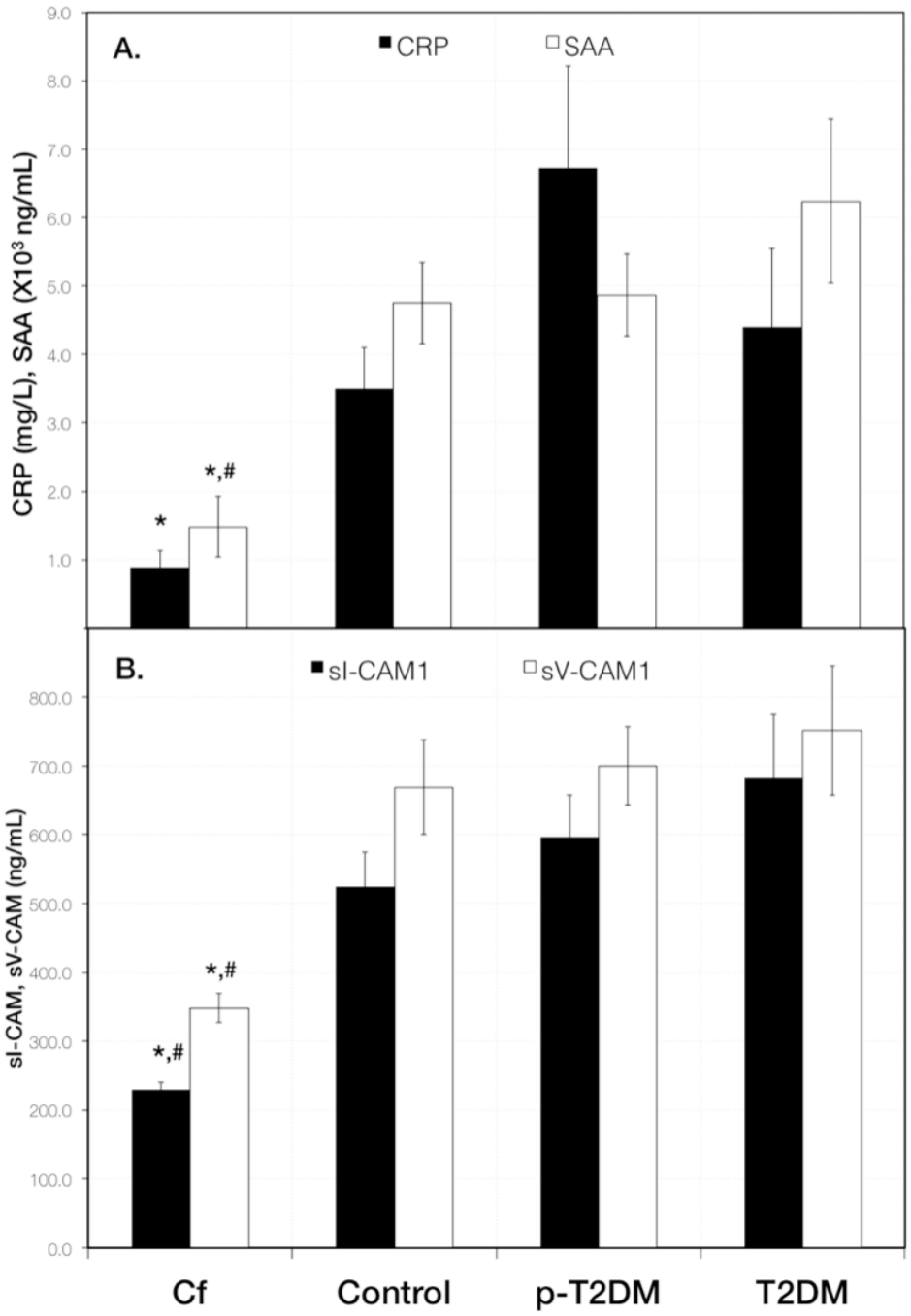


Figure 2.6. Baseline values for inflammation markers and soluble adhesion molecules mean baseline (A) CRP and SAA **(B)** sI-CAM1 and sV-CAM1 in each group. **p<0.05 versus T2DM and #p<0.05 versus p-T2DM.*

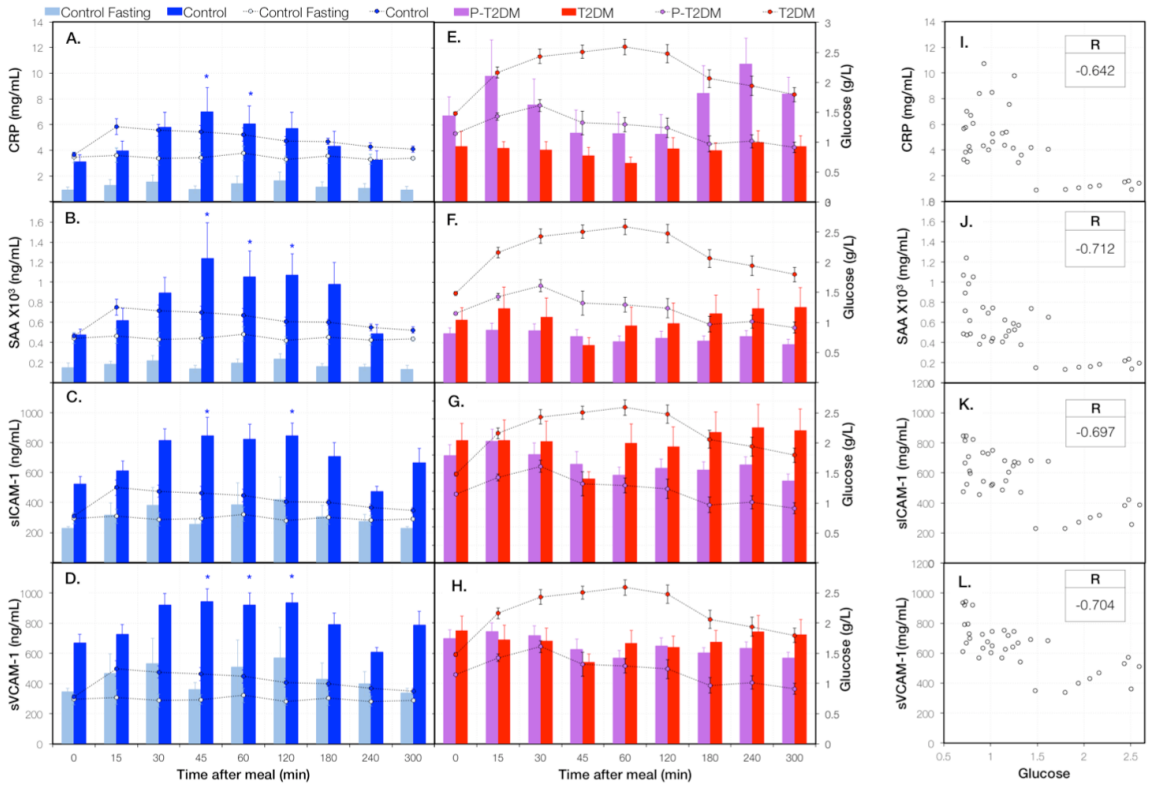


Figure 2.7. Postprandial inflammation marker levels (A) CRP, **(B)** SAA, **(C)** sICAM-1 and **(D)** sVCAM-1 levels in control and control fasting. **(E)** CRP, **(F)** SAA, **(G)** sICAM-1 and **(H)** sVCAM-1 levels in p-T2DM and T2DM after a meal for 5 hours; **p<0.05 when compared to time 0 for control participants*. **(I-L)** Linear correlation of blood glucose levels and **(I)** CRP (*p<0.0001*) **(J)** SAA (*p<0.0001*), **(K)** sICAM-1 (*p<0.0001*), and **(L)** sVCAM-1 (*p<0.0001*).

A.

	Pearson Correlation Coefficient Value				
	Age	Waist	BMI	DBP	SBP
MMP-2/9	0.45	0.46	0.39	0.29	0.72*
Elastase	0.65**	0.73**	0.68**	-0.01	0.65**
Trypsin	0.10	0.58*	0.65**	-0.33	0.04

Figure 2.8. Linear correlations of baseline protease activity levels and anthropometric characteristics of participants. (A) Table with Pearson correlation coefficient values for baseline protease activity values and baseline anthropometric characteristics of control participants. **(B-C)** Linear correlation of baseline trypsin activity values with **(B)** BMI and **(C)** waist circumference for control participants. **p<0.1* and ***p<0.05* Pearson correlation coefficient.

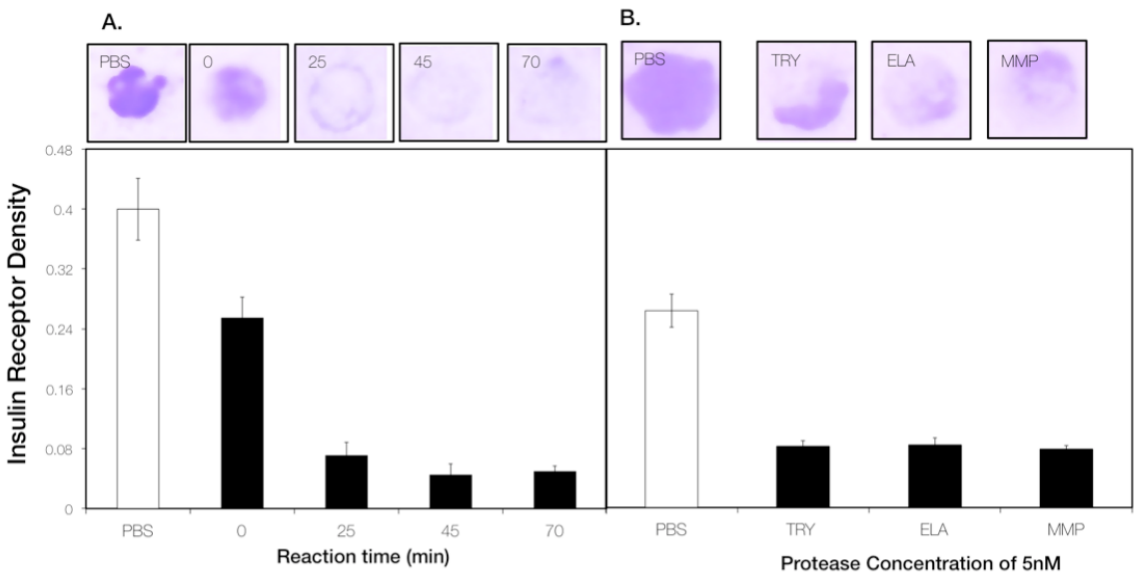


Figure 2.9. Insulin receptor density changes with reaction time and protease concentration. (A) Insulin receptor density depletion over reaction time with plasma from control participants collected 45 minutes after meal consumption. **(B)** Insulin receptor density after incubation with 1X PBS and 5nM of trypsin, elastase, and MMP-2 enzyme spiked in 1X PBS.

Table 2.1. Baseline characteristics of study participants. (mean±SEM)

Characteristics	Control	p-T2DM	T2DM	Control Fasting
Sample Size	7	10	10	3
Age (years)	53.0 ± 5.6	57.4±4.0	57±4.4	44±2.6
Females (%)	5 (0.71)	6 (0.6)	6 (0.6)	1
Weight (kg)	78.5±6.8*	86.6±6.7	99.6±7.4	56.3±1.3**,#
Waist (cm)	98.88±6.0	102±5.5	112.2±5.2	81.9±3.2*,#
BMI (kg/m^2)	29.1±2.5	31.76±2.5	33.4±2.8	21.5±0.62*,#
Fasting Glucose (mg/dL)	77.6±3.4**	114.1±1.8**	147.9±5.4	73.9±4.8**,##
SBP (mmHg)	118.0±4.7*	129.0±1.25	133.0±2.9	108.0±0.9**
DBP (mmHg)	69.4±1.5	71.3±4.6	75.7±3.0	73.3±1.1
MSI	1.28±0.3*	2.22±0.5*	3.60±0.2	0.33±0.2**,#
Race and Ethnicity				
Non Hispanic White	10 (0.91)	9 (0.9)	10 (1)	3 (1)
Hispanic	1 (0.09)	1 (0.1)	0 (0)	0 (0)

References

[1] Muoio DM, Newgard CB. Molecular and metabolic mechanisms of insulin resistance and β-cell failure in type 2 diabetes. Nature reviews Molecular cell biology. 2008 Mar 1;9(3):193-205.

[2] Donath MY, Shoelson SE. Type 2 diabetes as an inflammatory disease. Nature Reviews Immunology. 2011 Feb 1;11(2):98-107.

[3] DeLano FA, Zhang H, Tran EE, Zhang C, Schmid-Schönbein GW. New hypothesis for insulin resistance in hypertension due to receptor cleavage. Expert review of endocrinology & metabolism. 2010 Jan 1;5(1):149-58.

[4] DeLano FA, Schmid-Schönbein GW. Pancreatic digestive enzyme blockade in the small intestine prevents insulin resistance in hemorrhagic shock. Shock (Augusta, Ga.). 2014 Jan;41(1).

[5] Umehara A, Nishioka M, Obata T, Ebina Y, Shiota H, Hashida S. A novel ultra-sensitive enzyme immunoassay for soluble human insulin receptor ectodomain and its measurement in urine from healthy subjects and patients with diabetes mellitus. Clinical biochemistry. 2009 Sep 30;42(13):1468-75.

[6] Yuasa T, Amo K, Ishikura S, Nagaya H, Uchiyama K, Hashida S, Ebina Y. Development of in vitro model of insulin receptor cleavage induced by high glucose in HepG2 cells. Biochemical and biophysical research communications. 2014 Feb 28;445(1):236-43.

[7] Horton F, Wright J, Smith L, Hinton PJ, Robertson MD. Increased intestinal permeability to oral chromium (51Cr)-EDTA in human Type 2 diabetes. Diabetic Medicine. 2014 May 1;31(5):559-63.

[8] Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet–induced obesity and diabetes in mice. Diabetes. 2008 Jun 1;57(6):1470-81.

[9] Sampson M, Davies I, Gavrilovic J, Sussams B, Brown J, Astley S, Hughes DA. Plasma matrix metalloproteinases, low density lipoprotein oxidisability and soluble adhesion molecules after a glucose load in Type 2 diabetes. Cardiovascular diabetology. 2004 Jun 18;3(1):7.

[10] Lefkowitz RB, Marciniak JY, Hu CM, Schmid-Schönbein GW, Heller MJ. An electrophoretic method for the detection of chymotrypsin and trypsin activity directly in whole blood. Electrophoresis. 2010 Jan 1;31(2):403-10.

[11] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. Analytical chemistry. 2010 Sep 9;82(19):8251-8.

[12] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for trypsin activity using polyanionic focusing gel electrophoresis. Electrophoresis. 2010 Jul 1;31(14):2442-51.

[13] Camilleri M, Madsen K, Spiller R, Van Meerveld BG, Verne GN. Intestinal barrier function in health and gastrointestinal disease. Neurogastroenterology & Motility. 2012 Jun 1;24(6):503-12.

[14] Lake-Bakaar G, McKavanagh S, Redshaw M, Wood T, Summerfield JA, Elias E. Serum immunoreactive trypsin concentration after a Lundh meal. Its value in the diagnosis of pancreatic disease. Journal of clinical pathology. 1979 Oct 1;32(10):1003-8.

[15] Papazafiropoulou A, Perrea D, Moyssakis I, Kokkinos A, Katsilambros N, Tentolouris N. Plasma levels of MMP-2, MMP-9 and TIMP-1 are not associated with arterial stiffness in subjects with type 2 diabetes mellitus. Journal of diabetes and its complications. 2010 Feb 28;24(1):20-7.

[16] Signorelli SS, Malaponte G, Libra M, Pino LD, Celotta G, Bevelacqua V, Petrina M, Nicotra GS, Indelicato M, Navolanic PM, Pennisi G. Plasma levels and zymographic activities of matrix metalloproteinases 2 and 9 in type II diabetics with peripheral arterial disease. Vascular Medicine. 2005 Feb;10(1):1-6.

[17] Thrailkill KM, Bunn RC, Fowlkes JL. Matrix metalloproteinases: their potential role in the pathogenesis of diabetic nephropathy. Endocrine. 2009 Feb 1;35(1):1-0.

[18] Noji Y, Kajinami K, Kawashiri MA, Todo Y, Horita T, Nohara A, Higashikata T, Inazu A, Koizumi J, Takegoshi T, Mabuchi H. Circulating matrix metalloproteinases and their inhibitors in premature coronary atherosclerosis. Clinical chemistry and laboratory medicine. 2001 Jun 6;39(5):380-4.

[19] Lewandowski KC, Banach E, Bieńkiewicz M, Lewiński A. Matrix metalloproteinases in type 2 diabetes and non-diabetic controls: effects of short-term and chronic hyperglycaemia. Archives of medical science: AMS. 2011 Apr;7(2):294.

[20] Mazzone T, Chait A, Plutzky J. Cardiovascular disease risk in type 2 diabetes mellitus: insights from mechanistic studies. The Lancet. 2008 May 30;371(9626):1800-9.

[21] Vandivier RW, Fadok VA, Hoffmann PR, Bratton DL, Penvari C, Brown KK, Brain JD, Accurso FJ, Henson PM. Elastase-mediated phosphatidylserine receptor cleavage impairs apoptotic cell clearance in cystic fibrosis and bronchiectasis. The Journal of clinical investigation. 2002 Mar 1;109(5):661-70.

[22] Rodrigues SF, Tran ED, Fortes ZB, Schmid-Schönbein GW. Matrix metalloproteinases cleave the β2-adrenergic receptor in spontaneously hypertensive rats. American Journal of Physiology-Heart and Circulatory Physiology. 2010 Jul 1;299(1):H25-35.

[23] Talukdar S, Bandyopadhyay G, Li D, Xu J, McNelis J, Lu M, Li P, Yan Q, Zhu Y, Ofrecio J, Lin M. Neutrophils mediate insulin resistance in mice fed a high-fat diet through secreted elastase. Nature medicine. 2012 Sep 1;18(9):1407-12.

[24] Marzi C, Huth C, Herder C, Baumert J, Thorand B, Rathmann W, Meisinger C, Wichmann HE, Roden M, Peters A, Grallert H. Acute-phase serum amyloid A protein and its implication in the development of type 2 diabetes in the KORA S4/F4 study. Diabetes care. 2013 May 1;36(5):1321-6.

[25] Frankwich K, Tibble C, Torres-Gonzalez M, Bonner M, Lefkowitz R, Tyndall M, Schmid-Schönbein GW, Villarreal F, Heller M, Herbst K. Proof of Concept: Matrix metalloproteinase inhibitor decreases inflammation and improves muscle insulin sensitivity in people with type 2 diabetes. Journal of Inflammation. 2012 Oct 1;9(1):35.

[26] Lee SW, Song KE, Shin DS, Ahn SM, Ha ES, Kim DJ, Nam MS, Lee KW. Alterations in peripheral blood levels of TIMP-1, MMP-2, and MMP-9 in patients with type-2 diabetes. Diabetes research and clinical practice. 2005 Aug 31;69(2):175-9.

[27] Rawson ES, Freedson PS, Osganian SK, Matthews CE, Reed G, Ockene IS. Body mass index, but not physical activity, is associated with C-reactive protein. Medicine and science in sports and exercise. 2003 Jul;35(7):1160-6.

[28] Marfella R, Esposito K, Giunta R, Coppola G, De Angelis L, Farzati B, Paolisso G, Giugliano D. Circulating adhesion molecules in humans. Circulation. 2000 May 16;101(19):2247-51.

[29] Ceriello A, Falleti E, Motz E, Taboga C, Tonutti L, Ezsol Z, Gonano F, Bartoli E. Hyperglycemia-induced circulating ICAM-1 increase in diabetes mellitus: the possible role of oxidative stress. Hormone and Metabolic Research. 1998 Mar;30(03):146-9.

Chapter 3

Pilot study of circulating proteolytic activity and proteolytic receptor cleavage in schizophrenia and comparison groups

Jamie Joseph,[a] Elaine A. Skowronski,[b] Augusta E. Modestino,[c] Geert W. Schmid-Schönbein,[c] Michael J. Heller,[b,c] Colin A. Depp,[a] Dilip V. Jeste[a]

[a]*Department of Family Medicine and Public Health, University of California, San Diego.*

[b]*NanoEngineering Department, University of California, San Diego.*

[c]*Bioengineering Department, University of California, San Diego.*

Abstract

Intestinal permeability, insulin resistance, and hypertension have been associated with the pathogenesis of schizophrenia. In this study we investigate circulating proteolytic activity of digestive pancreatic proteases that would indicate intestinal permeability and provide a possible mechanism for insulin resistance via proteolytic cleavage of the ectodomain of the insulin receptor. We utilize a novel method for the detection of protease activities directly in fresh human whole blood samples. The density of insulin receptor (IR) and low density lipoprotein receptor-related protein 1 (LRP1) on the surface of peripheral blood mononuclear cells (PBMC) incubated with plasma from schizophrenia patients and control volunteers served to indicate the possibility that circulating proteolytic activity is responsible for certain cell dysfunctions. Inflammatory biomarkers and soluble adhesion molecules were also measured in schizophrenia patients and control volunteers.

3.1. Introduction

Schizophrenia is a severe chronic brain disorder with symptomatic onset in early adulthood that persists throughout life [3]. Schizophrenia affects approximately 0.5–1% of the world population with high heritability [9]. The diagnosis of schizophrenia remains subjective, based on interviews of the person and family members, as there are no empirical disease markers available [2]. There is a great clinical need for reliable diagnostic tests based on the identification of blood markers for schizophrenia. Such diagnostic tests would not only improve our understanding of the molecular characteristics underlying the disease processes of schizophrenia, but would also help diagnose the disease in the early-onset phase, identify disease subtypes, aid in predicting and monitoring treatment response and compliance, and identify novel drug targets.

Patients with first-episode psychosis (FEP) have high risk of weight gain, glucose dysregulation and dyslipidemias during the early stages of illness [1]. For example, circulating insulin-related peptides are elevated in first-onset schizophrenia subjects with no relative difference in glucose levels [8]. This suggests that hyperinsulinemia may have a role in the development of schizophrenia and that measurement of these peptides may have utility in diagnosis or stratification of patients before antipsychotic treatment. Moreover, drugs that improve insulin action may represent a novel treatment strategy. Alterations of other metabolic biomarkers indicative of perturbations in glucoregulatory pathways have also been identified in schizophrenia patients. In addition, schizophrenia patients show increased prevalence of impaired glucose tolerance and metabolic syndrome, irrespective of whether they received antipsychotic treatment [10]. Such metabolic dysfunction coupled with evidence of intestinal permeability in schizophrenia patients

suggests that digestive enzymes leaking into the circulatory system may play a role by leading to low levels of autodigestion of cell surface receptors.

In the general population, certain biomarkers are predictive of development of insulin resistance, non-alcoholic fatty liver disease and type 2 diabetes. All these conditions are known co-morbidities of psychotic disorders [12]. However, previous studies have not investigated protease activity and cell receptor cleavage profiles in schizophrenia patients, which might be valuable in elucidating the possible role of unchecked circulating protease activity in the metabolic abnormalities of schizophrenia patients.

3.2. Results

3.2.1 Study participants

This study is ongoing as of the submission of this dissertation, but so far 4 control volunteers, 17 schizophrenia patients, and 15 enrollees with blinded diagnosis have been enrolled in the study. Age, sex, body mass index (BMI), diagnosis, and other clinical variables including smoking and drug use were included in medical records for this study but were not made available prior to the submission of this dissertation.

3.2.3 Circulating protease activity

There were no significant differences between the circulating proteolytic activities of trypsin, chymotrypsin, elastase, or MMP-2/9 in the whole blood of schizophrenia patients (n=17) versus control volunteers (n=4) (**Figure 3.1**, **Figure 3.2**). Significant correlations may become apparent once the blinded diagnoses of the other study enrollees (n=15) become available.

3.2.3 Inflammatory markers and soluble adhesion molecules

Significant linear correlations were observed between various inflammatory markers and soluble adhesion molecules (**Figure 3.3**). A significant linear correlation occurred between MMP-2/9 activity and the inflammatory marker interleukin 6 (IL-6) (**Figure 3.4**).

3.3. Discussion

Molecular studies of chronic schizophrenia patients can be confounded since routinely used antipsychotic medications have several side effects, such as dysregulated glucose homeostasis. We were unable to circumvent this problem since our analyses of blood and plasma samples were not exclusively from first-onset, antipsychotic naïve patients. Recruitment of such patients is challenging, as large clinics diagnose only 20–30 such patients each year and few centers follow standard operating procedures for sample collection. Once we have full demographic data on the patients enrolled in this study we will attempt to match control subjects for age, gender, BMI and smoking [13].

One major confounder in this study that may frustrate the elucidation of the role of circulating protease activity in the pathogenesis of schizophrenia is the elevation of proteolytic activity associated with smoking. According to a study in hospital outpatients, 70% to 90% of patients with schizophrenia use nicotine. Heavy smokers (more than 20 cigarettes per day) had 4 times the risk for multiple substance abuse. Many patients with schizophrenia are recreational cannabis users, and cannabis has a known effect on glucoregulation; thus we examined this potential confounding factor very closely. One study found that recent cannabis use was associated with a significant reduction in serum glucose. These patients absorb nicotine more effectively and efficiently, leading to

increased nicotine levels in the body. High nicotine levels also increase P450 enzyme activity, resulting in the necessity of higher dose neuroleptics for effective treatment [14].

Other studies have shown that while drug-naïve patients with first-onset schizophrenia cluster very tightly together according to metabolomic profiles, a small number of samples did not [14]. These outliers may indicate the existence of schizophrenia subgroups, and it will be of interest to explore whether clinical parameters, such as disease progression, severity, and/or drug response relate to distinct proteolytic activity signatures. Although the sample size of this study is too small to make assumptions about patient subgroups, once enrollees with blinded diagnoses have their medical records revealed we hope to begin analyzing the enrolled patients for subgroups. Future studies with larger sample sizes over longer periods of follow-up are needed for deriving and validating diagnostic models for the identification of subjects at highest risk of metabolic co-morbidities and for the prediction of their metabolic outcomes.

3.4. Conclusion

These early results need to be tested in larger studies and confirmed before their clinical relevance will be known. It will be important for such follow-up studies to involve patients with other psychiatric diseases (not just schizophrenia), to see whether the biomarkers are specific to schizophrenia or whether they indicate a broader range of psychiatric diseases.

3.5. Materials and Methods

3.5.1 Study population

The Institutional Review Board of the University of California, San Diego reviewed and approved all human protocols. Human blood was obtained with informed consent from

healthy volunteers (n=4), schizophrenia patients (n=17), and enrollees with blinded diagnosis (n=15).

3.5.2 Sample acquisition

Blood (approximately 7.5 mL) was obtained from the forearm vein via venipuncture and drawn in lithium heparin-containing tubes and EDTA-containing tubes. Blood was kept at 4 °C from the time of venipuncture until it could be assayed (within 2 hours of venipuncture) and processed into plasma and frozen immediately at –80 °C.

3.5.3 Protease activity assays

Activities of MMP-2 and -9, elastase, trypsin, and chymotrypsin were measured in fresh whole blood samples of volunteers using the charge-changing protease substrates and electrophoretic method developed by our lab [5-7]. This method uses synthetic fluorescent peptides that are specifically cleaved by a protease; the peptides are originally negatively or neutrally charged; upon cleavage by the protease of interest, two fragments are produced, a negative one and a fluorescently labeled positive one, which allows for electrophoretic separation and detection in whole blood with no sample preparation (**Figure 3.1**). This method has been described in detail [5-7].

3.5.4 Insulin receptor labeling

Freshly isolated PBMC cells from a healthy volunteer were incubated with plasma from control fasting, control, p-T2DM and T2DM participants collected at different time points after the meal, or with spiked proteases (elastase, MMP-2, and trypsin). A 50μL PBMC suspension and 150μL plasma solution were incubated for 1 hour at 37^{o}C. The cells were then spread as a blood smear, fixed (10% formalin, neutral buffered) and labeled with a primary antibody against the extracellular domain of the insulin receptor (sc-710

polyclonal antibody mapping to the N-terminus, Santa Cruz Biotech) followed by the biotin/avidin Vector TMB peroxidase enzyme substrate. The cell images were collected using a bright field microscope (40X and 60X objective) and digitally analyzed (NIH Image J 1.440) to determine light absorption as a measure of the surface density of the insulin receptor extracellular domain (**Figure 3.6**).

3.5.5 LRP1 receptor labeling

Freshly isolated PBMC cells from a healthy volunteer were incubated with plasma from control fasting, control, p-T2DM and T2DM participants collected at different time points after the meal, or with spiked proteases (elastase, MMP-2, and trypsin). A 50μL PBMC suspension and 150μL plasma solution were incubated for 1 hour at 37^{o}C. The cells were then spread as a blood smear, fixed (10% formalin, neutral buffered) and labeled with a primary antibody against the extracellular domain of the LRP1 receptor (sc-450 polyclonal antibody mapping to the N-terminus, Santa Cruz Biotech) followed by the biotin/avidin Vector TMB peroxidase enzyme substrate. The cell images were collected using a bright field microscope (40X and 60X objective) and digitally analyzed (NIH Image J 1.440) to determine light absorption as a measure of the surface density of the insulin receptor extracellular domain (**Figure 3.6**).

3.5.6 Inflammatory markers and soluble adhesion molecules

Whole blood was preserved with EDTA. Following centrifugation at 4 ^{o}C, plasma was stored at -80 ^{o}C until assayed. Circulating levels of C-reactive protein (CRP), serum amyloid A (SAA), soluble intercellular cell adhesion molecule-1 (sICAM-1) and soluble vascular cell adhesion molecule-1 (sVCAM-1) were determined by commercial ELISA

(MSD, Rockville, MD). Intra- and inter-assay coefficients were <5%. Assays were performed in triplicate (**Figure 3.4**, **Figure 3.5**).

3.5.7 Statistical Analysis

ANOVA, ANCOVA, and Pearson linear correlations were performed on the data using SPSS software.

Acknowledgments

This work is funded by a project fellowship from UC San Diego Frontiers of Innovation Scholars Program (FISP).

Chapter 3, in part, is currently being prepared for submission for publication of the material. Jamie Joseph, Elaine A. Skowronski, Augusta E. Modestino, Geert W. Schmid-Schönbein, Michael J. Heller, Colin A. Depp, Dilip V. Jeste. “Pilot study of circulating proteolytic activity and proteolytic receptor cleavage in schizophrenia and comparison groups.” The dissertation author was the secondary investigator and secondary author of the material being prepared for submission for publication. The dissertation author designed and ran protease activity assays and receptor cleavage assays, analyzed data, and composed the text and figures of this chapter.

A. Target Protease	Charge-Changing Fluorescent Sequences and Cleavage Site	Charge	
		Substrate	C- Product
Trypsin	Ac-N-DGDAGR // AGAGK(ε-BFL)-NH_2	-1	+1
Elastase	Ac-N-DA//G//SV//A//G//A//GK(ε-BFL)-NH_2	-1	+1
MMP-2/9	Ac-NGDPVGL//TAGAGK(ε-BFL)-NH_2	-1	+1

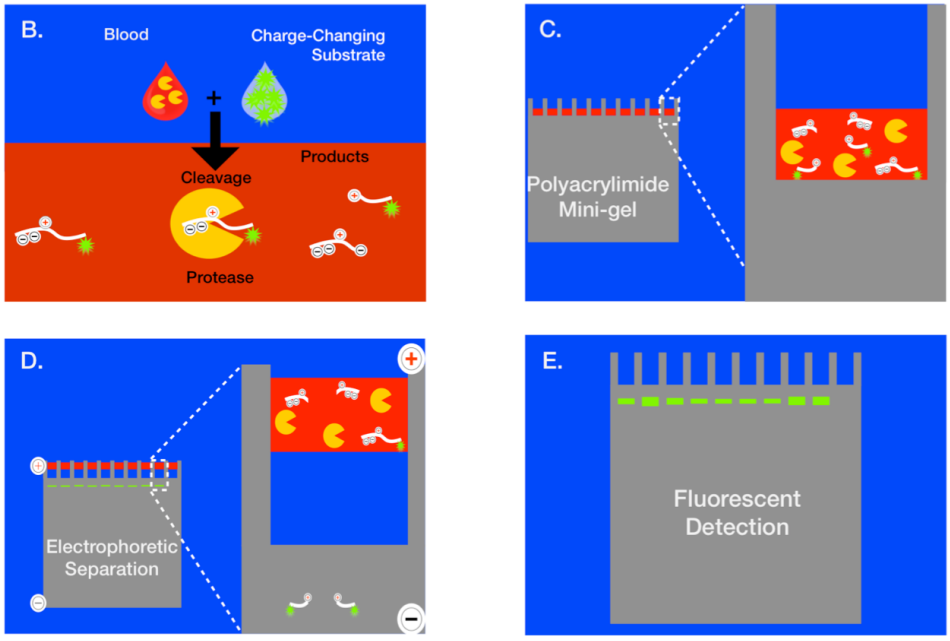


Figure 3.1. Protease activity detection. (A) Charge-changing fluorescently labeled substrate sequences and cleavage sites. **(B-E)** Novel electrophoretic assay for protease activity detection. **(B)** 4µL of whole blood is combined with 4µL of charge-changing substrate designed to be cleaved by a target protease. **(C)** After a 30 min reaction, 6µL aliquots of reaction mixture are loaded into a polyacrylamide mini-gel. **(D)** Gel is run at 500V for 10 minutes **(E)** Detection of fluorescence is carried out in a fluorescence scanner.

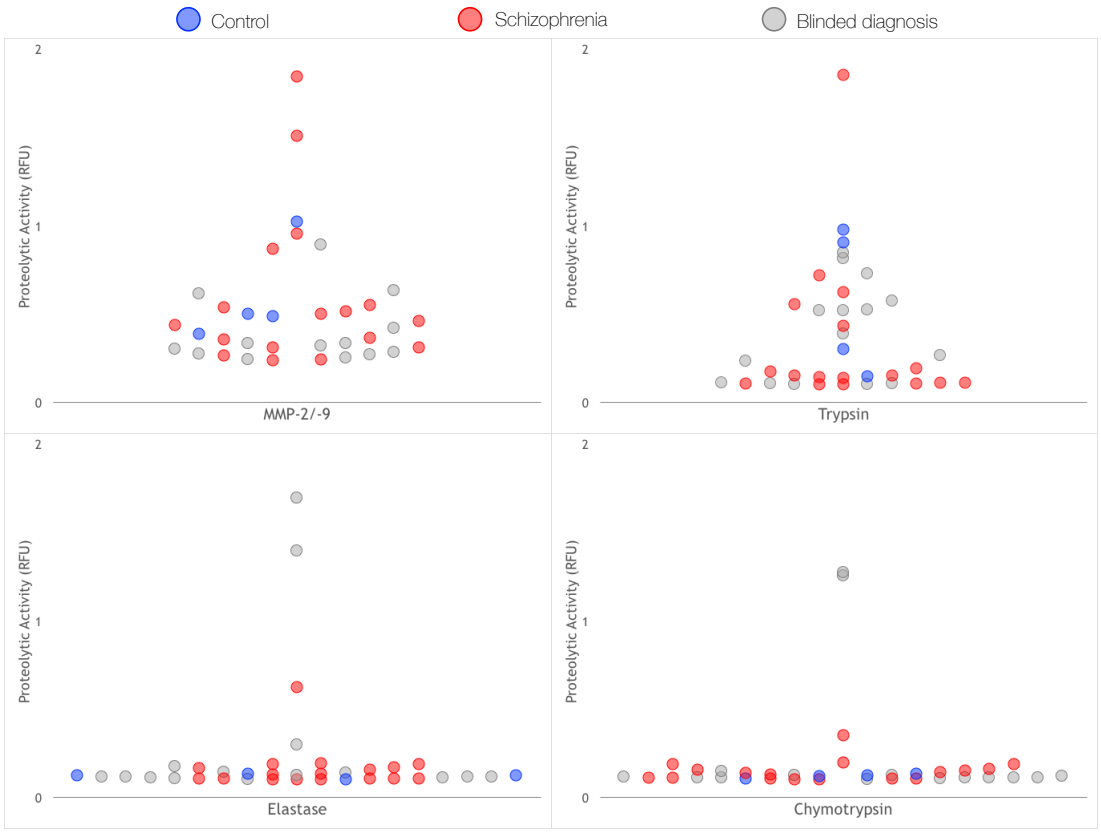


Figure 3.2. Protease activity in circulation. Clustering of control, schizophrenia, and diagnosis blinded patients according to the proteolytic activities measured in whole blood.

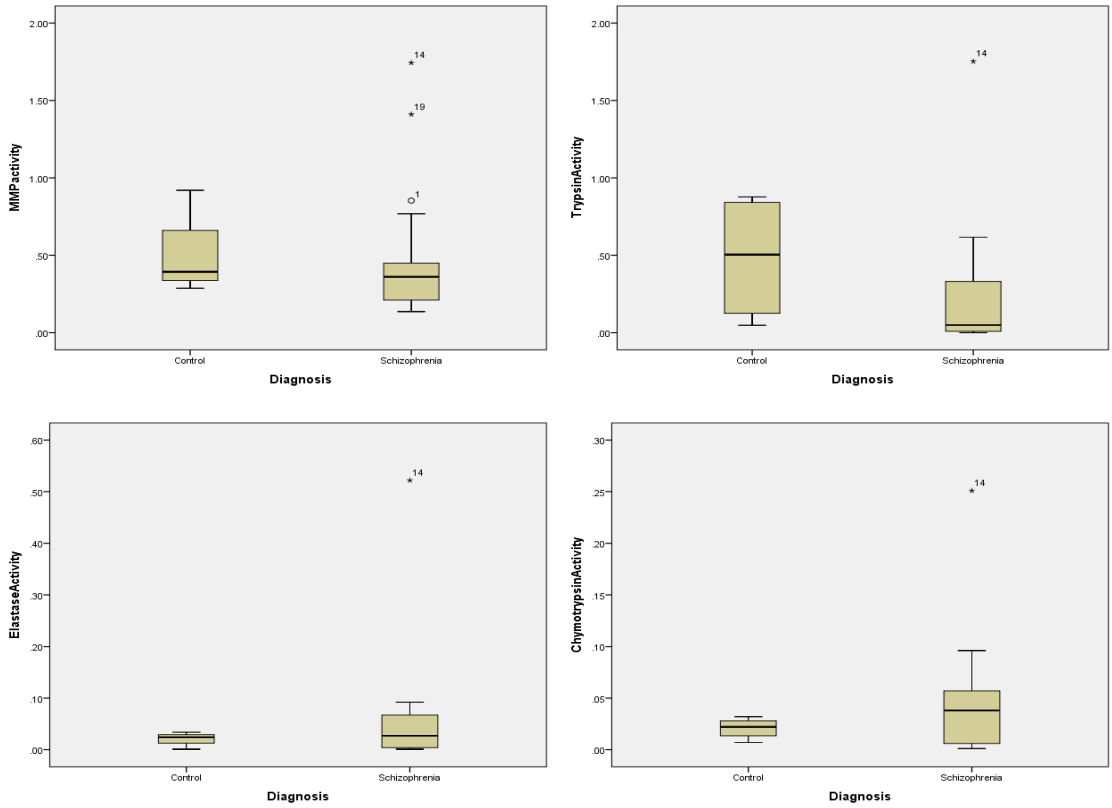
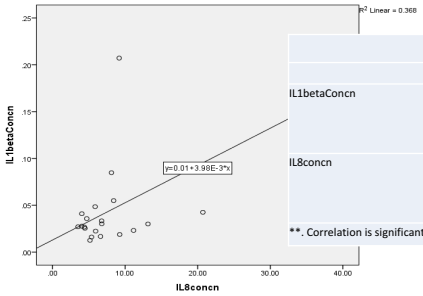


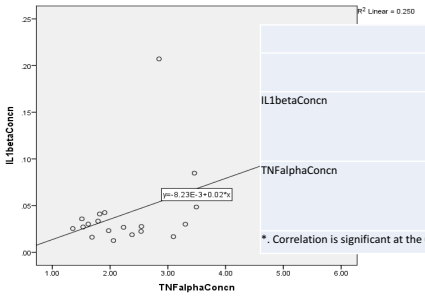
Figure 3.3. Mean of protease activity in circulation. No significant difference was seen between circulating proteolytic activities for control group and schizophrenia patients.



Correlations

		IL1betaConcn	IL8concn
IL1betaConcn	Pearson Correlation	1	.606**
	Sig. (2-tailed)		0.004
	N	21	21
IL8concn	Pearson Correlation	.606**	1
	Sig. (2-tailed)	0.004	
	N	21	21

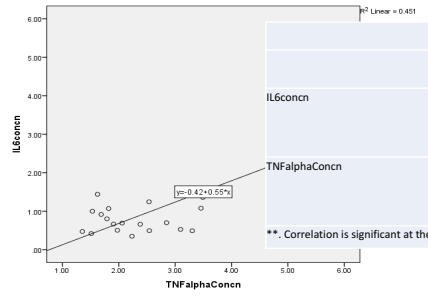
**. Correlation is significant at the 0.01 level (2-tailed).



Correlations

		IL1betaConcn	TNFalphaConcn
IL1betaConcn	Pearson Correlation	1	.500*
	Sig. (2-tailed)		0.021
	N	21	21
TNFalphaConcn	Pearson Correlation	.500*	1
	Sig. (2-tailed)	0.021	
	N	21	21

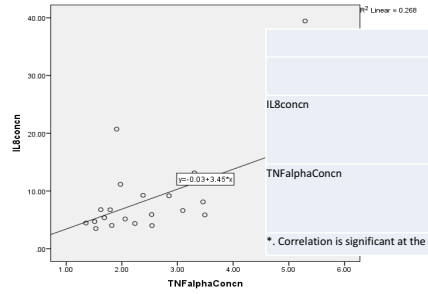
*. Correlation is significant at the 0.05 level (2-tailed).



Correlations

		IL6concn	TNFalphaConcn
IL6concn	Pearson Correlation	1	.672**
	Sig. (2-tailed)		0.001
	N	21	21
TNFalphaConcn	Pearson Correlation	.672**	1
	Sig. (2-tailed)	0.001	
	N	21	21

**. Correlation is significant at the 0.01 level (2-tailed).

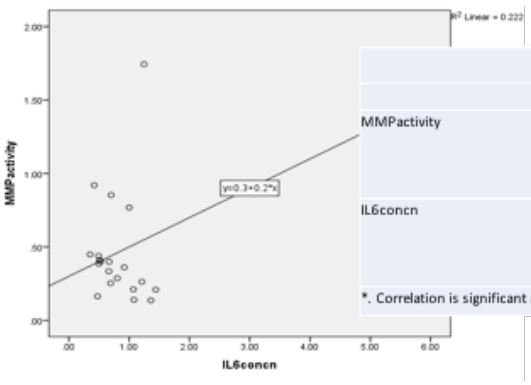


Correlations

		IL8concn	TNFalphaConcn
IL8concn	Pearson Correlation	1	.518*
	Sig. (2-tailed)		0.016
	N	21	21
TNFalphaConcn	Pearson Correlation	.518*	1
	Sig. (2-tailed)	0.016	
	N	21	21

*. Correlation is significant at the 0.05 level (2-tailed).

Figure 3.4. Pearson linear correlations for inflammatory markers and soluble adhesion molecules. Significant linear correlations were found for certain pairs of inflammatory markers and soluble adhesion molecules across control group and schizophrenia patients.



Correlations

		MMPactivity	IL6concn
MMPactivity	Pearson Correlation	1	.471*
	Sig. (2-tailed)		0.031
	N	21	21
IL6concn	Pearson Correlation	.471*	1
	Sig. (2-tailed)	0.031	
	N	21	21

*. Correlation is significant at the 0.05 level (2-tailed).

Figure 3.5. Pearson linear correlation for proteases and inflammatory markers. Of the proteases and inflammatory markers and soluble adhesion molecules, only circulating MMP-2/9 activity and interleukin-6 concentration showed significant linear correlation.

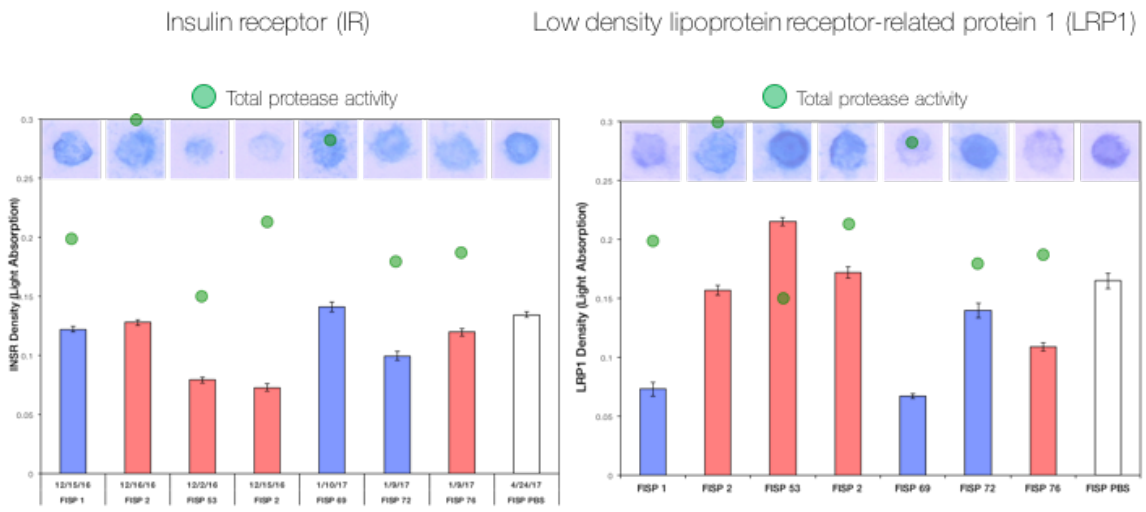


Figure 3.6. Density of cell receptors exposed to proteolytic activity. Cells were incubated with the plasma of schizophrenia patients (red) or the plasma of control group (blue). Staining with labeled antibodies specific to the cell receptors of interest revealed the degree to which the plasma incubation cleaved the ectodomains of the insulin receptor (IR) and the low density lipoprotein receptor-related protein 1 (LRP1).

References

[1] Guest PC, Wang L, Harris LW, Burling K, Levin Y, Ernst A, Wayland MT, Umrania Y, Herberth M, Koethe D, Van Beveren JM. Increased levels of circulating insulin-related peptides in first-onset, antipsychotic naive schizophrenia patients. Molecular psychiatry. 2010 Feb 1;15(2):118-20. doi:10.1038/mp.2009.81.

[2] He Y, Yu Z, Giegling I, Xie L, Hartmann AM, Prehn C, Adamski J, Kahn R, Li Y, Illig T, Wang-Sattler R, Rujescu D. Schizophrenia shows a unique metabolomics signature in plasma. Transl Psychiatry. 2012 Aug 14;2:e149. doi: 10.1038/tp.2012.76.

[3] Holmes E, Tsang TM, Huang JT, Leweke FM, Koethe D, Gerth CW, Nolden BM, Gross S, Schreiber D, Nicholson JK, Bahn S. Metabolic profiling of CSF: evidence that early intervention may impact on disease progression and outcome in schizophrenia. PLoS Med. 2006 Aug;3(8):e327.

[4] Kotronen A, Velagapudi VR, Yetukuri L, Westerbacka J, Bergholm R, Ekroos K, Makkonen J, Taskinen MR, Oresic M, Yki-Järvinen H. Saturated fatty acids containing triacylglycerols are better markers of insulin resistance than total serum triacylglycerol concentrations. *Diabetologia* 2009; **52**: 684–690.

[5] Lefkowitz RB, Marciniak JY, Hu CM, Schmid-Schönbein GW, Heller MJ. An electrophoretic method for the detection of chymotrypsin and trypsin activity directly in whole blood. Electrophoresis. 2010 Jan 1;31(2):403-10.

[6] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. Analytical chemistry. 2010 Sep 9;82(19):8251-8.

[7] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for trypsin activity using polyanionic focusing gel electrophoresis. Electrophoresis. 2010 Jul 1;31(14):2442-51.

[8] Liu ML, Zhang XT, Du XY, Fang Z, Liu Z, Xu Y, Zheng P, Xu XJ, Cheng PF, Huang T, Bai SJ, Zhao LB, Qi ZG, Shao WH, Xie P. Severe disturbance of glucose metabolism in peripheral blood mononuclear cells of schizophrenia patients: a targeted metabolomic study. J Transl Med. 2015 Jul 14;13:226. doi: 10.1186/s12967-015-0540-y.

[9] McEvoy J, Freudenreich O, McGee M, Vander Zwaag C, Levin E, Rose J. Clozapine decreases smoking in patients with chronic schizophrenia. Biol Psychiatry. 1995;37(8):550-552.

[10] Newcomer JW. Metabolic syndrome and mental illness. Am J Manag Care. 7007 Nov;13(7 Suppl):S170-7.

[11] Rhee EP, Cheng S, Larson MG, Walford GA, Lewis GD, McCabe E, Yang E, Farrell L, Fox CS, O'Donnell CJ, Carr SA, Vasan RS, Florez JC, Clish CB, Wang TJ, Gerszten RE. Lipid profiling identifies a triacylglycerol signature of insulin resistance and improves diabetes prediction in humans. *J Clin Invest* 2011; **121**: 1402–1411.

[12] Severance EG, Gressitt KL, Stallings CR, Origoni AE, Khushalani, Leweke FM, Dickerson FB, Yolken RH. Discordant patterns of bacterial translocation markers and implications for innate immune imbalances in schizophrenia. Schiz Res. 2013 Aug;148(1-3):130-137. http://dx.doi.org/10.1016/j.schres.2013.05.018.

[13] Spelman LM, Walsh PI, Sharifi N, Collins P, Thakore JH. Impaired glucose tolerance in first-episode drug-naïve patients with schizophrenia. Diabet Med. 2007 May;24(5):481-5. Epub 2007 Mar 22.

[14] Suvitaival T, Mantere O, Kieseppä T, Mattila I, Pöhö P, Hyötyläinen T, Suvisaari J, Orešič M. Serum metabolite profile associates with the development of metabolic co-morbidities in first-episode psychosis. Transl Psychiatry. 2016 Nov 15;6(11):e951. doi: 10.1038/tp.2016.222.

[15] Ziedonis DM, Smelson D, Rosenthal RN, Batki SL, Green AI, Henry RJ, Montoya I, Parks J, Weiss RD. Improving the care of individuals with schizophrenia and substance use disorders: consensus recommendations. J Psychiatr Pract. 2005 Sep;11(5):315-39.

Chapter 4

Associations of circulating proteolytic activity in neonates and length of stay in the neonatal intensive care unit

Elaine A. Skowronski,[a] Michael J. Heller,[a,b] Shelley M. Lawrence[c]

[a]*NanoEngineering Department, University of California, San Diego.*

[b]*Bioengineering Department, University of California, San Diego.*

[c]*Department of Family Medicine and Public Health, University of California, San Diego.*

Abstract

The intestine of premature neonates is not fully mature and may be permeable to digestive enzymes. In this study we monitored the circulating proteolytic activities of neonates and related this activity to the length of stay of patients in the neonatal intensive care unit (NICU). In addition to investigating the leakage of pancreatic proteases into the circulation, we also monitored the coagulation protease thrombin in the circulation. Coagulation dysregulation has been associated with severe inflammation, such as that caused by sepsis, and since premature neonates are at higher risk of sepsis, coagulation proteolytic activity was of interest.

4.1. Introduction

The advances in perinatal care over the last two decades have led to an improvement of neonatal survival worldwide [1, 2]. Adequate parenteral and enteral nutrition is associated with better outcomes for preterm infants [3]. Early introduction and rapid achievement of full enteral feeding (FEF) is a priority in the nutritional management of preterm infants, because it reduces the need for central venous catheters (CVCs) and thus the risk of infection and also reduces the length of hospital stay [4].

The need to attain FEF rapidly often conflicts with the physiologic immaturity of the GI function of preterm infants and also with the occurrence of various comorbidities in the neonatal period [10]. In this perspective, we focus on length of stay (LOS) of neonates in the neonatal intensive care unit (NICU) and whether LOS is associated with clinical variables and circulatory biomarkers for gastrointestinal immaturity and permeability [8]. We deploy novel protease activity assays to measure circulating proteolytic activity of pancreatic proteases as well as matrix metalloproteinases associated with tissue remodeling.

In addition to gastrointestinal immaturity, preterm infants also have a good hemostasis that is not reflected in conventional clotting tests such as prothrombin (PT) and partial thrombin time (PTT) using strong activators of clotting [9]. This is important to consider when pathological tests are obtained in neonates with bleeding complications or prior to invasive procedures. It may also be important for the definition of adequate doses of anticoagulants. The aim of the present study is to elucidate the role that some prenatal, neonatal and early postnatal variables play in favoring or delaying the achievement of

stable thrombin activity levels and to determine whether time to this achievement influences the LOS in the NICU.

4.2. Results

4.2.1 Study participants

This pilot study is ongoing but as of the submission of this dissertation has assay the blood of 8 neonates in the neonatal intensive care unit (NICU) after obtaining parental consent. Clinical variables including sepsis diagnosis and gestational age were provided after testing was completed for each patient (**Figure 4.1, Figure 4.2**).

4.2.2 Circulating protease activity

Protease activity was elevated mostly early in life and decreased with age, possibly reflecting the maturing of the intestinal lining with age (**Figure 4.3**).

4.3. Discussion

This study is the first to evaluate the effects of gestational age on circulating protease activity in neonates. Various prenatal, neonatal, and postnatal variables must also be considered when evaluating differences between circulating proteolytic activity of neonatal patients. Any clinical variable that disrupts adequate intestinal blood flow will most likely negatively impact the integrity and proper functioning of the intestine and in turn the proper containment of powerful digestive enzymes such as pancreatic proteases.

Patent ductus arteriosus (PDA) impacts blood flow to vital organs in premature infants and the presence of symptomatic PDA has been associated with numerous adverse clinical outcomes, including impaired feeding tolerance and necrotizing enterocolitis. The increased flow of blood through the lungs also can reduce blood flow to the rest of the body. This can damage other organs, especially the intestines and kidneys [9].

Our data suggest that there are clinical effects associated with increases in circulating protease activity in preterm neonates (**Figures 4.4, 4.5, 4.6**). Additional studies, confirming these findings and powered sufficiently to determine the association between circulating protease activity and the incidence of necrotizing enterocolitis are required.

A limiting factor in our study is the lack of exclusion criteria. It would be prudent to exclude neonates exposed to external variables potentially affecting circulating protease activity, e.g. protease inhibitor administration.

4.5. Materials and methods

4.5.1 Study design

The study protocol was approved by the institutional review board of the University of California, San Diego. From 2016 to 2017, we conducted a pilot study at University of California, San Diego Hillcrest Medical Center. In newborns and infants who needed intensive care, we monitored circulating protease activity. The primary endpoints of the study were morbidity. Measures of morbidity assessed were length of stay (LOS) in the neonatal intensive care unit (NICU); number of septic episodes; and hepatic damage as assessed by liver function tests. All physicians and nurses involved in the patients' care were blinded to all assay results. All researchers involved in running assays were blinded to all clinical information concerning patients.

4.5.2 Patients

Provided parental consent was obtained, patients admitted to the neonatal intensive care unit at the University of California, San Diego Hillcrest Medical Center were enrolled if they fulfilled all of the inclusion criteria and none of the exclusion criteria. Inclusion criteria were gestational age >20 weeks and age ≤1 year. Exclusion criteria were an inborn

error of metabolism, an immunodeficiency or a disease that entailed impaired growth (other than dysmaturity), the use of corticosteroid drugs, preexistent life expectancy less than 6 months.

4.5.3 Protease activity assays

Protease activity assays were performed on scavenged whole blood samples taken for complete blood count (CBC) testing of neonates. These scavenged samples were taken at different times of day, were not exclusively fasting or non-fasting. To obtain some information about thrombin generation in neonates in vivo, we investigated thrombin generation in shed blood from capillary incisions in neonates [5-8] (**Table 4.1**).

4.5.4 Length of stay

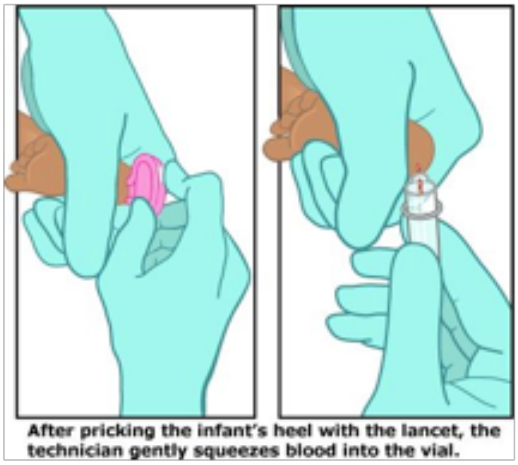
Length of stay in the NICU was expressed in days and calculated as the difference between the date of discharge and the start of NICU admittance.

4.5.5 Septic episodes

The Centers for Disease Control and Prevention criteria for nosocomial infections were used to identify septic events. For septic events to be taken into account, positive blood cultures were mandatory. Primary and secondary bloodstream infections were grouped together as "sepsis."

Acknowledgments

Chapter 4, in part, is currently being prepared for submission for publication of the material. Elaine A. Skowronski, Michael J. Heller, Shelley M. Lawrence. "Associations of circulating proteolytic activity in neonates and length of stay in the neonatal intensive care unit." The dissertation author is the primary investigator and author of this material.



Neonate	Days post-birth
FASTR-013	33–103
FASTR-014	19
FASTR-016	0–84
FASTR-017	3–27
FASTR-018	0–86
FASTR-019	0–56
FASTR-020	0–56
FASTR-021	42–56

Figure 4.1. Neonate sample acquisition and length of testing.

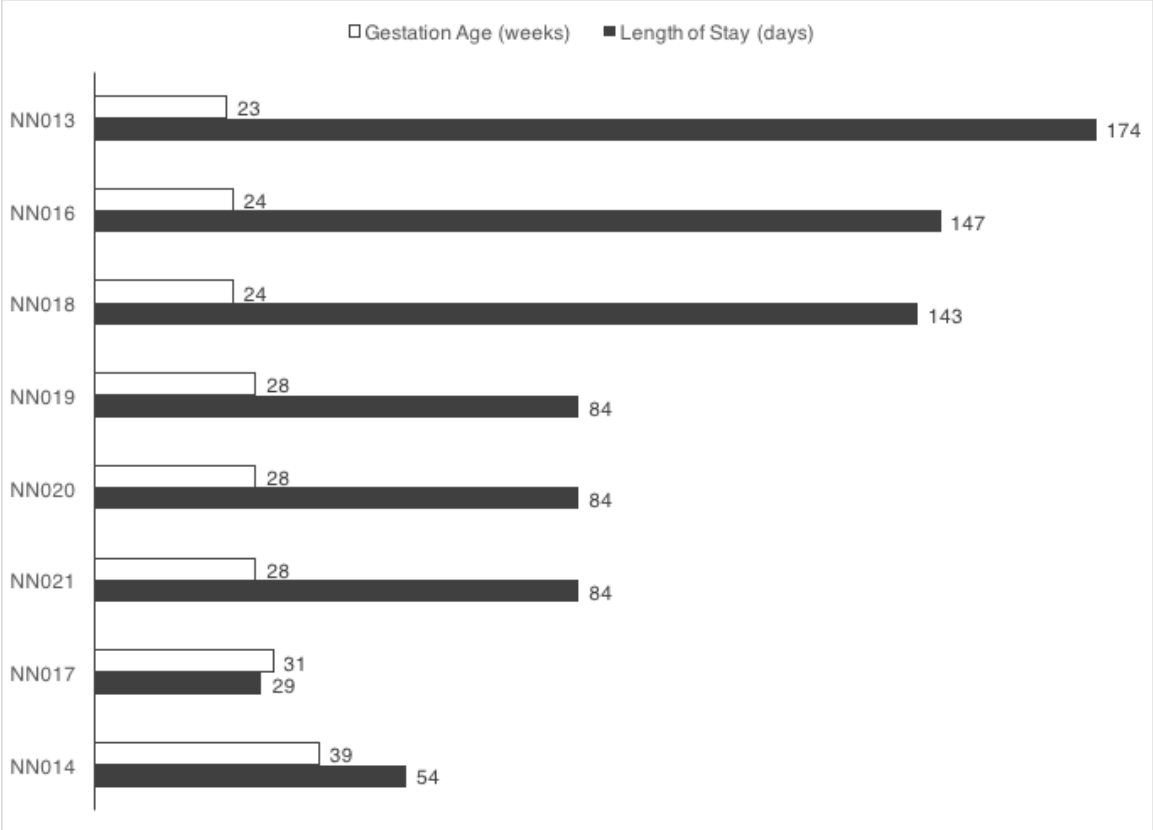


Figure 4.2. Gestational age and length of stay in the neonatal intensive care unit.

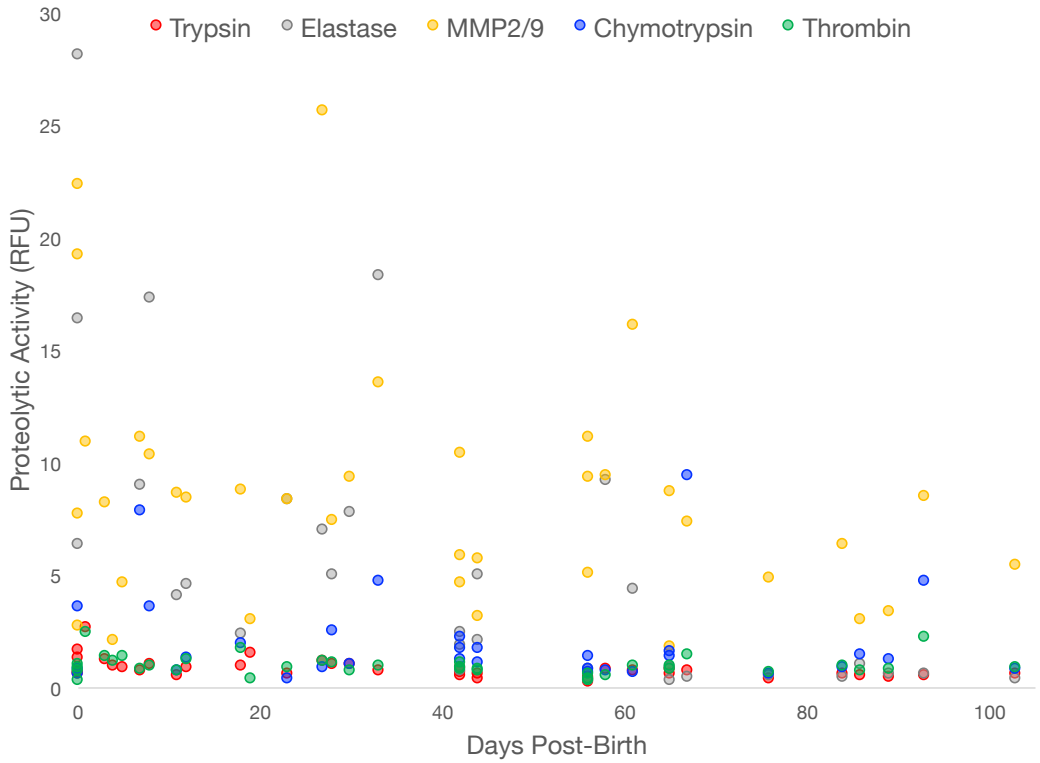


Figure 4.3. Time course of circulatory protease activity in neonates.

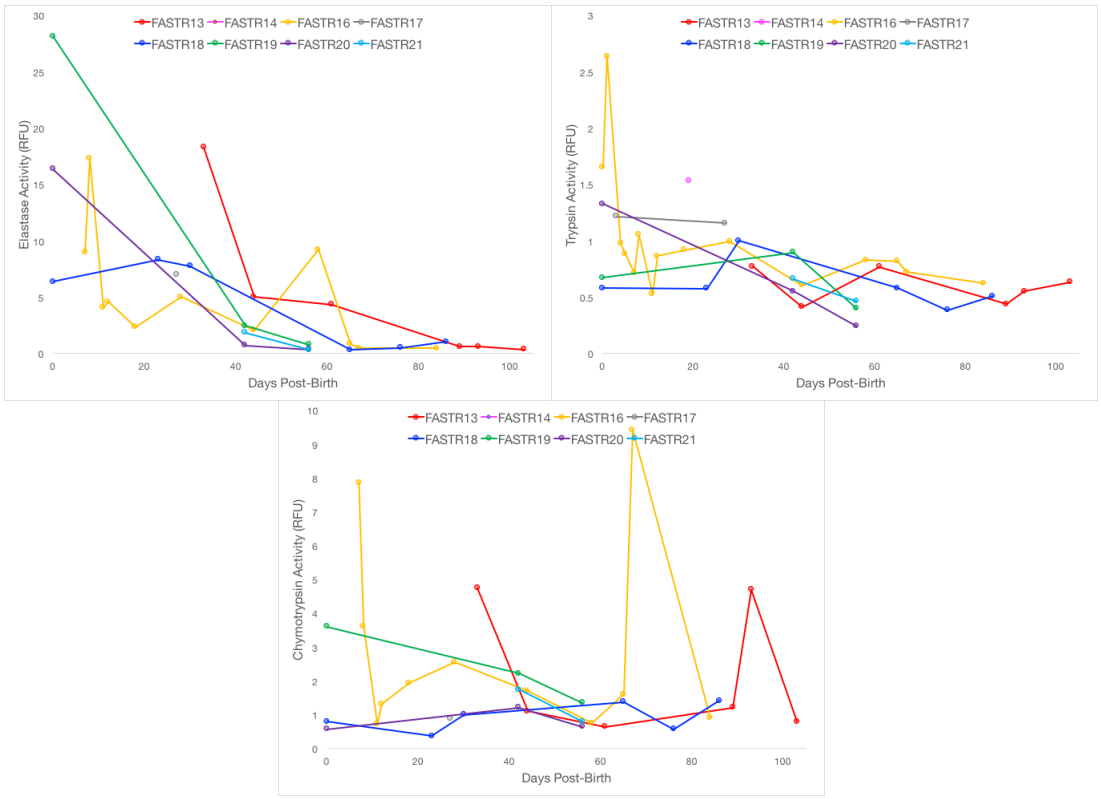


Figure 4.4. Activity of pancreatic proteases.

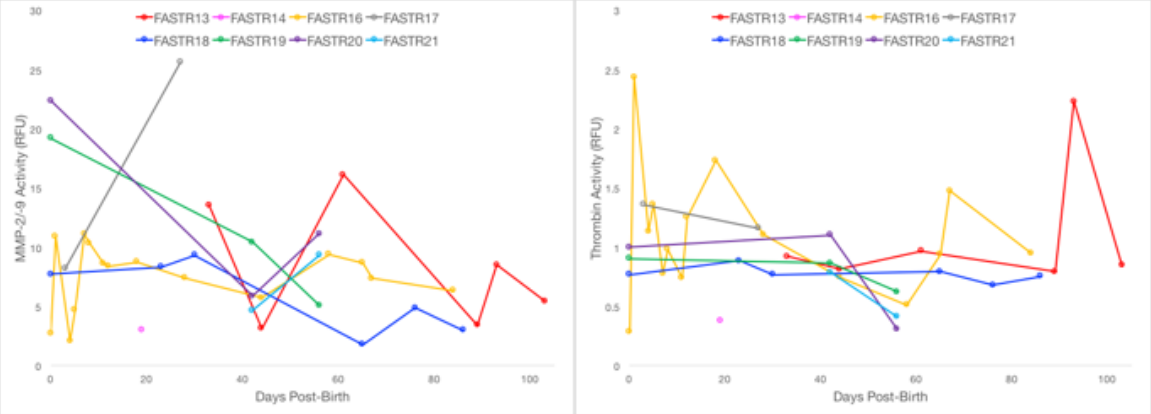
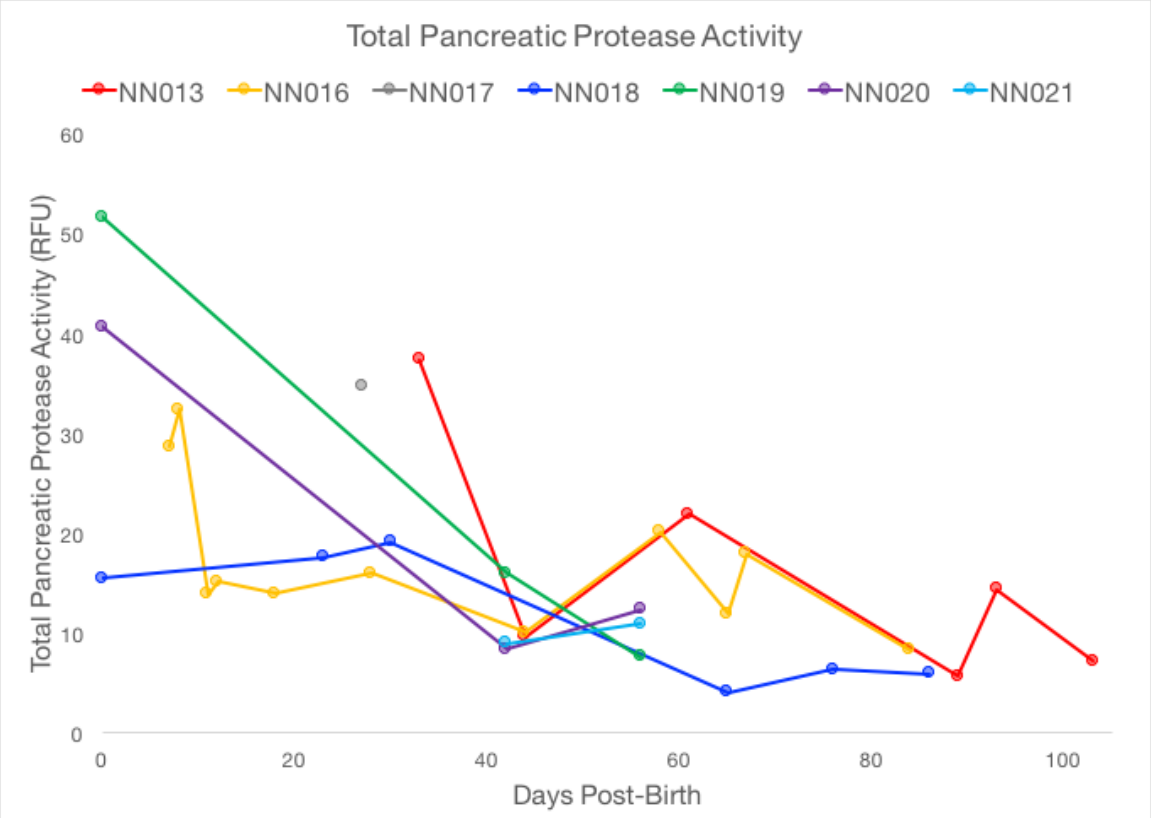


Figure 4.5. Activity of MMP-2/9 and thrombin.



NN013	Adrenal insufficiency, hyperglycemia, hypoglycemia, PDA	Klebsiella infection, Sepsis	Aspiration of formula, Surgery for PDA
NN016	IUGR, PDA	Staph, Sepsis ruled out	Surgery for PDA
NN017	IUGR, SGA, hypoglycemia	None reported	Surgery: none
NN018	IDM, Hernias: bilateral inguinal, PDA	Staph, Sepsis	Surgery: hernia repair
NN019	Hypoglycemia, PDA	Sepsis ruled out	Surgery: none
NN020	Hypoglycemia, PDA	Sepsis ruled out	Surgery: none
NN021	Hypoglycemia, PDA	Sepsis ruled out	Surgery: none

Figure 4.6. Proteolytic activity with diagnoses of neonates.

Table 4.1. Peptide substrates for protease activity detection.

Target Protease	Charge-Changing Fluorescent Peptide Sequences and Cleavage Site	Charge	
		Substrate	Cleavage Product
Trypsin	Ac-*N*-**Asp⁻**–Gly–**Asp⁻**–Ala–Gly–**Arg⁺**// Ala–Gly–Ala–Gly–Lys**(ε-BFL)**-NH_2	-1	+1
Elastase	Ac-*N*-**Asp⁻**–Ala//Gly//Ser–Val// Ala//Gly//Ala//Gly–Lys**(ε-BFL)**-NH_2	-1	+1
MMP-2/9	Ac-*N*-Gly–**Asp⁻**–Leu–Ala–Ala–Ile// Thr–Ala–Ala–Gly–Ala–Gly–Lys**(ε-BFL)**-NH_2	-1	+1
Chymotrypsin	Ac-*N*-**Asp⁻**–Gly–**Asp⁻**–Ala–Ala–Tyr// Ala–Ala–Tyr–Ala–Gly–Ala–Gly–diaminoethyl-**BFL**	-2	+1
Thrombin	Ac-*N*-**Asp⁻**–**Asp⁻**–Nle–Thr–Pro–**Arg⁺** //Gly-Ser-Ala-Gly-Ala-Gly-Ala-Gly-diaminoethyl-**BFL**	-1	+1

References

[1] Albers MJ, Steyerberg EW, Hazebroek FW, Mourik M, Borsboom GJ, Rietveld T, Huijmans JG, Tibboel D. Glutamine supplementation of parenteral nutrition does not improve intestinal permeability, nitrogen balance, or outcome in newborns and infants undergoing digestive-tract surgery: results from a double-blind, randomized, controlled trial. Annals of surgery. 2005 Apr 1;241(4):599-606.

[2] Corvaglia L, Fantini MP, Aceti A, Gibertoni D, Rucci P, Baronciani D, Faldella G. Predictors of full enteral feeding achievement in very low birth weight infants. PLoS one. 2014 Mar 19;9(3):e92235.

[3] Fellman V, Hellström-Westas L, Norman M, Westgren M, Källén K, Lagercrantz H, Marsál K, Serenius F, Wennergren M. One-year survival of extremely preterm infants after active perinatal care in Sweden. JAMA. 2009 Jun 3;301(21): 2225–33.

[4] Havranek T, Rahimi M, Hall H, Armbrecht E. Feeding preterm neonates with patent ductus arteriosus (PDA): intestinal blood flow characteristics and clinical outcomes. The Journal of Maternal-Fetal & Neonatal Medicine. 2015 Mar 24;28(5):526-30.

[5] Lefkowitz RB, Marciniak JY, Hu CM, Schmid-Schönbein GW, Heller MJ. An electrophoretic method for the detection of chymotrypsin and trypsin activity directly in whole blood. Electrophoresis. 2010 Jan 1;31(2):403-10.

[6] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for elastase, chymotrypsin, matrix metalloproteinase-2, and matrix metalloproteinase-9 activity. Analytical chemistry. 2010 Sep 9;82(19):8251-8.

[7] Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Whole blood assay for trypsin activity using polyanionic focusing gel electrophoresis. Electrophoresis. 2010 Jul 1;31(14):2442-51.

[8] Modestino A, Tyndall M, Yu J, Lefkowitz RB, Schmid-Schönbein GW, Heller MJ. Thrombin generation assay in untreated whole human blood. Electrophoresis. 2016 Aug;37(15-16):2248-56. doi: 10.1002/elps.201600061.

[9] Morgan J, Young L, Mcguire W. Delayed introduction of progressive enteral feeds to prevent necrotising enterocolitis in very low birth weight infants. Cochrane Database Syst Rev. 2013 May 31;(5):CD001970.

[10] Tyson JE, Parikh N, Langer J, Green C, Higgins RD. Intensive care for extreme prematurity-moving beyond gestational age. N Engl J Med. 2008 Apr 17;358(16): 1672–81.