Title
Studies toward the design, synthesis, and evaluation of new therapeutic and diagnostic agents for amyloid-associated diseases

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UNIVERSITY OF CALIFORNIA, SAN DIEGO

Studies toward the design, synthesis, and evaluation of new therapeutic and diagnostic agents for amyloid-associated diseases

A Thesis submitted in partial satisfaction of the requirements for the degree Master of Science

in

Chemistry

by

Jamie PP Do

Committee in charge:

Professor Jerry Yang, Chair
Professor Mana Parast
Professor Emmanuel Theodorakis

2015
This Thesis of Jamie PP Do is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Chair

University of California, San Diego

2015
DEDICATION

This Thesis is dedicated to my parents, Phuong Phan and Phi Do, and my little care bear of a sister, Diane Do – for never ceasing to understand, forgive, and love.

Thank you.
“IT IS THE QUALITY OF ONE’S CONVICTIONS THAT DETERMINES SUCCESS, NOT THE NUMBER OF FOLLOWERS.” – REMUS LUPIN
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<tr>
<td>Aβ</td>
<td>Amyloid-beta</td>
<td>NFT</td>
<td>Neurofibrillar tangles</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
<td>NMDA</td>
<td>N-methyl-D-aspartate receptors</td>
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<tr>
<td>ANCA</td>
<td>Amino naphthalenyl-2-cyano-acrylate</td>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
<td>PIGF</td>
<td>Placental growth factor</td>
</tr>
<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
<td>RAGE</td>
<td>Receptor for advanced glycation end products</td>
</tr>
<tr>
<td>BOC</td>
<td>Tert-butyl pyrocarbonate</td>
<td>sEng</td>
<td>Soluble endoglin</td>
</tr>
<tr>
<td>BTA</td>
<td>Benzothiazole aniline</td>
<td>sFlt-1</td>
<td>Soluble fms-like tyrosine kinase</td>
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<tr>
<td>CAM</td>
<td>Cerium ammonium molybdate</td>
<td>TEA</td>
<td>Triethylamine</td>
</tr>
<tr>
<td>DCM</td>
<td>Dichloromethane</td>
<td>TFA</td>
<td>Trifluoroacetic acid</td>
</tr>
<tr>
<td>DIPEA</td>
<td>Diisopropanylethylamine</td>
<td>TFAA</td>
<td>Trifluoroacetic anhydride</td>
</tr>
<tr>
<td>DMP</td>
<td>Dess martin periodinane</td>
<td>TFE</td>
<td>Trifluoroethanol</td>
</tr>
<tr>
<td>ESI-MS</td>
<td>Electrospray ionization mass spectrometry</td>
<td>ThT</td>
<td>Thioflavin T</td>
</tr>
<tr>
<td>GA</td>
<td>Gestational age</td>
<td>TLC</td>
<td>Thin layer chromatography</td>
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<tr>
<td>HSA</td>
<td>Albumin derived from human serum</td>
<td></td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
<td></td>
<td></td>
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<tr>
<td>LRP-1</td>
<td>Low-density lipoprotein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MVK</td>
<td>Methyl vinyl ketone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NFI</td>
<td>Normalized fold increase</td>
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Chapter 3 and chapter 4, in part, are currently being prepared for submission for publication. Jamie P Do, Kevin Cao, Louise Laurent, Mana Parast, and Jerry Yang “Amyloid-binding Fluorescence Diagnostic Assay for Pre-eclampsia.” I was the primary investigator and author.
ABSTRACT OF THE THESIS

Studies toward the design, synthesis, and evaluation of new therapeutic and diagnostic agents for amyloid-associated diseases

by

Jamie PP Do

Master of Science in Chemistry

University of California, San Diego, 2015

Professor Jerry Yang, Chair

Amyloid-misfolding is a characteristic of many different disorders. This thesis is separated into the focus of pathogenic amyloid entities evident in Alzheimer’s disease and pre-eclampsia.
Alzheimer’s disease (AD) is a neurodegenerative disorder that attributes its toxicity to the presence of amyloid-β aggregates. These aggregates have been documented to confer its toxicity by interfering with the functions of cellular proteins. To reduce toxicity, one of the strategies in the development of an AD therapeutic includes the design of a small molecule that can prevent the interaction of amyloid aggregates with cellular proteins. Current amyloid-binding molecules (BTA molecules) have restricted potential as an AD therapeutic because of their low solubility in aqueous media. To increase the utility of these BTA derivatives, the hydrophilicity of these molecules must be increased. Chapter 2 of this thesis is focused on addressing this solubility issue with the synthesis of a BTA derivative that is decorated with zwitterionic oligomers.

Pre-eclampsia is gestational disorder that has been recently characterized with the presence of amyloid aggregates. This disorder harms the mother and her unborn baby. Despite the alarms that this disorder raises, effective diagnostic measures are limited primarily by the heterogeneous manifestation of clinical symptoms exhibited by patients with pre-eclampsia. The second part of this thesis focuses on a different facet of amyloid misfolding diseases to develop diagnostic tools for pre-eclampsia.
Part I.

Studies toward the design of zwitterionic benzothiazole anilines
to enhance targeting of Alzheimer’s disease-related amyloids
Chapter 1.

Alzheimer’s disease: The rise of an age-dependent chronic disorder

1.1 Introduction

Since its discovery by Dr. Alois Alzheimer’s in November 1905, Alzheimer’s disease has been met with various controversies and different attempted therapeutics.\(^1\)-\(^3\) Among the variations of dementia, Alzheimer’s disease (AD) ranks as the most prevalent, unparalleled in inflicting irrevocable injury to both patients’ cerebral health and to the morale of their family members.\(^4\)

The hallmark damages of AD originate from the initial impairment of the hippocampus, a vital structure for long-term and short-term memory storage.\(^5\) Injuries to the brain impair the patients’ ability to retain memory, communicate, and execute daily functions.\(^5\) Strikingly, such decline in the brain’s function, as evidenced by the physical reduction in the brain size of an AD patient, results in the gradual death of other vital organs and, eventually, the patient.\(^6\)

These serious effects of AD affect 5% of the population over the age of 60, and its prevalence increases sharply with age.\(^4\) Demographic studies have projected a drastic increase in the frequency of the elderly population as the baby boomers generation reach those ages.\(^6\)-\(^9\)
As their numbers rise, predicted cases of AD in the baby boomer population will cause the nationwide frequency of AD cases to skyrocket, amounting to approximately one million cases annually.\textsuperscript{7,8} The future costs of AD in the forms of assisted care and from Medicare and Medicaid will experience a dramatic increase. This fiscal strain will place a heavy burden on American taxpayers, families, and other caregivers alike.\textsuperscript{7} Unfortunately, no therapeutic has been proven effective to prevent AD or reverse its fatal onset. These daunting predictions prompt the need for rapid action for effective therapeutic development since the toll of AD will strike heavily on society within the next 40 years.\textsuperscript{9}

1.2 Pathogenic mechanisms specific to AD

Currently, there are various hypotheses that reason the etiology and the pathology of AD.\textsuperscript{6,10–12} The widely accepted hypothesis among them is the amyloid cascade hypothesis.\textsuperscript{6,13} This hypothesis attributes the toxicity of AD to the accumulation of a peptide, amyloid-beta (Aβ).\textsuperscript{6,13,14} This small peptide is a product of the metabolism of a trans-membrane protein known as the amyloid precursor protein (APP).\textsuperscript{6} Under normal physiological conditions, this trans-membrane protein is cleaved by alpha secretase, followed by gamma secretase (Figure 1.1). Alternatively, this protein can also be cleaved by beta secretase and gamma secretase, releasing the pathogenic Aβ entity. Once formed, this metabolic product resists ubiquitination and subsequent proteolytic degradation.\textsuperscript{4}
Figure 1.1. Schematic for proposed amyloid cascade hypothesis. Reproduced from reference 14.

Furthermore, alternative clearance mechanisms that export Aβ across the blood brain barrier (BBB) are down regulated.$^{15-17}$ Specifically, the expression for low density lipoprotein receptor (LRP-1), a receptor of the low density lipoprotein family responsible for the export of Aβ across the BBB, is reduced.$^{15-17}$ Additionally, the import of Aβ by RAGE, receptor for advanced glycation end products, is up-regulated (Figure 1.2).$^{15-17}$ This imbalance of Aβ contributes to the accumulation of Aβ in the brain.
Figure 1.2. Schematic for the net import of Aβ monomers into the brain of an Alzheimer’s patient. Reproduced from reference 16.

As these Aβ peptides accumulate in the brain, they aggregate and form both soluble and insoluble oligomers and protofibrils.6,18,19 Additionally, these oligomers further aggregate into long fibrils composed of β-pleated sheets, which are energetically stable states due to multiple noncovalent interactions.20 These aggregates are toxic and are believed to play a central role in the development of AD.18,21 The accumulation of these collective aggregates induces various extracellular and intracellular structural changes. Extracellularly, they accumulate as cytoplasmic depositions in the cerebrovascular and leptomeningeal vessels.4 Extracellular aggregates may confer its toxicity by interacting with cellular proteins, such as catalase, and disabling it from reducing hydrogen peroxide to water.22

These Aβ peptides can also permeate the plasma membrane of the neuron and induce structural changes in tau, a protein abundant in neurons.23 This protein maintains the structural integrity of the axon and enables it to properly propagate electrical signals
and elicit a response in the functioning body.\textsuperscript{24,25} However, for an AD patient, tau can be subjected to hyper-phosphorylation because Aβ intervenes with signaling pathways that causes an imbalance between the phosphorylation and dephosphorylation mechanisms.\textsuperscript{23} These changes support the formation of neurofibrillary tangles (NFTs) and disrupts the cytoskeleton of the neuron.\textsuperscript{4,26,27} These changes ultimately endangers the neuron, leading to synaptic dysfunction and oxidative stress. These changes also parallel with the severity of symptoms in AD patients.\textsuperscript{6,25,27}

1.3 \textbf{A novel therapeutic approach: targeting amyloid aggregates using benzothiazole derivatives}

Despite the severe impact of AD in human health, there is no current therapeutic that is effective to prevent or cure AD.\textsuperscript{6} Current AD therapies only address the symptoms associated with AD, such as cognitive decline that results from either the lack of acetylcholine or neuronal death.\textsuperscript{10,28} Therapeutics that compensate for the dearth of acetylcholine act as inhibitors to the hydrolysis of acetylcholine, thereby maintaining sufficient concentrations of physiological acetylcholine so that an electrical signal can be elicited from one neuron to the next.\textsuperscript{10} Current FDA approved therapeutics that decelerate AD progression by inhibiting the hydrolysis of acetylcholine include donepezil, galantamine, and rivastigmine.\textsuperscript{6,10,25}

Aside from the cognitive disability resulting from the dearth of acetylcholine, cognitive decline has also been attributed to an over-excitation of N-methyl-D-aspartate receptors (NMDA).\textsuperscript{6} This receptor can be excited by glutamate, an excitatory
neurotransmitter. However, over-excitation of this receptor has been correlated with neuronal death, epilepsy, and brain damage. Therapeutics that prevent neuronal over-excitation are inhibitors to the NMDA receptor. Currently, an FDA approved therapeutic of this kind is memantine. Despite the aims of current therapeutics, these drugs neither prevent AD-related fatality nor address the causative malfunctions. Instead, these current therapeutics alleviate symptoms associated with the disorder in a small subset of patients.

Therefore, the rational design of new strategies for AD therapeutic is an attractive research area. Cellular targets of the amyloid cascade hypothesis have been explored for the development of amyloid β-centric therapeutics. These therapeutics have been aimed to prevent the formation of the pathogenic Aβ cleavage product or prevent the aggregation of Aβ (Table 1.1). However, these agents have had very limited success in clinical trials.
Table 1.1. Prospective amyloid β-centric therapeutics for AD that exhibited poor results in Phase III clinical trials.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
<th>Target</th>
</tr>
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<tbody>
<tr>
<td>Tramiprosate</td>
<td><img src="image" alt="Tramiprosate Structure" /></td>
<td>Soluble Aβ monomers as inhibitors of amyloid aggregation&lt;sup&gt;29&lt;/sup&gt;</td>
</tr>
<tr>
<td>Tarenflurbil</td>
<td><img src="image" alt="Tarenflurbil Structure" /></td>
<td>γ-secretase&lt;sup&gt;30&lt;/sup&gt;</td>
</tr>
<tr>
<td>Semagacestat</td>
<td><img src="image" alt="Semagacestat Structure" /></td>
<td>γ-secretase&lt;sup&gt;31&lt;/sup&gt;</td>
</tr>
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Because the suggested therapeutic agents shown in Table 1.1 have performed poorly in Phase III of clinical trials, alternative approaches, such as benzothiazole derivatives, have been explored to reduce Aβ-related toxicity (Table 1.2). Unlike the mechanisms of the compounds from Table 1.1, these benzothiazole derivatives have been shown to reduce amyloid-related toxicity by inhibiting the interactions between amyloid aggregates and cellular proteins (Figure 1.3).<sup>22,32</sup>
Table 1.2. Amyloid β-centric benzothiazole derivatives as potential therapeutics for AD.

<table>
<thead>
<tr>
<th>Name</th>
<th>Structure</th>
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<tr>
<td>ThT</td>
<td><img src="image" alt="ThT structure" /></td>
</tr>
<tr>
<td>BTA-EG₄</td>
<td><img src="image" alt="BTA-EG₄ structure" /></td>
</tr>
<tr>
<td>BTA-EG₆</td>
<td><img src="image" alt="BTA-EG₆ structure" /></td>
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The design of these small molecules has been constrained to satisfy requirements necessary for cerebral-targeted therapeutics. Specifically, these cerebral amyloid-targeting molecules must be able to satisfy Lipinski’s Rule of 5 and penetrate the BBB, have a large affinity for amyloid peptides, and be biocompatible. As depicted in Table 2, these small molecule derivatives have been designed to fulfill these criteria with the incorporation of a benzothiazole moiety. This benzothiazole functional group grants hydrophobicity to the cerebral therapeutic and enables passage across the BBB. This moiety is also the structural skeleton of an amyloid-binding molecule, Thioflavin T (ThT). ThT, commonly used as a histological stain, is a fluorophore that detects amyloid peptides in the brains of patients with AD post-mortem.
Despite its ability to bind to amyloid peptides and signal the presence of amyloid aggregates, the charge on ThT restricts its diffusion across the BBB and reduces its potential as a therapeutic for AD.\textsuperscript{36} This constraint has prompted various research groups to functionalize ThT to allow its diffusion across the BBB while retaining its affinity for amyloid peptides.\textsuperscript{22,36} Previously, efforts by Yang and coworkers have addressed this problem by decreasing the hydrophobicity of ThT with the syntheses and applications of
uncharged ThT derivatives. To maintain its biocompatibility in aqueous media, Yang and coworkers introduced ethylene glycol units to the resultant molecule so that it can be readily assayed for *in vivo* and *in vitro* studies. These resultant ThT derivatives became known as benzothiazole aniline (BTA) compounds (Table 1.3). When assayed *in vitro*, it was observed that uncharged ThT derivatives were able to bind to amyloid with a much greater affinity than ThT. Additionally, the binding of BTA derivatives showed cytoprotective effects by inhibiting the interactions between catalase and amyloids. Interestingly, *in vivo* studies with AD mice showed that the usage of these molecules induced the formation of dendritic spines and increased the cognitive ability of AD mice. These observations suggested that BTA derivatives are able to counter the effects of axonal damage during the early stages of AD. Additionally, explorations were made to substitute the aniline nitrogen for sulfur since the BTA analogue with the sulfur is more synthetically practical. The sulfur analogue of BTA has shown to be less toxic than the aniline analogue (unpublished work by coworker in Yang research group).

Despite the efforts to increase the biocompatibility of ThT, the utility of BTA derivatives have been limited due to their insolvability in pure aqueous medium. Thus, to analyze the cyto-protective effects of BTA derivatives at current experimental concentrations with *in vitro* and *in vivo* assays, it is necessary to prepare the BTA compounds in an aqueous solution with DMSO. Therefore, the usage of DMSO as a co-solvent limits the usage of these BTA derivatives as a therapeutic for AD patients since there is no physiological source of DMSO to maintain the solubility of BTA in the human body. In attempt to address this solubility limitation, the aim of this thesis is to functionalize BTA with zwitterionic oligomers, instead of ethylene glycol oligomers, to
enhance the hydrophilicity of the BTA derivatives and increase its therapeutic utility (Figure 1.4).

![Chemical structures of BTA-derivatives](image)

**Figure 1.4.** a) Chemical structure of BTA-EG4. b) Chemical structure of BTA-(carboxybetaine)4.

The inspiration of increasing the hydrophilicity of BTA derivatives with zwitterionic oligomers stems from Keefe’s and Jiang’s observation that, upon the conjugation of zwitterionic polymers to proteins, the protein exhibits an enhanced affinity for its substrate than when conjugated to ethylene glycol oligomers of comparable size.\(^3^9\) In the presence of the zwitterion, there are ion-dipole interactions with the zwitterionic oligomer and water molecules such that there is an entropic driving force that favors stronger hydrophobic-hydrophobic interaction between the substrate and the binding pocket of the protein.\(^3^9\) Incorporating zwitterionic functionality would address our need to increase the hydrophilicity of BTA. Additionally, it would, theoretically, increase the binding affinity between BTA and A\(\beta\) aggregates. This appending benefit and the resultant increased hydrophilicity encourage us to create additional BTA derivatives that can have increased utility to reduce the A\(\beta\)-related toxicity of AD.
1.4 Thesis goal #1

The aim of this research project is to increase the utility of BTA derivatives by functionalizing BTA with zwitterionic oligomers. With this successful derivatization, future studies could evaluate its potential as an AD therapeutic and make comparative assessments with its BTA-EG₄ predecessor. Chapter 2 details the methodology to design the synthesis of a BTA-carboxybetaine molecule.
Chapter 2.

Design and functionalization of benzothiazole aniline with betaines

2.1 Introduction

This chapter presents different synthetic strategies aimed to increase the hydrophilicity of the BTA derivatives, while also retaining biocompatibility and selectivity toward Aβ targeting. Preceding research conducted by Yang and coworkers has shown that amyloid-related toxicity can be attenuated with BTA derivatives. However, compounds such as BTA-EG₄, which exhibited promising in vitro and in vivo activity for treating AD-related cellular injury, suffers from poor solubility in pure aqueous media and a reduced usage as an AD therapeutic. To address this limitation, we set out to conjugate zwitterionic oligomers onto BTA to increase its water solubility. The central hypothesis is that this increased hydrophilicity, by replacing the tetra(ethylene glycol) group in BTA-EG₄ with a tetracarboxy betaine group, will also provide enhanced or comparable benefits as the BTA-EG₄ analogue for maintaining neuronal health.
2.2 The usage of zwitterions as an alternative to ethylene glycol units

Since 1970s, polyethylene glycol (PEG) oligomers and polymers have been introduced to alter the pharmacokinetics of therapeutic molecules and increase the hydrophilicity of an organic molecule, and to prevent self-aggregation of the therapeutic molecule.\textsuperscript{40–42} Despite the attractive features that PEG offers, the exploration and development of alternative hydrophilic appendages remained because the increased usage of PEG also revealed its inherent limitations.\textsuperscript{40} The drawbacks of PEG included the non-biodegradability of PEG adducts, its susceptibility to oxidation to yield hydroxyl acid metabolites, and its resultant toxicity observed with PEG-induced immunogenicity.\textsuperscript{43,44} These setbacks were rectified by using different hydrophilic oligomers, such as zwitterionic oligomers.\textsuperscript{43} The usage of zwitterionic oligomers display a reduced activation of the immune system compared to PEG derivatives.\textsuperscript{43} Additionally, because zwitterionic oligomers contain charge, they are able to form stronger electrostatic interactions with water and are able to decelerate the adsorption of biological entities onto the surface of the protein conjugate, a drawback inherent to PEG that limits its removal from the body.\textsuperscript{43,44}
Because Yang and coworkers have already shown that the synthesized BTA-EG₄ and BTA-EG₆ reduces toxicity in AD in vitro and in mouse models, we aspired to synthesize an analogous BTA derivative consisting of four zwitterionic functionalities for an equal comparison (Figure 2.1). We were initially inspired by the synthesis of the poly(carboxybetaine) by coupling β-propiolactone with a tertiary amine (Figure 2.1).⁴⁵ Such tertiary amine could theoretically readily be afforded from iterative reductive aminations. The final linkage of the tetra(amo) linker could then be conjugated onto BTA. However, it is this final linkage step has been greatly complicated due to the observed inert behavior of BTA, as explored in Section 2.3.2.
2.3 Synthetic design of the betaines

2.3.1 Design of the tetramine linker

We commenced the synthesis of the tetr(amino) linker 7 from commercially available N-aminoethanol 1. The secondary amine of 1 was protected with tert-butyl pyrocarbonate (BOC) and resulted in 82% yield of N-Boc aminoethanol 2 (Scheme 2.1).46 Following, the hydroxyl group of 2 was subjected to oxidation to yield the aldehyde 3. We initially chose to oxidize the hydroxyl group using Albright-Onodera conditions with P_{2}O_{5} as an oxidant.47 However, using Albright-Onodera conditions consistently resulted in 30-45% yields. Then, we looked into using Dess-Martin Periodinane (DMP) as an oxidant. When we performed the DMP oxidation at room temperature, consistently low yields (approximately 30 – 35%) were obtained. This observation led us to hypothesize that the DMP was degrading at room temperature.
Thus, we chose to perform the oxidation using DMP at 0°C. Additionally, we also catalyzed this reaction by performing the oxidation with 35% water in dichloromethane (DCM) (v/v).48 The additive water serves to hasten the rate of decomposition of the Periodinane intermediate and yield the aldehyde product.49 Performing the oxidation in this way afforded the aldehyde 3 in 50 – 60% yield.

The following step was to perform a reductive amination with the aldehyde 3 and the commercially available diamine 3.1 to generate a tertiary amine 4 (Table 2.1). We were initially inspired to perform the reductive amination using Borch conditions as detailed by Frontier and coworkers (entry 1, Table 2.1).50 However, the resultant amine from the crude reaction could not be isolated or visualized by thin layer chromatography (TLC). Because the experimental procedure involved performing aqueous workups, we suspected that a large percentage of the desired product remained in the aqueous phase. We also considered the possibility that the reaction was incomplete. Thus, we allowed the reaction to proceed for 2 days instead of 1 day at pH 7, and yet, we still obtained 25%. We then hypothesized that the low yield result may be a result of inadequate activation of the aldehyde, which would limit for imine formation. To ensure that we activated the aldehyde, we acidified reaction with formic acid, but the yield reduced to 15% (entry 1.1, Table 2.1).

Because we encountered many synthetic difficulties using Borch conditions, we decided to consider different alternatives to generate a tertiary amine. Instead of a reductive amination, we considered reactions in which we could substitute the commercially available diamine 3.1 with a leaving group. The easiest way we considered was to tosylate the hydroxyl group of N-Boc aminoethanol 2, but this was hampered by
16% yield of the tosylated product. This reaction was followed by the addition of the secondary amine 3.1 and an *in-situ* Finkelstein reaction. After 1 day, the reaction was incomplete by TLC, and thus, we refluxed it in acetone for 2 days and added a catalytic amount of 18-crown-6 ether to increase efficiency of the reaction. However, 5 – 10% yields were obtained with these conditions.

Finally, we encountered a published protocol to perform reductive amination in trifluoroethanol (TFE), which improves yield to 40% (entry 2, Table 2.1). This was a particularly exciting result, and we investigated for approaches to increase the yield further. We believed that the bottleneck of the reaction was still the formation of the imine. Our explorations led us to the usage of Lewis acids to activate aldehyde toward reductive amination (entry 3, Table 2.1). We then considered if the choice of the Lewis acid has a significant impact on the yield of the reaction. Because the iron of FeBr$_3$ coordinates to the carbonyl oxygen, we inquired if this interaction can be more favorable with a softer Lewis acid since the carbonyl oxygen is a soft base. Interestingly, the usage of PdCl$_2$ consistently afforded the triamine 4 in 50% yield (entry 4, Table 2.1). These improved yields may be due to a putative stronger soft acid-soft base coordination between Pd$^{2+}$ and carbonyl oxygen.
Table 2.1. Reductive amination strategies to convert aldehyde 3 to triamine 4. Note the commercially available diamine is abbreviated in the scheme and in the text as 3.1 for simplicity.

![Reductive amination scheme](image)

<table>
<thead>
<tr>
<th>Entry</th>
<th>Conditions</th>
<th>pH</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>NaCNBH₃ in MeOH</td>
<td>7</td>
<td>25</td>
</tr>
<tr>
<td>1.1</td>
<td>Same as 1, but catalyzed with formic acid</td>
<td>5</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>NaBH₄ in TFE</td>
<td>-</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>NaBH₄ in MeOH, catalyzed with FeBr₃</td>
<td>-</td>
<td>45</td>
</tr>
<tr>
<td>4</td>
<td>NaBH₄ in MeOH, catalyzed with PdCl₂</td>
<td>-</td>
<td>50-55</td>
</tr>
</tbody>
</table>

The protecting group of 4 was removed using trifluoroacetic acid (TFA), followed by another reductive amination (Scheme 2.1). This second reductive amination required the addition of triethylamine (TEA) to neutralize the TFA salt to afford the tetra(amo) linker 7. The resultant TFA salt needs to be neutralized prior to a conjugation reaction with BTA and then this react with β-propiolactone. We chose to react the molecule with β-propiolactone lastly to minimize the inevitable difficulties that accompany the purification of a zwitterionic molecule.
2.3.2 Functionalization of benzothiazole anilines and generation of BTA-carboxybетaines

![Chemical structure](image)

**Figure 2.2.** A proof-of-principle reductive amination with BTA and aldehyde 2.

Next, we needed to devise a synthetic strategy to conjugate the linker onto BTA. Because our reductive amination procedures had been optimized during the synthesis of the tetra(amine) linker 7, we wanted to utilize the same approach to conjugate the neutralized tetra(amine) linker 7 to BTA. To show that it is possible to conjugate our tetra(amine) linker 7 onto BTA, we performed a test reaction with BTA and aldehyde 2 to yield product 8 in 15% (Figure 2.2). Despite the low yield (attributed to unreacted BTA), this test reaction suggested that a reductive amination can be performed with a poor nucleophile such as aniline of BTA. By using a better nucleophile, such as the secondary amine of our tetra(amine) linker 7, the reductive amination between BTA and tetra(amine) linker 7 could be readily performed. Therefore, to improve our yield and efficiency in the conjugation reaction, we aimed to functionalize BTA with a synthetic handle such that our tetra(amine) linker 7 could be added in a reductive amination.
To perform a reductive amination with BTA, we first need to add an aldehyde for reductive amination with the tetra(amino) linker 7. To generate an aldehyde handle, we initially reacted tert-butyl 3-bromopropionate with BTA under basic conditions. With the ester, the goal was to reduce it to a primary alcohol and then oxidize it to an aldehyde. However, we were only able to isolate 10% of the ester 9 (Figure 2.3). By analyzing the TLC of the crude reaction, we found that the reaction was largely incomplete as evidenced by the unreacted BTA. We believed that a reason for an incomplete reaction is steric hindrance imposed by the tert-butyl group. Therefore, we used methyl 3-bromopropionate to make ester 10 under the same conditions at room temperature and in the microwave reactor (Figure 2.3). Yet, no significant change in the yield was observed because purification was complicated by the presence of many side products. We then
considered making a different electrophilic molecule, carboxylic acid 11. We attempted to couple 3-bromopropionic acid onto BTA with conditions described by Capule and coworkers. However, we encountered solubility difficulties with recrystallizing the carboxylic acid 11 (Figure 2.3). To directly yield alcohol 12, we reacted BTA with 2-bromoethanol at 40°C to obtain our desired alcohol in 30% (Figure 2.3). Following, we wanted to oxidize the hydroxyl functional group of 12 to an aldehyde 15 (Figure 2.4). However, using both DMP and the Albright-Onodera conditions, \(^1\)H NMR showed that aldehyde 15 was not generated. We also considered performing an ozonolysis with product 13 (Figure 2.3). To do this, we reacted BTA with allyl bromide under basic conditions. However, we were only about to isolate 30% because the di-alkylated product was also formed even when allyl bromide was the limiting reagent. However, we also attempted to react BTA with 1,2-dibromoethane under basic conditions to yield product 14 (Figure 2.3). However, with various bases (TEA, N, N-diisopropylethylamine, K\(_2\)CO\(_3\)) and solvents (THF, DMF, DCM), the reaction did not proceed and only starting BTA was observed by \(^1\)H NMR.
Figure 2.4. Schematic diagram that illustrates attempts to react the terminal hydroxyl group of 11.
Because we were able to add an alcohol to BTA to afford 12, we strategized activating the hydroxyl group with a tosylation or an iodination reaction (Figure 2.4). To obtain tosyl 16 and preclude the tosylation at the aniline, the reaction was performed with a catalytic amount of 4-Dimethylaminopyridine (4-DMAP) and TEA. No isolatable product was obtained as this reaction produced only a trace amount of product. We also explored other reactions that could yield products 17 by I$_2$ in the presence of triphenylphosphine or a chlorination reaction with SOCl$_2$ under basic conditions. However, no products were observed, and we suspected that the nucleophilic attack of the activated halogenating agent (phosphonium salt or thionyl chloride) was limited by the steric hindrance of the BTA moiety. We wanted to conjugate molecules 17 with tetra-amino linker 7, but the progress with these reactions was limited by the trace substitution of the hydroxyl group.

![Figure 2.5](image.png)

**Figure 2.5.** Schematic diagram illustrating models used to optimize conditions to functionalize tetra(amino) linker so that it could be conjugated onto BTA.

After unsuccessfully reacting BTA with various electrophiles, we concluded that the reduced reactivity of anilines compared to amines imposed a greater synthetic difficulty than we expected. Instead of generating an electrophilic group on BTA, we
decided to consider adding this moiety onto the tetra(amino) linker 7. We developed a series of simpler studies based on model reactions using a commercially available secondary amine 3.1 (Figure 2.5). Initially, we decided to react secondary amine 3.1 under basic conditions with 2-bromoethanol to yield amine 18 (Figure 2.5). By TLC, it was suspected that low yields of product were obtained, but attempts to purify and quantify these new products were prevented by the immobility of the crude products on normal phase silica. We also considered an alternative stationary phase, basic alumina, but we were unable to visualize the products on alumina. We also considered reacting the secondary amine 3.1 with allyl bromide to yield product 19 (Figure 2.5). Our aim was to react 18 in an ozonolysis reaction to obtain aldehyde 15 (aldehyde 15 shown in Figure 2.4). However, our efforts to perform an ozonolysis were precluded because 19 was only successfully coupled and isolated once with 10% yield. Reproducibility of this reaction could not be achieved by repetition of synthesis or by altering solvents, bases, temperatures, or using the Tsuji-Trost conditions with Pd(PPh3)4 and K2CO3.55
After trying various reactions with allyl bromide, we became inspired by the Michael addition of either the amine or the BTA onto the an \( \alpha,\beta \)-unsaturated ketone or aldehyde. Our synthetic explorations with Michael addition of BTA or an amine onto an \( \alpha,\beta \)-unsaturated system started with the usage of acrolein. The purpose of performing Michael addition reactions with both BTA and the diamine 3.1 was to determine the optimal conditions for Michael addition. When we reacted acrolein with BTA, our aim was to obtain adduct 20 (Figure 2.6). However, we did not observe any Michael reaction products, and only observed the presence of starting BTA. When we tried this Michael addition with acrolein and diamine 3.1 under the same reaction conditions, the reaction generated many products without any evidence of 21 (Figure 2.6). We hypothesized that these side products may have been attributed to the *in-situ* polymerization of acrolein,\(^{56}\) suggesting that polymerization reaction occurs quicker than the Michael addition. Thus,
we considered using an $\alpha,\beta$-unsaturated ketone instead, but this time, with a different and stronger nucleophile, a thiolate (Figure 2.7a).

![Chemical structures of starting materials, BTA and thiolate analogue.]

![Synthetic scheme for formation of thiolate 22 by Pummerer rearrangement and methanolysis and reactions with thiolate 22. c) Attempted reductive amination of ketone 25.]

**Figure 2.7.** a) Chemical structures of starting materials, BTA and thiolate analogue. b) Synthetic scheme for formation of thiolate 22 by Pummerer rearrangement and methanolysis and reactions with thiolate 22. c) Attempted reductive amination of ketone 25.

In order to synthesize BTA-(carboxybetaine)$_4$ using thiophenol BTA, we used a thiophenol BTA derivative that was graciously donated to me by a current coworker in
the Yang group, Kevin Sibucao (Figure 2.7a). Our initial journey with the thiophenol
BTA commenced with the Pummerer rearrangement of the sulfoxide in the presence of
trifluoroacetic anhydride (TFAA) to yield its corresponding acylalkoxy thioether (Figure
2.7b). This step is followed by methanolysis, which generates the hemithioacetal in situ
and loses formaldehyde to generate the thiolate 22. This thiolate 22 was reacted with 2-
bromoethanol to yield alcohol 23 in 75% yield over 3 steps. Attempts to oxidize 23 to
obtain aldehyde 26 using Albright Onodera conditions were complicated by additional
oxidation of the thiol into a sulfoxide and sulfone. Instead, thiolate 22 was used as a
nucleophile in a Michael addition reaction with methyl vinyl ketone (MVK) to yield the
resultant ketone 25 in 65% yield, over 3 steps (Figure 2.7b). This conjugate addition
adduct was then subjected to various reductive amination conditions.

Initially, we tested the reductive amination of the ketone 25 with secondary amine
3.1 before attempting to conjugate the desired tetra(amino) linker 7; we expected to
obtain product 27 (Figure 2.7c). Because the ketone 25 is less reactive to reductive
amination than the corresponding aldehyde, the reaction required a catalytic amount of
TiCl₄ to activate the electrophilic carbon and augment effective imine formation. Because
TiCl₄ is able to chelate alcohols and form complexes with borohydride species, the
reaction was performed in toluene and was removed in an aqueous workup prior to the
addition of sodium borohydride.⁵⁷,⁵⁸ The analysis of the intermediate revealed only
starting material by ¹H NMR without any trace of the secondary amine 3.1. This
observation suggested the absence of the imine formation or the dissolution of the imine
into the aqueous phase. Alternatively, the TiCl₄ may have coordinated at other sites in the
molecule. This could minimize the concentration of the activated ketone required for the
reductive amination. Alternatively, we tried the reductive amination using catalytic PdCl$_2$ in THF. Analogous to previous attempts, we were unable to observe the addition of 3.1 to compound 25. Using $^1$H NMR, it appeared that a degradation product was obtained since the NMR showed aromatic signals but a lack of the methylene protons from the starting material. Unfortunately, the functionalization of the BTA is at where this synthesis stands. Due to the various synthetic challenges that we faced during this synthetic study, we were unable to generate a zwitterionic derivative of BTA.

2.4 Concluding Remarks

During the course of this research, we aimed to synthesize a water-solubilizing derivative of BTA to increase its potential to be used as an AD therapeutic. To achieve this goal, our strategy was to incorporate zwitterionic oligomers as water solubilizing groups. To introduce zwitterionic oligomers, we had to address 3 points:

1. Synthesize the tetra(amino) linker
2. Functionalize BTA such that the tetra(amino) linker could be introduced
3. Add $\beta$-propiolactone to make the final zwitterionic product.

We successfully synthesized the tetra(amino) linker. We have attempted to introduce a synthetic handle onto BTA so that the tetra(amino) linker could be readily conjugated. The functionalization of BTA imposed various synthetic challenges for us. Because of the hindrances that resulted from the low nucleophilicity of the aniline, we used a better nucleophile, the thiophenol derivative of BTA. The usage of this derivative granted us success in incorporating a handle on BTA. Following the functionalization of BTA thiophenol, we encountered additional opportunities to determine the optimal
reaction conditions to conjugate the tetra(amino) linker onto BTA thiophenol. We experienced difficulties in performing a reductive amination with a ketone on BTA thiophenol and an amine (Figure 2.7c). We believed this synthetic obstacle was due to the inert behavior of ketones toward reductive amination. Using an aldehyde, instead of a ketone, is preferred for a reductive amination because an aldehyde is more reactive, as we have recently observed. We have been unable to add an aldehyde onto a BTA derivative through a Michael addition of BTA thiophenol and acrolein (24, Figure 2.7b) because acrolein is prone to in-situ oligomerization, which complicates the synthesis and purification of the desired product. An alternative strategy to introduce an aldehyde onto BTA is generating a Weinreb amide on BTA, followed by a reduction to yield an aldehyde, which could readily participate in a reductive amination to yield the desired product (Figure 2.8).
During this research, we aimed to address 3 points. By exploring how to generate the tetra(amino) linker 7, we have devised a potentially new method to make polyamines using iterative Lewis acid catalyzed reductive aminations. This method may be useful to chemists studying the total synthesis of natural products. While we were unable to conjugate the tetra(amino) linker 7 onto a BTA derivative, our study set the stage for future synthetic studies that aims to introduce zwitterionic functionality onto a BTA derivative.
2.5. Experimental details

2.5.1 Materials and Methods

All synthetic reagents were obtained from Sigma Aldrich, Fluka, Alfa Aesar, or Acros and were used without further purification. Microwave conditions were performed using a Biotage Initiator microwave reactor. Reactions were monitored by TLC using normal phase silica plates and visualized under UV light and/or stained with 10% basic KMnO₄ or cerium ammonium molybdate (CAM). Products were elucidated using a 400 Hz Varian Mercury Plus NMR or JEOL ECA-500 NMR in CDCl₃ or MeOD. Electrospray ionization mass spectrometry (ESI-MS) data was obtained using ThermoFinnigan LCQDECA-MS.

2.5.2 Synthesis for products shown in Scheme 1

Preparation of tert-butyl (2-hydroxyethyl)(methyl)carbamate (2).

2-(methylamino)ethan-1-ol (67 mmol, 6 mL) was dissolved in DCM (130 mL) at room temperature under an inert atmosphere. Following, TEA (83 mmol, 12 mL) and Boc₂O (12 g, 55 mmol) was added. After 2 hours, the reaction mixture was treated with 5% solution of citric acid and extracted with DCM. The organic layer was washed with water, followed by brine, dried with magnesium sulfate (MgSO₄), filtered and concentrated. Flash chromatography (75% hexanes, 25% ethyl acetate on normal phase silica) was used to isolate desired product. N-Boc-aminoethanol 2 was obtained after
concentration in vacuo (8 g, 89%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 3.74 - 3.76$ (t, 5.5 Hz, 2H), $\delta = 3.38 - 3.41$ (t, 5.3 Hz, 2H), $\delta = 2.92$ (s, 3H), $\delta = 1.46$ (s, 9H).

**Preparation of tert-butyl methyl(2-oxoethyl)carbamate (3).** Dess Martin Periodinane (1.4 g, 3.2 mmol) was stirred in 8 mL of DCM at 0°C. After 5 minutes, a solution of N-Boc-aminoethanol 2 (0.5 g, 2.9 mmol) in DCM (1 mL) was added dropwise to the reaction mixture and allowed to stir for 10 minutes. The reaction mixture was then diluted with a solution of 5 μL of H$_2$O in DCM (5 mL). Reaction proceeded overnight at 0°C. The reaction was quenched with the addition of saturated sodium bicarbonate solution (NaHCO$_3$, 25 mL) and saturated sodium thiosulfate solution (Na$_2$S$_2$O$_3$, 25 mL). The resultant mixture was stirred for 1 hr at room temperature, and then partitioned with DCM. The organic layer was partitioned with water, washed with brine, dried with MgSO$_4$, and filtered. The filtrate was concentrated to yield a crude oil, which was purified on normal phase silica (75% hexanes, 25% ethyl acetate to 50% hexanes, 50% ethyl acetate after 1 column volume). Desired product was isolated and concentrated in vacuo to afford N-Boc-aminoethanal 3 (316 mg, 63%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 9.60$ (s, 1H), $\delta = 3.91 - 4.02$ (d, 45.6 Hz, 2H), $\delta = 2.93 - 2.96$ (d, 14.7 Hz, 2H), $\delta = 1.42 - 1.47$ (d, 19.9 Hz, 9H).

**Preparation of tert-butyl (2-((3-dimethylamino)propyl)(methyl)amino)ethyl)(methyl)carbamate (4).**

N-Boc-aminoethanal 3 (500 mg, 2.9 mmol) was stirred in a MeOH (6 mL). The reaction mixture was cooled to 0°C for the addition of PdCl$_2$ (103 mg, 0.6 mmol). The resultant
mixture stirred at 0°C for 5 minutes prior to the addition of N,N,N′-Trimethyl-1,3-propanediamine (1 mL, 3.5 mmol) in 1 portion. The reaction mixture was warmed to room temperature for 10 minutes and then proceeded to stir at 40°C overnight. The reaction was cooled to 0°C prior to addition of NaBH₄ (165 mg, 4.3 mmol). Following the observation of effervescence, the reaction mixture was warmed to room temperature for 5 minutes. Then, the reaction mixture was warmed to 40°C and stirred overnight at 40°C. The reaction mixture was filtered over celite and then concentrated. The desired triamine 4 was isolated using silica gel chromatography (50% MeOH, 50% DCM with 1% TEA) and concentrated in vacuo (410 mg, 52%). ¹H NMR (400 MHz, CDCl₃): δ = 3.26 (s, 2H), 2.83 (s, 3H), 2.43 (s, 2H), 2.34 - 2.37 (t, 2H), 2.15 - 2.25 (m, 12), 1.56 - 1.62 (m, 2H, 1.41 (s, 9H). ESI-MS (m/z) calcd for C₁₄H₃₁N₃O₂ [M]+ 273.24; found [M+H]+ 274.18.

**Preparation of tert-butyl (2-((2-((3-)
(dimethylamino)propyl)(methyl)amino)ethyl)(methyl)amino)ethyl)(methyl)carbamate (7).**

a) Boc deprotection of 4. Prior to the reductive amination to yield product 7, the triamine 4 was deprotected using TFA. To a round bottom flask of 7 mL of DCM was added 4 (375 mg, 1.4 mmol) and 7 mL of TFA. The reaction proceeded for 4 hours at room temperature. Then, the reaction was concentrated using rotary evaporator and then concentrated in vacuo to yield the TFA salt 4.

b) Reductive amination to yield product 7. To a flame-dried round bottom flask with 3 mL of MeOH was added aldehyde 3 (308 mg, 1.8 mmol, 1.2 eq). Prior to adding the TFA
salt 4 to the reaction flask, the TFA salt 4 was treated separately with TEA until the TFA was neutralized. Then, this mixture was added to the reaction flask containing aldehyde 3. After, NaBH₄ (73 mg, 1.9 mmol, 1.3 eq) was added to the reaction flask and the reaction proceeded at room temperature for 24 hours. The reaction was filtered and concentrated via rotary evaporator. Crude reaction mixture was purified on normal phase silica and eluted using 50% MeOH/50% DCM with 1% TEA. Desired product 7 was obtained after concentrated in vacuo. ¹H NMR (400 MHz, CDCl₃): δ = 3.29 (s, 2H), 2.86 (s, 3H), 2.22 - 2.5 (m, 22H), 1.64 - 1.66 (m, 2H), 1.45 (s, 9H). ESI-MS (m/z) calcd for C₁₇H₃₈N₄O₂ [M]+ 330.30; found [M+H]+ 331.24 and [M-Boc]+ 231.26.

2.5.3 Synthesis for selected compounds in Figure 2.2 – 2.7

Preparation of tert-butyl methyl(2-((4-(6-methylbenzo[d]thiazol-2-yl)phenyl)amino)ethyl)carbamate (8). To a round bottom flask with 3 mL of TFE was acidified with 0.01 M HCl. After vigorous stirring, pH was checked using pH paper and the reaction was acidic. Following, aldehyde 2 (408 mg, 2.4 mmol) was added. After 10 minutes, BTA (850 mg, 3.5 mmol) was added as a solid in portions to the reaction mixture. To help dissolve BTA in the reaction mixture, 1 mL of DCM was added. After 7 hours, NaBH₄ (133 mg, 3.5 mmol) was added and the reaction proceeded overnight at 35°C. The crude product was filtered and concentrated by rotary evaporator. Desired product was isolated using normal phase column chromatography (90% DCM/10% hexanes) to afford 8 in 15%. ¹H NMR (400 MHz, CDCl₃): δ = 7.89 (m, 3H), 7.63 (s, 1H),
6.63 (s, 2H), 3.54 (br, 2H), 3.35(t, 2H), 2.90 (s, 3H), 2.47 (s, 3H), 1.46 (s, 9H). ESI-MS (m/z) calcd for C_{22}H_{27}N_{3}O_{2}S [M]+ 397.18; found [M+H]+ 398.23, [M+Na]+ 420.18, [M-Boc]+ 298.34.

Preparation of tert-butyl (4-(6-methylbenzo[d]thiazol-2-yl)phenyl)glycinate (9). To a Biotage microwave vial (2 – 5 mL) was added BTA (200 mg, 0.83 mmol, 1 eq) in 1 mL of THF. Following, DIPEA (0.5 mL, 3.3 mmol, 4 eq) and tert-butyl bromoacetate (0.5 mL, 3.33 mmol, 4 eq) were added and the contents were sealed using a Biotage cap with septum. Contents were vigorously vortexed before the reaction proceeded for 2 hours under at 130°C. Desired product was isolated by flash column chromatography using 75% hexanes:25% ethyl acetate as eluent. Desired product was concentrated in vacuo to afford the desired product 9 (10 mg, 3%). \(^1\)H NMR (400 MHz, MeOD): \(\delta = 7.81 - 7.83\) (d, 8.7 Hz, 2H), 7.75 - 7.77 (d, 8.3 Hz, 1H), 7.70 (s, 1H), 6.67 - 6.69 (d, 8.7 Hz, 2H), 3.89 (s, 2H), 2.46 (s, 3H), 1.47 (s, 9H).

Preparation of 2-((4-(6-methylbenzo[d]thiazol-2-yl)phenyl)amino)ethan-1-ol (12). To a flame-dried round bottom flask containing 4 mL of 2-bromoethanol was added BTA in portions (500 mg, 2.1 mmol, 1 eq). The reaction mixture was observed to be clear and amber in color. The reaction mixture proceeded overnight under reflux at 60°C. Following, 1 M NaOH (aq) was added to the reaction mixture, and partitioned with DCM. The organic layer was washed with H$_2$O, brine, dried with MgSO$_4$, and filtered over celite. Organic filtrate was concentrated by rotary evaporator and crude was purified using normal phase column chromatography (75% DCM:25% ethyl acetate). Solvent was
removed in *in vacuo* to give product 12 (192 mg, 32%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta =$ 7.90 - 7.92 (d, 8.5 Hz, 2H), 7.87 - 7.89 (d, 8.2 Hz, 1H), 6.69 - 6.71 (d, 8.7 Hz, 2H), 3.88 - 3.91 (t, 5.2 Hz, 2H), 3.38 - 3.40 (t, 5.3 Hz, 2H), 2.47 (s, 3H).

**Preparation of N-allyl-4-(6-methylbenzo[d]thiazol-2-yl)aniline (13).** To a flame-dried 10 mL round bottom flask was added BTA (394 mg, 1.65 mmol, 2 eq) in 4 mL of anhydrous THF to give 0.2 M reaction. The reaction proceeded for 10 minutes to completely solubilize BTA. Once BTA was fully dissolved, allyl bromide (100 mg, 72 μL, 0.83 mmol, 1 eq) was added to the reaction mixture and the reaction proceeded overnight at 60°C. Desired product was isolated using normal phase column chromatography (15% ethyl acetate:29% DCM:55% hexanes). Solvent was removed using a rotary evaporator and *in vacuo* to afford product 13 (21.61 mg, 21%). $^1$H NMR (400 MHz, CDCl$_3$): $\delta =$ 7.85 - 7.91 (m, 3H), 7.64 (s, 1H), 6.65 - 6.68 (m, 2H), 5.92 - 5.99 (br, 1H), 5.29 - 5.34 (d, 17.2 Hz, 1H), 5.20 - 5.23 (d, 10.2 Hz, 1H), 3.86 (s, 2H), 2.48 (s, 3H). ESI-MS ($m/z$) calcd for C$_{17}$H$_{16}$N$_2$S [M]$^+$ 280.10; found [M+H]$^+$ 281.31.

**Preparation of N1-allyl-N1,N3,N3-trimethylpropane-1,3-diamine (19).** To a round bottom flask with TEA (383 mg, 527 μL, 3.78 mmol, 2 eq) and 1 mL MeOH/DCM (10%/90%) was added diamine 3.1 (438 mg, 554 μL, 3.78 mmol, 2 eq) and allyl bromide (228 mg, 200 μL, 1.89 mmol, 1 eq) to give a clear yellow reaction. The reaction proceeded for 2 days at 35°C. Following, 9 mL of NaOH solution (aq) was added and the reaction was stirred vigorously. Desired product was extracted into DCM from the
aqueous layer. The organic layer was washed with H₂O, brine, and dried with MgSO₄. The filtrate was filtered with celite and concentrated. Desired product 19 was isolated column chromatography (90% DCM/10% MeOH, 1% TEA). Solvent was removed in vacuo to yield product 19 (31 mg, 10%). ¹H NMR (400 MHz, CDCl₃): δ = 5.81 - 5.89 (m, 1H), 5.11 - 5.18 (m, 2H), 2.98 - 3.00 (dt, 2H), 2.35 - 2.38 (t, 2H), 2.24 - 2.29 (t, 2H), 2.22 (s, 6H), 2.21 (s, 3H), 1.62 - 1.68 (m, 2H). ESI-MS (m/z) calcd for C₉H₂₀N₂ [M]+ 156.16; found [M+H]+ 157.10.

Preparation of 2-((4-(benzo[d]thiazol-2-yl)phenyl)thio)ethan-1-ol (23).

To a 50 mL round bottom flask with 8 mL of DCM was added 2-(4-(methylsulfinyl)phenyl)benzo[d]thiazole (200 mg, 0.66 mmol, 1 eq) and 8 mL of TFAA. The Pummerer rearrangement proceeded for 1 hour under reflux. Following, the excess TFAA was removed by rotary evaporated and the reaction mixture was concentrated in vacuo for 1 hour. Then, the reaction mixture, which consisted of bright yellow flakes, was purged with N₂. Following, 16 mL of TEA in MeOH (50%:50% v/v) was added slowly to the reaction mixture. The reaction mixture was agitated at room temperature until the reaction mixture was completely soluble. Solvent and TEA were removed using rotary evaporation and concentrated in vacuo for 1 hour to yield a yellow oil. This oil was dissolved in 8 mL of THF. Once dissolved, K₂CO₃ (273 mg, 1.98 mmol, 3 eq) and 2-bromoethanol (246 mg, 280 μL, 3.96 mmol, 6 eq) were added. The reaction mixture proceeded overnight at room temperature. K₂CO₃ was filtered over celite. Desired product was isolated using 90% DCM/10% ethyl acetate to give product 23 (141.94 mg,
75% over 3 steps. $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.04 - 8.07$ (d, 1H), 7.99 - 8.01 (d, 2H), 7.89 - 7.91 (d, 1H), 7.48 - 7.51 (td, 1H), 7.44 - 7.47 (d, 2H), 7.37 - 7.41 (td, 1H), 3.82 - 3.86 (q, 2H), 3.21 - 3.23 (t, 2H).

Preparation of 4-((4-(benzo[d]thiazol-2-yl)phenyl)thio)butan-2-one (25)

To a 50 mL round bottom flask with 8 mL of DCM was added 2-((4-(methylsulfinyl)phenyl)benzo[d]thiazole (200 mg, 0.66 mmol, 1 eq) and 8 mL of TFAA. The Pummerer rearrangement proceeded for 1 hour under reflux. Following, the excess TFAA was removed by rotary evaporated and the reaction mixture was concentrated in vacuo for 1 hour. Then, the reaction mixture, which consisted of bright yellow flakes, was purged with N$_2$. Following, 16 mL of TEA in MeOH (50%:50% v/v) was added slowly to the reaction mixture. The reaction mixture was agitated at room temperature until the reaction mixture was completely soluble. Solvent and TEA were removed using rotary evaporation and concentrated in vacuo for 1 hour to yield a yellow oil. This oil was dissolved in 8 mL of THF. Once dissolved, K$_2$CO$_3$ (273mg, 1.98 mmol, 3 eq) and methyl vinyl ketone (424 mg, 505 $\mu$L, 6.06 mmol, 9 eq) were added. The reaction mixture proceeded overnight at room temperature. K$_2$CO$_3$ was filtered over celite. Desired product was isolated using 90% DCM/10% ethyl acetate to give product 25 (126.30 mg, 65% over 3 steps). $^1$H NMR (400 MHz, CDCl$_3$): $\delta = 8.05 - 8.07$ (d, 8.2 Hz, 1H), 8.00 - 8.02 (d, 8.4 Hz, 2H), 7.89 - 7.91 (d, 8.2 Hz, 1H), 7.47 - 7.51 (t, 7.5 Hz, 1H), 7.37 - 7.41 (m, 3H), 3.21 - 3.24 (t, 7.3 Hz, 2H), 2.81 - 2.85 (t, 7.1 Hz, 2H), 2.18 (s, 3H).
Part II.

Studies to develop diagnostic tool for pre-eclampsia
3.1 Introduction

Pregnancy is an experience that is complete with immense wonder and change. During the 40 weeks of pregnancy, the woman’s body undergoes an array of crucial changes to ensure a successful pregnancy and fetal development.\textsuperscript{59,60} However, for approximately 3 – 5% of pregnant women, their pregnancy is complicated by pre-eclampsia.\textsuperscript{61–64} Pre-eclampsia is currently an incurable pregnancy complication that occurs during the third trimester and threatens the lives of the mother and her unborn baby.\textsuperscript{62,65,63,66,67}

The pathology of this disease is closely associated to the placenta, rather than the fetus.\textsuperscript{65,68} This association of the pathology with placenta has been confirmed because pre-eclampsia can develop in the presence of hydatidiform moles, irregular masses that form in the uterus at the beginning of pregnancy.\textsuperscript{65,68} Currently, the only solution for the pre-eclampsia is the removal of the placenta by delivery.\textsuperscript{69,70}

In order to expand the clinical understanding of pre-eclampsia, many researchers have studied its pathophysiology. The widely accepted factors in the development of this disease are the irregularities stemming from placentation.\textsuperscript{62,63,71} These abnormalities manifest as various symptoms exhibited by both the mother and the fetus. Pre-eclampsic women display a range of systemic complications such as renal insufficiency, liver injury, and cerebral perturbations.\textsuperscript{62,65} Many of her organs, including the placenta,
experience a reduction in blood circulation that results from the constriction in the blood vessels. This reduced circulation ultimately leads to organ damage. Furthermore, in the severe cases, the disorder can lead to eclampsia, threatening the mother with grave and unexpected brain seizures.\textsuperscript{62}

These maternal effects directly endanger the fetus as they potentially compromise the time required for the fetus to sufficiently grow and mature. Approximately, 10\% (and possible as much as 67\%) of fetuses experience intrauterine growth restriction.\textsuperscript{65} Furthermore, if the condition of the mother worsens such that a hasty delivery is required, 10 – 15\% fetuses experience preterm delivery, the birth of a unborn baby before 37 weeks of gestation.\textsuperscript{62} Neonates who are delivered before 37 weeks of gestation are more likely to suffer from neonatal respiratory distress and neurological impairments.\textsuperscript{72,73} Furthermore, pre-eclampsia is a dangerous complication to both the mother and her baby because both have a higher risk of developing cardiovascular and metabolic diseases in later life.\textsuperscript{63,69}

\section*{3.2 Paucity in prevention, diagnosis, and treatments}

Because of the alarming complication that pre-eclampsia has risen to physicians and mothers alike, scores of research has aimed to develop therapeutics, diagnostics, and preventions for this disorder.\textsuperscript{61,62,65,66,74,75} Currently, the onset of hypertension after 20 weeks of gestation and proteinuria are considered diagnostic criteria.\textsuperscript{61,62,65} Hypertension has been defined as the blood pressure reading of \( \geq 140/90 \) mmHg that is obtained from 2 different readings from at least 4 – 6 hours apart.\textsuperscript{62,65,76} Proteinuria describes any
urinary excretion that contains $\geq 300$ mg of protein/24 hour urine collection or $\geq 1^+$ from a urine dipstick test. However, the relevance of proteinuria as a diagnostic parameter has been debated by many researchers. Some research groups omit proteinuria as a diagnostic parameter because pre-eclampsia can develop before the renal insufficiency causes proteinuria. Furthermore, the usage of the dipstick test has been contested as a reliable method, especially since dipstick test results will vary tremendously throughout the day. Severe proteinuric patients can be readily detected, but patients with trace amounts of urinary proteins have been falsely regarded as negative. Furthermore, Verlohren and coworkers has reported that the combination of blood pressure readings and proteinuria lack the sensitivity and specificity to predict the severity of maternal and fetal outcomes.

In search for more effective detection methods for pre-eclampsia, the expression of different angiogenic and anti-angiogenic growth factors have been explored. Among the angiogenic factors screened during pregnancy is the placental growth factor (PlGF); this growth factor is released by the placenta and induces the development and perfusion of blood vessels during embryonic development. Clinicians have also looked for anti-angiogenic factors; among this category of biomarkers are soluble endoglin (sEng) and soluble fms-like tyrosine kinase (sFlt-1). The levels of these biomarkers are higher in patients with pre-eclampsia than the healthy counterpart. Powe and coworkers have reported these anti-angiogenic growth factors inhibit the action of PlGF. This leads to symptoms consistent with pre-eclampsia such as vasoconstriction, hypertension, and proteinuria. However, some researchers have differing perspectives on the diagnostic power of angiogenic biomarkers. Lehnen and coworkers have found the
usage of the ratio of sFlt-1/PlFG to exhibit high specificity, but compromised sensitivity. In their study, they showed that this diagnostic parameter is not a reliable predictor of pre-eclampsia, but only a predictor for severe pre-eclampsia.

Table 3.1. Clinical manifestations of presented by women with pre-eclampsia. (* denotes HELLP syndrome).

<table>
<thead>
<tr>
<th>Clinical Manifestations of Pre-eclampsia</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypertension (≥ 140/90 mm Hg)</td>
</tr>
<tr>
<td>Proteinuria (≥ 300/day)</td>
</tr>
<tr>
<td>Hemolysis*</td>
</tr>
<tr>
<td>Elevated levels of liver enzymes</td>
</tr>
<tr>
<td>(aminotransaminases)*</td>
</tr>
<tr>
<td>Thrombocytopenia*</td>
</tr>
<tr>
<td>Coagulation irregularities</td>
</tr>
<tr>
<td>Elevated serum uric acid levels</td>
</tr>
<tr>
<td>Reduced renal flow</td>
</tr>
<tr>
<td>Reduced plasma volume</td>
</tr>
</tbody>
</table>

These efforts to develop diagnostic agents have been hampered continually, and it is a direct result of the multitude of pre-eclampsic symptoms. The heterogeneity in the pre-eclampsic symptoms is shown in Table 3.1. The pre-existing complications – such as hypertension, diabetes mellitus, and other disorders – compromise the detection accuracy as well as hinder the development of effective diagnostics. Because the diagnosis of pre-eclampsia may be superimposed onto a pre-existing condition, such as hypertension, a combination of markers shown on Table 3.1 must be considered. Additionally, some patients exhibit different signs that have been accepted to be associated with pre-eclampsia; these symptoms are known as HELLP (hemolysis, elevated liver enzymes, and low platelets), which are also shown in Table 3.1.
Due to these variations in clinical symptoms, clinicians segregate the pre-eclampsic population into different subpopulation based on the severity of presented symptoms (Table 3.2).\textsuperscript{62,85}

**Table 3.2. Classifications of pre-eclampsia.\textsuperscript{62,65,86}**

<table>
<thead>
<tr>
<th>Classification</th>
<th>Clinical Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severe pre-eclampsia</td>
<td>• BP $\geq 160/110$ mm Hg</td>
</tr>
<tr>
<td></td>
<td>• $&gt; 5$ g/24 hr urine sample, or $+3$ dipstick</td>
</tr>
<tr>
<td></td>
<td>• Intrauterine fetal growth restriction, epigastric pain, any of HELLP symptoms, neurological disturbances</td>
</tr>
<tr>
<td></td>
<td>• BP $\geq 140/90$ mm Hg</td>
</tr>
<tr>
<td>Mild pre-eclampsia</td>
<td>• $&gt; 300$ mg/24 hr urine sample, or $+1$ dipstick</td>
</tr>
<tr>
<td></td>
<td>• Onset of proteinuria in woman with chronic hypertension before gestation</td>
</tr>
<tr>
<td>Superimposed pre-eclampsia</td>
<td>• Development of HELLP symptoms</td>
</tr>
</tbody>
</table>

It should be capitulated that symptomatic variability complicates the development of pre-eclampsic therapeutics since because these symptoms may suggest different hypertensive or renal complications, rather than specifically identify pre-eclampsia.\textsuperscript{86} Furthermore, it prevents the establishment of uniform diagnostic parameters by health professionals from international regions.\textsuperscript{87}

### 3.3 Bridging amyloid diseases with pre-eclampsia

In the midst of the enigma that pre-eclampsia presents to many, novel insight regarding pathogenic entities emerged when Buhimschi and coworkers discovered
amyloid aggregates in the placenta and urine from pre-eclampsic women who were both hypertensive and proteinuric. Their findings were supported with the analysis of the aggregates that exhibited “congophilia” for Congo Red (CR) and binding to ThT. CR and ThT are small molecules that bind to amyloid proteins, and the Buhimschi’s observed binding of the urinary aggregates to both CR and ThT are consistent with characteristics that are widely accepted for amyloids. These urinary aggregates were characterized to be fibrillar oligomers and protofibrils, precursors to the formation of aggregates in various protein misfolding disorders. Additionally, they showed that these aggregates originated from the placenta, offering new attributes to the placenta as its role in the pathophysiology of pre-eclampsia.

These initial findings suggest that the aggregates could act as a potential biomarker for pre-eclampsia. However, the diagnostic usage of these aggregates requires further evaluation. The presence of amyloid aggregates welcomes the investigation of the role of amyloid aggregates in the pathogenesis of pre-eclampsia. We were very enthused by the potential of Buhimschi’s initial reporting because, if amyloid aggregates are characteristic to the development of pre-eclampsia, we could potentially develop diagnostic tools for pre-eclampsia using probes intended for diagnosis of Alzheimer’s disease. The class of probes of interest were originally derived from Theodorakis, Yang and coworkers and are known as ANCA (amino naphthalenyl-2-cyano-acrylate). Molecules of this class are fluorophores that exhibit an enhancement in fluorescence intensity and an anti-Stokes shift in the presence of Alzheimer’s disease-related amyloids, Aβ (Figure 10). Specifically, the usage of ANCA-11 was explored because it exhibited the largest enhancement in fluorescence intensity in the presence of
Aβ (1-42).\textsuperscript{93} Efforts were also made to generate amide derivatives of ANCA, which are more stable than the ester analogues of ANCA (Figure 3.1b). These amide derivatives are more resistant to hydrolysis when analyzed using \textit{in vivo} and \textit{in vitro} assays. Additionally, Kevin Cao, a current member of the Yang group, explored the use of more stable derivatives of ANCA resulting in the birth of an additional amyloid-binding fluorescent probe, AMDX-201.\textsuperscript{94} Because of this amide derivative is more chemically stable and exhibits an enhanced fluorescence emission in the presence of amyloid aggregates compared to in absence of amyloid aggregates, we aimed to extend the usage of AMDX-201 from Alzheimer’s disease to pre-eclampsia.

![Figure 3.1. a) Structure of ANCA-11. b) Structure of amide derivative of ANCA-11. This derivative is called AMDX-201. c) Fluorescence emission spectrum of ANCA-11 in absence and in the presence of Aβ. Structures and spectrum were reproduced from reference 93.](image)

### 3.4 Thesis goals #2

Using AMDX-201, the goal of this thesis project was to investigate its diagnostic utility for pre-eclampsia. Consistent with the precedent observation that pre-eclampsic
women contain amyloid aggregates, we hypothesized that the application of Alzheimer’s disease-related amyloid probes can be extended to detect amyloid aggregates and distinguish pre-eclampsic patients from healthy patients. Our underlying hypothesis is that the ease of detection of these aggregates would be related to the total concentration of urinary protein. Chapter 4 details the feasibility phase of our investigation, during which we explored the utility of AMDX-201 to distinguish pre-eclampsic patients from healthy patients. We also assessed its detection performance with a standard amyloid-binding fluorophore, ThT. In section 4.3, we expanded utility of the amyloid-binding fluorophores in a blind prospective study as we assessed additional factors (such as gestational age, pre-existing conditions, and proteinuria) associated with the pathology of pre-eclampsia. Together, with these two phases of our study, we wanted to address the following questions:

- Can we discriminate healthy patients from pre-eclampsic patients using amyloid-targeting fluorescent probes?
- Does the usage of AMDX-201 grant superior detection performance than the standard amyloid-binding probe, ThT?

Notes about the chapter

Chapter 3, in part, is based on material that is currently being prepared for submission for publication. Jamie P Do, Kevin Cao, Louise Laurent, Mana Parast, and Jerry Yang “Amyloid-binding Fluorescence Diagnostic Assay for Pre-eclampsia.” I was the primary investigator and author.
Chapter 4.
Methodology of a diagnostic assay designed for pre-eclampsia-related urinary amyloid-peptides

4.1 Introduction

According to the American College of Obstetricians and Gynecologists, the only established diagnostic parameter for pre-eclampsia is chronic hypertension during pregnancy. However, this parameter may also suggest comorbidity or the presence of different disorders.\textsuperscript{67} Interestingly, Buhimschi and coworkers recently noted that patients with pre-eclampsia can be distinguished from a healthy cohort by the presence of misfolded amyloid-like proteins found in the urine and in the placenta of women with pre-eclampsia.\textsuperscript{86} To further extend this basic finding into a platform for diagnosis, we were excited to investigate the diagnostic application of fluorescent amyloid-binding probes such as AMDX-201 for detection of amyloid species in the urine of patients with pre-eclampsia. Additionally, we sought to compare if AMDX-201 offers any advantage in terms of sensitivity and specificity compared to the performance of the traditional amyloid-binding fluorescent probes such as ThT. This chapter details the initial steps taken to design a diagnostic assay for pre-eclampsia based on the detection of urinary amyloid proteins.
4.2 Feasibility phase to detect amyloid peptides in maternal specimen

The design of our fluorescence amyloid urine assay was based largely on the methods reported by Buhimschi, which we divided into a feasibility phase and a validation phase. During the feasibility phase, our goal was to determine whether we could discriminate a small number of healthy patients from pre-eclampsic patients using AMDX-201 in urine samples. These urine samples and those detailed in the following section were graciously provided by Dr. Mana Parast (UCSD Department of Pathology) and Dr. Louise Laurent (UCSD Department of Reproductive Medicine). The clinical characteristics for these patients and collection method are detailed in Section 4.5.1. With fluorescence spectroscopy, we analyzed the urine samples from healthy pregnant women (n = 4) and pre-eclampsic patients (n = 4). Additionally, we quantified the total concentration of urinary proteins in each urine sample. We excluded the use of a CR absorbance assay as described by Buhimschi due to the limited volume of urine samples that could be accessed. Briefly, Buhimschi and coworkers reported that urine samples were to be lyophilized and the total protein content of each sample were to be normalized to a concentration that showed linearity with the CR assay, 6 mg/mL total protein. However, because the average concentration of total protein from the urine samples we analyzed was 0.2 mg/mL, suggesting we would need > 30 mL of each urine sample on average in order to carry out the analysis with CR, which was well above the volume that was available to us.

A consequence of excluding the CR assay in our studies was that we would not be able to estimate the total concentration of urinary amyloids in our specimens. However, we were able to optimize conditions to generate the largest enhancement in observed
fluorescence when ThT or AMDX-201 bound to amyloids in solution. While we observed some nonspecific binding of the fluorophores to non-amyloid serum proteins, such as immunoglobulin G (IgG), albumin from human serum (HSA), and insulin, we observed a considerable enhancement in the fluorescent intensity for both fluorophores when they were exposed to aggregated Aβ (1-42) (Figure 4.1). In the presence of 0.5 mg/mL Aβ (1-42), we observed a 5-fold difference in the normalized fold increase (NFI) of fluorescence intensity compared to AMDX-201 in the presence of 0.5 mg/mL HSA (p = 0.3095, Figure 4.1a). Also, the NFI in fluorescence intensity of bound AMDX-201 in the presence of 0.5 mg/mL Aβ (1-42) was significantly (50-fold) larger than the NFI of AMDX-201 in the presence of the 0.5 mg/mL of IgG or 0.5 mg/mL of insulin (p = 0.0095, Figure 4.1a). Similar to AMDX-201, the NFI of ThT in the presence of Aβ (1-42) was significantly (7-fold (p = 0.0095) to 70-fold) greater than the NFI of ThT in the presence of the IgG or insulin (p = 0.0095, Figure 4.1b).

We were able to detect a fluorescence signal that corresponded to the bound probe when the probe was in the presence of ≥ 0.015 mg/mL of Aβ (1-42). Therefore, if the urine contained ≥ 0.015 mg/mL of urinary amyloids, then it could be readily detected and theoretically used to discriminate the healthy from pre-eclampsic patients. Collectively, the significant enhancement in the fluorescence intensity of the fluorophores in the presence of Aβ (1-42) compared to the other proteins demonstrates the preference of these probes for amyloid peptides.
Upon assessing urine samples in our feasibility phase, AMDX-201 in pre-eclampsic urine exhibited distinct spectroscopic characteristics from the characteristics of AMDX-201 in healthy urine (Figure 4.2a). In the presence of pre-eclampsic urine, there was an approximate significant enhancement in fluorescence intensity of AMDX-201 in pre-eclampsic urine compared to AMDX-201 in PBS and pre-eclampsic urine in PBS. This enhancement in fluorescence of AMDX-201 was absent when the fluorophore was assayed in healthy urine (Figure 4.2b) as there is no difference in the NFI between
AMDX-201 in PBS and AMDX-201 in healthy urine. Using the same sample preparation and fluorescence spectroscopic assay parameters, the other urine samples were analyzed and tabulated in Table 4.1. Fluorescence intensities for both pre-eclampsic and healthy urine samples are expressed as the NFI of the fluorophore (AMDX-201) in pre-eclampsic or healthy urine. NFI of AMDX-201 was computed as the ratio of the maximal fluorescence intensity of AMDX-201 in pre-eclampsic or healthy urine and the maximal fluorescence intensity of AMDX-201 in PBS.
Figure 4.2. Fluorescence spectra of AMDX-201 in a) pre-eclampsic urine and b) healthy urine.

The NFI of AMDX-201 from one of the urine samples, 1020_P1, appeared to be an outlier because the NFI of AMDX-201 of patient 1020_P1 appeared different from the NFI of the rest of the pre-eclampsic patients (Table 4.1). Because these data do not assume a Gaussian distribution, this data must be fitted to a Gaussian distribution using a logarithmic transformation before an outlier test is applied. Upon applying the Grubb’s detection method for outliers, we determined that this urine sample was not an outlier.
(Refer to the note regarding usage of Grubb’s method after fitting the data to a Gaussian distribution using logarithmic transformation of this pre-eclampsic cohort in Section 4.5.4).

**Table 4.1.** Summary of results from feasibility trial. * Patients who were normotensive and who were not proteinuric were defined as “healthy.”

<table>
<thead>
<tr>
<th>Urine specimen</th>
<th>Protein content (mg/mL)</th>
<th>NFI AMDX</th>
<th>NFI ThT</th>
<th>Outcome*</th>
</tr>
</thead>
<tbody>
<tr>
<td>0820_P1</td>
<td>-</td>
<td>4.32</td>
<td>-0.95</td>
<td>Severe pre-eclampsia, proteinuric</td>
</tr>
<tr>
<td>1020_P1</td>
<td>2.56 ± 0.07</td>
<td>19.12</td>
<td>0.68</td>
<td>Superimposed severe pre-eclampsia, proteinuric</td>
</tr>
<tr>
<td>1111_P1</td>
<td>1.20 ± 0.09</td>
<td>2.88</td>
<td>0.90</td>
<td>Severe pre-eclampsia, proteinuric</td>
</tr>
<tr>
<td>1114_P1</td>
<td>0.32 ± 0.06</td>
<td>2.53</td>
<td>0.97</td>
<td>Severe pre-eclampsia, proteinuric</td>
</tr>
<tr>
<td>1205_P1</td>
<td>0.04 ± 0.05</td>
<td>0.45</td>
<td>0.45</td>
<td>Healthy</td>
</tr>
<tr>
<td>1205_P2</td>
<td>0.07 ± 0.05</td>
<td>0.83</td>
<td>0.83</td>
<td>Healthy</td>
</tr>
<tr>
<td>1208_P1</td>
<td>1.01 ± 0.14</td>
<td>0.45</td>
<td>0.45</td>
<td>Healthy</td>
</tr>
<tr>
<td>1209_P1</td>
<td>0.40 ± 0.06</td>
<td>1.98</td>
<td>0.50</td>
<td>Healthy</td>
</tr>
</tbody>
</table>

Using the NFI of AMDX-201, the pre-eclampsic cohort was significantly different from the healthy cohort (Figure 4.3a, n = 4, p = 0.0286). Interestingly, when the two cohorts were compared using the NFI of ThT, the populations were not significantly different (Figure 4.3b, p = 0.4857). Although the comparison between the detection
performance of ThT and AMDX-201 are different, it is not justified to conclude that
AMDX-201 elicited a superior performance. With only 8 patients enrolled in our
feasibility trial, we were unsure if the observed difference would be representative of the
entire pre-eclampsic and healthy population. Additionally, the time of urine collection (as
these samples were collected at different times), the diet of the patient, other pre-existing
condition may contribute to the differences within and between the cohorts. Despite the
ambiguity that may stem from this feasibility phase, we were able to see that AMDX-201
was able to discriminate between the healthy and pre-eclampsic population. To assess its
diagnostic utility, we needed to expand the scope of our study.
Figure 4.3. a) NFI of AMDX-201 in pre-eclampsic and healthy patients. b) NFI of ThT for pre-eclampsic and healthy patients. Red line represents sample median as determined using Mann-Whitney test. The bracket and asterisk denotes significance.

4.3 Correlation of diagnostic efficiency of amyloid-binding fluorophores with gestational age and proteinuria

To further assess the implications from the feasibility phase, we continued our pre-eclampsic studies to obtain a definitive answer to our initial inquiry. Going forward with our pre-eclampsic analyses, we designed our study as a blind prospective study with a goal of including 100 urine samples. (The selection criteria for patient enrollment and
clinical characteristics are noted in Section 4.5.1). By designing the experiment in this way, we were able to survey a population assess if factors, such as gestational age (GA) or total urinary protein content, affected the detection performance of the probes. As we have furthered Buhimschi’s initial findings, our research interest has broadened from discriminating a healthy cohort from a pre-eclampsic cohort based on spectroscopic analyses of urine specimens. Now, our research questions have expanded to include: can proteinuria and gestational age affect our discrimination of healthy cohorts from pre-eclampsic cohorts? Such insight may explain our observations from our feasibility phase. Additionally, it may enable us to determine if our discrimination is only effective for pre-eclampsic patients who ultimately develop more severe cases of pre-eclampsia or if we are able to diagnose pre-eclampsia asymptotically, prior to the onset of pre-eclampsia-related hypertension that becomes evident after 20 weeks of gestation.

From the time we started our prospective study in March 2015, we acquired and analyzed 88 urine samples (out of which 39 patient outcomes have been adjudicated to date). At the beginning of this study, we hypothesized that we can use fluorophores to discriminate pre-eclampsic from healthy patients, and the ease of distinction would be based on the presence of urinary aggregates and severity of disease. To test our hypothesis, we considered the relationship of the NFI of the probes and total protein content. Since the more severe cases of pre-eclampsia are classified by increased blood pressure readings and higher levels of urinary proteins, we hoped to see that this representation of our data would show the more proteinuric patients correlated to higher NFI values, which we predicted to be attributed to more severe cases of pre-eclampsia.
Additionally, because pre-eclampsia is a gestational disease that manifests during the third trimester, we envisioned that the usage of fluorescent probes would offer more effective towards the end of the pregnancy.

In order to assess if our ease of diagnosis of pre-eclampsia was correlated by the gestational age or by total urinary protein, we first had to ensure if the pre-eclampsic population could be significantly discriminated from the healthy cohort using our methods. Using the median NFI of AMDX-201, the pre-eclampsic patients exhibited a significant 2-fold difference from the healthy cohort (p = 0.0035, Figure 4.4a). When the pre-eclampsic population was compared with the population with “other diseases” (UTI, diabetes mellitus, hypertension, or renal complications), the median NFI of AMDX-201 of the two cohorts was not significantly different (p = 0.0503, Figure 4.4b). When this pre-eclampsic population was divided in accordance of their classifications, we were only able to provide statistical analyses for the superimposed pre-eclampsic patients (n = 6), but not the mild pre-eclampsic (n = 1) nor the severe pre-eclampsic (n = 2) cohorts. The rationale of segregating the entire pre-eclampsic population into their sub-cohorts was to determine if we were able to distinguish different classifications of pre-eclampsia more effectively. After we separated the pre-eclampsic population into sub-cohorts, the median NFI of AMDX-201 of the superimposed pre-eclampsic cohort was significantly 3-fold higher compared to the healthy patients (p = 0.0242, Figure 4.4c). However, the superimposed pre-eclampsic cohort was not statistically different from the cohort with other diseases (p = 0.1447, Figure 4.4d).
Figure 4.4 Comparative representation of pre-eclampsic population and healthy patients using NFI of AMDX-201. a) Comparison of entire pre-eclampsic population and healthy patients. Non-pregnant patients, a paternal patient, and patients who were not diagnosed with hypertension, diabetes mellitus, or proteinuria were classified as “healthy.” b) Comparison of entire pre-eclampsic population and patients who presented “other diseases.” Patients who were diagnosed with hypertension, renal complications, diabetes, or a UTI were classified as patients with “other diseases.” c) Comparison of superimposed pre-eclampsic patients (suPE) and healthy patients. d) Comparison of superimposed pre-eclampsic population and patients who presented pre-eclampsia-like symptoms. For all of the graphs, the red line represented the median. The bracket and asterisk denotes significance.

The NFI of ThT for the pre-eclampsic population was also significantly different from the healthy cohort, but by a 1.5-fold difference (p = 0.0028, Figure 4.5a). Compared to the cohort with “other diseases”, the NFI of ThT of the entire pre-eclampsic population was not significantly different (p = 0.673, Figure 4.5b). Likewise, the superimposed pre-
eclampsic cohort was not significantly different from the cohort with other diseases (p = 0.9433, Figure 4.5d). However, the median NFI of ThT of the superimposed pre-eclampsic population, as observed with the NFI of AMDX-201, was significantly 1.5-fold higher than the median NFI of ThT of the healthy population (p = 0.0134, Figure 4.5c).

Figure 4.5. Comparative representation of pre-eclampsic population and healthy patients using NFI of ThT. a) Comparison of entire pre-eclampsic population and healthy patients. b) Comparison of entire pre-eclampsic population and patients who presented “other diseases.” c) Comparison of superimposed pre-eclampsic patients (suPE) and healthy patients. d) Comparison of superimposed pre-eclampsic population and patients who presented pre-eclampsia-like symptoms. For all of the graphs, the red line represented the median. The bracket and asterisk denotes significance.
Because proteinuria is a characteristic of pre-eclampsia, we also assessed whether these patients had significantly different protein content in their urine. The median of total protein content of the entire pre-eclampsic population had significantly 4-fold more protein in their urine compared to the median of the healthy population (p < 0.001, Figure 4.6a). Compared to the median concentration of total urinary protein in patients with diseases other than pre-eclampsia, the median concentration of urinary protein in the entire pre-eclampsic population was not significantly different (p = 0.0932, Figure 4.6b). Furthermore, after the pre-eclampsic population was divided, the superimposed pre-eclampsic population remained to be statistically different than the healthy population (p = 0.0008, Figure 4.6c). But, this superimposed pre-eclampsic population was not statistically different from the cohort with diseases other than pre-eclampsia (p = 0.0872, Figure 4.6d).
Figure 4.6. Comparative representation of pre-eclampsic population and healthy patients using the concentration of the total urinary proteins. a) Comparison of entire pre-eclampsic population and healthy patients. b) Comparison of entire pre-eclampsic population and patients who presented “other diseases.” c) Comparison of superimposed pre-eclampsic patients (suPE) and healthy patients. d) Comparison of superimposed pre-eclampsic population and patients who presented pre-eclampsia-like symptoms. For all of the graphs, the red line represented the median. The bracket and asterisk denotes significance.

Thus far, we observed that the collective pre-eclampsic population was statistically different from the healthy population using fluorescence of both probes and the concentration of total proteins. However, using these methods of discrimination, it was not possible to distinguish the entire pre-eclampsic population from the patients diagnosed with diseases other than pre-eclampsia. Furthermore, despite our efforts to offer more effective discrimination between different classifications of pre-eclampsia, we
were unable to complete this analysis due to a limited sample size of patients with confirmed pre-eclampsia in our study. Thus, we limited our comparisons solely to the superimposed pre-eclampsic cohort. By comparing the superimposed pre-eclampsic cohort with the healthy population, we were able to observe statistical differences between these two groups. Contrarily, we were unable to discriminate the superimposed pre-eclampsic patients from patients with pre-eclampsia-like symptoms. Our inability to discriminate the pre-eclampsic cohort from the patients with other diseases is consistent with the reported difficulty to differentiate between patients who actually develop pre-eclampsia and patients who exhibit pre-eclampsia-like symptoms.65,83,95 Furthermore, our observations accentuate widely observed diagnostic challenges as this surreptitious disease manifests in various features that are often complicated with the presence of pre-existing conditions.

This difficulty to discriminate pre-eclampsic patients from patients presenting other disorders complicated our assessment of how gestational age and total protein content contribute to the diagnostic efficiency of pre-eclampsia using fluorescent probes. Although we previously showed that the entire pre-eclampsic population was significantly different from the healthy population, we also see that there are no clear boundaries that segregate the different populations of patients (Figure 4.7 and 4.8).
Because there is no clear trend observed between total protein content and the NFI of the fluorophores, these two measures tell different pieces of information. Currently, we are unsure if the detection efficiency based on urinary proteins – specifically urinary amyloids – would provide accurate discrimination of different severities of pre-eclampsia or distinguish between pre-eclampsia and pre-eclampsia-like patients (Figure 4.7 and 4.8). Specifically, using either the NFI of AMDX-201 or ThT, we saw no significant difference between the healthy population and the population with “other diseases” (p = 0.4094 and p = 0.673, respectively). Their similarity was not expected because only pre-eclamptic patients were predicted to exhibit a significantly different reaction to the fluorophores. However, we previously have shown that, using AMDX-201 and ThT, the pre-eclamptic patients and the “other patients” were also not
significantly different. We believe that our current inability to distinguish these two populations is also a result from the limited number of confirmed pre-eclampsic patients in our study.

Figure 4.8. a) Correlation of NFI of ThT and total urinary protein (mg/mL). Blue outline encloses the content magnified for (b).

Furthermore, we were unable to abstract more telling details regarding the effects of gestational age on the detection efficiency since we were unable to see a clear trend between the NFI of either fluorophore and the gestational age (Figure 4.9 and 4.10). No clear trend was evidenced since these are not true representations of the effects of the course of pregnancy on the detection ability.
Figure 4.9. a) Correlation of NFI of AMDX-201 and gestational age of patients. Blue outline encloses the content magnified for (b).

In order to obtain more insightful information and to assess the possible correlation, it is necessary to study patient specimen collected for the same patient throughout the course of her pregnancy and consistently during the same time of day. We believe this experimental design would minimize the variability inherent to the urine analysis from patients of varying gestational age.
While it may be unclear how both gestational age and proteinuria affects the fluorescence diagnostic method of pre-eclampsia, we have been able to distinguish pre-eclamptic patients from healthy patients using fluorophores and total protein. Thus, we wanted to determine if the usage of AMDX-201 grants superior sensitivity and specificity as a diagnostic agent than both ThT and proteinuria. To assess their diagnostic utility, we constructed receiver operating characteristic (ROC) curves. With these curves, we were able to further qualify our spectroscopic findings and to establish at what NFI threshold value do the probes exhibit high sensitivity (the proportion of the diagnostic tool to identify the true positives) and high specificity (the proportion of diagnostic tool to identify the true negatives). We selected an optimal cut-off value by calculating the value that maximizes Youden’s index ($J$), which is defined:

$$J = \text{maximum (sensitivity + selectivity} - 1).$$

Equation 4.1. Equation for Youden’s index, used to determine the optimal cut-off value based on computed sensitivity and specificity values.
By using Youden’s index to select a cut-off, we placed equal emphasis on the specificity and sensitivity in our selection. Thus, with an optimal cut-off value, we were able to determine at what NFI value separates the healthy population and the pre-eclampsic population. Due to the small number of mild pre-eclampsic patients (n = 1) and severe pre-eclampsic patients (n = 2), we were unable to construct ROC curves for these cohorts and compare them to the healthy cohort based on AMDX-201 NFI, ThT NFI, and total protein content. Instead, we focused on the probes’ ability to discriminate the healthy population from the entire pre-eclampsic population and discriminate the healthy from the superimposed pre-eclampsic patients. However, we are currently unable to determine cut-off values that discriminate the patients who presented “other disorders” and pre-eclampsic patients because those two populations are not significantly different.

When we constructed a ROC curve based on the NFI of AMDX-201 to determine the clinical utility of AMDX-201 as a predictive measure for pre-eclampsia using the collective pre-eclampsic population, we found that an AMDX-201 cut-off value > 2.6 exhibited 78% sensitivity and 85% specificity (AUC: 0.8302, p = 0.006, Figure 4.11). At this cut-off value, 16% of our healthy population received an “abnormal result,” a result observed above the cut-off value. However, 22% of the pre-eclampsic population were received a “normal result,” a result observed below the cut-off value. For patients who received an abnormal result, we can predict the likelihood that such patient will actually develop pre-eclampsia. This probability is known as the positive predicative value (PPV) and is computed using Equation 4.2.
\[ PPV = \frac{True \ positive \ results}{True \ positive \ results + False \ negative \ results} \]

**Equation 4.2.** Equation for positive predicative value, used to determine the probability of disease development given a positive result.

For patients who received a “normal result,” we can also determine the probability that the diagnosis will remain negative by calculating the negative predicative value (NPV). This probability is defined using Equation 4.3.97

\[ NPV = \frac{True \ negative \ results}{True \ negative \ results + false \ positive \ results} \]

**Equation 4.3.** Equation for negative predicative value, used to determine the probability of the absence of disease development if “normal” result is obtained.

Furthermore, because the prevalence of pre-eclampsia among pregnant women is 3 – 5%, we can also predict a patient’s likelihood to develop a disease if they receive an abnormal diagnostic result based on our cut-off value.97 For from the NFI of AMDX-201, if a patient receives an “abnormal” diagnostic result, the patient has 14 – 21% chance of actually having a disease since pre-eclampsia affects 3 – 5% of pregnant women. For patients who receive a “normal result,” we are able to predict that there is a 98 – 99% chance that a “normal” diagnostic result will remain normal using our cut-off value.
Interestingly, when we assessed the NFI of ThT for clinical utility, the threshold NFI that exhibited the best combination of sensitivity and specificity was determined to be $>1.395$, corresponding to $89\%$ sensitivity and $71\%$ specificity (AUC = 0.8545, $p = 0.002$, Figure 4.12). By using this optimized threshold, $29\%$ of our healthy patients obtained an abnormal test result and $22\%$ of our pre-eclampsic patients obtained a normal result. At this cut-off value, those misdiagnosed patients have a $9\%$ – $14\%$ chance of actually developing the disease, while there is a $99\%$ that the patients with negative results will remain healthy.

**Figure 4.11.** ROC curve for computed for discrimination of healthy patients from entire pre-eclampsic population using NFI of AMDX-201.
When compared the fluorescent diagnostic performance to the diagnostic measurement based on total urinary protein using the optimal threshold value of 0.239 mg/mL, we obtained the specificity of 85% and a sensitivity of 100% (AUC = 0.963, p < 0.0001, Figure 4.12). Using this cut-off value, 10% of our negative control as diagnosed with an abnormal result. While a small percentage of our negative control received a positive result, none of our pre-eclampsic population was misdiagnosed with a normal result. Using this cut-off value, the patients who were diagnosed with an abnormal result have a 25% of developing pre-eclampsia, while 100% of the patients with a normal result will remain healthy.

**Figure 4.12.** ROC curve for computed for discrimination of healthy patients from entire pre-eclampsic population using NFI of ThT.
Collectively, both AMDX-201 and ThT performed comparably to discriminate a pre-eclampsic patient from a healthy patient as evidenced by their similar AUC values. Additionally, at their optimal cut-off values, they both have comparable false negative and false positive frequencies.

Interestingly, using proteinuria as a diagnostic parameter resulted in the best sensitivity and specificity in discriminating healthy and pre-eclampsic patients. However, this parameter for pre-eclampsia diagnosis should be used with caution because proteinuria is not a specific symptom of pre-eclampsia, but also a result of pre-existing hypertension, pre-eclampsic-related hypertension, diabetes, and renal complications. Therefore, it would be inaccurate to state that proteinuria accurately discriminates pre-eclampsic patients from healthy patients. Therefore, we believe that it would be more accurate to propose that the ROC curve based on total urinary protein concentration actually discriminates the healthy patients from patients with a hypertensive-related disease, rather than pre-eclampsia. Our comparative findings

**Figure 4.13.** ROC curve for computed for discrimination of healthy patients from entire pre-eclampsic population using total protein concentration.
for ROC curves obtained for the discrimination of healthy and pre-eclamptic patients are summarized in Table 4.2.

**Table 4.2.** Summarized findings of the diagnostic performances of AMDX-201, ThT, and proteinuria to discriminate the entire pre-eclamptic population and the healthy population.

<table>
<thead>
<tr>
<th>Diagnostic parameter</th>
<th>Cut-off value</th>
<th>AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>False Neg. result (%)</th>
<th>False Pos. result (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMDX-201</td>
<td>&gt; 2.745</td>
<td>0.8302</td>
<td>78</td>
<td>85</td>
<td>33</td>
<td>15</td>
</tr>
<tr>
<td>ThT</td>
<td>&gt; 1.395</td>
<td>0.8545</td>
<td>89</td>
<td>71</td>
<td>22</td>
<td>28</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>&gt; 0.239</td>
<td>0.9603</td>
<td>100</td>
<td>85</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

Because we previously showed that our entire pre-eclamptic population consists of mild pre-eclampsia (n = 1), severe pre-eclampsia (n = 2), and superimposed pre-eclampsia (n = 6), we were also interested to determine which diagnostic test would provide a cut-off value for superimposed pre-eclamptic patients and healthy patients and with the highest specificity and sensitivity. For these analyses, we tabulated our findings in Table 4.3. By discriminating between the superimposed pre-eclamptic cohort and the healthy patients, we observed a reduction in the ability of AMDX-201 to accurately detect superimposed pre-eclamptic patients. However, the detection of pre-eclampsia using ThT improved when we compared the superimposed pre-eclamptic patients from the healthy patients. Additionally, the discrimination between healthy and superimposed pre-eclamptic patients using total urinary protein concentration had the same sensitivity and specificity as the distinction between the healthy and pre-eclamptic population. However, since superimposed pre-eclamptic patients have a pre-existing condition of
hypertension, it is plausible that these patients also have higher levels of urinary proteins compared to healthy patients. Like our previous comment regarding the origin of urinary protein, the usage of urinary protein levels is not an accurate distinction parameter for healthy and superimposed pre-eclampsic patients since patients with pre-eclampsia-like symptoms may also exhibit higher levels of urinary protein than healthy patients. Although discriminating healthy and pre-eclampsic patients using proteinuria initially shows superior discriminating power, this measure should not be used to compare those two groups since proteinuria is not a symptom that is solely specific to pre-eclampsia. Currently, our data suggests that NFI of ThT may provide superior diagnostic utility than either the NFI of AMDX-201 or proteinuria. However, because both of the NFI of ThT and NFI of AMDX-201 for the pre-eclampsic patients have a wide range of values and the limited number of confirmed cases, we need to unmask the outcomes of the remaining patients to obtain a better comparison of the fluorescence diagnostic performance of the two fluorophores.
Table 4.3. Summarized findings of comparative diagnostic performances of AMDX-201, ThT, and proteinuria to discriminate superimposed pre-eclampsic patients from healthy patients.

<table>
<thead>
<tr>
<th>Diagnostic parameter</th>
<th>Cut-off value</th>
<th>AUC</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>False Neg. result (%)</th>
<th>False Pos. result (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMDX-201</td>
<td>&gt;2.905</td>
<td>0.8125</td>
<td>67</td>
<td>90</td>
<td>38</td>
<td>20</td>
</tr>
<tr>
<td>ThT</td>
<td>&gt; 1.395</td>
<td>0.8714</td>
<td>100</td>
<td>71</td>
<td>0</td>
<td>33</td>
</tr>
<tr>
<td>Proteinuria</td>
<td>&gt; 0.239</td>
<td>0.9603</td>
<td>100</td>
<td>85</td>
<td>0</td>
<td>10</td>
</tr>
</tbody>
</table>

4.4 Concluding Remarks

Before this work, studying pre-eclampsia with the presence of urinary amyloids was based on a nascent observation that suggested the possible correlation between the development of the disease and the accumulation of misfolded proteins and/or renal complications. Thus, we felt compelled to ascertain whether the presence of urinary amyloids can be a diagnostic parameter for development of pre-eclampsia.

During the course of this research, we have been able distinguish pre-eclampsic patients from healthy patients based on the detection of urinary amyloids with NFI of ThT, AMDX-201, and proteinuria. We assessed the performance of a synthesized amyloid-binding fluorophore in comparison to the performance of a leading amyloid-binding probe by comparing their discriminating power.

Among the 88 urine samples in our prospective study, AMDX-201 and ThT exhibited comparable discriminating abilities with adequate specificity and sensitivity. Neither probes were able to discriminate pre-eclampsic patients from patients who
presented pre-eclampsia-like symptoms. Proteinuria, while capable of accurately classifying the healthy patients from the pre-eclampsic patients, was not a specific diagnostic parameter for pre-eclampsia and should not be used solely to discriminate patients.

As noted previously, our ROC curves were analyzed by using Youden’s index to compute the optimal cut-off value for the NFI of AMDX-201 and ThT. Traditionally, Youden’s index is optimally used for clinical studies with large sample sizes (> 200 subjects). However, in smaller clinical trials, the sensitivity and specificity values obtained from ROC analyses can be inflated from their actual values. Thus, applying the Youden’s index to small clinical trials, such as our clinical trial, may result in falsely enhanced specificity and sensitivity values and may skew our comparative analyses of ThT and AMDX-201.

Our comparative, prospective study has suggested that AMDX-201 and ThT can be reliable predicative values for the development of severe cases of pre-eclampsia. Furthermore, this study has suggested that the usage of proteinuria as a “gold standard” diagnostic for pre-eclampsia should be used with caution as it is not an unequivocal telling parameter as we have observed. The small positive correlation that we observed with NFI of the fluorophores and the total protein content suggests that the usage of amyloid-binding fluorophores would provide additional diagnostic information compared to current diagnostic parameters. Since these two measures provide different information, as a future study, we are interested to see how early in the pregnancy are we able to obtain an enhancement in the NFI of the fluorophores in the presence of pre-eclampsic
urine. Perhaps, these probes could detect a signal before proteinuria or hypertension is diagnosed.

Additional confirmed cases of pre-eclampsia in our current study need to be evaluated to provide a more conclusive comparison between the two diagnostic capabilities of the probes. Additional future studies include the identification of the entity responsible of the fluorescence enhancement of the fluorophores. This knowledge can deepen the investigation in the pathophysiology of pre-eclampsia so that an effective therapeutic could be designed to target the fundamental aberrations that manifests in the development of pre-eclampsia. Understanding the ulterior mechanism associated with disease development may also aid in the design of a diagnostic tool that can accurately recognize all classifications of pre-eclampsic patients, but also diagnose patients asymptomatically. Such tool would be a great asset to clinicians and patients alike as it could alleviate the number of unchecked maternal and fetal morbidity and mortality that pre-eclampsia currently claims.

4.5 Experimental Details

4.5.1 Study participants

This study was conducted in accordance to the approval of UCSD IRB. Informed consent was obtained from all of the patients before they partook in the study. Urine specimens from all participants were graciously obtained in collaboration with the research groups of Dr. Mana Parast (UCSD Department of Pathology) and Dr. Louise Laurent (UCSD Department of Reproductive Medicine). During the feasibility trial for
this study, conducted from August 2014 – December 2014, the urine specimens were obtained from patients who were admitted into labor and delivery at the UCSD Medical Center in Hillcrest. These urine samples were collected as spot collections, a classification for urine samples collected at a random time during the day. The collection criteria were women who were healthy, based on normotension, and women who were positive for pre-eclampsia based on presence of hypertension. The gestational age of the women presented during this timeframe varied between 32 – 40 weeks. All specimens were aliquoted prior to analysis to prevent repetitive episodes of freezing and thawing. Specimens were stored at –80°C and analyzed within 48 hours from their collection.

Patients recruited into the blind prospective study from March 2015 – June 2015 were classified with one of the following categories: patients with normal pregnancies but have low risk for pre-eclampsia; patients with normal pregnancies, but have high risk for pre-eclampsia, patients with pre-eclampsia; and patients with proteinuria, but no pre-eclampsia. To avoid contamination of amniotic fluid with the urine specimens, the urine of patients who experienced premature rupture of membranes were excluded from the study. Urine specimens were collected from patients presented at the labor and delivery department and the fetal center and genetics department at the UCSD Medical Center in Hillcrest. The gestational age of women in this prospective study ranged from 11 – 39 weeks. These samples were centrifuged at 2000 xg and the supernatants were retained for analyses. All specimens were aliquoted prior to analysis to prevent repetitive episodes of freezing and thawing. Specimens were stored at –80°C before analysis. Because of the frequency in sample collection, Specimen were collected, centrifuged, and stored in Dr. Parast’s – 80°C freezer prior to pick-up. Samples were retrieved every 2 weeks and
characterized within 48 hours. The outcomes of these patients were obtained following delivery.

4.5.2 Instrumental analysis

Total urinary protein concentrations were ascertained from a standard curve from the Bradford assay using SpectraMax 190 microplate reader (Molecular Devices). Fluorescence assays were performed using PTI spectroflurorimeter in 1 nm increments from 490 – 650 nm and EnSpire 2300 Multimode plate reader (Perkin Elmer). All assays were performed in duplicate.

4.5.3 Spectrophotometric methods

4.5.3a Quantification of total urinary protein in specimens

The Bradford protein quantification assay was performed in accordance to the Bio-Rad instruction’s manual. Briefly, a working solution of the Bradford dye concentrate was prepared by diluting 1 part of the Bio-rad dye concentrate with 4 parts of MilliQ water. Following, 200 uL of this working solution 10 uL of standard BSA solution or 10 uL of undiluted urine. Contents were vortexed afterwards and allowed to incubate at room temperature for 10 minutes. Then, samples were vortexed again to prevent any proteins from settling at the bottom prior to analysis. Optical densities were measured at 595 nm on a microplate reader. Analyses were performed in triplicate.
4.5.3b Analysis of ThT fluorescence in specimens

ThT fluorescence studies were conducted in accordance to a protocol described by Buhimschi et al. Briefly, 70 uL of 114 uM ThT solution in 1X PBS was added to 30 μL of urine supernatant. The final concentration of ThT in the sample was 80 uM. Samples were measured within 5 minutes of adding ThT. Measurements were made in duplicate using a fluorescence plate reader with excitation and emission wavelengths set at 444 nm and 485 nm, respectively. Each specimen was analyzed in triplicate. The auto-fluorescence of urine was corrected by calculating the difference of the fluorescence intensity of ThT in the urine and the fluorescence intensity of urine without the fluorophore.

4.5.3c Analysis of AMDX-201 fluorescence in specimens

AMDX-201 measurements were obtained by adding 5 μL of 60 μM AMDX-201 in dimethyl sulfoxide (DMSO) to 30 μL of supernatant urine in 65 μL of 1X PBS. Samples were incubated at room temperature for 10 minutes prior to fluorescence analyses. The final composition of these samples consists of 5% DMSO to help solubilize AMDX-201 in the aqueous medium. The final concentration of the fluorophore was 3 μM in PBS or patient urine specimen. Measurements were made in duplicate using a fluorescence plate reader with excitation and emission wavelengths set at 450 nm and 525 nm, respectively. Each specimen was analyzed in triplicate. The auto-fluorescence of urine was corrected by calculating the difference of the fluorescence intensity of AMDX-201 in the urine and the fluorescence intensity of urine without the fluorophore.
4.5.4 Statistical Analyses

Statistical analyses were performed in GraphPad Prism 5. In this study, 2 populations of pregnant women were being compared at a time. Because the experimental measurements during pregnancy are known to deviate from a non-Gaussian distribution, our statistically tests were calculated accordingly. While many different cohorts of patients were qualitatively compared, only 2 populations of patients were compared for statistical analyses. To determine statistical significance between 2 population medians, we performed the Mann-Whitney test. Regarding outlying data, common methods of outlier detection (such as the Dixon Q-test, Grubb’s test, and Rout test) could not be performed directly since these statistical tests assume that the data conforms to a normal distribution. Instead, the Grubb’s test for the outlier was applied after the data was fitted to a Gaussian distribution using a logarithmic transformation. A two-tailed p-value < 0.05 was considered to be significant.

Notes about the chapter

Chapter 4, in part, is featured in material that is currently being prepared for submission for publication. Jamie P Do, Kevin Cao, Louise Laurent, Mana Parast, and Jerry Yang “Amyloid-binding Fluorescence Diagnostic Assay for Pre-eclampsia.” I was the primary investigator and author.
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