Increased Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in Type 2 Diabetic Patients

Sridevi Gutta
Wright State University

Follow this and additional works at: https://corescholar.libraries.wright.edu/etd_all

Part of the Pharmacology, Toxicology and Environmental Health Commons

Repository Citation
Gutta, Sridevi, "Increased Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in Type 2 Diabetic Patients" (2014). Browse all Theses and Dissertations. 1459.
https://corescholar.libraries.wright.edu/etd_all/1459

This Thesis is brought to you for free and open access by the Theses and Dissertations at CORE Scholar. It has been accepted for inclusion in Browse all Theses and Dissertations by an authorized administrator of CORE Scholar. For more information, please contact library-corescholar@wright.edu.
Increased Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in Type 2 Diabetic Patients

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science

By

SRIDEVI GUTTA
B. Pharm, K.V.S.R Siddhartha College of Pharmaceutical Sciences,
Andhra Pradesh, India 2011

2014
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Sridevi Gutta ENTITLED “Increased Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in Type 2 Diabetic Patients” BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

______________________________
Khalid M. Elased, PharmD, Ph.D.
Thesis Director

______________________________
Norma Adragna, Ph.D., Interim Chair
Department of Pharmacology and Toxicology

Committee on Final Examination

______________________________
Khalid M. Elased, PharmD, Ph.D.

______________________________
Nadja Grobe, Ph.D.

______________________________
Mohammad Saklayen, M.D.

______________________________
Robert E.W. Fyffe, Ph.D.
Vice President for Research and Dean of Graduate School
Gutta, Sridevi, M.S., Department of Pharmacology and Toxicology, Wright State University.

Increased Urinary Angiotensin Converting Enzyme 2 (ACE2) and Neprilysin (NEP) in Type 2 Diabetic Patients

Chronic Kidney Disease (CKD) has reached epidemic proportions affecting more than 20 million adults in the US. CKD is routinely diagnosed and defined as reduced glomerular filtration rate (GFR), increased urinary albumin creatinine ratio (UACR) or both. Diabetes, hypertension and obesity share a major part in causing CKD. Angiotensin (1-7) (Ang (1-7)) is a vasodilator that plays an important renoprotective role in the renin angiotensin system, counteracting the vasoconstrictor and proliferative effects of Ang II. ACE2 and NEP which form Ang (1-7) by degrading Ang II and Ang I, respectively. A Disintegrin and Metalloproteinase (ADAM) 17, which is responsible for the ectodomain shedding of transmembrane proteins, sheds renal ACE2 and causes release of NEP from urinary exosomes. In this study, we aimed to investigate the levels of ACE2 and NEP in urine and to find their correlations with well-established markers of CKD in diabetic patients at different stages of albuminuria. Baseline UACR and estimated GFR (eGFR) were determined three months before initiation of the study in twenty nondiabetics (ND) and forty diabetic patients with normoalbuminuria, microalbuminuria, and macroalbuminuria. Based on their visits over the previous year, patients with history of microalbuminuria and whose UACR reversed to normal were considered as diabetic patients with normoalbuminuria. Metabolic and renal characteristics were measured in all groups. Enzyme activity of urinary ACE2 and NEP were measured using fluorogenic and mass spectrometric assays. We developed a sensitive and specific assay to measure ACE2 and NEP activities in human urine using matrix assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS). Significant inhibition of urinary ACE2 and NEP activities by MLN-4760 (specific ACE2 inhibitor) and thiorphan (specific NEP inhibitor) proved the specificity of the enzyme assays. Urinary ACE2 and NEP levels were significantly higher in diabetic patients compared to nondiabetic individuals, even before the onset of microalbuminuria ($p<0.05$).
Immunoblotting confirmed increased urinary ACE2 and NEP expression in diabetic patients prior to the onset of microalbuminuria compared to nondiabetic patients. Unlike plasma ACE2 activity, which was not detectable, plasma NEP was increased in subjects with diabetes. Increase in urinary ADAM17 levels were observed in diabetic patients compared to nondiabetic individuals (p<0.05). Urinary ACE2 activity correlated with eGFR, blood glucose, glycated hemoglobin (HbA1C), and BUN (all p<0.05), but not UACR and urinary protein-to-creatinine ratio (UPCR). Regression analysis of urinary NEP concentration with various metabolic parameters showed correlations with blood glucose, BMI, HbA1C, and creatinine clearance. In conclusion, alterations in kidney function can be more precisely indicated by shedding of ACE2 and NEP in urine of diabetic patients prior to onset of microalbuminuria. These new findings provide novel insights for the possible role of urinary ACE2 and NEP as non-invasive biomarkers for diabetic kidney disease prior to the onset of microalbuminuria.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>1. INTRODUCTION</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prevalence and epidemiology of Diabetes mellitus</td>
<td>1</td>
</tr>
<tr>
<td>Diabetic kidney disease</td>
<td>2</td>
</tr>
<tr>
<td>Role of Renin angiotensin system (RAS) in Kidney Disease</td>
<td>5</td>
</tr>
<tr>
<td>Angiotensin (1-7)</td>
<td>7</td>
</tr>
<tr>
<td>Angiotensin (1-7) forming enzymes</td>
<td>8</td>
</tr>
<tr>
<td>Angiotensin converting enzyme 2</td>
<td>8</td>
</tr>
<tr>
<td>Neprilysin</td>
<td>9</td>
</tr>
<tr>
<td>ADAM17</td>
<td>10</td>
</tr>
<tr>
<td>Biomarkers for DKD</td>
<td>12</td>
</tr>
<tr>
<td>Albuminuria</td>
<td>13</td>
</tr>
<tr>
<td>Estimated glomerular filtration rate</td>
<td>14</td>
</tr>
<tr>
<td>Biomarkers for DKD</td>
<td>16</td>
</tr>
<tr>
<td>Biomarkers of glomerular injury</td>
<td>16</td>
</tr>
<tr>
<td>Biomarkers of tubular injury</td>
<td>17</td>
</tr>
<tr>
<td>Inflammatory and fibrotic markers as diabetic kidney injury markers</td>
<td>19</td>
</tr>
<tr>
<td>Urinary RAS components as biomarkers</td>
<td>20</td>
</tr>
<tr>
<td>2. HYPOTHESIS AND SPECIFIC AIMS</td>
<td>23</td>
</tr>
<tr>
<td>3. MATERIALS AND METHODS</td>
<td>24</td>
</tr>
<tr>
<td>Study Protocol</td>
<td>24</td>
</tr>
<tr>
<td>Metabolic, renal and cardiovascular parameters</td>
<td>24</td>
</tr>
<tr>
<td>Sample collection</td>
<td>25</td>
</tr>
<tr>
<td>Urinary albumin assay</td>
<td>25</td>
</tr>
<tr>
<td>Urinary creatinine assay</td>
<td>26</td>
</tr>
<tr>
<td>Urinary and plasma ACE2 enzyme activity</td>
<td>27</td>
</tr>
<tr>
<td>Urinary and plasma NEP ELISA</td>
<td>27</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>------</td>
</tr>
<tr>
<td>Urinary ADAM17 ELISA</td>
<td>28</td>
</tr>
<tr>
<td>ACE2 and NEP activity determination using mass spectrometry</td>
<td>29</td>
</tr>
<tr>
<td>Western blot</td>
<td>30</td>
</tr>
<tr>
<td>Statistical analysis</td>
<td>31</td>
</tr>
<tr>
<td><strong>4. RESULTS</strong></td>
<td>58</td>
</tr>
<tr>
<td>Characteristics of general metabolic and renal functional parameters</td>
<td>58</td>
</tr>
<tr>
<td>Assessment of renal function</td>
<td>59</td>
</tr>
<tr>
<td>ACE2 enzyme activity</td>
<td>59</td>
</tr>
<tr>
<td>Correlation of urinary ACE2</td>
<td>60</td>
</tr>
<tr>
<td>Protein expression of ACE2</td>
<td>61</td>
</tr>
<tr>
<td>ADAM17 concentration analysis using ELISA</td>
<td>62</td>
</tr>
<tr>
<td>Neprilysin protein concentration using ELISA</td>
<td>62</td>
</tr>
<tr>
<td>Correlation of urinary NEP</td>
<td>63</td>
</tr>
<tr>
<td>Protein expression of NEP</td>
<td>64</td>
</tr>
<tr>
<td>Protein expression of urinary albumin</td>
<td>65</td>
</tr>
<tr>
<td>Mass spectrometric analysis of urinary ACE2 and NEP activities</td>
<td>65</td>
</tr>
<tr>
<td>Detection of ACE2 and NEP in urine at early stages of renal injury</td>
<td>66</td>
</tr>
<tr>
<td><strong>5. DISCUSSION</strong></td>
<td>68</td>
</tr>
<tr>
<td><strong>6. CONCLUSION</strong></td>
<td>78</td>
</tr>
<tr>
<td><strong>7. APPENDICES</strong></td>
<td>79</td>
</tr>
<tr>
<td><strong>8. REFERENCES</strong></td>
<td>84</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Renin angiotensin system cascade</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Classification of patients according to GFR and albuminuria</td>
<td>35</td>
</tr>
<tr>
<td>3</td>
<td>Urinary ACE2 activity assay in nondiabetic and diabetic patients</td>
<td>36</td>
</tr>
<tr>
<td>4</td>
<td>Confirmation of ACE2 activity assay using inhibitors</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>Plasma ACE2 activity assay in all patients</td>
<td>38</td>
</tr>
<tr>
<td>6</td>
<td>Correlation between eGFR and urinary ACE2</td>
<td>39</td>
</tr>
<tr>
<td>7</td>
<td>Correlation between UACR and urinary ACE2</td>
<td>39</td>
</tr>
<tr>
<td>8</td>
<td>Correlation between UPCR and urinary ACE2</td>
<td>40</td>
</tr>
<tr>
<td>9</td>
<td>Correlation between urinary ACE2 and HbA1C</td>
<td>40</td>
</tr>
<tr>
<td>10</td>
<td>Urinary ACE2 expression at normal GFR</td>
<td>41</td>
</tr>
<tr>
<td>11</td>
<td>Urinary ACE2 expression at low GFR</td>
<td>42</td>
</tr>
<tr>
<td>12</td>
<td>Urinary ADAM17 concentration using ELISA</td>
<td>43</td>
</tr>
<tr>
<td>13</td>
<td>Urinary NEP concentration in all patients</td>
<td>44</td>
</tr>
<tr>
<td>14</td>
<td>Plasma NEP concentration in nondiabetic and diabetic patients</td>
<td>45</td>
</tr>
<tr>
<td>15</td>
<td>Correlation between urinary NEP and plasma NEP</td>
<td>46</td>
</tr>
<tr>
<td>16</td>
<td>Correlation between eGFR and urinary NEP</td>
<td>46</td>
</tr>
<tr>
<td>17</td>
<td>Correlation between UACR and urinary NEP</td>
<td>47</td>
</tr>
<tr>
<td>18</td>
<td>Correlation between UPCR and urinary NEP</td>
<td>48</td>
</tr>
<tr>
<td>19</td>
<td>Correlation between urinary NEP and HbA1C</td>
<td>49</td>
</tr>
<tr>
<td>20</td>
<td>Urinary NEP expression at normal GFR</td>
<td>50</td>
</tr>
<tr>
<td>21</td>
<td>Urinary NEP expression at low GFR</td>
<td>51</td>
</tr>
<tr>
<td>22</td>
<td>Urinary albumin expression at normal GFR</td>
<td>52</td>
</tr>
<tr>
<td>23</td>
<td>Urinary albumin expression at low GFR</td>
<td>53</td>
</tr>
<tr>
<td>24</td>
<td>Western blot analysis of ACE2 at early stages of renal injury</td>
<td>54</td>
</tr>
<tr>
<td>25</td>
<td>Western blot analysis of NEP at early stages of renal injury</td>
<td>55</td>
</tr>
<tr>
<td></td>
<td>Mass spectrometric analysis of ACE2</td>
<td>56</td>
</tr>
<tr>
<td>---</td>
<td>-----------------------------------</td>
<td>----</td>
</tr>
<tr>
<td>27.</td>
<td>Mass spectrometric analysis of NEP</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>LIST OF TABLES</td>
<td>Page</td>
</tr>
<tr>
<td>---</td>
<td>-------------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>1</td>
<td>Stages of CKD</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>Baseline characteristics of all patients</td>
<td>32</td>
</tr>
<tr>
<td>3</td>
<td>Correlations of urinary ACE2 and NEP with various metabolic and renal functional parameters</td>
<td>34</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

Prevalence and epidemiology of Diabetes mellitus

In 2011, The International Diabetes Federation (IDF) estimated that 366 million people had diabetes worldwide (Whiting et al., 2011) and, in 2035, this number is expected to almost double (Guariguata et al., 2014). Recent statistical analysis in 2012 reported 29.1 million Americans with diabetes (Centers for Disease Control and Prevention, 2014). According to 2012 annual reports of American Diabetes Association, the economic costs for diabetes care was estimated at 245 billion dollars for diagnosed diabetic patients (American Diabetes Association, 2013).

Diabetes mellitus is a metabolic disorder caused by alterations in insulin production, insulin action or both, resulting in increased blood glucose and changes in carbohydrate, fat, and protein metabolisms. Based on the etiological types, the American Diabetes Association expert group classified diabetes into four types (Gavin et al., 1997). The most common categories are type 1 and type 2 diabetes mellitus. Type 1 diabetes mellitus or juvenile diabetes is characterized by acute deficiency in insulin secretion caused by destruction of pancreatic beta cell, specific to insulin, through an autoimmune or idiopathic process. It is usually diagnosed in children and young adults. Type 2 diabetes mellitus is characterized by insulin resistance and beta cell dysfunction, which is seen in patients having family history of diabetes, hypertension, older age, obesity, improper food, or lack of exercise (American Diabetes Association, 2014). Type 1 and type 2 diabetes account for about 5-10% and 90%, respectively, of total diabetic cases (American Diabetes Association, 2014). The prevalence of hyperglycemia during pregnancy, called gestational diabetes mellitus, is between 4.6-9.2% according to a 2014 analysis of the Central Disease Control and Prevention report (DeSisto et al., 2014). Other types of diabetes includes genetic defects of beta-cell function called maturity onset diabetes in youth (MODY) (Ben et al., 2011), genetic defects in insulin action,

Complications of type 2 diabetes

In the long run, diabetes mellitus causes damage to various organs such as the kidney, heart, nerves, eyes, and blood vessels. Complications caused by diabetes are divided into macrovascular and microvascular complications. Microvascular complications include neuro-, retino-, and nephropathy whereas macrovascular complications include cardio- and cerebrovascular diseases (Fowler, 2008). It has been well documented that patients with type 2 diabetes are at increased risk for developing cardiovascular disorders, including hypertension, coronary artery disease, and stroke (American Diabetes Association, 2013).

Diabetic Kidney Disease

Diabetic nephropathy, a major microvascular complication of diabetes, is one of the leading causes of end-stage renal failure in many countries (Kinney, 2006). Diabetic nephropathy is defined by progressive albuminuria, hyperfiltration that has a role in development and progression of the disease. Kidney disease caused by diabetes, often named diabetic kidney disease, occurs rarely in the first 10 years of diabetes. Male sex, older age, increased HbA1C, retinopathy, and cholesterol are some of the risk factors of diabetic nephropathy. In diabetes, hyperglycemia activates the renin-angiotensin system (RAS).

Juxtaglomerular cells from glomerulus, which are capable of synthesizing RAS components stimulate intracellular renin and produce angiotensin II (Ang II) (Vidotti et al., 2004). Increased Ang II production results in mesangial hypertrophy, which is linked to structural
and functional changes in glomerular filtration barrier and basement membrane (Mauer et al., 1984; Haraldsson et al., 2008). This process causes several proteins to pass through the glomerular filtration barrier more easily. Further, alteration in the anti-oxidant system function causes chemical modification of proteins and lipids with excess glucose, leading to increase in Advanced Glycation End products (AGE), Advanced Oxidized Lipid End Products (ALE), and Advanced Oxidation Protein Products (AOPP). The interaction of AGE’s with its receptors (RAGE) in the kidney induces tubulointerstitial fibrosis (Yamamoto et al., 2001). AGE’s and ALE’s increase proteinuria in all stages of diabetic nephropathy (Mohan et al., 2012). Transforming growth factor β1 (TGF-β1), connective tissue growth factor (CTGF), insulin-like growth factor (IGF), and vascular endothelial growth factor (VEGF) are some of the cytokines that induce tissue injury, thickening of the glomerular basement membrane, and alterations of the slit diaphragm permeability producing proteinuria and scarring (Jayakumar, 2012; New et al., 2014). All these factors cause oxidative stress that induces renal damage.

Microalbuminuria is an indicator for diabetic nephropathy, which is also know as incipient nephropathy. Two separate measurements of microalbuminuria 3-6 months apart must be done to confirm diabetic nephropathy. This is considered stage 1 of chronic kidney disease (CKD) with normal or mildly elevated GFR.

CKD is a condition in which damage to kidney function leads to decrease in filtration of blood and thus loss of waste material management. Until major renal damage occurs, the pathogenesis of renal injury cannot be assessed. Thus, it has been estimated that a large portion of individuals at early stages of CKD have been undiagnosed and/or untreated (Collins et al., 2006; Cueto-Manzano et al., 2005). End stage renal failure (ESRD), or simply kidney failure, is the final stage of CKD. CKD has risen to one of the most prevalent non-communicable diseases in the last two decades. Highlighting CKD as the third largest growth
among the top 25 causes of global mortality, the Global Burden Disease and International Society of Nephrology reported that deaths have gone up by 82.3% (Lozano et al., 2012). In the United States alone more than 20 million people have CKD at various levels of severity, and 113,136 patients started treatment for ESRD in 2011 (Levey et al., 2011). An increase in the prevalence of CKD is caused by clusters of unknown origin. Diabetes and hypertension are the major causes of CKD accounting for 44% and 28% of the total cases, respectively, according to the National Kidney Foundation 2011 facts sheet.

Table 1: Stages of CKD classified based on estimated glomerular filtration rate (GFR) and albuminuria according to KDIGO (Levin & Stevens, 2014).

The current hallmarks of CKD progression are decline in eGFR, increased urinary albumin excretion, or both. According to the United States National Kidney Foundation’s 2002 guidelines (KDOQI), CKD is defined as the decline in eGFR less than 60 ml/min/1.73m² for
more than 3 months, often accompanied by albuminuria (Eknoyan & Levin, 2002; Levin & Stevens, 2014). Microalbuminuria has been considered as an early noninvasive biomarker for CKD to date. In contrast, studies have shown presence of CKD in type 1 diabetes and type 2 diabetes without albuminuria (MacIsaac & Jerums, 2011). Additional research suggested that microalbuminuria may not be a specific marker for CKD (Cerasola et al., 2010; McKenna & Thompson, 1997). Currently, the reliability of albuminuria in early diagnosis of diabetic kidney disease has been questioned (MacIsaac et al., 2014). Therefore, there is an urgent need for better biomarkers to predict diabetic kidney disease.

Various biomarkers have been proposed for detection of CKD including urinary RAS components (Levin et al., 2014). Activation of RAS pathway has been heavily implicated in progression of CKD (Afkarian et al., 2014). In addition, the use of RAS blockers for the management of kidney disease suggests a role of the RAS in renoprotection (Levin & Stevens, 2014).

**Role of Renin angiotensin system (RAS) in Kidney Disease**

The RAS is a multi-enzymatic cascade and one of the most important endocrine hormone system which controls fluid balance, tissue repair, and blood pressure. The RAS is expressed in many local tissues including the kidney (Kimbrough et al., 1977), heart (Dzau, 1988), brain (Baltatu et al., 1998; de et al., 2013), retina (Moravski et al., 2000), liver (Bataller et al., 2003), pancreas (Lau et al., 2004), reproductive system (Leung & Sernia, 2003), lymphatic tissue, adipose tissue (Engeli et al., 2000; Engeli et al., 2003), and in the circulation (Dzau, 1988).

In the classical RAS, decrease in blood volume activates renin, which is secreted from juxtaglomerular cells in the kidney. Secretion of renin acts as a primary determinant for RAS activity (Davis & Freeman, 1976). The renin substrate angiotensinogen is released through
the hepatic circulation (Clauser et al., 1983). Cleavage of angiotensinogen by plasma renin forms the decapeptide, angiotensin I (Ang I) (Peach, 1977). Ang I is converted to Ang II by the action of angiotensin converting enzyme (ACE).

Figure 1: Renin angiotensin system cascade.

ACE, which is predominantly expressed in the pulmonary circulation, is a peptidyl dipeptidase that belongs to the gluzincin family of metalloproteases (Ryan et al., 1976). Activation of Ang II by binding to its receptor, Ang II type I receptor (AT1R), leads to vasoconstriction, sodium retention, aldosterone release, hyperplasia, and hypertrophy. Angiotensin converting enzyme 2 (ACE2) counteracts the vasoconstrictive actions of Ang II by catalyzing the enzymatic degradation of Ang II to Ang (1-7) (Donoghue et al., 2000;
Crackower et al., 2002). Ang (1-7) has a vasodilator (Chappell et al., 1998) effect mediated through binding to the Mas receptor (Santos et al., 2003). Apart from ACE2, Ang (1-7) is also formed by the degradation of Ang I. Neprilysin (NEP) form Ang (1-7) by degrading Ang I (Santos et al., 1988; Ferrario et al., 1997). Abnormal activation of RAS and alterations within the RAS can contribute to the pathogenesis of hypertension, organ damage, and diabetic complications particularly diabetic kidney disease (Mezzano et al., 2003).

**Angiotensin (1-7)**

Ang (1-7), a heptapeptide, is a truncated form of Ang II lacking phenylalanine in the eighth position. It is well known that Ang (1-7) counter-balances the actions of Ang II, including its vasoconstrictor (Li et al., 1997), mitogenic (Schorb et al., 1993), arrhythmogenic (Chen et al., 2006), and prothrombic (Brown & Vaughan, 1999) effects. Ang (1-7) induces an natriuretic and diuretic (DelliPizzi et al., 1994) effect that opposes water and sodium retention by Ang II. Ang (1-7) actions are mediated by coupling with a G-protein coupled receptor called Mas receptor. Ang (1-7) concentration is 6 times higher in the kidney compared to plasma (Campbell et al., 1991). Formation of Ang (1-7) involves many pathways including enzymes like ACE2, ACE, NEP, chymase, and prolyl carboxy peptidase (PRCP) (Varagic et al., 2014). Of all the enzymes, ACE2 and NEP are the main Ang (1-7) forming enzymes (Chappell et al., 2004). Ang (1-7) suppresses high glucose-induced protein synthesis via activation of tyrosine phosphthase in proximal tubular cells (Gava et al., 2009). Dose-dependent treatment of Ang (1-7) showed attenuation in progression of diabetic nephropathy in streptozocin (STZ)-induced rats (Singh et al., 2010). Decrease in lipotoxicity, oxidative stress, and inflammation by treatment with Ang (1-7) in diabetic mice improved the condition of diabetic nephropathy (Mori et al., 2014). On the other hand, inhibition of ACE2 induced increase in albuminuria while treatment with Ang (1-7) showed no effect on albuminuria in a mouse model of CKD (Dilauro et al., 2010). A recent study in hypertensive patients showed
reduced Ang (1-7) in stenotic kidney compared to contralateral kidney controls (Van Twist et al., 2014). Therapeutic combination of Ang (1-7) along with ACE inhibitor has no synergistic effect on renal injury whereas dose dependent treatment of Ang (1-7) ameliorated STZ-induced diabetic renal injury, suggesting use of Ang (1-7) as a renoprotective therapeutic agent (Zhang et al., 2014). The ACE2/ Ang (1-7)/ Mas receptor axis has become a potential target in treatment of several diseases (Iwai & Horiuchi, 2009; Fraga-Silva et al., 2013).

**Angiotensin (1-7) forming enzymes**

**Angiotensin Converting Enzyme 2 (ACE2)**

ACE 2 was cloned from a human heart failure cDNA library and human lymphoma cDNA library that shares 42% homology with ACE (Tipnis et al., 2000; Donoghue et al., 2000). It consists of 805 amino acids and is a member of M2 family of metalloproteases containing a HEXXH +E motif that functions as a zinc-binding domain. A study on ACE2 activity using different concentrations of zinc (0 μM, 10 μM, 100 μM, 1000 μM) suggested a range that lies between >0 μM and <10 μM zinc as sufficient amount for enzymatic function (Speth et al., 2014). ACE2 metabolizes Ang II and Ang I to Ang (1-7) and Ang (1-9), respectively. The catalytic efficacy of ACE2 in the formation of Ang (1-7) from Ang II is 400-fold greater than Ang (1-9) formation from Ang I (Vickers et al., 2002; Donoghue et al., 2000). A study in cultures of mouse proximal tubular cells prepared from C57BL6 mice and ACE2 KO mice (C57BL6 background) found full-length ACE2 at ≈100 kDa, ≈90 kDa and ≈70 kDa in cell lysates. This study also suggests Met706 as a cleavage site for ACE2 (Xiao et al., 2014). Studies in human and animal model showed increased urinary ACE2 in diabetic patients, suggesting that ACE2 could be used as a biomarker for renal diseases (Mizuiri et al., 2011a; Chodavarapu et al., 2013; Salem et al., 2014; Somineni et al., 2014; Xiao et al., 2012). In
addition, attenuation of ACE2 by H5N1 infection and SARS-CoV infection results in severe lung injury suggesting the protective role of ACE2 in H5N1 and SARS-CoV-induced lung pathologies. Marked increase in serum Ang II levels in these patients may address a potential treatment strategy for these infections (Li et al., 2003; Zou et al., 2014). A recent study in diabetic kidney of STZ-induced C57BL6 mice compared to ACE2 KO mice explained the key role of ACE2 in the maintenance of hyperfiltration associated with diabetes. Absence of hyperfiltration in response to high-protein diet in ACE2 KO mice suggests that ACE2 may help in recruitment of renal reserve (Tikellis et al., 2014).

**Neprilysin (NEP)**

Neprilysin (NEP) or neutral endopeptidase, another prominent Ang (1-7) forming metalloprotease, was first identified and isolated from renal epithelial brush border of proximal tubule as insulin B chain cleavage peptide (Erdős & Skidgel, 1989; Kerr & Kenny, 1974). It is considered a prototype of the M13 family of membrane bound zinc-dependent endopeptidases with a short end cytoplasmic NH₂ and a large extracellular domain (Zraika et al., 2007; Malfroy et al., 1988). NEP encodes a 90-110 kDa type II transmembrane glycoprotein (Malfroy et al., 1988; Turner & Tanzawa, 1997). It is involved in the degradation of many peptides, including Ang I, beta-amyloid (Aβ), and bradykinin, which gained some clinical importance in many diseases, such as neurodegenerative (Guan et al., 2009) and cardiovascular disease (Erdős & Skidgel, 1989).

One of the most recent advances in the treatment of hypertension is the use of NEP inhibitor along with ARB (McMurray et al., 2014). NEP is also known as CD10 in the fields of oncology and stem cell biology (Maguer-Satta et al., 2011). NEP plays a role in the treatment of Alzheimer’s disease (AD), which is caused by beta-amyloid (Aβ) degradation in the brain (Park et al., 2013). Deficiency of NEP in the brain contributes to the accumulation of Aβ
plaques in cerebral regions responsible for memory and cognition, pathology of Alzheimer’s disease.

In diabetes, formation of islet amyloid polypeptide deposits results in beta cell dysfunction and mass reduction (Hull et al., 2004). In such diseases, treatment with NEP enhancers showed amelioration in pancreatic beta cell dysfunction (Zhao & Townsend, 2009). At early stages of diabetes, attenuation of renal NEP activity was observed in hypertensive rats suggesting that the loss of renoprotective enzyme may contribute to disease progression (Yamaleyeva et al., 2012). In addition, elevated plasma and adipose tissue NEP levels were seen in obese patients associated with insulin resistance, suggesting a biomarker function for NEP in diabetes (Standeven et al., 2011). Recently, it has been shown that down-regulation of renal NEP in diabetic mice is reversed by exercise training or rosiglitazone treatment (Elased et al., 2013). However, the role of renal NEP in diabetes and its presence in urine or plasma of CKD patients has not yet been elucidated.

**A Disintegrin And Metalloproteinase 17 (ADAM17)**

A Disintegrin and Metalloproteinases (ADAM’s) are type I multidomain transmembrane proteins that cleave the extracellular domains of membrane-bound proteins in a process known as ectodomain shedding. ADAMs belong to the adamalysin family of metalloproteinases. ADAMs are about 750 amino acids in length and are important in cell fate determination, cell migration, neurite and axon guidance, immunity, wound healing, cell proliferation, sperm-egg interaction, heart development, and angiogenesis (Seals & Courtneidge, 2003; Gooz, 2010). Out of 25 human ADAMs, ADAM17 and 12 other ADAMs have a prometalloprotease domain of reprolysin-type active site for binding of zinc atom indicating zinc dependent mechanisms (Blobel, 2005).
ADAM17 or CD156q is a cell surface protein, which is also known as tumor necrosis factor-α-converting enzyme (TACE) or transforming growth factor-α. Due to its action on various transmembrane proteins, growth factors, cytokines, receptors, and adhesive molecules, ADAM17 has become a therapeutic target in a broad spectrum of diseases such as cancer, inflammation, kidney disease, Alzheimer's disease, and respiratory and cardiorenal diseases (Arribas & Esselens, 2009). The specific role of ADAM17 in the pathophysiology of these diseases is very complex. To date, evidence suggests that activation of Ang II mediates the increase of ADAM17 levels and causes its redistribution to the apical membrane of distal renal tubules (Lautrette et al., 2005). Moreover, studies in transfected HEK293 cells, Huh7 cells, and CHK-2 cells showed ACE2 as one of the target substrates in the RAS for ADAM17 (Lambert et al., 2005; Jia et al., 2009; Salem et al., 2014). A recent study in the endothelial cell line Ea.hy926 showed ADAM17-dependent release of NEP from endothelial cells via exosomes (Kuruppu et al., 2014). Previous evidence suggests a role of ADAM17 in insulin resistance and hyperglycemia (Federici et al., 2005; Fiorentino et al., 2010). Further, our study in type 1 diabetic mice showed treatment with insulin normalized ACE2 shedding by restoring renal ADAM17 and ACE2 expression to levels comparable to baseline (Salem et al., 2014). This study also showed that ACE2 shedding into the media of human proximal tubular HK-2 cells was reduced upon treatment with ADAM17 inhibitor TAPI. Attenuation in ADAM17 levels were observed in type 2 diabetic mice treated with rosiglitazone or undergoing daily exercise training (Somineni et al., 2014; Chodavarapu et al., 2013). In renal diseases, administration of ADAM17 inhibitor showed beneficiary effects by attenuating interstitial kidney fibrosis and glomerulo-tubular lesions (Lautrette et al., 2005).

Tissue inhibitors of metalloproteinases (TIMPs) are considered as endogenous inhibitors of ADAMs (Reiss & Saftig, 2009). TIMP3 deficiency resulted in increased ADAM17 activity (Federici et al., 2005), which aggravates diabetic nephropathy (Basu et al., 2012). Increase in
renal injury was observed in TIMP3-/- animals with interstitial kidney fibrosis when compared to wild type (Kassiri et al., 2009). In type 2 diabetic patients, clinical studies demonstrated increased insulin receptor resistance due to TIMP3 decrease and increased ADAM17 activity in the circulation (Cardellini et al., 2011; Cardellini et al., 2009).

**Biomarkers for Diabetic Kidney Disease**

Biological samples that can be extracted or excreted from the body are used as primary tools for the diagnosis of many diseases. Among them, urine and blood components are mostly preferred for the diagnosis of DKD. In clinical proteomics, most researches prefer urine due to its ready availability and non-invasive method of collection. In clinical practice, various methods of urine collection are considered. This is because albuminuria follows a circadian rhythm. 24-hour urine collection, spot urine collection, and first morning void urine are currently considered as proper methods for urine collection. A difference between macro and micro albuminuria can be measured using 24hr urine. The disadvantages of the 24-hour urine collection method are that the collection is tedious and needs more care (Lambers Heerspink et al., 2010). Hence, simpler and more accurate methods of urine collection have been suggested (Rodby et al., 1995; Parving et al., 2014) and collection of spot urine and first morning void urine has become an alternative method (Boloor et al., 2014). Collection of first morning void urine after a complete overnight rest is more preferred than spot urine due to lower influence of hydration status and physical activity (Mogensen et al., 1995; Boloor et al., 2014). In the clinic, use of spot urine is more preferable due to its accuracy in measurement of protein-to-creatinine ratio, which has proven to be in accordance with 24-hr urine data (Chitalia et al., 2001; Montero et al., 2012; Nayak et al., 2013). A systemic review in most recent studies recommends measurement of albuminuria and proteinuria in 24 hour urine before renal biopsy (Akbari et al., 2014).
Albuminuria

Albuminuria has become an established biomarker and paradigm for diabetic nephropathy (Mogensen, 1987; Bakris & Glassock, ). Microalbuminuria, or excretion of albumin into urine that ranges between 30 to 300 mg/day, is considered as the first sign of renal impairment (Mogensen et al., 1983). The word micro refers to albumin excretion in small amounts, below the threshold of urine dipstick test for proteinuria (Poulsen et al., 1992; Berry, 2003). Microalbuminuria has become an optional asymptomatic intermediate (MacIsaac et al., 2014) during the development of dipstick-positive proteinuria that occurs in parallel to decline in glomerular filtration rate (Newman et al., 2005). Positive dipstick proteinuria also termed as macroalbuminuria or overt proteinuria leads to subsequent evolution of kidney failure. Macroalbuminuria, which is evident after significant kidney damage, is a condition where the amount of albumin excreted exceeds 300 mg/day (Mogensen et al., 1983). In summary, progression of microalbuminuria to proteinuria leads to renal function loss and, ultimately, to ESRD. Studies in diabetic patients found one-third of the patients develop advanced chronic kidney disease before the progression of microalbuminuria to proteinuria, suggesting that microalbuminuria is not conditional (Perkins et al., 2010). A ten year follow-up study in microalbuminuria patients suggested that 40% of the patients may develop proteinuria (Caramori et al., 2000). Decline in renal function of patient with type 1 diabetes mellitus (Perkins et al., 2010) and type 2 diabetes mellitus (MacIsaac et al., 2004) with normal albumin levels questions the reliability of microalbuminuria as a surrogate marker for decline in renal function. Microalbuminuria is also used as a marker in cardiovascular diseases. Presence of microalbuminuria in hypertensive non-diabetic patients (Parving et al., 1974) led many studies (Adachi, 2014; Noyes & Eckardt, 2013; Ibsen et al., 2008) to suggest albuminuria as a risk marker for cardiovascular diseases. Reduction in albuminuria by use of antihypertensive agents and RAS
inhibitors reduces mortality due to cardiovascular risks in diabetic nephropathic patients (de Zeeuw et al., 2004; Holtkamp et al., 2011).

Proteinuria may elicit interstitial inflammation and tubular effects that lead to fibrosis, providing a link with tubulointerstitial injury (Eddy, 2004). Studies have shown that inhibition of proteinuria protects the kidney from renal injury and eventual kidney failure (Rossing et al., 1994; Apperloo et al., 1997). With the goals of treating hypertension and proteinuria, inhibition of the RAS using angiotensin-converting enzyme inhibitors (Ruggenenti et al., 1999), angiotensin II receptor blockers (Brenner et al., 2001), aldosterone antagonists (Verdugo et al., 2014), and renin inhibitors (Wang et al., 2014) are currently used treatment methods (Damman & Lambers-Heerspink, 2014). Studies involving patients with overt nephropathy showed decrease in blood pressure and remission of proteinuria using RAS inhibitors (Satirapoj, 2013). But a recent study in type 2 diabetic patients has shown that only one third of the patients achieved remission of proteinuria due to the effort to achieve normal blood pressure (Tan et al., 2014). Increased risk of ESRD together with severe diabetic retinopathy was associated with failure to attain remission of proteinuria in diabetic patients (Tan et al., 2014). In support to the reasons mentioned earlier, the diagnostic role of proteinuria as an independent biomarker has also been questioned due to toxicity of albumin and albumin-bound lipids to the proximal tubule and formation of glomerular crescents as a result of glomerular pathology (Christensen & Verroust, 2008). Thus albuminuria needs more evaluation to confirm its specificity as an independent biomarker for kidney disease (Williams, 2005).

**Estimated glomerular filtration rate (eGFR)**

Glomerular filtration rate (GFR) is a well-established marker for cardiovascular (Mathisen et al., 2011) and kidney function. Blood passes through the glomeruli of the kidney from the afferent arteriole to the efferent arteriole. This mechanism creates a pressure bed to filter
blood at a certain flow rate called glomerular filtration rate. A decline in eGFR indicates advanced glomerular lesions in normoalbuminuric patients with diabetes (Caramori et al., 2003; Iqbal & Alam, 2013). In proteinuric patients with initially normal GFR, a sharp progressive decline in GFR has been observed (Halbesma et al., 2006). Proteinuria along with estimated GFR can predict cardiorenal outcomes (Brantsma et al., 2008). In clinical practice, GFR cannot be measured easily (Stevens et al., 2006), and estimation of GFR has become an alternate method by using serum creatinine level, age, race, sex, and BMI (Levey et al., 2011). But the accuracy of eGFR in predicting CKD is imprecise as GFR estimates are based on many parameters. For instance, serum creatinine production is dependent on lean body mass (Perrone et al., 1992). Cystatin C, a unique protein secreted by most of the cells and filtered through the glomerulus, is less affected by muscle mass and diet which has become an alternate for serum creatinine. But serum cystatin C is also affected by non-GFR determinants such as age, gender, albumin, and serum C-reactive protein (Eriksen et al., 2010; Knight et al., 2004). In addition, evaluation of eGFR with serum creatinine is more associated with CKD compared to GFR estimation with cystatin C (Rule et al., 2013). Recent studies have shown that eGFR calculations based on both markers, i.e. serum creatinine and cystatin C (eGFRcr-cys), are more precise than eGFR calculations using only serum creatinine or cystatin C (Inker et al., 2012; Ma et al., 2007).

Current updates of the Kidney Disease Improving Global Outcomes (KDIGO) guidelines recommend GFR estimation using serum creatinine (eGFR-cr) followed by cystatin C (eGFR-cys) in cases with inaccurate GFR estimate using serum creatinine (Levin & Stevens, 2014). Most of the clinical laboratories use the MDRD (Modification of Diet in Renal Diseases) Study formula when serum creatinine measurement is used for estimation of GFR, and this equation is widely accepted in assessment of CKD (Levey et al., 2006). The current issue of the international KDIGO guidelines has an updated classification using a new
equation called CKD-EPI (Chronic Kidney Disease Epidemiology Collaboration) based on creatinine or cystatin C (Levey et al., 2009; Iqbal & Alam, 2013). To recognize the difference between cardiovascular outcomes, KDIGO in 2013 changed the eGFR categories by modifying G3 stage as G3a and G3b (Iqbal & Alam, 2013). Recent studies in ESRD patients showed an increased mortality rate in patients before reaching ESRD, suggesting a need for alternative endpoints and markers that could better predict the stage of dialysis (Coresh et al., 2014; Rosansky & Glassock, 2014).

**Biomarkers for Diabetic Kidney Disease (DKD)**

DKD is the most common form of CKD leading to ESRD and cardiovascular complications. Development and progression of kidney injury can be assessed by changes in nephron function. Glomerular dysfunction and tubular damage are thought to be affected in the pathogenesis of kidney dysfunction (Nauta et al., 2011). In diseased condition, presence of abnormal levels of proteins in nephron classifies their designation as glomerular or tubular markers.

**Biomarkers of glomerular injury**

Proteinuria is a sign of glomerular injury. However, recently new biomarkers for kidney podocytes injury such as urinary podocin, podocalyxin, and nephrin have been discovered.

**Transferrin**

Transferrin is a plasma protein, which is similar to albumin in molecular weight and charge. Presence of elevated urinary transferrin in type 2 diabetic patients predicts development of microalbuminuria (Zhou et al., 1997) and onset of nephropathy (Hellemons et al., 2012). However, increase in urinary transferrin in glomerulonephritis questions its use as a specific marker for DKD (Mackinnon et al., 2003).
Podocalyxin

Podocalyxin is a sialomucin that maintains podocyte shape and organization of the slit diaphragm. It is expressed on the surface of podocytes. Presence of podocytes in urine indicates various kidney injuries. Detachment of podocyte from glomerular basement membrane was observed at early stages DKD (Weil et al., 2012). Cross-sectional studies in type 2 diabetic patients with normoalbuminuria showed increased urinary podocalyxin levels (Hara et al., 2012; Ye et al., 2014), which could be used as an early marker for DKD.

Podocin

Podocin is a podocyte protein that maintains structural integrity of slit diaphragm (Boute et al., 2000). It is a membrane bound protein that binds components of slit diaphragm and recruits them to increase signaling properties (Schwarz et al., 2001). Presence of increased levels of podocin in urine of diabetic patients indicates progression in renal disease (Zheng et al., 2011; Simon et al., 2014).

Nephrin

Nephrin is a transmembrane protein that is expressed in the glomerular filtration slit diaphragm (Langham et al., 2002). Clinical studies in type 1 and type 2 diabetic patients with normoalbuminuria, microalbuminuria, and macroalbuminuria showed presence of nephrin in urine (Ebihara et al., 2000; Nakamura et al., 2000; Patari et al., 2003; Jim et al., 2012).

Biomarkers of tubular injury

Liver Fatty Acid Binding Protein (L-FABP)

L-FABP is expressed in proximal tubules (Su et al., 2004) that functions as an endogenous antioxidant during conditions of oxidative stress (Kamijo-Ikemori et al., 2014). Recent cross-
sectional studies and longitudinal studies in type 2 (Maeda et al., 2014) and type 1 diabetic patients (Panduru et al., 2013) with normoalbuminuria reported a significant increase in L-FABP levels.

**Connective Tissue Growth Factor (CTGF)**

CTGF is a profibrotic growth factor protein that is expressed in podocytes (Okada et al., 2005). In patients with type 2 diabetes and advanced renal injury, CTGF is also expressed in proximal tubules of kidney suggesting its role as a tubular marker in diseased condition (Kobayashi et al., 2006).

**Neutrophil Gelatinase- Associated Lipocalin (NGAL)**

NGAL is a lipocalin iron-carrying protein that is highly expressed in the tubular epithelium of the distal nephron (Bolignano et al., 2009). In type 2 diabetic patients with normo- or microalbuminuria, increased urinary NGAL showed negative correlation with eGFR (Jain et al., 2005). Studies in type 1 diabetic patients showed elevated urinary NGAL and its association with albuminuria (Otu et al., 2007), suggesting that urinary NGAL could indicate DKD.

**Kidney Injury Molecule 1 (KIM-1)**

KIM-1 is a type 1 transmembrane protein present on the apical membrane of proximal tubular cells. Recent cross-sectional study in diabetic patients showed increased KIM-1 levels when compared to control (Fu et al., 2012; van Timmeren et al., 2007). Moreover, urinary KIM1 predicted decline in eGFR levels of type 2 diabetic patients (Nielsen et al., 2012) independent of albuminuria (Fu et al., 2012). These finding propose KIM-1 as a marker for tubular kidney injury.
**Cystatin C**

Cystatin C is a cysteine protease inhibitor that is freely filtered by glomerulus. This protein is used for calculation of eGFR. In normal conditions, serum cystatin C is filtered through glomerulus, which is noted to be reabsorbed in proximal tubule. But independent of serum cystatin C, urinary cystatin C was increased in patients with renal tubular damage, suggesting impaired reabsorption (Herget-Rosenthal et al., 2004). In DKD patients at stage 3, urinary cystatin C was found to be associated with decline in GFR levels (Kim et al., 2013).

**Inflammatory and fibrotic markers as diabetic kidney injury markers**

Circulating TNF receptors 1 and 2 help in the prediction of CKD stage 3 and ESRD in type 1 and type 2 diabetes mellitus (Gohda et al., 2012; Niewczas et al., 2012). A pro-fibrotic cytokine, transforming growth factor beta (TGFβ) has been used for detection of experimental and human diabetic nephropathy (Yamamoto et al., 1993). Independent of albuminuria, Naura et al., found heart fatty acid binding protein (H-FABP) in urine due to kidney dysfunction (Nauta et al., 2011). Tamm-Horsfall protein (uromodulin), progranulin, clusterin, and alpha-1 acid glycoprotein were found in urine at early stages of renal injury in type 1 diabetes (Rossing et al., 2008). Hyperglycemia stimulates synthesis of collagen IV, which is a substantial component of tubular and glomerular basement membrane (Ziyadeh, 1993). Increase in urinary collagen type IV is associated with renal disease in type I and type II diabetes mellitus (Cohen et al., 2001; Araki et al., 2010). In diabetic nephropathy, urinary IL-6, IL-8, monocyte attractant protein-1, interferon gamma-inducible protein and macrophage inflammatory protein-1 were associated with renal function decline in type 1 diabetes (Wolkow et al., 2008). Increased haptoglobin levels in urine detected microalbuminuria and early decline in renal function of type 2 diabetic patients (Bhensdadia et al., 2013). A study in diabetic kidney disease patients showed biomarkers in the pathways...
of Fibroblast Growth Factor (FGF)-23 and Vascular Endothelial Growth Factor (VEGF)-A, which are independent of albuminuria levels (Agarwal et al., 2014).

**Urinary RAS components as biomarkers**

An activated intrarenal RAS plays a major role in the progression of renal injury (Kobori et al., 2007). Indeed, recent studies suggest urinary angiotensinogen as a novel biomarker (Xu et al., 2014; Kobori et al., 2008b; Mills et al., 2012). Increased urinary angiotensinogen levels were found in CKD patients perhaps as a specific consequence of proteinuria (Kobori et al., 2008b). Presence of undetectable circulating angiotensinogen led to the hypothesis that urinary angiotensinogen originates from proximal tubules of kidney (Kobori et al., 2002; Kobori et al., 2008a). A study in rats with diabetic nephropathy showed that urinary angiotensinogen excretion precedes albuminuria (Saito et al., 2009) and may be a marker for intrarenal Ang II, associated with CKD progression (Yamamoto et al., 2007). In addition, a recent study in CKD stage 3 patients with metabolic acidosis found dietary alkali treatment reduced urinary angiotensinogen and preserved glomerular filtration rate (Goraya et al., 2014). However, an investigation using kidney-specific angiotensinogen knockout mouse model revealed that renal angiotensinogen has no role in podocyte injury where renal Ang II level remained unchanged. This study suggests that renal angiotensinogen has no effect on renal angiotensin levels (Matsusaka et al., 2012). In addition, a recent peer review discussing the source for urinary angiotensinogen questioned its origin from renal tissue (Roksnoer et al., 2013).

In addition to angiotensinogen, the renin-prorenin and aldosterone axis has emerged as a new target in the field of biomarkers. Hyperglycemia induces increase in rate of renin excretion reflecting the status of the diabetic kidney prior to angiotensinogen excretion (van den Heuvel et al., 2011). Blockade of the RAS with ACE inhibitors and ARBs decreased the
renin excretion rate independent of alterations in plasma renin, suggesting a role of the renal renin-prorenin axis in the activation of the renal RAS (van den Heuvel et al., 2011; Lansang et al., 2001; Persson et al., 2009; Hollenberg et al., 2003). Van den Heuvel et al. also found decreased plasma angiotensinogen levels without changes in plasma aldosterone by use of RAS blockers (van den Heuvel et al., 2011). Synthesis of aldosterone is observed in diabetic kidneys, suggesting aldosterone synthesis may occur in the kidney (Xue & Siragy, 2005). Continuing in this vein, many studies have addressed the use of aldosterone blockers along with ACE inhibitors and ARBs as a treatment strategy for CKD (Bolignano et al., 2014). Stimulation of collecting duct prorenin by diabetes via Ang II caused hyperplasia in renin-producing connecting segments (Kang et al., 2008). Elevated plasma pro-renin levels in microvascular complications of diabetes, including nephropathy, suggest pro-renin may have a role as early biomarker (Batenburg et al., 2014; Danser et al., 1989; Franken et al., 1990).

Recent study on urinary ACE, which is expressed at 90 kDa, has been described as a genetic marker for hypertension (Casarini et al., 2001; Maluf-Meiken et al., 2012). In addition, presence of soluble ACE in pregnant women with pure preeclampsia suggested the 90 kDa N-domain isoform of ACE could serve as a urinary biomarker for preeclampsia (Krauspenhar et al., 2014).

Recent studies showed increased urinary ACE2 in diabetic patients with CKD (Mizuiri et al., 2011a) and in renal transplantation recipients (Xiao et al., 2012). Previous students in our lab have shown increased urinary ACE2 and decreased renal ACE2 in type 1 and type 2 diabetic mice. Normalizing hyperglycemia with antidiabetic medications and/or exercise attenuated microalbuminuria and the increase shedding of ACE2 in the urine of diabetic mice (Chodavarapu et al., 2013; Salem et al., 2014; Somineni et al., 2014).
Clinical studies in kidney transplant recipients (Nortier et al., 1992) and acute renal nephropathic patients (Vlaskou et al., 2000) have shown increased levels of NEP in urine and proposed this enzyme as a tubular marker (Nortier et al., 1993). Our studies in type 2 diabetic mice suggest that decreased urinary NEP may indicate development and progression of diabetic nephropathy (Chodavarapu et al., 2012; Elased et al., 2013).

This study was undertaken to test the hypothesis that urinary ACE2 and NEP are elevated in diabetic patients. For this purpose, we measured the levels of these markers and several metabolic and renal factors in urine and plasma samples obtained from non-diabetic individual and patients with type 2 diabetic patients.
2. HYPOTHESIS AND SPECIFIC AIMS

Hypothesis
There is an increase in urinary ACE2 and NEP prior to the onset of microalbuminuria in diabetic patients with CKD. Urinary ACE2 and NEP could be used as biomarker for CKD induced by type 2 diabetes.

Specific aims:

1. To test the hypothesis that urinary ACE2 and its sheddases, ADAM 17 are increased in type 2 diabetic patients with kidney injury and prior onset of microalbuminuria. Fluorogenic and mass spectrometry-based assays will be used to measure ACE2 activity. ELISA and western blot will be used to identify, quantify urinary ADAM17 and ACE2 protein expression.

2. To test the hypothesis, that urinary NEP is increased in type 2 diabetic patients with kidney injury and prior onset of microalbuminuria. Mass spectrometry-based assay will be used to measure NEP activity. ELISA and western blot will be used to identify, quantify NEP protein expression.

3. To test the hypothesis that, urinary ACE2 and NEP could be used as a biomarker to predict incidence or progression of CKD in type 2 diabetic patients.
3. MATERIAL AND METHODS

Study Protocol

This cross-sectional study was performed at the outpatient clinic of Dayton VA hospital, from 2008 to 2011. A total of 60 patients of which 40 patients had a history of microalbuminuria (30 to 300 mg albumin/g creatinine) and macroalbuminuria (>300 mg albumin/g creatinine) were considered. Based on their urinary albumin per creatinine ratio (UACR) at previous visits, the patients were classified into four groups: 1) Nondiabetic patients (ND); 2) Diabetic patients with normoalbuminuria (Dnormo); 3) Diabetic patients with microalbuminuria (Dmicro); 4) Diabetic patients with macroalbuminuria (Dmacro). Type 2 diabetic patients with hypertension were included. At the time of enrollment, patients with albuminuria >30 mg/g were under anti-hypertensive, anti-diabetic and diuretic medication.

This study was approved by Wright State University Institutional Review Board (IRB) and Dayton VA IRB Committee. Patients with liver dysfunction, heart failure, malignancy, pregnancy, and glomerulonephritis were excluded from the study.

Metabolic, cardiovascular, and renal parameters

Metabolic, cardiovascular and renal parameters including age, sex, height, weight, body mass index, mean arterial pressure, creatinine clearance, serum creatinine, serum albumin, glycated hemoglobin (HbA1c), blood glucose, spot albumin-to-creatinine ratio (UACR), protein creatinine ratio (UPCR), blood urea nitrogen (BUN), estimated glomerular filtration rate (eGFR) and electrolytes sodium, potassium, chloride, calcium were analyzed in Dayton VA Medical Center. Patients with active urinary tract infection and history of renal disease were excluded from the study. Determination of eGFR was done by Modification of Diet in Renal
Disease (MDRD) formula: \[ \text{MDRD} = 186 \times \text{SerumCr(mg/dL)}^{-1.154} \times \text{age}^{-0.203} \times 1.212 \ (\text{if patient was black}) \] or \( \times 0.742 \ (\text{if patient was female}). \)

**Sample collection**

Spot urine was collected directly by the patients (in the morning) into a sterile container containing 50 \( \mu \)l of protease inhibitor (Roche Diagnostics, Indianapolis, IN, USA). Medical history and anthropometric measurements were also recorded the same day. Venous blood was withdrawn from the antecubital vein from all subjects and centrifuged at 10,000 rpm for 10 min at 4°C on the same day. Blood samples were collected when patients were in sitting position after a 30-min rest. Urine and plasma samples were aliquoted and stored at -80°C.

**Urinary albumin assay**

Increased urinary albumin excretion is well-established predictor of CKD progression and may reflect both glomerular and tubulointerstitial kidney injury. To monitor kidney function, quantitative estimation of urinary albumin was performed using an Enzyme Linked Immuno Sorbent Assay kit purchased from Bethyl Laboratories (Montgomery, TX, USA). Urine samples were collected and stored as described above. In a 96-well plate, wells were coated with 100 \( \mu \)l affinity purified goat anti-human antibody diluted 1:100 in carbonate-bicarbonate buffer for 1hr at room temperature (RT). After incubation, the antibody solution was aspirated from each well and washed 3 times with 200 \( \mu \)l Tris-buffered saline containing 0.05M Tris, 0.138M NaCl, 0.0027M KCl and 0.05% Tween 20 (TBS-T buffer). Then each well was incubated with 200 \( \mu \)l blocking buffer (Tris buffered saline with 1% BSA) overnight at 4°C and washed 4x with TBS-T buffer the following morning. Two microliters of urine samples were diluted 1:1000 in conjugate buffer. One hundred microliters of diluted urine sample were added to wells in duplicate. At the same time, 100 \( \mu \)l of standards were added to assigned wells in duplicate followed by 1 hour incubation at RT. Wells were then washed...
4 times with TBS-T buffer. Then 100 µl HRP-conjugated detection antibody diluted 1:30,000 in conjugate buffer was added to each well and incubated for 1 hour at R.T. Wells were then washed 5 times with TBS-T buffer. Equal volumes of TMB substrate A and substrate B (KPL, Gaithersburg, MD) were mixed and 100 µl of combined substrate was added to each well and incubated in dark for 15 min. HRP cleaves the substrate to produce color. The intensity of the color produced is directly proportional to the amount of albumin present in the sample. 2N H₂SO₄ was added to each well to stop the reaction. The absorbance values were measured at 450 nm using a Fusion® Packard plate reader. Standard curve was plotted using standards in the range of 6.25-400 ng/ml to determine unknown urinary albumin concentrations.

**Urinary Creatinine Assay**

Creatinine is excreted into urine by glomerular filtration in a healthy individual at constant rate. Thus, urinary creatinine levels are useful in detecting renal disease and estimating the extent of impairment of renal function. Urinary creatinine assay was performed using a kit purchased from Quidel (San Diego, CA, USA). The assay is based on modified Jaffe reaction where alkaline picrate forms a colored solution in presence of creatinine. Urine samples and standards were diluted 1:40 in distilled water and 50 µl was loaded in duplicates into a 96-well plate. One hundred and fifty µl of working color reagent (7 mL picric acid + 1 mL 1N Na OH) was added to each well. This mixture was incubated for 30 min at RT. The intensity of the color was measured at 490 nm using a Fusion® Packard plate reader. A standard curve was plotted using assay standards to determine unknown creatinine concentrations. Urinary albumin was normalized to urinary creatinine to account for variability in glomerular filtration rate among mice. Urinary albumin excretion (UAE) is the urinary albumin concentration (µg/ml) divided by urinary creatinine (mg/ml) resulting in UAE (µg/mg).
**Urinary and plasma ACE2 activity**

Urinary ACE2 enzyme activity was measured by incubating sample volumes equivalent to 10 μg of urinary creatinine with reaction buffer (50 mM Tris, 5 mM ZnCl₂, 150 mM NaCl₂ and 10 μM Lisinopril) mixed with synthetic, fluorogenic peptide 7-Mca-APK-(Dnp) (Enzo Life Sciences, Farmingdale, NY). Upon cleavage by ACE, this fluorogenic peptide emits fluorescence which was measured at excitation (λₓₑₓ): 328 nm and emission (λₑₘₑ): 393nm using a Fusion® Packard instrument. The fluorogenic peptide is specific for ACE2 as described before (Chodavarapu et al., 2013; Salem et al., 2014; Somineni et al., 2014). Creatinine concentrations in the urine samples were used to normalize the assay results. The specificity of ACE2 enzyme fluorogenic assay was confirmed by inhibition with 10 μM of specific inhibitors such as ACE2 inhibitor, MLN-4760 (a gift from the former Millennium Pharmaceuticals, Cambridge, MA, USA), and comparison to prolyl endopeptidase/prolyl carboxypeptidase inhibitor Z-prolyl-prolinal (ZPP; Enzo Life Sciences, Farmingdale, NY, USA) and neprilysin inhibitor thiorphan (Sigma Aldrich, St. Louis, MO, USA). Plasma ACE2 fluorogenic assay was performed with 20 μl of plasma sample using the same assay.

**Urinary and plasma NEP ELISA**

Levels of NEP in the plasma and urine of patients were determined using the Human NEP Duoset ELISA Development Kit (Catalog # DY1182) purchased from R&D Systems (Minneapolis, MN, USA). Assays were performed according to the manufacturer instructions. Samples were stored at -80°C until assay. Samples and standards were assayed in duplicates. Hundred microliters of goat anti-human NEP capture antibody were used to coat 96 wells-plate overnight. Urine and plasma samples (100 μl) were added directly to the 96- well plate, and incubated at RT for 2-hr. Wells were washed 3 x with 300 μl PBS containing 0.05 % Tween 20. Wells were blocked for 1 hr at RT using diluent buffer (PBS...
contain 1% BSA). Hundred microliters of standards and diluted samples were loaded to the wells and incubated for 2 hours at RT. Then, biotinylated goat anti-human NEP detection antibody was added and incubated for 2 hrs at RT followed by binding of streptavidin-horse radish peroxidase (HRP) for 20 min. Unbound streptavidin-HRP was washed off and 3,3′,5,5′-Tetramethylbenzidine substrate was added. 2N H₂SO₄ was added to each well to stop the reaction. The absorbance at 450 nm was measured using a Fusion Packard plate reader (Packard Bioscience, Meriden, CT, USA). Unknown urinary and plasma NEP concentrations were determined from a standard curve plotted using assay standards in the range of 0.175–8 ng/ml.

**Urinary ADAM17 ELISA**

Levels of ADAM17 in the urine of patients were determined using the Human ADAM 17 Duoset ELISA Development Kit (Catalog # DY930) purchased from R&D Systems (Minneapolis, MN, USA). Assays were performed according to the manufacturer instructions. Samples were stored at -80°C until assay. Samples and standards were assayed in duplicate. Hundred microliters of mouse anti-human ADAM17 capture antibody (Cat# AF1182) were used to coat 96 well plate and incubated overnight at RT. After incubation, the antibody solution was aspirated from each well and washed 3 times with 300 μl of wash buffer (0.05% Tween_20 in PBS, pH 7.2 - 7.4). Three hundred microliters of Reagent Diluent were used to block the plate for 1 hour at RT. One hundred microliters of standard and urine samples were added to each well and incubated for 2 hr at RT. Plates were washed with 300 μl wash buffer 3 times. One hundred microliters of biotinylated goat anti-human ADAM17 detection antibody was added to each well and incubated for 2 hr at RT. One hundred microliters of streptavidin conjugated to horseradish-peroxidase (HRP) was added to each well of the assay plate and incubated for 20 min at room temperature in the dark. Unbound
streptavidin-HRP was washed off with 300 μl wash buffer 3 times. Equal volumes of TMB substrate A and substrate B (KPL, Gaithersburg, MD, USA) were mixed and 100 μl of combined substrate was added to each well and incubated in dark for 15 min. Equal volumes of TMB substrate A and substrate B (KPL, Gaithersburg, MD, USA) were mixed and 100 μl of combined substrate was added to each well and incubated in dark for 15 min. One hundred microliter of 1:1 mixture of Color Reagent A (H2O2) and Color Reagent B (Tetramethylbenzidine) (R&D Systems Catalog # DY999) were added to each well and incubate for 20 min at RT. The reaction was stopped using 2 N sulfuric acid. The absorbance was measured using a Fusion Packard plate reader (Packard BioScience, Meriden, CT, USA) at 450 nm. Unknown urinary ADAM17 concentration was determined from a standard curve plotted using assay standards in the range of 0.157–10 ng/ml.

**ACE2 and NEP activity determination using Mass Spectrometry**

Urinary ACE2 and NEP activity were measured using matrix assisted laser desorption/ionization (MALDI) time of flight (TOF) mass spectrometry (MS) with assistance of Dr. Nadja Grobe (wright state University, Dayton, OH). Urine sample equivalent to 25 μg of creatinine was incubated with 0.4 M MES buffer pH 6.75 and 10 μM Ang II or 70 μM Ang I for 2 hr at 37°C along with shaking at 800 rpm. For Ang II assays, 0.5 mM 4-aminophosphonobutyric acid (APBA) and 0.1 mM ZPP were added and ACE2 activity was tested using 0.1 mM MLN-4760. For Ang I assays, 0.5 mM APBA, 0.1 mM MLN-4760, and 0.1 mM Lisinopril were added and NEP activity was tested using 0.1 mM Thiorphan. The reaction was stopped by acidification with 1% trifluoroacetic acid (TFA) and 1:20 dilution using 90% acetonitrile containing 0.3% TFA. These samples were spotted on a MALDI target plate together with MALDI matrix consisting of 10 mg/ml α-cyano-4-hydroxy-cinnamic acid in 60% methanol, 10% acetone and 0.3% TFA. Mass spectra were obtained
using an Autoflex III smartbeam MALDI TOF/TOF instrument (Bruker Daltonics, Billerica, 
MA, USA) operated with positive polarity in reflectron mode. A total of 3000 laser shots 
were acquired randomly for each spot in the range of \( m/z \) 500–3000 at a laser frequency of 
100 Hz. Spectra were mass calibrated using a Bruker peptide calibration standard II.

**Western blot Analysis**

Urine samples were thawed on ice and 50 µl was added to equal volume of Laemmli Sample 
Buffer with 5% β-mercaptoethanol (Bio-Rad, Hercules, CA, USA) and boiled for 10 min. 
Urine samples equivalent of 10 µg creatinine were loaded on 10% SDS-PAGE gel and 
separated by electrophoresis at 130 V for 1 hr. Proteins on gel were then transferred to a 0.2 
µm PVDF membrane (Millipore, MA, USA) for 2 hrs 45 min at 72 V with Mini Trans-Blot 
Electrophoretic Tranfer cell (BioRad, Hercules, CA, USA). The membrane was blocked for 
1 h with 10% non-fat milk made in 10 mM Tris buffered saline with Tween-20 (TBS-T) at RT. For protein expression analysis, membranes were probed with goat anti-mouse ACE-2 
(1:1000 dilution, R&D systems, USA), goat anti mouse Neprilysin/CD10 (1:500 dilution, 
R&D systems, USA), goat anti-human albumin (1:500 Bethyl laboratories, Montgomery, 
USA) primary antibodies for 2 days at 4°C. The membranes were then washed with TBS-T 
buffer 3 times for 5 minutes at RT followed by incubation with horse radish peroxidase 
(HRP) conjugated rabbit anti-goat secondary antibody (1:2000, R&D systems, USA). 
Supersignal chemiluminescent substrate (Thermo Scientific, IL, USA) was used to detect the 
signals which were analyzed using ChemiDoc imaging system (BioRad, Hercules, CA, 
USA). The relative intensities of protein bands were quantified by Image lab 4.0 software 
(BioRad, USA). Signal intensities from ACE2, NEP, and albumin were calculated relative to 
amount of creatinine loaded.
**Statistical Analysis:**

Analysis was performed using Graph Pad Prism 5.01 software. For multiple comparisons between two or more groups, one-way ANOVAs were carried out followed by Bonferroni’s multiple comparison tests. A linear regression for Pearson’s population correlation was constructed to identify the association between ACE2 and NEP with selected metabolic and renal parameters. A p-value of $p<0.05$ was considered statistically significant.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Nondiabetic controls (n=19)</th>
<th>Diabetic patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nondiabetic controls (n=19)</td>
<td>Normoalbuminuria (n=13)</td>
</tr>
<tr>
<td>Age --yr</td>
<td>53.0±14.3</td>
<td>63.7±11.1</td>
</tr>
<tr>
<td>Male Sex-no. (%)</td>
<td>18 (93.7)</td>
<td>12 (91.7)</td>
</tr>
<tr>
<td>Race-no. (%)</td>
<td>White 18 (93.7)</td>
<td>8 (58.3)</td>
</tr>
<tr>
<td></td>
<td>Black 1 (6.2)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>Body-mass index-lbs/in2</td>
<td>31.5±5.5</td>
<td>34.2±7.3</td>
</tr>
<tr>
<td>Mean Arterial Pressure - mmHg</td>
<td>92.7±13.5</td>
<td>92.58±10.4</td>
</tr>
<tr>
<td>Glycated Hemoglobin-%</td>
<td>5.2±0.3</td>
<td>6.47±0.7</td>
</tr>
<tr>
<td>Serum Creatinine-mg/dl</td>
<td>0.9±0.2</td>
<td>1.0±0.2</td>
</tr>
<tr>
<td>Estimated GFR</td>
<td>90.6±21.0</td>
<td>85.2±18.8</td>
</tr>
<tr>
<td>Mean - ml/min/1.73m² Category-no./ Total no. ≥60</td>
<td>19/19</td>
<td>13/13</td>
</tr>
<tr>
<td></td>
<td>59.9-45.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>44.9-30.0</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>&lt;30</td>
<td>-</td>
</tr>
<tr>
<td>Urinary albumin-to creatinine ratio(mg/g)</td>
<td>3.10</td>
<td>10.10</td>
</tr>
<tr>
<td>Median</td>
<td>1.7-5.9</td>
<td>4.4-11.85</td>
</tr>
<tr>
<td>Urinary protein-to-creatinine Ratio(mg/g)</td>
<td>0.02</td>
<td>0.14</td>
</tr>
<tr>
<td>Median</td>
<td>0.0-0.1</td>
<td>0.1-0.24</td>
</tr>
<tr>
<td>Variable</td>
<td>Median</td>
<td>Interquartile Range</td>
</tr>
<tr>
<td>----------------------</td>
<td>--------</td>
<td>---------------------</td>
</tr>
<tr>
<td>Interquartile Range</td>
<td>114.6-147.8</td>
<td>78.5-155.4</td>
</tr>
<tr>
<td>Glucose-mg/dl</td>
<td>146.5#</td>
<td>130</td>
</tr>
<tr>
<td>Medications-no. (%)</td>
<td>92.0-109.0</td>
<td>91.0-155.0</td>
</tr>
<tr>
<td>ACE inhibitor or ARB</td>
<td>4(25)</td>
<td>9(75)</td>
</tr>
<tr>
<td>Diuretics</td>
<td>4(25)</td>
<td>4(33.4)</td>
</tr>
<tr>
<td>Calcium Channel</td>
<td>1(6.2)</td>
<td>5(41.7)</td>
</tr>
<tr>
<td>Blockers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta Blockers</td>
<td>2(12.2)</td>
<td>8(66.7)</td>
</tr>
</tbody>
</table>

Variables are expressed as no. (%), mean ± S.D. or medians and interquartile range and \(^* p<0.05 \) vs. nondiabetic, \(^# p<0.0001 \) vs. nondiabetic, \(^1 p<0.05 \) vs microalbuminuria, \(^@ p<0.0001 \) vs. microalbuminuria, \(^\parallel p<0.05 \) vs. macroalbuminuria, \(^\\parallel p<0.0001 \) vs macroalbuminuria, \(^\parallel\parallel p<0.0001 \) vs normoalbuminuria.
Table 3: Correlations of urinary ACE2 and NEP with various metabolic and renal functional parameters

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Urinary ACE2 activity (nmoles/hr/mg creatinine)</th>
<th>Urinary Neprilysin/creatinine (ng/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r</td>
<td>P value</td>
</tr>
<tr>
<td>Age</td>
<td>0.19</td>
<td>0.07</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.025</td>
<td>0.42</td>
</tr>
<tr>
<td>Mean arterial pressure (MAP)</td>
<td>0.19</td>
<td>0.07</td>
</tr>
<tr>
<td><strong>Biochemical Data</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>eGFR (ml/min per 1.73 m²)</td>
<td>-0.22</td>
<td>0.04 *</td>
</tr>
<tr>
<td>Glycated hemoglobin (HbA1C) (%)</td>
<td>0.42</td>
<td>0.0003 *</td>
</tr>
<tr>
<td>UACR (mg/g)</td>
<td>0.18</td>
<td>0.07</td>
</tr>
<tr>
<td>Blood Urea Nitrogen (mg/dl)</td>
<td>0.28</td>
<td>0.0014 *</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>0.31</td>
<td>0.008 *</td>
</tr>
<tr>
<td>Serum Creatinine (mg/dl)</td>
<td>0.25</td>
<td>0.03 *</td>
</tr>
</tbody>
</table>

Abbreviations: eGFR: estimated glomerular filtration rate; UACR: Urinary albumin-to-creatinine ratio
Figure 2: Classification of patients according to their GFR and albuminuria

- Total Patients (n=60)
  - Nondiabetic (ND) (n=20)
  - Diabetic with history of microalbuminuria (Dnormo) (n=12)
  - Diabetic with normoalbuminuria (Dnormo) (n=12)
  - Diabetic with microalbuminuria (Dmicro) (n=8)
  - Diabetic with macroalbuminuria (Dmacro) (n=20)
**Figure 3:** Urinary angiotensin converting enzyme 2 (ACE2) activity assay in nondiabetic control subjects and diabetic patients using fluorogenic assay method. One-way ANOVA showed a significant increase in urinary ACE2 activity of Dnormo, Dmicro and Dmacro compared to ND. *p*<0.05 vs ND. Each bar represents mean ± SEM of patients from ND (n=19), Dnormo (n=13), Dmicro (n=8), Dmacro (n=20).
Figure 4: Confirmation of specificity of fluorogenic activity assay for ACE2 using MLN-4760 (ACE2 inhibitor), Z-pro prolinal (ZPP; prolyl endopeptidase/prolyl carboxypeptidase inhibitor) and Thiorphan (NEP inhibitor). Urinary ACE2 activity without addition of inhibitors was set at 100%. *P<0.0001 vs. control assays without inhibitor. Each bar represents mean ± SEM of patients from ND (n=19), Dnormo (n=13), Dmicro (n=8), Dmacro (n=20).
Figure 5: Plasma angiotensin converting enzyme 2 (ACE2) activity assay in nondiabetic control subjects and diabetic patients. Urine sample from type 2 diabetic mice (db/db) was used to confirm and compare the ACE2 activity assay. There is no detectable ACE2 activity in plasma samples of non-diabetic and diabetic patients. Each bar represents mean ± SEM of patients from ND (n=19), Dnormo (n=13), Dmicro (n=8), Dmacro (n=20).
Figure 6: Correlation between estimated glomerular filtration rate and urinary ACE2 activity in nondiabetic and diabetic patients. Pearson correlation factor analysis showed significant negative correlation between eGFR and urinary ACE2 excretion. $r = -0.22$, $p = 0.04$ (n=60).

Figure 7: Correlation between urinary albumin per creatinine ratio and urinary ACE2 activity in nondiabetic and diabetic patients. Pearson correlation factor analysis showed no correlation between UACR and urinary ACE2 excretion. $r = 0.18$, $p = 0.07$ (n=60).
**Figure 8:** Correlation between urinary protein to creatinine ratio and urinary ACE2 activity in nondiabetic and diabetic patients. Pearson correlation factor analysis showed no correlation between protein-to-creatinine ratio and urinary ACE2 excretion. $r = 0.18, p = 0.08$.

**Figure 9:** Correlation between glycated hemoglobin and urinary ACE2 activity in nondiabetic and diabetic patients. Pearson correlation factor analysis showed significant positive correlation between glycated hemoglobin (%) and urinary ACE2 excretion. $r = 0.42, p = 0.0003$. 
**Figure 10:** Western immunoblot blot analysis of urinary ACE2 protein expression in nondiabetic and diabetic patients with various levels of albuminuria. Dual precision protein marker from bio-rad- Marker; Nondiabetic with GFR >90 ml/min/1.73m$^2$ – ND; Diabetic with microalbuminuria with GFR ≥60 ml/min/1.73m$^2$ - Dmicro; Diabetic with macroalbuminuria with GFR < 60 ml/min/1.73m$^2$ – Dmacro.
Figure 11: Western immunoblot analysis of urinary ACE2 protein expression in nondiabetic and diabetic patients with various levels of albuminuria. Dual precision protein marker from Bio-rad- Marker;

Nondiabetic with GFR >90 ml/min/1.73m² – ND;

Diabetic with microalbuminuria with GFR ≥45 ml/min/1.73m² - Dmicro;

Diabetic with macroalbuminuria with GFR < 30 ml/min/1.73m² – Dmacro.
Figure 12: Urinary ADAM17 concentration in non-diabetic subjects and diabetic patients using ELISA. One-way ANOVA showed a significant increase in urinary NEP/creatinine concentration of Dnormo, Dmicro and Dmacro compared to non-diabetics. *p < 0.05 vs ND controls. Each bar represents mean ± SEM of patients from ND (n=19), Dnormo (n=13), Dmicro (n=8), Dmacro (n=20).
Figure 13: Urinary nephrilysin concentration in non-diabetic subjects and diabetic patients using ELISA. One-way ANOVA showed a significant increase in urinary NEP/creatinine concentration of Dnormo, Dmicro, Dmacro compared to non-diabetics. *p < 0.0001 vs ND controls. Each bar represents mean ± SEM of patients from ND (n=19), Dnormo (n=13), Dmicro (n=8), Dmacro (n=20).
Figure 14: Plasma neprilysin concentration in non-diabetic subjects and diabetic patients using ELISA. One-way ANOVA showed a significant increase in plasma NEP concentration of diabetic patients with microalbuminuria when compared to macroalbuminuria. *p < 0.05 vs Dmicro. Each bar represents mean ± SEM of patients from ND (n=19), Dnormo (n=13), Dmicro (n=8), Dmacro (n=20).
**Figure 15:** Correlation between plasma NEP concentration and urinary NEP excretion in nondiabetic and diabetic patients. Pearson correlation factor analysis showed significant positive correlation between plasma NEP concentration and urinary NEP excretion. $r = 0.26, ^* p = 0.02$.

**Figure 16:** Correlation between estimated glomerular filtration rate (eGFR) and urinary NEP excretion in nondiabetic and diabetic patients. Pearson correlation factor analysis showed no correlation between eGFR and urinary NEP excretion. $r = -0.0045, ^* p = 0.37$. 


Figure 17: Correlation between urinary albumin-to-creatinine ratio and urinary NEP excretion in nondiabetic and diabetic patients. Pearson correlation factor analysis showed no correlation between urinary albumin-to-creatinine ratio and urinary NEP excretion. $r = 0.005$, $p = 0.48$. 

r = 0.005  
$p = 0.48$
Figure 18: Correlation between urinary protein-to-creatinine ratio (UPCR) and urinary NEP excretion in nondiabetic and diabetic patients. Pearson correlation factor analysis showed significant positive correlation between UPCR and urinary NEP excretion. $r = 0.13$, $p = 0.15$. 
Figure 19: Correlation between glycated hemoglobin (%) and urinary NEP excretion in nondiabetic and diabetic patients. Pearson correlation factor analysis showed significant positive correlation between glycated hemoglobin (%) and urinary NEP excretion. $r = 0.33$, $p < 0.01$. 
Figure 20: Western immunoblot analysis of NEP in unconcentrated urine of nondiabetic and diabetic patients with various levels of albuminuria. Dual precision protein marker from Bio-rad- Marker; Nondiabetic with GFR >90 ml/min/1.73m² – ND; Diabetic with microalbuminuria with GFR ≥60 ml/min/1.73m² - Dmicro; Diabetic with macroalbuminuria with GFR < 60 ml/min/1.73m² – Dmacro.
**Figure 21:** Western immunoblot analysis of NEP in unconcentrated urine of nondiabetic and diabetic patients with various levels of albuminuria. Dual precision protein marker from Bio-rad- Marker; Nondiabetic with GFR 80-90 ml/min/1.73m² – ND; Diabetic with microalbuminuria with GFR ≥45 ml/min/1.73m² - Dmicro; Diabetic with macroalbuminuria with GFR < 30 ml/min/1.73m² - Dmacro.
Figure 2: Western immunoblot analysis of urinary albumin in nondiabetic and diabetic patients with various levels of albuminuria. Dual precision protein marker from Bio-rad- marker; Nondiabetic with GFR $>90 \text{ ml/min/1.73m}^2$ – ND;

Diabetic with microalbuminuria with GFR $\geq 60 \text{ ml/min/1.73m}^2$ -lanes Dmicro;

Diabetic with macroalbuminuria with GFR $> 60 \text{ ml/min/1.73m}^2$ –lanes Dmacro.
Figure 23: Western immunoblot analysis of urinary albumin in nondiabetic and diabetic patients with various levels of albuminuria. Dual precision protein marker from Bio-rad- Marker; Nondiabetic with GFR $>90$ ml/min/1.73m$^2$ –ND;

Diabetic with microalbuminuria with GFR $\geq 45$ ml/min/1.73m$^2$ -Dmicro;

Diabetic with macroalbuminuria with GFR $< 30$ ml/min/1.73m$^2$ –Dmacro.
Figure 24: Western immunoblot analysis of urinary ACE2 [panel A] and albumin [panel B] in nondiabetic and diabetic patients with micro and normoalbuminuria. Dual precision protein marker from bio-rad Marker; Nondiabetic with GFR >90 ml/min/1.73m² – ND; Diabetic with microalbuminuria with GFR ≥45 ml/min/1.73m² – Dmicro; Diabetic with normoalbuminuria with GFR =70-90 ml/min/1.73m² – Dnormo.
**Figure 25:** Western immunoblot analysis of urinary NEP [panel A] and albumin [panel B] in nondiabetic and diabetic patients with normo and microalbuminuria. Dual precision protein marker from Bio-rad-Marker;

Nondiabetic with GFR >90 ml/min/1.73m² – ND;

Diabetic with microalbuminuria with GFR ≥45 ml/min/1.73m² – Dmicro;

Diabetic with normoalbuminuria with GFR = 70-90 ml/min/1.73m² – Dnormo.
Figure 26: Mass spectrometric analysis of Ang (1-7) formation (m/z 899) from Ang II (m/z 1046) A) Mass spectrometric analysis of Ang (1-7) formation (m/z 899) from Ang II (m/z 1046) in urine (25 μg creatinine) incubated for 2 hr at 37°C in 0.4 M MES buffer pH 6.75 containing 10 μM Ang II, 0.5 mM APBA and 0.1 mM ZPP in nondiabetic and diabetic patients. B) Inhibition of urinary Ang (1-7) formation (m/z 899) from Ang II (m/z 1046) by 0.1 mM MLN-4760 added to urine (25 μg creatinine) incubated for 2 hr at 37°C in 0.4 M MES buffer pH 6.75 containing 10 μM Ang II, 0.5 mM APBA and 0.1 mM ZPP.
Figure 27: Mass spectrometric analysis of Ang (1-7) formation (m/z 899) from Ang I (m/z 1296) A) Mass spectrometric analysis of Ang (1-7) formation (m/z 899) from Ang I (m/z 1296) in urine (25 μg creatinine) incubated for 2 hr at 37°C in 0.4 M MES buffer pH 6.75 containing 70 μM Ang I, 0.5 mM APBA, 0.1 mM MLN-4760 and 0.1 mM Lisinopril in nondiabetic and diabetic patients. B) Inhibition of urinary Ang (1-7) formation (m/z 899) from Ang I (m/z 1296) by 0.1 mM Thiorphan added to urine (25 μg creatinine) incubated for 2 hr at 37°C in 0.4 M MES buffer pH 6.75 containing 70 μM Ang I, 0.5 mM APBA, 0.1 mM MLN-4760 and 0.1 mM Lisinopril.
4. RESULTS

4.1. Characteristics of general metabolic and renal functional parameters

This study was undertaken to test the hypothesis that urinary ACE2 and NEP are elevated in diabetic patients. For this purpose, we measured the levels of these markers and several metabolic and renal factors in urine and plasma samples obtained from non-diabetic individual and patients with type 2 diabetic patients. The demographic and laboratory data are illustrated in table 2. The following parameters were measured.

a) Age and body mass index (BMI):

Subjects in ND, Dnormo, Dmicro, and Dmacro were well-matched in age and BMI (Table 2). However, most of the patients were male, and were either black or white in race (Table 2).

b) Blood glucose: There was a significant increase of casual blood glucose levels in Dmacro compared to ND (Table 2, \(^6\) \(p < 0.0001\)), but the glucose levels were normal in Dnormo and Dmicro.

c) Mean arterial Pressure (MAP): Some of the non-diabetic and diabetic participants were taking anthihypertensive medications (e.g. diuretics, ACEi, Calcium channel blocker and beta blockers). Blood pressure was controlled in all the participants and there was no difference in MAP between ND and Dnormo patients. However, MAP was significantly higher in Dmacro compare to Dmicro (Table 2, \(^1\) \(p < 0.05\))

d) Glycated hemoglobin (HbA1C):

Nondiabetic patients and type 2 participants were similar, aside from the expected higher levels for glucose. Since the duration of diabetes was longer than 5 years, a progressive increase of HbA1C levels was observed in ND, Dnormo, and Dmicro patients. Dmicro patients showed
significant increase in HbA1C levels (8.01%) compared to Dmacro (6.7%) and ND (6.5%) (Table 2, \( p<0.0001, \# p < 0.0001 \))

4.2. Assessment of renal function:

To investigate the incidence or progression of CKD in diabetic patients, the following parameters were measured.

a) Urinary albumin-to-creatinine ratio (UACR): There was a significant increase in the urinary albumin excretion of Dmicro compared to Dmacro (Table 2, \( \$ p<0.05 \)). Urinary albumin excretion levels were significantly increased in Dmacro compared to ND, Dnormo, and Dmicro (Table 2, \#p < 0.0001, \@ p < 0.0001, \¶ p<0.0001 \)). However, there was no difference in UACR between ND and Dnormo patients.

b) Estimated Glomerular Filtration Rate (eGFR): One of the important characteristics of CKD is lowered eGFR. There was a significant decrease in mean eGFR of Dmicro and Dmacro groups when compared to ND and Dnormo (Table 2, \( p < 0.0001 \)). Dnormo and ND exhibited normal eGFR levels (Table 2).

c) Urinary protein-to-creatinine ratio (UPCR): Urinary protein excretion levels were significantly increased in Dmacro compared to ND, Dnormo and Dmicro (Table 2, \#p < 0.0001, \@ p < 0.0001, \¶ p<0.0001 \)). There was no significant difference in urinary protein excretion between ND, Dnormo and Dmicro.

d) Serum creatinine: Serum creatinine levels showed no significant difference between ND, Dnormo and Dmicro. However, Dmacro had significantly increased serum creatinine levels when compared to ND and Dnormo (Table 2, \^ p < 0.05, \& p < 0.0001 \).

4.3. ACE2 enzyme activity:
To investigate the effect of early or progressive renal disease in nondiabetic and diabetic patients, ACE2 was measured. Urine and plasma samples were analyzed using a fluorogenic substrate. This assay was validated and confirmed using ACE2 inhibitor along with other enzyme inhibitors.

a) **Urinary ACE2 activity**: To demonstrate the presence of ACE2 activity in urine, samples were incubated with Mca-APK (Dnp) fluorogenic substrate. There was a significant difference for urinary ACE2 in Dnormo (median 3.1, IQR 1.17-5.52), Dmicro (median 5.8, IQR 2.65-7.8 nmol/hr/mg creatinine) and Dmacro (median 3.85, IQR 2.0-6.75) compared to ND (median 1.2, IQR 0.9-3.0 nmol/hr/mg creatinine) (Figure 3, \( p<0.01 \)).

b) **Confirmation of ACE2 fluorogenic assay specificity**: To validate the specificity of ACE2 fluorogenic assay, different enzyme inhibitors were used. Results show that MLN-4760 significantly inhibited urinary ACE2 activity while thiorphan and ZPP had no effect (Figure 4, \( * p<0.0001 \))

c) **Plasma ACE2 activity**: To investigate the presence of ACE2 activity in plasma, samples were incubated with Mca-APK (Dnp) fluorogenic substrate. We found that there was no detectable ACE2 activity in plasma (Figure 5), confirming our and other previous results (Elased et al., 2005; Chodavarapu et al., 2013; Salem et al., 2014) using plasma samples from healthy and diabetic animals or patients with CKD. Urine sample from type 2 diabetic mice (\( db/db \)) model was used to confirm that the assay is working.

### 4.4. Correlation of urinary ACE2 with metabolic and renal functional parameters:

To investigate whether urinary ACE2 could be a potential biomarker to predict CKD, its activity was correlated with the following metabolic and renal parameters.
a) **Glycated hemoglobin (HbA1C):** To identify a statistically significant relationship between urinary ACE2 and HbA1C, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. There was a significant positive correlation between urinary ACE2 and glycated hemoglobin (Table 3, Figure 9, r=0.42, p=0.0003).

b) **Estimated glomerular filtration rate (eGFR):** To identify a statistically significant relationship between urinary ACE2 and eGFR, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. There was a significant negative correlation between urinary ACE2 and eGFR (Table 3, Figure 6, r=-0.22, p=0.04).

c) **Urinary albumin-to-creatinine ratio (UACR):** To identify a statistically significant relationship between urinary ACE2 and UACR, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. UACR was positively associated with urinary ACE2, but there was no significant correlation (Table 3, Figure 7, r= 0.18, p=0.07).

d) **Urinary protein-to-creatinine ratio (UPCR):** To identify a statistically significant relationship between urinary ACE2 and UPCR, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. UPCR was positively associated with urinary ACE2 but no significant correlation was observed (Table 3, Figure 8, r= 0.18, p=0.08).

4.5. **Urinary ACE2 protein expression at low and normal levels of GFR:**
To investigate the effect of well-established CKD markers on urinary ACE2 protein expression at normal (eGFR > 60 ml/min) and low levels (eGFR < 60 ml/min), immunoblotting analysis was performed.

a) **Normal eGFR**: Western blotting of human unconcentrated urine detected ACE2 protein at 50 kDa, 65 kDa, 85 kDa and 120 kDa (Figure 10). Expression of bands varied among patient groups. Dmicro expressed bands at 50 kDa and 65 kDa while Dmacro expressed additional bands at 85 kDa and 120 kDa when compared to Dmicro.

b) **Low eGFR**: Immunoblotting in unconcentrated urine exhibited ACE2 bands at 50 kDa, 65 kDa, and 120 kDa (Figure 11). Dmicro and Dmacro expressed highly intensified ACE2 bands when compared to Figure 10. Expression of 85 kDa was absent in Dmacro samples with low GFR and the expression of band at 50 kDa was higher compared to Dmacro in Figure 10. ND had no visible ACE2 bands.

**4.6. Measurement of ADAM17 concentration using ELISA**:

To investigate a possible mechanism in the shedding of urinary ACE2, urinary ADAM17 concentration was measured using enzyme linked immune sorbent assay (ELISA).

**Urinary ADAM17**: Urinary ADAM17 concentration was analyzed in unconcentrated urine samples and values were normalized using urinary creatinine concentration (μg/μl). A significant increase in urinary ADAM17 levels was observed in Dmicro (median 15.06, IQR 8.82-19.28 ng/μg creatinine), Dnormo (median 8.55, IQR 3.65-11.89 ng/μg creatinine) and Dmacro (median 9.28, IQR 1.86-17.18 ng/μg creatinine) when compared with ND (median 5.23, IQR 2.82-8.54 ng/μg creatinine) (Figure 12, p < 0.05).

**4.7. Measurement of Neprilysin concentration using ELISA**:
To detect and evaluate the effect of progressive renal disease on urinary and plasma neprilysin concentration using enzyme linked immune sorbent assay.

a) **Urinary Neprilysin:** Urinary neprilysin concentration was analyzed in unconcentrated urine samples, and values were normalized using urinary creatinine concentration (μg/μl). A significant increase in urinary neprilysin levels was observed in Dnormo (median 562.0, IQR 197.9-1022 ng/μg creatinine), Dmicro (median 1064.0, IQR 807.6-2079 ng/μg creatinine), and Dmacro (median 541.8, IQR 134.8-1051 ng/μg creatinine) compared with ND subjects (median 268.1, IQR 112.2-503.9 ng/μg creatinine) (Figure 13, \(p < 0.0001\)).

b) **Plasma Neprilysin:** There was no significant difference between plasma NEP levels in Dnormo, Dmicro, and Dmacro compared to ND although plasma NEP levels were significantly higher in Dmicro compared to Dmacro (Figure 14, \(p < 0.05\)).

c) **Association of urinary NEP with plasma NEP:** To identify a statistically significant relationship between urinary NEP and plasma NEP, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. There was a significant positive correlation between urinary and plasma NEP (Figure 15, \(r=0.26, p=0.02\)).

### 4.8. Correlation of urinary NEP with metabolic and renal functional parameters:

To investigate whether urinary NEP could be a potential biomarker to predict CKD, its activity was correlated with the following metabolic and renal parameters.

a) **Glycated hemoglobin (HbA1C):** To identify a statistically significant relationship between urinary NEP and glycated hemoglobin, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. There was a
significant positive correlation between urinary NEP and HbA1C (Table 3, Figure 19, r=0.33, p < 0.01).

b) *Estimated glomerular filtration rate (eGFR):* To identify a statistically significant relationship between urinary NEP and eGFR, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. There was no significant correlation between eGFR and urinary NEP (Table 3, Figure 16, r= -0.045, p=0.37).

c) *Urinary albumin-to-creatinine ratio (UACR):* To identify statistically significant relationship between urinary NEP and UACR, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. No correlation was observed between UACR and urinary NEP (Table 3, Figure 17, r = 0.005, p=0.48).

d) *Urinary protein-to-creatinine ratio (UPCR):* To identify statistically significant relationship between urinary NEP and UPCR, Pearson correlation and linear regression were calculated between these variables in nondiabetic and diabetic groups. UPCR was positively associated with urinary NEP, but there was no significant correlation (Table 3, Figure 18, r= 0.13, p=0.15).

### 4.9. Urinary NEP protein expression at low and normal levels of GFR:

To investigate the effect of well-established CKD markers on urinary NEP expression at normal (eGFR > 60 ml/min) and low (eGFR <60 ml/min) levels of eGFR using immunoblotting analysis.

a) *Normal eGFR:* Western blotting of human unconcentrated urine detected NEP protein at 50 kDa, 65 kDa, 94 kDa, 100 kDa, and 120 kDa (Figure 20). Expression of bands varied among patient groups. Dmacro expressed bands at 50 kDa, 65 kDa, and 100 kDa, whereas
Dmicro expressed additional bands at 120 kDa but not at 50 kDa compared to Dmacro. Visible NEP bands were observed at 65 kDa and 94 kDa in ND samples.

b) **Low eGFR:** Immunoblotting in unconcentrated urine exhibited NEP bands at 50 kDa, 65 kDa, 94 kDa, and 100 kDa (Figure 21). Dmicro and Dmacro expressed highly intensified NEP bands compared to Figure 20. Expression of 120 kDa was absent in Dmicro samples with low GFR and the expression of band at 65 kDa was higher compared to Dmacro. ND expressed visible NEP bands at 65 kDa and 94 kDa.

**4.10. Urinary protein expression albumin at low and normal GFR levels:**

To demonstrate the variations in albuminuria levels for the above urinary ACE2 and NEP westerns, the following immunoblotting was shown.

a) **Normal eGFR:** Western blotting of human unconcentrated urine detected albumin protein at 66kDa (Figure 22). Expression of bands varied among patient groups. Dmacro expressed bands at the highest intensity, followed by Dmicro and ND.

b) **Low eGFR:** Western blotting of human unconcentrated urine identified albumin bands at 66 kDa (Figure 23). Expression of bands varied among patient groups. Dmacro expressed bands at the highest intensity, followed by Dmicro and ND.

**4.11. Mass spectrometric analysis of ACE2 and NEP activities:**

To detect and confirm the presence of urinary ACE2 and NEP activities using Ang II and Ang I as substrates, a sensitive and more specific analysis was done using mass spectrometry.

a) **Urinary ACE2 activity:** To detect ACE2 activity, urine was incubated with Ang II. Formation of Ang (1-7) indicates the presence of ACE2 activity in urine. Disappearance of
Ang (1-7) at m/z 899 when incubated with Ang II and MLN-4760, an inhibitor of ACE2, in diabetic and nondiabetic urine confirms the ACE2 specificity of this assay (Figure 26).

g) Urinary NEP activity using mass spectrometry: To detect urinary NEP activity, the sample was incubated with Ang I. Formation of Ang (1-7) indicates the presence of NEP activity in urine. For Ang I incubations, the disappearance of the Ang (1-7) peak at m/z 899 in urine of diabetic and nondiabetic patients when incubated with thiorphan, an inhibitor of NEP, confirmed the activity of NEP (Figure 27).

4.12. Detection of ACE2, NEP and ADAM17 in patients at early stages of renal injury:

To investigate the potentiality of urinary ACE2 and NEP at early stages of renal injury in ND and diabetic patients, following parameters were measured.

a) Urinary ACE2 activity: A significant increase in urinary ACE2 activity of Dnormo was observed when compared to ND patients (Figure 3, p<0.01).

b) Urinary Neprilysin: Urinary NEP significantly increased in Dnormo when compared to ND patients (Figure 13, p < 0.0001).

c) Urinary ADAM17: There was a significant increase in urinary ADAM17 concentration when compared to ND patients (Figure 12, p < 0.05).

d) Urinary ACE2 expression: ACE2 bands were expressed at 50 kDa and 65 kDa in ND and Dnormo groups. These ACE2 bands were expressed in Dmicro with more intensity when compared to Dnormo. Immunoblotting using albumin as the primary antibody for the same samples showed presence of albumin bands in ND and an increase in the molecular weight and band intensity was observed according to the level of albuminuria (Figure 24 Panel B)
e) **Urinary NEP expression**: Western blot of NEP expressed bands at 50 kDa, 94 kDa, 100 kDa, and 120 kDa. Neprilysin bands were expressed in Dnormo at 120 kDa and 100 kDa, whereas Dmicro expressed faint bands at 120 kDa and 94 kDa. ND expressed faint NEP bands at 94 kDa (Figure 25 Panel A). Immunoblotting using anti-albumin as the primary antibody for the same samples showed presence of albumin bands in ND and an increase in the molecular weight and band intensity was observed according to the level of albuminuria (Figure 25 Panel B).

f) **Urinary ACE2 activity using mass spectrometry**: Formation of Ang (1-7) when detected with mass spectrometry indicates the presence of ACE2 activity in urine. Disappearance of Ang (1-7) peak at m/z 899 in diabetic and nondiabetic patients using MLN-4760, an inhibitor of ACE2, confirmed this assay (Figure 26).

g) **Urinary NEP activity using mass spectrometry**: Formation of Ang (1-7) when detected with mass spectrometry indicates the presence of NEP activity in urine. Disappearance of Ang (1-7) peak at m/z 899 in diabetic and nondiabetic patients when thiorphan, an inhibitor of NEP, was incubated with Ang I and urine confirmed the activity of NEP (Figure 27).
5. DISCUSSION

Because of the major clinical and economic burden of diabetic kidney disease (DKD), considerable effort has been made to identify ways to prevent its onset and to delay its progression. Currently the best available non-invasive method for the early recognition of pending kidney disease in diabetic patients is the estimation of GFR and assessment of albumin excretion rate (AER). However, the value of assessing levels of urinary albumin and its sensitivity and specificity for the prediction of pending kidney disease has been debated (Brosius & Pennathur, 2013). The present research program was designed to identify ACE2 and NEP as novel early non-invasive biomarkers for DKD with the overall goal to improve diagnosis and monitor treatment response in patients with DKD.

ACE2 is highly expressed in the kidney and known to be renoprotective by degrading Ang II to Ang-(1-7). Previous studies demonstrated increased urinary ACE2 shedding in murine model of type 1 and type 2 diabetes (Chodavarapu et al., 2013; Salem et al., 2014; Somineni et al., 2014; Batlle et al., 2012). However, there are very limited clinical studies on urinary ACE2. Higher levels of ACE2 excretion in urine have been shown in diabetic patients with CKD (Mizuiri et al., 2011a) and in renal transplantation patients compared to healthy individuals (Xiao et al., 2012). However, the difference of in the level of urinary ACE2 in diabetic patients with different degree of albuminuria has not yet been investigated. In consistent with previous studies, we demonstrated increased urinary ACE2 in diabetic patients with microalbuminuria and macroalbuminuria compared with non-diabetic individuals. In addition, the data showed increased urinary ACE2 in diabetic patients before the onset of albuminuria. As shown in figure 3, urinary ACE2 in Dnormo patients was significantly higher compared to non-diabetics individuals (p<0.01). There was a significant correlation of urinary ACE2 activity with renal parameters such as eGFR, serum creatinine and BUN. In addition, urinary ACE2 significantly positively correlated with blood glucose
and HbA1C. This data indicate that diabetes-associated CKD strongly correlates with urinary ACE2 levels.

NEP has a molecular weight of approximately 90-110 kDa with a short N-terminal cytoplasmic and a large extracellular domain (Sexton et al., 2012). Initially, NEP was discovered in the brush border membranes of the rabbit kidney as a cleavage peptide of the insulin B chain (Kerr & Kenny, 1974). NEP is expressed in several other tissues such as intestines, adrenal glands, lungs, and, with lower levels, in the brain and spinal cords (Li et al., 1995). NEP has broad substrate specificity but a preference for peptides (Webster et al., 2014). NEP plays a crucial role in the formation of Ang-(1-7) from Ang I. Apart from Ang I, NEP is responsible for the degradation of amyloid β protein in the brain (Iwata et al., 2001) suggesting a promising new therapy for Alzheimer’s disease (Hemming et al., 2007; Leissring et al., 2003). NEP is also involved in the degradation of bradykinin, atrial natriuretic peptide (Cruden et al., 2004) and C-type natriuretic peptide (Thong et al., 2014). It has been suggested that ACE2 and NEP contribute to the renoprotective effects of Ang II type 1 receptor blockers (ARBs) by increasing the formation of Ang-(1-7) (Whaley-Connell et al., 2006). Recently, use of a combination of ARB and NEP inhibitor decreased the incidence of mortality and hospitalizations among patients with a history of heart failure (McMurray et al., 2014). However, the physiological function of NEP in the heart remains poorly understood, and the role of NEP on DKD has not been identified yet. This is the first study that identified and analyzed NEP in urine and confirmed its presence using a newly developed mass spectrometry activity assay. In addition to urine analysis, NEP concentration and ACE2 activity were analyzed in plasma of non-diabetic and diabetic patients. Dmacro patients showed significant decrease in plasma NEP concentration compared to Dmicro, although no detectable ACE2 activity was observed in plasma of all patients.
Increased prevalence of CKD may be closely related with diabetes, hypertension, obesity, hyperuricemia, hyperlipidemia, and other comorbid conditions (Lin et al., 2014). Recent studies conducted on the causes of CKD found diabetes as one of the main comorbidity (Pani et al., 2014). Albuminuria is a well-established diagnostic tool used for the detection of CKD and diabetic nephropathy. Its use as a biomarker for the prediction of various kidney diseases depends on the amount of albumin excreted in to urine. However, in clinically healthy patients, microalbuminuria was observed most likely due to elevated hydraulic pressure, increased glomerular filtration coefficient and changes in sieving properties of the glomerular filter but not glomerular hyperfiltration (Jensen et al., 1995; Parving et al., 2014). Sieving properties of glomerular filter are characterized by size and charge selectivity. Neutral particles with a molecular radius >4.2 nm and anion particles with molecular radius <3.4 nm are restricted to pass through glomerular filtration (Brenner et al., 1978a; Brenner et al., 1978b; Pollak et al., 2014). In normal healthy conditions, the negatively charged albumin which has a molecular radius of 3.6 nm, is minimally filtered (Brenner et al., 1978b; Brenner et al., 1978a; Pollak et al., 2014). But in diabetic patients with normoalbuminuria, stage 3 CKD was observed which indicates low specificity of albuminuria in detecting CKD (Middleton et al., 2006; MacIsaac & Jerums, 2011; MacIsaac et al., 2014). In the present study, correlation analysis demonstrated that increased urinary ACE2 and NEP in diabetic patients were independent of albuminuria.

High blood glucose, elevated risk of cardiovascular diseases, and activated RAS causes structural (e.g. increased in the glomerular pore size) or biochemical (e.g. loss of negatively charged molecules) changes in the glomerular filter that lead to excretion of albumin (Kamiyama et al., 2012; Parving et al., 2014). Ameliorating glomerular sieving dysfunction and albuminuria by use of RAS inhibitors indicates the role of the RAS in causing microalbuminuria and kidney disease (Ruggenenti et al., 2012). Glomerular sieving
dysfunction can be measured by glomerular sieving coefficient (GSC), which is the concentration of a given molecule in Bowman’s space divided by that in plasma. GSC ranges between 0 and 1 where low GSC implies a severe restriction of glomerular filtration barrier (Deen & Lazzara, 2004). In activated RAS, an acute threefold increase of ~44% in albumin GSC was measured after Ang II infusion. Decrease in albumin GSC by infusion of AT1 receptor (AT1R) blocker after Ang II infusion indicates AT1R-mediated Ang II action on albumin excretion (Schiessl & Castrop, 2013). In addition, disruption of the ACE2 gene and increase in renal Ang II resulted in augmentation of nephrin, which may reflect their role in the early onset of albuminuria (Bichu et al., 2009; Shiota et al., 2010).

Studies in diabetic patients with controlled glycemia found that a regression of microalbuminuria to normoalbuminuria prevents or delays the risk of developing microalbuminuria (Perkins et al., 2003; Shiota et al., 2010). Long term clinical diagnosis of microalbuminuria reported 40% regression to normoalbuminuria (de Boer et al., 2011). In our study, presence of normal levels of urinary albumin in Dnormos who have history of microalbuminuria support the regression of microalbuminuria to normoalbuminuria (Perkins et al., 2003; Bakris & Glassock, ). In diabetic patients, there is a chance of progression of microalbuminuria to macroalbumunuria (Parving et al., 2014). One of the factors that causes progression of microalbuminuria to macroalbuminuria is increased HbA1C (Giorgino et al., 2004). In the present study, Dmicro patients showed increased HbA1C and ACE2 activity compared to Dmacro patients which may be due to poor glycemic control. Additionally, regression analysis of urinary ACE2 showed positive correlation with HbA1C, suggesting its use as an indicator for progressive diabetes. Long-term cohort study of HbA1C and urinary ACE2 in diabetic patients with controlled glycemia may lead to new insights for the possible role of controlled glycemia in CKD patients with diabetes. Additionally, we observed a
strong correlation of urinary ACE2 with eGFR, confirming the predictive power for estimating the risk in diabetic patients to develop CKD.

Many proteins are shed into the urine due to dysfunction of the glomerular barrier. ACE2 was first observed in urine of healthy individuals (Lew et al., 2006). It had initially been proposed that shedding of urinary ACE2 may be due to glomerular filtration from plasma. Plasma ACE2 is detected in sheep serum (Shaltout et al., 2007), and plasma of diabetic rodents (Nadarajah et al., 2012). In support to these animal studies, increased levels of circulating ACE2 were reported in patients with type I diabetes having vascular complications (Soro-Paavonen et al., 2012). There are conflicting data regarding the presence of actively circulating ACE2 and the source of urinary ACE2 in patients with CKD and mouse model of diabetic nephropathy. Infusion of soluble recombinant ACE2 in db/db diabetic mice failed to increase urinary ACE2. In addition, earlier studies using SELDI-TOF-MS, has shown that circulating ACE2 is not active in mouse plasma (Elased et al., 2006). Using a different approach, Lew et al., also demonstrated lack of plasma ACE2 activity and hypothesized the presence of an endogenous inhibitor of ACE2 in plasma (Lew et al., 2008). It worth mentioning that in humans, ACE2 activity could only be detected in 7.5% of a large cohort of subjects (Rice et al., 2006). Detection of unchanged plasma ACE2 levels in chronic kidney injured rats (Burrell et al., 2012; Rice et al., 2006), indicates that an endogenous inhibitor may be restricted to human plasma. Thus, low plasma ACE2 activity is observed in CKD patients when the presumably endogenous inhibitor is removed (Roberts et al., 2013). Discrepancy in results may be due to the variations in the method of analysis used for detection i.e. incubation for more than 12 hours. On other hand, location of ACE2 gene on X chromosome (Tipnis et al., 2000) questioned the possibility of differences in plasma ACE2 activity between sexes. Assessment of plasma ACE2 activity in patients undergoing hemodialysis (Roberts et al., 2013) and kidney transplantation patients (Soler et al., 2012)
showed lower plasma ACE2 activity in females compared to males. But in patients with stage III/IV CKD, plasma ACE2 activity was increased (Roberts et al., 2013). In contrast, reduction in plasma ACE2 activity was observed in patients with ESRD during dialysis, compared to predialysis CKD patients (Roberts et al., 2013). In our study, no detectable plasma ACE2 activity was observed in the patient population which may be due to presence of an endogenous inhibitor. However, this is unlikely to be the reason for lack of active circulating ACE2, because urinary ACE2 was detected in patients with macro albuminuria.

Decreased expression of ACE2 in tubules of diabetic patients compared to healthy controls establishes ACE2 as tubular marker. Protein expression of ACE2 is reported in both tubulointerstitium and glomeruli (Mizuiiri et al., 2011b). Immunostaining results of normal and diabetic animal models with respect to glomerular ACE2 and tubular ACE2 expression demonstrates that tubular ACE2 increases in diseased state (Somineni et al., 2014; Salem et al., 2014; Chodavarapu et al., 2013). Studies in diabetic animal models with kidney disease showed an early increase in renal ACE2 mRNA activity, and expression to compensate hyperglycemia-induced Ang II accumulation (Wysocki et al., 2006; Ye et al., 2004; Ye et al., 2006). Our study is the first to demonstrate an early detection of urinary ACE2 activity in Dnormo patients where a slight decline in eGFR is observed in the respective patient group. However, as diabetes and kidney disease progress (Mizuiiri et al., 2011b; Reich et al., 2008), decrease in renal ACE2 expression was observed. In our study, increased accumulation of ACE2 in urine of Dmacro patients and Dmicro patients may imply renal disease progression in diabetic patients. Further time dependent follow-up studies in patients at early stages of diabetes are needed to confirm ACE2 as an early biomarker for CKD. Formation of Ang (1-7) from Ang II using a mass spectrometric approach supports the results obtained with the ACE2 fluorogenic assay. Immunoblot analysis of ACE2 in concentrated urine of healthy subjects detected a full length ACE2 at ≈120 kDa and a deglycosylated band at 85 kDa along
with an unaltered band at ≈75 kDa (Lew et al., 2006). Clinical studies in CKD patients reported ACE2 immuno-reactive bands at 120 kDa and 75 kDa (Mizuiri et al., 2011a). In support of these conclusions, ACE2 bands in urine of diabetic renal transplant patients were shown at 120 kDa and 90 kDa. But addition of deglycosylase enzyme in urine of both patient groups exhibited deglycosylated bands at 85 kDa and 65 kDa (Xiao et al., 2012). Continuing in this vein, we found ACE2 bands at 120kDa, 85kDa, 75kDa and 65kDa in unconcentrated urine of diabetic patients with renal disease at different levels of albuminuria. Expression of ACE2 band in diabetic patients with normoalbuminuria could be an early sign of CKD presence. But sequential analysis of urinary ACE2 using longitudinal studies in diabetic patients with renal dysfunction could confirm ACE2 as a novel biomarker for CKD.

Presence of smaller, soluble and active fragments of ACE2 in diabetic urine support a role for active proteolytic shedding of renal enzyme, most likely due to ADAM17. We reported increased renal ADAM-17 expression in diabetic animal models, which strengthens the hypothesis that ADAM17 is involved in the proteolytic shedding of ACE2 (Chodavarapu et al., 2013; Salem et al., 2014; Somineni et al., 2014). To confirm ADAM17 mediated shedding of ACE2, recent studies in Elased’s lab showed pharmacological inhibition of ADAM17 decreased shedding of ACE2 in human renal proximal tubular HK-2 cells (Salem et al., 2014). The present study showed increased urinary ADAM17 in diabetic patients compared to nondiabetic controls. Further characterization or sequence analysis of urinary ACE2 fragments could give new insights into sources and mechanisms underlying ACE2 shedding.

ACE2 exhibits the highest catalytical efficiency in the class of Ang (1-7) forming enzymes (Schindler et al., 2007; Chappell et al., 2014). NEP also contributes to the formation of Ang (1-7) from Ang I (Rice et al., 2004; Welches et al., 1993). NEP is expressed in the kidney
brush border especially in glomerulus and proximal tubules. In glomerulus, presence of active NEP regulates degradation of natriuretic peptides and natriuresis, which may alter the activity of juxtaglomerular synthesis of renin (Bae et al., 2011). In proximal tubules, NEP has been shown to be involved in the metabolism of renal kinins which participate in short-term regulation of water and sodium excretion. In kidney transplant recipients, urinary NEP levels were increased at the 1st and/or the 2nd post-operative days which turned to normal levels between the 3rd and 5th post-operative days (Nortier et al., 1992). Interestingly, another study in renal transplant patients proposed urinary NEP as a marker for proximal tubular injury (Nortier et al., 1993). Increased urinary NEP levels in patients with acute tubulotoxic renal nephropathy and acute renal nephropathy compared to patients with chronic nephropathy showed the potential diagnostic value of urinary NEP in renal diseases (Vlaskou et al., 2000). This novel finding is in consensus with increased NEP levels in patients with kidney injury (Han et al., 2008). Studies in animal model and humans speculate that NEP inhibitors could slow the progression of CKD (Judge et al., 2014). In our study, increased excretion in NEP levels was identified in human urine of diabetic patients with kidney disease where circulating NEP or renal NEP could serve as main sources.

Low levels of NEP in blood and the similarity in molecular weights of renal and urinary NEP suggests that NEP may not be filtered through the glomerulus, which might support the kidney as origin for urinary NEP (Skidgel et al., 1987). It was observed in our study that plasma NEP concentration was decreased in Dmacro patients compared to Dmicro and. NEP activity was observed in urine of Dmicro and Dmacro patients. A serial analysis of renal NEP, plasma NEP and urinary NEP in humans could provide a platform to identify the source of urinary NEP.

In clinical studies, the molecular weight of urinary NEP was determined to be 94 kDa (Erdős & Skidgel, 1989; Vlaskou et al., 2000). In our study, Western blot analysis of urinary NEP
revealed an increase in NEP molecular weight from 94 kDa to 100 kDa in diabetic subjects, which is assumed as a result of partial glycosylation. Additionally, high intensity NEP bands were observed in diabetic patients compared to nondiabetic controls. Tissue specific differences in glycosylation of NEP ranged from 90-120 kDa (Muller et al., 2010). In diabetic patients with microalbuminuria, a second band at ≈120 kDa, corresponded to complete glycosylation product of mature NEP (Sato et al., 2012). Furthermore, the appearance of faint bands at 120 kDa in Dnormo may represent an early change in kidney function. Until now, release of full-length NEP in biological fluids has been proposed (Muller et al., 2010; Sato et al., 2012). In contrast, we detected a smaller immunoreactive band at ≈65 kDa in urine of our patient population which is in accordance to a study by Vlaskou and co-workers (Vlaskou et al., 2000). The variations in the intensities of these bands at 65 kDa and 100 kDa in patients with low and normal GFR need to be analyzed.

A recent clinical trial in healthy subjects showed an increase in NEP activity during differentiation of human pre-adipocytes to adipocytes (Standeven et al., 2011). Increased plasma and adipose tissue NEP in association with insulin resistance in obese murine models illustrates the effect of obesity on NEP (Standeven et al., 2011). In the present study, correlation analysis showed association of urinary NEP with BMI and HbA1C. Further in-depth studies of urinary and kidney NEP are necessary to establish urinary NEP as a clinical diagnostic tool.

To the best of our knowledge, this study is the first in the literature to investigate whether urinary NEP could be a predictor for CKD in diabetic patients. Urinary NEP levels were found to be elevated in diabetic patients with CKD, and its activity was present in diabetic patients with microalbuminuria and macroalbuminuria. On the other hand, urinary ACE2 activity has been shown to increase in diabetic patients and is associated with metabolic and renal parameters. Levels of ADAM17 are increased in urine of diabetic patients, implicating
its role in the shedding of ACE2. Increased urinary ACE2 and NEP could be used as a biomarker for intrarenal RAS activation.
6. CONCLUSION

In conclusion, significantly increased urinary ACE2 was observed in type 2 diabetic patients with a history of microalbuminuria, and its association with low eGFR might imply a link between urinary ACE2 and the severity of kidney disease progression. Presence of urinary ACE2 activity and protein expression in type 2 diabetic patients with normoalbuminuria reflects its potential use as an early marker for the detection of pending kidney disease. Our study also provides a unique approach for the investigation of urinary NEP and confirmed the presence of urinary NEP activity in type 2 diabetic patients with microalbuminuria and macroalbuminuria. In summary, NEP seems to be following the ACE2 trend in diabetic patients with CKD. These findings could further help establish the role of ACE2 and NEP as non-invasive biomarkers to assess kidney damage in diabetic patients.
APPENDIX A

Role of Physical Exercise Training on Renal and Urinary Neprilysin Protein Expression in db/db mice

Sridevi Gutta, Hari K. Somineni, Gregory P. Boivin, Khalid M. Elased

Wright State University Boonshoft School of Medicine

Angiotensin II (Ang II), a potent vasoactive peptide cleaved from Ang I, plays a pivotal role in the progression of cardiovascular and renal diseases. Diabetic nephropathy (DN) is one of the main microvascular complications of uncontrolled diabetes leading to end stage renal disease eventually. Activation of Renin-Angiotensin cascade by hyperglycemia up regulates Ang II level. Enzymatic cleavage of Ang I to form Ang (1-7) by endopeptidases opposes its actions. Neutral endopeptidase or neprilysin (NEP), a thermolysin like zinc-containing metallopeptidase group is currently considered as a potential target in renal pathology, forms Ang (1-7) by degrading Ang I. Recent evidence suggests that exercise training improves renal function which also acts as a real polypill in prevention of diabetes. The aim of this study is to test the hypothesis that exercise training imparts renoprotection by upregulating renal and urinary NEP protein expression in db/db mice. Seven weeks old lean and db/db male mice were subjected to exercise training for 10 weeks. Exercise groups were run on a mouse forced exercise walking wheel system at a speed of 8 m/min, 1 hour a day, 7 days a week. Weekly monitoring included blood glucose, 24-hr urinary volume, albumin and creatinine levels. Exercise training significantly lowered blood glucose level and urinary albumin excretion in db/db mice. Western blot analysis demonstrated significant increase in renal and urinary NEP protein expression in 17wk db/db mice subjected to exercise training compared to untreated db/db mice (p<0.0001). In addition, exercise training decreased plasma triglycerides and increased plasma adiponectin and insulin levels in db/db mice. In conclusion, upregulated renal NEP by exercise could be responsible for improved renal functioning at least in partial and could be considered as a renoprotective mechanism in db/db mice. Urinary NEP reflecting its changes in the kidney could be used as a marker to study intrarenal status.

Oral and Poster presentation in 28th Ohio Physiological Society: Ohio, 2014
APPENDIX B

Effect of exercise and rosiglitazone on neprilysin protein expression in db/db diabetic mice

Khalid M Elased, Sridevi Gutta, Laale F. Alawi, Hari K Somineni, Gregory P. Boivin

Department of Pharmacology & Toxicology, Wright State University, Boonshoft School of Medicine 3640 Colonel Glenn Hwy. Dayton, OH, 45435

Angiotensin II (Ang II) plays a pivotal role in kidney disease progression in diabetes. Diabetic nephropathy is one of the main microvascular complications of uncontrolled diabetes, which eventually lead to end stage renal disease. Glycemic control and blockade of renin angiotensin system has been shown to attenuate diabetic nephropathy. Angiotensin converting enzyme (ACE) 2 is highly expressed in the kidney and has been shown to be renoprotective by degrading Ang II to Ang-(1-7). We have shown previously increased urinary ACE2 excretion contribute to the pathogenesis of diabetic nephropathy. Neutral endopeptidase (NEP) belonging to the M13 family of zinc containing metallopeptidase group converts Ang I to Ang-(1-7) which is also a potential target for drug development. We tested the hypothesis that physical exercise training and treatment with rosiglitazone decrease the shedding of urinary ACE2 and upregulate NEP protein expression. Six wks old normal and db/db mice were subjected either to physical exercise training or rosiglitazone treatment (20 mg/kg/day) for 10 wks. Exercise training significantly lowered blood glucose, urinary albumin and ACE2 excretion in db/db mice. The decreased renal NEP levels in db/db mice was increased by exercise training and rosiglitazone. In addition, exercise training reduced plasma triglycerides and increased plasma adiponectin and insulin levels in db/db mice. In conclusion, exercise training and rosiglitazone upregulate renal NEP protein expression. Urinary NEP could serve as a prognostic tool in the progression of kidney damage and reflects intrarenal NEP status.

Poster presented in 49th European Association for Study of Diabetes (EASD), 2013:

Barcelona, Spain
APPENDIX C

Increased Urinary Angiotensin Converting Enzyme 2 (ACE2) in Diabetic patients with CKD

Sridevi Gutta, Nadja Grobe, Hassan Osman, Mohammad Saklayan, Khalid M. Elased

Department of Pharmacology & Toxicology, Wright State University, Boonshoft School of Medicine, 3640 Colonel Glenn Hwy. Dayton, OH, 45435

Type 2 diabetes and its associated Chronic Kidney Disease (CKD) have become a major health burden. CKD is associated with tenfold increase in cardiovascular mortality and risk multiplayer in hypertensive and diabetic patients. There is a need for new sensitive biomarkers for detection and monitoring CKD since albuminuria may be nonspecific and insensitive. Angiotensin converting enzyme 2 (ACE2) is highly expressed in renal tubules and has been proposed to be renoprotective. ACE2 is a monocarboxy peptidase mediating degradation of angiotensin (Ang) II. The aim of the study is to investigate the association between urinary ACE2 and incidence or progression of CKD at early stages among individuals with type 2 diabetes. Participants were recruited from Dayton VA Medical Center. Using Western blot and ACE2 enzyme activity, we investigated ACE2 shedding in urine samples of 16 nondiabetic controls and 40 diabetic patients with various levels of albuminuria. Urinalysis included protein, albumin, creatinine and glucose. There was no evidence of urinary ACE2 in nondiabetic healthy patients with normal GFR. However, there was significantly increased urinary ACE2 activity in diabetic microalbuminuric patients compared with macroalbuminurics and nondiabetic controls (p<0.05). In support of our ACE2 activity assay, presence of ACE2 immunoreactive bands in urine of diabetic patients with inverted microalbuminuria confirmed early detection of CKD in diabetics. In conclusion, urinary ACE2 is increased in diabetic patients with inverted microalbuminuria compared to nondiabetic healthy patients. The present study identified a significant difference in urinary ACE2 between diabetic patients with history of microalbuminuria and non-diabetic subjects suggesting that urinary ACE2 could be used as an early, noninvasive biomarker for early stage of CKD.

Poster presentation in Wright State University Celebration of Research, Scholarship and Creative activities, 2014: Dayton, OH, USA
APPENDIX D

INCREASED URINARY ANGIOTENSIN CONVERTING ENZYME 2 (ACE2) in TYPE 2 DIABETIC PATIENTS (T2DM)

Sridevi Gutta1, Nadja Grobe1, Hassan Osman2, Mohammad Saklayan2, Khalid M. Elased1

1Wright State University, Boonshoft School of Medicine, Dayton,
2Dayton Veterans Affairs Medical Center, Dayton, OH 45428

Diabetes and its associated chronic kidney disease (CKD) is a major health burden and there is an urgent need for new sensitive biomarkers to detect and monitor the progression of CKD. Albuminuria is still the gold standard for the evaluation of kidney function. However, its sensitivity and reliability have recently been questioned. ACE2 is highly expressed in renal tubules and has been shown to be shed in the urine of diabetic patients with CKD. The aim of the study was to investigate whether urinary ACE2 is increased in diabetic patients with CKD before the onset of microalbuminuria. Participants were recruited from Dayton VA Medical Center (Dayton, OH, USA). Baseline urinary albumin creatinine ratio (UACR) and estimated glomerular filtration rate (eGFR) were determined three months before initiation of the study in non-diabetic patients (UACR <30 mg/g, eGFR=97.40±16 ml/min/1.73 m²), and in diabetic patients with normoalbuminuria (UACR <30 mg/g, eGFR=83.08±17 ml/min/1.73 m²), microalbuminuria (UACR = 30-300 mg/g, eGFR=47.13±23 ml/min/1.73 m²), and macroalbuminuria (UACR >300 mg/g, eGFR=39.68±20 ml/min/1.73 m²). Using fluorogenic and mass spectrometry-based enzyme assays, we measured urinary and plasma ACE2 activity in patients. Urinary ACE2 activity was significantly increased in diabetic patients with normoalbuminuria (0.58±0.2 nmol/hr/mg creatinine), microalbuminuria (1.19 ±0.5 nmol/hr/mg creatinine), and macroalbuminuria (2.265±0.4 nmol/hr/mg creatinine) compared with non-diabetic controls (0.06 ± 0.02 nmols/hr/mg creatinine) (p<0.0001). These results were confirmed by detecting the ACE2 product Ang-(1-7) (m/z 899) in incubations of urine samples with the natural substrate Ang II (m/z 1046) using mass spectrometry-based enzyme assays. In addition, urinary ACE2 expression was significantly increased in diabetic patients as determined by western blot analysis (p<0.05). Plasma ACE2 activity was not detectable in control and diabetic patients. In conclusion, urinary ACE2 is increased in diabetic patients with CKD which suggests that urinary ACE2 could be used as an early, noninvasive biomarker for diabetic nephropathy before the onset of microalbuminuria.

Poster presented at High Blood Pressure Research meeting, 2014: San Francisco, California, USA
APPENDIX E

Use of mass spectrometry for the development of new biomarkers for diabetic nephropathy

Nadja Grobe, Sridevi Gutta, Hassan Osman, Mohammad Saklayen, Khalid M. Elased

Wright State University, Boonshoft School of Medicine, Dayton, Dayton Veterans Affairs Medical Center, Dayton, OH 45428.

Diabetes and its associated chronic kidney disease (CKD) is a major health burden and there is an urgent need for new sensitive biomarkers to detect and monitor the progression of CKD. Albuminuria is still the gold standard for the evaluation of kidney function. However, its sensitivity and reliability have recently been questioned. Angiotensin converting enzyme 2 (ACE2) and neprilysin (NEP) are highly expressed in renal tubules and responsible for the generation of renoprotective angiotensin (Ang) 1-7. The aim of the study was to investigate whether urinary ACE2 and NEP are increased in diabetic patients with CKD indicating early renal damage before the onset of microalbuminuria. Participants were recruited from Dayton VA Medical Center (Dayton, OH, USA). Baseline urinary albumin creatinine ratio (UACR) and estimated glomerular filtration rate (eGFR) were determined three months before initiation of the study in non-diabetic patients (UACR <30 mg/g, eGFR=97.40±16 ml/min/1.73 m²), and in diabetic patients with normoalbuminuria (UACR <30 mg/g, eGFR=83.08±17 ml/min/1.73 m²), microalbuminuria (UACR = 30-300 mg/g, eGFR=47.13±23 ml/min/1.73 m²), and macroalbuminuria (UACR >300 mg/g, eGFR=39.68±20 ml/min/1.73 m²). Mass spectrometry-based enzyme assays were used to detect the ACE2 and NEP product Ang(1-7) (899) in incubations of urine samples with natural NEP substrate Ang I(m/Z 1296). Urinary ACE2 and NEP were significantly increased in diabetic patients compared with non-diabetic controls (p < 0.01). Western blot analysis, ELISA and fluorogenic substrate assays confirmed these findings. Ang II or Ang I enzyme activities were blocked by specific ACE2 inhibitor MLN-4760 or NEP inhibitor thiorphan, respectively. NEP, but not ACE2, was detected in plasma of patients. In conclusion, urinary ACE2 and NEP are increased in diabetic patients with CKD before the onset of microalbuminuria which suggests their use as early, noninvasive biomarker for diabetic nephropathy.

Oral presentation at International Society of Hypertension(ISH), New investigator symposium, 2014: San Francisco, California, USA


Gooz, M., 2010. ADAM-17: the enzyme that does it all, Critical reviews in biochemistry and molecular biology 45: 146-169.


aldosterone, reflects the renal renin-angiotensin-aldosterone system activity and the efficacy of renin-angiotensin-aldosterone system blockade in the kidney, J Hypertens. 29: 2147-2155.


