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# Inhibition of inflammatory cytokines - potential new treatment for diabetic nephropathy

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Thesis

**INHIBITION OF INFLAMMATORY CYTOKINES – POTENTIAL NEW  
TREATMENT FOR DIABETIC NEPHROPATHY**

by

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B.S., Providence College, 2012

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TREATMENT FOR DIABETIC NEPHROPATHY**

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**ABSTRACT**

Type II diabetes mellitus is currently on the rise and reaching epidemic proportions in the United States. In addition to this increase, the number of cases of diabetic complication such as kidney disease has increased. Currently diabetic kidney disease is the leading cause for end stage renal disease in the United States accounting for nearly half of all cases. Type II diabetes is the result of metabolic, hemodynamic and inflammatory alterations within the body. Currently there is a standard of care to treat both metabolic and hemodynamic perturbations by enforcing tight glycemic control and utilizing anti-hypertensive drugs, most notably RAS inhibitors. These therapeutic interventions however, are not sufficient as many patients with type II diabetes will still develop diabetic kidney disease therefore another treatment option is imperative. Currently there are no treatments available to counteract the adverse inflammatory responses associated with type II diabetes which are strong contributors to the progression of the diabetic kidney disease. Among the inflammatory parameters studied as potential targets for therapeutic intervention the inflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) stands out among the rest for its multifaceted role in disease progression.

TNF- $\alpha$  has been shown to both directly and indirectly involved in development and progression of diabetic kidney disease. The inflammatory cytokine itself is toxic to renal cells initially increasing the permeability of the glomerular filtration barrier and contributing to proteinuria which eventually causes cellular apoptosis. TNF- $\alpha$  also activates second messengers and up-regulates transcription factors that further contribute to the progression of diabetic kidney disease.

Two TNF- $\alpha$  inhibitors, pentoxifylline and chrysin have stood out among the other investigational drugs which have been studied as potential therapeutic options to delay the progression of diabetic kidney disease. Pentoxifylline is a methyl-xanthine derivative that is currently used to treat peripheral vascular disease. It has shown good effect in clinical trials decreasing both urinary TNF- $\alpha$  concentrations as well as urinary protein excretion. Chrysin is a natural plant derivative belonging to the flavonoid family and is known for its anti-inflammatory and anti-oxidant properties. Currently chrysin has only been studied in animal models of diabetic kidney disease but has shown to not only decrease concentrations of inflammatory cytokines to control levels and improve renal functions but also prevented the histopathological changes associated with diabetic kidney disease suggesting that chrysin has the ability to not only slow the progression of disease and preserve renal function, it has the ability to prevent the disease from ever taking root.

Diabetic kidney disease is a devastating disease affecting millions of people worldwide. It is important for further investigation with these investigational drugs to be

performed in large scale clinical trials to produce safety and efficacy data with the end goal of becoming approved as new treatments for diabetic kidney disease.

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## LIST OF ABBREVIATIONS

AGE.....	Advanced Glycation End Product
AI.....	Angiotensin I
AII.....	Angiotensin II
ACEI.....	Angiotensin Enzyme Converting Inhibitor
ARB.....	Angiotensin II Receptor Blocker
AGT.....	Angiotensinogen
CTGF.....	Connective Tissue Growth Factor
DKD.....	Diabetic Kidney Disease
ESRD.....	End Stage Renal Disease
eGFR.....	Estimated Glomerular Filtration Rate
E/C.....	Extracellular
ECM.....	Extracellular Matrix
GFR.....	Glomerular Filtration Rate
ICAM-1.....	Intercellular Adhesion Molecule
IL-1.....	Interleukin-1
IL-6.....	Interleukin-6
I/C.....	Intracellular
MCP-1.....	Monocyte Chemoattractant Protein
MMF.....	Mycophenolate Mofetil
NO.....	Nitric Oxide

NF- $\kappa$ B.....	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
PTX.....	Pentoxifylline
PAI-1.....	Plasminogen Activator Inhibitor
PFD.....	Pirfenidone
PKC.....	Protein Kinase C
PM.....	Pyridoxamine
ROS.....	Reactive Oxygen Species
RAGE.....	Advanced Glycation End Product Receptor
RAS.....	Renin Angiotensin System
SOCS.....	Suppressors of Cytokine Signaling Proteins
TGF- $\beta$ .....	Transforming Growth Factor Beta
TNF- $\alpha$ .....	Tumor Necrosis Factor Alpha
UAE.....	Urinary Albumin Excretion
VEGF.....	Vascular Endothelial Growth Factor

## INTRODUCTION

### **Energy Metabolism**

#### *Hormones of Energy Metabolism*

Insulin is an anabolic, peptide hormone composed of 51 amino acids and produced by the beta cells of the Islets of Langerhans in the pancreas. Insulin plays a major role in energy metabolism and is responsible for maintaining concentrations of blood glucose and free fatty acids, insuring these levels do not exceed the upper limit of normal. Insulin is stored within secretory granules and upon stimulation by glucose (primary stimulus), amino acids and free fatty acids is released into the blood stream via exocytosis. Insulin first enters circulation via the hepatic portal vein where 50% of the hormone is degraded in its first pass through the liver, thus the peripheral tissues in which insulin exerts its effects do not experience the full concentration of insulin. This hormone has a half-life of 5-8 minutes and in addition to being degraded by insulinases in the liver it is also degraded in the kidney (Koeppen & Stanton, 2009).

Insulin receptors are located on the surface of cells where the hormone exerts its mechanism of action: the liver, skeletal muscle and adipose tissue. In the liver insulin promotes glycogen synthesis and storage and activates both glycolysis and lipogenesis. Insulin also works to inhibit the opposing pathways thus inhibiting gluconeogenesis, glycogenolysis, ketogenesis and fatty acid oxidation. In skeletal muscle insulin promotes glucose uptake, glycogen synthesis and protein synthesis. Insulin also works to inhibit

glycogenolysis. Finally in adipose tissue insulin activates the storage of triacylglycerides and inhibits lipolysis (McPhee & Hammer, 2009).

Glucagon is a catabolic, peptide hormone composed of 29 amino acids and is produced by the alpha cells of the Islets of Langerhans in the pancreas. Glucagon is the antagonizing hormone to insulin in energy metabolism. Similar to insulin, glucagon has a short half-life of 3-6 minutes with a quarter being metabolized in its first pass through the liver and the remaining 75% metabolized later on by both the kidneys and liver. The release of glucagon from alpha cells is stimulated by amino acids and low blood glucose and is inhibited by both glucose and insulin (Koeppen & Stanton, 2009).

Unlike insulin, glucagon exerts its actions solely on one organ, the liver. As a catabolic hormone glucagon works to break down stored energy to utilize as fuel. Glucagon promotes multiple processes in the liver to achieve this including gluconeogenesis, glycogenolysis, fatty acid oxidation and ketogenesis (McPhee & Hammer, 2009). In a similar fashion to insulin, glucagon also suppresses the opposing actions including glycolysis, glycogen synthesis and lipid synthesis. (Koeppen & Stanton, 2009)

### **Normal Energy Metabolism**

Under normal conditions the body experiences two states, fed and fasting. In the fed state from a primarily carbohydrate meal the body experiences an increase in insulin production while glucagon is inhibited. This promotes glycogen synthesis and storage in both the liver and skeletal muscle as well as fat storage in adipose tissue. At the same

time glucose production and ketogenesis in the liver are both inhibited. In the fed state from a protein rich meal both insulin and glucagon increase due to stimulation from the amino acids released during protein breakdown. Insulin exerts the same actions as during a carbohydrate meal but the presence of glucagon allows for the liver to produce glucose in order to counterbalance the effects of insulin. In the fasting state the primary hormone in action is glucagon. Glucagon stimulation exerts its effects primarily on the liver leading to glycogen breakdown. Free fatty acids are also released during fasting and are used for fuel by skeletal muscle and the liver for ketogenesis. Although fasting, there is enough insulin present to prevent hyperglycemia and ketoacidosis (McPhee & Hammer, 2009).

### **Disrupted Energy Metabolism**

During a prolonged fast normal energy metabolism is interrupted and if not corrected can be extremely dangerous and potentially life threatening. The disruption in energy metabolism due to prolonged fasting is similar to what is observed in a patient with diabetes mellitus. This perturbation of metabolism leads to further increased levels of glucagon and further decreased levels of insulin compared to what is observed during a normal fasting state. At this point glycogen stores have been depleted and the only source of glucose is from hepatic gluconeogenesis using amino acids as the substrate. Eventually the body will switch to ketogenesis to meet its minimal energy requirements (McPhee & Hammer, 2009). In diabetic patients glucagon levels may be inappropriately high thus mimicking the prolonged fasting state. In type II diabetes mellitus this is the result of insulin resistance and pancreatic beta cell dysfunction. This state prevents the inhibition

of gluconeogenesis in the liver by insulin leading to hyperglycemia. Insulin resistance is also observed in skeletal muscle and adipose tissue leading to increased fat breakdown and hyperlipidemia as well as decreased glycogen storage (Medscape, 2014; Scheen, 2003).

## **Type II Diabetes Mellitus**

Diabetes mellitus is a metabolic disorder characterized by an absolute or relative deficiency of insulin resulting in hyperglycemia (Gardner & Shoback, 2011). Insulin deficiency is the result of various deficits in insulin production, insulin function or both (Center for Disease Control and Prevention (CDC), n.d.). There are four known classifications of diabetes mellitus, type 1, type 2, gestational and other specific types (Gardner & Shoback, 2011).

Commonly known as non-insulin dependent or adult onset diabetes, type 2 diabetes is the clinical manifestation of insulin resistance and impaired insulin secretion due to pancreatic beta cell dysfunction (Leahy, 2005; CDC, n.d.; Scheen, 2003). The metabolic disturbance observed in type II diabetes causes a response from the immune system adding on the classification of not only a metabolic disorder but also an immune disorder (Navarro & Mora, 2005). Long-term activation of the immune system due to hyperglycemia results in a chronic state of low grade inflammation in patients with type II diabetes that results in disease instead of repair (Navarro & Mora-Fernández, 2006).

Type II diabetes mellitus is the result of many contributing factors, both genetic and environmental (Table 1), which together stress glucose homeostasis causing an

imbalance between the insulin required by the body and the insulin produced by pancreatic beta cells (Gardner & Shoback, 2011). Regardless of how type II diabetes develops a common phenotype known as the type II diabetes triad is present in all patients. The triad consists of pancreatic beta cell dysfunction, insulin resistance manifested as impaired glucose clearance by skeletal muscle and overproduction of hepatic glucose (Leahy, 2005). Once insulin resistance takes root hyperglycemia will ensue and continue to exacerbate this condition thus creating a pathological feedback mechanism (Scheen, 2003).

**Table 1 – Factors Contributing to Insulin Resistance** (Table amended from Gardner & Shoback, 2011)

Pre-receptor	Insulin autoantibodies Reduced transendothelial transit
Primary defect in insulin signaling	Insulin receptor mutations Leprechaunism (complete) Rabson-Medenhall syndrome (partial) Defects in other genes involved in insulin signaling Type A (mild) Defects in other genes involved in insulin signaling Insulin receptor autoantibodies (Type B) Ataxia telangiectasia syndrome
Secondary to other endocrine disorders	Cushing syndrome Acromegaly Pheochromocytoma Glucagonoma Hyperthyroidism Insulinoma
Secondary to other disorders	Visceral obesity Stress (infection, surgery, etc) Uremia Hyperglycemia (mild resistance seen in type 1 diabetes) Liver disease Cytogenetic disorders (Down, Turner, Klinefelter) Neuromuscular disorders (muscular dystrophies, ataxias, muscle inactivity) Congenital lipodystrophies/lipoatrophy Acquired lipodystrophy
Secondary to normal physiologic states	Puberty Pregnancy Starvation
Secondary to medications	Glucocorticoids Atypical antipsychotic drugs Antiretroviral protease inhibitors Nicotinic acid Thiazide diuretics Oral contraceptive Progesterone $\beta$ blockers

Insulin resistance and pancreatic beta cell dysfunction work together to potentiate type II diabetes, thus one does not cause the other as previously believed. Insulin resistance is an important risk factor for the development of type II diabetes however it is not causative. Patients with insulin resistance can also have plasma glucose levels within normal limits, the transition from normoglycemic to impaired glucose tolerance and eventually diabetes is the result of beta cell dysfunction (Leahy, 2005).

Insulin resistance is noted in the cells of skeletal muscle, liver and adipose tissue. Insulin resistance is caused by a multitude of factors, however, there seems to be three commonalities among the majority of cases: aging, lack of exercise and poor diet (Scheen, 2003). There are three postulated mechanisms for the cause of insulin resistance. First, insulin down regulates its own receptor via negative feedback and receptor mediated endocytosis. Second, insulin activates serine/threonine kinases which in turn inactivate insulin receptors and/or insulin receptor proteins. Finally it is believed insulin receptors and/or insulin receptor proteins concentrations may be decreased due to suppressors of cytokine signaling proteins, key regulatory proteins of the inflammatory process (Koeppen & Stanton, 2009; Tamiya, Kashiwagi, Takahashi, Yasukawa, & Yoshimura, 2011). The suppressors of cytokine signaling proteins are a family of proteins composed of SOCS-1 through SOCS-7. SOCS-1 and SOCS-3 are the two members involved in insulin resistance. SOCS-1 and SOCS-3 bind to different domains of the insulin receptor preventing insulin receptor substrate binding thereby interfering with insulin signaling leading to resistance (Ueki, Kondo, & Kahn, 2004).

Obesity also plays a major role in insulin resistance as well as the development of type II diabetes with an emphasis on body fat distribution. People with increased central fat mass experience decreased insulin sensitivity compared to people with fat distributed elsewhere throughout the body. Type II diabetics also experience ectopic fat accumulation meaning adipocytes are unable to accommodate increasing amounts of triglycerides therefore these are stored in other tissues of the body including insulin sensitive tissue (skeletal muscle, liver and endocrine pancreas) thus contributing to insulin resistance (Scheen, 2003). Obesity has also been associated with enhanced production of suppressors of cytokine signaling proteins, which as previously mentioned, work to contribute to insulin resistance (Ueki et al., 2004).

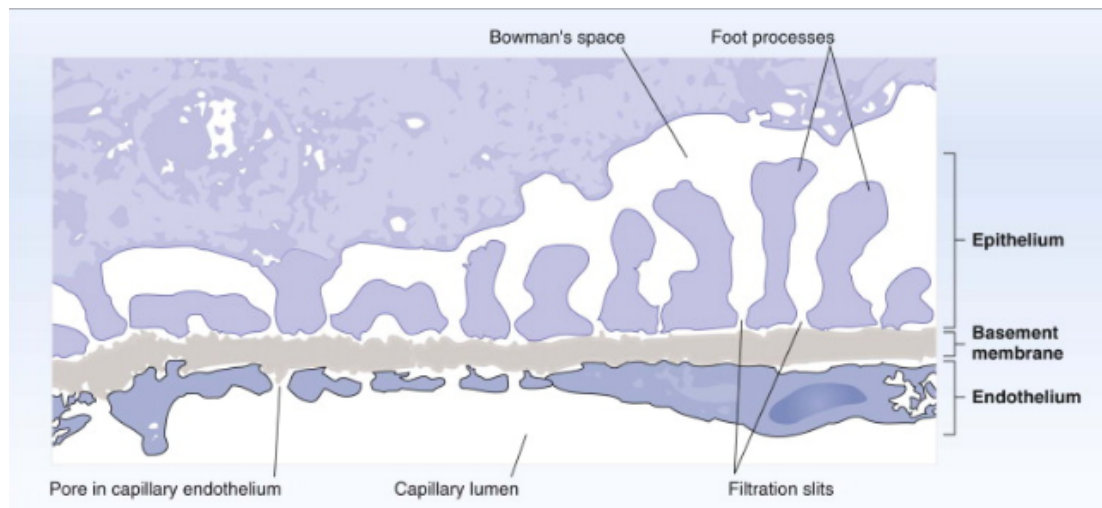
The ability of adipose tissue to produce pro-inflammatory cytokines also contributes to insulin resistance in obesity related type II diabetes (Gardner & Shoback, 2011). Adipose tissue releases the main inflammatory cytokines involved in diabetes and is responsible for maintaining the chronic low grade inflammation observed in type II diabetes mellitus (Navarro & Mora, 2005). As fat stores increase in the body the amount of free fatty acids and proinflammatory adipokines increase. As a result macrophages are recruited to the adipose tissue and subsequently activated. Activated macrophages release multiple cytokines such as tumor necrosis factor-  $\alpha$  (TNF-  $\alpha$ ), interleukin-6 (IL-6), and nitric oxide (NO). These molecules increase adipocyte resistance to insulin further potentiating the release of proinflammatory cells thereby initiating a positive feedback mechanism promoting chronic inflammation and insulin resistance (Gardner & Shoback, 2011).

Insulin deficiency is also present in patients with type II diabetes due to pancreatic beta cell deficit and apoptosis. There are three main mechanisms by which this insulin deficiency occurs. First there is a genetic defect, although this has not yet been confirmed in patients with type II diabetes (Scheen, 2003). The next proposed mechanism is the “thrifty phenotype hypothesis” which suggests that malnutrition while in utero effects the development of beta cells resulting in deficient insulin secretion (Scheen, 2003). Finally it is believed than an unfavorable metabolic environment contributes to insulin deficiency and the overall development of type II diabetes (Scheen, 2003). The increased concentrations of glucose and non-esterified free fatty acids observed in type II diabetes are toxic to cells. The ectopic deposition of fat in beta cells results in increased synthesis of nitric oxide that causes damage to the cells eventually leading to apoptosis thus contributing to beta cell and insulin deficiency (Scheen, 2003).

### **Glomerular Filtration Barrier**

The glomerular filtration barrier (Figure 1) is composed of three layers whose physical characteristics determine the rate of filtration as well as the composition of the filtrate (Costanzo, 2013). The first layer of the barrier is the glomerular endothelium. The glomerular endothelium is covered by a glycocalyx composed of proteoglycans and glycosaminoglycans (Maezawa, Takemoto, & Yokote, 2015). Endothelial cells have pores ranging from 70-100nm in diameter and allow the passage of fluid, dissolved solutes and plasma proteins. The second layer of the glomerular filtration barrier is the basement membrane which itself has three layers: lamina rara interna, lamina densa, and

lamina externa. The lamina rara interna is fused to the glomerular endothelium, the lamina densa lies in the middle of the basement membrane and the lamina externa is fused to the final layer of the filtration barrier composed of specialized epithelial cells known as podocytes.



**Figure 1: Glomerular Filtration Barrier.** The glomerular filtration barrier is composed of three layers: endothelium, basement membrane and terminally differentiated epithelium known as podocytes. These three layers impose both size and electrostatic barriers preventing the passage of large, negatively charged molecules from passing out of the blood and into the urine. (Costanzo, 2013)

The basement membrane permits fluid and dissolved solutes to pass through however plasma proteins are not filtered through this layer (Costanzo, 2013). As previously mentioned, podocytes are specialized epithelial cells that form the final layer of the glomerular filtration barrier. Podocytes provide physical support to the glomerulus and contain branching, interdigitating structures known as pedicels. The pedicels form filtration slits 25-60 nm in diameter, that are covered by a thin diaphragm composed of nephrin. The filtration slits impose the greatest size restriction of the glomerular filtration barrier (Costanzo, 2013; Satchell & Tooke, 2008).

In addition to a size restriction imposed by the glomerular filtration barrier, there is also an electrostatic restriction to the filtration process. Negatively charged glycoproteins are found on all layers of the glomerular filtration barrier impeding the filtration of similarly charged particles (Costanzo, 2013). Heparan sulfate is the major glycoprotein found attached to both glomerular endothelial cells and basement membrane providing the negative charge for the electrostatic filtration barrier. The glomerular basement membrane also contains proteins such as laminin, fibronectin and collagen that are negatively charged thus contributing to the electronic component of glomerular filtration (Ovalle, PhD & Nahirny, 2013). Electrostatics are not as important in regards to the filtration of small solutes however it is important in regards to plasma proteins as they are also negatively charged and are therefore repelled by the filtration barrier thus are not filtered into the urine (Costanzo, 2013).

### **Diabetic Kidney Disease**

There are three key characterizations of diabetic kidney disease (DKD): persistent albuminuria which is defined as greater than 300 mg of albumin per day in the urine over the course of 3 to 6 months, decline in glomerular filtration rate (GFR) and elevated blood pressure. These diagnostic criteria are the clinical manifestations of cellular changes occurring in the kidney as a result of hyperglycemia and chronic inflammation associated with diabetes (Medscape, 2014). Thickening of the glomerular basement membrane is the earliest morphological change associated with diabetic nephropathy. The extent of thickening observed is an indication of the severity of disease progression

(Medscape, 2014). Hyperglycemia also induces mesangial cell expansion. This is believed to be caused by either increased production of matrix proteins or glycosylation of matrix proteins. The third cellular change is the result of intraglomerular hypertension which causes hardening of the glomeruli (Medscape, 2014).

Diabetes directly effects the filtering units of the kidneys causing them to breakdown leading to kidney disease and if not properly treated the need for renal replacement therapy (National Kidney Foundation, n.d.). The microalbuminuria observed in patients with type II diabetes and DKD is the result of ultra-structural changes to the glomerular filtration barrier leading to loss of charge selectivity. Reactive oxygen species (ROS) are increased in patients with type II diabetes and are known to disrupt the glycocalyx of the endothelial layer of the glomerular filtration barrier. ROS decrease the production of heparan sulfate thereby decreasing the concentration of negative charge on the filtration barrier. It is this alteration to the endothelial layer of the glomerular filtration barrier that is believed to initiate microalbuminuria (Satchell & Tooke, 2008).

In addition to diabetes mellitus there are many other contributing factors that put a patient at risk for developing DKD. These can be broken down in modifiable and non-modifiable risk factors (Table 2). To decrease the risk of developing DKD it is important to control as many modifiable risk factors as possible thus the strong emphasis on controlling blood pressure and blood glucose in addition to following a balanced diet and exercise regimen (Harjutsalo & Groop, 2014).

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<b>Nonmodifiable</b>
Genetic factors
Male sex
Age at onset of diabetes between 5 and 15 years
Long duration of diabetes
Increasing age
Family history of diabetic kidney disease, type 2 diabetes, hypertension, and insulin resistance
<b>Modifiable</b>
Poor glycemic control
Hypertension
Lipid abnormalities
Smoking
Metabolic syndrome
Insulin resistance
Low-grade inflammation
Endotoxins
Advanced glycation end products
Low intensity of physical activity
Salt intake

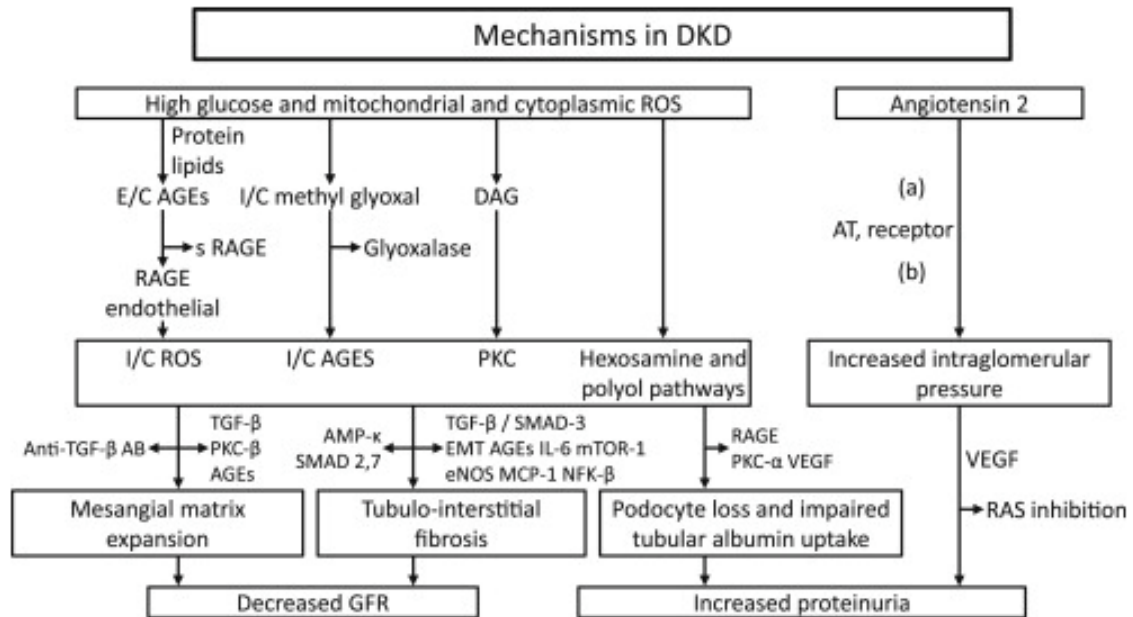
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**Table 2: Non-modifiable and modifiable risk factors contributing to diabetic kidney disease**  
(Harjutsalo & Groop, 2014)

### **Pathophysiology of Diabetic Kidney Disease**

Diabetic kidney disease is not the result of a single perturbation but rather multiple pathways are implicated in the progression of this disease. The three major pathways involved are metabolic, hemodynamic and inflammatory. Hyperglycemia initiates the disease process by activating many deleterious intracellular mechanisms as well as activating the inflammatory pathway. Hemodynamic alterations play a similar role as hyperglycemia in that they also activate harmful intracellular pathways as well as

enhancing inflammatory mechanisms (Macisaac, Ekinici, & Jerums, 2014). The interplay of these pathological mechanisms is illustrated in Figure 2.



**Figure 2: Mechanisms involved in the pathogenesis of diabetic kidney disease.** The metabolic alterations observed in diabetic kidney disease are induced by hyperglycemia. Hemodynamic alterations associated with disease are incited by the activation of the renin angiotensin system. Metabolic and hemodynamic alterations contribute to disease progression in their own right as well as enhancing the inflammatory response (Macisaac et al., 2014).

### *Renin and Angiotensin II*

The intrarenal renin angiotensin system produces two mediators in the pathogenesis of diabetic kidney disease: renin and angiotensin II (AII). The hyperglycemia associated with diabetes mellitus stimulates the production of AII as well as angiotensinogen (AGT), an inactive precursor to AII. As the concentration of AII

increases and it begins to accumulate in the tissue, it stimulates the expression of angiotensinogen mRNA. This creates a positive feedback resulting in increased amounts of AII which is deleterious to renal cells (Carey, Giacchetti). Renin and AII stimulate the production of pro-inflammatory cytokines as well as pro-fibrotic factors including TNF- $\alpha$ , IL-1  $\beta$ , TGF-  $\beta$ , plasminogen activator inhibitor (PAI-1) fibronectin and collagen 1, (Alicic & Tuttle, 2014). AII also plays a direct role in the progression of DKD as it induces podocyte injury via the NAPDH oxidase pathway which increases production of ROS. Renin can also have damaging effects on the kidneys by activating a variety of effectors including ERK 1 & 2, TGF-  $\beta$ , as well as hypertrophic and proliferative effects, all of which contribute to the pathology of diabetic nephropathy (Mathew, Cunard, & Sharma, 2011).

#### *Advanced Glycation End Products*

Advanced glycation end (AGEs) products directly and indirectly contribute to the progression of DKD. AGEs have the ability to cross-link their proteins which directly affects the glomerulus causing extracellular matrix expansion as well as glomerulosclerosis. AGEs also bind to proinflammatory cytokine receptors on various cells including endothelial cells and mesangial cells as well as receptors located on the glomerular basement membrane. These receptors are known as RAGEs (receptors for advanced glycation products) and the formation of the AGE-RAGE complex has multiple implications in disease progression. Receptor binding causes increased gene expression of growth factors (TGF-  $\beta$ , VEGF, CTGF), increased inflammatory cytokine (IL-1, IL-6, TNF-  $\alpha$ ) production as well as forming ROS (Alicic & Tuttle, 2014).

### *Protein Kinase C, Nox and JAK/STAT Pathway*

Protein Kinase C (PKC) is found in several different isoforms, some of which have roles in the development and progression of DKD, namely PKC-  $\alpha$  and PKC-  $\beta$ . PKC-  $\alpha$ , when activated, up-regulates vascular endothelial growth factor (VEGF) leading to proteinuria. PKC-  $\beta$  is directly involved in the renal hypertrophy and sclerosis observed in DKD as it upregulates pro-fibrotic factors such as TGF-  $\beta$ , type IV collagen, laminin and fibronectin. There are a number of inciting molecules for the activation of PKC most notably, hyperglycemia, AGEs, AII and ROS (Alicic & Tuttle, 2014). PKC has also been shown to increase expression of Intercellular Adhesion Molecule – 1 (ICAM-1) on the surface of glomerular and tubulo-interstitial cells. Increased expression of ICAM-1 enhances inflammation by promoting intracellular macrophage infiltration. In the presence of high glucose these macrophages will produce TGF- $\beta$  thereby also contributing to the glomerular and tubulo-interstitial fibrosis associated with DKD (Tuttle, 2005).

Nox is composed of several multimeric enzyme complexes and itself is a part of NADPH oxidase. Nox functions to generate ROS in reaction to increased glucose, AGEs and increased amino acids. Nox2 can be found primarily in podocytes, mesangial cells and endothelial cells while Nox 4 is found primarily in the glomerulus, proximal tubule and distal tubule (Alicic & Tuttle, 2014). The generation of ROS by Nox leads to mesangial expansion by inducing extracellular matrix protein synthesis as well as podocyte injury, both of which contribute to the albuminuria observed in DKD. ROS also up-regulate pro-fibrotic factors leading to fibrosis and apoptosis. ROS are especially

harmful as they not only directly cause histological changes in the glomerulus but also cause indirect injury with the ability to activate other pathways implicated in the pathogenesis of DKD such as PKC (Alicic & Tuttle, 2014).

The JAK/STAT pathway has also been shown to play a role in the development and progression of DKD. JAK is a tyrosine kinase which activates STAT in the JAK/STAT pathway leading to cellular proliferation and growth. It has been observed in patients with diabetes that JAK/STAT pathway mRNA is overexpressed leading to increased collagen production and fibrosis. This pathway is activated by increased glucose, AGEs and AII (Alicic & Tuttle, 2014).

Finally the transcription factor NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells), a downstream target activated by inflammatory cytokines such as TNF- $\alpha$ , plays an important role in the disease progression of diabetic nephropathy. Under normal conditions, NF- $\kappa$ B exists as a heterodimer of two proteins, p65 and p50, which is bound to inhibitory  $\kappa$ B (I $\kappa$ B) proteins. In this inactive state the p65/p50 dimer is located in the cytosol of the cell. Upon cellular stimulation by inflammatory cytokines the I $\kappa$ B proteins are degraded via I $\kappa$ B kinases thereby activating NF- $\kappa$ B. Once activated, the p65 subunit of NF- $\kappa$ B translocates into the nucleus initiating transcription of key players in DKD including, TGF- $\beta$ . In animal models of DKD NF- $\kappa$ Bp65 is markedly increased, thereby confirming the central role this protein plays in disease pathogenesis and marking it has a potential therapeutic target (Ahad, Ganai, Mujeeb, & Siddiqui, 2014).

## **Inflammatory Cytokines in Diabetic Kidney Disease**

Cytokines are peptides which exert effects over neighboring cells in a paracrine or juxtacrine fashion as well as retaining the ability to affect itself thus acting in an autocrine fashion. The actions of cytokines are carried out upon cytokine binding to surface receptors on cells for which they have a high affinity. Cytokines target a vast array of cells and produce different actions depending on type of cell and cytokine involved (Navarro-González & Mora-Fernández, 2008).

The hypothesis that inflammatory cytokines played a role in diabetic nephropathy was first proposed in 1991 by Hasegawa et al. when the levels of TNF-  $\alpha$  and IL-1 found in glomerular basement membranes was compared between diabetic and non-diabetic rats. It was discovered that the glomerular basement membranes of diabetic rats contained a significantly increased amount of TNF-  $\alpha$  and IL-1 when compared to non-diabetic rats (Hasegawa et al., 1991; Navarro-González & Mora-Fernández, 2008).

It is now known that interleukin-6 (IL-6), interleukin-1 (IL-1), interleukin-18 (IL-18) and tumor necrosis factor-  $\alpha$  (TNF-  $\alpha$ ) are the major contributing cytokines involved in the pathogenesis of DKD (Navarro-González & Mora-Fernández, 2008). These inflammatory mediators are responsible for activating a series of mechanisms causing the observed histological damage in patients with diabetic nephropathy. Some of the pathologies caused by inflammatory cytokine action include but are not limited to “beta

cell dysfunction and apoptosis, insulin signaling impairment, systemic endothelial dysfunction and altered vascular flow,” (Pan et al., 2013).

IL-6 has been shown to increase the width of the glomerular basement membrane, the expression of fibronectin as well as the permeability of endothelial cells, all of which occur in the early stages of disease progression (Elseweidy, Elswefy, Younis, & Zaghoul, 2013; Navarro-González & Mora-Fernández, 2008). IL-1 also increases the permeability of endothelial cells as well as increasing the thickness of the glomerular basement membrane by stimulating both mesangial cell proliferation and extracellular matrix synthesis. The effects of IL-18 are not definitely known; however it is believed IL-18 up-regulates other inflammatory cytokines and is involved in the apoptosis of glomerular endothelial cells (Duran-Salgado & Rubio-Guerra, 2014). IL-18 has also been associated with urinary albumin excretion rate (Navarro & Mora-Fernández, 2006).

TNF-  $\alpha$  plays a central role in the pathogenesis of diabetic kidney disease, contributing to many of the observed pathologies of DKD (Table 3). It is synthesized by monocytes and macrophages as well as intrinsically in the kidney (Navarro & Mora-Fernández, 2006). TNF-  $\alpha$  exerts its deleterious effects through multiple mechanisms such as activating second messengers, up-regulating transcription factors and growth factors as well as stimulating the production of cell adhesion molecules (Duran-Salgado & Rubio-Guerra, 2014). TNF-  $\alpha$  alters key regulatory genes involved in glucose and lipid metabolism thereby perturbing insulin signaling and cellular glucose uptake. Hemodynamics are also altered by this cytokine as TNF- $\alpha$  stimulates the release of vasoconstricting compounds from mesangial cells. This action decreases glomerular

blood flow thereby decreasing GFR (Navarro & Mora-Fernández, 2006). In addition to the various mechanisms TNF- $\alpha$  activates this cytokine in and of itself is harmful to the kidneys. TNF- $\alpha$  is cytotoxic to mesangial and epithelial cells leading to apoptosis as well increasing the permeability of the glomerular filtration barrier (Elseweidy et al., 2013; Navarro & Mora-Fernández, 2006).

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Cell contraction
Increment of tubular sodium reabsorption
Increment of renal protein content and glomerular volume
Inhibition of endothelium-dependent relaxation
Reduction of glomerular blood flow and glomerular filtration rate
Disruption of glomerular permeability barrier
Increment of albumin permeability
Stimulation of plasminogen-activator inhibitor type-1 tissue factor production
Downregulation of tissue factor pathway inhibitor mRNA
Reduction of thrombomodulin expression
Stimulation of polymorphonuclear leukocytes and monocytes recruitment
Stimulation of adhesion molecules expression
Stimulation of synthesis and release of chemokines/cytokines/growth factors: MCP-1, RANTES, IP-10, IL-6, IL-8, fibronectin, PDGF, NGF
Induction of major histocompatibility complex antigen expression
Stimulation of the production of diverse pro-inflammatory and hemodynamic mediators: complement components, reactive oxygen species, nitric oxide, adenosine, platelet activating factor, prostaglandins
Proliferation, cytotoxicity and regulation of cell death related genes
Induction of apoptosis

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**Table 3: Effects of TNF-  $\alpha$  on the Kidneys** (Navarro & Mora-Fernández, 2006)

The inflammatory cytokines transforming growth factor –  $\beta$  (TGF-  $\beta$ ) and vascular endothelial growth factor (VEGF) are also directly involved in the pathogenesis of diabetic nephropathy. TGF-  $\beta$  expression is increased in the presence of hyperglycemia, and in conjunction with VEGF contributes to the increased in size of the glomerular cells as well as the increased collagen synthesis observed in patients with diabetic nephropathy (“Medscape, 2014,” 2014).

### **Intrarenal Renin Angiotensin System (RAS)**

The kidneys utilize the renin angiotensin system to perform one of its major functions: that of regulating blood pressure. Intrarenal RAS contains all of the same components of systemic RAS including renin, angiotensinogen (AGT), angiotensin I (AI) and angiotensin II (AII). Activation of the RAS begins with the production of the enzyme renin by the juxtaglomerular cells found in the renal afferent arteriole. The production and secretion of renin is stimulated by low blood pressure which is sensed by the afferent arteriole. Renin then enters the blood stream where it encounters AGT which is produced by cells of the proximal tubule. Renin cleaves AGT into AI which is subsequently hydrolyzed into the biologically active AII. AII is a potent vasoconstrictor thereby increasing peripheral resistance and blood pressure in order to correct the inciting hypotensive state (Carey & Siragy, 2003; Singh & Williams, 2009).

AII has two receptors in the kidneys, AT<sub>1</sub> which is located throughout the kidneys on vascular smooth muscle cells and AT<sub>2</sub> which is also located throughout the kidney on afferent arterioles, endothelial cells, interstitial cells and proximal tubule cells. The

actions of AII are mainly carried out via AT<sub>1</sub> receptors including arteriole vasoconstriction, sodium reabsorption by the tubules, and inhibition of pressure-natriuresis. Binding of AII to AT<sub>2</sub> receptors causes the opposing actions produced by binding to AT<sub>1</sub> receptors thus promoting vasodilation and decreased renal vascular resistance (Carey & Siragy, 2003).

### **RAS Inhibition in Treatment of Diabetic Kidney Disease**

In addition to strict glycemic control the current standard of care for diabetic nephropathy is treatment with a RAS inhibitor, either an angiotensin II receptor blocker (ARB) or an angiotensin converting enzyme inhibitor (ACEI). Angiotensin II preferentially vasoconstricts the postglomerular (efferent) arterioles causing an increase in glomerular pressure which then contributes to the progression of renal disease. Thus inhibition of the RAS assists in slowing the progression of chronic nephropathies (Remuzzi, Perico, Macia, & Ruggenti, 2005).

ARBs perform effective RAS inhibition by preventing the binding of AII to the AT<sub>1</sub> receptor. This directly inhibits the vasoconstriction of the postglomerular arteriole thereby preventing an increase in glomerular pressure (Remuzzi et al., 2005). Multiple studies, most notably the Irbesartan in Diabetic Nephropathy Trial and the RENAAL trial, have found that implementing the use of an ARB in treating diabetic nephropathy reduced the risk of serum creatinine doubling, i.e., of a reduced GFR, end stage renal disease and death (Brenner et al., 2001; Lewis et al., 2001; Remuzzi et al., 2005). The use

of ARBs also promotes AT<sub>2</sub> activity providing added renal protection (Carey & Siragy, 2003).

ACE inhibitors interfere with the RAS by preventing the conversion of AI to AII (Remuzzi et al., 2005). The inhibition of ACE also prevents the degradation of bradykinin. Bradykinin is an endogenous vasodilator that has been shown to have renoprotective properties due to its ability to decrease blood pressure (Carey & Siragy, 2003). In addition to lowering blood pressure, ACEIs decrease proteinuria associated with diabetic nephropathy (Remuzzi et al., 2005).

Combination therapy, dosing with an ARB and an ACEI poses great risk for the patient. Many studies have shown combination therapy increases the subjects risk for adverse cardiovascular events, hyperkalemia and acute kidney injury. In comparison to single ACEI or ARB therapy, combination therapy does not provide greater renal protection or antiproteinuric effect. The risk of using combination therapy outweighs the benefit thereby eliminating this as a potential therapy for patients with DKD (Fried et al., 2013; Mann et al., 2008; Parving et al., 2012).

## **Epidemiology**

Currently 29 million Americans are estimated to have diabetes mellitus, accounting for slightly over 9% of the US population. An additional 86 million adults in the United States are estimated to have pre-diabetes, a condition of elevated blood sugar but not so high as to be diagnosed with type II diabetes. Of those 86 million adults, 15% - 30% will develop type II diabetes over the next five years (CDC, n.d.).

As the rates of diabetes mellitus continue to increase so do the number of cases of DKD. Diabetic kidney disease reaches peak incidence 15-20 years post DM diagnosis (Harjutsalo & Groop, 2014). Due to the current obesity epidemic the United States is facing, type II diabetes mellitus is on the rise, especially in the younger population (Harjutsalo & Groop, 2014; CDC, n.d.). With disease onset occurring at younger ages more diabetic patients will develop DKD and eventually End Stage Renal Disease (ESRD) (Harjutsalo & Groop, 2014). Currently DKD is the leading cause of end stage renal disease in the United States accounting for nearly 50% of all ESRD cases (de Boer et al., 2011; Mitka, 2004). Rates of ESRD due to type II diabetes mellitus have increased the greatest in the youngest age group, patients aged 30-44 (Harjutsalo & Groop, 2014).

Despite increased patient use of glucose lowering medications as well as medications that inhibit the RAS, the incidence of DKD is increasing, clearly conveying the need for additional agents to combat this devastating disease (de Boer et al., 2011). Of the three major pathways that contribute to the progression of DKD, two (metabolic and hemodynamic) currently have drugs on the market and are proving not to be enough to treat disease progression. With this in mind, implementing therapy to interfere with and inhibit the inflammatory contribution to this disease is the next logical step.

## Published Studies

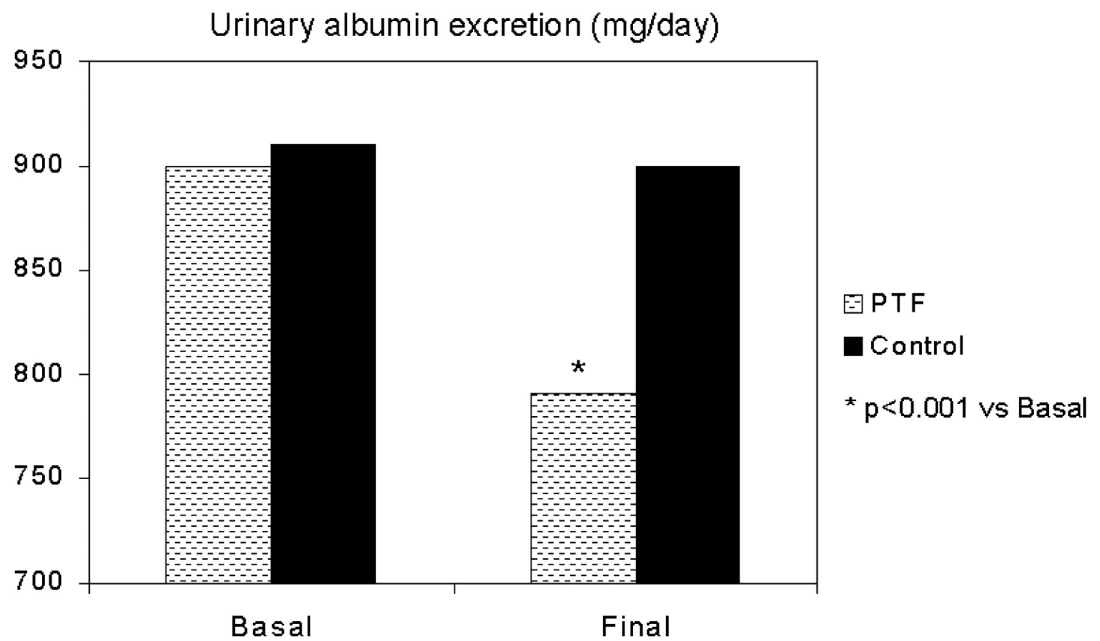
### Pentoxifylline

Pentoxifylline (PTX) is an erythrocyte phosphodiesterase inhibitor derived from methylxanthine and is currently on the market to treat peripheral vascular disease (Navarro et al., 1999; Navarro, Mora, Muros, & García, 2005). The inhibition of phosphodiesterase results in an increase in intracellular cyclic AMP. This promotes the integrity of the erythrocyte membrane and increases resistance to deformity. In addition to its effects on erythrocytes, PTX also increases fibrinolytic activity thus decreasing blood viscosity allowing blood to flow more easily (Behcets, n.d.).

PTX also has known immunoregulatory and anti-inflammatory properties (Navarro et al., 1999). The mechanism behind which PTX derives its renoprotective function is largely unknown however investigators have come to attribute it to PTX's ability to decrease concentrations of proinflammatory cytokines (McCormick et al., 2008). PTX inhibits TNF-  $\alpha$  mRNA. In doing so PTX prevents transcription and synthesis of TNF-  $\alpha$ . Decreasing the concentration of TNF-  $\alpha$  greatly aids in protecting renal function due to the vast role TNF-  $\alpha$  plays in the pathogenesis of DKD (Navarro-González & Mora-Fernández, 2008).

A 2005 study by Navarro et al. showed that the addition of Pentoxifylline to normotensive patients on an ARB provided a greater renoprotective and antiproteinuric effect compared to those patients on an ARB and placebo. This study utilized a sample of 61 subjects with nephropathy due to type II diabetes who were normotensive and were

taking an ARB at the recommended dose to treat diabetic nephropathy. The subjects were then randomized in a double blind fashion, 30 subjects received treatment with Pentoxifylline and 31 subjects received treatment with placebo. Prior to initiation of treatment all subjects were noted to have similar baseline renal function, blood pressure, metabolic control, albuminuria as well as serum and urinary TNF-  $\alpha$  concentrations. At the end of the study the treatment group was noted to have decreased albuminuria as well as decreased serum and urinary TNF-  $\alpha$  concentrations compared to the control group as depicted in Figure 4. Renal function, blood pressure and metabolic control remained the same across both treatment and control groups. This is an important discovery because it means that the decrease in albuminuria and TNF-  $\alpha$  concentrations is due to Pentoxifylline and not due to hemodynamic or metabolic changes (Figure 3) (Navarro et al., 2005).



**Figure 3: Effect of pentoxifylline treatment on urinary albumin excretion.** Baseline urinary albumin excretion rates for both placebo and pentoxifylline treatment groups were similar at about 900mg/day. End of study data collection showed a significant decrease in urinary albumin excretion rate for patients treated with pentoxifylline compared to placebo as noted by the p value of less than 0.001. (Navarro et al., 2005)

A short clinical trial was run by Ghorbani et al. (2011) to test the hypothesis that the addition of Pentoxifylline to combination therapy with an ARB and an ACEI would result in a further decrease in urinary protein excretion than combination therapy alone. This trial took place over the course of 6 months with 100 subjects and was conducted in a double blind and randomized fashion. All patients received 50mg of losartan (ARB) and 15mg of enalapril (ACEI) daily. In addition to this treatment regimen subjects in the treatment group received 400mg of PTX daily. The treatment group experienced significant decreases in urinary protein excretion after three months and six months whereas proteinuria did not change throughout the course of the study for the control

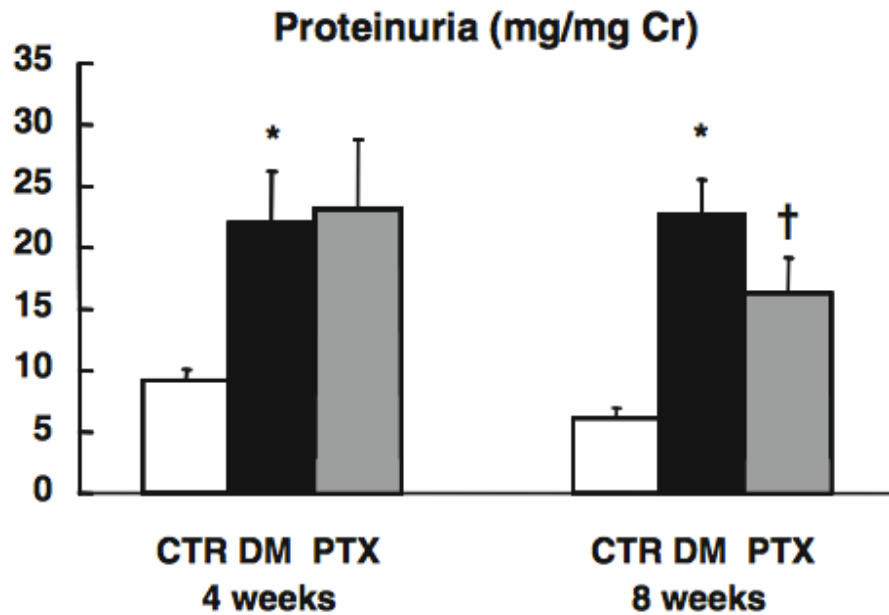
group. The treatment group also experienced renal protection from PTX treatment as noted by the increase in creatinine clearance after six months of treatment. The control group did not experience any improvement in creatinine clearance through the duration of the study. These results suggest PTX may attenuate disease progression as well as maintain and potentially improve renal function in patients with type II diabetes (Ghorbani, Omidvar, Beladi-Mousavi, Lak, & Vaziri, 2012).

In 2014 Navarro et al. once again investigated PTX over the course of two years in patients with type II diabetes and hypothesized that the addition of PTX to a patient maximum tolerated daily dose of an ARB or ACEI would delay the progression of diabetic nephropathy as well as restore kidney function. In an open label study with PTX investigators compared estimated glomerular filtration rate (eGFR), urinary albumin excretion (UAE) and urinary TNF-  $\alpha$  concentrations in patients taking PTX and patients not taking PTX. As seen previously, the addition of PTX to an ACEI or ARB regimen significantly decreased proteinuria. All subjects had a similar baseline UAE with the control group having an average UAE of 1000mg/d and the experimental group having an average UAE of 1100mg/d. End of study measurements showed that the control group average UAE increased by 5.7% to 1117mg/d while the PTX group average UAE decreased by -14.9% to 973mg/d. Thus there was an overall 20.6% difference in average UAE between groups in favor of those subjects taking PTX. As prior studies have shown, urinary TNF-  $\alpha$  concentrations decreased in subjects taking PTX and did not change in control subjects. Finally investigators in this study looked at PTX effects on eGFR to determine if PTX could protect renal function. End of study measurements showed a

significant mean difference in eGFR of 4.3 ml/min per 1.73m<sup>2</sup> in favor of the PTX group. The between group difference in eGFR did not reach statistical significance until 1 year into the trial and remained statistically significant until the trial concluded. This suggests that long term administration of PTX is necessary in order to preserve renal function. Blood pressure and glycemic control did not differ significantly in either group thus attributing the decrease in proteinuria and preservation of renal function to PTX (Navarro-González et al., 2015).

As increasing evidence was published indicating Pentoxifylline as a promising new drug therapy for DKD it was important to establish just how long a subject would need to be dosed to observe the renoprotective effect of PTX. In a 2009 study involving streptozotocin induced diabetic rats, Han et al. hypothesized PTX is most effective when administered for a period of time greater than four weeks (Figure 4). The rats were split into three groups, control, diabetic and untreated, and diabetic treated with PTX. Researchers compared body weight, kidney weight, glucose levels, urinary protein excretion and urinary monocyte chemoattractant protein (MCP-1) among the three groups at four weeks and again at 8 weeks. It was observed that at four weeks of treatment, diabetic rats treated with PTX did not experience a decrease in urinary protein excretion when compared to untreated diabetic rats; however, urinary protein excretion was attenuated in PTX treated rats after eight weeks of treatment. In contrast, PTX treated rats did experience a decrease in urinary MCP-1 at four weeks and this continued through the eight weeks of treatment. Investigators concluded that the anti-inflammatory effect of

PTX is apparent within the early stages of treatment and the antiproteinuric effect is experienced with prolonged treatment (Han, Han, Kim, Kang, & Cha, 2010).

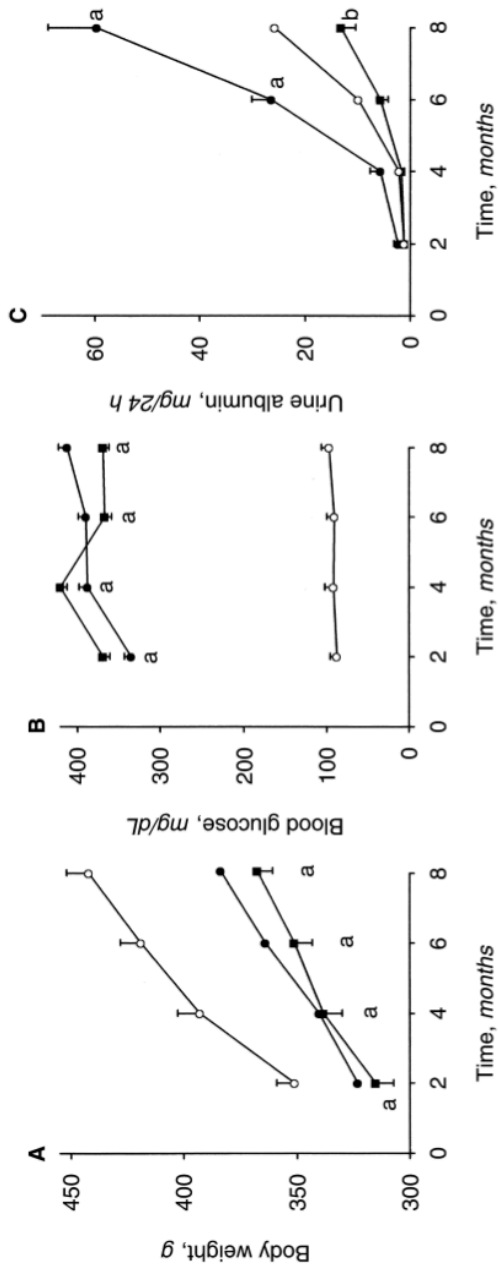


**Figure 4: Effect of prolonged administration of PTX.** Administration of pentoxifylline for a period of four weeks does not provide significant renal protection for rat models of type II diabetes as evidenced by the significant amount of proteinuria. Renal protection is observed after eight weeks of administration of pentoxifylline in rat models of type II diabetes as seen by the significant decrease in proteinuria compared to untreated diabetic rats. These results support the hypothesis that prolonged administration of pentoxifylline heightens its renoprotective nature. CTR: control group, DM: diabetic control group, PTX: pentoxifylline treatment group (Han et al., 2010)

### **Mycophenolate Mofetil**

Mycophenolate Mofetil (MMF) is an immunosuppressive drug with known antilymphocyte and anti-inflammatory properties. MMF's anti-inflammatory property

derives from its ability to prevent leukocyte recruitment at sites of inflammation ( Rx List, The Internet Drug Index, n.d.). In a 2002 study by Utimura et al. MMF was used to treat Munich-Wistar rats with streptozotocin induced diabetes. This study compared three groups of rats, a control (non-diabetic rats), diabetic rats treated with insulin and finally diabetic rats treated with both insulin and MMF. The study took place over a period of eight months during which it was observed that diabetic rats treated with both insulin and MMF had significantly decreased albuminuria in comparison to diabetic rats treated with only insulin (Figure 5). MMF treated rats did not display any changes in blood pressure, blood glucose concentration or glomerular dynamics when compared to the control group and diabetic rats treated with insulin group, thus the antiproteinuric effect of MMF can be solely attributed to the immunosuppressive properties of this drug (Utimura et al., 2003).



**Figure 5: Effect of Mycophenolate Mofetil on Body Weight, Blood Glucose and UAE in animal models of type II diabetes.** A. Streptozotocin induced diabetic rats maintained a lower body weight compared to control throughout the duration of the study regardless of treatment with MMF. B. Streptozotocin induced diabetic rats remained hyperglycemic throughout the duration of the study compared to control regardless of treatment with MMF. C. Urinary albumin excretion was significantly decreased in streptozotocin induced diabetic rats treated with MMF compared to untreated diabetic rats. UAE in MMF treated rats was comparable to that of non-diabetic control. (Utamura et al., 2003)  
 Key: Open circles: control, Filled circles: untreated diabetic rats, Filled squares: MMF treated diabetic rats

It was also observed that MMF treated rats did not have glomerular macrophage infiltration nor did these rats experience glomerulosclerosis. Interestingly, it was noted that although at two months the glomeruli had been infiltrated with macrophages in rats with diabetes, the glomeruli did not express any other injury associated with diabetes. Thus glomerular macrophage infiltration is an early step in the pathogenesis of diabetic nephropathy and precedes glomerular injury. Further investigation is warranted to determine if early treatment with MMF can prevent glomerulopathy (Utamura et al., 2003).

### **Infliximab**

Infliximab is a TNF- $\alpha$  inhibitor that is currently approved to treat autoimmune diseases such as rheumatoid arthritis, Crohn's disease and ulcerative colitis (Medline Plus n.d.). This drug is also currently under investigation as a potential treatment for DKD. In a 2007 study involving streptozotocin induced diabetic rats, investigators hypothesized TNF- $\alpha$  inhibition would decrease urinary albumin excretion thus delaying the progression of diabetic nephropathy. Post induction of diabetes, infliximab treated rats received injections of the study drug every four weeks for a total study duration of twelve weeks. Infliximab treatment decreased urinary TNF- $\alpha$  excretion significantly on weeks 1, 4 and 12 when compared to untreated diabetic rats. However, infliximab treatment was unable to reduce urinary TNF- $\alpha$  excretion to levels comparable to non-diabetic control rats. On the other hand, treatment with infliximab did decrease urinary protein excretion

significantly that achieved similar concentrations as non-diabetic control rats. Although other parameters of disease progression were not evaluated in this study the results do suggest that targeting TNF- $\alpha$  with infliximab has the potential to attenuate some of the disease process of diabetic nephropathy (Moriwaki et al., 2007).

### **FR167653**

In the previously mentioned study by Moriwaki et al. (2007) the investigators also looked at another inhibitor of TNF- $\alpha$  to potentially decrease urinary protein excretion in streptozotocin induced diabetic rat models of DKD. FR167653, a known potent inhibitor of p38 MAPK, not only has been shown to inhibit TNF- $\alpha$  but also IL-1 in *in vitro* studies. In contrast to infliximab, FR167653 was administered orally to streptozotocin induced diabetic rats over a period of twelve weeks. FR167653 treated rats experienced very similar results as those treated with infliximab. They displayed decreased urinary TNF- $\alpha$  concentrations that did not reach control concentrations and also experienced decreased urinary protein excretion which were comparable to control concentrations (Moriwaki et al., 2007).

### **Pirfenidone**

TGF-  $\beta$  is another cytokine of interest in treating DKD for not only its pro-inflammatory properties but also its pro-fibrotic properties. Pirfenidone (PFD) is an anti-inflammatory, anti-fibrotic agent derived from pyridone which has been investigated as a possible treatment for DKD. Pirfenidone is a known TGF-  $\beta$  inhibitor and in animal

models has shown to also decrease concentrations of TNF-  $\alpha$ . Pirfenidone exerts its inhibitory effects on TGF-  $\beta$  in a multitude of ways. PFD can act to block the TGF-  $\beta$  promoter, inhibit the secretion of TGF-  $\beta$  proteins as well as inhibit the phosphorylation of SMAD2, a downstream target of TGF-  $\beta$ . This inhibition results in decreased matrix protein accumulation as well as reduced expression of mesangial matrix genes (Mathew et al., 2011). It is hypothesized that the inhibition of TGF-  $\beta$  will decrease renal fibrosis thereby sustaining optimal renal function for a longer period of time thus delaying the progression of diabetic kidney disease (Sharma et al., 2011).

### **Pyridoxamine**

Pyridoxamine (PM) is an investigational product currently being tested for the treatment of diabetic kidney disease. PM is derived from vitamin B and has been shown to inhibit the formation of AGEs by trapping their intermediate form (Chen & Francis, 2012; Elseweidy et al., 2013). PM also scavenges ROS which in addition to inhibiting AGEs may provide renal protection in DKD (Lewis et al., 2012). In a study evaluating PM in alloxan induced diabetic rats; it was shown that after six weeks of treatment with PM, pro-inflammatory cytokine gene expression was significantly reduced. In this study researchers found that PM did not improve renal function but rather attenuated some of the key players in the pathogenesis of DKD (Elseweidy et al., 2013).

Although pyridoxamine did not seem to have an apparent renoprotective effect in animal studies, this potential treatment made its way into human clinical trials. One such study was a double blind, randomized, placebo controlled study by Lewis et al. in which

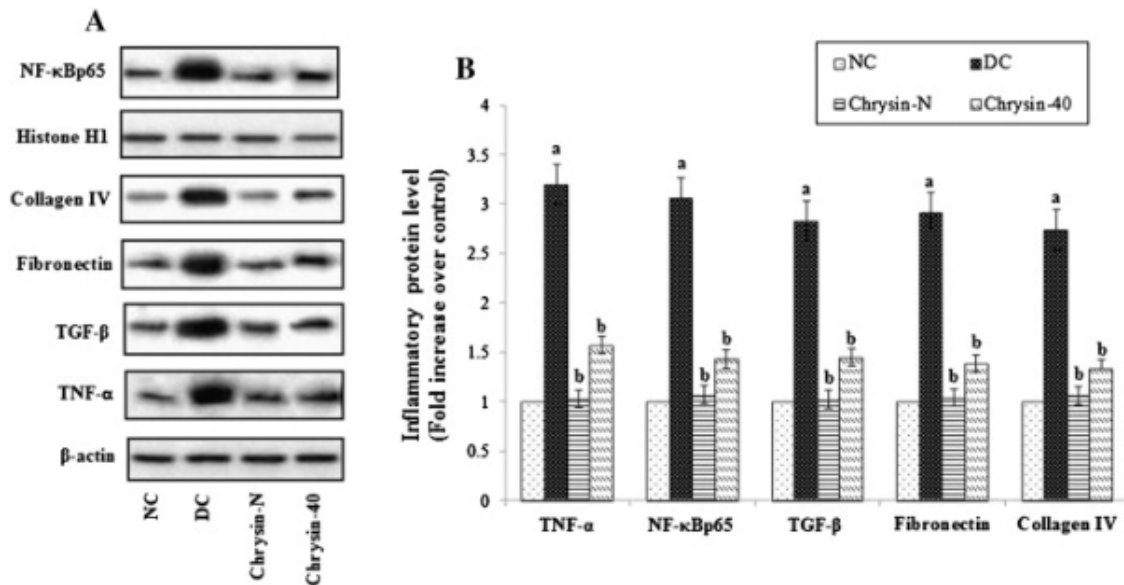
subjects were placed into one of three groups: placebo, pyridoxamine (Pyridorin) 150 mg twice a day, or pyridoxamine 300 mg twice a day. Subjects in each group had similar baseline characteristics and after one year of therapy it was determined that pyridoxamine failed to protect renal function and prevent an increase in serum creatinine. Accordingly, with the investigators' secondary endpoints each group was broken down into tertiles based on baseline creatinine. When examined in these groups, it was observed that subjects in the first tertile (those with the lowest baseline serum creatinine and thus the greatest baseline renal function) of both treatment groups did experience a slowing of disease progression. From this, investigators concluded that pyridoxamine is effective in protecting renal function in those subjects with greater baseline renal function. This was based on both the observed data and the fact that AGE formation occurs early in the disease process. Therefore those subjects who have the greatest intact renal function will benefit more from pyridoxamine than those who are far more progressed in the disease (Lewis et al., 2012).

### **Natural Derivatives of Plants**

Researchers have not only looked towards synthetic chemical compounds to ameliorate diabetic kidney disease but have also investigated natural compounds such as flavonoids and curcumin. These products are known for the anti-inflammatory and antioxidant effects and are currently widely used in pharmacy with little known side effects thus presenting as potential therapeutic agents to attenuate or even prevent the chronic

inflammation and its side effects observed in DKD (Ahad et al., 2014; Fang et al., 2015; Pan et al., 2013).

One such flavonoid that has been investigated for its potential therapeutic use is chrysin. Chrysin is a naturally occurring compound with anti-inflammatory and anti-oxidant effects found in plant extracts and honey. Ahad et al treated streptozotocin induced diabetic rats for 16 weeks to determine the potential benefits of chrysin. At the end of the 16 week treatment period, rats treated with chrysin not only showed significantly reduced concentrations of inflammatory cytokines but had concentrations of these cytokines similar to that of control, non-diabetic rats (Figure 6). Compared to untreated diabetic rats, chrysin treated rats had decreased serum creatinine, BUN and proteinuria as well as increased creatinine clearance thus implying chrysin has the ability to improve renal function in diabetic kidney disease. At the end of the treatment period the rats were sacrificed and all had the left kidney removed in order to determine the histological effects of chrysin. Untreated diabetic rats displayed the expected histopathology of diabetic nephropathy with thickened glomerular basement membranes and expanded mesangial matrices. In comparison, chrysin treated diabetic rats displayed histology resembling the control, non-diabetic rats, with normal glomerular basement membranes and normal mesangial matrices. Researchers believe that through the inhibition of TNF- $\alpha$ , chrysin was not only able to attenuate the disease process, it was able to prevent it (Ahad et al., 2014).



**Figure 6: Effect of chrysin on inflammatory biomarkers.** Treatment with chrysin in type II diabetic induced rats maintained non-diabetic control levels of inflammatory biomarkers expressed by renal cells. A. Western blot representing protein concentrations of inflammatory biomarkers after 16 weeks of treatment.  $\beta$ -actin served as the loading control for cytoplasmic proteins and Histone H1 served as the loading control for nuclear proteins. B. Graphical representation of inflammatory protein levels expressed by cultured renal cells after 16 weeks of treatment. NC: normal control, DC: diabetic control, Chrysin-N: normal rats treated with chrysin at 40mg/kg bw/day, Chrysin-40: diabetic rats treated with chrysin at 40mg/kg bw/day (Ahad et al., 2014)

Flavonoid derivatives have also been studied for the treatment of diabetic kidney disease with one such derivative being L6H21 (Fang et al., 2015). L6H21 is a derivative of chalcone and can be found in plants, spices and teas. *In vitro* studies have shown therapeutic benefit of L6H21 in preventing the progression of diabetic nephropathy. In renal NRK-52E cells, L6H21 has been shown to inhibit the expression of inflammatory cytokines induced by hyperglycemia. Investigators showed that hyperglycemia induces the expression of inflammatory cytokines at both the mRNA and protein level and,

pretreatment of NRK-52E cells with L6H21 showed significant inhibition, in a dose dependent manner, of inflammatory cytokine mRNA as well as proteins. L6H21 also significantly decreases chemokine and cell adhesion molecule expression in addition to inhibiting the activation of NF- $\kappa$ B by preventing the degradation of I $\kappa$ B proteins. Investigators also compared L6H21 to a known anti-inflammatory agent, curcumin. In all cases L6H21 had a greater inhibitory effect than curcumin. L6H21 worked in a dose dependent manner, thus higher concentrations had greater inhibitory effect (Fang et al., 2015).

As previously stated curcumin does have an inhibitory effect *in vitro*, however large concentrations are required due to its poor bioavailability (Fang et al., 2015; Pan et al., 2013). A curcumin derivative, B06, was engineered to have the same anti-inflammatory properties with a greater pharmacokinetic profile. Both *in vitro* and *in vivo* studies have been done which look at the efficacy of B06 in the prevention of DKD. *In vitro*, pretreatment with B06 decreased both the concentrations and transcription of inflammatory cytokines, TNF-  $\alpha$  and IL-6. B06 also prevented the phosphorylation and thus degradation of I $\kappa$ B proteins thereby preventing the activation of NF- $\kappa$ B. *In vivo* study was performed using streptozotocin induced diabetic rats to determine the therapeutic effect of B06. Serum concentration of TNF-  $\alpha$  and nitric oxide are key indicators of inflammation, the greater the concentration of these biomarkers the greater the inflammatory process taking place. In B06 treated rats there was no significant increase in these inflammatory biomarkers. B06 treated rats also did not experience a rise in plasma creatinine nor an increase in kidney weight to body weight ratio as is observed

in models of diabetic nephropathy. Finally the histopathological changes observed in DKD (GBM thickening, mesangial matrix expansion, and inflammatory cell infiltration) were not seen in B06 treated rats (Pan et al., 2013).

## DISCUSSION

The current standard of care for treating diabetic kidney disease consists of antiproteinuric agents such as angiotensin II receptor blockers and angiotensin converting enzyme inhibitors in addition to strict glycemic control. However these agents alone have proven not sufficient when it comes to combating diabetic nephropathy. Therefore many patients progress to end stage renal disease and experience significant cardiovascular events. In some cases ARBs and ACEIs cannot be used due to intolerable side effects such as hyperkalemia and angioedema thereby creating an even greater need for additional agents to combat this disease (McCormick et al., 2008).

With increasing research the role inflammation plays in type II diabetes mellitus as well as the progression of diabetic kidney disease has come to light causing researchers to target hallmarks of inflammation as possible treatments. Tumor necrosis factor- $\alpha$  is one such inflammatory biomarker that has been extensively studied in regards to the progression of DKD. TNF- $\alpha$  not only causes vast cellular damage accounting for the characteristic histopathological changes of this disease but also contributes to the metabolic and hemodynamic perturbations observed in patients with diabetes mellitus (Elseweidy et al., 2013). Multiple studies have also shown a positive correlation of urinary TNF- $\alpha$  concentration and urinary protein excretion (Moriwaki et al., 2007; Navarro-González et al., 2015; Navarro et al., 2003). With such an extensive role in disease pathogenesis it is no wonder why TNF- $\alpha$  has been the target of many investigational treatments for DKD.

Pentoxifylline has been extensively studied over the course of many years as a potential therapy for diabetic kidney disease. Data from multiple studies show that addition of PTX to a previously established antiproteinuric regimen (ACEI or ARB) not only decreases proteinuria even further but also provides greater renal protection (Ghorbani et al., 2012; Navarro-González et al., 2015; Navarro & Mora, 2005). PTX is able to exert such effects due to its inhibitory action on TNF- $\alpha$  at the level of transcription (Navarro-González et al., 2015). In decreasing the concentration of TNF- $\alpha$ , PTX prolongs the deleterious effects of this cytokine on the kidney thereby maintaining greater renal function for a longer period of time and delaying time to end stage renal disease.

Pentoxifylline has shown great promise in studies conducted in type II diabetes patients with subsequent diabetic nephropathy; however, these studies are greatly limited. Many of these studies come from the same research group who do not always utilize the classic double blind, randomized, placebo controlled clinical study protocol. By not controlling all parameters of the study as well as knowing which patients are receiving experimental treatment introduces the possibility of investigator bias. Also it is important for this work to not only be reproducible by the same research group but by multiple investigators. These studies also often only looked at a small patient population, this decreases the power of these studies and can also lead to type I error in statistical analysis. On the other hand, since these studies have produced positive results the next step should be to establish a protocol for administration of Pentoxifylline in patients with type II diabetes and diabetic nephropathy, which would incorporate a large number of

patients and run in a double blind, randomized placebo controlled fashion to increase the power of the statistics and minimize potential bias. Long term, large scale trials would also produce safety and tolerability data of pentoxifylline in human subjects which would reveal to investigators and industry sponsors if this is a drug that warrants further investigation.

Infliximab and FR167653 are also potential therapeutic options for the treatment of DKD due to their inhibitory effect on TNF- $\alpha$  (Moriwaki et al., 2007). These drugs have not been introduced into the human population but they have been studied in animal models of DKD due to type II diabetes. Both infliximab and FR167653 were shown to decrease urinary concentrations of TNF- $\alpha$  as well as restore urinary protein concentrations to non-diabetic control levels. Further animal studies are warranted for both infliximab and FR167653 to reproduce the results of this study as well as to obtain safety information for these drugs. Infliximab is currently used in humans to treat autoimmune diseases therefore it has a known safety and tolerability profile which may allow for a quicker transition from animal to human subjects (Moriwaki et al., 2007).

An additional TNF- $\alpha$  inhibitor has also been studied in regards to diabetic kidney disease. Chrysin is a member of the flavonoid family and is known for its anti-inflammatory properties with little known adverse effect. Although chrysin has not been studied in humans, data from animal studies have shown promising results. Not only did streptozotocin induced diabetic rats treated with chrysin have improved renal function compared to untreated diabetic rats, their kidneys showed no evidence of pathology due to DKD. This led researchers to conclude that chrysin could not only delay disease

progression, it prevented the disease from taking root (Ahad et al., 2014). With such promising results animal studies should be repeated to reproduce the data as well as to determine its safety and tolerability. If these results were to be reproduced the next step would be to investigate the safety and efficacy of chrysin in the treatment of diabetic nephropathy in patients with type II diabetes. It is important for a drug like chrysin to be investigated in humans due to its potential to prevent DKD. This would have an astronomical effect on the way diabetic patients are currently treated and potentially prevent numerous patients from reaching end stage renal disease and the need for renal transplantation.

Transforming growth factor- $\beta$  is another inflammatory cytokine that plays a central role in the inflammatory contribution to DKD progression and has been targeted by therapeutic agents to treat this disease. Many of the pathological mechanisms activated by hyperglycemia lead to enhanced production of TGF- $\beta$  including AGE-RAGE complex binding and the protein kinase C pathway. Activation of the renin angiotensin system also contributes to the production of TGF- $\beta$ . The enhanced production of TGF- $\beta$  results in glomerular cell hypertrophy as well as increased collagen synthesis leading to fibrosis and glomerulosclerosis (Medscape, 2014).

Pirfenidone is a known anti-fibrotic, anti-inflammatory agent that is currently used to treat idiopathic pulmonary fibrosis and has been investigated as a potential treatment for DKD ( Scleroderma Research Foundation, n.d.). Animal models have shown pirfenidone may inhibit TNF- $\alpha$  in addition to TGF- $\beta$  thereby acting in a multifaceted way to combat disease progression (Mathew et al., 2011). Prifenidone has

been studied in diabetic patients; however those involved were a mix of patients with type I and type II diabetes. Sharma et al. showed that subjects taking Pirfenidone at 1200 mg a day had better renal outcomes compared to subjects taking placebo as well as subjects taking Pirfenidone at 2400 mg a day. The primary endpoint of this study was change in eGFR after one year of treatment with either placebo or one of two doses of Pirfenidone. At the end of the twelve-month treatment period the subjects taking 1200 mg of Pirfenidone experienced a mean increase in eGFR of 3.3 ml/min per 1.73m<sup>2</sup> whereas subjects in both the placebo group and 2400 mg of Pirfenidone daily group did not experience any significant changes in eGFR. These results suggest Pirfenidone as a viable therapeutic agent for DKD. However, renal biopsies were not performed in this study, therefore the improvement in eGFR cannot be definitively attributed to the anti-fibrotic properties of Pirfenidone (Sharma et al., 2011). The etiology of type I and type II diabetes is different. Therefore to determine the therapeutic benefit of pirfenidone for DKD due to type II diabetes a study would need to be conducted utilizing only a type II diabetic population.

The increased blood glucose concentration associated with diabetes mellitus leads to the formation of advanced glycation end products that directly and indirectly contribute to the progression of diabetic kidney disease. On their own AGEs contribute to glomerular hypertrophy and sclerosis thereby damaging the filtering unit of the kidney and promoting proteinuria. AGEs also increase the expression of pro-inflammatory cytokines and growth factors thus enhancing their deleterious effects on the kidney

(Alicic & Tuttle, 2014). For their multifactorial contribution to the progression of DKD, AGEs serve as an excellent target for therapeutic intervention.

Pyridoxamine is a vitamin B derivative that is hypothesized to prevent formation of AGEs thus delaying the progression of DKD as well as preserving renal function. Data show that pyridoxamine is only effective in patients with greater baseline renal function thus the only patients that would benefit from potential treatment are those who have their DKD discovered early in the disease process (Lewis et al., 2012). The manufacturing of a novel therapeutic drug is an expensive and timely process. With that in mind the focus for DKD treatment should remain on therapeutic options that serve a broader group of patients. Diabetic kidney disease is on the rise and unfortunately will not always be caught early therefore it is important that potential agents for this disease be able to work across a broad spectrum of renal function in order to benefit the greatest number of patients.

In addition to being harmful to renal cells on their own, inflammatory cytokines take on another role in DKD progression in their ability to activate the transcription factor NF- $\kappa$ B. NF- $\kappa$ B, once activated, initiates the transcription of more inflammatory cytokines thereby enhancing inflammation and tissue destruction. Two natural derivatives of plants, L6H21 and B06 have been shown to inhibit enhanced inflammatory cytokine production in *in vitro* studies by inhibiting NF- $\kappa$ B (Ahad et al., 2014; Fang et al., 2015). When B06 was introduced into an animal study, investigators observed no histopathological changes in rats treated with B06 (Pan et al., 2013). L6H21 was studied in animal models of type I diabetes and yielded positive results. Diabetic induced rats that were treated with L6H21

expressed decreased concentrations of inflammatory cytokines and did not display the histopathological changes associated with DKD (Fang et al., 2015). Both of these compounds have not been extensively studied for the treatment of DKD nor have they been introduced to humans for this purpose but they both show great promise. The next step would be to continue animal studies in subjects induced with type II diabetes in attempts to reproduce these results as well as begin to ascertain a safety and tolerability profile.

The chronic inflammation associated with type II diabetes is not isolated to one area of the body such as the kidneys but rather is the result of the activation of the immune system in its entirety. Mycophenolate mofetil is an immunosuppressant that is currently used to treat autoimmune diseases and has been studied in animal models for the treatment of DKD. Data from Utimura et al. 2003 revealed MMF has antiproteinuric properties however it is with great caution that this drug should be used for treating patients with DKD. As an immunosuppressant, this treatment option would not only suppress immune activation in the kidney but in the entire body leaving patients susceptible to illness. Mycophenolate mofetil was also observed to inhibit glomerular macrophage infiltration which investigators determined to be an early step in disease pathogenesis. This would imply that the therapeutic effects of MMF are most beneficial to those who are in the early stages of disease progression. It is unknown whether MMF can reverse the disease and therefore MMF may not be effective in patients with decreased renal function. Further investigation would need to be done in animal models to confirm the action of MMF in regards to treating DKD and determine if the risk of

utilizing an immunosuppressant for the treatment of DKD is outweighed by the potential benefit (Utamura et al. 2003).

## CONCLUSIONS

Diabetic nephropathy is a complex and devastating disease that is difficult to definitively diagnose in patients. This is the result of varying definitions as to what exactly is diabetic nephropathy. It is widely accepted to use three defining characteristics for diagnosis: chronic albuminuria, defined as greater than or equal to 300 mg of albumin in the urine per day, decreased GFR and increased blood pressure (Medscape, 2014). These markers are not enough however as they have each their own limitations that may result in delayed diagnosis and treatment. In order to be able to treat these patients there needs to be a defined standard that physicians can utilize to diagnose DKD. This would also aid in early intervention thereby improving renal outcomes for patients and delaying as well as possibly preventing progression to end stage renal disease and the need for renal replacement therapy.

There is a great need for additional treatment to the current standard of care in order to prevent the progression of disease and avoid total kidney failure. Not only would this improve quality of life but would also save millions of dollars in health care spending. The studies presented all have their own limitations including: small patient population or no human population at all, lack of placebo and lack of investigator blinding to study treatment. These studies lack power and data. In order for these drugs to be viewed as serious candidates for the treatment of DKD there need to be larger trials that can produce not only positive, reproducible results but also safety and tolerability data in human subjects.

It is also important to note the drugs being studied are interfering with the immune system, a system that is ubiquitous throughout the body and suppressing it and its components can have serious side effects. These drugs may leave patients susceptible to serious illness that is why long-term data need to be established to determine if the benefits of these drugs outweigh the risk.

Urinary concentrations and renal cortical mRNA concentrations of TNF- $\alpha$  are some of the earliest clinical signs of a patient that has DKD and are extremely important in regards to patients progressing to end stage renal disease (Navarro & Mora-Fernández, 2006). Rise in TNF- $\alpha$  concentrations often precede microalbuminuria, the first clinical sign of DKD. Patients with type II diabetes and microalbuminuria are at a 42% greater risk of developing overt nephropathy and DKD compared to those who are normoalbuminuric (Bruno et al., 2003). The importance of TNF- $\alpha$  to disease progression is evident, thereby making it the best inflammatory biomarker to target in order to combat DKD. Routine testing of urinary TNF- $\alpha$  concentration in addition to the current battery of tests that patients with diabetes must undergo would increase the chances of early disease detection allowing for early intervention.

As early detection is critical to treatment and prevention of disease progression the investigational products that fall under the category of TNF- $\alpha$  inhibitors are those that should be further pursued in the treatment of DKD. In comparison to other potential therapeutic interventions, PTX has been more extensively studied in patients with DKD due to type II diabetes. Although each study has its respective limitations, PTX has consistently shown to delay the progression of DKD as well as provide greater renal

protection compared to the current standard of care for DKD (Ghorbani et al., 2012; Navarro-González et al., 2015; Navarro & Mora, 2005). PTX can also be used in addition to ACEI and ARB therapy which is beneficial to diabetic nephropathy patients as this therapeutic regimen would not only delay disease progression from an inflammatory standpoint but also help to control the hemodynamic perturbations associated with this disease. At this point in time efforts to combat DKD should be focused on research regarding treatment with PTX to determine long-term effects of PTX in subjects with DKD.

Among the other TNF- $\alpha$  inhibitors chrysin stands out the most for its hypothesized ability to prevent diabetic kidney disease. Chrysin was administered immediately upon induction of diabetes in the presented study (Ahad et al., 2014). Those rats that received chrysin treatment did not show any clinical manifestations of DKD nor were there histopathological changes observed in these rats. This study implies that chrysin is a compound that could be given upon diagnosis of type II diabetes and would prevent nephropathy from taking place. This is drastically different from PTX that is administered to subjects with a previously established diagnosis of DKD. At this point in time animal studies with chrysin need to be repeated to provide a supportive foundation for investment into this drug as a potential therapeutic for diabetic nephropathy. With continued positive results from pre-clinical studies arises the possibility of moving chrysin into clinical trials with the end goal of becoming FDA approved for the treatment of DKD.

The target for therapeutic intervention for diabetic kidney disease has been identified. It is now the time for extensive time and effort to be invested into TNF- $\alpha$  inhibitors to produce a novel therapeutic agent to combat this globally devastating disease. The ideas and foundation are there, the extensive patient population is present; it is now up to industry and sponsoring health agencies to invest research and financial support in these drugs which have the potential to greatly impact modern medicine.

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## VITA

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1990

#### Technical Skills

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- Proficient in Microsoft Word, Excel and PowerPoint
- Proficient in Spanish

#### Work Experience

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- **Clinical Research Assistant**
  - July 2012 – August 2013; June 2014 to present
  - **Rhode Island Hospital** - Providence, Rhode Island
  - Responsible for assisting the clinical research coordinator with seeing patients, running labs and data entry.
- **EMT Basic**
  - November 2011 – January 2013
  - **New England Ambulance** – Johnston RI
  - Responsible for transporting patients to and from hospitals and providing necessary medical interventions based on patient assessment and knowledge of the situation.
- **General/Organic Chemistry Lab Teaching Assistant**
  - September 2009 to May 2011
  - **Providence College** – Providence RI
  - Responsible for setting up the experiment, running and cleaning various pieces of equipment and assisting the students as needed.
- **Dietary Aide/Resident Program Assistant**
  - April 2007 to present
  - **Sakonnet Bay Manor** – Tiverton, Rhode Island
  - Responsible for serving, clearing and cleaning the dining room. After a year gained a position of greater responsibility and leadership within the dietary staff as an opener and closer. In 2011 I switched to the activities department where my new job entailed working directly with the residents to improve their quality of life.

#### Education and Training

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- **Boston University School of Medicine, 2015**
  - Boston, Massachusetts, United States

- Degree: Masters of Science
- Major: Medical Science
- **Providence College, 2012**
  - Providence, Rhode Island, United States
  - Degree: Bachelor of Science
  - Major: Biology
  - Alpha Epsilon Delta pre-medical national honor society member
- **American Safety Programs, 2010**
  - Providence, Rhode Island, United States
  - Emergency Medical Technician - Basic
  - Licensed EMT-Basic in the state of Rhode Island.
  - Nationally certified EMT-Basic.
  - CPR Certified

### **Clinical Research Experience**

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- Role: Research Assistant/Coordinator
  - SAVOR: Saxagliptin Assessment of Vascular Outcomes Recorded in Patients with Diabetes Mellitus. A Multicenter, Randomized Double Blind Placebo Controlled Phase IV Trial to Evaluate the Effect of Saxagliptin on the Incidence of Cardiovascular Death, Myocardial Infarction or Ischemic Stroke in Patients with Type 2 Diabetes
    - TIMI Study Group
    - 07/2012 – 08/2013
  - A Randomized Double-Masked, Place Controlled, Multicenter Phase 2 Study to Evaluate the Safety and Renal Efficacy of LY2382770 in Patients with Type 2 Diabetes.
    - Eli Lilly
    - 07/2012 – 08/2013, 06/2014 – 08/2014
  - A Phase III Multicenter, Two-phase, Multi-dose, Prospective, Randomized, Double-blind, Placebo-Controlled Study to Investigate the Safety and Efficacy of ZS (Microporous, Fractionated, Protonated Zirconium Silicate) and Oral Sorbent, in Subjects with Mild to Moderate Hyperkalemia
    - ZS Pharma
    - 11/2012 – 08/2013
  - Randomized Double Blind Parallel-Group MultiCenter Study to Evaluate the Efficacy and Safety of HX575 Epoetin Alfa vs. US Licensed Epoetin Alfa (Epogen/Procrit) in the treatment of Anemia Associated with Chronic Kidney Disease
    - Sandoz
    - 06/2013 – 08/2013, 06/2014 – 01/2015
  - An exploratory Phase 2, randomized, double-blind, placebo-controlled, parallel design study to evaluate the safety, tolerability, and pharmacodynamics of AZD1722 in CDK patients with type 2 diabetes

