THE RENOPROTECTIVE EFFECT
OF FOODS HIGH IN OMEGA-3 FATTY ACIDS
IN A RAT MODEL OF TYPE 1 DIABETES

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By

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The renoprotective effect of foods high in omega-3 fatty acids
in a rat model of type 1 diabetes

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ABSTRACT

**Background:** Dietary omega-3 polyunsaturated fatty acids (n-3 PUFAs) have demonstrated anti-inflammatory actions that are beneficial in several diseases, but their potential benefit and mechanism of action in the treatment of diabetic nephropathy is not yet established.

**Methods:** Type 1 diabetes was induced in Sprague-Dawley rats using a single injection of streptozotocin (STZ), and animals were divided into five groups; non-diabetic, diabetic eating unsupplemented chow, diabetic eating n-3 PUFA rich canola or walnut diets, and diabetic eating n-6 PUFA rich corn oil diet. For all supplemental diets, added fat contributed 40% of calories, and animals were fed *ad libitum*. In the first study (“prevention” study) supplementation started 2 weeks before induction of diabetes, while in the second study (“delayed-treatment” study) supplementation started 7 weeks after induction of diabetes. The duration of the studies were 30 weeks of diabetes (prevention study), or 45 weeks (delayed-treatment study). Various measures of kidney damage were assessed including albuminuria, and glomerular and tubular damage, and inflammatory markers including renal TGF-β, IL-6 and MCP-1.
**Results:** In both studies, the n-3 PUFA and n-6 PUFA rich diets prevented the increases in urine albumin excretion (UAE) and blood pressure, and the development of glomerulosclerosis, and tubulointerstitial fibrosis characteristic of diabetic kidney disease. In most cases, the inflammatory markers (cytokines TGF-β, MCP-1, and IL-6) observed in kidneys of the diabetic rats fed normal chow were reduced with both n-3 and n-6 PUFA rich diet. The lack of inflammation in kidneys from rats fed n-3 PUFA rich diets prevented the increase in collagen I and IV, and a loss of nephrin and nestin typically associated with diabetic renal disease.

**Conclusion:** Dietary n-3 and n-6 PUFA rich foods appear to be beneficial in both the prevention and treatment of diabetic renal complications through suppression of pro-inflammatory cytokines. Further studies are needed to determine the amount of n-3 or n-6 PUFA needed to obtain these results in humans.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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</thead>
<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid (20:4n-6)</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>ACEI</td>
<td>angiotensin converting enzyme inhibitor</td>
</tr>
<tr>
<td>ADA</td>
<td>American Diabetes Association</td>
</tr>
<tr>
<td>AGE</td>
<td>advanced glycation end-products</td>
</tr>
<tr>
<td>AHA</td>
<td>American Heart Association</td>
</tr>
<tr>
<td>ALA</td>
<td>a-linolenic acid (18:3n-3)</td>
</tr>
<tr>
<td>Ang-II</td>
<td>angiotensin-II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ARB</td>
<td>angiotensin receptor blocker</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CHD</td>
<td>coronary heart disease</td>
</tr>
<tr>
<td>CRD</td>
<td>chronic renal disease</td>
</tr>
<tr>
<td>D</td>
<td>diabetic</td>
</tr>
<tr>
<td>D+C/W</td>
<td>diabetic with canola or walnut supplemented diet</td>
</tr>
<tr>
<td>D+canola</td>
<td>diabetic with canola supplemented diet</td>
</tr>
<tr>
<td>D+corn</td>
<td>diabetic with corn oil supplemented diet</td>
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<tr>
<td>D+walnuts</td>
<td>diabetic with walnut supplemented diet</td>
</tr>
<tr>
<td>DAB</td>
<td>3’-3’-diaminobezidin tetrahydrochloride hydrate</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglyceride</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid (22:6n-3)</td>
</tr>
<tr>
<td>DN</td>
<td>diabetic nephropathy</td>
</tr>
<tr>
<td>DPA</td>
<td>docosapentaenoic acid (22:5n-3)</td>
</tr>
<tr>
<td>DRI</td>
<td>daily recommended intake</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid (20:5n-3)</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
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<tr>
<td>T2D</td>
<td>type 2 diabetes</td>
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<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TIFI</td>
<td>tubulointerstitial fibrotic index</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>UAE</td>
<td>urinary albumin excretion</td>
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INTRODUCTION

Rational for the study

Diabetic nephropathy, a form of chronic progressive kidney disease, is the leading cause of chronic renal disease (CRD), end-stage renal disease (ESRD) and the need for kidney dialysis and transplantation in the Western World. The best currently available treatments for diabetic nephropathy are glucose lowering and anti-hypertensive drugs which slow the progression of renal disease, but do not completely halt or reverse existing disease.

Previous investigators have reported that omega-3 polyunsaturated fatty acids (n-3 PUFA) have anti-inflammatory effects, lower blood pressure, and are beneficial in diseases including cardiovascular disease, and IgA nephropathy. Epidemiological studies suggest that n-3 PUFA slow the decline in renal function, e.g. decline in creatinine clearance in healthy older people, and increase in albuminuria in older type 1 and type 2 diabetics. While the facts suggest that dietary n-3 PUFAs should be useful in the treatment and prevention of diabetic nephropathy, this is still not established; clinical studies show no consistent benefit in either type 1 or type 2 diabetic patients suggesting that further studies are needed to determine the actual effect of n-3 PUFAs in diabetic kidney disease.

The objective of this work is to examine the benefits of a diet rich in n-3 PUFA on diabetic kidney disease and provide insights into the mechanisms by which dietary
supplementation with n-3 PUFAs might exert renoprotective effects in diabetic kidney disease. *The overall hypothesis is that n-3 PUFAs are renoprotective, preventing or attenuating the decline of renal disease in an experimental model of type 1 diabetes.* Dietary n-3 PUFAs are thought to act through a number of mechanisms, including attenuation of hyperglycemia, inflammation, and hypertension. This work evaluates the renoprotective ability of dietary n-3 PUFAs, and highlights some of the mechanistic pathways through which this protection might be conferred. These studies show that dietary n-3 PUFAs are beneficial in the prevention and treatment of diabetic renal disease.
BACKGROUND

I. Diabetic Nephropathy

Diabetic nephropathy (DN) is the leading cause end-stage renal disease (ESRD) and the need for kidney dialysis and transplantation in the Western World.\textsuperscript{1} DN is a progressive microvascular end-organ complication that affects approximately 20-40\% of patients with type 1 and type 2 diabetes.\textsuperscript{12} The modifiable risk factors for DN include glycemic control, blood pressure, dyslipidemia, diet and smoking. Unmodifiable risk factors include male sex, duration of diabetes and familial, genetic and ethnic factors. DN is defined by both functional and structural criteria, with the structural criteria being predominantly used in experimental studies, while the functional criteria are generally used in clinical practice.

A. Clinical Features of Diabetic Nephropathy

Diabetic nephropathy is defined by macroalbuminuria (> 300 mg/day) in the presence of long standing hyperglycemia. Clinically, diabetic nephropathy is characterized by progressive increase in albuminuria (from microalbuminuria, through macroalbuminuria to ESRD), progressive decline in renal function, and hypertension. These clinical elements will be discussed below.
a) **Albuminuria**

Microalbuminuria, defined as urinary albumin excretion of 150-300 mg/day, is an early indicator of incipient diabetic nephropathy. Increased glomerular permeability causes plasma proteins, principally albumin, to enter the ultrafiltrate. While some of this filtered protein is reabsorbed by the proximal tubules, any filtered protein remaining will appear in the urine. Albumin is the most common plasma protein, and the appearance of albumin in the urine is commonly used as an indicator of glomerular damage. Macroalbuminuria is defined as urinary albumin excretion >300 mg/day, and is associated with overt nephropathy. Increasing albuminuria is a clear indicator of progressive renal damage, and is also a strong predictor of non-renal morbidity and mortality, for instance it is the best single predictor of cardiovascular disease progression.\(^\text{13}\)

The classical theory of albuminuria, as described above, involves damage to the glomerular membrane and loss of negative charge from the filtration slit resulting in increased filtration of albumin. However, a more recent theory posits that the filtered load of albumin is high even in the healthy kidney, and that the filtered albumin is reabsorbed by the proximal epithelia.\(^\text{14}\) In this modern theory, damage to the glomerular basement membrane and loss of negative charge from the filtration slit plays little or no role in albuminuria\(^\text{15}\), but rather damage to the tubules causes increased
albuminuria. In either case, albuminuria is an indicator of kidney damage, and a requirement for the diagnosis of diabetic nephropathy.

b) Altered Glomerular Filtration Rate

Glomerular hyperfiltration, i.e. an increase in glomerular filtration rate (GFR), is often seen early in diabetic nephropathy possibly due to increased glomerular capillary pressure and/or leaky glomerular capillaries, as well as impaired tubuloglomerular feedback. As the glomerular damage progresses, the thickened capillary basement membrane ultimately decreases the rate of diffusion and results in a decrease in GFR. Additionally, a loss of total number of nephrons contributes to decrease in GFR. A decrease in GFR is the diagnostic criteria for the determination of the severity of kidney damage. Additionally, reduced GFR is a predictor of non-renal disease, for instance cardiac events.

c) Hypertension

Hypertension is one of the clinical hallmarks of diabetic nephropathy, as well as a risk factor for developing DN. Hypertension in the absence of diabetes is capable of causing kidney damage. The most important intervention for slowing the progression of diabetic nephropathy is anti-hypertensive treatment, even in normotensives diabetics.
B. Morphological Features of Diabetic Nephropathy

Numerous morphological changes are seen in biopsy or autopsy of kidneys with diabetic kidney disease. Primary changes are the presence of glomerulosclerosis and tubulointerstitial fibrosis, hallmarks of diabetic nephropathy. Glomerulosclerosis is characterized by thickening of the capillary basement membrane, increase in mesangial extracellular matrix (ECM), mesangial cell proliferation, glomerular capillary dilatation, and occasionally glomerular nodules (Figure 1). Tubulointerstitial fibrosis is damage to the renal tubules, and includes tubular atrophy, tubular dilatation, and increased deposition of ECM. Additionally, inflammation and the presence of inflammatory cells are found in both the glomerulus and tubules. Hyperplastic arteriolosclerosis of the afferent arterioles, seen in numerous kidney diseases, is also seen in DN.32
Figure 1. Morphological features of glomerulosclerosis.

- mesangial cell proliferation & increased mesangial extracellular matrix
- tubular dilatation & atrophy
- glomerular capillary dilatation
- glomerular nodule
- thickening of basement membrane
a) Glomerular Basement Membrane Thickening

In diabetic nephropathy, the basement membranes of the glomerular capillaries are thickened (Figure 1), by concentric layers of hyaline material composed primarily of collagen IV. Collagen IV is secreted by epithelial cells and is the main component of the basal laminae, along with glycoproteins (e.g. laminin) and proteoglycans (e.g. heparan sulfate). Increased deposition of collagen IV is found in the thickened glomerular capillaries and contributes to tubulointerstitial fibrosis. It is well correlated with renal damage.

b) Mesangial Expansion

In addition to the glomerular basement thickening, expansion of the mesangium is commonly seen in DN (Figure 1). This expansion is caused by both an increase in mesangial cells and an increase in ECM secretion by mesangial cells. The increase in mesangial expansion is strongly correlated with clinical features of DN, including albuminuria and decreased GFR.

c) Microaneurysm (glomerular capillary dilatation)

An increase in glomerular capillary pressure causes dilatation of the glomerular capillaries. This increase in capillary diameter is commonly seen in glomeruli subject
to DN (Figure 1), and represents a loss of surface area. Additionally capillary dilatation causes a loss of structural integrity as the dilated capillaries are subjected to greater wall tension. The loss of surface area and structural integrity contributes to both the increase in albuminuria and the decrease in GFR.

d) Inflammation

Chronic inflammation contributes to many chronic diseases\textsuperscript{41-43}, including cardiovascular disease\textsuperscript{44}, and is commonly seen diabetic kidney disease\textsuperscript{45-48}. Chronic inflammation is characterized by infiltration of inflammatory cells, tissue destruction, and tissue repair including fibrosis. The inflammatory cells are the mononuclear cells of the immune system, including macrophages, lymphocytes, and plasma cells. These inflammatory cells are largely responsible for the tissue destruction, and macrophages in particular direct the tissue repair and fibrosis\textsuperscript{33}.

e) Kimmelstiel-Wilson Nodules

In 1936 Paul Kimmelstiel and Clifford Wilson identified nodules in the kidneys of diabetics who were admitted to hospital and subsequently died from diabetic complications\textsuperscript{49} (Figure 2). These nodules, since named Kimmelstiel-Wilson nodules, are commonly found in human kidneys subjected to DN. Kimmelstiel-Wilson nodules are composed of mesangial matrix proteins subject to non-enzymatic glycosylation\textsuperscript{50}. 
and are very rarely found outside the context of diabetes. Mesangiolysis, the degeneration of mesangial cells and dissolution of mesangial matrix, leads to glomerular microaneurysms with accumulation and concentric compression of lysed mesangial matrix. While mesangiolysis is common to many kidney diseases, in the presence of chronic hyperglycemia it leads to advanced diabetic glomerulosclerosis and Kimmelstiel-Wilson nodules. Kimmelstiel-Wilson nodules generally do not develop in STZ-induced diabetic kidney disease in mice or rats; however, Kimmelstiel-Wilson-like nodules have been reported in animal models of diabetic nephropathy involving eNOS knock-out animals. This suggests that endothelial dysfunction and decreased NO production is essential for Kimmelstiel-Wilson nodule formation.
Figure 2. Kimmelstiel-Wilson nodules.

A) from Kimmelstiel & Wilson\cite{49}, reprinted from Am J Pathol 1935 12:83-98.7 with permission from the American Society for Investigative Pathology. B) public domain photo from CDC.
Figure 3. Morphological features of tubulointerstitial fibrosis.
f) Tubulointerstitial Fibrosis

In addition to the glomerular damage described above, DN is also associated with tubulointerstitial fibrosis (Figure 3). In fact, this tubulointerstitial fibrosis is better correlated with the extent of kidney damage than glomerulosclerosis. This observation gives credence to the modern theory of albuminuria, in which tubular damage is responsible for albuminuria rather than glomerular damage. The damaged renal tubules show dilatation and atrophy of epithelial cells. The basement membranes are often thickened, and frank fibrosis is often seen in the interstitium, along with an increase in fibroblasts. This damage severely reduces the tubular function; in particular proximal tubule reabsorption is reduced contributing to decreased GFR.

g) Hyperplastic Arteriolosclerosis

Hyperplastic arteriolosclerosis, which affects the afferent arteriole of the kidney, is a form of arteriosclerosis often seen in kidneys subject to high blood pressure. In hyperplastic arteriolosclerosis, the arteriole walls are thickened by increased layers of smooth muscle, while the lumen is narrowed. This compensation protects the glomerulus by providing a drop in pressure in the afferent arteriole sufficient to prevent intraglomerular hypertension. Unfortunately, the stiffened arteriolar wall and narrowed lumen can also cause both glomerular ischemia and a loss of autoregulation.
C. Pathophysiology of Diabetic Nephropathy

Hyperglycemia is considered the key causative factor in etiology of diabetic nephropathy (DN) (Figure 5). In the presence of high ambient glucose, mesangial cells, endothelial cells, podocytes, and tubular epithelial cells all exhibit maladaptive responses, including excessive ECM, angiotensin II (Ang-II) and TGF-β secretion, oxidative stress, as well as apoptosis. Additionally, hyperglycemia increases the rate of production of advanced glycation end-products (AGEs). AGEs cause dysfunction via interference with normal protein function, as well as activate inflammatory processes via receptors for AGE (RAGEs). Finally, chronic high glucose can cause glucotoxicity through increased production of reactive oxygen species (ROS), which can cause cell dysfunction or increased cellular apoptosis. Given this wide range of powerfully damaging mechanisms, the central role of hyperglycemia in the development of DN is without question. As additional evidence for the central role of hyperglycemia, good glycemic control prevents the development of DN.

Of course as noted above, most diabetics do not develop DN, so hyperglycemia alone is not sufficient to cause DN. Other contributing factors are hypertension and hyperlipidemia. Albuminuria, which is component of DN, is also an independent risk factor for the progression of DN.
a) Hyperglycemia

Hyperglycemia acts through a variety of mechanisms to cause long term diabetic kidney complications\(^6^8\) (Figure 4). Hyperglycemia increases the rate of non-enzymatic glycosylation and the resulting advanced glycation end-products (AGEs) prevent the normal functioning of proteins, decrease the turnover rate for proteins, and trigger inflammatory responses. Hyperglycemia also results in accelerated function of normal enzymatic pathways that use glucose as a substrate. This results in deleterious buildup of products, including diacylglyceride (DAG), sorbitol, and hexosamines. Overproduction of ATP via oxidative phosphorylation results in increased production of reactive oxygen species (ROS). ROS, AGEs, and DAG increase Protein kinase C (PKC) activation. For instance PKC activation is increased in mesangial cells exposed to chronic high ambient glucose. These factors stimulate inflammation and local TGF-β production, and result in fibrosis. Indeed, mesangial cells convert to extracellular matrix-overproducing myofibroblasts in response to high ambient glucose and TGF-β.\(^3^9\) Additionally, hyperglycemia stimulates local Ang-II secretion in mesangial cells, podocytes, and tubular epithelial cells.\(^6^9,^7^0\)
Figure 4. Mechanisms of hyperglycemic damage.
b) Angiotensin II (Ang-II)

The systemic role of Ang-II in maintaining blood pressure is well known. However, the intrarenal function of Ang-II possibly plays a more important role in the progression of DN.\textsuperscript{71} As mentioned above, hyperglycemia stimulates the local synthesis of Ang-II and enhanced intrarenal Ang-II is involved in the deterioration of renal function.\textsuperscript{72} Ang-II stimulates production of TGF-β, and adversely alters renal hemodynamics, principally by causing efferent arterial constriction. Blockade of the Ang-II production by use of angiotensin converting enzymes inhibitors (ACEI) or Ang-II receptor blockers (ARBs) significantly slows the progression of DN, even in the absence of hypertension. Additionally, urinary angiotensinogen, a marker of the intrarenal RAS and Ang-II, is increased in hypertensive patients, and is decreased by RAS blockade.\textsuperscript{73}

c) Hemodynamic Factors

Increased systemic blood pressure, loss of local autoregulation, and constriction of the efferent arteriole can each independently cause an increase in glomerular capillary pressure (GCP). If multiple problems with pressure regulation occur simultaneously, the increase in GCP is even greater. Increased GCP produces a mesangial stress and provokes the secretion of TGF-β and accompanying secretion of ECM by mesangial cells.\textsuperscript{74} This response by mesangial cells is exaggerated by high
glucose and Ang-II. Additionally, increased GCP is thought to produce increased GFR and microalbuminuria seen early in the development of DN.

d) Transforming Growth Factor-β (TGF-β)

TGF-β secretion is stimulated by mesangial stretch, hyperglycemia, inflammation, and Ang-II. TGF-β has a wide array of biological activities, including controlling cell proliferation and apoptosis, stimulating ECM accumulation and angiogenesis, and regulating effects of other cytokines. The role of TGF-β in response to glomerular damage is to stimulate the synthesis and inhibit the degradation of ECM. This increase in ECM is an appropriate response to acute injury; however, with chronic hyperglycemia, the response becomes maladaptive and results in fibrosis. The increase in TGF-β and its accompanying fibrosis are central to both glomerulosclerosis and tubulointerstitial fibrosis seen in DN.

e) Albuminuria

As mentioned above, albuminuria is a component of DN and is also an independent risk factor for progression of DN. Protein that escapes into the ultrafiltrate can be reabsorbed by the epithelial cells of the proximal tubules. However, in the classical theory of albuminuria, as the glomerular membrane becomes leaky and the filtered load of protein increases, the reabsorbed protein becomes harmful. When
exposed to albumin, epithelial cells of the proximal tubule produce matrix proteins and TGF-β, and potentially undergo an epithelial mesenchymal transformation. Consequently, an increase in albuminuria is an indicator of renal decline, while reduction in albuminuria is an appropriate therapeutic goal in management of kidney disease. It is also interesting to note that once damage becomes sufficiently severe, glycemic control is unable to prevent further decline. For instance, albuminuria can cause additional damage without hyperglycemia.

f) Inflammation

As mentioned above, hyperglycemia can cause inflammation by increased ROS production and through AGE-RAGE interactions. Chronic hyperglycemia leads to chronic inflammation and the recruitment of monocytic immune cells, e.g. macrophages and lymphocytes. CD68-positive cells, a marker of activated macrophages, accumulate in chronically inflamed tissues. Transforming growth factor-β (TGF-β) is secreted by activated macrophages and stimulates collagen expression by neighboring cells. CD68-positive activated macrophage infiltration and TGF-β expression are markers of damaged diabetic kidneys. Interleukin 6 (IL-6) is a pro-inflammatory cytokine that is secreted locally by macrophages and T-cells (as well as circulating systemically). IL-6 has also been shown to be increased in the damaged kidney.
g) Other Vascular Problems

Intrarenal Ang-II is elevated in hyperglycemia, leading to increased glomerular capillary pressure (GCP). Additionally, hypertension is a component of DN, and an independent risk factor for disease progression. Increased myogenic tone in the afferent arteriole protects the glomerulus from hypertension, but chronic hypertension leads to hyperplastic arteriolosclerosis. With arteriolosclerosis comes a loss of autoregulation and increased risk of ischemia.

h) Podocytopathy

Although the traditional view of diabetic glomerulosclerosis and its resulting albuminuria has focused on the role of mesangial cells, recent work suggests that damage to the podocyte is actually more important to development of albuminuria. Hyperglycemia can cause apoptosis in podocytes as well as loss of podocyte foot process proteins. For instance, nephrin, a structural protein of the podocytes foot process, is decreased in DN, contributing to the loss of filtration barrier integrity. The loss of nephrin appears to result from hyperglycemia. Additionally, nestin, an intermediate filament, is present in podocytes from healthy kidneys, but is significantly reduced in podocytes from kidneys subject to IgA nephropathy, membranous nephropathy, and focal segmental glomerular sclerosis with albuminuria. This suggests that loss of nestin is associated with albuminuria.
Figure 5. Diabetic Nephropathy.
D. Treatment of Diabetic Nephropathy

If left untreated, diabetic nephropathy is a disease with a high rate of mortality and morbidity, but with proper detection and treatment, DN can be avoided or delayed. Microalbuminuria is an early indicator of incipient diabetic kidney damage and carries a high risk of cardiovascular disease. Aggressive management by inhibiting the pathological pathways described above can greatly improve the outlook for these patients. This section will describe the treatments currently available for DN.

a) Intensive Glycemic Control

Given that hyperglycemia is the keystone that is responsible for initiating the deleterious events of diabetes, it is only reasonable that intensive glycemic control delays the onset and slows the progression of diabetic nephropathy as well as other diabetic complications, e.g. retinopathy and neuropathy. Intensive glycemic control is most effective when started early. When near normal glycemia is maintained in normoalbuminuric patients, prevention of nephropathy is possible. However, when intensive glycemic control is started after incipient or overt nephropathy, results are discouraging. Once sufficient albuminuria is present, pathological processes are underway that no longer require hyperglycemia, and the disease process becomes self-sustaining.
b) Renin-angiotensin system (RAS) Blockade

**ACE inhibitors (ACEIs)**, which include captopril (Capoten®), enalapril (Vasotec®), ramipril (Altace®), as well as many others, inhibit angiotensin converting enzyme (ACE). **Angiotensin receptor blockers (ARBs)**, which include candesartan (Cilexetil®), losartan (Cozaar®), valsartan (Diovan®), are antagonist to angiotensin II (Ang-II) receptors. Both ACEIs and ARBs block the renin-angiotensin system (RAS).

Elevated systemic Ang-II can cause hypertension. While hypertension is an independent risk factor for progression to DN, and RAS blockade is a cornerstone in the treatment of hypertension, the benefits of ACEIs and ARBs in treating DN are independent of their antihypertensive effects. Blockade of RAS by use of angiotensin-converting enzyme inhibitors (ACEIs) and angiotensin receptor blockers (ARBs) has proven useful in slowing the progression of DN. RAS mediates kidney damage through a wide range of mechanisms and the actions of RAS blockade provide benefits through pleiotropic mechanisms including, antihypertensive, hemodynamic, and antiproteinuric effects.

ACEIs and ARBs are the first line anti-hypertensive drugs for diabetic patients. RAS blockade has been shown to decrease albuminuria and limit progression of DN, but it has not been shown to reverse DN. Therefore, RAS blockade is most useful early in DN; however, the benefit of RAS blockade in non-proteinuric patients is unclear.
As an added bonus, ACEIs and ARBs reduce the risk of developing diabetes, while both thiazide diuretics and β-blockers accelerate the appearance of new-onset T2D in patients with hypertension. Ang-II decreases mitochondrial content of skeletal muscle, and blockade of RAS helps to preserve insulin sensitivity by preserving skeletal muscle mitochondria. Additionally, recent studies have identified that ACEIs and ARBs also attenuate AGE accumulation. It is postulated that ACEIs and ARBs reduce the accumulation of AGE in diabetes partly by increasing the production and secretion of soluble RAGE into plasma.

**c) Other Anti-hypertensives**

Hypertension is both a component of DN and an independent risk factor for DN progression. Reduction of blood pressure is beneficial in preventing progression of DN. As mentioned above, ACEIs and ARBs exhibit benefits in DN that are independent of their anti-hypertension effects, and are therefore the first line anti-hypertensive medicines for DN. Additionally, other anti-hypertensive medications including thiazide, loop, and K⁺ sparing diuretics, as well as aldosterone blockers, calcium channel blockers, and β-blockers. Achieving the goal blood pressure is more important than the agents used, and often more than one anti-hypertensive agent is needed. For patients with albuminuria < 1 g/d, the goal blood pressure is 130/80 mmHg, while for patients with albuminuria > 1 g/d the goal pressure is 125/75 mmHg.
d) Diet Modification: Low Sodium, Low Protein

Dietary sodium reduction has been shown to reduce blood pressure, and prevent the onset of hypertension.\textsuperscript{109,110} Additionally, maintaining high dietary potassium consumption is beneficial for preventing or reducing hypertension,\textsuperscript{111} but high potassium diets are contraindicated in patients with renal failure.

Some studies suggest that dietary protein restriction can prevent or reduce the progression of diabetic and non-diabetic kidney diseases\textsuperscript{112-114}, while other studies do not find a benefit for low protein diets.\textsuperscript{115,116} Consequently, at this time there is insufficient evidence to recommend low protein diets for the prevention or treatment of DN.\textsuperscript{117}

E. Animal Models of Diabetic Nephropathy

Numerous animal models of diabetes have been used in research.\textsuperscript{118,119} The streptozotocin (STZ) model is a commonly used model of type-1 diabetes. Some models of type-2 diabetes include db/db, ob/ob, high-fat/high-fructose, non-obese diabetic, agouti.\textsuperscript{120}

STZ is cytotoxic and is avidly transported into cells via the glucose transporter type 2 (GLUT2). Consequently, STZ is selectively toxic to cells that express GLUT2. STZ toxicity to pancreatic β-cells causes β-cell failure\textsuperscript{121} and hypoinsulinemia with hyperglycemia. Unfortunately, other cells expressing GLUT2 are also affected,
including intestinal mucosal cells, epithelia cells of the renal proximal tubules, and growth hormone releasing hormone (GHRH) secreting cells. The effect of STZ on GHRH production is the most problematic of these. The loss of GHRH production leads to a loss of growth hormone (GH) production. In contrast with human diabetes, GH secretion in STZ-diabetic rats is decreased compared to non-diabetics.\textsuperscript{122, 123} Additionally, supplemental GH in the STZ model of DN exacerbates the kidney damage\textsuperscript{122}, possibly through the action of growth hormone on podocytes\textsuperscript{124}, suggesting that damage to GH secretion alters the natural history of kidney disease progression in the animal compared to the human diabetic.

The STZ model of diabetes and DN has numerous advantages.\textsuperscript{120} It is fast; diabetes occurs within 24 hours after injection. It is reliable and reproducible; almost every rat injected becomes diabetic with almost no surviving β-cells, or insulin production, giving a near uniform level of diabetes. It is widely used and well established; results are comparable between labs and across time. Additionally, STZ can be used to induce diabetes in any strain of rat or mouse, and therefore animals with genetic predispositions to kidney disease can be used. For instance, spontaneously hypertensive rats (SHR) can be used to produce diabetic rats with a more aggressive progression of kidney disease.\textsuperscript{125} Given these advantages, STZ-induced diabetes is a very popular model of diabetic complications including diabetic kidney disease.

While STZ can be used on any strain of either rats or mice, the current research was done using the Sprague-Dawley rat. Kidney disease resulting from STZ-induced
diabetes in the Sprague-Dawley rat is reminiscent of diabetic nephropathy, see Table 1. The biggest shortcomings of this model are the lack of reliable kidney failure, e.g. decreasing GFR, and the lack of reliable hypertension. Albuminuria consistently develops at 4-8 weeks and continues to increase with longer periods of diabetes. Morphological changes in the STZ model also closely resemble the human kidney subject to DN, including glomerulosclerosis, and tubulointerstitial fibrosis (Figure 1).

In humans, despite the different etiology, both type 1 and type 2 diabetes produce nephropathy that follows the same pattern, and the resulting kidney disease looks very similar. Thus, while the STZ diabetic rat is a model of type 1 diabetes, information that is learned from it should be applicable to diabetic nephropathy in both type 1 and type 2 diabetics.
The streptozotocin (STZ) model of diabetic nephropathy (DN)

<table>
<thead>
<tr>
<th>Clinical Features</th>
<th>STZ Rat model</th>
</tr>
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<tbody>
<tr>
<td>albuminuria</td>
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<tr>
<td>↑GFR (early)</td>
<td>Variable</td>
</tr>
<tr>
<td>↓GFR (late)</td>
<td>Variable</td>
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<td>Hypertension</td>
<td>Variable</td>
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<table>
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<tr>
<th>Morphological Features</th>
<th>STZ Animal model</th>
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<tr>
<td>GBM thickening</td>
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</tr>
<tr>
<td>Mesangial expansion</td>
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<td>Microaneurysms</td>
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</tr>
<tr>
<td>Inflammation</td>
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</tr>
<tr>
<td>Kimmelstiel-Wilson Nodules</td>
<td>No</td>
</tr>
<tr>
<td>Tubulointerstitial fibrosis</td>
<td>Yes</td>
</tr>
<tr>
<td>Arteriolosclerosis</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1. The STZ animal model of diabetic nephropathy.
II. Dietary Polyunsaturated Fatty Acids

Polyunsaturated fatty acids (PUFAs) are a major class of fatty acids. The other classes of fatty acids are monounsaturated and saturated fatty acids. A carbon in a fatty acid is said to be unsaturated if it has a double bond. A saturated fatty acid has no double bonds, while a monounsaturated fatty acid has one double bond. PUFAs have more than one double bond.

PUFAs are essential for life. Double bonds introduce kinks into the fatty acid structure, and this less orderly structure causes the melting point to be lower. Because of the less orderly structure and lower melting point, PUFAs play an important role in providing the proper fluidity for cell membranes. PUFAs form a necessary component of the phospholipid bilayer of cell plasma membranes and without PUFAs construction of properly functioning cell membranes would not be possible. While PUFAs are essential for life, they cannot be synthesized by humans, or any other higher animal, and therefore must be consumed in the diet. Dietary sources and interconversion of PUFAs will be discussed in section II.A.

In addition to their role in maintaining proper membrane fluidity, PUFAs play a number of other roles in maintaining health. For instance, certain PUFAs serve as substrates for enzymatic conversion to eicosanoids, short lived hormones with 20 carbons. Additionally, both PUFAs and eicosanoids bind to certain nuclear receptors, e.g. PPAR-γ, and alter gene regulation and protein expression. Finally, PUFAs serve as
a source of dietary energy and can displace calories from other, potentially harmful, sources.

Given the roles that PUFAs play in the normal physiology and biochemistry of the body, it is not surprising that PUFAs play a significant role in good health. Dietary PUFAs have been shown to provide a myriad of health benefits. These benefits will be discussed in detail in section II.B.

A. Introduction to Polyunsaturated Fatty Acids

Because they are essential for life and must be consumed in the diet, PUFAs are commonly referred to as essential fatty acids (EFA). The essential PUFAs encompass omega-3 (n-3) and omega-6 (n-6) PUFAs. Omega-9 PUFAs are also biologically active, but can be synthesized de novo and are not considered essential fatty acids. Because they can be synthesized, elevated n-9 PUFAs are a potential sign of EFA deficiency.

The designation of n-3 or n-6 is determined by the position of the carbon double bond closest to the end (omega) of the PUFA. PUFAs can be lengthened or shortened and carbon double bonds can be added or removed. However, n-3 and n-6 PUFAs cannot be inter-converted. All n-3 PUFAs in the body must have originated as dietary n-3 PUFAs; likewise all n-6 PUFAs must have originated as dietary n-6 PUFAs.
a) Dietary Sources of Polyunsaturated Fatty Acids

Short chain (18 carbons) PUFAs are generally found in plants while long chain (20+ carbons) PUFAs are found almost exclusively in animal products. Linoleic acid (LA), 18:2n-6\(^1\), and \(\alpha\)-linolenic acid (ALA), 18:3n-3, are the most common dietary short chain PUFAs, while arachidonic acid (AA), 20:4n-6, eicosapentaenoic acid (EPA), 20:5n-3, and docosahexaenoic acid (DHA), 22:6n-3, are the most common dietary long chain fatty acids.

LA is the most common PUFA in the modern Western diet. Rich sources of ALA, e.g. chia and flaxseed (Table 2), are consumed in much lower quantities than high LA sources (e.g. corn and wheat products). Canola and walnuts are good sources of short chain n-3 PUFA, while cold water fish, e.g. salmon, are good sources of long chain n-3 (Table 2).

b) Conversion of Polyunsaturated Fatty Acids

Dietary fatty acids are efficiently absorbed and enter cells via fatty acid transporters.\(^{127-129}\) The short chain PUFAs, linoleic acid (LA) and \(\alpha\)-linolenic acid (ALA), can be converted to AA and EPA, respectively, by the actions of \(\Delta6\)-desaturase,

\(^1\) The notation 18:2n-6 indicates that the fatty acid contains 18 carbons with 2 double bonds starting on the 6\(^{th}\) carbon from the end.
elongase, and Δ5-desaturase. Although over 30% of ingested ALA is consumed by β-oxidation,\textsuperscript{130} ALA is readily converted to EPA.\textsuperscript{131-133}

Because LA and ALA use the same enzymes for conversion of short chain PUFA to long chain PUFA, high LA intakes decrease plasma phospholipid EPA.\textsuperscript{134} On the other hand, an increase in dietary ALA will result in a decrease in conversion of LA to AA.\textsuperscript{135} Therefore, an increase in dietary ALA will not only increase EPA but will also decrease AA concentration in the plasma membrane and should provide anti-inflammatory benefits which will attenuate the deleterious effects of chronic inflammatory diseases.

While ALA is readily converted to EPA and also docosapentaenoic acid (DPA), 22:5\textit{n}-3, it is not efficiently converted to DHA, at least in males and post-menopausal women.\textsuperscript{132,136} Supplementation with EPA also does not result in elevated DHA. In fact supplementation with either ALA or EPA can result in a decrease in DHA content of RBC membranes.\textsuperscript{133} Conversion from DPA to DHA is not straight-forward, and conversion to DHA is very inefficient in men. Conversion of DPA to DHA is much higher in young women than men.\textsuperscript{137} Additionally, DHA in plasma fatty acids is increased in post-menopausal women on HRT\textsuperscript{138}, suggesting that estrogen increases conversion to DHA. DHA is required for proper fetal brain development and DHA production increases during pregnancy, especially during the 3\textsuperscript{rd} trimester, to support rapid fetal brain growth.\textsuperscript{139} This beneficial increase in DHA does not seem to extend to lactating women; dietary flaxseed oil, rich in ALA, increased the breast-milk, plasma,
and erythrocyte contents of ALA, EPA, and DPA but had no effect on breast-milk, plasma, or erythrocyte DHA contents.\textsuperscript{140}

Although DHA is poorly synthesized from ALA or EPA, DHA is the most common n-3 PUFA in most tissues and is thought to be essential for proper development of the brain and retina.\textsuperscript{128} However, vegetarians and vegans have much lower concentrations of DHA\textsuperscript{141, 142} without any obvious clinical manifestations.\textsuperscript{143} Increased DPA content compensates for low DHA content, and brings into question the essentiality of DHA.\textsuperscript{144} Not withstanding the evidence from vegans, the current research suggests that DHA is essential, and nutritional groups are advocating a daily recommended intake (DRI) for both EPA and DHA.\textsuperscript{145} Further confounding this situation, a recent study in rats, using more precise measurement of conversion of ALA to DHA found that the DHA synthesis rate exceeded the daily rat brain DHA consumption rate, suggesting that synthesis from ALA could maintain brain DHA homeostasis in the absence of dietary DHA, at least in rats.\textsuperscript{146}

The consumption of ALA-rich foods or supplements is sufficient to elevate cell membrane EPA and DPA content, which shows the effectiveness of ALA conversion to long chain n-3 PUFAs. The amount of ALA required is easily achieved with modest dietary modification.\textsuperscript{131, 140, 147, 148} However, supplementation with ALA or EPA does not generally increase availability of DHA.
### Table 2. Sources of dietary PUFAs.

Values are milligrams per 100 grams of food or oil.
B. Benefits of n-3 Polyunsaturated Fatty Acids

Attesting to their pleiotropic effects, n-3 PUFAs appear protective in many diseases. Supplementation with n-3 PUFAs shows protective benefit in a wide range of diseases, including heart disease, rheumatoid arthritis, and psychiatric diseases.\textsuperscript{149} For instance, cognitive decline and overall mortality in elderly patients is inversely associated with cellular EPA concentrations.\textsuperscript{150,151} While the benefit of n-3 PUFAs in some clinical applications requires further investigation, it is clear that habitual dietary consumption of n-3 PUFAs bring certain health benefits.\textsuperscript{151} This section will examine some of these health benefits in more detail.

a) Cardiovascular Benefits

Consumption of fish and fish oil, rich in n-3 PUFAs decrease the incidence and severity of cardiovascular disease\textsuperscript{5} and plasma n-3 PUFA levels are inversely correlated with the risk of sudden death from heart attacks.\textsuperscript{152} For secondary prevention of cardiovascular disease, fish oil has been shown to reduce overall and cardiovascular mortality, myocardial infarction, and sudden cardiac death. Fish oil also lowers triglycerides.\textsuperscript{153} Compelling evidence for the cardiovascular benefit comes from numerous trials testing n-3 PUFA supplements containing ALA, EPA, and/or DHA. These trials showed reductions in cardiovascular events of 19\% to 45\%.\textsuperscript{154}
The evidence for a beneficial effect of n-3 PUFAs is compelling, and as early as 1996, the American Heart Association (AHA) recommended n-3 PUFA consumption for prevention of cardiovascular disease. Dietary or supplemental n-3 PUFAs show benefits in a range of cardiovascular related clinical signs, including improved lipid profile, favorable effect on coronary blood flow and coronary heart disease (CHD), decreased arterial stiffness, decreased sudden cardiac death, decreased arrhythmias, and reduced infarct size. Electrical characteristics of the heart are also beneficially affected by consumption of n-3 PUFAs.

Given the broad and compelling nature of the cardiovascular benefits of n-3 PUFAs, in 2003, the AHA strengthened their endorsement of n-3 PUFAs, see Table 4. Despite the clinical evidence of benefit, the precise mechanisms of protection are unclear. The data suggest several routes by which n-3 PUFAs provide cardiovascular protection, see Table 3.

In addition their dietary or supplemental benefits, other uses for n-3 PUFAs are under investigation. Pericardial injection of n-3 PUFAs might protect the myocardium from ischemic damage, prevent arrhythmias and reduce infarct size, if delivered early enough. Additionally, low n-3 PUFA content of RBCs has been proposed as cardiovascular risk factor. This proposed omega-3 index is the percentage of EPA + DHA of total fatty acids in RBCs. A high omega-3 index, i.e. EPA + DPA > 8% of total fatty acids in RBCs, was associated with 90% less risk for sudden cardiac death, compared to a low omega-3 index, i.e. < 4%. The omega-3 index is also associated
with depression.\textsuperscript{165} Unfortunately, subsequent research could not confirm this finding in cardiovascular disease.\textsuperscript{166} Clearly, more research is needed in all aspects of n-3 PUFA benefit in cardiovascular disease, but for now, eat more n-3 PUFAs.
Proposed cardiovascular benefits of n-3 PUFAs; research suggests they can:

- Decrease risk for arrhythmias, which can lead to sudden cardiac death.
- Decrease risk for thrombosis, which can lead to heart attack and stroke.
- Decrease triglyceride and remnant lipoprotein levels.
- Decrease rate of growth of the atherosclerotic plaque.
- Improve endothelial function.
- Reduce adhesion molecule expression
- Reduce platelet-derived growth factor
- Promote nitric oxide-induced endothelial relaxation
- (Slightly) lower blood pressure.
- Reduce inflammatory responses.

Table 3. Proposed cardiovascular benefits of n-3 PUFAs.

Adapted from Connor.⁴,¹⁶²
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>Patients without documented CHD:</td>
<td>Eat a variety of (preferably oily) fish at least twice a week. Include oils and foods rich in (\alpha)-linolenic acid (flaxseed, canola, and soybean oils; flaxseed and walnuts).</td>
</tr>
<tr>
<td>Patients with documented CHD:</td>
<td>Consume (\approx 1) g of EPA+DHA per day, preferably from oily fish. EPA+DHA supplements could be considered in consultation with the physician.</td>
</tr>
<tr>
<td>Patients with hypertriglyceridemia:</td>
<td>Two to four grams of EPA+DHA per day provided as capsules under a physician’s care.</td>
</tr>
</tbody>
</table>

[^167]: Adapted from AHA recommendations.
b) Immune System Benefits

n-3 PUFAs are powerful immunomodulators. As described above, n-3 PUFAs are generally anti-inflammatory and reduce chronic inflammation.\textsuperscript{3, 168-170} They typically reduce production of inflammatory eicosanoids, e.g. \textit{LTB}_4, \textit{LTC}_4-LTE\textsubscript{4}, and \textit{PGD}_2,\textsuperscript{171, 172} as well as reduce other inflammatory cytokines, e.g. IL-1\textbeta, and TNF-\alpha,\textsuperscript{171} they alter membrane fluidity and lipid raft composition, thereby affecting intercellular signaling,\textsuperscript{173} and they activate PPARs.\textsuperscript{174, 175} Consequently, both animal and human studies have shown benefit in various autoimmune and hypersensitivity diseases, including rheumatoid arthritis\textsuperscript{153, 172, 176} and asthma.\textsuperscript{171, 176}

The actions of n-3 PUFAs affect both innate and adaptive immunity.\textsuperscript{172, 173, 177} Although n-3 PUFAs are generally anti-inflammatory and reduce chronic inflammation, they do not reduce the effectiveness of the immune system to fight off pathogens. Animal studies demonstrate actual benefit in resistance to infectious disease in animal fed high n-3 PUFA diets.\textsuperscript{178, 179} Additionally, n-3 PUFAs have shown some potential benefit in reducing cancer, either by reduced inflammation or increased immunosurveillance. For instance, actinic keratosis, a pre-malignant cell transformation, was reduced in people consuming the most n-3 PUFAs compared to the lowest.\textsuperscript{180}
c) Renal Benefits

The kidneys appear to respond to the effects of dietary n-3 PUFAs, however the mechanisms of action are unclear. IgA nephropathy is a renal disease with chronic inflammation that is improved by treatment with n-3 PUFAs, usually in the form of fish oil.\textsuperscript{6, 7, 181-186} Despite increasing knowledge of the beneficial effects of fish oils, little is known for certain about the mechanisms of action of n-3 PUFAs. Both EPA and DHA down-regulate inflammation via a PPAR-\(\gamma\)–dependent pathway in human kidney (HK-2) cells, suggesting that PPAR-\(\gamma\) activation as a possible mechanism for the protective effect of fish oil in IgA nephropathy.\textsuperscript{185}

Plasma n-3 PUFA levels are inversely correlated with decline of renal function in healthy older patients.\textsuperscript{8} Similar to the reduced decline in cognitive function, n-3 PUFAs play a protective role in the kidney. One possibility is that n-3 PUFAs reduce inflammation that would otherwise damage renal cells. Clinical studies suggest that long-term treatment with n-3 PUFAs improve renal function and lower risk of renal disease.\textsuperscript{187}

Similar to many of the diseases discussed in this section, diabetic nephropathy is a disease with a chronic inflammatory component. Studies suggest that PUFA might be protective in animal models of diabetic nephropathy\textsuperscript{188, 189}, but human studies have been inconsistent and discouraging.\textsuperscript{190-192} The current study will investigate the use of dietary n-3 PUFA in reducing diabetic kidney complications, and determine the effects
of dietary n-3 PUFAs on pro-inflammatory and matrix accumulating pathways of kidney damage.
III. Hypothesis and General Aims

A. Hypothesis

The object of this work is to determine whether foods high in dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) confer protective effects on the diabetic kidney, preventing and possibly reversing renal damage. The overall hypothesis is that n-3 PUFAs are renoprotective, preventing or attenuating the decline of numerous indicators of renal damage. Dietary n-3 PUFAs are thought to act through a number of mechanisms, including attenuation of hyperglycemia, attenuation of inflammatory cytokine increase, and attenuation of blood pressure increase. This work evaluates the renoprotective ability of dietary n-3 PUFAs, and highlights some of the mechanistic pathways through which this protection might be conferred.

B. General Aims

In the STZ-induced diabetic rat, the hypotheses are that dietary n-3 PUFA:

1A. Prevent or reverse the decline in renal dysfunction associated with diabetic renal disease; this was determined by measuring urine albumin excretion, and creatinine clearance.
1B. Prevent the renal structural changes associated with diabetic renal disease; this was determined by measuring glomerulosclerosis, tubulointerstitial fibrosis, and collagen I, collagen IV, and nephrin protein expression.

1C. Attenuate renal inflammation as determined by preventing an increase in renal transforming growth factor beta (TGF-β), interleukin 6 (IL-6), and monocyte chemotactic protein type 1 (MCP-1) protein expression.

1D. Prevent or attenuate an increase in blood pressure typically seen with diabetic kidney disease; this was determined by measuring systolic blood pressure.

1E. Attenuate the increase in blood glucose typically seen with diabetes; this was determined by measuring blood glucose and HbA1c concentrations.
MATERIALS AND METHODS

I. General Experimental Procedures

The streptozotocin (STZ) induced model of diabetes was used in the current study. STZ destroys the pancreatic β-cells and produces a disease that is similar to type 1 diabetes (T1D). The STZ diabetic rat develops diabetic complications quickly and reliably (see Table 1), and is the most widely used model of diabetic renal disease currently in use.

A. Experimental Animals

Prevention Study: To examine the effects of n-3 PUFA diets on prevention of diabetic renal disease, male Sprague-Dawley (S-D) rats (8 wk, Harlan, Madison, WI) were randomly divided into 5 groups and fed experimental diets: non-diabetic (ND), diabetic (D), diabetic fed a canola rich diet (D+canola), diabetic fed a corn oil rich diet (D+corn), and diabetic fed a walnut rich diet (D+walnuts). Diet supplementation started at the time of arrival into the animal facility. After 2 weeks, diabetes was induced as described below. Animals were fed ad libitum. The study was carried out for 30 weeks.

Delayed-treatment Study: To examine whether n-3 PUFA diets affect diabetic renal disease in animals with established diabetes, diabetes was induced in male S-D
rats as indicated below, and after 7 weeks of diabetes, rats were randomly placed on different diets: normal chow (D); canola (D+canola); walnut (D+walnuts); or corn oil (D+corn). Non-diabetic controls were maintained on normal chow. All analysis was performed as indicated below. The study was carried out for 45 weeks.

B. Induction of Diabetes

After an overnight fast, diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ, Sigma, St. Louis, MO) at a dose of 55 mg/kg body weight in 0.1M citrate buffer (pH 4.5). Non-diabetic rats were given a single intraperitoneal injection of citrate buffer only. 24h following induction of diabetes, blood glucose was measured using the FreeStyle glucometer, and only rats with blood glucose > 200 mg/dl were included in the study. Throughout the study, the rats were injected twice weekly with glargine insulin (Lantus, Aventis Pharmaceuticals Inc., Kansas City, MO) to prevent excessive weight loss and mortality. Glargine is a long-acting insulin that affects blood glucose levels for 18-24 hours after administration.
C. Maintenance of Diabetic Animals

All rats were fed a low salt rat diet (Harlan 7034, 0.10% Na) either without supplementation (ND and D), or supplemented with canola (D+canola, Mazola, ACH Food Companies, Memphis, TN), corn oil (D+corn, Mazola, ACH Food Companies), or walnuts (D+walnuts, Mt Lassen Farms, CA). Canola and corn oil were supplemented at 200 g/kg, e.g. 200 g of oil plus 800 g of powdered rat chow. Walnuts were supplemented at 300 g/kg, e.g. 300 g of walnuts plus 700 g of powdered rat chow. Added canola, corn oil, or walnuts provided approximately 40% of total calories from added fat. Canola and corn oil contain 8.8 Cal/g from fat, while walnuts contain 5.8 Cal/g from fat, and therefore more walnuts were required to add the same amount of fat. The base diet contained 25%, 60%, and 15% of calories from protein, carbohydrates, and fat, respectively; while the canola and corn oil supplemented diet contained approximately 15%, 35%, and 50% of calories from protein, carbohydrates, and fat, respectively and the walnut diet contained approximately 17%, 35%, and 48% of calories from protein, carbohydrates, and fat, respectively. Both canola and walnuts are a good source of n-3 PUFA, and no significant differences in physiologic or pathophysiologic findings were observed between these groups; therefore, the canola and walnut animals were combined into an omega-3 rich group, D+C/W, and all results will be given for the combined group. The corn oil group served as a macronutrient control for the canola and walnut groups. Food and water were given *ad libitum*. Given
the anticipated increase in food intake in the diabetic rats, low sodium chow was chosen in order to prevent kidney damage due to sodium load, while still providing a diet with sufficient sodium\textsuperscript{193}.

D. Metabolic Studies and Measurement of Blood Glucose and HbA1c

Body weight (BW) and blood glucose (FreeStyle, TheraSense, Alameda, CA) were measured weekly. The animals were placed in metabolic cages for 24h for measurement of food and water consumption, urine output and urine collection for subsequent measurement of urine albumin content. For the prevention study, rats were placed in the metabolic cages at weeks 4, 10, 14, 18, 23, and 28, and for the delayed-treatment study, rats were placed in the metabolic cages at weeks 6, 18, 35, 43. HbA1c was measured at week 44 using A1cNow (Metrika/Bayer HealthCare – Diabetes Care, Sunnyvale, CA) following the manufacturers instructions.

E. Measurement of Blood Pressure

Systolic blood pressure was measured by non-invasive tail cuff sphygmomanometry\textsuperscript{194} (Narco Bio-Systems, Houston, TX) one week prior to the termination of the study. Telemetric blood pressure monitoring systems (telemetry) are the most accurate method for measuring normal blood pressure in an intact conscious rat. However, the most commonly used method for blood pressure measurement in
long term STZ-induced diabetes studies is tail-cuff sphygmomanometry. Tail-cuff sphygmomanometry, which was used in both the prevention and delayed-treatment studies, has been found to support telemetry findings. Femoral artery catheterization, another method for blood pressure measurement, can only be used in anesthetized animals. Because the anesthesia used for our studies, Nembutal®, alters hemodynamics, blood pressure measurement taken under anesthesia will reflect depth of anesthesia rather than conscious blood pressure, and thus cannot be used as indicators of conscious blood pressure.

F. Collection of Blood and Tissues

At the termination of the study, 30 weeks for the prevention study and 45 weeks for the delayed-treatment study, the animals were weighed, anesthetized with sodium pentobarbitone (40 mg/kg IP) and blood was collected via cardiac puncture. The kidneys were excised, weighed and then either snap frozen in liquid nitrogen for protein analysis or immersion-fixed with HistoChoice (Amresco, Solon, OH) for histological analysis. All experiments were performed according to the guidelines recommended by the National Institutes of Health and approved by the Georgetown University Animal Care and Use Committee.
II. Analysis

The tissue, blood, urine, and other samples collected using the methods described above were analyzed using the following techniques.

A. Urine Albumin Excretion (UAE) and Creatinine Clearance

Urine albumin concentration was measured using the Nephrat II albumin kit (Exocell, Inc., Philadelphia PA), according to the manufacturer’s protocol. The rate of UAE was calculated as the product of urine albumin concentration and the 24h urine output. To estimate the glomerular filtration rate (GFR) of the animals, urine and plasma creatinine concentrations were measured using a kit (BioAssay Systems, Hayward, CA), according to manufacturer’s instructions. Creatinine clearance, as a measure of estimated GFR was calculated as the urinary creatinine excretion ($U_cV$) divided by the plasma creatinine concentration.

B. Glomerulosclerotic Index (GSI)

PAS (Periodic acid Schiff) stained kidney paraffin sections (4 µm) were examined under a light microscope to assess the degree of glomerular damage, defined as mesangial matrix expansion, capillary dilation, and glomerular tuft occlusion. Approximately 60 glomeruli per sample were randomly selected and evaluated at x400 using a semi-quantitative scoring method. Briefly, stained sections from each animal
were assessed using the following grading scale: grade 0, no obvious sclerosis (normal); grade 1, sclerotic area < 25% (minimal sclerosis); grade 2, sclerotic area 25% to 50% (moderate sclerosis); grade 3, sclerotic area 50% to 75% (moderate-severe sclerosis); grade 4, sclerotic area 75% to 100% (severe sclerosis). The glomerulosclerotic index (GSI) was calculated using the weighted average of the evaluated glomeruli, as previously described. All evaluations were performed with the observer masked to the treatment group.

C. Tubulointerstitial Fibrotic Index (TIFI)

Masson’s trichrome-stained kidney paraffin sections (4 μm) were examined under a light microscope to assess the degree of tubulointerstitial fibrosis defined as tubular atrophy or dilatation, presence of inflammatory cells and deposition of extracellular matrix (ECM). Approximately 30 fields (at 400x) per sample were randomly selected and evaluated for degree of tubulointerstitial damage, in both the renal cortex and medulla using a semi-quantitative scoring method similar to GSI scoring: grade 0, no obvious damage; grade 1, affected area < 10%; grade 2, affected area 10% to 25%; grade 3, affected area 25% to 75%; grade 4, affected area 75% to 100%. The tubulointerstitial fibrotic index (TIFI) was calculated using the weighted average of the evaluated fields as previously described. All evaluations were performed with the observer masked to the treatment group.
D. Determination of Protein Expression

Western blotting was used to quantify changes in concentration of specific proteins in kidney tissue from control and diabetic rats. Tissue was homogenized in 80mM Tris buffer (pH 7.3) containing protease inhibitor Cocktail Set III (Calbiochem, EMD Chemicals Inc., San Jose, CA). Proteins were denatured as needed, see Table 5, by adding β-mercaptoethanol (2.5%) and heating at 95°C for 15 minutes. Protein samples were loaded onto 4-15% precast SDS-PAGE gels (Bio-Rad, Hercules, CA) and transferred to a nitrocellulose membrane. The membranes were incubated first with 5% non-fat milk for blocking non-specific reactions and then with primary antibody, see Table 5, at 4°C overnight. The membranes were washed, incubated with anti-mouse IgG (1:10,000; KPL, Gaithersburg, MD), anti-rabbit IgG (1:10,000; KPL, Gaithersburg, MD) or anti-goat IgG (1:10,000; Vector, Burlingame, CA) conjugated to horseradish peroxidase and proteins visualized by enhanced chemiluminescence (KPL; Gaithersburg, MD) (see Table 5). The membrane was stripped and incubated with anti-β-actin (1:3000; Cell Signaling Technology). Because the samples used for collagen I and collagen IV were not heat-denatured, β-actin was not accessible for probing. Therefore for collagen I and collagen IV, to verify equal levels of protein loading, another gel with identical loading was stained with Coomassie blue. The densities of specific bands were quantitated by densitometry using the Scion Image version 4.02
(Scion Corporation, www.scioncorp.com). The densities of specific bands were then normalized to the total amount of protein loaded in each well following densitometric analysis of β-actin or Coomassie blue staining, as appropriate.
Antibodies used in IHC & Western blotting

<table>
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<th>Antibody</th>
<th>Manufacturer</th>
<th>Catalog #</th>
<th>Source</th>
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<th>AR</th>
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<td>Collagen IV</td>
<td>Chemicon</td>
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<td>Abcam</td>
<td>ab7202</td>
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Table 5. Antibodies used in immunohistochemistry and Western blotting.
E. Immunohistochemistry (IHC)

To identify the sites of protein expression, IHC was performed in kidney tissues in parallel to western blotting. Paraffin sections (4 µm) were dewaxed and hydrated. Antigen retrieval (AR) was performed as needed, see Table 5, by heating the slides in citrate buffer (1.8 mM citric acid, 8.2 mM trisodium citrate) in a microwave for 10 minutes, at approximately 90°C. For collagen IV, antigen retrieval was performed by incubating the slides in 1% trypsin with 0.1% albumin for 15 minutes. The slides were then serially incubated with 6% H₂O₂ in methanol for 10 minutes for blocking endogenous peroxidase, avidin and biotin (Vector; Burlingame, CA) for 15 minutes (for blocking endogenous avidin/biotin), and 10% normal goat serum or 0.1% albumin (for antibodies raised in goat) for 30 minutes at room temperature (RT) (for blocking non-specific reactions). Sections were rinsed 3x with phosphate buffered saline (PBS) between each incubation and then incubated with primary antibody, as listed in Table 5 overnight at 4°C. Slides were rinsed with PBS, incubated with biotinylated IgG raised against mouse (Sigma, St. Louis, MO), rabbit (Sigma, St. Louis, MO), or goat (Dakopatts, Glostrup, Denmark), as appropriate, for 1 hour at RT, rinsed with PBS, and incubated with avidin-biotin complex (Vector; Burlingame, CA) for 1 hour at RT. Positive immunoreaction was identified after incubation with 3’-3’-diaminobezidin tetrahydrochloride dehydrate, DAB Chromogen (Dako; Carpenteria, CA) and counterstaining with Mayer’s hematoxylin.
F. Statistical Analysis

Statistical analysis was performed using Prism version 4.00 (GraphPad, San Diego, CA). Unless otherwise stated, data were analyzed using a one-way ANOVA with a post hoc comparison using Tukey’s Multiple Comparison Test. A value of $P < 0.05$ was considered statistically significant. Unless otherwise indicated, all values are presented as mean ± SEM.
Aim 1 – THE PREVENTION STUDY

I. Experimental Protocol

A. Specific Aim

The object of this study was to determine whether foods high in dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) confer protective effects on the diabetic kidney, *preventing* the decline in renal function and the worsening of the renal inflammation and pathology associated with diabetic renal disease, and attenuating the rise in blood pressure that accompanies long-term diabetes. In the STZ-induced diabetic rat, the hypotheses were that dietary n-3 PUFA:

1A. Prevent the decline in renal function associated with diabetic renal disease; this was determined by measuring urine albumin excretion and creatinine clearance.

1B. Prevent the renal structural changes associated with diabetic renal disease; this was determined by measuring glomerulosclerosis, tubulointerstitial fibrosis, collagen I, collagen IV and nephrin protein expression.

1C. Attenuate renal inflammation as determined by preventing an increase in renal levels of interleukin 6 (IL-6), transforming growth factor beta (TGF-β) and monocyte chemotactic protein type 1 (MCP-1) protein expression.

1D. Attenuate the increase in blood pressure characteristic of long-term diabetes
B. Specific Methods

Male Sprague-Dawley rats (8 weeks, Harlan, Madison, WI) were randomly divided into 5 groups:

1. non-diabetic (ND, n=4)
2. diabetic (D, n=5)
3. diabetic fed canola oil-supplemented diet (D+canola, n=5)
4. diabetic fed corn oil-supplemented diet (D+corn, n=5)
5. diabetic fed walnut-supplemented diet (D+walnuts, n=4).

As mentioned in Experimental Animals (section I.A), both canola and walnuts are a good source of n-3 PUFA, and no significant differences in physiological or pathophysiological findings were observed between these groups; therefore, the canola and walnut animals were combined into an omega-3 rich group, D+C/W (n=9), and all results will be given for the combined group.

Originally, all n-3 PUFA data (D+canola and D+walnuts) were analyzed separately against the control (ND), diabetic (D), and corn oil (D+corn) animals. Because the n-3 PUFA data was very consistent over the many parameters tested, the n-3 PUFA groups were combined. While this gave a power advantage over the D+corn group, we also analyzed the separate n-3 PUFA groups to fully examine the potential
differences between n-3 and n-6 PFUA. The 5 group analysis is given in section “FIVE GROUP ANALYSIS” starting on page 120.

Diet was supplemented as described in the methods section above. Diet supplementation started 2 weeks before induction of diabetes and continued for the duration of the study (30 weeks after induction of diabetes).

Diabetes was induced as described and rats were maintained for 30 weeks using 5U of glargine insulin twice per week as described in General Experimental Procedures. Body weight, blood glucose, food consumption, urinary albumin excretion, creatinine clearance, and blood pressure were measured as described.

At 30 weeks, animals were sacrificed and blood and tissue collected as described. Glomerulosclerosis, tubulointerstitial fibrosis, collagen I, collagen IV, nephrin, TGF-β, IL-6, and MCP-1 were measured as described above.

**II. Results**

The results of the prevention study confirmed the renoprotective benefits shown in our preliminary studies. The n-3 PUFA rich diet almost completely prevented the diabetic complications seen in the diabetic animals fed normal rat chow. Surprisingly, n-6 PUFA rich diet had similar effects on diabetic renal disease, suggesting that both are effective in preventing the pathological consequences of diabetes in the kidney. As mentioned above, results for the n-3 PUFA rich diets are presented for the combined
group (D+C/W). In cases where the separate analysis of the n-3 PUFA rich diet groups resulted in any change, the 5 group analysis is show in “FIVE GROUP ANALYSIS” starting on page 120.

A. Basic characteristics of animals at time of sacrifice

After 30 weeks, diabetes (D) was associated with a 5.6-fold increase in blood glucose (see Table 6), while D+C/W and D+corn were associated with 4.0-fold, and 3.9-fold increases, respectively. These differences were consistent between groups throughout the experimental period. The D animals weighed 156 grams less than the ND animals despite a 2.3 fold greater food consumption, while there was a trend toward greater weight with less food consumption in the D+C/W and D+corn groups compared to D (see Table 6). Additionally, there was a 1.6-fold increase in kidney weight and a 2.3-fold increase in kidney weight normalized to body weight among D compared with ND, indicating diabetic renal hypertrophy. This change was significantly attenuated in both D+C/W animals, while only the change in kidney weight was attenuated in D+corn animals.
<table>
<thead>
<tr>
<th>PARAMETER (units)</th>
<th>ND n = 4</th>
<th>D n = 5</th>
<th>D+C/W n = 9</th>
<th>D+corn n = 5</th>
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<tr>
<td>Blood glucose (mg/dl)</td>
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<td>395±23#</td>
<td>280±20 *$</td>
<td>274±22#,$</td>
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<tr>
<td>Body weight (g)</td>
<td>538±13</td>
<td>382±30#</td>
<td>430±17#</td>
<td>430±8#</td>
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<td>Food intake (kcal/day)</td>
<td>55±4</td>
<td>128±9#</td>
<td>100±7#,$</td>
<td>104±7#</td>
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<td>Kidney/body weight (g/100g)</td>
<td>0.50±0.01</td>
<td>1.16±0.08#</td>
<td>0.72±0.06$</td>
<td>0.78±0.05#,$</td>
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<tr>
<td>Kidney weight (g)</td>
<td>2.70±0.04</td>
<td>4.32±0.17#</td>
<td>3.02±0.14$</td>
<td>3.36±0.20$</td>
</tr>
</tbody>
</table>

Table 6. Basic characteristics of animals at the time of sacrifice.
Values are mean ± SEM. One-way ANOVA with Tukey post-test; # P < 0.05 vs. ND, $ P < 0.05 vs. D. Abbreviations: ND – non-diabetic, D – diabetic, D+C/W – diabetic fed either canola or walnut supplemented diets, D+corn – diabetic fed corn oil supplemented diet.
B. Renal parameters

After 28 weeks, there was a 5.5-fold increase in urinary albumin excretion (UAE) in D compared with ND animals, indicating significant renal damage (see Figure 6). This increase in UAE was completely attenuated in the n-3 PUFA group, D+C/W. While the increase in UAE was also attenuated in the corn oil group, UAE among the D+corn was still 3.2-fold higher than observed in the D+C/W group.

The diabetic animals also exhibited a 45 mmHg increase in systolic blood pressure compared with ND (see Figure 7), as measured by tail cuff sphygmomanometry. This increase in systolic blood pressure was completely attenuated in the D+C/W animals, but only partially attenuated in the D+corn animals.

There was no difference in creatinine clearance normalized for body weight between any of the treatment groups (see Figure 8). As mentioned in the background section, GFR does not consistently decline in the STZ rat model of diabetic nephropathy.
Figure 6. 24-hour Urine Albumin Excretion at the time of sacrifice – prevention study.

After 28 weeks of diabetes, UAE was significantly increased in diabetic (D) animals: dietary supplementation with canola/walnuts prevented this increase. UAE was also attenuated in the D+corn animals, although albuminuria was significantly greater in this group than in the D+C/W animals.
Figure 7. Systolic Blood Pressure – prevention study.

Blood pressure was measured by tail-cuff sphygmomanometry. There was a significant increase in blood pressure (BP) in D rats, compared with ND controls. The increase in blood pressure was prevented in the D+C/W animals, while BP in diabetic rats fed corn oil was not different than the diabetic controls.
Figure 8. Creatinine clearance for prevention study.

Creatinine clearance normalized for body weight was not significantly different between any groups.
C. Morphological Parameters

Moderate levels of glomerular and tubulointerstitial fibrosis were evident in the D animals fed normal diet (see Figure 9 A&B, Figure 11 A&B, Figure 13 A&B). The glomerular and tubular damage was completely prevented in the D+C/W animals, but only partially attenuated in the D+corn animals.

The semiquantitative measurement of glomerulosclerosis (GSI) showed a 3.0-fold increase in GSI among D compared with ND (see Figure 9). Representative periodic acid Schiff (PAS) staining indicated glomerulosclerosis among the D animals was associated with mesangial expansion, accumulation of ECM, capillary expansion, and accumulation of inflammatory cells (see Figure 10).

The semiquantitative measurement, tubulointerstitial fibrotic index (TIFI), showed a 1.7-fold increase in TIFI in kidneys from D compared with ND animals in the cortex (see Figure 11A) and a 2.1-fold increase in the inner stripe of the outer medulla (see Figure 13B). Tubulointerstitial fibrosis among the D animals was associated with tubular dilation, accumulation of ECM, atrophy of tubular epithelial cells, accumulation of inflammatory cells, and capillary dilation in both the renal cortex (Figure 9B) and medulla (Figure 11B). Again, these changes were completely absent in the D+C/W animals, while only partially attenuated in the D+corn animals.
Figure 9. Glomerulosclerotic Index (GSI) in control and diabetic rats – prevention study.

GSI, a measure of glomerulosclerosis, was elevated in kidneys from diabetic (D) rats compared to non-diabetic (ND) rats. This glomerular damage was prevented in diabetic rats fed canola or walnuts (D+C/W). Supplementation with corn oil (D+corn) partially attenuated this glomerular damage.
Figure 10. Glomerulosclerotic Index (GSI) in control and diabetic rats – prevention study.

Representative PAS staining indicating glomerular damage in diabetic (D) rats, and normal appearance of glomeruli from non-diabetic (ND) and D+C/W rats. g, glomerulus; p, proximal tubule; d, distal tubule; +, dilated capillaries; *, dilated tubules; green ellipse, extracellular matrix expansion. Original magnification ×400.
Figure 11. Cortical Tubulointerstitial Fibrotic Index (TIFI) in control and diabetic animals – prevention study.

TIFI, a measure of tubulointerstitial fibrosis, was elevated in the kidney cortices from diabetic (D) compared to non-diabetic (ND) rats, but this tubular damage was completely prevented by supplementation with canola or walnuts (D+ C/W). Damage was partially attenuated by corn oil (D+corn).
Figure 12. Cortical Tubulointerstitial Fibrotic Index (TIFI) in control and diabetic animals – prevention study.
Representative Masson’s trichrome staining indicating tubular damage in diabetic (D) rats, and normal appearance of glomeruli from non-diabetic (ND), and D+C/W rats. Kidneys from diabetic rats fed corn oil (D+corn) showed modest damage. g, glomerulus; p, proximal tubule; d, distal tubule; +, dilated capillaries; *, dilated tubules; green ellipse, extracellular matrix expansion. Original magnification ×400.
Figure 13. Medullary (ISOM) Tubulointerstitial Fibrotic Index (TIFI) in control and diabetic animals – prevention study.

TIFI in the inner stripe of the outer medulla (ISOM) was elevated in kidneys from diabetic (D) rats compared to non-diabetic (ND) rats. This tubular damage was prevented in diabetic rats fed canola or walnuts (D+C/W). Supplementation with corn oil (D+corn) partially attenuated this damage.
Figure 14. Medullary (ISOM) Tubulointerstitial Fibrotic Index (TIFI) in control and diabetic animals – prevention study.

Representative Masson’s trichrome staining indicating tubular damage, in particular ECM expansion, in diabetic (D) rats, and normal appearance of tubules from non-diabetic (ND) and D+C/W rats. Supplementation with corn oil (D+corn) partially attenuated this tubular damage. green ellipse, extracellular matrix expansion. Original magnification ×200.
D. Structural Proteins

Consistent with the morphological changes presented above, changes in expression of ECM proteins also indicate moderate damage to the kidneys of the D animals (see Figure 15, Figure 16, and Figure 18). Collagen I protein expression was elevated 2.4-fold in D animals compared with ND (see Figure 15), and the same pattern was observed for collagen IV (1.6-fold increase, see Figure 16). Increased accumulation of collagen I and IV, as observed after immunohistochemical localization, see Figure 16, was consistent with the increase in tubulointerstitial fibrosis shown in Figure 9 and Figure 11. The increase in collagen I was completely attenuated in the D+C/W group, while the D+corn group is significantly greater than the ND. The accumulation of collagen IV was partially attenuated in both D+C/W and D+corn.

Nephrin, component of the podocyte foot process, shows a small but significant 0.82-fold decrease in the D animals compared with the ND animals (Figure 18). Loss of nephrin is in part responsible for increased leakage of protein across the glomerular basement membrane and consequent albuminuria. The current data support this, there is a negative correlation between nephrin and albuminuria (Figure 18). This decrease in nephrin was partially attenuated in both D+C/W and D+corn. Nestin, an intermediate filament found in normal podocytes, was also decreased in D versus ND (Figure 20). This decline in nestin expression was completely attenuated in the D+C/W animals.
Figure 15. Collagen I in kidneys from control and diabetic animals – prevention study.

The increase in collagen I observed in diabetic (D) compared with non-diabetic (ND) rats was prevented in diabetic rats fed canola or walnuts (D+C/W). Collagen I in kidneys from corn oil (D+corn) diet animals was significantly greater than ND levels.
Figure 16. Collagen IV in kidneys from control and diabetic animals – prevention study.

The increase in collagen IV observed in diabetic (D) compared with non-diabetic (ND) rats was partially attenuated in diabetic rats fed canola or walnuts (D+C/W) and corn oil diets (D+corn). D+C/W and D+corn were not different from ND controls.
Figure 17. Collagen IV in kidneys from control and diabetic animals – prevention study.

Representative IHC staining of collagen IV in the cortex. Collagen IV is localized to glomerular and peritubular basement membranes as well as Bowman’s capsule. These are all increased in diabetic (D) compared to non-diabetic (ND) rats. But this increase is not evident in rats fed canola or walnuts (D+C/W) and corn oil (D+corn). g, glomerulus; p, proximal tubule; d, distal tubule; green ellipse, collagen IV expansion. Original magnification ×400.
Nephrin, a component of the podocyte foot processes, was decreased in diabetic (D) compared to non-diabetic (ND) rats. This loss of nephrin was partially attenuated in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.

Figure 18. Nephrin in kidneys from control and diabetic animals – prevention study.
Figure 19. Nephrin vs. Urine Albumin Excretion -- prevention study.

Nephrin protein content was inversely correlated with urine albumin excretion. The loss of nephrin is thought to render the glomerular filtration slits more permeable to albumin and lead to an increase in albumin filtration and urine albumin excretion.
Figure 20. Nestin expression in kidneys from control and diabetic animals – prevention study.

Representative IHC staining of renal nestin. Nestin was localized to podocytes. Nestin staining in diabetic (D) rats is decreased compared to non-diabetic (ND) rats. Expression of nestin in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) is similar to ND and shows no loss of nestin. g, glomerulus; brown stain, nestin. Original magnification ×400.
E. Markers of Tissue Inflammation

In addition to the renal structural damage demonstrated by increased GSI and TIFI, and changes in ECM proteins (collagen I & IV) and podocyte (nephrin and nestin) proteins, markers of tissue inflammation were also consistently elevated in our D versus ND (Figure 21, Figure 23, and Figure 24), supporting previous reports of chronic inflammation in diabetic animals or humans with albuminuria.\textsuperscript{85, 203-205}

After 30 weeks, transforming growth factor beta (TGF-β) was increased 2.3-fold in kidneys from D compared with ND rats (see Figure 21). This increase was partially attenuated in both D+C/W and D+corn.

Interleukin 6 (IL-6), an indicator of inflammation\textsuperscript{206, 207}, was elevated 1.7-fold in kidneys from D compared with ND rats (see Figure 23). This increase was completely attenuated in both the D+C/W and D+corn.

Monocyte chemotactic protein 1 (MCP-1), is an inflammatory protein that recruits immune cells to tissues.\textsuperscript{204, 205, 208, 209} Figure 24 illustrates a 1.6-fold increase in renal MCP-1 in D compared with ND rats. This increase was completely attenuated in both the D+C/W and D+corn groups. Consistent with an increase in MCP-1, CD68-positive cells, a marker of activated macrophages, have been shown to be increased in diabetic kidney damage.\textsuperscript{202}
Figure 21. TGF-β in kidneys from control and diabetic animals – prevention study.

TGF-β was increased in kidneys from diabetic (D) compared with non-diabetic (ND) rats. This increase in TGF-β was partially attenuated in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.
Figure 22. TGF-β in kidneys from control and diabetic animals – prevention study. Representative IHC staining of renal TGF-β, shown as brown, indicating increased TGF-β in diabetic (D) compared with non-diabetic (ND) rats. TGF-β expression was attenuated in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn). g, glomerulus; green ellipse, excessive TGF-β. Original magnification ×400.
Figure 23. IL-6 in kidneys from control and diabetic animals – prevention study. IL-6, a marker of inflammation, was increased in diabetic (D) compared with non-diabetic (ND) rats. This increase was completely prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn).
Figure 24. MCP-1 in kidneys from control and diabetic animals – prevention study.

MCP-1, monocyte chemoattractant protein-1, increased in diabetic (D) compared with non-diabetic (ND) rats. This increase was completely prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.
Figure 25. MCP-1 in kidneys from control and diabetic animals – prevention study.

Representative IHC staining for MCP-1 indicating increased staining in diabetic (D) compared with non-diabetic (ND) and attenuated staining from rats fed canola or walnuts (D+C/W) and corn oil (D+corn). g, glomerulus; green ellipse, elevated MCP-1 stain. Original magnification ×400.
Aim 2 – THE DELAYED-TREATMENT STUDY

In Aim 2, the *delayed-treatment* study, two experimental parameters were varied as compared to Aim 1: first, supplementation with high fat diets was not started until week 7 after induction of diabetes, and second, the study was conducted for 45 weeks of diabetes as compared to 30 weeks.

The results from Aim 1 support the hypotheses that n-3 PUFA rich diets prevent the inflammation and damage associated with diabetic kidney disease in an STZ-induced model of diabetes. Additionally, n-6 PUFA rich diet also prevented diabetic kidney disease. In fact, the complete prevention of diabetic kidney damage in the prevention study strongly suggests that treatment with both n-3 and n-6 PUFA rich diets could be beneficial in treatment of diabetes in human. Unfortunately, treatment of diabetic complications in humans generally does not start until after diabetes has been well established. In our second experiment, treatment with supplemental diets was not started until after establishment of early diabetic kidney disease. In the STZ model of diabetic kidney disease, elevated urinary excretion of albumin is seen by week 6\(^{210}\), with morphological damage present by week 8.\(^{211}\) By week 12, morphological damage is extensive.\(^{212-217}\) In our second experiment, diet supplementation was started 7 weeks after induction of diabetes, after increased urine albumin excretion was observed giving time for the development of early kidney damage, but not extensive damage. Additionally, the second study was conducted over 45 weeks versus 30 weeks, allowing
more time for the development of renal complications. These experimental variations were intended to attempt to recapitulate human diabetic nephropathy. Significant renoprotection from dietary n-3 or n-6 PUFAs under these conditions would strongly support a potential therapeutic role for dietary PUFAs in patients with diabetic renal disease.
III. Experimental Protocol

A. Specific Aim

The object of this study was to determine whether dietary omega-3 polyunsaturated fatty acids (n-3 PUFA) are renoprotective, reversing established albuminuria and attenuating the decline in renal function, inflammation and pathology associated with existing diabetic renal disease, and attenuating the diabetes-induced increase in blood pressure. In the STZ-induced diabetic rat, the hypotheses were that dietary n-3 PUFA:

1A. Reverse the decline in established renal dysfunction associated with diabetic renal disease and prevent further decline; this was determined by measuring urine albumin excretion and creatinine clearance.

1B. Prevent the renal structural changes associated with diabetic renal disease; this was determined by measuring glomerulosclerosis, tubulointerstitial fibrosis, and collagen I, collagen IV and nephrin protein expression.

1C. Attenuate renal inflammation as determined by preventing an increase in renal transforming growth factor beta (TGF-β), interleukin 6 (IL-6) and monocyte chemotactic protein type 1 (MCP-1) protein expression.

1D. Prevent or attenuate an increase in blood pressure typically seen with diabetic kidney disease.
B. Specific Methods

Male Sprague-Dawley rats (10 weeks, Harlan, Madison, WI) were randomly divided into 5 groups:

1. non-diabetic (ND, n=7)
2. diabetic (D, n=5)
3. diabetic fed canola oil-supplemented diet (D+canola, n=4)
4. diabetic fed corn oil-supplemented diet (D+corn, n=5)
5. diabetic fed walnut-supplemented diet (D+walnuts, n=4).

As mentioned in Experimental Animals (section I.A), both canola and walnuts are a good source of n-3 PUFA, and no significant differences in physiological or pathophysiological findings were observed between these groups; therefore, the canola and walnut animals were combined into an omega-3 rich group, D+C/W (n=8), and all results are given for the combined group.

Diet was supplemented as described in the methods section above, and began 7 weeks after induction of diabetes and continued for the duration of the study (45 weeks).
Diabetes was induced as described and rats were maintained for 45 weeks using 2.5U of glargine insulin twice per week as described. The dose for glargine was reduced from 5U twice per week to 2.5U twice per week, after determining that the response for 2.5U was similar to the response for higher doses. Body weight, blood glucose, food consumption, urinary albumin excretion, creatinine clearance, and blood pressure were measured as described. At 45 weeks, animals were sacrificed and blood and tissue collected as described and analysis performed as previously described.
IV. Results

A. Basic animal characteristics at the time of sacrifice

After 45 weeks, diabetes (D) was associated with a 5.0-fold increase in blood glucose (see Table 7), while both D+C/W and D+corn were associated with a 3.5-fold increase. HbA1c, a measure of glycated hemoglobin and an indicator of average blood glucose value, was significantly correlated with blood glucose levels ($r^2 = 0.79$, $p < 0.01$). HbA1c was 2.6-fold higher for D compared with ND, while D+C/W and D+corn both showed a 2.1-fold increase. While the D+C/W and D+corn animals showed decreased blood glucose levels, these animals were still well above the cutoff for diabetes in experimental animals (i.e. > 200 mg/dl).

D animals weighed 178 grams less than the ND animals despite 2.0 fold increase in food consumption. While there was a trend toward greater weight with decreased food consumption in the D+C/W and D+corn groups compared to D (Table 7), this trend did not achieve significance.

Additionally, there was a 1.5-fold increase in kidney weight and a 2.4-fold increase in kidney weight normalized to body weight in D compared with ND, which is most likely due to renal hypertrophy caused by hyperglycemia. This change was partially attenuated in both D+C/W and D+corn (Table 7).
<table>
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<th>Parameter (units)</th>
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<th>D ( n = 5 )</th>
<th>D+C/W ( n = 8 )</th>
<th>D+corn ( n = 5 )</th>
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</thead>
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<tr>
<td>Blood glucose (mg/dl)</td>
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<td>438±42#</td>
<td>305±30#,$</td>
<td>309±9#,$</td>
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<tr>
<td>HbA1c (%)</td>
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<td>12.1±0.6#</td>
<td>9.7±0.6#,$</td>
<td>9.7±0.4#,$</td>
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<td>386±29#</td>
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<td>119±14#</td>
<td>78±6$</td>
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<tr>
<td>Kidney/body weight (g/100g)</td>
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<td>1.21±0.14#</td>
<td>0.94±0.20</td>
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<tr>
<td>Kidney weight (g)</td>
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<td>3.95±0.40#</td>
<td>3.42±0.36</td>
<td>3.16±0.16</td>
</tr>
</tbody>
</table>

Table 7. Basic characteristics of animals at the time of sacrifice.

Values are mean ± SEM. One way ANOVA with Tukey post-test. \# P < 0.05 vs. ND, \$ P < 0.05 vs. D.
B. Renal Parameters

UAE increased significantly over the 45 week period in diabetic (D) rats (see Figure 26). By week 6, diabetes was associated with a 4.3-fold increase in UAE (ND 6.8±1.9; D 29.4±6.0; p < 0.05). All treatments (diets) that were started on week 7 decreased UAE (see Figure 27), suggesting the delayed-treatment of diabetic renal injury.

After 45 weeks of diabetes, D was associated with 7.5-fold increase in UAE compared with ND (see Figure 29). This increase in UAE was completely attenuated in the n-3 PUFA group, D+C/W. The increase in UAE was partially attenuated in the D+corn group; UAE among the D+corn was still 3.2-fold higher than for ND.

In addition to the increase in UAE, the diabetic animals exhibited a 49 mmHg increase in systolic blood pressure compared with ND (see Figure 30). This increase in systolic blood pressure was attenuated in the D+C/W and D+corn animals.

Similar to the prevention study, creatinine clearance normalized for body weight was not significantly different between any groups (see Figure 31).
Figure 26. Urine Albumin Excretion (UAE) in control and diabetic rats – delayed-treatment study.

UAE for diabetic (D) rats was significantly greater than for non-diabetic rats (ND) at week 6. Dietary supplementation with canola, walnuts or corn oil was started at week 7. At week 18, UAE decreased for rats eating canola or walnuts (D+C/W) and corn oil (D+corn), while UAE continued to increase for D and ND. D and ND were significantly different at all time points. ND and D+C/W were not different at any time point and did not have significantly different trend lines, while D+corn and ND did have significantly different trends by 2-way ANOVA. By week 43, ND, D+C/W, and D+corn all had significantly lower UAE than D.
Figure 27. Regression of UAE in diabetic rats fed C/W and corn oil diets.
While UAE continued to increase for both diabetic (D) and non-diabetic (ND) rats between weeks 6 and 18, UAE declined for rats fed canola or walnuts (D+C/W) and corn oil (D+corn) after supplementation began on week 7.
Figure 28. Regression of UAE after treatment with high PUFA diets.
Rats started on high PUFA diets on week 7 showed an overall decline in UAE by week 18. Pair-wise t-test comparing UAE at week 6 and week 18 showed a significant decline in UAE (p < 0.05).
Figure 29. Final 24-hour Urine Albumin Excretion (UAE) – delayed-treatment study.

After 43 weeks of diabetes, UAE was significantly increased in diabetic (D) animals; dietary supplementation with canola or walnuts (D+C/W) prevented this increase. UAE was also attenuated by corn oil (D+corn), but not to the same degree.
Figure 30. Systolic blood pressure in control and diabetic rats – delayed-treatment study.

Blood pressure (BP) was measured by tail-cuff sphygmomanometry. There was a significant increase in BP in diabetic (D) animals compared with non-diabetic (ND) controls. The increase in BP was prevented by canola and walnuts (D+C/W), and corn oil (D+corn).
Figure 31. Creatinine clearance for delayed-treatment study.

Creatinine clearance normalized for body weight was not significantly different between any of the treatment groups.
C. Morphological Parameters

Moderate to severe glomerulosclerosis and tubulointerstitial fibrosis was evident in the D animals after 45 weeks of diabetes (see Figure 32, Figure 34, and Figure 36). Glomerulosclerosis and tubulointerstitial fibrosis were attenuated in both the D+C/W animals and the D+corn animals.

The semiquantitative measurement of the degree of glomerular damage, the glomerulosclerotic index (GSI), indicated a 2.6-fold increase in glomerulosclerosis in kidneys from D compared with ND animals (see Figure 32A). Glomerulosclerosis among the D animals was associated with mesangial expansion, accumulation of ECM, capillary expansion, and accumulation of inflammatory cells (Figure 26B).

The semiquantitative measurement of tubulointerstitial fibrosis, the tubulointerstitial fibrotic index (TIFI) was increased by 2.7-fold in kidneys from D compared with ND animals in the renal cortex (see Figure 34A) and by 2.3-fold in the inner stripe of the outer medulla (see Figure 36A). Tubulointerstitial fibrosis in the D animals was associated with tubular dilation, accumulation of ECM, atrophy of tubular epithelial cells, accumulation of inflammatory cells, and capillary dilation (Figure 34B and Figure 36B). These changes were completely absent in the D+C/W animals, and almost completely attenuated in the D+corn animals.
Figure 32. Glomerulosclerotic Index (GSI) in control and diabetic animals – delayed-treatment study.

GSI, a measure of glomerulosclerosis, was elevated in kidneys from diabetic (D) rats compared to non-diabetic (ND) rats. This glomerular damage was prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.
Figure 33. Glomerulosclerotic Index (GSI) in control and diabetic animals – delayed-treatment study.
Representative PAS stained sections indicating glomerular damage in diabetic (D) rats, and normal glomeruli from non-diabetic (ND) rats. Glomerular damage was prevented and glomeruli appear normal for diabetic rats eating canola or walnut (D+C/W) and corn oil (D+corn) supplemented diets. g, glomerulus; p, proximal tubule; d, distal tubule; +, dilated capillaries; *, dilated tubules; green ellipse, extracellular matrix expansion. Original magnification ×400.
Figure 34. Cortical Tubulointerstitial Fibrotic Index (TIFI) in control and diabetic animals – delayed-treatment study.

TIFI, a measure of fibrosis, was elevated in kidneys from diabetic (D) rats compared to non-diabetic (ND) rats. This damage was prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.
Figure 35. Cortical Tubulointerstitial Fibrotic Index (TIFI) in control and diabetic animals – delayed-treatment study.

Representative Masson-Trichrome stained sections showing fibrotic damage in diabetic (D) rats, and normal appearance in non-diabetic (ND) rats. Kidney sections from diabetic rats fed canola or walnut (D+C/W) and corn oil (D+corn) appear normal. g, glomerulus; p, proximal tubule; d, distal tubule; +, dilated capillaries; *, dilated tubules; green ellipse, extracellular matrix expansion. Original magnification ×400.
Figure 36. Medullary (ISOM) Tubulointerstitial Fibrotic index in control and diabetic animals – delayed-treatment study.

TIFI in the inner stripe of the outer medulla (ISOM) was elevated in kidneys from diabetic (D) compared to non-diabetic (ND) rats. This tubular damage was prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn).
Figure 37. Medullary (ISOM) Tubulointerstitial Fibrotic index in control and diabetic animals – delayed-treatment study.

Representative Masson’s trichrome staining indicating tubular damage in diabetic (D) rats, and normal appearance from non-diabetic (ND), as well as in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn). Note that these pictures are in the same plane of section and area of the kidney, but D looks different because of the extent of tubular dilatation and cellular atrophy. *, dilated tubules; green ellipse, extracellular matrix expansion. Original magnification ×200.
D. Extracellular matrix and podocyte Proteins

Consistent with the morphological changes presented above, changes in ECM and podocyte proteins also indicated diabetic renal injury after 45 weeks of STZ-induced diabetes (see Figure 38, Figure 39, and Figure 41). There was a 3.3-fold increase in collagen I protein expression in D compared with ND animals (see Figure 38); likewise, there was a 4.4-fold increase in collagen IV protein expression (see Figure 39A). The increase in collagen I was partially attenuated in both the D+C/W group and the D+corn group. Increased accumulation of collagen IV was observed by immunohistochemistry, and representative stained sections are illustrated in Figure 39B. The accumulation of collagen IV was completely attenuated in both D+C/W and D+corn.

There was a 0.44-fold decrease in nephrin protein expression in kidneys from D animals compared with the ND animals (see Figure 41). Loss of nephrin is in part responsible for increased leakage of protein across the glomerular basement membrane and consequent albuminuria. $^{89, 91, 200, 210, 218}$ This is demonstrated by the inverse relationship between UAE and nephrin shown in Figure 42. This decrease in nephrin is partially attenuated in both D+C/W and D+corn. Additionally, staining for nestin was greatly reduced in the D animals compared to ND (see Figure 43) indicating podocyte damage in diabetes. This change was not seen in D+C/W and D+corn.
Collagen I content was increased in the kidney cortex of diabetic (D) rats compared to non-diabetic (ND) rats, indicating fibrosis. This increase was partially prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn).

Figure 38. Collagen I expression in kidneys form control and diabetic animals – delayed-treatment study.
Collagen IV content was increased in the kidney cortex of diabetic (D) rats compared to non-diabetic (ND) rats, indicating fibrosis. This increase was prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn).

Figure 39. Collagen IV expression in kidneys from control and diabetic animals – delayed-treatment study.
Figure 40. Collagen IV expression in kidneys from control and diabetic animals – delayed-treatment study.

Representative IHC staining of collagen IV in the renal cortex indicating ECM expansion and fibrosis in diabetic (D) compared with non-diabetic (ND) rats. Damage was prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn). Note the distribution of collagen IV in the basement membranes of tubules and glomeruli in ND, D+C/W, and D+corn compared to the expansion of collagen IV and damaged tubules in D. g, glomerulus; *, dilated tubules; green ellipse, extracellular matrix expansion. Original magnification ×200.
Nephrin, a protein found on the filtration slits of podocytes, was decreased in diabetic (D) compared to non-diabetic (ND) rats. This loss of nephrin was partially prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.

**Figure 41. Nephrin protein expression in kidneys from control and diabetic animals – delayed-treatment study.**

Nephrin, a protein found on the filtration slits of podocytes, was decreased in diabetic (D) compared to non-diabetic (ND) rats. This loss of nephrin was partially prevented in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) diets.
Figure 42. Nephrin vs. Urine Albumin Excretion – regression study. Nephrin protein content was inversely correlated with urine albumin excretion. The loss of nephrin is thought to render the glomerular filtration slits more permeable to albumin and lead to an increase in albumin filtration and urine albumin excretion.
Figure 43. Nestin expression in kidneys from control and diabetic animals – delayed-treatment study.

Representative IHC staining of renal nestin, which is localized to podocytes. Podocytes from diabetic (D) rats showed decreased nestin staining compared to non-diabetic (ND) rats. Nestin staining in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn) was similar to ND, and showed no loss of nestin. g, glomerulus; brown stain, nestin. Original magnification ×400.
E. Markers of Tissue Inflammation

As observed in Aim 1, in addition to the diabetic damage demonstrated by GSI and TIFI and changes in structural proteins, inflammatory markers were elevated in D versus ND animals (see Figure 44, Figure 46, Figure 47).

Transforming growth factor beta (TGF-β) renal cortical expression was increased 2.0-fold in kidneys from D compared with ND animals (see Figure 44); Interleukin 6 (IL-6) protein expression was elevated 1.9-fold in D compared with ND (see Figure 46). The increases in TGF-β and IL-6 protein expression were partially attenuated in both the D+C/W and D+corn animals.

Monocyte chemotactic protein 1 (MCP-1) renal cortical protein expression was also elevated 2.4-fold in kidneys from D compared with ND animals (see Figure 47). This increase was not attenuated in D+corn, and only partially attenuated in D+C/W.
Figure 44. TGF-β expression in kidneys from control and diabetic animals – delayed-treatment study.

TGF-β expression in diabetic (D) rats was increased compared to non-diabetic (ND) rats, suggesting chronic inflammation and fibrosis. This increase in TGF-β was partially attenuated in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn).
Figure 45. TGF-β expression in kidneys from control and diabetic animals – delayed-treatment study.

Representative IHC staining of renal TGF-β indicating increased TGF-β expression in damaged glomerulus and tubules of diabetic (D) rat compared to non-diabetic (ND) rat. This increased TGF-β expression was reduced in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn). g, glomerulus; p, dilated proximal tubule; +, dilated capillaries; green ellipse, excess TGF-β expression in damaged area. Original magnification ×400.
Figure 46. IL-6 renal cortical expression in kidneys from control and diabetic animals – delayed-treatment study.

IL-6, a marker of inflammation, was increased in diabetic (D) compared to non-diabetic (ND) rats. This increase was partially attenuated in diabetic rats fed canola or walnuts (D+C/W) and corn oil (D+corn).
Figure 47. MCP-1 renal cortical expression in kidneys from control and diabetic animals—delayed-treatment study.

MCP-1, monocyte chemoattractant protein 1, was increased in diabetic (D) compared to non-diabetic (ND) rats. This increase was partially attenuated in diabetic rats fed canola or walnuts (D+C/W), but not in diabetic rats fed corn oil (D+corn).
Figure 48. MCP-1 renal cortical expression in kidneys from control and diabetic animals—delayed-treatment study.

Representative IHC staining of renal MCP-1 indicating increased expression in damaged tubules of diabetic (D) rats compared to non-diabetic (ND) rat. This increased MCP-1 expression was reduced in diabetic rats fed canola or walnuts (D+C/W). g, glomerulus; p, proximal tubule; d, distal tubule; +, dilated capillaries; #, extracellular matrix expansion; brown, MCP-1 staining. Original magnification ×400.
FIVE GROUP ANALYSIS

I. Introduction

The data presented in the previous sections were analyzed using a combined n-3 PUFA treatment group that included both the D+canola and D+walnut animals. This section presents information on the separate canola and walnut groups when the analysis differed from the n-3 PUFA grouped data. Table 8 presents a concise comparison between the 4 group analysis (i.e. ND, D, D+C/W, and D+corn) and the 5 group analysis (i.e. ND, D, D+canola, D+walnut, and D+corn).

In general, because the data were analyzed by one way ANOVA with a Tukey post-test, and the Tukey post-test discounts the power of any analysis as the number of groups increases, it was expected that some previously significant differences will lose significance. Additionally, because the number of animals in the D+canola and D+walnut groups are roughly half the number in the D+C/W group, a loss of significance was particularly expected in the D+canola and D+walnut groups. Indeed this is what Table 8 shows; while most of the significant differences remain with the 5 group analysis confirming the effects of n-3 and n-6 PUFA in diabetic kidney disease, when differences were observed they usually involved loss of significance for the D+walnut or D+canola versus the D group.
Comparison of 4 group analysis and 5 group analysis

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<td>MCP-1</td>
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</table>

Table 8. Comparison of 4 group analysis and 5 group analysis

- – no change in significance, i.e. both D+canola & D+walnut have same significance as D+C/W, and no other groups showed changes in significance

*C – D+canola lost significance against D, no other changes in significance

*W – D+walnut lost significance against D, no other changes in significance

X – other change in significance

NA – not applicable
II. The prevention study

Overall the 5 group analysis results for the prevention study are comparable to the 4 group analysis. Ten out of sixteen parameters received the same significance, while another three parameters showed only minor changes not related to the renoprotective effects of dietary PUFAs. The overall results were consistent, confirming that dietary n-3 PUFAs significantly prevented the pathophysiologic changes associated with diabetic kidney disease. Furthermore, the data confirm that dietary n-6 PUFA was also effective at reducing most parameters associated with diabetic kidney disease.

A. Diabetic parameters

For parameters that indicate diabetes (i.e. blood glucose, body weight, and food consumption) the 5 group analysis was consistent with the 4 group analysis. Statistical differences were observed for body weight and food consumption. For body weight (Figure 49) the trend remains unchanged, although D+walnut was no longer significantly lower the ND. For food consumption (Figure 50) the omega-3 groups (i.e. D+canola and D+walnut) were no longer significantly different from D, while all diabetic groups continued to have significantly greater food consumption than ND. These results confirm that the D+canola, D+walnut, and D+corn animals were diabetic.
B. Functional parameters
For functional parameters of diabetic kidney complications (i.e. kidney weight, creatinine clearance, systolic blood pressure, and UAE) again the 4 group analysis was consistent with the 5 group analysis. Both kidney weight and creatinine clearance showed no differences in significance. For systolic blood pressure (Figure 51) both D+walnut and D+canola lost significance against D; D+corn was not significantly different in either analysis.

C. Morphological parameters
When the n-3 PUFA diets were analyzed separately, the morphological parameters (i.e. GSI, cortical TIFI, and medullary TIFI) were consistent with the combined n-3 PFUA analysis with only a modest loss of significance. For GSI there was no change in significance between the 5 group and 4 group analyses. In both cortical (Figure 52) and medullary (Figure 53) TIFI, there was a loss in significance for D+walnut. Both D+canola and D+corn were unchanged in all three parameters.

D. Structural parameters
For the structural parameters (i.e. collagen I, collagen IV, and nephrin) there was no loss of significance among the omega-3 groups. Among the structural parameters, the
only change in significance occurred for nephrin (Figure 54); ND was no longer significantly better than D in the 5 group analysis.

E. Markers of inflammation

There were no differences in significance for the markers of inflammation (i.e. TGF-β, IL-6, and MCP-1) between the 4 group analysis and 5 group analyses. These markers of inflammation were significantly lower in diabetic rats fed high n-3 PUFA diets compared to diabetic rats fed unsupplemented chow where the data were analyzed together (i.e. 4 group) or separately (i.e. 5 group). The rats fed high n-6 PUFA diet also showed significantly lower markers of inflammation.
Figure 49. Five group analysis of body weight for the prevention study.
While D+C/W was significantly lower than ND in the 4 group analysis, D+walnut was not significantly lower than ND in the 5 group analysis. D+corn was unchanged. $ – P < 0.05$ vs D, # – $P < 0.05$ vs ND.
Figure 50. Five group analysis of food consumption for the prevention study. While D+C/W was significantly lower than D in the 4 group analysis, neither D+canola nor D+walnut was lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05 \text{ vs D}, \# – P < 0.05 \text{ vs ND.}$
Figure 51. Five group analysis of systolic blood pressure for the prevention study. While D+C/W was significantly lower than D in the 4 group analysis, neither D+canola nor D+walnut was significantly lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05 vs D, # – P < 0.05 vs ND.
Cortical Tubulointerstitial Fibrosis

Figure 52. Five group analysis of cortical tubulointerstitial fibrosis for the prevention study.

While D+C/W was significantly lower than D in the 4 group analysis, only D+canola was significantly lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05$ vs D, $\# – P < 0.05$ vs ND.
Figure 53. Five group analysis of medullary tubulointerstitial fibrosis for the prevention study.

While D+W+C was significantly lower than D in the 4 group analysis, only D+canola was significantly lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05$ vs D, # – $P < 0.05$ vs ND. D+corn was unchanged. $ – P < 0.05$ vs D, # – $P < 0.05$ vs ND.
Figure 54. Five group analysis of nephrin for the prevention study.

While D was significantly lower than ND in the 4 group analysis, after 5 group analysis, there were no significant differences between any groups.
III. The delayed treatment study

Overall the analysis of 4 groups results for the delayed treatment study were consistent with the analysis of separate n-3 PUFA groups (5 group analysis) (Table 8). The differences are summarized below.

A. Diabetic parameters

For parameters that indicate diabetes (i.e. blood glucose, HbA1c, body weight, and food consumption) the 4 group analysis was consistent with the 5 group analysis. Minor differences were observed in D+canola which was no longer significantly lower than D for the 5 group analysis for blood glucose (Figure 55), despite significantly different HbA1c and body weights (as also observed in the 4 group analysis). For food consumption (Figure 56) only D+walnut remained significantly different from D in the 5 group analysis, while D+corn no longer had significantly greater food consumption than ND.

B. Functional parameters

For functional parameters of diabetic kidney complications (i.e. kidney weight, creatinine clearance, systolic blood pressure, UAE, and regression of UAE) the 5 group analysis was almost unchanged from the 4 group analysis. Kidney weight, creatinine
clearance, systolic blood pressure, and UAE showed no differences in significance. For regression of UAE after initiation of treatment (Figure 57), D+walnut was no longer significantly better than D; both D+canola and D+corn remained significantly better than D.

C. Morphological parameters

For the morphological parameters (i.e. GSI, cortical TIFI, and medullary TIFI) again the trend remains with only a modest loss of significance when the n-3 PUFA groups were analyzed separately. For GSI there was no change in significance between the 4 group and 5 group analyses; while for cortical TIFI (Figure 58), there was a loss in significance for D+walnut, and for medullary TIFI (Figure 59) both D+canola and D+walnut lost significance against D. D+corn were unchanged in all three parameters.

D. Structural parameters

For the structural parameters (i.e. collagen I, collagen IV, and nephrin) there were no changes in significance for collagen I and nephrin. For collagen IV (Figure 60) both D+C/W and D+corn were significantly better than D in the 4 group analysis, but in the 5 group analysis only D+walnut was still significantly better than D. Additionally, in the 5 group analysis for collagen IV, D+canola was significantly greater than ND.
E. Markers of inflammation

For the markers of inflammation (i.e. TGF-β, IL-6, and MCP-1) only MCP-1 showed a difference between the 4 group analysis and 5 group analyses with no significant differences found between any groups for MCP-1 in the 5 group analysis.
Figure 55. Five group analysis of blood glucose for the delayed treatment study.
All diabetic groups fed high PUFA diets had significantly higher blood glucose compared to ND, for both the 4 group and 5 group analyses. While D+C/W was significantly lower than D in the 4 group analysis, only D+walnut was significantly lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05$ vs D, # – $P < 0.05$ vs ND.
Figure 56. Five group analysis of food consumption for the delayed treatment study.

While D+C/W was significantly lower than D in the 4 group analysis, only D+walnut was significantly lower than D in the 5 group analysis. D+corn, which was significantly higher than ND in the 4 group analysis, was no longer higher in the 5 group analysis. $ – P < 0.05$ vs D, # – $P < 0.05$ vs ND.
Figure 57. Five group analysis of change in UAE for the delayed treatment study. While D+C/W was significantly lower than D in the 4 group analysis, only D+canola was significantly lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05$ vs D, $\# – P < 0.05$ vs ND.
Figure 58. Five group analysis of tubulointerstitial fibrosis for the delayed treatment study.

While D+C/W was significantly lower than D in the 4 group analysis, only D+canola was significantly lower than D in the 5 group analysis. D+corn was unchanged. $ – P < 0.05 \text{ vs } D, \# – P < 0.05 \text{ vs } \text{ND}$.
While D+C/W was significantly lower than D in the 4 group analysis, neither D+canola nor D+walnut was significantly lower than ND in the 5 group analysis. D+corn was unchanged. $ – P < 0.05 \text{ vs } D, \# – P < 0.05 \text{ vs } ND.
Figure 60. Five group analysis of collagen IV for the delayed treatment study.
While D+C/W was significantly lower than D in the 4 group analysis, only D+walnut was significantly lower than D in the 5 group analysis. Additionally, in the 5 group analysis, D+canola was significantly greater than ND and D+corn was no longer significantly better than D. $ – P < 0.05 \text{ vs D, } \# – P < 0.05 \text{ vs ND.}
Figure 61. Five group analysis of MCP-1 for the delayed treatment study.

No groups were significantly different in the 5 group analysis.
DISCUSSION

This thesis investigated the potential for dietary n-3 polyunsaturated fatty acid (PUFAs) interventions to prevent diabetic renal damage. Dietary n-3 PUFAs have been found to be beneficial in a wide range of diseases, such as cardiovascular\textsuperscript{219} and psychiatric diseases.\textsuperscript{220-226} However, clinical trials in diabetic kidney disease have generally failed to show benefit.\textsuperscript{190,227-232} Our studies tested the hypothesis that diets high in n-3 PUFAs are beneficial in attenuating diabetes-related renal damage and found that diets high in n-3 PUFA prevent renal damage in an animal model of type 1 diabetes. Additionally, dietary n-6 PUFA was also investigated, and the efficacy of n-3 and n-6 rich diets were compared.

The STZ-induced Sprague-Dawley diabetic rat develops diabetic kidney disease characterized by albuminuria, glomerulosclerosis, tubulointerstitial fibrosis, hypertension, and inflammation. Treatment with dietary canola or walnuts prevented an increase in urinary albumin excretion (UAE), an increase in blood pressure, and significantly attenuated or prevented pro-inflammatory mechanisms (e.g. TGF-β, IL-6, MCP-1) which have been shown to lead to increased collagen I and collagen IV, and decreased nephrin associated with renal damage.

Our findings show that diets rich in walnuts and canola beginning prior to the induction of diabetes resulted in complete prevention of diabetic kidney disease typical of long-term diabetes (30 weeks) (Figure 6). Surprisingly, dietary n-6 PUFA, in the
form of corn oil, was also effective in preventing diabetic kidney disease. In addition, when the n-3 and n-6 PUFA rich diets were given to animals after 6 weeks of diabetes, early albuminuria was reversed (Figure 27), suggesting that early diabetic renal damage was reversed (Figure 29). This provides strong support for both dietary n-3 PUFA and dietary n-6 PUFA in the prevention and treatment of diabetic kidney disease. The details of these findings will be discussed below.

Dietary n-6 PUFAs are predominantly linoleic acid (LA), e.g. average US intake of LA is ~15 g/day, while intake of arachidonic acid is ~0.15 g/day.\textsuperscript{233} Dietary n-3 PUFAs are predominantly α-linolenic acid (ALA), e.g. average US intake of ALA is ~1.5 g/day, while intake of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) total ~0.15 g/day.\textsuperscript{234} The ratio of dietary n-6 PUFAs to n-3 PUFAs in the US is typically ~10:1; consequently studies that look at dietary PUFAs without differentiating them are primarily investigating linoleic acid.

**Effect of dietary PUFAs on hyperglycemia**

In our studies, dietary PUFAs were associated with significantly lowered blood glucose and HbA1c (Table 6 and Table 7). While the diabetic animals eating PUFA supplemented diets were still diabetic, i.e. blood glucose > 200 mg/dl, they did have a significant decrease in blood glucose compared to untreated diabetic animals.

Low carbohydrate-high fat diets have been shown to improve blood glucose control\textsuperscript{226,235} and this effect is independent of the type of fat; saturated,
monounsaturated, and polyunsaturated fatty acids will all work for blood glucose reduction. In our work, canola, corn oil, and walnuts each produced comparable results with respect to blood glucose. While substituting fat for carbohydrates improves glucose control\textsuperscript{236}, high fat diets which include high levels of saturated fat and trans-fat can contribute to the development of metabolic syndrome\textsuperscript{237} and atherosclerosis.\textsuperscript{238} Additionally, dietary saturated fat is positively associated with development of microalbuminuria.\textsuperscript{239} PUFAs, on the other hand, show benefit in preventing metabolic syndrome and atherosclerosis\textsuperscript{4, 240}, possibly through preventing β-cell damage\textsuperscript{241} and maintaining healthy lipid profiles.\textsuperscript{170, 242-247} For instance in hypertensive rats, lard which is high in saturated fats decreased average lifespan, while linoleate (18:2n-6) increased average lifespan compared to a high carbohydrate diet.\textsuperscript{248}

Dietary PUFAs can provide the benefits of reducing blood glucose without the adverse effects of saturated-fat and trans-fat. This suggests that, at least in part, the renoprotective benefits of the PUFA supplemented diet were mediated through blood glucose lowering via decreased carbohydrate consumption. However, this cannot be the sole mechanism because these animals were still diabetic and the striking renoprotective effects could not be accounted for by this mechanism alone.

In our work, added fat accounted for 40% of calories, and the high fat diets resulted in decreased blood glucose and HbA1c. In numerous clinical trials involving n-3 PUFAs, the amount of added fat accounted for 1% to 3% of calories and did not result in decreased blood glucose or HbA1c.\textsuperscript{10, 228, 245-247, 249-252} Displacement of
carbohydrate calories reduces the rise in blood glucose seen in diabetes, but most studies of n-3 PUFAs fail to detect this effect because the amount of added fat is too small. Our work suggests that large quantities of supplemental dietary PUFAs may be needed in order to elicit a significant reduction in hyperglycemia. Whether this is a reasonable dietary regimen or whether a smaller amount of supplemental fat will suffice are questions which require further study.

**Dietary PUFAs prevent an increase in albuminuria**

In our studies, albuminuria was present in the diabetic animals eating unsupplemented chow, while the diabetic animals eating diets supplemented with canola, walnuts or corn oil showed no increase in urine albumin excretion compared to non-diabetic animals (Figure 6 and Figure 29). This finding supports earlier studies in STZ diabetic rats showing that dietary PUFAs reduced albuminuria and glomerular damage.

In diabetics, albuminuria is a hallmark of diabetic nephropathy and a strong predictor of cardiovascular disease and progressive loss of kidney function. In the STZ model of diabetic renal disease, albuminuria is well correlated with renal injury, both glomerulosclerosis and tubulointerstitial fibrosis. Increased filtered load of albumin results from glomerular damage and plays a central role in the development of tubular damage because increased endocytosis of
filtered protein can damage to tubular epithelial cells. Additionally, albumin-bound fatty acids may induce deleterious or protective effects on the renal tubule after ultrafiltration of albumin. One study found that albumin-bound PUFAs, including linoleic acid, were potent survival factors, while another study found that linoleic acid complexed with albumin was associated with severe cytotoxicity.

In clinical trials and epidemiological studies, n-3 PUFAs show similar benefits in humans. In young type 1 diabetics, treatment with n-3 PUFAs lowered the risk of developing albuminuria and slowed the development of albuminuria in older type 2 diabetics. This suggests that glomerular damage is being prevented and the findings from our studies support this idea. Additionally, consumption of dietary PUFA, particularly from plant oils, and PUFA content, especially n-6 PUFAs, of serum triglycerides were inversely associated with microalbuminuria in type 2 diabetics. In type 1 diabetics, supplemental cod liver oil, a rich source of n-3 PUFAs, reduced albuminuria. These data strongly suggest that dietary PUFAs can be effective in the treatment of diabetic nephropathy. However, numerous clinical trials that have investigated n-3 PUFAs in diabetic kidney disease have failed to show significant benefit. Often these studies show a trend toward improvement of albuminuria with dietary n-3 PUFA, but the result does not reach significance; a meta-study of these clinical trial also failed to show a significant result. The current work helps to clarify these inconsistent findings by showing that dietary PUFAs are effective in attenuating
or preventing albuminuria in diabetic animals, suggesting that the disparity in clinical findings is related more to dosage than to efficacy of dietary PUFAs.

**Attenuation of blood pressure increase in rats fed PUFA**

STZ-induced diabetic rats showed a significant increase in systolic blood pressure, approximately 50 mmHg, compared to non-diabetic rats. Diabetic rats consuming chow supplemented with canola or walnuts showed no increase in blood pressure compared to non-diabetic rats and approximately 50 mmHg reduction in systolic blood pressure compared to untreated diabetics. Diets high in PUFAs have established cardioprotective and antihypertensive benefits. For example, after only 8 weeks, supplemental cod liver oil not only reduced albuminuria but also reduced blood pressure in diabetic patients. In a wide range of animal models, n-3 PUFAs have demonstrated benefit in preventing increased blood pressure. Supplementation with n-3 PUFAs reduced hypertension in TGR(mRen-2)27 and Dahl salt-sensitive rats, and in partially nephrectomized dogs.

Numerous human studies of dietary PUFAs have seen benefits involving improved lipid profiles, and reduced hypertension. However, the reduction in blood pressure is typically less than 5 mmHg for both systolic and diastolic pressure. This is a modest reduction compared to the reduction seen in our work. In general, the greater the blood pressure, the greater the blood pressure lowering effect of dietary n-3 PUFAs. For instance, spontaneously hypertensive rats (SHR) treated with dietary n-3 PUFAs...
PUFAs have reductions of blood pressure comparable to the current studies, i.e. greater than 30 mmHg reduction. The blood pressure of untreated diabetic rats in the current study, as well as for the untreated SHR rats, was approximately 200 mmHg. This is much higher than typically seen in human studies, and greater baseline blood pressure leads to greater blood pressure lowering effect of n-3 PUFAs. The anti-hypertensive effects of n-3 PUFAs arise from a number of varied actions of n-3 PUFAs, including effects on the vasculature, heart, kidneys, and adrenal glands.

The actions of n-3 PUFAs on vascular smooth muscle cells (VSMCs) result in reduced tension and subsequently reduced vascular resistance to blood flow which results in reduced blood pressure and cardiac workload. Treatment with n-3 PUFAs decreases angiotensin II (Ang-II) binding to VSMCs. This decreased Ang-II binding results in increased K$^+$ efflux out of VSMCs and subsequent hyperpolarization. Additionally, Ca$^{++}$ influx into VSMC is reduced, and without Ca$^{++}$ muscle contraction is reduced. With reduced Ang-II binding to VSMCs, VSMC proliferation and migration is also decreased.

Mesangial cells are modified VSMCs and the influence of n-3 PUFAs on mesangial cells is similar to the actions given above for VSMCs. In short, n-3 PUFAs decrease the effect of Ang-II on mesangial cells, including reducing the vasoconstriction of the afferent and efferent arterioles. As mentioned in the background section, intra-renal Ang-II causes constriction of both the afferent and efferent arterioles with a net increase in glomerular capillary pressure and decrease in renal and
peritubular blood flow. A decrease in the action of intra-renal Ang-II will blunt this response, and spare the glomerulus from the deleterious hemodynamic factors associated with the diabetic kidney.

In the adrenal cortex, treatment with n-3 PUFAs decreases aldosterone and cortisol production.\textsuperscript{303, 304} Aldosterone and cortisol both prevent the loss of sodium in the kidney, so decreased production of both aldosterone and cortisol prevent increase in blood volume associated with sodium retention. In addition to its effects on sodium retention, cortisol acts to increase blood pressure by increasing VSMC response to epinephrine and norepinephrine. Additionally, cortisol acts to increase blood glucose, and reducing cortisol production might help lower blood glucose.

In endothelial cells, n-3 PUFAs increase NO production and decrease TXA2 production.\textsuperscript{305, 306} The increased NO production stimulated VSMC relaxation and vasodilation, as well as decreased platelet activation and thrombogenesis. Additionally, the decreased TXA2 reduces thrombogenesis.

Treatment with n-3 PUFAs beneficially modulates cardiac electrophysiology and baroreceptor response. Dietary n-3 PUFAs reduce heart rate\textsuperscript{161}, which can result in a reduction of cardiac output and blood pressure. Additionally, dietary n-3 PUFAs reduce arrhythmias and reperfusion injury in ischemia. While reductions in arrhythmias and reperfusion injury are beneficial and can reduce cardiovascular mortality and morbidity, these do not necessarily reduce blood pressure. Thus the multiple effects of PUFAs through many systems can help maintain blood pressure.
Given the preponderance of data in favor of the antihypertensive and cardioprotective benefits of n-3 PUFAs, the AHA recommends consumption of n-3 PUFAs for prevention of both primary and secondary adverse cardiac events (Table 4).\textsuperscript{162} Despite the established cardiac benefits and lack of demonstrated harm, the ADA is still reluctant to recommend consumption of n-3 PUFAs.\textsuperscript{12} Our studies help to establish the benefits of dietary n-3 PUFAs to diabetics, and move the ADA closer to making recommendations that can prevent unnecessary cardiac and renal damage.

Dietary n-6 PUFAs, in particular linoleic acid (18:2n-6), have also been shown to be antihypertensive, although the results are far more controversial than with dietary n-3 PUFAs.\textsuperscript{307} It has been proposed that linoleic acids lowers blood pressure through conversion to arachidonic acid (20:4n-6) and synthesis of series 2 prostanoids. However studies involving linoleic acid do not reliably find an antihypertensive benefit.\textsuperscript{308} This may be due in part to the very modest benefit of linoleic acid, e.g. a 2 standard deviation increase in linoleic acid was associated with a 1.9 mmHg decrease in systolic blood pressure.\textsuperscript{307} Additionally, essential hypertension is associated with a decrease in the desaturase and elongase enzymes needed to convert linoleic acid to arachidonic acid.\textsuperscript{309} While the loss of conversion of linoleic acid to arachidonic acid may be a cause of essential hypertension, it also prevents treatment with dietary linoleic acid from reducing essential hypertension and this loss of desaturase and elongase activity is a predictor of both cardiovascular mortality and total mortality.\textsuperscript{310}
In the current studies, the antihypertensive benefits of corn oil were comparable to those of canola and walnuts. While the proposed mechanisms of action and reliability of dietary n-3 vs n-6 PUFAs differ, in our study the effects were similar suggesting that dietary n-6 PUFAs are possibly more beneficial than earlier studies indicate. A recent science advisory from the AHA reiterates the health benefits of dietary n-6 PUFAs, principally linoleic acid. and a yet another recent study, this one using pooled data from 11 American and European cohort studies including 5249 coronary events and 2155 coronary deaths, found again that dietary PUFAs consumption is correlated with decreased risk of coronary events and death, while SFAs and carbohydrate consumption were correlated with higher risk of coronary events and death. Evidence for monounsaturated fatty acids (MUFAs) is more controversial; in the US MUFA consumption is correlated with animal fat consumption and therefore SFA consumption and increased risk, while in Mediterranean countries, e.g. Italy, Greece, Spain, MUFA consumption is typically from olive oil rather animal products and is correlated with lower risk of coronary events and death.

**Dietary PUFAs prevent diabetic renal damage**

Morphologically, diabetic nephropathy is characterized by glomerulosclerosis and tubulointerstitial fibrosis. In our prevention and delayed-treatment studies, diabetic animals showed significant glomerulosclerosis and tubulointerstitial fibrosis after 30 and 45 weeks of diabetes, respectively. This was associated with increases in
albuminuria and blood pressure. Supplementing chow with PUFA rich walnuts, canola or corn oil attenuated this increase in albuminuria and blood pressure, and completely prevented the increase in glomerulosclerosis and tubulointerstitial fibrosis (Figure 9, Figure 11, Figure 13, Figure 32, Figure 34, and Figure 36). While we hypothesized that there would be a significant reduction in renal damage with diabetic rats fed n-3 PUFA supplemented diet, the findings that both the n-3 and n-6 PUFA rich diets completely prevented, and even reversed early UAE were unexpected. This suggests that dietary intervention is an effective treatment against diabetic renal damage and that clinical studies have shown only modest or no protection possibly because the amount of supplemental fat was too small.

**Dietary PUFAs prevent collagen accumulation**

A key aspect of our studies is that the supplemental diets blocked typical profibrotic pathways common in diabetic complications. In both the prevention study and the delayed-treatment study, collagen I and collagen IV protein accumulation was increased in diabetic rats, but treatment with dietary n-3 PUFA ameliorated this change (Figure 15, Figure 16, Figure 38, and Figure 39). Accumulation of ECM proteins, including collagen I and collagen IV, is central to the development of glomerulosclerosis and tubulointerstitial fibrosis and loss of renal function.\(^{35, 268, 270, 279, 313-320}\) Reports of increased collagen are nearly universal in studies of STZ-induced diabetic kidney disease, and treatments that show benefit routinely show a decrease in
collagen protein expression compared to untreated diabetic.\textsuperscript{35, 108, 270, 277, 279, 315-318, 320-323}

The fact that the diets prevented excessive collagen accumulation is consistent with the reduction in inflammation and the prevention of albuminuria.

**Dietary PUFAs prevent podocytopathy**

Recent studies have shown that podocyte damage contributes to development of diabetic renal disease.\textsuperscript{324, 325} In particular, disturbances of the podocyte foot processes and filtration slits are strongly correlated with proteinuria and progression of glomerular disease.\textsuperscript{326} Loss of nephrin, a cell adhesion molecule found on the filtration diaphragm,\textsuperscript{327, 328} is centrally involved in the development of proteinuria and sclerosis of renal disease.\textsuperscript{218} Loss of nestin\textsuperscript{201} is also seen with podocytopathy and glomerular damage.\textsuperscript{90}

In our studies, untreated diabetes resulted in loss of nephrin, nestin, proteinuria, and glomerulosclerosis, while these pathologies were not observed in diabetic animals treated with dietary n-3 PUFAs. Our findings suggest that dietary n-3 PUFAs are renoprotective, at least in part, by preserving podocyte integrity.

**Suppression of proinflammatory pathways by dietary PUFAs**

In addition to the anti-hyperglycemic and anti-hypertensive effects of dietary n-3 PUFAs, there is an anti-inflammatory action of n-3 PUFAs, reducing the production and secretion of inflammatory cytokines, including TGF-β, IL-6, and MCP-1. In our studies, these cytokines were significantly increased in diabetic animals eating
unsupplemented chow. This increase was attenuated or prevented by treatment with canola and walnuts (Figure 21, Figure 23, Figure 24, Figure 44, Figure 46, and Figure 47).

TGF-β, an inflammatory cytokine, plays a central role in chronic inflammation and fibrosis (Figure 62). Increased expression of TGF-β is a key process in the development and progression of diabetic complications including diabetic nephropathy.\textsuperscript{314, 329, 330} As mentioned above, TGF-β appears to be increased by a number of factors, including Ang-II, PKC activation, and inflammatory cytokines. TGF-β is commonly increased in STZ-induced diabetic kidney disease\textsuperscript{35, 100, 272, 273, 275, 279, 280, 317, 320, 321, 331-336}, and increased TGF-β is instrumental in stimulating production of extracellular matrix (ECM) proteins and causing fibrosis. Numerous treatments that reduce diabetic kidney damage also show reduction of TGF-β expression\textsuperscript{35, 100, 199, 276, 279, 320, 321, 333, 334, 337} compared to untreated diabetics, but the current work is the first to show this effect with dietary n-3 PUFAs.\textsuperscript{338}

In addition to TGF-β, other inflammatory markers, e.g. IL-6 and MCP-1, are typically increased in chronic inflammation and diabetic complications.\textsuperscript{339-341} The increase in both IL-6 and MCP-1, which occurred with diabetes, was reduced by treatment with dietary n-3 PUFAs. The current work identified an important role of n-3 PUFAs on attenuating renal TGF-β, IL-6, and MCP-1 expression. This suggests that the ability of n-3 PUFAs to block the cascade of inflammatory cytokines is an important renoprotective mechanism.
In the glomerulus, TGF-β is produced primarily by mesangial cells. However, monocytes that migrate into the glomerulus and become macrophages can also secrete high levels of TGF-β. MCP-1 is a monocyte attractant and stimulates the infiltration of monocytes into the glomerulus.\textsuperscript{342, 343} Additionally, MCP-1 can promote macrophage-mediated tubular damage.\textsuperscript{344} Often, TGF-β and MCP-1 levels are expressed nearly concurrently in acute inflammation\textsuperscript{345}, and increased TGF-β expression can contribute to an increase in MCP-1\textsuperscript{346} suggesting a regulatory loop between TGF-β and MCP-1.\textsuperscript{347}

Hyperglycemia can also induce secretion of TGF-β and MCP\textsuperscript{348-350}, suggesting that the anti-hyperglycemic effects of dietary fats may contribute, in addition to the anti-inflammatory effects of dietary n-3 PUFAs, to reduce TGF-β and MCP-1. Additionally, renin-angiotensin blockade also lowers MCP-1 expression, suggesting that reduction of Ang-II or adverse hemodynamics factors might also be responsible for decreased MCP-1 expression.\textsuperscript{339}

Because arachidonic acid (AA) is the precursor for the generally proinflammatory eicosanoids, the n-6 PUFAs have been considered to be proinflammatory fatty acids and the ratio of n-6 to n-3 fatty acids is believed to be associated with inflammation.\textsuperscript{351, 352} However work looking at the inflammatory nature of n-6 PUFAs have found that they can also be anti-inflammatory.\textsuperscript{353, 354

**Summary of benefits of dietary PUFAs**
The potential mechanisms of action by which dietary n-3 or n-6 PUFAs might exert their renoprotective effects are shown in Figure 62. Items in red indicate potential mechanisms of action of PUFAs. Items in green represent deleterious effects of diabetes that were benefited by treatment with dietary PUFAs. In an STZ-induced diabetic Sprague-Dawley rat model of type 1 diabetes, dietary PUFAs reduce blood glucose, reduced blood pressure, and reduced inflammatory cytokines compared to untreated diabetic animals. This had the effect of preventing collagen I and IV accumulation, as well as preventing loss of the podocyte proteins nephrin and nestin. This prevented glomerulosclerosis, tubulointerstitial fibrosis, and albuminuria. In short, the kidneys from diabetic animals eating n-3 or n-6 PUFA rich diet were not distinguishable from the kidneys of non-diabetics.
Figure 62. Potential mechanisms of action of dietary PUFAs.
CONCLUSION

In the current studies, treatment with diets high in either n-3 or n-6 PUFAs significantly reduced kidney disease associated with diabetes. Additionally, PUFA rich diets were able to reverse early diabetic kidney damage. These data support the concept that dietary PUFA are effective in the treatment and prevention of diabetic kidney disease. Using a high dose of supplemental dietary fat from walnuts, canola or corn oil, i.e. 40% of calories, resulted in significant protection from diabetic kidney disease in STZ-induced diabetic rats. Thus in this model, prevention of the development of diabetic kidney disease is attainable; however, the dietary model used in these studies may not be optimal, i.e. similar results might be achieved with less supplemental fat.

Because STZ induced diabetes involves destruction of the pancreatic β-cells, it is similar to type 1 diabetes (T1D). Therefore we predict that this research may be extended to T1D in humans and provide a method to prevent diabetic nephropathy or reverse early kidney damage. The applicability of this work to type 2 diabetes (T2D) is less obvious, because T2D represents an energy overabundance, and the best treatment for T2D is negative energy balance, e.g. reduction of total calorie consumption. If negative energy balance can be achieved and carbohydrate calories can be substituted for n-3 or n-6 PUFAs, these studies suggest that dietary PUFA rich foods might be beneficial in T2D as well.

These studies were conducted in rats. While the rat model of diabetic renal disease is a good representation of diabetic nephropathy in humans in both the clinical
manifestations and disease mechanisms, it is still an animal model. In addition to the inherent differences between rats and humans, the rats in the study had very poorly controlled hyperglycemia; blood glucose was well > 200 mg/dl, and HbA1c was > 9%. While it is reasonable to predict that the results from our studies are applicable to similarly poorly controlled human diabetics, it is not clear how these data translate to humans with good glycemic control, e.g. HbA1c < 7%. The best treatment for T1D is good glycemic control which can greatly delay the development of diabetic complications. The obvious hypothesis is that n-3 and n-6 PUFA rich foods would still be beneficial in further reducing or delaying diabetic renal complications in patients with well controlled T1D.

These studies did not use purified components, but rather used commonly available food items that are a mixture of numerous components. Canola, the n-3 PUFA rich oil, contains both n-3 and n-6 PUFAs, as well as monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA), while walnuts contain all those components and many others. Corn oil, the n-6 rich oil, also contains very modest amounts of n-3 PUFA, as well as MUFA and SFA. The benefits seen in our studies have been attributed to dietary n-3 PUFAs, while in the strictest sense these benefits should be attributed to the whole food, i.e. canola, walnuts or corn oil. However, because numerous sited literature using purified components have established the actions of PUFAs and other studies have shown associations between
the presence of PUFAs and beneficial health outcomes\textsuperscript{3, 165, 222, 224, 306}, it is not unreasonable to attribute benefits to these components.

The current studies did not block specific pathogenic or proinflammatory pathways, but rather relied on previous work which looked at the effects of n-3 and n-6 PUFAs. Specifically, this work did not measure any eicosanoids. Additionally, the concentrations and actions of Ang-II were not examined. For both of these topics, there is a significant literature detailing their effects. The discussion reviewed the potential effects of n-3 and n-6 PUFAs on these pathways.

An important consideration in the current studies is that 40% of calories came from supplemental fat. While this seems like an unpalatably high fat diet, the rats ate it with gusto, and the “standard American diet” gets approximately 40% of calories from fat, unfortunately the wrong fats.\textsuperscript{244} The difficulty of this high fat protocol is not that people won’t eat high fat diets, but rather that people are reluctant to eat the supplemental fat in place of other calories. For instance, the high-fat, low-carbohydrate Atkins diet has no better long term compliance than other diets. Clearly with T2D, negative energy balance, i.e. food consumption less than energy expenditure, is the most effective treatment, and increasing fat consumption will only be beneficial if other food consumption is reduced. However, fat consumption can be a useful part of weight reduction program; high fat foods slow gastric emptying and can prolong the feeling of satiety. For example, habitual nut eaters tend to be leaner than non-nut eaters\textsuperscript{356};
suggesting that walnuts could be an extremely healthy snack\textsuperscript{357, 358} – providing healthy PUFAs and reducing net calorie consumption.

For our studies, a high dose was chosen in order to provide a high likelihood of seeing a treatment effect. At 40\% of calories, the supplemental fat from canola, walnuts, and corn oil significantly reduced diabetic kidney complications. This suggests that a lower dose of canola or walnuts might still provide protection. Future research could investigate this dose response and determine the minimum amount of fat needed to prevent diabetic kidney complications.

While the focus of this work was on the renoprotective effects of dietary n-3 PUFAs, in the current studies, n-6 PUFA rich corn oil also showed significant protective benefit with regard to glomerulosclerosis and tubulointerstitial fibrosis. This is controversial, previous studies have shown that n-6 PUFAs are pro-inflammatory and therefore could be detrimental in many forms of chronic disease including cardiovascular disease and diabetes.\textsuperscript{129, 352, 359-361} For instance, in the dog model of renal insufficiency\textsuperscript{289}, while n-3 PUFA had a protective effect, n-6 PUFA had a deleterious effect. However, more recently, n-6 PUFAs have been largely exonerated\textsuperscript{233} and the AHA endorses consumption of dietary n-6 PUFAs. The present studies support a beneficial effect of n-6 PUFAs in treating diabetic renal disease. In our studies dietary n-6 PUFA rich corn oil provided comparable protection from diabetic kidney complications, compared with walnuts and canola. Taken together with previous studies and the controversial nature of treatment with n-6 PUFAs, the current studies
suggest that dietary n-6 PUFAs are renoprotective and that further study of n-6 PUFAs is warranted in an effort to resolve any controversies.

In conclusion, diabetic rats fed canola or walnuts were protected from kidney complications. Treatment with dietary corn oil also significantly reduced diabetic complication. In all cases, supplemental fat contributed 40\% of calories, and average blood glucose and HbA1c were reduced, demonstrating that substituting fat calories for carbohydrate calories reduces blood glucose. Overall, the success of these studies indicates that diets high in beneficial PUFAs may be effective in preventing and treating diabetic kidney complications.
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