# LONG-TERM EFFECTS OF DIETARY HIGH PROTEIN ON RENAL HEALTH IN THE PIG MODEL

BY

**YONG JIA**

A Thesis

Submitted to the Faculty of Graduate Studies of

The University of Manitoba

in Partial Fulfillment of the Requirements

for the Degree of

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Department of Human Nutritional Sciences

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## ABSTRACT

The impact of habitually consuming a high protein (HP) diet at the upper limit of the acceptable macronutrient distribution range (AMDR) on kidney health is unknown. The current study was designed to test the hypothesis that long-term consumption of a diet providing 35% of energy as protein will have negative consequences on renal health, as assessed in a pig model. Methods: Adult female, non-pregnant, commercial pigs (Genesus) were randomized to receive either NP (15% energy from protein) or HP (35% energy from protein) isocaloric diets for either 4 or 8 months. Diets contained whole protein sources with an animal: plant ratio of 2:1 in the NP diet to mimic the average Canadian diet. The increased protein in the HP diet was achieved by increasing egg and dairy protein sources. Body composition was measured by dual-energy X-ray absorptiometry. Glomerular volume and kidney fibrosis were evaluated on kidney sections by quantitative image analysis. The inflammatory marker monocyte chemoattractant protein-1 (MCP-1) and the growth factor transforming growth factor beta-1(TGFβ1) were assessed in renal tissue using commercial ELISA kits. Results: Pigs given the HP diet had lower body weights and percentage of body fat. Pigs consuming the HP diet had significantly higher glomerular filtration rates (GFR) and larger kidneys. Renal MCP-1 levels and renal fibrosis also were significantly higher in pigs given the HP diet, while proteinuria and renal TGFβ1 expression did not differ. Conclusion: These findings suggest that, despite the potential benefit of the HP diet on body composition, long-term intakes of protein at the upper limit of the AMDR may compromise renal health in healthy female pigs.

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## LIST OF ABBREVIATONS

| | |
|---|---|
| **AGEs** | advanced glycation end products |
| **AMDR** | Acceptable Macronutrient Distribution Range |
| **BMDC** | bone marrow-derived cells |
| **CKD** | chronic kidney disease |
| **DEXA** | dual energy x-ray absorptiometry |
| **DRI** | Dietary Reference Intakes |
| **ECM** | extracellular matrix |
| **ESRD** | end-stage renal disease |
| **GBM** | glomerular basement membrane |
| **GFR** | glomerular filtration rate |
| **GH** | growth hormone |
| **H&E** | hematoxylin and eosin |
| **HP** | high protein |
| **IGF** | insulin-like growth factor |
| **IOM** | Institute of Medicine |
| **K/DOQI** | Kidney Disease Outcome Quality Initiative |
| **KFC** | Kidney Foundation of Canada |
| **LBW** | lean body weight |
| **MA** | microalbuminuria |
| **MCP-1** | monocyte chemoattractant protein-1 |
| **NHANES III** | Third National Health and Nutrition Examination Survey |
| **NKF** | National Kidney Foundation |
| **NP** | normal protein |
| **OC** | osteocalcin |
| **PTH** | parathyroid hormone |
| **ROS** | reactive oxygen species |
| **TAL** | thick ascending limb |
| **TG** | triglyceride |
| **TGFβ1** | transforming growth factor beta-1 |
| **VEGF** | vascular endothelial growth factor |
| **WHO** | World Health Organization |

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# 1 LITERATURE REVIEW

## 1.1 High Protein Diets

### 1.1.1 Introduction

The popularity of high protein (HP) diets among the population and health care providers has surged as obesity has become more and more common in Canada, where more than 60% of adults are overweight or obese (Health Canada, 2004). However, there is lack of sufficient data to prove the long-term safety of these diets, especially on kidney health. This is reflected in the Institute of Medicine's (IOM) report on Dietary Reference Intakes (DRI) for Macronutrients (IOM, 2002) which stated:

> "There was insufficient evidence to suggest an Upper Level for protein and insufficient data to suggest an upper limit for an Acceptable Macronutrient Distribution Range (AMDR) for protein. To complement the AMDRs for fat and carbohydrate for adults, protein intakes may range from 10 to 35 percent of energy intake to ensure a nutritionally adequate diet" (p 661), and
>
> "Research is needed on high protein intakes (>145 mg N/kg/day) in relationship to: positive nitrogen balance and requirement estimates; metabolic and possible toxic effects…and pathways impacted by these high intakes" (p 573).

The Recommended Dietary Allowance (RDA) for protein is 0.8g/kg body weight/day (10% of energy) and the World Health Organization (WHO) recommends that protein intake should be between 10 to 15% of energy (WHO, 2003). Typically Canadians consume approximately 1.3 g/kg body weight/day, which comprises about 16% of total caloric intake, and of which the ratio of animal to plant protein is 2:1(Statistics Canada, 2007). Furthermore, approximately 300,000 Canadians (about 1% of the population)

consume more than 23% of their energy as protein (IOM, 2002). These numbers are likely higher since most individuals underreport their protein intakes (Rosell,M.S. 2003; Subar,A.F. 2003).

Although HP weight-loss diets have existed in North America for decades, there is no consensus on what constitutes an HP diet. Based on the composition of some popular HP diets, such as Atkins, South Beach, Zone, Protein Power and Stillman (Atkins, R.1999; Agatston, A.2003; Sears, B.1995; Eades,M. 1996; Stillman, I.1967), a HP diet refers to (1) a diet that has people consuming more than the general populations' average intake of ~15% of energy from protein, e.g., as much as 30%-35%, which is within the AMDR as laid out in the DRIs, or (2) daily protein consumption greater than 1.5 g /kg body weight. The philosophy of these diets is: eating too much carbohydrate causes obesity and other health problems; protein can leads to decreased hunger and weight loss. Those diets recommend eating lean meat, fish, eggs and cheese and that the protein comprise 26%-64% total energy intake.

In addition to weight loss, HP diets may improve the blood lipid profile, reduce the risk of cardiovascular disease (CVD), improve insulin sensitivity and prevent muscle loss in the obese population (Hu,F.B. 2005;300 Layman,D.K. 2004). However, the long-term safety of HP diets is uncertain. Reported short-term symptoms include constipation, headaches, muscle cramps and halitosis. Increased animal protein consumption also is associated with hyperuricosuria, hypercalcuria and a reduction in urinary pH, all risk factors for stone formation. Other long-term effects may include altered cognitive function (a complication of ketosis), osteoporosis, vitamin deficiencies, and progression of chronic renal insufficiency (Friedman,A.N. 2004). The inclusion guidelines for supplementation with multiple vitamins and minerals in these

diets suggest an admission of the nutritional inadequacy of the diets.

### 1.1.2 Effects of High Protein Diets on Weight Loss

A HP diet is often recommended as one of the management strategies for weight loss and weight control in overweight or obese individuals. HP weight loss diets enhance loss of body mass, fat mass, percentage body fat, and retain lean mass compared with lower-protein diets in the short term (within 6 months) (Halton,T.L. 2004;34 Cunningham,W. 2006). Possible mechanisms include (1) increased satiety, decreased subsequent energy intake; (2) refined carbohydrate displacement with higher dietary protein, thus reducing glycemic load and postprandial insulin level; (3) higher thermic effect of protein. Because the body has no storage capacity for protein, it needs to be metabolically processed immediately. The synthesis of protein, as well as urea production and gluconeogenesis can all lead to higher energy expenditure. It is likely that these mechanisms work together and are related to each other (Noakes,M. 2005; Hu,F.B. 2005; Krieger,J.W. 2006).

In the long-term, however, studies of greater than 6 months show that the relative advantage of HP, low-carbohydrate diets in weight loss is not sustained for longer periods of time, compared to high-carbohydrate “conventional” diets. Recently, several long-term randomized controlled trials to evaluate efficiency of HP diets on weight loss among overweight or obese populations have been published (Foster,G.D. 2003; Due,A. 2004; Brinkworth,G.D. 2004; Dansinger,M.L. 2005; McAuley,K.A. 2006) . The duration of these trials varied from 24 to 216 weeks and 25-30% of energy was from protein. Weight loss from these diets was relatively small, ranging from 2.1% to 7.1% of body weight and no study showed a statistically significant difference in weight loss between HP and “conventional” diets.

## 1.2 Chronic Kidney Disease

### 1.2.1 An Overview of Chronic Kidney Disease

Chronic kidney disease (CKD) is defined as the presence of kidney injury and impaired kidney function. The National Kidney Foundation (NKF) definition includes the following criteria: 1) the presence for 3 or more months of kidney damage which is defined as structural or functional abnormalities of the kidney, with or without decreased GFR. Kidney abnormalities can manifest by either kidney pathological changes or markers of kidney damage; 2) GFR<60 mL/min/1.73 $m^2$ for 3 or more months, with or without kidney damage (NKF, 2002). Race, gender, age (older than 60 years), diabetes, hypertension, cardiovascular disease and family history of these diseases or kidney disease are all risk factors for CKD. The NKF classification defines five stages of CKD by increasing degree of kidney impairment (Table 1). CKD is very common and according to the National Health and Nutrition Examination Survey (NHANES) III the prevalence of CKD in the US adult population is 11% (19.2 million) (Coresh, 2003). Assuming a similar prevalence, it is estimated that 2 million Canadians have CKD. Patients with CKD have a lower quality of life, higher health care costs, and face the prospect of end-stage renal disease (ESRD) requiring dialysis or transplantation. According to the Kidney Foundation of Canada (KFC), there were 32,375 Canadians on renal replacement therapy in 2005 and this number is expected to double over by 2015 (KFC, 2008).

**Table 1** Stages and protein recommendation of chronic kidney disease[1]

| Stage | Description | GFR mL/min/1.73 $m^2$ | Protein recommendation (g/kg body weight/day) |
|---|---|---|---|
| 1 | Kidney damage with normal or elevated GFR | ≥ 90 | 0.75 |
| 2 | Kidney damage with normal with mild decrease GFR | 60–89 | 0.75 |
| 3 | Moderate decrease GFR | 30–59 | 0.75 |
| 4 | Severe decrease GFR | 15–29 | 0.6-0.75 |
| 5 | Kidney failure/ESRD[2] | < 15 or dialysis | 0.6(non-dialyzed) 1.2-1.3(dialyzed) |

[1]Adapted from National Kidney Foundation, 2002; Stall, S. 2008

[2] ESRD=end stage renal disease

### 1.2.2 Pathogenesis of Chronic Kidney Disease

Traditionally, it has been assumed that once substantial renal scarring occurs there is an inevitable progression to end-stage kidney disease, a process leading to chronic progressive renal function failure. Glomerular and tubulointerstitial inflammation are known precursors of glomerulosclerosis and tubulointerstitial fibrosis, respectively. Activation of glomerular and tubular cells following injury leads to the release of cytokines, proinflammatory chemokines and growth factors, such as endothelin (ET-1), monocyte chemoattractant protein-1 (MCP-1), regulated upon activation, normal T-cell expressed and secreted (RANTES) and transforming growth factor-beta 1(TGFβ1), which then further attract inflammatory cells. These inflammatory cells include macrophages, lymphocytes, dendritic cells and natural killer (NK) cells, each of which is in varying degrees of activation. Inflammatory cells interact with intrinsic renal cells (epithelial cells, mesangial cells and myofibroblasts), the latter become activated, proliferate, transform and synthesize excessive extracellular matrix (ECM). Ultimately, glomerulosclerosis and tubulointerstitial fibrosis develop (El Nahas,M. 2005).

Recent studies focusing on extra-renal cells involved in renal remodeling suggest the potential role of bone marrow-derived cells (BMDC) in the repair of glomeruli and tubules following injury. BMDC may contribute to the repopulation of the mesangial and tubular cells. Some observers estimate, based on bone marrow transplantation experiments, that around 10–12% of mesangial cells in the recovering glomeruli are from bone marrow origin. Although it may contribute relatively little to the development of glomerulosclerosis and tubulointerstitial fibrosis, it provides a new view and challenges the role of renal remodeling research (El Nahas,M. 2006).

### 1.2.3 Protein Intake and Risk Factors for Chronic Kidney Disease

High dietary protein can induce hemodynamic changes in the kidney and perhaps the Brenner hypothesis (hyperfiltration hypothesis) is the most frequently cited reference with regard to the potentially harmful effects of dietary protein intake on renal function. It states that habitual consumption of excessive dietary protein negatively impacts kidney function by causing a sustained increase in glomerular hyperfiltration and glomerular hypertension. These situations lead to renal injury and glomerulosclerosis which in turn reduce functioning nephron mass and aggravate glomerular hyperfiltration, ultimately causing progressive renal failure (Brenner,B.M. 1982). However, this evidence is from patients with co-existing renal disease, so the question of whether HP intake results in adverse effects in healthy persons without renal disease is not well established. Moreover, according to Brenner and other researchers, it is suggested that HP intake plays a central role in the decline of renal function with age and that protein restriction might prevent this decline.

Limited data exist regarding the role of dietary protein intake as an independent risk factor for either the initiation or progression of CKD. It is already known that dietary protein can modulate renal hemodynamics by increasing glomerular filtration rate (GFR) and renal blood flow (Friedman,A.N. 2004). Whether this hyperfiltration is a risk factor in the healthy population, leading to glomerular injury and developing CKD in the long-term, is unknown. There are some data, however, showing that long-term high protein intake may accelerate renal function decline in individuals with mild renal insufficiency and these individuals usually are unaware of their kidney situation and considered “healthy” (Knight,E.L. 2003; Coresh,J. 2003). On the other hand, short-term human studies have shown some benefits of HP diets in delaying the

progression of CKD. These studies demonstrate an inverse relationship between dietary protein intake and the known risk factors for CKD, including systemic blood pressure, triglyceride (TG) and obesity (Hu,F.B. 2005; Noble,C.A. 2006).

### 1.2.4 Dietary Protein Restriction for Chronic Kidney Disease Patients

The reason for manipulating the diet of CKD patients is that they will develop protein intolerance when eating an excess of dietary protein. The manifestations of protein intolerance in CKD patients include the multitude of problems identified as uraemic symptoms; other problems are hyperparathyroidism and bone disease, reduced insulin sensitivity, and increased breakdown of protein and amino acids, and augmentation of proteinuria (Walser,M. 1999; Gansevoort,R.T. 1995). In patients with uncomplicated CKD (i.e., no catabolic illness or metabolic acidosis), diets are recommended with the minimum daily requirement for protein (Table 1), or a very-low-protein diet containing ~ 0.3 g protein/kg body weight/day. The composition of this diet is predominantly vegetable protein supplemented with a mixture of essential amino acids and ketoacids to produce neutral nitrogen balance and maintain normal serum protein levels and lean body mass (Mitch, W.E.2002). It is widely accepted that in CKD patients, low-protein diets, especially with ketoacid supplements, can slow the loss of renal function by decreasing the accumulation of urea and other nitrogen-containing compounds of protein metabolism. In addition, low-protein diets will limit the tendency for hyperphosphatemia and metabolic acidosis (Kent,P.S. 2005; Mandayam,S. 2006).

## 1.3 High Protein Diet in Human Studies

### 1.3.1 Effects of High Protein Diets on Normal Kidney Function

Although the popularity of HP diets has surged recently in developed nations where obesity has become more and more common, there is insufficient evidence on the potential effects, especially in the long term, of high dietary protein on the normal kidney. Early studies on the effects of high dietary protein on renal health are mainly focused on the renal hemodynamic responses to HP diets and mostly are single-meal studies. For example, in Chan and colleagues' study (Chan,A.Y. 1988) HP diets (1.5 g/kg) were given to 12 healthy volunteers. Three hours after the meal there was an elevation of urine concentration. The rate of peak postmeal renal plasma flow (RPF) was elevated by 13% and peak increases in postmeal GFR exceeded baseline by 10%. Other studies also show that one-time HP meals lead to an acute rise in RPF, GFR, and proteinuria (Kontessis,P. 1990; Viberti,G. 1987; Bergstrom,J. 1985).

Limited clinical trials studying the effects of HP diets on kidney in the healthy population yield conflicting results. Short-term studies show similar results to single-meal studies, i.e. there is an increase in GFR after the HP compared to low-protein diet, especially in the older population (Wagner,E.A. 2007). Whether this hyperfiltration leads to glomerular injury in healthy populations over the long term is still uncertain. Proteinuria (or albuminuria) is recognized as a predictor of kidney disease progression in persons with CKD, as well as a risk factor in healthy populations (Gerstein,H.C. 2001; Furtner,M. 2005). Therefore, whether HP diets alter urinary protein excretion is of direct clinical importance. A cross-sectional Dutch survey among a diverse healthy population reported that the risk for microalbuminuria progressively increased with greater daily protein ingestion, especially the group

whose daily protein consumption was more than 1.5g/kg body weight (Hoogeveen,E.K. 1998). In contrast, assessing data from NHANES III, high dietary protein intake (110.9 g/day) is not associated with microalbuminuria among healthy persons (Wrone,E.M. 2003). Another 4-month study of 88 subjects with normal renal function consuming protein ranging from 0.29 g/kg/day to 2.6 g/kg/day showed the correlation between urinary albumin excretion rate and nitrogen excretion rate (an indicator for daily protein intake) was not significant, although there was a positive, nonlinear relationship between protein intake and creatinine clearance (Brandle,E. 1996). The 10-year cohort Nurses' Health Study followed middle-aged women with protein intake which ranged from 60 g to 93 g /day and found no association between protein intake and change in GFR in women with normal renal function (Knight,E.L. 2003). Further, in an intervention study, Skov et al studied 65 overweight, but otherwise healthy subjects who adhered to low (12% of energy from protein) or HP (25% of energy from protein) diets for six months. In the HP group, both kidney size and GFR were significantly increased from that measured at baseline. No significant changes in serum or urinary creatinine and albumin excretion were noted for either group (Skov,A.R. 1999).

However, in most studies subjects consume about 20%-30% of energy intake as protein (daily protein intake from 1.5 to 2.4g/kg body weight), while the upper range of the AMDR is 35%, so data to suggest the safety of high dietary protein intake is insufficient. Lack of long-term follow up is another problem, because the effect of the diet is subtle and the progression of the disease is relatively slow. Most of the reported studies examine single-meals or short-term clinical effects and data from long-term studies are very limited and conflicting. Furthermore, previous studies focus mainly on the relationship between level of dietary protein and renal health by evaluating clinical

parameters of kidney function, including urinary albumin and creatine measurement as an estimation of GFR. However, there are some critical cytokines and proinflammatory chemokines released in the early stages of progressive renal injury, which are present before the decline of kidney function and may indicate effects of protein on kidney health. Assessments of these inflammatory markers at the gene and protein level are scant. Without question, deeper studies are needed to demonstrate whether there is a link between protein intake and the initiation or progression of renal disease in healthy individuals.

### 1.3.2 Effects of High Protein Diets on Mild Renal Insufficiency

Mild renal insufficiency is defined as patients with GFR between 55 and 80mL/min/1.73 $m^2$, which mostly equals to CKD stage 2 or to stage 3 in a small portion. It is not a small population because according to NHANES III, 23-32% of people older than 20 years of age have mild renal insufficiency and 41-47% of people more than 40 years of age have a GFR between 60-89 mL/min/1.73 $m^2$ (Coresh,J. 2003). It also indicates that the prevalence of CKD in the United States in 1999-2004 is higher than it was in 1988-1994. The prevalence estimates of CKD stages in 1988-1994 and 1999-2004, respectively, were 2.7% and 3.2% for stage 2; 5.4% and 7.7% for stage 3. A higher prevalence of diagnosed diabetes and hypertension and higher body mass index partly explain the increase in the prevalence of decreased GFR (Coresh,J. 2007). Interventions may be most beneficial in patients with mild renal insufficiency compared with other CKD stages. In patients with mild renal insufficiency medicines can significantly reduce the incidence of cardiovascular events and coronary revascularization but in stage 4 CKD patients those medicines are not as effective (Tonelli,M. 2004).

Populations with mild renal insufficiency usually have no apparent symptoms so they are often considered healthy and unaware of their condition. However, data show that over a 5-year period, mortality increases by CKD stage from 19.5% in stage 2 to 45.7% in stage 3 (Keith,D.S. 2004). Moreover, there are no clear protein intake guidelines for patients with mild insufficiency. Therefore, it is not known how many people with CKD adhere to HP diets and those people may be adversely affected by high dietary protein intake. Knight's study mentioned before also indicated that in women with mild renal insufficiency high total protein intake, particularly high intake of nondairy animal protein, may accelerate renal function decline in GFR (Knight,E.L. 2003).

### 1.4 High Protein Diets in Animal Studies

Although there is limited research regarding the long-term effects of HP intake on renal health in humans, animal models have provided important insight into this question. In long-term animal studies, HP diets can cause the development of functional and structural impairments of the kidney. It is well established that HP diets can elevate the excretion rate of creatinine, which is caused by changes in RPF and GFR responding to increased protein intake. In one study, GFR measured by creatinine clearance in rats given HP diets (32% casein) was almost double that in the rats on low protein diets (10% casein) (Bouby,N. 1988). Animal on HP diets also have increased rates of protein excretion and proteinuria, kidney weight and size (Bertani,T. 1989; Hostetter,T.H. 1986; Itoh,H. 2002; Piepsz,A. 1994). In Bertani and colleagues' study rats on 42% protein diet developed higher serum creatinine by the end of the study compared to the other two diets (7% and 23% respectively) that remained unchanged over time. They also developed proteinuria and the average proteinuria

was higher than rats given 23% protein as energy. The molecular mechanism underlying HP-induced kidney hypertrophy is possibly attributed to insulin-like growth factor (IGF) and growth hormone (GH), which have pleiotropic effects on metabolism, cellular proliferation and differentiation. Serum and glomerular IGF levels are found to be elevated in rats fed HP diets and displaying renal hypertrophy (Hirschberg,R. 1991; Chin,E. 1994; Schrijvers,B.F. 2002).

Research in rodent animal models also demonstrates histopathologic evidence to support the finding of adverse effects of HP diets on the kidney in the long term. Rats and mice with intact kidneys exposed to diets with higher protein content (protein percent ranged from 23.2% to 44%) have a greater prevalence of developing nephropathy than those on diets with lower protein content. These histological changes include hypercellular glomeruli, thickening of capillary loops of the glomeruli, glomerular sclerosis, chronic inflammatory cell infiltration, tubular dilation or atrophy, tubule regeneration, tubular protein casts and tubulointerstitial fibrosis (Rao,G.N. 2001; Bertani,T. 1989; Hostetter,T.H. 1986; Itoh,H. 2002). Furthermore, some studies also demonstrated that HP–induced renal hypertrophy chiefly involves the inner stripe of the outer medulla, and the thick ascending limb (TAL) (Bouby,N. 1988; Goldstein,D.L. 2002). On the other hand, some other HP experiments did not demonstrate nephropathy after long-term follow-up (Lacroix,M. 2004; Piepsz,A. 1994). A recent study showed that after being given 50% HP diets for 6 months there was not any noticeable lesion in HP rat kidney. However, two rats of the HP group did exhibit renal interstitial lymphocytic inflammation, which is a known precursor of tubulointerstitial fibrosis. Moreover, some strains of rats remain small even when fed *ad libitum,* such as the Wistar, Long-Evans or Brown Norway strains, and are less susceptible to chronic progressive nephrosis. It means food intake may have less of an

effect on the development of CKD in these strains of rat compared with that in other strains (Baylis,C. 1998).

### 1.5 Early Mediators of Renal Disease

#### 1.5.1 Monocyte Chemoattractant Protein-1

##### 1.5.1.1 Introduction

The human monocyte chemoattractant protein-1 (MCP-1) gene (CCL2) is located on the 17q11.2-q21.1 and it consists of two exons (Reape,T.J. 1999). The MCP-1 protein belongs to the CC subfamily of chemokines and mediates chemotaxis of monocytes to inflammatory sites primarily through interactions with its CC chemokine receptor 2 (CCR2), which is a seven transmembrane G-protein-coupled receptor. MCP-1 is secreted by monocytes and various non-leukocytic cells, including endothelial cells, vascular smooth muscle cells, and renal cells, such as mesangial cells and tubular epithelial cells. The ligating of MCP-1 with CCR2 can promote macrophage adhesion and chemotaxis in disease sites. MCP-1 has been implicated in a number of diseases such as atherosclerosis, rheumatoid arthritis and cancer. In addition to being a vigorous chemoattractant to recruit monocytes/macrophages, MCP-1 has the following capacity: (1) recruit memory T cells and NK cells; (2) stimulate cytokine production by inflammatory and other cells; (3) induce tissue factor and other procoagulant effects; (4) activate and migrate endothelial cells; and (5) induce the proliferation and migration of smooth muscle cells (Daly,C. 2003; Charo,I.F. 2004).

### 1.5.1.2 Monocyte Chemoattractant Protein-1 and Kidney

The normal kidney expresses low amounts of MCP-1, but in glomerulonephritis, diabetic nephropathy, and partial nephrectomy MCP-1 levels become increased (Rovin,B.H. 1996; Lianos,E.A. 1994). MCP-1 plays an important role in the pathogenesis of progressive glomerulosclerosis and tubulointestitial fibrosis in different animal models. In a study that examined the relationship between rat renal tubular cells and proteinuric exposure, unstimulated renal cells expressed very low levels of MCP-1 mRNA. The expression of MCP-1 mRNA reached a peak (six fold greater than control) within 4 hours of exposure to albumin and was maintained for at least 24 hours with continued exposure. Removal of albumin from the media led to a rapid decline in MCP-1 mRNA expression (Wang,Y. 1997). In animal models, blocking MCP-1/CCL2 or CCR2 is associated with reduced interstitial macrophage infiltration and tubulointerstitial damage. MCP-1 gene deficient animals demonstrate diminished fibronectin deposition in the glomerulus and renal cortical fibronectin mRNA levels. (Kanamori,H. 2007; Chow,F.Y. 2007; Giunti,S. 2008).

There is conflicting evidence regarding the cell type which expresses MCP-1 in the kidney. Ota et al. find that mouse proximal tubular cells are the only site to express MCP-1 and are accompanied by significant macrophage infiltration to the tubulointerstitium. No significant expression of MCP-1 protein or mRNA is noted in the glomeruli (Wada,T. 2000; Ota,T. 2002). On the other hand, some studies show that MCP-1 also is capable of being synthesized in glomerular mesangial cells. In mesangial cells, MCP-1 binding to CCR2 induces a 2.5-fold increase in fibronectin protein levels at 24 h followed by a rise in pericellular fibronectin (Giunti,S. 2008). This may have importance in glomerulosclerosis induced by persistent glomerular

hypertrophy. However, no matter the site of MCP-1 expression, the underlying mechanism of increased MCP-1 synthesis might involve activation of NF-κB and TGFβ1 production in these cells (Donadelli,R. 2000; Giunti,S. 2008).

The expression of MCP-1 in urine can predict the severity of tubulointerstitial disease and the risk for loss of kidney function. Eardley and colleagues studied 215 patients with CKD, including diabetic nephropathy and found that the albumin–creatinine ratio correlated with urinary MCP-1, interstitial macrophage numbers, and index of chronic damage. The highest levels of MCP-1 and macrophage infiltratiton were observed in patients with the highest range of albuminuria. Urinary MCP-1 and interstitial macrophage numbers were highly correlated with the degree of chronic damage in patients. There is also a correlation between proteinuria and urinary MCP-1, and urinary MCP-1 and macrophage infiltration at the early stage of kidney damage (<20% chronic damage) (Cunningham,W. 2006; Conrad,K.P. 2004; Corsi,M.M. 1999; Eardley,K.S. 2008; Eardley,K.S. 2006). Both clinical and animal studies indicate that urinary levels of MCP-1 may respond quickly to anti-inflammatory therapies, which correlate with a decline in immunostaining for MCP-1 and CD68+ macrophages in the kidney. Treatment of patients with the angiotensin-converting enzyme (ACE) inhibitor reduced urine MCP-1 in type 2 diabetic nephropathy, and this correlated with the decline in proteinuria. Therefore, urinary MCP-1 has obvious clinical importance and is regarded as a new marker for follow up (Amann,B. 2003; Han,S.Y. 2004; Szeto,C.C. 2005; Tesch,G.H. 2008).

### 1.5.2 Transforming Growth Factor-beta1 (TGFβ1)

#### 1.5.2.1 Introduction

Transforming growth factor-beta (TGFβ) was the first discovered member of the TGFβ superfamily, which encompasses a large group of soluble extracellular proteins including TGFβ, activins, inhibins, growth and differentiation factors (GDF) and bone morphogenetic proteins (BMP) (Chang,H. 2002).There are three isotypes of TGFβ in mammals, TGFβ1, -2 and -3, all exhibiting similar biological activity. TGFβ1 is the most abundantly expressed isoform and is found predominantly in the immune system (Gorelik,L. 2002). TGFβ1 plays an important role in regulating many biological processes: (1) modulation of inflammatory cell function, (2) cell proliferation, differentiation and apoptosis; (3) control of extracellular matrix production. After TGFβ1 binding to its receptors, it activates a cascade of signal transduction events from the cell surface to the nucleus. Smad proteins, which modulate the activity of TGFβ ligands, are the central components of TGFβ signaling (Mehra,A. 2002; Feng,X.H. 2005).

#### 1.5.2.2 Transforming Growth Factor-beta1 and Kidney

Within the kidney, TGFβ1 is recognized among the TGFβ isoforms to play an important role in renal fibrogenesis and progressive kidney disease. TGFβ1 is known to mediate the formation of fibrotic kidney after chronic injury through Smad signalling pathways. Briefly, TGFβ1 activates the receptor-associated Smad-2 and Smad-3 which associate to form a heteromultimer with Smad-4. This complex is then translocated to the nucleus, where it can regulate target gene expression (Border,W.A. 1997; Liu,Y. 2006).

TGFβ1 has emerged as an important downstream mediator for the development of renal hypertrophy and the accumulation of mesangial extracellular matrix components. When plasma protein leaks across the glomerular basement membrane (GBM) into Bowman's space, the protein induces elevated TGFβ1expression in tubular cells, which can facilitate the development of interstitial fibrosis and tubular atrophy. Besides direct effects of albuminuria on tubular cells, glomerular hyperfiltration may lead to the changes in glomerular filtration barrier, and then lead to an increased filtration of TGFβ1, that may further alter the function of tubular cells. Wang and colleagues used a rat model with glomerular hyperfiltration and found that TGFβ1 was present in plasma in high molecular weight forms and was translocated into tubular fluid in rats with glomerular hyperfiltration states. At the same time, they incubated NRK-49F cells (an in vitro model of rat renal interstitial myofibroblasts) with TGFβ1 and found that it increased the expression of interstitial extracellular matrix proteins collagen $\alpha_2$ I, collagen $\alpha_1$ III and fibronectin about 2.5- to 4-fold, respectively (Wang, 2000). In addition, under locally high concentrations of TGFβ1and other growth factors, tubular cells may change their phenotype and become fibroblasts by a process called epithelial to mesenchymal transition (EMT). This is an important process which contributes to interstitial fibrosis and tubular atrophy. High dietary protein also can induce the production of advanced glycation end products (AGEs), which cause activation of TGFβ1 systems. An *in vitro* study showed that when elevated levels of amino acids designed to resemble the plasma profile produced by protein feeding were added to rat mesangial cell culture medium, it induced formation of AGEs and reactive oxygen species (ROS) in mesangial cells. The accumulation of AGEs produced an increased expression of TGFβ1 at both the protein and mRNA level (Tuttle,K.R. 2005). TGFβ1 also can cause podocyte apoptosis, and as a consequence,

the GBM can adhere to Bowman's capsule, which can contribute to the development of glomerulosclerosis (Wolf,G. 2007; Ziyadeh,F.N. 2008).

Human studies indicate that urinary excretion of TGFβ1 might be a good non-invasive prognostic factor for assessing the severity of renal damage. In one study, urinary TGFβ1 mRNA level was compared with the degree of histological damage from kidney biopsy and renal function indicators. The results showed that urinary TGFβ1 mRNA expression correlated significantly with estimated GFR and the degree of tubulointerstitial fibrosis. Moreover, urinary TGFβ1 gene expression correlated with its intra-renal expression in glomeruli and tubulointerstitium (Szeto,C.C. 2005). Other studies also indicate that participation of TGFβ1 in the development of diabetic nephropathy. TGFβ1 excretion correlates with GFR, thickness of glomerular basal membrane. TGFβ1 level even increases obviously in urine of patients with stage 1-2 CKD compared to that of normal controls (Liu,Y. 2006). An increased level of TGFβ1 in the urine is associated with worse clinical outcomes (Chen,S. 2003).

Furthermore, MCP-1 and TGFβ1 can affect each other's expression in kidney cells. Cheng's study demonstrated that TGFβ1 stimulated MCP-1 mRNA and protein expression of mesangial cells in a time- and dose-dependent manner. When mesangial cells were exposed to TGFβ1 (10 ng/ml), the MCP-1 level was increased significantly after 2-h treatment and maximal induction was observed at concentrations ≥5 ng/ml TGF-β1. The stimulatory effect of TGFβ1 persisted through 48 h (Cheng,J. 2005). Conversely, in a mesangial proliferative glomerulonephritis model, MCP-1 was rapidly induced after acute injury, promoted macrophage influx, and increased TGFβ1 expression (Schneider,A. 1999; Viedt,C. 2002). Viedt's study also found that MCP-1

was itself directly proinflammatory on the proximal tubule and at the same time it activated TGFβ1and other proinflammatory pathways in proximal tubular epithelial cells.

### 1.5.3 Endothelin-1

#### 1.5.3.1 Introduction

The endothelin (ET) family comprises 21-amino acid peptides and there are three members (ET-1, ET-2, and ET-3). ET-2 is very similar to ET-1, while ET-3 differs from ET-1 at 6 out of 21 positions (Goraca,A. 2002). ETs are synthesized not only in vascular endothelial and smooth muscle cells but also in neural, renal, pulmonary and inflammatory cells (Spieker,L.E. 2001). ET-1 has a major influence on the function and structure of the vasculature, which acts as the natural counterpart of the vasodilator nitric oxide (NO). ET-1 also contributes to regulating cell proliferation. ET-1 acts through $ET_A$ and $ET_B$ receptors to affect cellular reactions. Additionally, $ET_B$ receptors in the lung are a major pathway for the clearance of ET-1 from plasma (Masaki,T. 1994; Nakas-Icindic,E. 2004).

Physical factors such as stress or stimuli including thrombin, epinephrine, angiotensin II (Ang II), growth factors, cytokines and free radicals enhance secretion of ET-1. By contrast, mediators like NO, cyclic GMP, atrial natriuretic peptide, and prostacyclin reduce the release of endogenous ET-1. Thus, under normal conditions, the effects of the ET-1 are carefully regulated through inhibition or stimulation of ET-1 release from cells (Nakas-Icindic,E. 2004).

### 1.5.3.2 Endothelin-1 and Kidney

ET-1 is the major renal endothelin isoform produced by and the sources of ET-1 are not only the endothelium but also the mesangial and renal epithelial cells (Sorokin,A. 2003). Since 1991, many articles have been published which discuss the biological actions of ET-1 in renal disease. ET-1 involves the progression of kidney disease in the following ways: (1) Increases vascular resistance, decreases renal flow and glomerular filtration. There is a high density of binding sites for ET-1 in the glomeruli and ET-1 is a more potent vasoconstrictor than Ang II, another potent vasoconstrictor, and to have a greater effect on the GFR than Ang II (Nambi,P. 1992; van de Water,F.M. 2006). In addition, ET-1 can constrict peritubular capillaries and then lead to peritubular areas of ischaemia and adjacent tubules damage, which also contributes to tubulointerstitial fibrosis (Neuhofer,W. 2006). (2) Inhibits sodium and water reabsorption by the nephron. (3) Enhances glomerular cell proliferation and stimulates extracellular matrix formation. ET-1 activates a variety of signaling systems in mesangial cells to affect cell hypertrophy, proliferation, and increase renal cell fibronectin and collagen production, which lead to excessive accumulation of EMC and fibrosis (Naicker,S. 2001). Chronic treatment with an ET receptor antagonist attenuates increases in glomerular mRNA levels of collagen, laminin, tumor necrosis factor (TNF), TGFβ1 and basic fibroblast growth factor in diabetic rats (Ding,S.S. 2003). (4) Pro-inflammatory effect. Studies suggest that TGFβ is one of the most potent regulators of ET-1 expression in kidney tissue. In human fibroblasts, when combining ET-1 with TGFβ1, collagen production is more significant than with either of the substances alone (Dube,J. 2000). Daily administration of high doses of TGFβ2 in mice can result in significantly elevated ET-1 expression in kidney tissue and at the

same time cortical tubulointerstitial fibrosis and vasoconstriction is observed (Ledbetter,S. 2000).

In addition, increased urinary excretion of ET-1 has been observed in animal models and humans with glomerular disease and proteinuria, as well as some physical situations, such as pregnancy (Roccatello,D. 1994; Vlachojannis,J. 1997; Jeyabalan,A. 2007). In fact, instead of the increase in clearance from the circulation ET-1, the increase in urinary excretion is more likely associated with intra-renal production and tubular secretion. Adding bovine serum plasma into proximal tubular cells medium induced a significant dose-dependent increase in proximal tubular cell ET-1 synthesis (Zoja,C. 1995).

### 1.5.4 Regulated upon Activation, Normal T-cell Expressed and Secreted (RANTES)

#### 1.5.4.1 Introduction

Regulated upon activation, normal T-cell expressed and secreted (RANTES) (gene name CCL5) is a small protein of 68 amino acids and is also a kind of pro-inflammatory chemokine. It differs from other chemokines in that it is derived from a “late” expressed gene in T lymphocytes, normally expressed 3-5 days after T-cell activation, which is important for maintaining inflammation and facilitating expansion of the inflammatory cell infiltration. It regulates the activation and directs the migration of leukocytes in immune and inflammatory responses to the site of inflammation by binding to specific receptors CCR1, CCR3, CCR4 and CCR5 (Appay,V. 2001). Increased RANTES expression has been associated with a wide range of inflammatory, autoimmune and allergic disorders and pathologies, including allogenetic transplant rejection,

atherosclerosis, arthritis, atopic dermatitis, inflammatory airway disorders such as asthma, delayed-type hypersensivity reactions, glomerulonephritis, endometriosis, some neurological disorders (such as Alzheimer's disease) and certain malignant diseases (Graziano,F.M. 1999; Conti,P. 2001; Stasikowska,O. 2007). In addition to T cells and monocytes, it also acts on a range of other cells, including basophils, eosinophils, NK cells, dendritic cells and mast cells (Hebert,C.A.1999).

### 1.5.4.2 Regulated upon Activation, Normal T-cell Expressed and Secreted and Kidney

Like MCP-1, RANTES also belongs to CC chemokine beta subfamily and plays an important role in recruitment of lymphocytes and monocytes into tissue during kidney disease. RANTES is secreted basolaterally by tubular epithelial cells during proteinuria, and its expression attracts chronic inflammatory cells to the interstitium, modulating interactions between renal resident and inflammatory cells and participating in the chain of events leading to kidney fibrosis (Wang,S.N. 2000). When proximal tubular fluid from diabetic rats is added to cultured proximal tubular cells, there is significant increase in RANTES mRNA levels as well as protein levels. In the same study, there also was increased macrophage infiltration and interstitial collagen and fibronectin expression in kidneys from diabetic rats (Ruster,C. 2008). In a 10-year retrospective study of patients with type 2 diabetes, an independent positive correlation was found between the CCR5 59029A (+) genotype, one of RANTES receptors, and the onset or progression of diabetic nephropathy (Mizuno,M. 2006). Blockade of CCR1, another RANTES receptor, can reduce interstitial macrophages and T lymphocytes accumulation in rat with nephrotic syndrome and the renal fibrosis also was significantly reduced (Vielhauer,V. 2004).

## 1.6 Pig Model

Pigs have been regarded as an important mammalian model for human biology. One of the advantages of using the pig as a biomedical model is the average lifespan of 12-18 years allows long-term experiments evaluating the safety and efficacy of therapies, including dietary interventions the effects of which are relatively slow and subtle in the progression of disease.

Another advantage of pig model is the organ development. In primates, nephrogenesis is complete before birth. However, in rodents, kidneys are immature at birth and there is considerable postnatal renal development. Although in pigs, there are some immature glomeruli visible in the outer cortex at birth, glomeruli maturation is completed in the pig by the eighth week of postnatal life. The time frame for the maturation of pig renal circulation also is similar to that of human. The physiological kidney filtration barrier is already prominent in late fetal life (Buckley,N.M. 1986; Lumbers, E.R.1995).

Pig kidneys have similar anatomy and physiology to human kidneys, as is evidenced by the many xenotransplantation studies of pig kidney into human. These studies indicate that in addition to similar structure and relative size, kidney function also is similar between pig and human kidneys. The value of GFR of pig is close to that of human kidneys, which are 5 ml/min/kg and 4 ml/min/kg, respectively (Valentin,J.F. 1999; Kirkman,R.L. 1989). In addition, pig kidneys also have similar ability to handle body fluid volume, osmolarity and metabolites, such as urea, creatinine, ammonia and electrolytes (Valentin,J.F. 1999; Kirkman,R.L. 1989).However, they do have difference in handling proteins. In adult pigs, a normal level of proteinuria is 6 to 20 mg/100 ml. In contrast, the normal values are 0 to 8 mg/100 ml in healthy adult human’s urine, and protein in urine, even in small amounts, is considered to be a pathological situation

(Ibrahim,Z. 2006).

The pig has been widely used as an important model for human physiology studies. For example, it is reported that pig kidney is a good model to study metabolic pathways in the regulation of glomerular inflammatory and hemodynamic events. Livio and colleagues studied the pattern of arachidonic acid metabolism in isolated glomeruli from pig kidney by RIA, HPLC and GC-MS. By all these methodologies, they found that the main COX product was 6-keto-PGF1α and pig glomeruli converted arachidonic acid via the lipoxygenase pathway into mono-HETEs, which indicates the functional similarity between human and pig kidney (Livio,M. 1988).

The pig also is regarded as a good model of different human diseases since it can reliably recapitulate disease pathogenesis and reflects the morphological and biochemical aspect of diseases. There are many successful pig models of diseases afflicting humans, including diabetes, cancer, gastrointestinal, infectious and metabolic diseases (Schook,L. 2005; Cooper,D.K. 2002).

**1.7 Hypothesis and Objectives**

Therefore, adult female pigs were used as an animal model to test the following hypotheses:

Long-term protein intake at the upper end of the AMDR (35% of energy intake from protein) is detrimental to renal health. Specifically,

(1) Long-term HP diets will have harmful effects on renal function;

(2) Long-term HP diets will cause increased glomerular size and development of kidney fibrosis; and

(3) Long-term HP diets will increase the renal production of early mediators of progressive renal disease.

The alternative hypothesis for this study is that long-term high dietary protein intake

has no adverse effects on renal health.

To test these hypotheses, female, non-pregnant pigs (Genesus) at 7 months will receive diets with either 15% or 35% of energy as protein. The objectives are as follows:

(1) To determine the long-term safety of a diet containing 35% of energy as protein by determining effects on renal function, using proteinuria, creatinine clearance and inulin clearance as indicators;

(2) To evaluate the possible renal histological changes caused by long term consumption of HP diets, including glomerular volume and kidney cortical fibrosis as indicators;

(3) To identify the effect of dietary protein on renal production of MCP-1 and TGFβ1, which may contribute to renal damage.

# 2 LONG TERM EFFECTS OF DIETARY HIGH PROTEIN ON RENAL HEALTH IN THE PIG MODEL

## 2.1 Abstract

The impact of habitually consuming a high protein (HP) diet at the upper limit of the acceptable macronutrient distribution range (AMDR) on kidney health is unknown. The current study was designed to test the hypothesis that long-term consumption of a diet providing 35% of energy as protein will have negative consequences on renal health, as assessed in a pig model. **Methods:** Adult female, non-pregnant, commercial pigs (Genesus) were randomized to receive either NP (15% energy from protein) or HP (35% energy from protein) isocaloric diets for either 4 or 8 months. Diets contained whole protein sources with an animal: plant ratio of 2:1 in the NP diet to mimic the average Canadian diet. The increased protein in the HP diet was achieved by increasing egg and dairy protein sources. Body composition was measured by dual-energy X-ray absorptiometry. Glomerular volume and kidney fibrosis were evaluated on kidney sections by quantitative image analysis. The inflammatory marker monocyte chemoattractant protein-1 (MCP-1) and the growth factor transforming growth factor beta-1(TGF$\beta$1) were assessed in renal tissue using commercial ELISA kits. **Results:** Pigs given the HP diet had lower body weights and percentage of body fat. Pigs consuming the HP diet had significantly higher glomerular filtration rates (GFR) and larger kidneys. Renal MCP-1 levels and renal fibrosis also were significantly higher in pigs given the HP diet, while proteinuria and renal TGFβ1 expression did not differ. **Conclusion:** These findings suggest that, despite the potential benefit of the HP diet on body composition, long-term intakes of protein at the upper limit of the AMDR may compromise renal health in healthy female pigs.

Key Words: Pig model; High protein diet; Kidney health

## 2.2 Introduction

The AMDR for protein has been set at 10-35% of energy. Nevertheless, the Institute of Medicine (IOM) committee for the Dietary Reference Intakes (DRI) for macronutrients indicated that there is lack of sufficient data on the long-term safety of the upper limit of this range (IOM, 2002). HP diets are increasingly being recommended as one of the management strategies for weight loss and weight control in overweight and obese individuals. Reduced calorie HP diets are as effective as low fat diets with respect to loss of body mass, fat mass, and retention of lean mass, especially in the short term (within 6 month) (Halton, T.L. 2004; Cunningham, W. 2006). However, in view of the high prevalence of chronic kidney disease (CKD) among the general population, it is important to understand the potential effect of HP diets on kidney health. According to the National Health and Nutrition Examination Survey (NHANES) III the prevalence of CKD in the US adult population is 11% (19.2 million). Assuming a similar prevalence, it is estimated that 2 million Canadians have CKD (Coresh,J. 2003; KFC, 2007).

Habitual consumption of HP leads to increased renal workload and hemodynamic changes in the kidney by causing an increase in GFR and morphologic changes, including renal and glomerular hypertrophy (Piepsz,A. 1994; Pullman,T.N.1954; Skov,A.R. 1999; Goldstein,D.L. 2002; Itoh,H. 2002). Proteinuria is recognized as a risk factor for kidney disease in healthy populations (Gerstein,H.C. 2001; Furtner,M. 2005). Some reports indicate that the risk for microalbuminuria among the healthy population progressively increases with greater daily protein ingestion (Hoogeveen,E.K. 1998), but others have not (Skov,A.R. 1999; Knight,E.L. 2003).

Animal studies also indicate adverse effects of HP diets on the kidney in the long term. Rats and mice exposed to HP diets have a greater prevalence of developing

nephropathy, including glomerular hypertrophy, glomerulosclerosis, tubulo-interstitial fibrosis, tubule regeneration, and chronic inflammatory cell infiltration (Rao,G.N. 2001; Bertani,T. 1989; Hostetter,T.H. 1986; Itoh,H. 2002). Proinflammatory chemokines and growth factors such as MCP-1 and TGFβ1 may play an important role in early stages of kidney damage induced by HP diets, as kidney cells exposed to a high concentration of albumin have elevated MCP-1 and TGFβ1 expression (Wang,Y. 1997; Wang,S.N. 2000). Chronic inflammatory cells attracted by MCP-1 can interact with renal cells, and can become activated, proliferate, transform and synthesize excessive extracellular matrix (ECM), and ultimately, lead to kidney fibrosis (El Nahas,M. 2005). Suppression of MCP-1 or TGFβ1 expression can slow down the progress of kidney damage, demonstrating their role in the progression of renal injury development (Chow,F.Y. 2007). In addition, endothelin-1(ET-1) and regulated upon activation, normal T-cell expressed and secreted (RANTES), another pro-inflammatory chemokine, also are involved in the progression of kidney disease. They may enhance glomerular cell proliferation, attract chronic inflammatory cell accumulation and stimulate fibronectin and collagen production, which lead to excessive accumulation of EMC and kidney fibrosis (Dube,J. 2000;Ledbetter,S. 2000; Wang,S.N. 2000; Ruster,C. 2008).

Ethical issues and the length of study required to examine the effects of HP diets precludes human studies. The pig kidney has been proposed as a model for the human kidney because, like other primates and unlike rodent kidneys, nephrogenesis is complete before birth. Pig kidneys also have similar anatomy, physiology and ability to handle body fluid volume, osmolarity and metabolites, such as urea, creatinine, ammonia and electrolytes (Valentin,J.F. 1999; Kirkman, R.L.1989). In addition, the pig kidney is reported to be a good model to study metabolic pathways in the regulation of

glomerular inflammatory and hemodynamic events (Livio,M. 1988). Therefore, the long term effect of dietary protein intake at the upper limit of the AMDR on kidney health was examined in female adult pigs. Females were selected because this population is often the target of weight loss and has more risk factors for bone disease (Ersoy,F.F. 2007).

## 2.3 Materials and Methods

### 2.3.1 Animal and Diet

Adult female non-pregnant pigs at 7 months of age were randomized to receive isocaloric diets containing either 15% of energy as protein (NP diet) or the 35% of energy as protein (HP diet) (Table 2, Appendix 6.1.1-6.1.2). These levels were selected since 15% mimics the average protein intake of Canadians (Statistics Canada, 2007) while 35% represents the upper end of the AMDR (IOM, 2002). Whole protein sources in a 2:1 ratio of animal to plant proteins in the NP diet were used (Appendix 6.1.3), which represents the typical Canadian dietary protein intake. The proportion of meat, dairy and egg protein also reflected the average population dietary intake (Smit,E. 1999). HP diets were achieved by increasing egg albumin and skim milk content, which are the common ways to increase protein intake by individuals. These two diets were balanced for fat, minerals and vitamins and the nutrient levels met the requirements of the pig (National Research Council, 1998).

Pigs were housed by diet group and body weights were recorded every two weeks and feed disappearance every week. There were 30 pigs on each diet at the beginning of this study but 8 from the NP and 5 from the HP diet were removed from the study because of feet problems. Eight pigs on each diet were terminated after 4 months, and 14 from the NP diet and 17 from the HP diet were terminated after 8 months. All the

procedures were approved by the University of Manitoba Animal Care Committee and followed the guidelines of the Canadian Council on Animal Care.

### 2.3.2 Body Composition

The right ham from each pig was analyzed for composition by dual energy x-ray absorptiometry (DEXA) (Lunar BX-1 L-8743, GE Healthcare). The ham was put in the leg position on the scanning bed and the “human” module was used for scanning. In addition, all of the right carcasses from the 4-month termination were divided into five parts and scanned, including the shoulder, posterior and anterior loins, belly and ham, and also were analyzed by DEXA. The data from different parts were added up to determine whole body composition. Comparison of the whole body data with that from the ham verified that the ham represented the entire carcass (Appendix 6.5.1-6.5.3). The software used to analyze composition was Encore 2005 (GE Healthcare).

**Table 2** Diet ingredient and composition

| Ingredient | NP Diet | HP Diet |
|---|---|---|
| | g/100 g diet | |
| **Wheat**[1] | 30.4 | 30.4 |
| **Barley**[1] | 7.6 | 7.6 |
| **Low Ash Poultry Meal**[2] | 4.4 | 4.4 |
| **Pork Meal**[2] | 4.4 | 4.4 |
| **Egg Albumen**[3] | 0.8 | 22.2 |
| **Skim Milk Powder**[4] | 7.8 | 13.5 |
| **Sucrose**[5] | 14.6 | 6.1 |
| **Corn Starch**[6] | 23.1 | 9.6 |
| **Lactose**[7] | 3.13 | 0 |
| **Lard**[8] | 0.57 | 0 |
| **Canola**[9] | 1.42 | 1.42 |
| **Mineral and Vitamin Mix**[10] | 0.25 | 0.25 |
| **Composition** | | |
| **Protein** | 13 | 31 |
| **Fat** | 4 | 4 |
| **Carbohydrate** | 83 | 65 |
| **NDF**[11] | 1.14 | 1.14 |
| **ME**[12]**(kcal)** | 3472 | 3528 |
| **Calcium** | 0.75 | 0.75 |
| **Phosphorus** | 0.49 | 0.49 |
| **Potassium** | 0.54 | 0.54 |
| **Sodium** | 0.25 | 0.25 |
| **Zinc(ppm)** | 187 | 190 |
| **CP/ME**[12] **(%)** | 15 | 35 |

[1] The Puratone Corporation, Niverville, MB
[2] Rothsay, Winnipeg, MB
[3] Micheal Foods, Minnetonka, MN
[4] Parmalat, St. Claude, MB
[5] Upper Canada Malt, Burlington, ON
[6] Casco Inc., Etobicoke, ON
[7] Davisco, Eden Prairie, MN
[8] White Cap Frozen Foods, Winnipeg, MB

[9] Canbra Foods, Lethbridge, AB
[10]Landmark Feeds, Winnipeg, MB
[11] NDF= neutral detergent fiber
[12]CP = crude protein; ME = metabolizable energy

### 2.3.3 Renal Function

One week prior to termination, animals were individually housed in metabolic crates. Two days before termination pigs were lightly sedated and a foley catheter was introduced into the bladder for urine collection and a 22 ga-25 mm cannula was introduced into an ear vein for inulin infusion. After one day, 24-hour urine was collected and weighed to calculate the volume and a sample was frozen at -80ºC for further analysis. On the day of termination, a primed (60 mg/kg)-continuous (2 mg/kg/min) infusion of inulin was initiated to evaluate GFR (Ling,W.D. 1989). After allowing 2 hours to reach steady state, timed urine was collected to calculate urine flow (Appendix 6.2.1). At the end of the collection, a blood sample was obtained from the ear vein. After infusion, animals were terminated with an overdose of xylazine and azaperone.

Urinary protein concentrations were determined by protein assay using the Bradford method (Bradford, 1976; Appendix 6.2.3). Urinary and serum creatinine concentrations were determined by a method based on the creatinine-picrate reaction (Heinegard,D. 1973; Appendix 6.2.2).

### 2.3.4 Morphology

At termination, kidneys, liver and spleen were removed and weighed. Kidneys were measured before a transverse incision of the kidney from the middle portion was made to allow optimal examination of the renal pelvis, renal papilla and the junction with the ureter. An integrated section of kidney from the upper pole of the left kidney was sampled and included portions of both the cortex and medulla. Kidney tissues were fixed in 10% formalin prior to embedding in paraffin and sectioned at 5 microns.

Sections stained with hematoxylin and eosin or Masson's Trichrome to evaluate glomerular volume or kidney cortical fibrosis, respectively (Appendix 6.3.1-6.3.2). Randomly selected areas (30 per kidney) with glomeruli were captured using the 10X objective. Image Pro version 6.0 software was used to measure the largest diameter of each glomerulus. Glomerular volume was calculated as described (Sankaran,D. 2007; Appendix 6.3.1). To evaluate fibrosis, the collagen density was determined as described by Sung and O (Sung,F.L. 2002; Appendix 6.3.2). In brief, images were randomly captured from different zones of the kidney containing both glomeruli and tubulointerstitial areas, using a 40X objective. Analysis was done in Adobe Photoshop CS3 Extended program. All blue colors in the picture were selected using magic wand tool and the density was measured.

### 2.3.5 Renal MCP-1, TGFβ1, RANTES and ET-1

A portion of cortex sampled from the upper pole of the left kidney was lyophilized and 30 mg was homogenized on ice in 3 mL of ice-cold homogenization buffer with Triton X-100 for a total of 60 seconds using a Polytron homogenizer (Cuozzo,F.P. 2002; Appendix 6.4.1-6.4.2). Renal TGFβ1 (MB 100B, R&D Systems, Minneapolis, MN), MCP-1 (KHC1012, BioSource International, Camarillo, California), RANTES (DRN00B, R&D Systems, Minneapolis, MN) and ET-1(BI-20052, BioMedica, Austria) levels were determined following Enzyme-Linked ImmunoSorbent Assay (ELISA) kit instructions (Appendix 6.4.4.1-6.4.4.4)and data were presented based on protein content (Bradford, 1976).

### 2.3.6 Statistical Analysis

Data were analyzed by 2X2 ANOVA, with diet and time as factors, using the GLM procedure of SAS software (SAS, version 9.1, Cary, NC). Normality of the data was assessed using the Shapiro-Wilk's Statistic (W >0.05). If the data did not follow a normal distribution, transformation was used to achieve normality of the data. If data could not be normalized the MIXED procedure was used. Diet and time effects were considered significant at $p < 0.05$. If interactions were present ($p<0.05$), Tukey-Kramer comparisons were used to test differences among groups. Body weights from 0 to 8 months were analyzed by repeated measures analysis, using GLM procedure. Values were expressed as mean ± SE.

## 2.4 Results

### 2.4.1 Feed Intake, Body Weight and Body Composition

Feed intakes in pigs on the HP and NP diets were similar and pigs on HP diets had higher daily protein intake (Table 3, Figure 1), but overall, pigs given the HP diets had ~ 5% lower body weight than NP diets (Table 3). Repeated measures analysis of body weights from 0 to 4 months (n=24-25) revealed that pigs on HP diet weighed less at all time points except at week of 0 (Figure 2). Repeated measures analysis of body weights of only pigs that were terminated at the 8 month time point (n=16-17) revealed that pigs on HP diet weighed less than on NP diet only at weeks of 2 and 4 (Figure 3). Further, hams from pigs on the HP compared to the NP diet had lower fat percent, fat mass and higher lean percent. Post hoc tests revealed that the difference in body weight, ham fat percent, lean percent and fat mass between pigs on HP and NP diets was significant at 4 months, not at 8 months. There were no significant differences in

ham lean mass, bone mineral density (BMD) and bone mineral content (BMC) between pigs on HP and NP diets (Table 3).

When the ham data was used to estimate whole body composition, pigs given HP had lower body fat mass than those given NP diets and there were no difference in lean body weight and body BMC (Table 3).

### 2.4.2 Renal Function

Overall, pigs given the HP had higher inulin clearance rates than the NP diets when expressed relative to body weight. Protein intake did not appear related to changes in urinary protein excretion per 24 hours, urinary protein per urinary creatinine and creatinine clearance (Table 4).

### 2.4.3 Morphologic Studies

Gross examination showed that kidneys from pigs given the HP diets were heavier and larger compared to those given NP diets. Since the pigs given the HP diets weighed less, kidney weights relative to body weights in pigs given the HP diets were ~18% higher compared to those given NP diets. In addition, liver weights relative to body weights were ~10% higher in pigs on HP compared to NP diets. There was no difference in spleen weights (Table 3). Diet by time interaction was present when analyzing kidney volume. Post hoc analysis revealed that renal hypertrophy occurred at both 4 and 8 months in pigs given the HP diets (Figure 4, Appendix 6.3.3). Cysts with clear yellow fluid and smooth walls were observed in kidney cortex from pigs given both HP and NP diets. Pigs with cysts were randomly distributed in each group, 11 in HP and 9 in NP.

Under microscopic examination, it was observed that some glomeruli had obviously

larger diameters and the Bowman's space was occupied by enlarged glomeruli in the kidneys from pigs given the HP diets. Hypercellular glomeruli also were detectable and open capillary loops were not noticeable in kidneys from pigs given HP diets. Obvious collagen deposition around the glomerular and tubular areas and chronic inflammatory cell infiltration in the tubulointerstitial area also were observed in kidneys from pigs given the HP diets. Compared with scattered chronic inflammatory cell infiltration at 4 months, chronic inflammatory cell foci in the tubulointerstitial area were observed in kidneys from 8 month pigs on HP diets. Therefore, glomerular volume and cortical fibrosis were quantitated. Post hoc analysis revealed that glomerular hypertrophy occurred at both 4 and 8 month when relative to body weight and only at 8 month when relative to kidney weight in kidneys from pigs given the HP diets (Figures 5-7; Appendix 6.3.3). Further, the density of collagen staining revealed that cortical fibrosis was 28% higher in kidneys from HP compared to NP diets (Figures 8-9; Appendix 6.3.3). In addition, the walls of the cysts all were lined with flattened epithelium, indicating renal simple cysts which is not associated with diseased kidneys (Bisceglia,M. 2006).

### 2.4.4 Early Mediators of Renal Disease

Renal MCP-1 levels were higher in kidneys from pigs given the HP diets (Figure 10; Appendix 6.6), consistent with the apparent increase in chronic inflammatory cells observed in sections from these pigs. With respect to renal TGFβ1, there was no significant dietary effect between pigs on HP and NP diets (Figure 11; Appendix 6.6). The concentrations of RANTES and ET-1 were below the limit of detection (Appendix 6.7).

**Table 3** Food intake, body and organ weights and body composition in pigs given normal protein (NP) or high protein (HP) diet[1]

| | 4-month | | 8-month | | Main Effects | |
|---|---|---|---|---|---|---|
| | NP | HP | NP | HP | Diet | Time |
| | n= 8 | n= 8 | n= 14 | n= 17 | p | p |
| **Food intake[2] (kg/day)** | 2.7±0.4[3] | 2.2±0.3[4] | 2.8±0.1[5] | 2.5±0.2[6] | 0.1430 | 0.5898 |
| **Protein intake[2](g/day)** | 357±57[3] | 693±81[4] | 366±16[5] | 764±61[6] | <0.0001 | 0.5362 |
| **Body weight (kg)** | 254±10 | 219±10 | 274±8 | 270±8 | 0.0495 | 0.0004 |
| **Kidney weight (g)** | 302±13 | 331±26 | 314±13 | 347±14 | 0.0793 | 0.4320 |
| **Kidney weight (g/kg BW)** | 1.25±0.04 | 1.60±0.14 | 1.15±0.03 | 1.30±0.04 | 0.0002 | 0.0029 |
| **Liver weight (g)** | 2887±178 | 2859±110 | 2680±73 | 2942±114 | 0.3561 | 0.6198 |
| **Liver weight (g/kg BW)** | 12±0.7 | 13±0.8 | 10±0.2 | 11±0.4 | 0.0111 | <0.0001 |
| **Spleen weight (g)** | 936±71 | 1006±78 | 940±96 | 990±89 | 0.5591 | 0.9565 |
| **Spleen weight (g/kg BW)** | 3.7±0.3 | 4.6±0.3 | 3.4±0.4 | 3.7±0.4 | 0.1599 | 0.1177 |
| **Carcass weight[7] (kg)** | 105±3 | 90±4 | 118±3 | 116±4 | 0.0378 | <0.0001 |
| **Ham lean (%)** | 68.4±1.7 | 74.8±2.1 | 62.7±1.6 | 63.8±1.5 | 0.0482 | <0.0001 |
| **Ham lean mass (kg)** | 16.82±0.71 | 16.09±0.47 | 15.89±0.32 | 16.10±0.52 | 0.6353 | 0.4121 |
| **Ham fat (%)** | 31.6±1.7 | 25.2±2.1 | 37.3±1.6 | 36.3±1.5 | 0.0490 | <0.0001 |
| **Ham fat mass (kg)** | 7.80±1.78 | 5.79±1.87 | 9.74±2.88 | 9.32±2.78 | 0.0460 | 0.0004 |
| **Ham BMC (g)** | 605±21 | 601±25 | 738±21 | 766±32 | 0.7081 | <0.0001 |
| **Ham BMD(g/cm$^2$)** | 1.40±0.03 | 1.45±0.03 | 1.46±0.03 | 1.49±0.04 | 0.3090 | 0.1434 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **Body fat mass (kg)[8]** | 100±6 | 74±8 | 121±8 | 114±6 | 0.0445 | 0.0004 |
| **Lean body weight (kg)[8]** | 157±5 | 152±2 | 161±4 | 163±4 | 0.8034 | 0.1395 |
| **Body BMC(g)[8]** | 5477± 200 | 5269±173 | 6679±6715 | 6715±277 | 0.7614 | <0.0001 |

[1]Values are the means±SE, NP diet contained 15% and HP diet contained 35% protein as energy.
[2]Data from metabolic crates
[3] n=6; [4] n=4; [5] n=8; [6] n=12, outliers excluded if the plotted residuals exceeded 3x root mean squared error.
[7] Values are right side of carcass
[8]Values are estimated from ham data.

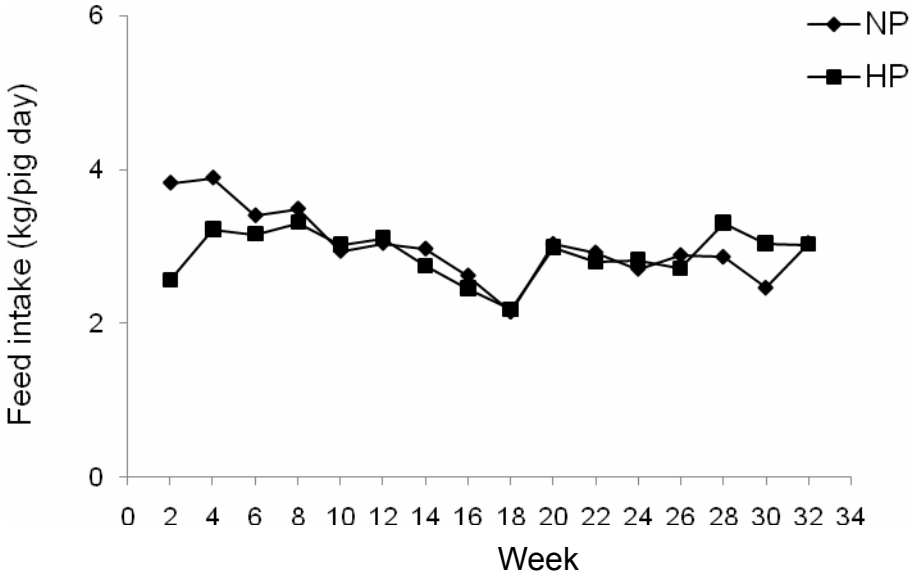


**Figure 1**: Average biweekly feed intakes of pigs given normal protein (15% of energy) and high protein (35% of energy) diets (n=17-30).

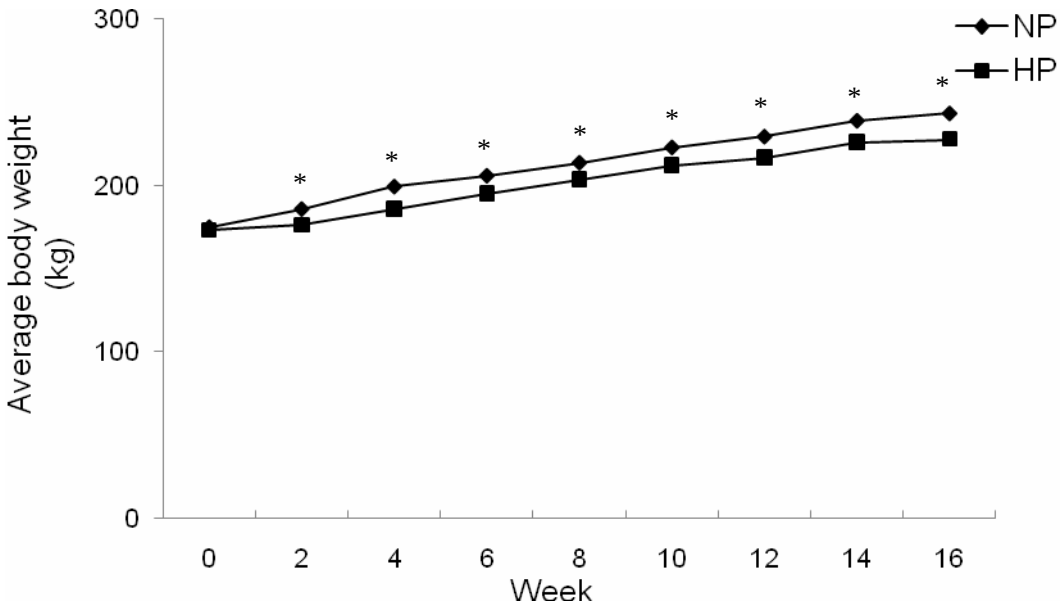


**Figure 2**: Repeated measures of body weights from 0 to 4 months. * $p<0.05$ (n=24-25).

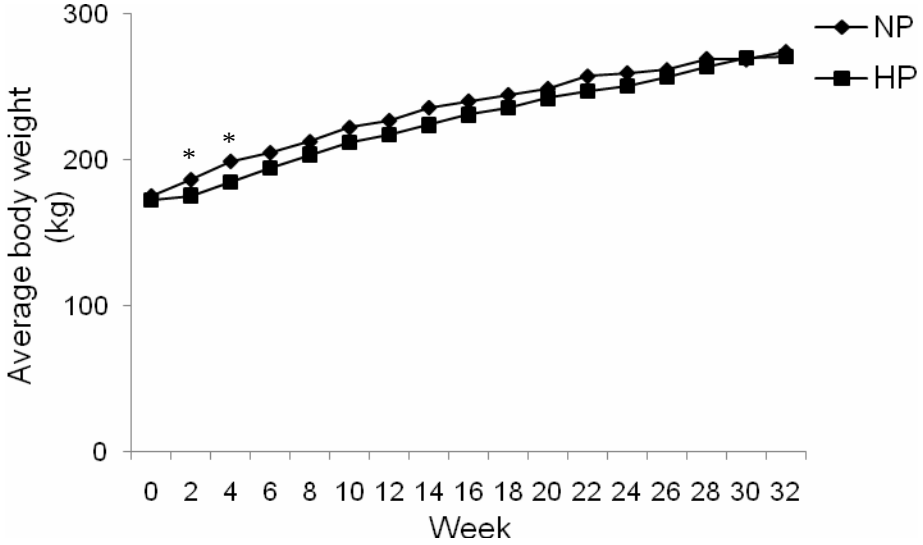


**Figure 3**: Repeated measures of body weights from 0 to 8 months of only pigs that were terminated at the 8 month time point.
* $p<0.05$ (n=16-17).

**Table 4** Parameters for renal function

| | 4-month | | 8-month | | Main Effects | |
|---|---|---|---|---|---|---|
| | NP n=8 | HP n=8 | NP n=12 | HP n=12 | Diet p | Time p |
| **Inulin clearance (ml/min)** | 412±167[1] | 541±141[2] | 154±22[3] | 197±40[4] | 0.2586 | 0.0009 |
| **Inulin clearance (ml/min/kg BW)** | 1.50±0.52 | 2.61±0.60 | 0.58±0.09 | 0.76±0.16 | 0.0495 | 0.0001 |
| **Inulin clearance (ml/min/kg LBW[5])** | 2.74±1.12 | 3.72±1.17 | 1.00±0.15 | 2.74±1.12 | 0.2811 | 0.0001 |
| **Creatinine clearance (ml/min)** | 381±85 | 530±119 | 516±62 | 555±73 | 0.2682 | 0.3448 |
| **Creatinine clearance (ml/min/kg BW)** | 1.60±0.37 | 2.36±0.50 | 1.86±0.21 | 1.99±0.27 | 0.1815 | 0.8656 |
| **Creatinine clearance (ml/min/kg LBW[5])** | 2.42±0.53 | 3.14±0.77 | 3.18±0.36 | 3.49±0.45 | 0.3245 | 0.2817 |
| **Urinary protein (mg/24 hr)** | 767±309 | 1021±297 | 3640±861 | 4890±2291 | 0.6242 | 0.0001 |
| **mg Urinary protein/ g creatinine** | 98±28 | 101±16 | 382±81 | 485±176 | 0.7501 | <0.0001 |

[1]n=4; [2]n=6; [3]n=11; [4]n=10; [5]LBW=lean body weight

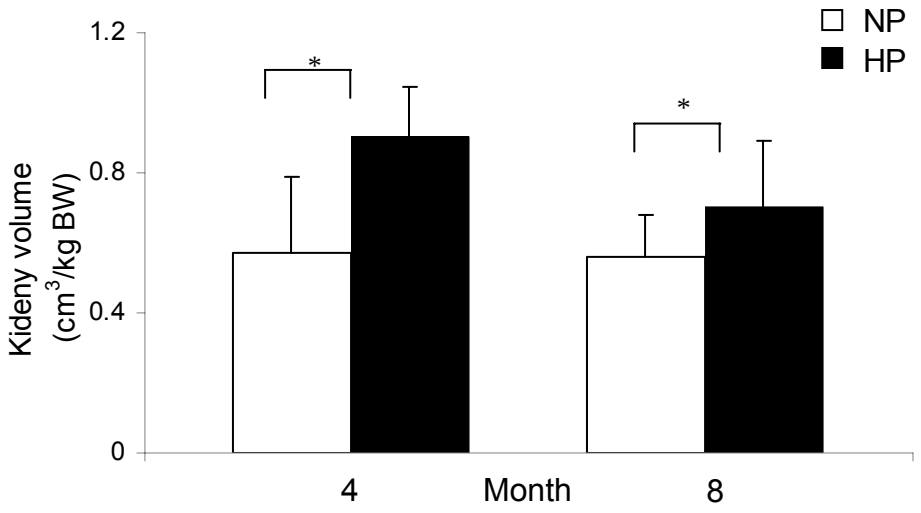


**Figure 4:** Kidney volume per kg body weight of normal protein (NP) and high protein (HP) pigs. Diet, p<0.0001, Time, p=0.0179, Diet*Time=0.035, n=14-17. * p<0.05. The data is expressed in table from Appendix 6.3.3.

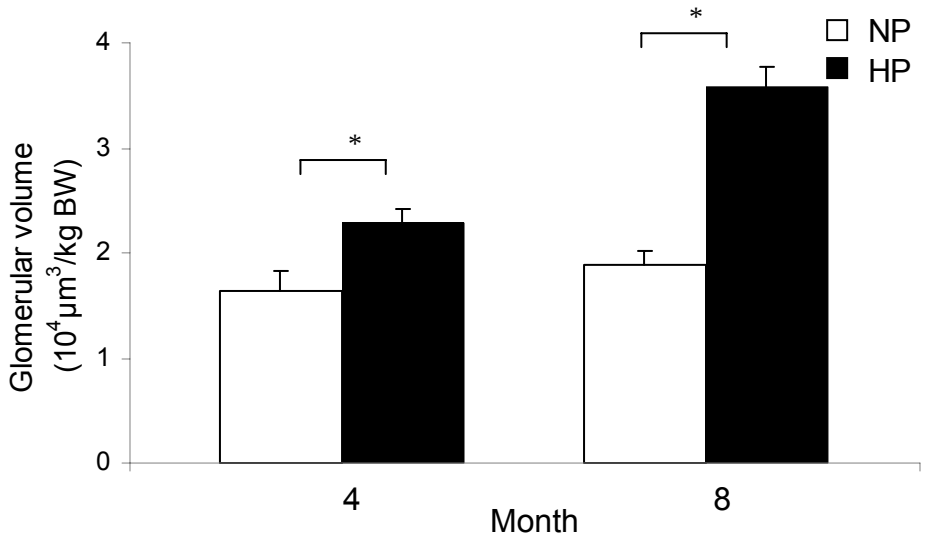


**Figure 5:** Glomerular volume of kidneys from pigs consuming normal protein (NP) or high protein (HP) diets, expressed relative to body weight. Diet, $p<0.0001$, Time, $p<0.0001$, Diet*Time=0.0034, n=14-17. * $p<0.05$. The data is expressed in table from Appendix 6.3.3.

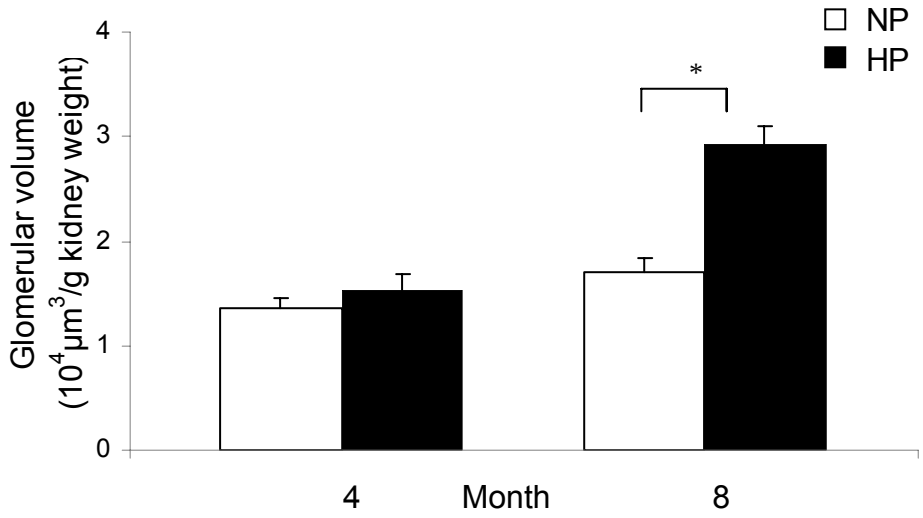


**Figure 6:** Glomerular volume of kidneys from pigs consuming normal protein (NP) or high protein (HP) diets, expressed relative to kidney weight. Diet, p=0.0001, Time, p<0.0001, Diet*Time=0.0133, n=14-17. * p<0.05. The data is expressed in table from Appendix 6.3.3.

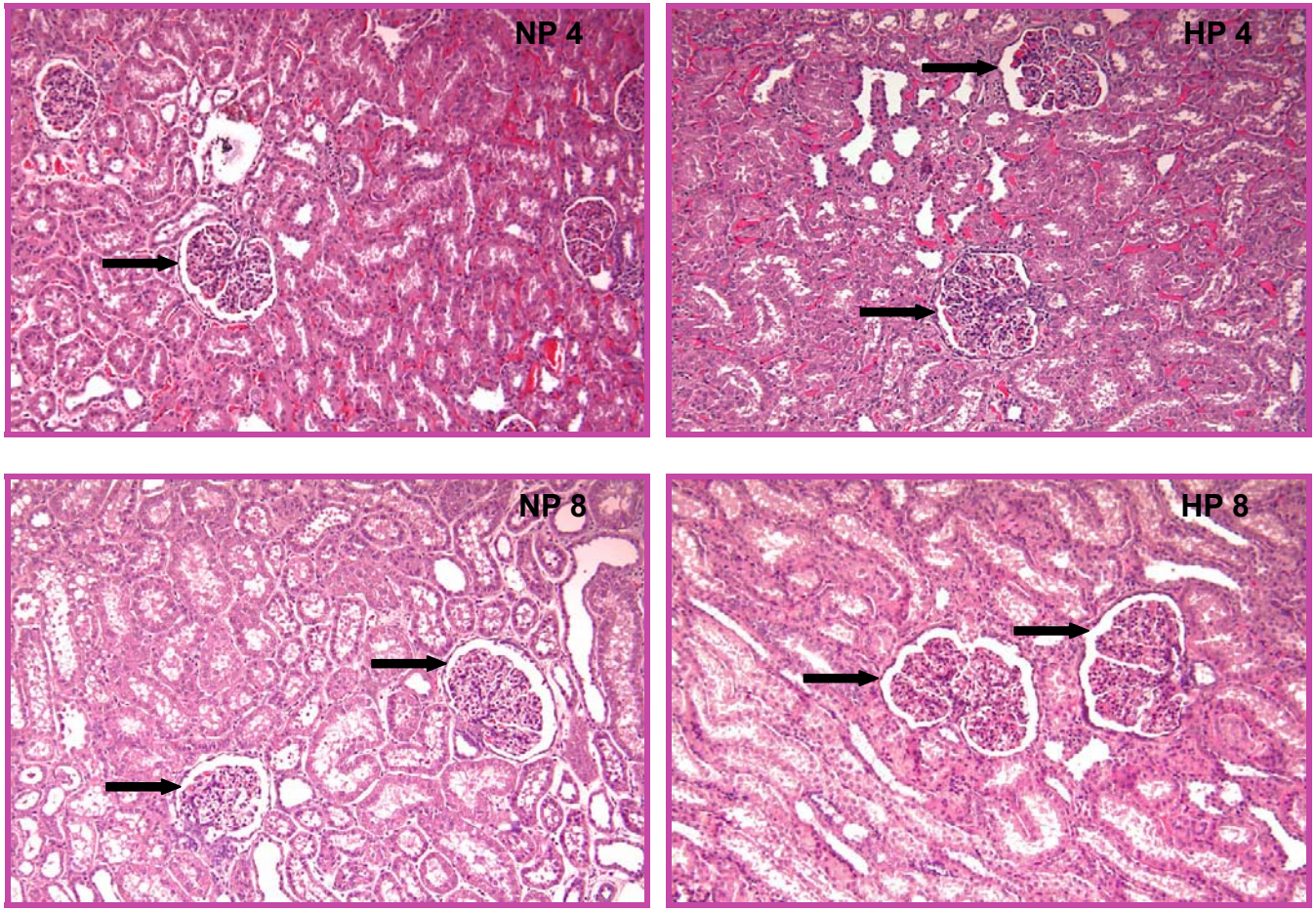


**Figure 7:** Cross section of glomeruli at 4 and 8 months stained with H&E, at 10X magnification. Arrows point to glomeruli.

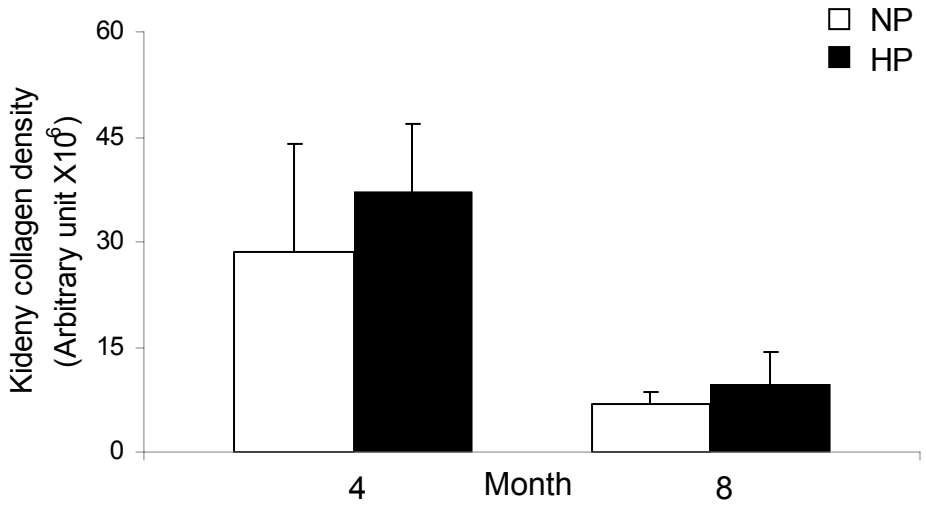


**Figure 8:** Density of collagen staining in kidneys from pigs consuming normal protein (NP) or high protein (HP) diets. Diet, p=0.0128, Time, p<0.0001, Diet*Time=0.8842, n=14-17. The data is expressed in table from Appendix 6.3.3.

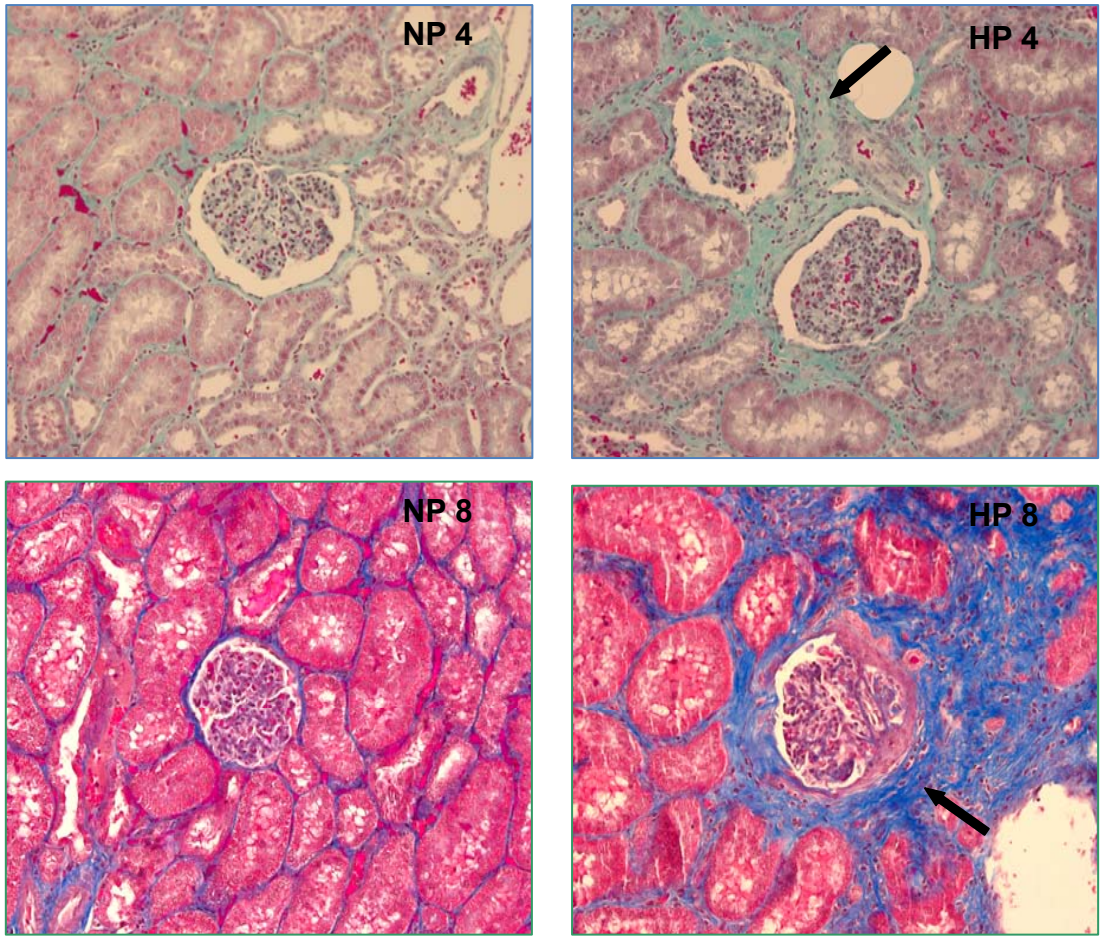


**Figure 9:** Kidney cortex at 4 and 8 months stained with Masson's TriChrome at 10X magnification. Arrows point to fibrosis.

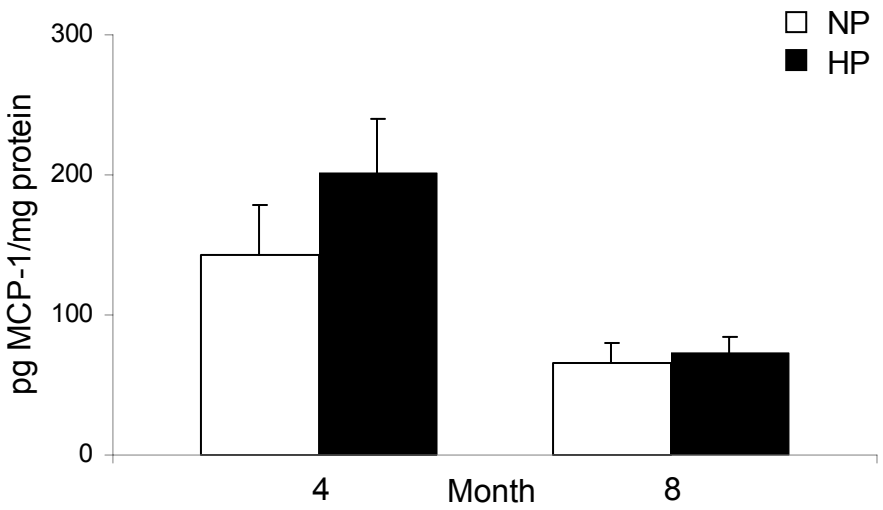


**Figure 10:** Renal MCP-1 levels in pigs given normal protein (NP) or high protein (HP) diets. Diet, p=0.0218 and Time, p <0.0001, n=14-17. The data is expressed in table from Appendix 6.6.

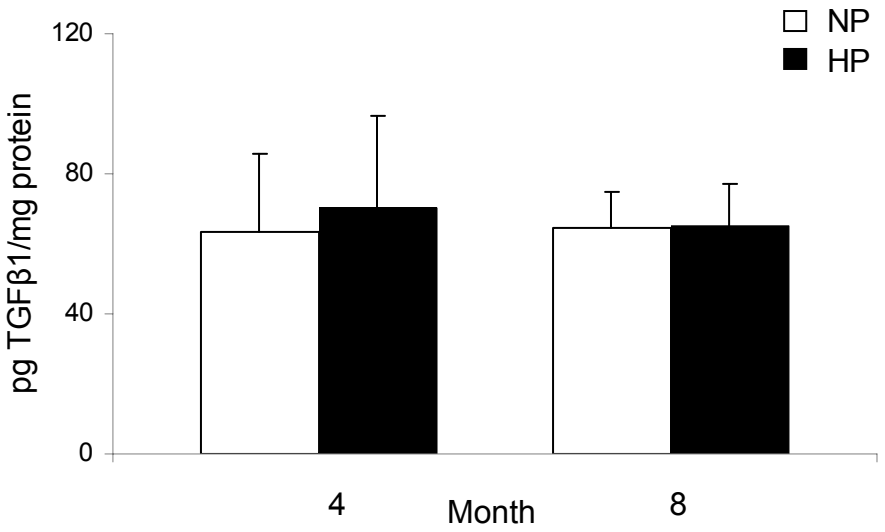


**Figure 11:** Renal TGFβ1 levels in pigs given normal protein (NP) or high protein (HP) diets. Diet, p=0.6165 and Time, p=0.3228, n=14-17. The data is expressed in table from Appendix 6.6.

## 2.5 Discussion

This study demonstrates that long-term dietary protein intake at the upper range of the AMDR for protein may have detrimental effects on renal health in adult female pigs. High dietary protein resulted in increased GFR, renal and glomerular hypertrophy, increased renal MCP-1 expression and kidney cortical fibrosis (including glomerulosclerosis and tubulointerstitial fibrosis).

The Brenner hypothesis (hyperfiltration hypothesis) provides a framework for the effect of protein intake on the progression of kidney disease. It states that habitual consumption of excessive dietary protein negatively impacts kidney health by causing a sustained increase in glomerular hyperfiltration and glomerular hypertension. These situations can be reflected by changes in GFR and renal hypertrophy, which then might lead to renal inflammation and renal cell proliferation, and ultimately cause progressive renal failure (Brenner,B.M. 1982).

Long-term high dietary protein ingestion can increase renal blood flow, glomerular capillary pressure and GFR, which is qualitatively similar to the acute effects of short-term HP intake on renal hemodynamics (Friedman,A.N. 2004). The present study demonstrated the inulin clearance of pigs on the HP was higher than that of those on the NP diets, which agreed with previous studies. Both animal and human studies indicate that chronic increases in protein intake are associated with increases in GFR (Pullman, T.N.1954; Hegsted,M. 1981; Piepsz,A. 1994; Robertson,J.L. 1986; Skov,A.R. 1999). In these studies, different filtration markers were measured to determine the GFR, including creatinine (Pullman, T.N. 1954; Hegsted,M. 1981; Robertson,J.L. 1986) and radiotracer ($^{99m}$Tc-DTPA or $^{51}$Cr-EDTA) (Piepsz,A. 1994; Skov,A.R. 1999). Theoretically inulin is the perfect marker to determine the “true” GFR

in healthy adults because it is eliminated exclusively by the kidneys, and is neither secreted nor reabsorbed by tubules (Sterner,G. 2007). However, in the present study, some inulin clearance data was lost because of loss of ear vein catheters during infusion, resulting in the small sample size and relatively large variability in the 4 months data, which is a limitation. Further, creatinine clearance also was assessed to evaluate GFR in the present study and although the trends were similar to the inulin data, there were no statistical differences between NP and HP groups. Creatinine clearance is the most commonly used method to evaluate GFR, but creatinine is filtrated by glomeruli and also secreted by tubules. The extent of tubular creatinine secretion is not constant between individuals. Moreover, serum creatinine levels may be altered by total muscle mass and dietary meat intake (Rahn,K.H. 1999; Sterner,G. 2007). However, there is no difference in meat intake in HP and NP groups in the present study. Therefore, the present study indicated that HP diets may change renal hemodynamics and increase GFR.

Adaptive mechanisms are assumed to occur in response to HP diet-induced increases in GFR. This was observed in the current study by increases in kidney weight, kidney volume and glomerular volume after consumption of HP diets. The results from this study demonstrated overall ~18% heavier kidney weights, larger kidney and glomerular volume at both 4 and 8 months in pigs given the HP diets, which was consistent with previous studies. In a long-term HP diet study among overweight/obese otherwise healthy subjects, after six months' intervention the kidney volume increased by 9.1 $cm^3$ in the HP group (~2.5% larger than that at baseline) and the correlation between kidney volume and dietary protein intake was stronger than that at baseline (Skov,A.R. 1999). Animal studies also indicate a linear relationship between protein intake rate and kidney weights/kidney volume/glomerular volume.

Kidney weights corrected by body weights were significantly greater and kidney volumes were 20-26% larger in the animals given HP diets compared to those given low or normal protein diets (Bouby,N. 1988; Smith,L.J. 1993; Reyes,A.A. 1994; Hammond,K.A. 1998; Goldstein,D.L. 2002). Further, in a rat study using the same study design as the current pig study, there also was significant renal (28% higher when relative to body weight) and glomerular (22% larger) hypertrophy in rats given HP diets (Wakefield, A. 2007). In the current study glomeruli appeared hypercellular in kidneys from pigs on HP diets, indicating increased cell numbers in glomeruli. It has been suggested that the intrinsic glomerular cells (including epithelial cells, endothelial cells and mesangial cells) play a role in renal and glomerular hypertrophy. For example, a significant enhancement of proliferation of mesangial cells was observed in HP-fed rats (Weissgarten,J. 1998; Weissgarten,J. 2000). Other animal studies also observed that rats and mice with intact kidneys exposed to long-term HP diets had hypercellular and enlarged glomeruli (Rao,G.N. 2001; Bertani,T. 1989; Hostetter,T.H. 1986; Itoh,H. 2002). The proliferation of these glomerular cells is probably mediated by various growth factors, such as growth hormone or insulin-like growth factors (IGF). In a previous study, both serum and glomerular IGF levels were higher in animals fed HP diets (36%-60% protein). When adding serum from animals fed HP diets to culture medium, it significantly increased the proliferative effect on mesangial cells (Hirschberg,R. 1991; Weissgarten,J. 1998; Weissgarten,J. 2000). Recent studies also show that high dietary protein induced glomerular hypertrophy is vascular endothelial growth factor (VEGF)-dependent. Increased expression of VEGF in glomeruli directly caused the glomerular hypertrophy in transgenic rabbits. Moreover, the administration of a neutralizing VEGF-antibody in mice fed a short-term high protein diet completely prevents the glomerular hypertrophy (Flyvbjerg,A. 1999; Schrijvers,B.F. 2002; Liu,E.

2007; Yao,B. 2006). The present study confirmed that a long term HP diet can cause renal and glomerular hypertrophy but further mechanisms leading to renal and glomerular hypertrophy needed to be studied.

Persistent glomerular hypertrophy is regarded as an early sign of kidney disease. Rats and mice with intact kidneys exposed to long-term HP diets have glomerular hypertrophy and a greater prevalence of developing nephropathy than those on low protein diets (Rao,G.N. 2001; Bertani,T. 1989; Hostetter,T.H. 1986; Itoh,H. 2002; Wakefield,A. 2007). Decreasing dietary protein in rats has been reported to attenuate glomerular tuft total cell count, glomerular hypertrophy and glomerulosclerosis associated with both age and partial nephrectomy (Wiggins,J.E. 2005; Steffes,M.W. 2001). For example, increased glomerular volume was prevented and the increase in glomerular sclerotic index was delayed in moderate (~26.5% decrease) and marked (~50% decrease) protein intake decreased rats, compared with ad libitum rats which daily protein intake was ~4.4g (21% protein) (Keenan,K.P. 2000). Glomerular hypertrophy might trigger the progression of kidney disease, leading to renal inflammation, tubular atrophy and kidney fibrosis. Renal inflammation is a key contributor to the development of kidney fibrosis. It can be induced through increasing the release of proinflammatory chemokines, such as MCP-1, which plays an important role in the recruitment of inflammatory cells, especially macrophages, into the kidney (El Nahas, A.M. 2005; Ruster,C. 2008). In the present study, glomerular hypertrophy, as well as an increase in renal MCP-1 levels and renal chronic inflammation (especially at 8 months) in the tubulointerstitial area was observed, which suggests a role of MCP-1 in glomerular hypertrophy induced renal inflammation. A chronic inflammatory cell, especially macrophage, accumulation around cortical tubules is associated with chronic renal damage induced by glomerular hypertrophy, which leads

to declining renal function. The accumulating chronic inflammatory cells can interact with renal cells, the later become activated, proliferate, transform and synthesize excessive ECM, and ultimately, kidney fibrosis develops (El Nahas, A.M. 2005). Other studies also support the role of MCP-1 in fibronectin deposition in kidney. Rats injected with bovine serum albumin daily developed interstitial inflammation and fibrosis. MCP-1 mRNA and protein levels were increased and the macrophages showed distinct tubular patterns of distribution (Eddy,A.A. 1995). Human kidney cell culture showed that MCP-1 binding to CCR2 had a modest effect on cell proliferation and increased fibronectin protein levels (Burt,D. 2007; Giunti,S. 2008). Analysis of patient biopsies has identified expression of MCP-1 mRNA and protein correlates with the accumulation of CD68+ macrophages (Wada,T. 2000). Renal biopsies also demonstrate that macrophage numbers closely correlate with the degree of kidney damage (Szeto,C.C. 2005; Eardley,K.S. 2006; Tesch,G.H. 2008). In the present study, renal chronic inflammation was accompanied by significantly increased cortical fibrosis in the kidneys from pigs given the HP diets, which supported the connection between chronic inflammatory cell accumulation and kidney fibrosis. The importance of MCP-1 in the development of kidney fibrosis has been determined by examining effects of the MCP-1 receptor (CCR2) in MCP-1 gene deficient obese mice. In these mice, kidney macrophage accumulation and the progression of renal injury (albuminuria, histopathology, renal fibrosis) was substantially lowered in CCL2(-/-) compared with CCL2(+/+) db/db mice with equivalent diabetes. At the same time, fibronectin deposition in the glomerulus and renal cortical fibronectin mRNA level was diminished. Macrophage infiltration was inhibited in the mice by CCR2 antagonist treatment (Kanamori,H. 2007; Chow,F.Y. 2007; Giunti,S. 2008). A very recent study showed that MCP-1 binding to CCR2 induced a 2.5-fold increase in fibronectin protein levels at 24

hours, followed by a rise in pericellular fibronectin in the cultured medium of human mesangial cells (Giunti,S. 2008). Since the present study revealed a connection between persistent glomerular hypertrophy and the progression of kidney disease, it would be worthwhile to evaluate correlations between macrophage numbers and the degree of kidney damage.

There was no dietary effect on renal TGFβ1 levels in this study and one possible reason is that TGFβ1 may be more involved in later stages of fibrosis. TGFβ1 is an important growth factor associated with kidney fibrosis, especially tubulointerstitial fibrosis. It also is associated with other tubulointerstitial injuries, including an increase in tubular cell damage index, tubular dilatation and atrophy (Wang,S.N. 2000). These injuries normally occur later than glomerular injury. For example, uninephrectomied diabetic mice having increased albuminuria and severe glomerulosclerosis in 37% of glomeruli displayed more significant tubulointerstitial fibrosis compared to sham-operated diabetic mice, which had glomerulosclerosis only in 8% of glomeruli (Ninichuk,V. 2007). Another human study also found that little or no active TGFβ1 immunostaining was detected prior to manifest diabetic nephropathy (Wahab,N.A. 2005). In the present study, the collagen deposition was around the glomerular area and there was no obvious tubular cell damage, tubular dilatation or atrophy, in kidneys from pigs on HP diets, indicating an early stage of fibrosis. Further, in a rat study, using the same study design as the current pig study, there was more glomerulosclerosis in rats given HP diets but no significant difference in tubulointerstitial fibrosis between different diets (Wakefield,A. 2007). Moreover, urinary protein is regarded as the factor to induce elevated TGFβ1 expression in tubular cells, which can then facilitate the development of interstitial fibrosis and tubular atrophy (Liu,B.C. 2006; Phillips,A.

2007). There was no increased proteinuria in the current study and it might be another reason to explain the unchanged renal TGFβ1 levels.

Proteinuria is recognized as predictor of kidney disease progression in persons with CKD, as well as a risk factor in healthy populations (Gerstein,H.C. 2001; Furtner,M. 2005). Therefore, whether HP diets alter urinary protein excretion is of direct clinical importance. Long-term studies using rat and mouse models show that animals on HP diets have increased rates of urinary protein excretion (Hostetter,T.H. 1986; Bertani,T. 1989; Piepsz,A. 1994; Itoh,H. 2002). Conversely, the current study demonstrated that protein intake did not appear to be related to changes in urinary protein excretion. Long-term clinical trials and nutrition surveys support the present study. No significant changes in urinary protein excretion were found in healthy or overweight/obese but otherwise healthy individuals consuming HP diets. However, the protein intake in these studies ranged from 107.8g/day to 110.9g/day (~25% of energy from protein), which were lower than that of the current study (Skov,A.R. 1999; Wrone,E.M. 2003). In contrast, a cross-sectional Dutch survey in the healthy population reported that the risk for microalbuminuria progressively increased with greater daily protein ingestion, especially the group whose daily protein consumption was more than 1.5g/kg body weight (Hoogeveen,E.K. 1998). Actually, the underlying mechanism of HP consumption altering urinary protein excretion is not well understood. Urinary protein is affected by both filtration at the glomeruli and reabsorption by renal tubular epithelial cells. Dextran-sieving analyses showed that HP intake did not disrupt the glomerular filtration barrier; therefore impaired or altered renal tubular reabsorption is more likely to be associated with proteinuria (Chan,A.Y. 1988). As mentioned, there was no obvious tubular damage in the present study and it might explain the lack of changes in urinary protein. Another possible reason for the lack of changes in proteinuria is the

source of protein. In the present study, egg white was one of the sources to achieve HP diets, which also is one of the most common methods to achieve HP diets in people. Egg white has a relatively small amount of lysine compared to other animal proteins (Adam,M. 2007). Oxidative reaction of lysine can increase amounts of advanced glycation end products (AGEs), which have been implicated in the incidence of increased proteinuria by generating intracellular reactive oxygen species (ROS) (Forbes,J.M. 2007). Mice injected with lysine had higher circulating and renal AGE levels and more significant proteinuria compared with $p66^{Shc}$ (a central regulator of oxidative stress) knockout mice (Menini,S. 2007). In the present study, the increase in egg white content in HP diets did not induce a high level of lysine intake and it might be another possible reason to explain the lack of significant effects of diet on proteinuria. Therefore, the current study indicates that long term HP diets do not alter urinary protein excretion and, that the amino acid composition of the HP diets may be important in the dietary protein effect on proteinuria.

As mentioned, there was a similar study, using the same study design as the current pig study but using a rat model to observe the effects of HP diets on kidney health (Wakefield,A. 2007). It is worthwhile to compare the results from both the rat and the present pig studies with that from human studies. Human studies indicate that chronic increases in protein intake are associated with increases in GFR (Pullman, T.N.1954; Hegsted,M. 1981; Skov,A.R. 1999), kidney weight and volume (Skov,A.R. 1999). There were increases in GFR (creatinine clearance relative to body weight in rats and inulin clearance relative to body weight in pigs, respectively) and kidney weight, and also significant renal hypertrophy in both rats and pigs given HP diets, which is consistent with human studies. Proteinuria is another important predictor of kidney disease in populations. Nutrition surveys report that the risk for microalbuminuria

progressively increases with greater daily protein intake adjusted for age, diabetes, hypertension and homocysteine (Hoogeveen,E.K. 1998), although there are no significant changes in urinary protein excretion in healthy individuals with ~25% of energy from protein (Skov,A.R. 1999; Wrone,E.M. 2003). Wakefield's rat study demonstrated that urinary protein excretion was 4.8 times higher in HP compared to NP rats, which is consistent with other rodent animal studies (Wakefield,A. 2007; Hostetter,T.H. 1986; Bertani,T. 1989; Piepsz,A. 1994; Itoh,H. 2002). Conversely, no significant dietary effects on urinary protein were found in pigs given the same upper limit of energy from protein as the rats. Therefore, the rat's kidney might be more similar to human's in response to HP diets and might be a better model to study the underlying mechanisms of proteinuria induced by HP diet.

With respect to kidney histology, although there are no human studies indicating the relationship between HP diets and changes of kidney histology or mediators associated with kidney fibrosis in healthy populations, studies of human with different renal diseases have demonstrated that HP diets accelerate the pre-existing renal disease progression (Brenner,B.M. 1982; Uribarri, J. 2006). In addition, human kidney cell culture studies show a positive correlation between MCP-1 and increased fibronectin protein levels (Burt,D. 2007; Giunti,S. 2008). Both rat and pig studies revealed a connection between HP diets and the progression of kidney disease, including glomerular hypertrophy and increased renal fibrosis. However, there was no dietary effect on renal TGFβ1 levels (relative to renal protein content). Interesting, renal MCP-1 levels were higher in the current study but lower in the rat study in the animals given HP compared to NP diets. Overall, it is hard to say which is the best animal model to study the effects of HP diets on the human kidney since it depends on which factors are considered to be the most important.

In addition to examing the effects of HP diets on kidney health, the present study also evaluated the effects of HP diets on body composition. As the prevalence of overweight and obesity among the population continues to increase (Health Canada, 2004), HP diets are one of the options in the management of weight loss and weight control in overweight or obese individuals. Low calorie HP diets can enhance loss of body mass, fat mass and percentage body fat in the short term (within 6 month) (Halton,T.L. 2004; Cunningham,W. 2006). Some mainstream organizations, such as the American Diabetes Association, also recommend that pre-diabetic or diabetic individuals consider HP diets as a strategy of weight control (American Diabetes Association, 2008). The present study indicated that overall, pigs on HP had lower BW and fat mass than those on NP diets, which agrees with other animal and human studies (Lacroix,M. 2004; Halton,T.L. 2004; Cunningham,W. 2006). However, the advantage of HP diets in weight loss is not sustained for longer periods of time (more than 6 months) according to clinical trials. Weight loss in these studies was relatively small, ranging from 2.1% to 7.1% of body weight (Foster,G.D. 2003; Due,A. 2004; Brinkworth,G.D. 2004; McAuley,K.A. 2006; Dansinger,M.L. 2005). Careful examination the results from the present study found that the difference in body weight between pigs on HP and NP diets was less obvious at 8 than at 4 months. At the end of 4 months, the difference in average body weight was 35±10 kg. However, at the end of 8 months, the difference was only 4±8 kg. The same trend also was observed in the ham fat percent and fat mass. Furthermore, repeated measures analysis of body weight from 0 to 8 months revealed that the significant difference of body weight between pigs on HP and NP diets happened only at weeks of 2 and 4. Therefore, the sustainable effect of HP diets on weight loss is questionable. Another repeated measures of body weights from 0 to 4 months revealed that pigs on HP diet weighed

less at all time points. The possible reason for the differences in these two analyses is that the latter had a larger sample size.

The effect of HP diets on bone health also has been debated. Some studies indicate that HP intake might be detrimental to bone health because the catabolism of sulfur amino acids produces excessive sulphuric anions, which need more calcium to neutralize and consequently decreases calcium reabsorption (Zwart,S.R. 2005; Amanzadeh,J. 2003). The current study showed no change in ham BMD in response to HP diets, which was consistent with previous long-term observations in rats (26%~60% dietary protein) (Whiting,S.J. 1981; Calvo,M.S. 1982; Mardon,J. 2008). Some human studies also found that there were no high dietary protein-induced effects on net bone balance and the majority of the rise in urinary calcium in response to an increase in dietary protein was due to an increase in intestinal calcium absorption (Promislow,J.H. 2002; Dawson-Hughes,B. 2004; Kerstetter,J.E. 2005). In the present study, considering that each diet was balanced for minerals, the two groups should have the same intestinal calcium asorption. Moreover, HP diets may have a favorable effect on bone health since insulin-like growth factor 1(IGF-1) is an osteotrophic factor and its anabolic action is stimulated by dietary protein intake (Thissen,J.P. 1994). This positive effect might reduce or offset the possible negative effect of HP diet-generated acid load on bone mass, therefore, resulting in no significant change in BMD.

In conclusion, the main findings of the present study (Table 5) indicate that long-term high dietary protein intake from whole protein sources are associated with kidney damage in normal female pig kidneys. High dietary protein can induce renal and glomerular hypertrophy, lead to an increase in renal release of the

proinflammatory chemokine MCP-1, which leads to chronic inflammatory cell recruitment, and ultimately, development of renal cortical fibrosis.

This may have implications for individuals on HP diets. A significant portion of the population on HP diets is pre-diabetic or diabetic. Pre-diabetes and diabetes may cause glomerulopathy and tubular damage, which is an independent risk factor for CKD. In addition, among the general population, the percentage of people with mild renal insufficiency is not small and the prevalence keeps increasing (Coresh,J. 2003). It is very common that these individuals are unaware of their compromised renal function and choose HP diet for weight loss or other purpose, such as muscle building. Therefore, the AMDR upper limit of 35% for protein appears not to be safe considering the negative effects on healthy kidneys in the current study and it might accelerate the progression of CKD in patients unaware their renal condition.

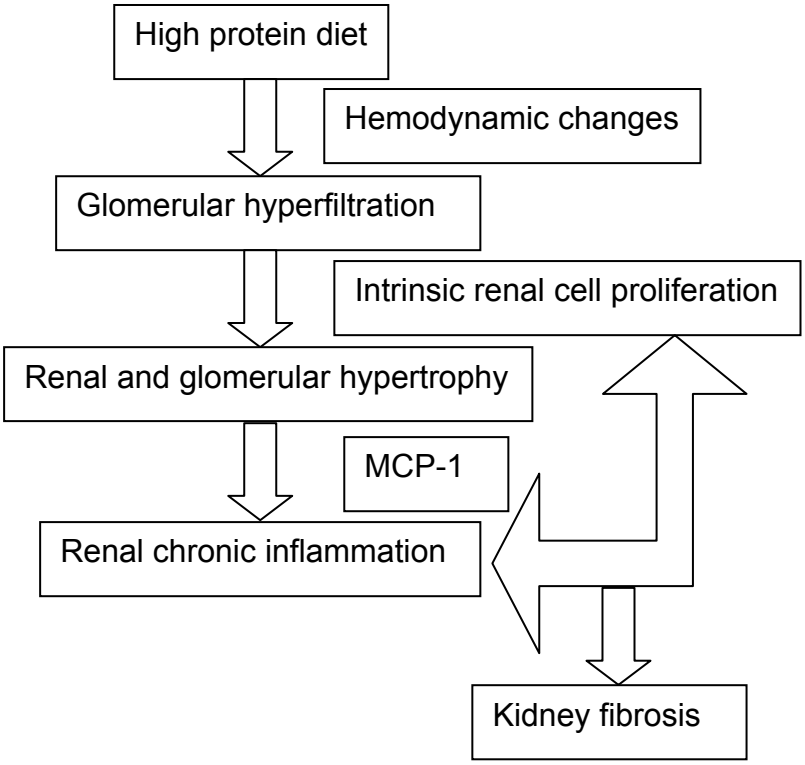


**Figure 12:** Summary of main findings of the present study. The present study indicates the relationship between high dietary protein and the cascade of events in renal inflammation and fibrosis in the pig model. HP diets may lead to glomerular hyperfiltration, which is caused by hemodynamic changes in the kidney. Glomerular hyperfiltration induces renal and glomerular hypertrophy, which leads to an increase in renal release of the proinflammatory chemokine MCP-1. The hypercellular glomeruli in the present study indicate the proliferation of intrinsic renal cells, which involves renal and glomerular hypertrophy. Increased expression of MCP-1 leads to chronic inflammatory cell recruitment, interactions between inflammatory cells and intrinsic renal cells, and ultimately, development of renal fibrosis.

## 3 Strengths and Limitations

A primary strength of this study was the animal model we used. To our knowledge, this is the first study using adult healthy pigs to explore the effect of the upper range of AMDR for protein on normal kidneys over long-term period. This study provided an opportunity to compare the pig and rodent model in studying renal disease progression. Compared with rodents, the pig kidney is more similar to human in regard to organ development, kidney size, renal hemodynamics and physiology. The effects of the HP diet on kidney weight, kidney and glomerular volume are consistent, whether in pig, rodent or human studies. However, the effect on proteinuria is different between the pig and rodent studies. In rat and mouse studies, HP diets result in increased proteinuria. Conversely, the current pig study protein intake was not related with changes in proteinuria, which is consistent with long-term human studies. Further, the present pig study indicated an increased expression of renal MCP-1 in response to the HP diet. Interestingly, in a rat study, using the same study design as the pig study, we previously have found a decrease in renal MCP-1 level. Although, there is no human study that has examined the relationship between dietary protein and renal MCP-1, *in vitro* studies have found that MCP-1 expression increases with increasing protein content in kidney cell culture medium. Therefore, the pig model might be a better model for humans to study the mechanisms of kidney changes induced by HP diet over long time.

A second strength was the protein source of each diet. In the present study whole protein sources were used instead of purified protein. In previous studies, the most common protein source is casein, which is only a part of the daily protein source in the human population. Animal, dairy and plant protein might have different effects on kidney health and daily protein sources are not normally limited to one source in the

general population. Therefore, whole protein sources may better reflect the real effects of daily protein intake. In addition, we increased skim milk powder and egg albumin content to achieve HP diets, which are common sources to increase protein intake in the population.

The third strength was the level of protein in each diet. In the NP group, we chose 15% as energy from protein, which represents not only the normal protein requirement in pigs but also the average Canadian daily protein intake. The level of 35% protein as energy in HP group is the upper range of the AMDR and seldom has been used in clinical studies. Therefore, the current study furnished new data regarding the safety of this upper limit.

Fourthly, in the current study, GFR was assessed by both creatinine clearance and inulin clearance. Although creatinine clearance is an easy way to evaluate GFR in clinical practice, inulin is regarded as the perfect marker to reflect the true GFR.

Several limitations exist in the present study. One of them was the limitation of the space to raise the animal. After 4 months, the space became crowded and part of the pigs from both group had to be terminated because of leg problems. At first, the study was designed as a 12 months study but it had to be stopped at 8 months due to insufficient number of pigs in each group due to the earlier mortalities.

Secondly, this study included only female pigs so there was no way to address any possible gender differences. As we know, the male is more prone to developing CKD and the effect of HP diets in males is unknown from this study.

Thirdly, we had limited experience of inulin infusion in large size animals therefore some of the ear vein catheters were lost during the infusion resulting in some lost data at 4 months. However, we modified the method to introduce the catheter at 8 months and the percentage of successful infusions of inulin improved.

Lastly, the feed intake was measured indirectly according to feed disappearance. The study was designed to measure the feed intake of each pig using a computerized feeding system. However, the composition of the ingredients precluded pellet formation. Therefore, individual feed intake data was collected when the pigs were transferred into metabolic crates. The stress of this new environment caused some pigs to consume very little feed resulting in lost data.

## 4 Future Research

For further investigation of high dietary protein intake and kidney health, there are several potential directions:

Use different levels of protein intake (eg. 20%, 25%, 30%, 35%) and observe at what level negative effects on renal health occurs. Or use the same protein level (35%) but different ME levels, which means using different amount of protein intake per body weight per day and observe the effects on renal health.

Measure urinary level of MCP-1 and TGFβ1. In regard to clinical practice, changes in urinary level of inflammatory markers will be an easier way to detect renal disease and a good marker for following up therapeutic effect and progression of disease.

To explore the distribution of MCP-1, TGFβ-1 and to determine the exact site/cell type to express these inflammatory markers in the kidney at the protein or mRNA levels, using immunohistochemistry or *in situ* hybridization analysis.

Since MCP-1 can stimulate macrophage accumulation in kidney tissue, immunohistochemistry, such as with anti-CD68, could be used to evaluate macrophage infiltration. This data would shed light on the relationship between renal or urinary MCP-1 levels and macrophage numbers and evaluate correlations between macrophage numbers and the degree of kidney damage.

One of the possible mechanisms of glomerular hypertrophy is glomerular cell proliferation, which is mediated by various factors. The role of IGF and VEGF in glomerular cell proliferation and whether neutralizing these factors would prevent glomerular hypertrophy in subjects consuming HP diet could also be studied.

Future studies also should explore further mechanisms leading to renal inflammation and fibrosis induced by HP diets. Dietary protein-derived AEGs are thought to contribute to inflammatory and pro-oxidative processes in kidney. Another interesting direction would be to explore the relationship between AGEs and the cascade of events in renal inflammation and fibrosis.

Lastly, to further analyze the effect of HP diet on bone health, biochemical markers, such as plasma IGF-1, parathyroid hormone (PTH), osteocalcin (OC) and urinary calcium levels, could be measured and mechanical testing of femurs also could be performed.

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# 6 Appendix

**Appendix 6.1.1** Contribution of individual feed ingredients to the macro- and micronutrient composition of the NP diet (Refer to 2.3.1)[1]

| | Protein (%) | Fat (%) | ME (Kcal/kg) | NDF[2] (%) | Calcium (%) | Available phosphorous (%) | Potassium (%) | Sodium (%) | Zinc (ppm) |
|---|---|---|---|---|---|---|---|---|---|
| Wheat | 3.4 | 0.6 | 997 | 0.73 | 0.012 | 0.047 | 0.115 | 0.006 | 0 |
| Barley | 0.8 | 0.2 | 239 | 0.32 | 0.005 | 0.011 | 0.031 | 0.002 | 0 |
| Poultry Meal | 2.9 | 0.7 | 168 | 0.03 | 0.086 | 0.062 | 0.033 | 0.021 | 0 |
| Pork Meal | 2.5 | 0.4 | 141 | 0.07 | 0.287 | 0.127 | 0.024 | 0.023 | 4.11 |
| Egg Albumen | 0.6 | 0 | 30 | 0 | 0.004 | 0.003 | 0.004 | 0.005 | 0.01 |
| Skim Milk | 2.7 | 0 | 278 | 0 | 0.092 | 0.073 | 0.131 | 0.036 | 3.27 |
| Sucrose | 0 | 0 | 531 | 0 | 0 | 0 | 0 | 0 | 0 |
| Corn Starch | 0.1 | 0 | 805 | 0 | 0 | 0.005 | 0 | 0.005 | 0 |
| Mineral & Vitamin | 0 | 0 | 0 | 0 | 0.037 | 0 | 0 | 0 | 180.03 |
| $CaCO_3$[3] | 0 | 0 | 0 | 0 | 0.005 | 0 | 0 | 0 | 0 |
| $CaHPO_4$[4] | 0 | 0 | 0 | 0 | 0.219 | 0.160 | 0 | 0 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| KCl[5] | 0 | 0 | 0 | 0 | 0 | 0 | 0.201 | 0.002 | 0 |
| NaCl[6] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.150 | 0 |
| Lactose | 0 | 0 | 125 | 0 | 0 | 0 | 0 | 0 | 0 |
| Solid Fat | 0 | 0.6 | 46 | 0 | 0 | 0 | 0 | 0 | 0 |
| Canola | 0 | 1.4 | 113 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 13 | 4 | 3472 | 1.14 | 0.75 | 0.49 | 0.54 | 0.25 | 187 |

[1]data were calculated as: macro/micronutrient = ingredient weight (g/100g diet) x chemical composition of ingredient (%). Chemical compositions of each ingredient were analyzed by commercial lab (Bodycoat Testing, Lethbridge, AB).
[2]NDF= Neutral detergent fiber
[3]Calcium Carbonate
[4]Dicalcium Phosphorus
[5]Potassium Chloride
[6]Sodium Chloride

**Appendix 6.1.2** Contribution of individual feed ingredients to the macro- and micronutrient composition of the HP diet (Refer to 2.3.1)[1]

| | Protein (%) | Fat (%) | ME (Kcal/kg) | NDF[2] (%) | Calcium (%) | Available phosphorous (%) | Potassium (%) | Sodium (%) | Zinc (ppm) |
|---|---|---|---|---|---|---|---|---|---|
| Wheat | 3.4 | 0.6 | 997 | 0.73 | 0.012 | 0.047 | 0.115 | 0.006 | 0 |
| Barley | 0.8 | 0.2 | 239 | 0.32 | 0.005 | 0.011 | 0.031 | 0.002 | 0 |
| Poultry Meal | 3.0 | 0.7 | 168 | 0.03 | 0.086 | 0.062 | 0.033 | 0.021 | 0 |
| Pork Meal | 2.5 | 0.4 | 141 | 0.07 | 0.287 | 0.127 | 0.024 | 0.023 | 4.11 |
| Egg Albumen | 16.6 | 0.6 | 832 | 0 | 0.111 | 0.075 | 0.004 | 0.005 | 0 |
| Skim Milk | 4.6 | 0 | 480 | 0 | 0.159 | 0.125 | 0.131 | 0.036 | 5.67 |
| Sucrose | 0 | 0 | 221 | 0 | 0 | 0 | 0 | 0 | 0 |
| Corn Starch | 0 | 0 | 336 | 0 | 0 | 0.002 | 0 | 0.005 | 0 |
| Mineral & Vitamin | 0 | 0 | 0 | 0 | 0.037 | 0 | 0 | 0 | 180.03 |
| $CaCO_3$[3] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| $CaHPO_4$[4] | 0 | 0 | 0 | 0 | 0.053 | 0.039 | 0 | 0 | 0 |
| KCl[5] | 0 | 0 | 0 | 0 | 0 | 0 | 0.201 | 0.002 | 0 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| NaCl[6] | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0.1503 | 0 |
| Lactose | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Solid Fat | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Canola | 0 | 1.4 | 113 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 31 | 4 | 3528 | 1.14 | 0.75 | 0.49 | 0.54 | 0.25 | 190 |

[1]data were calculated as: macro/micronutrient = ingredient weight (g/100g diet) x chemical composition of ingredient (%). Chemical compositions of each ingredient were analyzed by commercial lab (Bodycoat Testing, Lethbridge, AB).
[2]NDF= Neutral detergent fiber
[3]Calcium Carbonate
[4]Dicalcium Phosphorus
[5]Potassium Chloride
[6]Sodium Chloride

**Appendix 6.1.3** Ratio of animal protein to plant protein in NP and HP diets (Refer to 2.3.1)

| | Plant protein (%) | Meat protein (%) | Poultry protein (%) | Dairy protein (%) | Egg protein (%) | Animal : Plant protein |
|---|---|---|---|---|---|---|
| NP | 33 | 19 | 23 | 20 | 4 | 2:1 |
| HP | 14 | 8 | 10 | 15 | 54 | 6:1 |

## 6.2 Assessment of Renal Function

### 6.2.1 Inulin Clearance

Timed urine collections and inulin clearance rates were determined on termination day (Refer to 2.3.3). One week prior to termination, animals were individually housed in metabolic crates. Two days before termination pigs were lightly sedated and a transurethral foley catheter was introduced into the bladder. A 22 ga-25 mm cannula was introduced into an ear vein. Heparin was given through the cannula to prevent clotting. After allowing 24 hours for recovery, a prime dose of inulin (60mg/kg) was infused using a Harvard infusion pump (Harvard Apparatus, Holliston, Massachusetts, model No. 22) through the ear vein catheter. After that, the infusion rates were adjusted to achieve 2mg/kg/min continuous infusion, 2.5 mL/min and 4 mL/min for 4 and 8 months, respectively. After allowing 2 hours to reach steady state, a 30 minutes urine collection was initiated in a pre-weighted 4L sample container (Fisher, Pittsburgh, PA). At the end of the collection, a blood sample was obtained from the ear vein with sodium heparin coated vacutainer (BD, Franklin Lakes, NJ). Urine and plasma inulin concentration was measured by spectrophotometer. GFR was calculated using the following equation:

**GFR (mL/min) = urine flow (mL/min) X urine/plasma inulin ratio**

where, **urine flow =** $\dfrac{\text{urine volume (mL)}}{\text{collection time(min)}}$

$$\text{urine/plasma inulin ratio} = \frac{\text{urine inulin concentration (mg\%)}}{\text{plasma inulin concentration (mg\%)}}$$

### 6.2.2 Creatinine Clearance

Both urinary and serum creatinine concentrations (mg/dL) were determined determined by a method based on the creatinine-picrate reaction (Heinegard and Tiderstrom, 1973, Refer to 2.3.3). A Costar 96-well microplate (Corning Incorporatio,

Corning, New York), was used and wells were labeled as blank, standard or sample. Standard concentrations of 0.5,1, 2, 3, 5, 6, 8 and 10 mg/dL were made using a Creatinine Standard Set (Sigma, C3613) for urine samples. Since serum creatinine concentration is low, standard concentrations of 0.25, 0.5, 1, 3, 5, 6, 8 and 10mg/dL were used. Urinary samples were diluted 10 fold. 20 μL of blank (deionized water), standard and samples were added to the wells in triplicate. 200 μL of Picric solution (0.05 M Sodium Phosphate: and 0.05 M Sodium Borate: 4% aqueous SDS: 1.3% Picric Acid=2:2:1) was added to each well and mixed thoroughly on an orbital shaker (Fisher Scientific, Fair Lawn, NJ, Model No. 361) at room temperature for 45 minutes. The plate was read at 500 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporatio, Sunnyvale, California). Following this, 20 μL of 60% Acetic Acid solution was added to each well and mixed thoroughly and allowed to stand for 6 minutes at room temperature. A second absorbency reading was taken at 500 nm. Using Microsoft Excel, a standard curve with absorbance on the X-axis and concentration on the Y-axis was constructed. Concentration of creatinine in the sample was calculated using the equation given by the Excel when adding the trend line.

Creatinine clearance was calculated using the following equation:

**Creatinine Clearance (mL/min) =**

$$\frac{\text{urine creatinine (mg/dL) X urine volume (dL/24 hr)}}{\text{serum creatinine (mg/dL)}}$$

where, 24 hr was expressed in minutes.

### 6.2.3 Microassay for Urinary Protein

Urinary protein concentrations were determined by protein assay using the Bradford method (Bradford, 1976, Refer to 2.3.3). A Costar 96-well microplate (Corning Incorporation, Corning, New York), was used and wells were labeled as blank,

standard or sample. Standard concentrationns of 0.05, 0.10, 0.20, 0.30, and 0.50 mg/mL were made using Bovine Serum Albumin (Sigma, St. Louis, Missouri). 10 μL of blank (deionized water), standard, and 20~200 fold diluted (with deionized water) urine samples were added to wells in triplicate. 200 μL of room temperature Bradford Reagent (Sigma, St. Louis, Missouri) was added to each well and mixed on an orbital shaker (Fisher Scientific, Fair Lawn, New Jersey, Model No. 361) for approximately 15 minutes until there is no precipitate. The plate was read at 595 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporatio, Sunnyvale, California). The standard curve was plotted by the SOFTmax PRO software (version 1.20, Molecular Devices Corporatio, Sunnyvale, California) and the unknowns were calculated from the line of the standard curve.

### 6.3 Assessment of Renal Histology

At termination, kidneys were removed and weighed. Kidneys were measured before a transverse incision of the kidney from the middle portion was made to allow optimal examination of the renal pelvis, renal papilla and the junction with the ureter. An integrated section of kidney from the upper pole of the left kidney was sampled and included portions of both the cortex and medulla. Kidney tissues were fixed in 10% formalin prior to embedding in paraffin. The tissue was sectioned at 5 microns with a Microtome (American Optical 820, Southbridge, Massachusetts).

#### 6.3.1 Assessment of Glomerular Volume

Sections from kidneys for evaluating glomerular volume were stained with hematoxylin and eosin (Refer to 2.3.4). Paraffin sections were deparaffinized and rehydrated in xylene, different concentration of ethanol and deionized $H_2O$. While sections were in water, surfaces were skimmed with a Kimwipe to remove oxidized particles. The sections were then stained with the alum haematoxylin. Sections were

rinsed in running tap water and then dipped in 0.3% acid alcohol to destain. The sections were rinsed in running tap water and deionized water again. After that, the sections were stained with eosin for 2 mins and then dipped into ethanol and xylene to be dehydrated. Finally, the sections were covered with a coverslip using Permount (Fisher Scientific, Fair Lawn, NJ). As a result, nuclei were stained blue and cytoplasm red or purple.

There are several methods for estimating glomerular volume, of which two are Cavalieri (considered the "gold standard") (Gundersen, H. 1987) and Weibel-Gomez (Weibel, E.R.1979). Studies indicate that both Cavalieri and Weibel-Gomez methods can provide reliable estimates of glomerular volume. However, the Cavalieri method is time consuming as it involves counting consecutive serial sections of complete glomeruli and requires knowing the average thickness of biopsy sections. In contrast, the Weibel-Gomez method is an assumption based method requiring only one kidney section and is easier to perform (Buzello, M. 2000). Lane found that the Weibel-Gomez method did correlate with the Cavalieri method ($r = 0.68$; $p < 0.05$). At least 15 profiles are needed to provide a dependable estimate of glomerular volume by Weibel-Gomez (Lane, P.H. 1992; Lane, P.H.1995). Therefore the Weibel-Gomez method is widely used in animal and human studies (Samuel, T. 2007; Alperovich, G. 2004; Maddox, D.A. 2002; Hirose, K. 1982). In the present study, 30 profiles in each kidney were measured. The basic theory of the Weibel-Gomez method is to estimate the glomerular volume by approximating the shape as a sphere. Since very rarely will the shape of glomeruli be actually described as sphere, Weibel introduced the definition of “volume-equivalent sphere”, which describes the “best fit” shape for glomeruli and uses the mean tangent diameter, which is close to that of similar solids, such as an ellipsoid (Weibel, E.R. 1979). Further, the sphere has the smallest tangent diameter of

all solids of equal volume, so that the error introduced in substituting a sphere for another solid will always be in the direction of underestimation. This method requires only determination of the mean glomerular random cross-sectional areas to estimate glomerular volume and uses maximal caliper diameter of each profile when measuring. Because this method uses “equivalent sphere” and assumes glomerular sphericity, the area can be estimated from the formula for circle. To obtain r, the average of glomerular diameters (maximal caliper diameter) is divided by two. Therefore, the mean glomerular volume (MGV) is calculated from the following formula:

$$\mathbf{MGV = \beta/K \times (\pi r^2)^{3/2}}$$

Where, β = 1.38, which is the shape coefficient for spheres, K = 1.10, which is the size distribution coefficient of glomeruli, and $\pi r^2$ is the estimation of the area of the glomerular section.

Images were captured using SPOT Advanced software version 3.0.1 (Diagnostic Instruments Inc, Sterling Heights, Michigan). A Spot CCD high-resolution camera (Diagnostic Instruments Inc, Sterling Heights, Michigan) was connected with an Olympus BX60 microscope. 30 randomly selected areas with glomeruli were captured using the 10X objective. Image Pro version 6.0 was used to measure the largest diameter of each glomerulus. Before measuring, each image had to be calibrated to the objective grid choosing the proper calibration set in the software. Measurement was across the visceral layer of Bowman’s capsule (podocytes), not including Bowman’s space or the parietal layer of Bowman’s capsule.

### 6.3.2 Assessment of Kidney Cortical Fibrosis

Sections from kidneys for evaluating cortical fibrosis were stained with Masson's Trichrome Staining for collagen (Refer to 2.3.4). Sections were deparaffinized and

rehydrated through 100% alcohol, 95% alcohol 70% alcohol. After being washed in distilled water, sections were stained in Weigert's iron hematoxylin working solution for 10 minutes. After rinsing in running warm tap water (37□) for 10 minutes and washing in distilled water for 30 seconds, sections were stained in Biebrich scarlet-acid fuchsin solution for 15 minutes. The sections were washed in distilled water for 30 seconds and then stained in phosphomolybdic-phosphotungstic acid solution for 15 minutes. After that, sections were transferred directly (without rinse) to aniline blue solution and stained for 5-10 minutes. After rinsing briefly in distilled water and staining in 1% acetic acid solution for 2-5 minutes, the sections were washed in distilled water for 30 seconds and then dehydrated very quickly (within 20 seconds) through 95% ethyl alcohol and absolute ethyl alcohol to wipe off Biebrich scarlet-acid fuchsin staining. They were then cleared by soaking in xylene for 20 seconds. Finally, slides were mounted with Permount (Fisher Scientific, Fair Lawn, NJ). As a result, collagen was stained blue, nuclei black and cytoplasm red.

Images were acquired using a Zeiss Axioskop2 microscope equipped with an Axiovision digital camera. Images were randomly captured from different zones of the kidney cortex, containing both glomeruli and tubulointerstitial areas, using a 40X objective. Analysis was done in Adobe Photoshop CS3 Extended program. Briefly, all blue colors in the picture were selected using magic wand tool and then measured.

**Appendix 6.3.3** Parameters for renal morphology (Refer to Figure 4-9)

| | 4-month | | 8-month | | Main effects | p |
|---|---|---|---|---|---|---|
| | NP n=8 | HP n=8 | NP n=14 | HP n=17 | | |
| **Kidney volume ($cm^3$)** | 143±11 | 198±20 | 155±8 | 195±11 | Diet | 0.0006 |
| **Kidney volume ($cm^3$/kg BW)** | 0.57±0.05 | 0.70±0.03 | 0.56±0.03 | 0.90±0.08 | Diet*Time Interaction | 0.0352 |
| **Glomerular volume ($10^6 \mu m^3$)** | 4.04±0.31 | 4.99±0.39 | 5.23±0.32 | 9.98±0.65 | Diet*Time Interaction | 0.0065 |
| **Glomerular volume ($10^4 \mu m^3$/kg BW)** | 1.63±0.14 | 2.28±0.15 | 1.88±0.11 | 3.59±0.19 | Diet*Time Interaction | 0.0340 |
| **Glomerular volume ($10^4 \mu m^3$/g kidney weight)** | 1.35±0.11 | 1.54±0.14 | 1.70±0.13 | 2.92±0.19 | Diet*Time Interaction | 0.0133 |
| **Cortical fibrosis ($10^6$ Arbitrary unit)** | 28.65±5.45 | 37.17±3.39 | 6.72±0.53 | 9.67±1.13 | Diet<br>Time | 0.0128<br><0.0001 |

### 6.4 Assessment of Early Mediators of Renal Disease

#### 6.4.1 Lyophilization of Kidneys

In order to prepare samples for enzyme-linked immunosorbent assay (ELISA), a portion of kidney cortex sampled from the upper pole of the left kidney was lyophilized. Frozen portions of kidney were weighed initially and placed in a weighing bowl covered by foil with pierced holes on the top, and then placed into a freeze drying apparatus (Virtis, Model No 10-145MR-BA, Gardiner, New York). Tissue samples were dried until two consecutive equal weights were obtained. Dried kidneys were stored at -80°C (Refer to 2.3.5).

#### 6.4.2 Homogenization of Kidneys

Lyopholized kidney cortex was homogenized using a modification of the method of Cuozzo et al (Cuozzo,F.P. 2002, Refer to 2.3.5). 30 mg pre-weighed lyophilized left kidney cortex was pulverized using a spatula and then transferred to a test tube. Three mL of particulate homogenization buffer (see below) was added to each tube and homogenized on ice for a total of 60 seconds using a Polytron homogenizer (Brinkmann Instruments, Mississauga, Ontario). After each homogenization, the rotor was cleaned with ethanol and dd$H_2O$ and wiped dry using a Kimwipe. The buffer contained 50 mM Tris-HCl (pH 7.2), 250 mM sucrose, 2 mM ethylene-diamine-tetraacetic acid (pH 7.6),1 mM ethylene glycol-bis (β-aminoethyle ether) N,N,N',N'-tetracetic acid (pH 7.5), 50 μM NaF, 0.5% Triton X-100, 100 μM sodium orthovanadate, 25 μg/ml aprotinin, 25 μg/ml pepstatin, 25 μg/ml leupeptin, 1 μg/ml soybean trypsin inhibitor, 10 mM β-mercaptoethanol, and 144 μM 4-(2-aminoethyl) benzene sulfonyl fluoride. The homogenate was transferred into a 5 ml ultracentrifuge tube and pairs of tubes were balanced against each other. Samples were centrifuged at 100,000 g for 35 minutes at 4°C using the Beckman L5-50B

ultracentrifuge (Mississauga, Ontario). The supernate fraction was collected with pipette and stored immediately at -80°C. Whole cell homogenates were used since the location of intracellular inflammatory proteins is uncertain in kidney.

### 6.4.3 Determination of Protein Content in Homogenates

Protein contents in kidney cortical homogenates were determined by the Bradford method described in Appendix 6.2.3 and there was no difference between kidneys from HP and NP diets (see figure: Appendix 6.4.3).

### 6.4.4 Enzyme-Linked ImmunoSorbent Assay (ELISA) Kits

The basic principle of ELISA is: a monoclonal specific antibody is pre-coated onto a microplate. When adding an unknown amount of antigen (such as cytokine and chemokine) into the wells, any antigen present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for this antigen is added to the wells to sandwich the antigen immobilized during incubation, and in the final step a substance is added that the enzyme can convert to some detectable signal. Renal levels of TGFβ1, MCP-1, RANTES and ET-1 were determined by using commercial ELISA kits. The total amount of protein in the kidney homogenates was measured using the Bradford method. TGFβ1, MCP-1, RANTES and ET-1levels in the kidney were expressed per mg of kidney protein.

#### 6.4.4.1 Measure Renal TGFβ1 Levels

A porcine TGFβ1 ELISA kit was used for analysis (MB 100B, R&D Systems, Minneapolis, MN). Wells in a 96-well microplate coated with a TGFβ1 specific monoclonal antibody were labeled as blank, standard, or sample. TGFβ1 Standard was reconstituted with Calibrator Diluent and the final concentrations of standard were 0, 31.2, 62.5, 125, 250, 500, 100, and 2000 pg/mL. 100 μL kidney cortex homogenates were activated with 20 μL of 1N HCl and neutralized with 13 μL of 1.2N NaOH/0.5 M

HEPES after a 10 min incubation period. 50 μL of assay diluent RD1-21 (for cell culture supernates) was added to each well except for the blank. Then 50 μL of control (TGFβ1 kit control), standard and activated kidney sample were added to each well in duplicate, tapped for 1 minute to mix, covered and incubated at room temperature for 2 hours. Each well was aspirated and washed 4 times with diluted wash buffer using an eight-channel plate washer (Nunc, Roskilde, Denmark). Then 100 μL of TGFβ1 conjugate was added to each well and incubated at room temperature for 2 hrs. The same washing process was completed a second time and 100 μL of substrate solution was added to each well. The plate was incubated for 30 minutes at room temperature and protected from light. 100 μL of stop solution was added and the plate was mixed with gentle tapping. The absorbance of each well was read at 450 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporatio, Sunnyvale, California). A standard curve was created using SOFTmax PRO software version 1.20 (Molecular Devices Corporatio, Sunnyvale, California) and a four-parameter logistic curve-fit was generated. Because the samples were activated with 20 μL of 1N HCl and neutralized with 13 μL of 1.2N NaOH/0.5 M HEPES, the final concentration of TGFβ1 (pg/ml) of the unknown kidney samples was multiplied by the dilution factor of 1.3 (Refer to 2.3.5).

**6.4.4.2 Measure Renal MCP-1 Levels**

A human MCP-1 ELISA kit was used (KHC 1012, BioSource International, Camarillo, California). In another published study, human MCP-1 ELISA kit from the same company also was successfully used in pig samples (Ekekezie, 2001).Wells in a 96-well microplate were labeled as blank, standard, or sample. Standard was reconstituted and diluted from purified MCP-1 standard and the final concentration was 0, 15.6, 31.2, 62.5, 125, 250 and 500 pg/mL. All the samples were diluted 4 fold

according to the kit instructions. 50 μL of incubation buffer was added to all the wells, then 50 μL control, standard and samples were added to the appropriate wells in duplicate. 50 μL biotinylated anti-MCP-1 was added to each well except the blank and the side of the plate was tapped gently to mix. The plate was covered and left to incubate for 2 hours at room temperature. Following this the plate was aspirated and washed 4 times with the provided wash buffer. 100 μL of streptavidin-HRP working solution was added to each well except the blank, covered and incubated at room temperature for 30 minutes. The above wash cycle was repeated and 100 μL of stabilized chromogen was added to each well, covered and incubated in the dark at room temperature for another 30 minutes. After this time period 100 μL of stop solution was added to each well and the plate was tapped gently to mix. The plate was read at 450 nm using a SpectroMax Microtiter Plate Reader (Molecular Devices Corporatio, Sunnyvale, California). A standard curve was created using SOFTmax PRO software version 1.20 and a four-parameter logistic curve-fit was generated. The final concentration of MCP-1 (pg/ml) of the unknown kidney samples was multiplied by the dilution factor of 4 (Refer to 2.3.5).

**6.4.4.3 Measure Renal RANTES Levels**

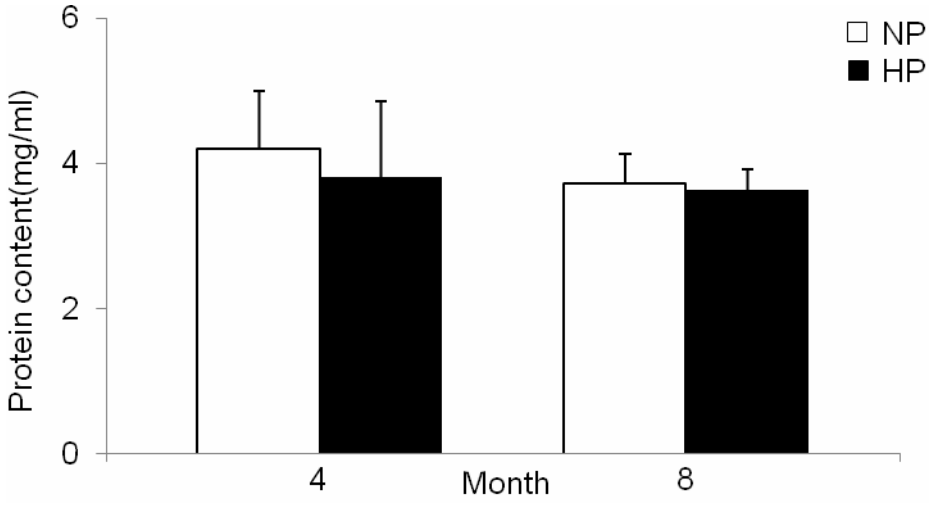
Human RANTES ELISA kit was used for analysis (DRN00B, R&D Systems, Minneapolis, MN). Wells in a 96-well microplate were labeled as blank, standard, or sample. Standard concentrations were 0, 31.2, 62.5, 125, 250, 500, 1000, and 2000 pg/mL. One hundred μL of Assay Diluent RD1W was added to each well. After that, One hundred μL of standard, kidney samples were added to the appropriate wells in duplicate, and the plate was tapped gently to mix. The plate was then covered and incubated at room temperature for 2 hours. Following this the plate was aspirated and washed 3 times with the provided wash buffer. Two hundred μL of conjugate was

added to each well except the blank, covered and incubated at room temperature for 1 hour. The wash cycle was then repeated and 200 μL of substrate solution was added to each well and incubated at room temperature for 20 minutes, protecting from light. After this time period 50 μL of stop solution was added to each well and the plate was tapped gently to mix. The absorbance of each well was read at 450 nm using a SpectroMax Microtiter Plate Reader and a four parameter logistic standard curve was plotted with SOFTmax PRO software version 1.20 (Refer to 2.3.5).

**6.4.4.4 Measure Renal and Urinary ET-1 Levels**

The amino acid sequence of ET-1 used in this kit is highly conserved through species so a human ET-1 ELISA kit was used for analysis (BI-20052, BioMedica, Austria). Wells in a 96-well microplate were labeled as blank, standard, control or sample. Standard concentrations were 0, 0.625, 1.25, 2.5, 5, and 10 fmol/mL. Two hundred μL of detection antibody was added to each well, except blank. After that, fifty μL of standard, control, kidney or urine samples were added to the appropriate wells in duplicate, and the plate was tapped gently to mix. The plate was then covered and incubated overnight at room temperature. Following this the plate was aspirated and washed 5 times with the provided wash buffer. Two hundred μL of conjugate was added to each well except the blank, covered and incubated at room temperature for 1 hour. The wash cycle was then repeated and 200 μL of substrate solution was added to each well and incubated at room temperature for 30 minutes, protecting from light. After this time period 50 μL of stop solution was added to each well and the plate was tapped gently to mix. The absorbance of each well was read at 450 nm using a SpectroMax Microtiter Plate Reader and a linear standard curve was plotted with SOFTmax PRO software version 1.20 (Refer to 2.3.5).

**Appendix 6.4.5** Protein contents in kidney cortex homogenates.




Diet, p=0.2347, Time, p=0.1073, Diet*time, p=0.4445

## 6.5 Verify Representation of Ham DEXA Data

All of the right carcasses from the four months termination were divided into five parts and scanned, including the shoulder, posterior and anterior loins, belly and ham, and also were analyzed by DEXA. The data from different parts were added up to determine whole body composition. To verify whether the ham represented the entire carcass, comparison of the whole body data with that from the ham was made (Appendix 6.5.1-6.5.3, Refer to 2.3.2).

**Appendix 6.5.1** Comparison of the right carcass fat mass and fat percent with ham fat mass and fat percent at 4-month (n=6-8).

| Fat Mass(g) | NP | HP | p | Fat Percent (%) | NP | HP | p |
|---|---|---|---|---|---|---|---|
| Ham | 7807±628 | 5441±593 | 0.0176 | Ham | 32±2 | 25±2 | 0.0311 |
| Shoulder | 10834±1519 | 7816±778 | 0.0898 | Shoulder | 39±2 | 31±2 | 0.0470 |
| Anterior loin | 9897±396 | 7023±1478 | 0.0672 | Anterior loin | 35±2 | 28±4 | 0.1317 |
| Posterior loin | 4844±544 | 3476±634 | 0.1232 | Posterior loin | 36±2 | 28±3 | 0.0377 |
| Belly | 5830±537 | 4391±592 | 0.0992 | Belly | 53±2 | 49±3 | 0.2155 |
| Right carcass | 39212±2959 | 30426±3009 | 0.0638 | Right carcass[1] | 37±2 | 32±2 | 0.1201 |

[1] fat%= fat (g)/tissue (g) x100%

**Appendix 6.5.2** Comparison of the right carcass lean mass and lean percent with ham lean mass and lean percent at 4-month (n=6-8).

| Lean Mass(g) | NP | HP | p | Lean Percent (%) | NP | HP | p |
|---|---|---|---|---|---|---|---|
| Ham | 16732±707 | 15908±504 | 0.3733 | Ham | 68±2 | 75±2 | 0.0311 |
| Shoulder | 16660±993 | 17067±780 | 0.7575 | Shoulder | 61±2 | 69±2 | 0.0470 |
| Anterior loin | 18782±1280 | 16405±810 | 0.1534 | Anterior loin | 65±2 | 72±4 | 0.1317 |
| Posterior loin | 8311±618 | 8289±645 | 0.9812 | Posterior loin | 64±2 | 72±3 | 0.0377 |
| Belly | 5113±270 | 4520±587 | 0.2170 | Belly | 47±2 | 49±3 | 0.2155 |
| Right carcass | 65598±1466 | 62872±1110 | 0.1885 | Right carcass[1] | 63±2 | 68±3 | 0.1201 |

[1] lean%= lean (g)/tissue (g) x100%

**Appendix 6.5.3** Comparison of the right carcass BMC and BMD with ham BMC and BMD at 4-month (n=6-8).

| BMC (g) | NP | HP | p | BMD(g/cm$^2$) | NP | HP | p |
|---|---|---|---|---|---|---|---|
| Ham | 605±21 | 601±25 | 0.8955 | Ham | 1.39±0.02 | 1.45± | 0.1250 |
| Shoulder | 1476±102 | 1334±48 | 0.2525 | Shoulder | 1.56±0.06 | 1.37±0.04 | 0.0267 |
| Anterior loin | 412±36 | 391±33 | 0.6763 | Anterior loin | 0.74±0.02 | 0.73±0.03 | 0.8547 |
| Posterior loin | 245±16 | 268±28 | 0.4675 | Posterior loin | 1.18±0.05 | 1.13±0.04 | 0.5051 |
| Right carcass | 2738±100 | 2634±87 | 0.4666 | Right carcass | 1.27±0.03 | 1.22±0.03 | 0.2289 |

**Appendix 6.6** The markers of renal disease progression in pigs on normal protein (NP) or high protein (HP) diets as determined by MCP-1 and TGFβ1 (Refer to Figure 10-11)

| | 4-Month | | 8-Month | | Main Effects | |
|---|---|---|---|---|---|---|
| | NP n=8 | HP n=8 | NP n=12 | HP n=12 | Diet p | Time p |
| MCP-1 (pg/mg Renal Protein) | 143.23±34.93 | 201.11±38.55 | 65.33±15.21 | 72.75±11.52 | 0.0215 | <0.0001 |
| MCP-1 (pg/mg Dry Kidney) | 58.64±3.14 | 69.2±5.61 | 24.05±1.32 | 26.32±1.10 | 0.0424 | <0.0001 |
| MCP-1 (pg/mg Wet Kidney) | 12.03±0.64 | 14.19±1.15 | 4.95±0.27 | 5.40±0.22 | 0.0424 | <0.0001 |
| MCP-1 (µg/mg Kidney) | 3.63±0.24 | 4.36±0.32 | 1.58±0.09 | 1.96±0.12 | 0.0050 | <0.0001 |
| TGFβ1 (pg/mg Renal Protein) | 63.7±7.84 | 70.47±25.88 | 64.58±2.90 | 65.29±3.42 | 0.6165 | 0.3228 |
| TGFβ1 (pg/mg Dry Kidney) | 26.49±3.14 | 21.05±3.61 | 23.97±1.13 | 23.57±1.07 | 0.1302 | 0.4982 |
| TGFβ1 (pg/mg Wet Kidney) | 5.43±0.64 | 4.32±0.74 | 4.92±0.23 | 4.83±0.22 | 0.1302 | 0.4982 |
| TGFβ1 (µg/mg Kidney) | 1.68±0.24 | 1.33±0.24 | 1.56±0.05 | 1.74±0.09 | 0.4848 | 0.0786 |

### 6.7 Renal RANTES, ET-1and Urinary ET-1 Levels

Renal homogenates as described in Appendix 6.4.2 were used to determine RANTES and ET-1 levels and 24-hour urine sample was used to determine urinary ET-1 levels, following ELISA kit instructions (Appendix6.4.4.3 and Appendix 6.4.4.4). The concentrations of RANTES and ET-1 were below the limit of detection.

With respect to the very low concentrations of RANTES and ET-1 in the present study (Refer to 2.4.4), the early stage of kidney damage might be the reason. RANTES is secreted basolaterally by tubular epithelial cells during proteinuria (Wang,S.N. 2000). Studies also suggest that TGFβ1 is one of the most potent regulators of ET-1 expression in kidney tissue (Dube,J. 2000). As mentioned, the current study showed signs of early stage kidney damage and there was no dietary effect on renal TGFβ1 levels or on urinary protein excretion. Therefore, ET-1 and RANTES might be at a relatively low level at this stage of kidney damage and below the limit of detection. In addition, there is no information regarding species cross-reactivity of these human kits with pig samples, although the amino acid sequence of ET-1 used in this kit is highly conserved through species. This might be another possible reason to explain the undetectable levels of these two mediators.