Prevalence of Hyperhomocysteinemia in Patients with Predialysis Chronic Kidney Disease after Folic Acid Food Fortification of the Canadian Food Supply

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
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Elevated plasma total homocysteine (ptHcy) or hyperhomocysteinemia (hHcy) independently predicts cardiovascular disease in predialysis chronic kidney disease (pCKD). Folate status is one of the known nutritional determinants of ptHcy. In the era of folic acid food fortification, this cross-sectional study aimed to describe in pCKD subjects (n=48): 1) Prevalence of hHcy. 2) Intake and status of nutrients involved in homocysteine metabolism. 3) Determinants of ptHcy. The prevalence of hHcy was 93.8% (95% CI: 81.8 to 98.4). Median (25th, 75th percentile) total folate intake from food and supplements was 389 (282,640) µg DFE/d. No subject was folate deficient (red blood cell < 317 nmol/L). Red blood cell folate (r = - 0.406, p=0.004) and energy-protein undernutrition (r = 0.357, p=0.013) independently predicted ptHcy. To conclude, total folate intake among subjects with pCKD was sufficient to prevent folate deficiency but not able to prevent a high prevalence of hHcy.
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<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CVD</td>
<td>Cardiovascular disease</td>
</tr>
<tr>
<td>DB&lt;sub&gt;6&lt;/sub&gt;E</td>
<td>Dietary vitamin B&lt;sub&gt;6&lt;/sub&gt; equivalent</td>
</tr>
<tr>
<td>DFE</td>
<td>Dietary folate equivalent</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>hHcy</td>
<td>Hyperhomocysteinemia</td>
</tr>
<tr>
<td>pCKD</td>
<td>Predialysis chronic kidney disease</td>
</tr>
<tr>
<td>ptHcy</td>
<td>Plasma total homocysteine</td>
</tr>
<tr>
<td>RBC</td>
<td>Red blood cell</td>
</tr>
<tr>
<td>SGA</td>
<td>Subjective global assessment</td>
</tr>
<tr>
<td>TSF</td>
<td>Tricep skinfold thickness</td>
</tr>
<tr>
<td>USA</td>
<td>United States of America</td>
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Chapter 1

1 Introduction

People with chronic kidney disease, not yet on dialysis, have a high prevalence of cardiovascular disease (CVD), and mortality from CVD (Collins et al. 2003; Astor et al. 2008). In 1999, clinical practice guidelines developed by the Canadian Society of Nephrology recommended the establishment of multidisciplinary predialysis chronic kidney disease (pCKD) clinics to promote early recognition of disease and introduction of measures to prevent development or exacerbation of CVD (Churchill et al. 1999). The positive outcome of this recommendation was demonstrated in a study, conducted in Canada, which found that dialysis patients who received multidisciplinary care at a pCKD clinic prior to the initiation of dialysis had a significantly lower mortality rate compared to dialysis patients who did not receive such care (Goldstein et al. 2004).

Elevated plasma concentration of the amino acid homocysteine has been found to be associated with CVD, in both the general population and in people with pCKD (Selhub 1999; Jungers et al. 1997). Studies conducted before folic acid food fortification, or in countries without fortification, have shown that a large majority of people with pCKD had an elevated plasma total homocysteine (ptHcy) concentration (Menon et al. 2005; Nerbass et al. 2006). Nutritional determinants of elevated ptHcy, or hyperhomocysteinemia (hHcy), in pCKD, include B vitamin status (folate, B₁₂, B₆), protein intake and energy-protein status (Nerbass et al. 2006; Parsons et al. 2002; Menon et al. 2005; Suliman et al. 2002).

In 1998, Health Canada mandated the addition of folic acid to the food supply to reduce the occurrence of neural tube defects (Canada Gazette 1998). This health strategy has been effective as it was reported that the prevalence of neural-tube defects significantly decreased after fortification compared to before fortification (De Wals et al. 2007). Another positive consequence of folic acid food fortification has been the reduction in the prevalence of hHcy in the general population (Pfeiffer et al. 2008). As in pCKD, folate status is a determinant of ptHcy
in the general population (Ganji et al. 2009). The prevalence of hHcy in the pCKD population, however, has not been reported post-folate fortification.

The present study, conducted in the era of folic acid food fortification, describes, in subjects with pCKD attending a multidisciplinary pCKD clinic, the prevalence of hHcy, intake and status of the nutrients involved in homocysteine metabolism, and the determinants of ptHcy.
Chapter 2

2 Literature Review

2.1 Chronic Kidney Disease and Burden of Cardiovascular Disease

An estimated 1.5 million Canadians have pCKD (Stigant et al. 2003). The leading causes of kidney failure are diabetes (35%), renal vascular disease (18%), including hypertension, and glomerulonephritis (11%) (The Kidney Foundation of Canada 2010). It is predicted that the incidence of chronic kidney disease (CKD) will increase in tandem with the expected increase in the incidence of type 2 diabetes because as previously mentioned diabetes is a leading cause of CKD (Atkins 2005). Between 2007 and 2017, an estimated 1.9 million Canadians aged 20 and older will be diagnosed with diabetes (Manuel et al. 2010).

Chronic kidney disease is defined as the presence of kidney damage or a reduced glomerular filtration rate (< 60 ml/min/1.73m$^2$) for ≥ 3 months (National Kidney Foundation 2002). Table 2.1 describes the stages of CKD. There are five stages, with the stages being defined by the estimated glomerular filtration rate (eGFR). In kidney disease, kidney function can progressively decline to stage 5 CKD, indicated by an eGFR of <15 ml/min/1.73m$^2$ and represents kidney failure. When a person has kidney failure, they require dialysis or a kidney transplant to sustain life.

The term CKD refers to people who have kidney disease who are not yet receiving dialysis as well as to people who are receiving dialysis (National Kidney Foundation 2002). To differentiate between these two populations the term predialysis CKD (pCKD) is used to refer to people who have kidney disease but are not yet receiving dialysis.
Table 2.1 Stages of Chronic Kidney Disease by Glomerular Filtration Rate (GFR)
(Adapted from National Kidney Foundation, 2002)

<table>
<thead>
<tr>
<th>Stage</th>
<th>Description</th>
<th>GFR (ml/min/1.73m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Kidney damage with normal or ↑ GFR</td>
<td>≥90</td>
</tr>
<tr>
<td>2</td>
<td>Kidney damage with mild ↓ GFR</td>
<td>60-89</td>
</tr>
<tr>
<td>3</td>
<td>Moderate ↓ GFR</td>
<td>30-59</td>
</tr>
<tr>
<td>4</td>
<td>Severe ↓ GFR</td>
<td>15-29</td>
</tr>
<tr>
<td>5</td>
<td>Kidney Failure</td>
<td>&lt;15 (or dialysis)</td>
</tr>
</tbody>
</table>

Elderly adults (≥ 67 years old) in the Medicare population in the United States of America (USA), with pCKD, were found to be 5 to 10 times more likely to die than to reach kidney failure and require treatment (Collins et al. 2003). This is attributable, in large part, to the high burden of CVD in the pCKD population. Collins et al. (2003) reported the prevalence of CVD to be 80% and 44%, in elderly adults (≥ 67 years old), identified with and without pCKD, respectively. Mortality, secondary to CVD, was found to be 2.36 times more likely among adults (≥ 20 years old) in the general population, identified with an eGFR between 15 to 59 ml/min/1.73m², than those identified with an eGFR ≥ 90 ml/min/1.73m² (Astor et al. 2008).

2.2 Hyperhomocysteinemia

2.2.1 Homocysteine Metabolism in Health

Homocysteine is a sulfur containing amino acid that is generated from methionine via the transmethylation pathway (Selhub 1999). Figure 2.1 depicts the metabolism of homocysteine in the body. Methionine itself is an indispensable amino acid that is derived from dietary protein or the breakdown of endogenous protein. Methionine is condensed with adenosine triphosphate to form S-adenosylmethionine, the universal methyl group donor (Finkelstein 1998; Selhub 1999). S-adenosylhomocysteine is formed when S-adenosylmethionine donates its methyl group, with S-adenosylhomocysteine subsequently being hydrolyzed to homocysteine (Selhub 1999). Homocysteine is remethylated to methionine via the remethylation pathway by acquiring a methyl group from folate, in the form of 5-methyltetrahydrofolate, or from betaine (Selhub 1999). The reaction with 5-methyltetrahydrofolate requires the enzyme methionine synthase,
which is vitamin B₁₂ dependent. Alternatively, homocysteine is transsulfurated to form cysteine via the transsulfuration pathway which requires two reactions, both of which are vitamin B₆ dependent. In the first reaction, homocysteine condenses with serine to form cystathionine in the presence of cystathionine β-synthase. This reaction is irreversible, making the transsulfuration pathway the elimination route of homocysteine. Cystathionine is hydrolyzed to cysteine and α-ketobutyrate in the presence of γ-cystathionase. Cysteine is oxidized via the cysteine catabolic pathways to the end product sulfate, which is excreted in the urine (Stipanuk 2004).

In methionine excess, for example after a meal, concentrations of methionine, S-adenosylmethionine and S-adenosylhomocysteine are elevated and can act to inhibit the transmethylation and remethylation pathways, and activate the transsulfuration pathway (Finkelstein 2007). A high concentration of methionine inhibits methionine synthase and betaine homocysteine methyltransferase, the enzymes that remethylate homocysteine to methionine. High concentration of S-adenosylmethionine inhibits: 1) Methionine adenosyltransferase, the enzyme converting methionine to S-adenosylmethionine. 2) Methylene tetrahyrofolate reductase, the enzyme required for the formation of 5-methyltetrahydrofolate. 3) The gene expression of betaine homocysteine methyltransferase. A high concentration of S-adenosylhomocysteine inhibits methionine synthase, betaine homocysteine methyltransferase and the numerous adenosylmethionine-dependent transmethylases, which convert S-adenosylmethionine to S-adenosylhomocysteine. High concentrations of either S-adenosylmethionine or S-adenosylhomocysteine activate cystathionine β-synthase.

The hydrolysis of S-adenosylhomocysteine to homocysteine is a reversible reaction (Finkelstein 1990). Thermodynamics favor synthesis of S-adenosylhomocysteine. As such, in homocysteine excess S-adenosylhomocysteine accumulates. Hoffman et al. (1979) demonstrated, in vitro, that methylation reactions are inhibited by increasing the concentration of S-adenosylhomocysteine or by decreasing the ratio of S-adenosylmethionine to S-adenosylhomocysteine.
Figure 2.1 Homocysteine Metabolism (Adapted from Finkelstein 1998; Selhub 1999)
ATP = adenosine triphosphate; SAM = S-adenosyl methionine; SAH = S-adenosyl homocysteine
Homocysteine metabolism occurs intracellularly (Gariboto et al. 2003). All cells are capable of transmethylation and remethylation, but not all cells are capable of transsulfuration (Stipanuk 2004). In humans, the complete set of transsulfuration enzymes has been found in the liver, kidney, pancreas and adrenal glands (Bao et al. 1998; Sturman et al. 1970; Zlotkin et al. 1982). Tissue not capable of transsulfuration export excessive homocysteine to the plasma for removal by tissue capable of transsulfuration (Stipanuk 2004; Finkelstein 2007). Plasma total homocysteine concentration represents the amino acid in transit from a site of production to a site of catabolism (Finkelstein 1998).

In plasma, homocysteine is present in its free reduced form as a sulfhydryl (2%), its free oxidized form as a disulfide (16%) bound to homocysteine or cysteine molecules, and its protein bound form as a disulfide, bound predominantly to albumin (82%) (Mansoor et al. 1992; Refsum et al. 1985). Plasma total homocysteine reflects free and bound homocysteine (Refsum et al. 1985). In the general population, normal ptHcy is 5 to 13 µmol/L (Pfeiffer et al. 2008).

2.2.2 Hyperhomocysteinemia and Cardiovascular Disease

There is evidence that CVD is related to an elevated ptHcy concentration in both CKD and non CKD populations (Jungers et al. 1997; The Homocysteine Studies Collaboration 2002; Boushey et al. 1995; Danesh et al. 1998). Mild, moderate and severe hHcy have been defined as a ptHcy concentration of 15 to 25 µmol/L, 25 to 50 µmol/L and > 50 µmol/L, respectively (Perla-Kajan et al. 2007).

In 1969, McCully suggested that hHcy is related to atherosclerosis based on autopsy evidence from children with severe hHcy, homocysteinuria and premature atherothrombotic disease, secondary to an inborn error of homocysteine metabolism. Subsequent experimental studies provide evidence that homocysteine is involved in atherogenesis and thrombogenesis, leading to vascular dysfunction (Lonn 2007; Kaul et al. 2006). These studies postulate several potential mechanisms by which homocysteine may cause vascular dysfunction, including: 1) Auto-oxidation of homocysteine to cytotoxic reactive oxygen species, including superoxide and hydrogen peroxide. 2) Activation of inflammatory mediators by homocysteine, such as nuclear factor-kappa β, responsible for the transcriptional regulation of proinflammatory genes. 3)
Metabolism of homocysteine to homocysteine-thiolactone which induces cell apoptosis. 4) Reaction of homocysteine-thiolactone with protein lysine residues (protein N-homocysteinylation) or formation of disulfide bonds between homocysteine and protein cysteine residues (protein S-homocysteinylation), both of which alter the function of the proteins. 5) Deoxyribonucleic acid (DNA) hypomethylation. 6) Endoplasmic reticulum stress secondary to hHcy which results in the accumulation of unfolded proteins in the endoplasmic reticulum (Welch et al. 1998; Antoniades et al. 2009; Perla-Kajan et al. 2007; Perna AF 2009; van Guldener et al. 2007; Urquhart et al. 2007).

Observational studies and meta-analyses of observational studies conducted in healthy, cardiac and CKD populations have provided evidence for an association between ptHcy and CVD (The Homocysteine Studies Collaboration 2002; Boushey et al. 1995; Danesh et al. 1998; Jungers et al. 1997). The Homocysteine Studies Collaboration trial (2002), a meta-analysis of 30 observational studies involving healthy subjects, found that a lower ptHcy concentration (25% or, typically, 3 μmol/L) was associated with a significant reduction in the risk of coronary artery disease (OR 0.89; 95% CI: 0.83 to 0.96) and stroke (OR 0.81; 95% CI: 0.69 to 0.95).

The Homocysteine Lowering Trialists’ Collaboration (2005) conducted a meta-analysis of 25 randomized controlled trials and reported that 0.8 mg/d folic acid significantly, and maximally, reduces ptHcy by 23% (95% CI: 21-26). Studies included in the meta-analysis were conducted in countries without folic acid food fortification of the food supply. Based on the finding of this study and evidence that countries with folic acid food fortification have a higher folate status and a lower ptHcy concentration, the authors predicted that supplementation with 0.8 mg of folic acid, in countries with folic acid food fortification, will lower ptHcy concentration only by 15%.

In pCKD subjects, intervention with 5mg/d folic acid has been found to significantly, and maximally, decrease ptHcy by 49 ± 3.2% (Bernasconi et al. 2006). It is noteworthy that in the study by Bernasconi et al. (2006) 44% of the subjects still had hyperhomocysteinemia despite folic acid supplementation. The inability of a supraphysiological dose of folic acid to normalize homocytseine concentration in pCKD is related to the fact homocysteine metabolism is altered in CKD (Stam et al. 2004; van Guldener 2006).
Despite evidence for the ability of intervention with folic acid to reduce ptHcy concentration, overall, randomized control trials conducted in cardiac and CKD populations have not been able to conclusively demonstrate a beneficial outcome in terms of decreasing the risk of CVD events (Lonn et al. 2006; Bonaa et al. 2006; Zoungas et al. 2006; Mann et al. 2008; House et al. 2010).

The HOPE 2 trial included subjects with a prior stroke and found a significant difference in the change from baseline in ptHcy levels between those who received intervention with folic acid (2.5 mg/d) and those who did not. However, intervention did not significantly decrease the relative risk (0.95; 95% CI: 0.84 to 1.07) of death from a composite of cardiovascular causes, myocardial infarction, or stroke (Lonn et al. 2006). Bonaa et al. (2006) found a significant increased risk for a composite of myocardial infarction or stroke of 1.22 (95% CI: 1.00 to 1.50) after intervention with 0.8 mg folic acid, among subjects with a prior myocardial infarction. This increased risk occurred despite the finding that ptHcy concentration was significantly lower in the intervention group than the placebo group after 2 months and at the end of the study.

In subjects with stage 4 and 5 CKD, including those receiving dialysis, intervention with 15mg/d folic acid did not significantly decrease the risk (HR 0.93; 95% CI: 0.58 to 1.48) of first myocardial infarction, stroke or death from CVD (Zoungas et al. 2006). This study may not have been able to find a significant risk reduction in CVD events as the difference in mean ptHcy levels between the folic acid group and the placebo group was significantly different at 24 months (-7.1; 95% CI: -10.2 to -4.0) but not at 36 months (-2.4; 95% CI: -6.8 to 1.9) or 48 months (-4.7; 95% CI: -9.4 to -0.1), the endpoint of the study. A post-hoc analysis of the HOPE 2 trial which included only subjects with pCKD found no significant difference in the relative risk (1.19; 95% CI: 0.88 to 1.61) of death from a composite of cardiovascular causes, myocardial infarction, or stroke between those who received vitamin intervention (2.5 mg/d folic acid) and those who did not (Mann et al. 2008). This result occurred despite the finding of a significantly lower ptHcy concentration in the vitamin intervention group compared to the placebo group. House et al. 2010 reported a significant increase in the risk (HR 2.0; 95% CI: 1.0 to 4.0) of a composite of myocardial infarction, stroke, revascularization and all-cause mortality among subjects with diabetic nephropathy (stage 1, 2 and 3 CKD) after intervention with 2.5mg/d folic acid. This increased risk occurred despite the finding that change in the concentration of ptHcy
from baseline was significantly different (-4.8; 95% CI: -6.1 to -3.7) between the intervention and placebo groups, with the intervention group having a greater decrease in ptHcy.

Folic acid supplementation may or may not have a role specific to primary and secondary stroke prevention. The Hope 2 trial, a randomized control trial, found intervention with 2.5mg/d folic acid in subjects with a prior stroke resulted in a significant reduced risk of recurrent stroke (RR 0.75; 95% CI: 0.59 to 0.97) (Lonn et al. 2006). A meta-analysis of randomized control trials reported a significant decrease in risk (RR 0.82; 95% CI: 0.68 to 1.00) of the occurrence of a first stroke, after folic acid intervention (Wang et al. 2007). It is important to note that in the study by Wang et al. (2007) the upper end of the confidence interval of the relative risk was 1.00 suggesting that it is possible that folic acid intervention did not reduce the risk of stroke. House et al. 2010 reported an increase in the risk (HR 6.6; 95% CI: 0.8-54.4) of stroke among subjects with diabetic nephropathy (stage 1, 2 and 3 CKD) after intervention with 2.5mg/d folic acid, but this did not reach statistical significance.

Several theories have been proposed as to why randomized controlled trials with vitamin intervention have not shown a benefit, in terms of a cardiovascular outcome. One theory suggests that intervention with folic acid may be atherogenic. Folate is a cofactor in one-carbon metabolism, providing a methyl group for DNA methylation and, as such, folate supplementation may increase DNA methylation (Sauer et al. 2009). DNA hypermethylation has been shown to be associated with atherosclerosis (Dong et al. 2002). A second theory suggests that the vascular lesions among subjects in secondary prevention trials are so far advanced that they may no longer be susceptible of reversal upon homocysteine-lowering therapy (Ingrosso et al. 2009). A third theory is that individual trials lacked the power required to detect a significant difference. Many vitamin intervention trials were designed to detect 30% reductions in coronary heart disease or stroke based on observational studies published by the late 1990s, while a later meta-analysis of observational studies reported that a lower ptHcy was associated with only an 11% lower risk of coronary heart disease and only a 19% lower risk of stroke (B-Vitamin Treatment Trialists’ Collaboration 2006; Danesh et al. 1998; The Homocysteine Studies Collaboration trial 2002). The B-Vitamin Treatment Trialists’ Collaboration (2006) reviewed the design and power of many large randomized vitamin intervention trials and found that the individual trials did not have adequate power to determine whether lowering ptHcy reduces the risk of CVD events. The
three theories just described regarding the reason why randomized controlled trials with vitamin intervention have not shown a benefit, in terms of a cardiovascular outcome have yet to be substantiated.

In summary, randomized controlled trials to date have not been able to demonstrate that lowering ptHcy with vitamin supplementation reduces the risk of CVD in CKD and non CKD populations. Therefore, the causal relationship between hHcy and CVD has yet to be proven. However, experimental and observational studies have provided evidence of an association between elevated ptHcy concentration and CVD (Lonn et al. 2006; Bonaa et al. 2006; Zoungas et al. 2006; Mann et al. 2008; House et al. 2010; Lonn 2007; Kaul et al. 2006; Welch et al. 1998; Antoniades et al. 2009; Perla-Kajan et al. 2007; Perna AF 2009; van Guldener et al. 2007; Urquhart et al. 2007; Jungers et al. 1997; The Homocysteine Studies Collaboration 2002; Boushey et al. 1995; Danesh et al. 1998).

2.2.3 Hyperhomocysteinemia and Chronic Kidney Disease

Studies conducted in pCKD populations not exposed to folic acid food fortification found mean ptHcy concentration to consistently reflect mild or moderate hHcy (Arnadottir et al. 1996; Chauveau et al. 1992; Hong et al. 1998; Hultberg et al. 1993; Jungers et al. 1999; Nanayakkara et al. 2008; Nerbass et al. 2006; Parsons et al. 2002; Samuelson et al. 1999; Sarnak et al. 2002). As an example, Sarnak et al (2002) studied a pCKD population in the USA with an eGFR of 13 to 24 ml/min/1.73m$^2$ and found median ptHcy concentration to be 20.5µmol/L. In the era of folic acid food fortification, in Canada, mean ptHcy concentration, in subjects with diabetic nephropathy (stage 1, 2 and 3 CKD) was found to reflect mild hHcy (15.5 ± 5.2 µmol/L) (House et al. 2010).

Menon et al. (2005) studied a pCKD population with an eGFR of 13 to 24 ml/min/1.73m$^2$ in the USA before fortification, and reported the prevalence of hHcy (>15 µmol/L) to be 85%. Nerbass et al. (2006) studied a pCKD population with a creatinine clearance of 29.8 ± 14.3 ml/min/1.73m$^2$ in Brazil, a country without folic acid food fortification, and reported the prevalence of hHcy (≥ 14 µmol/L) to be 89%. A review of the literature revealed that the prevalence of hHcy in the pCKD population after the initiation of folic acid food fortification has not been reported.
2.2.4 Determinants of Plasma Total Homocysteine in Non Chronic Kidney Disease and Chronic Kidney Disease Populations

2.2.4.1 Kidney Function

Elevated ptHcy concentration has been reported to be related to decreased kidney function. A national study, conducted in the general population in the USA, prior to the initiation of folic acid food fortification, found the prevalence of hHcy (≥ 15 µmol/L) to be significantly higher in those with an eGFR between 15 to 59 ml/min/1.73m² (32.9%; 95% CI: 25.6 to 41.4) than those with an eGFR ≥ 90 ml/min/1.73m² (5.8%; 95% CI: 4.7 to7.3) (Muntner et al. 2004). A national study, conducted in the general population in the USA after fortification reported kidney function, as assessed by serum creatinine, to be one of the strongest determinants of ptHcy (Ganji et al. 2009).

Numerous studies conducted in pCKD populations living in countries without folate fortification have demonstrated an inverse association between kidney function level and ptHcy and some studies have reported kidney function to independently predict ptHcy (Samuelson et al. 1999; Parsons et al. 2002; Chauveau et al. 1992; Sarnak et al. 2002; Arnadottir et al. 1996; Nerbass et al. 2006).

The mechanism by which kidney function contributes to the variability in ptHcy concentration is not clear. Excretion of homocysteine in the urine via the healthy human kidney is not a significant route for the elimination of ptHcy and therefore decreased kidney function with decreased homocysteine output in the urine is an unlikely mechanism of hHcy (Urquhart et al. 2007; Fux et al. 2005). Specifically, in the healthy human, it has been found that < 1% of total body homocysteine is eliminated in the urine and in experimentally induced hHcy only 2.3% of the homocysteine dose was recovered in the urine.

Decreased renal metabolism of homocysteine, in kidney disease, may be the mechanism of hHcy in rats but unlikely the mechanism in humans. In the healthy rat kidney, in vivo data, derived by measuring renal arteriovenous difference for homocysteine as well as urinary excretion of this amino acid, demonstrated a significant uptake of homocysteine, with substantial homocysteine metabolism and minimal urinary excretion (Bostom et al. 1995; House et al. 1998). These studies are supported by in vitro data that demonstrated, in the rat kidney, the
presence of transport systems for cellular entry of homocysteine and the transsulfuration enzymes, cystathionine β-synthase and γ-cystathionase (Foreman et al. 1982; House et al. 1997). In vitro data also demonstrated that, in the rat kidney, homocysteine metabolism occurs mainly via the transsulfuration pathway, the elimination route of homocysteine (House et al. 1997). In the healthy human kidney, in vivo data, derived by measuring renal arteriovenous difference for homocysteine did not demonstrate significant uptake of this amino acid (van Guldener et al. 1998; Garibotto et al. 2003). The difference in the uptake and metabolism of homocysteine found between rats and humans may relate to the difference in the proportion of free plasma homocysteine available for filtration at the glomerulus, as only the free fraction of ptHcy is thought to be freely filtered (Friedman et al. 2001). The proportion of free plasma homocysteine in rats and humans is 75% and 18%, respectively (Friedman et al. 2001; Mansoor et al. 1992). The difference may also relate to the finding that even though the full set of transsulfuration enzymes is found in the human kidney, the activity of these enzymes is low (Sturman et al. 1970).

Whole body clearance of ptHcy may be altered in CKD as an isotope study found subjects with kidney failure to have significantly lower whole body homocysteine clearance via the remethylation and transsulfuration pathways than healthy controls (Stam et al. 2004).

Altered whole body metabolism of homocysteine by elevated plasma sulfate, due to decreased urinary sulfate excretion, may contribute to hHcy in kidney disease (Nakanishi et al. 2002). Homocysteine is catabolized via the transsulfuration pathway to sulfate (Stipanuk 2004). Nakanishi et al. (2002) demonstrated that compared to healthy controls, people with pCKD have significantly higher plasma tHcy and sulfate concentrations, and a significantly lower urine sulfate concentration. In the same study, among people with pCKD, both plasma and urine sulfate independently predicted ptHcy. The researchers of this study postulated that accumulation of plasma sulfate in pCKD may affect the transsulfuration pathway, resulting in an elevated ptHcy concentration.

Another possibility is that altered whole body metabolism of homocysteine by elevated s-adenosylhomocysteine, due to decreased kidney metabolism of s-adenosylhomocysteine, may contribute to hHcy in kidney disease (Garibotto et al. 2009; Jabs et al. 2006; Finkelstein 2007).
The healthy human kidney may be involved in s-adenosylhomocysteine metabolism as in vivo data, derived by measuring renal arteriovenous difference for s-adenosylhomocysteine, demonstrated significant uptake of s-adenosylhomocysteine (Garibotto et al. 2009). It is possible, then, that in kidney disease, a reduced kidney mass results in decreased metabolic disposal of s-adenosylhomocysteine. In support of this idea is the finding that plasma s-adenosylhomocysteine was inversely associated with kidney function in children with an eGFR of 18 to 150 ml/min/1.73m$^2$ (Jabs et al. 2006). As s-adenosylhomocysteine concentration regulates enzymes involved in all pathways in homocysteine metabolism, elevated plasma s-adenosylhomocysteine level may act to alter whole body metabolism of homocysteine (Finkelstein 2007).

2.2.4.2 Nutritional Determinants of Hyperhomocysteinemia

In the general population, in the era of folic acid food fortification, folate status, as assessed by red blood cell (RBC) folate or by serum folate, has been reported to independently predict ptHcy (Ganji et al. 2009).

Studies conducted in pCKD populations living in countries without folic acid food fortification have reported folate status, as assessed by plasma or RBC folate, to independently predict ptHcy (Nerbass et al. 2006; Parsons et al. 2002). To the writers knowledge the relationship between folate status and ptHcy in pCKD has not been described in the era of folic acid food fortification.

The mechanism by which folate contributes to the variability in ptHcy is related to folate’s role in the remethylation of homocysteine to methionine (Selhub 1999).

Elevated ptHcy concentration has also been reported to be related to vitamin B$_{12}$ status. A meta-analysis of randomized controlled trials found that intervention with vitamin B$_{12}$ (0.4 mg/d) significantly lowered ptHcy by 7%, after ptHcy lowering with supplementation of folic acid (Homocysteine Lowering Trialists’ Collaboration 2005). Vitamin B$_{12}$ status, in the era of folic acid food fortification, may be the dominant nutritional determinant of ptHcy in the general population. Evidence for this comes from an intervention trial conducted in healthy subjects who did not take food fortified with folic acid or personal B-vitamin supplements before or during the
study (Quinlivan et al. 2002). In this trial, healthy subjects received increasing doses of folic acid supplement (0, 100, 200 and 400 µg/d) with each dose of folic acid taken for approximately 6 weeks. With each increase in dose of folic acid supplement the association between ptHcy and serum folate became weaker and the association between ptHcy and serum vitamin B\textsubscript{12} became stronger. Further evidence comes from a cross sectional study conducted in the general population, after fortification, that found vitamin B\textsubscript{12} status, as assessed by serum methylmalonic acid concentration, to be the strongest predictor of ptHcy (Ganji et al. 2009).

In pCKD subjects, living in a country without folic acid food fortification, vitamin B\textsubscript{12} status, as assessed by serum vitamin B\textsubscript{12}, was reported to independently predict ptHcy (Nerbass et al. 2006). Of note, plasma methylmalonic acid is not a valid marker of vitamin B\textsubscript{12} status in CKD as it has been found to be positively associated with serum creatinine (Vogiatzoglou et al. 2009).

The mechanism by which vitamin B\textsubscript{12} contributes to the variability in ptHcy is related to its role as a cofactor for methionine synthase, the enzyme responsible for the remethylation of homocysteine to methionine (Selhub 1999).

Vitamin B\textsubscript{6} status, as assessed by fasting serum pyridoxal 5\textquotesingle-phosphate, has been found to be significantly and inversely associated with fasting ptHcy in the general population (Jacques et al. 2001). However, studies have not been able to provide evidence that vitamin B\textsubscript{6} intervention lowers fasting ptHcy. Ubbink et al. (1996) supplemented non CKD subjects, who were vitamin B\textsubscript{6} deficient (plasma pyridoxal 5\textquotesingle-phosphate < 32.0 nmol/L), with pyridoxine and reported that, compared to baseline, plasma pyridoxal 5\textquotesingle-phosphate significantly increased, but there was no significant difference in fasting ptHcy. In a meta-analysis of intervention trials, the addition of vitamin B\textsubscript{6} to folic acid did not significantly decrease fasting ptHcy further (Homocysteine Lowering Trialists’ Collaboration 2005).

In the pCKD population, vitamin B\textsubscript{6} status, as assessed by fasting serum pyridoxal 5\textquotesingle-phosphate, has been found to be significantly and inversely associated with ptHcy (Menon et al. 2005).
The mechanism by which a vitamin B₆ deficiency results in hHcy may be related to its role as a cofactor for enzymes in the transsulfuration pathway, the elimination route of homocysteine.

Non fasting ptHcy concentration, in healthy subjects, has been found to increase significantly after a meal high in animal protein (Guttormsen et al. 1994). The mechanism by which this occurs may be related to the fact that animal protein is rich in the essential amino acid methionine, the precursor of homocysteine in humans (Guttormsen et al. 1994).

Fasting ptHcy has been found to be inversely, and independently, associated with usual protein intake in the general population (Stolzenberg-Solomon et al. 1999). The mechanism by which a low protein diet results in an elevated fasting ptHcy is not clear. It has been suggested that a vitamin B₁₂ deficiency accounts for the negative association found between protein intake and fasting ptHcy, as animal foods are high in both protein and vitamin B₁₂, and serum vitamin B₁₂ has been found to independently predict ptHcy (Stolzenberg-Solomon et al. 1999; Allen 2009; Ganji et al. 2009). It has also been suggested that a low protein diet results in decreased removal of ptHcy via the transsulfuration and remethylation pathways. This may be due to the fact that intake of serine and choline, metabolites involved in the removal of homocysteine via the transsulfuration and remethylation pathways, respectively, can be low in diets that are low in animal protein (Stolzenberg-Solomon et al. 1999). The mechanism by which a low protein diet results in an elevated fasting ptHcy may also be due to the fact that the activity of enzymes, involved in the removal of ptHcy, via the transsulfuration (cystathionine synthase) and remethylation (betaine-homocysteine methyltransferase) pathways have been found to be significantly lower in the liver of rats fed a low methionine diet compared to those fed a high methionine diet (Finkelstein et al. 1986). In contrast to the finding, by Stolzenberg-Solomon et al. (1999), of an inverse association between fasting ptHcy concentration and usual protein intake, an intervention trial that randomly assigned healthy subjects to a low protein, low methionine diet (12% of total energy, 1.4 g methionine/d) or a high protein, high methionine diet (22% of total energy, 2.7 g methionine/d) diet did not find a significant difference in fasting ptHcy between the two diet groups after 6 months (Haulrik et al. 2002).

In the pCKD population, observed protein intake has been found to be inversely associated with fasting ptHcy (Menon et al. 2005).
There is conflicting evidence as to whether energy-protein status in pCKD subjects is a determinant of ptHcy and as to the direction of the relationship. Serum albumin has consistently been shown to be positively associated with ptHcy and to independently predict ptHcy (Menon et al. 2005; Parsons et al. 2002). The positive association found between serum albumin and ptHcy is more likely related to the role of albumin bound to homocysteine in plasma than to its role as a marker of energy-protein status (Mansoor et al. 1992; Refsum et al. 1985; Keys et al. 1950). Suliman et al. (2002) found that compared to subjects who were well-nourished, as assessed by subjective global assessment (SGA), subjects who were undernourished had a significantly lower ptHcy concentration. In a subsequent study, Suliman et al. (2004) found that low ptHcy was related to a combination of both undernutrition (SGA) and inflammation (C-reactive protein), not undernutrition (SGA) alone. Undernutrition, as assessed by tricep skinfold thickness (TSF) or body mass index (BMI) may or may not be related to a high ptHcy concentration. Plasma tHcy was found to be inversely associated with TSF ($r = -0.17, p<0.05$) by Menon et al. (2005). Parsons et al. (2002) found BMI to inversely, and independently predict ptHcy. In contrast, Nerbass et al. (2006) did not find a significant association between ptHcy and TSF or BMI. It was demonstrated by Duenhas et al. (2003) that energy-protein status, as assessed by TSF (% of standard) or BMI (kg/m$^2$), decreases as kidney function decreases. Specifically, the researchers found TSF (97.4 ± 42.7%) and BMI (25.0 ± 4.2 kg/m$^2$) of pCKD subjects with a creatinine clearance < 19.9 ml/min/1.73m$^2$ to be significantly lower than the TSF (111.4 ± 52.8%) and BMI (27.1 ± 4.7 kg/m$^2$) of pCKD subjects with a creatinine clearance ≥ 43 ml/min/1.73m$^2$. The studies by Menon et al. (2005) (eGFR: 13 to 55 ml/min/1.73m$^2$) and Parsons et al. (2002) (eGFR: < 10 to 75 ml/min/1.73m$^2$) each included subjects with a wide range of kidney function levels which may have resulted in a large variability in energy-protein status. The study by Nerbass et al. (2006) may have included subjects with less variation in kidney function (creatinine clearance: 29.8 ± 14.3 ml/min/1.73m$^2$) and thus perhaps less variation in energy-protein status. As such, Nerbass et al. (2006) may not have been able to find a significant association between ptHcy and TSF or BMI due to less variability in energy-protein status among their subjects, than the studies by Menon et al. (2005) (n=804) and Parsons et al. (2002) (n=197).
2.2.4.3 Other Determinants

The most common genetic cause of mild hHcy is a mutation in the gene encoding the methylenetetrahydrofolate reductase enzyme (Uehara et al. 2008). The mutation is a substitution of cytosine (C) by thymine (T) at nucleotide 677 that changes alanine to valine at position 222 of the amino acid sequence (Frosst et al. 1995). Compared to individuals without this mutation (CC), individuals homozygous (TT) for the substitution have been shown to have significantly lower MTHFR activity and significantly higher ptHcy. Jacques et al. (1996) studied the general population (n=365) in the USA and found the prevalence of the homozygous (TT) genotype to be 12.3%. In the same study, the researchers found a significant interaction between MTHFR genotype and folate status. Specifically, in the subjects with plasma folate levels below the sample median, individuals homozygous for the substitution (TT) had a significantly higher ptHcy concentration (12.1 µmol/L) than individuals without the mutation (CC) (9.8 µmol/L) (p<0.05). In subjects with plasma folate levels at or above the sample median, there was no significant difference in ptHcy concentration between individuals without the mutation (CC) (7.8 µmol/L) and individuals homozygous for the substitution (TT) (7.9 µmol/L). In the general population, the absolute difference in ptHcy between MTHFR genotypes (TT vs. CC) was found to be higher (2.5 µmol/L) before folic acid food fortification compared to after folic acid food fortification (0.7 µmol/L) (Tsai et al. 2009).

The prevalence (12.8%) of the homozygous (TT) genotype in the CKD population appears to be similar to that (12.3%) in the general population (Fodinger et al. 1997; Jacques et al. 1996). A significant interaction between genotype and folate status has been demonstrated in the CKD population (Jacques et al. 1996; Fodinger et al. 1997).

In a study conducted in the general population (n=16,254), age has been found to independently predict ptHcy (Ganji et al. 2009). The increase of ptHcy with age is thought to be due to age-dependent causes which include a decline in kidney function, a decrease in enzymes that metabolize homocysteine and an increase in vitamin B_{12} deficiency as result of intestinal malabsorption (Ganji et al. 2009). The authors also found gender to be a determinant of ptHcy in the general population, with males (8.23 ± 1.01 µmol/L) having a significantly higher ptHcy than females (7.50 ± 1.01 µmol/L) (Ganji et al. 2009). This gender difference may be related to the
fact that mean cystathione β synthase activity is significantly lower in males than females (Vitvitsky et al. 2007). The difference in the activity level of this enzyme may be related to testosterone as this hormone has been found to regulate cystathione β synthase activity (Vitvitsky et al. 2007).

In another study of the general population (n=10,537), coffee consumption has been found to be significantly and positively associated with ptHcy (Ulvik et al. 2008). Verhoef et al. (2002) conducted a crossover intervention trial whereby healthy subjects received three treatments, in random order, for two weeks each. Treatments included coffee, caffeine pill (no coffee), and placebo pill (no coffee). With the exception of caffeine provided by the treatments all other caffeine containing products were prohibited. The coffee and caffeine pill treatments each provided 870 mg caffeine/day. The outcome measure was fasting ptHcy. Compared to placebo, coffee significantly increased ptHcy by 0.9 ± 1.2 µmol/L or 11%. Compared to placebo, caffeine (pill) significantly increased ptHcy by 0.4 ± 1.0 µmol/L or 5%. The effect of caffeine (pill) on ptHcy was significantly less than the effect of coffee, suggesting that compounds in coffee, other than caffeine, must also be contributing to the homocysteine raising effect of coffee.

In the general population (n=6,545), alcohol consumption has been found to independently predict ptHcy (Ganji et al. 2003). Gibson et al. (2008) conducted a crossover intervention trial whereby healthy subjects received three treatments in random order for two weeks each. Treatments included red wine, vodka, and no alcohol. The outcome measure was fasting ptHcy. Compared to no alcohol, red wine significantly increased mean ptHcy while vodka did not.

In the general population (n=16,254) serum cotinine, a biochemical marker of smoking, was found to independently predict ptHcy (Ganji et al. 2009). The mechanism by which smoking increases ptHcy is not clear.

Medications have also been found to influence ptHcy concentration (Appendix A) (Atar et al. 2005; Desouza et al. 2002; Jackson et al. 1998; Kilicdag et al. 2005; Luftjohann et al. 2001; Milionis et al. 2003; Miller et al. 2003; Morris et al. 2000; Nilsson et al. 2004; Rin Suh et al. 2001; Toffoli et al. 2003; Varela-Moreiras 2001; Wulffele et al. 2003).
2.3 Folic Acid Food Fortification

2.3.1 Folate Metabolism in Health

Figure 2.2 depicts the chemical structure of folic acid or pteroylmonoglutamic acid, which includes a pteridine ring, a para-aminobenzoic acid and a glutamic acid residue (Lucock 2000; Fowler 2001). This is the parent structure for a large family of folates (Lucock 2000). Folates differ in the oxidation state of the pteridine ring, the one-carbon substituent at the N5 and N10 positions and the number of glutamic acid residues (Lucock 2000).

![Figure 2.2 Structure of Folic Acid](Reproduced from Shane, with permission. Copyright Elsevier 2000)

Natural folate in food does not exist as folic acid, but rather exists mainly as 5-methyl-tetrahydropolyglutamic acid and 10-formyltetrahydropolyglutamic acid (Lucock 2000; Fowler 2001). Food polyglutamates are not able to cross cell membranes. As such, in the intestine, they are hydrolyzed to monoglutamates by pteroyl-γ-glutamylhydrolase (folate conjugase) and enter the enterocyte by a saturable active transport system (Lucock 2000; Fowler 2001; Food and Nutrition Board, Institute of Medicine 1998b). In the enterocyte, tetrahydromonoglutamates are metabolized to 5-methyl-tetrahydromonoglutamic acid and enter the portal circulation. Much of this folate is taken up by the liver where it can be released to the systemic circulation. 5-methyl-tetrahydromonoglutamic acid is the plasma form of this vitamin (Lucock 2000; Fowler 2001). An exception to this is the presence of unmetabolized folic acid in the plasma (Troen et al. 2006; Food and Nutrition Board, Institute of Medicine 1998b). Plasma 5-methyl-tetrahydromonoglutamic acid can be incorporated into the developing erythroblast during
erythropoiesis in the bone marrow. Red blood cell folate is the storage form of folate and is largely in the form of 5-methyl-tetrahydropolyglutamic acid and formyl-tetrahydropolyglutamic acid (Lucock 2000). Alternatively, plasma 5-methyl-tetrahydromonoglutamic acid can be taken up by peripheral tissue where it is metabolized to tetrahydromonoglutamic acid and subsequently, tetrahydropolyglutamic acid (Lucock 2000).

In the tissue cell, folates must be in their reduced and polyglutamate forms in order to be biologically active (Food and Nutrition Board, Institute of Medicine 1998b; Bailey et al. 1999; Beaudin et al. 2009). Figure 2.3 depicts the metabolism of folate in the body. Folate, as a cofactor in one-carbon metabolism, is involved in DNA methylation and nucleotide synthesis pathways (Sauer et al. 2009). With regard to DNA methylation, folate, in the form of 5-methyl-tetrahydropolyglutamic acid, donates a methyl group to homocysteine to form methionine (Bailey et al. 1999). Methionine is activated by adenosine triphosphate to form S-adenosylmethionine and S-adenosylmethionine, in turn, serves as the universal donor for a variety of methylation reactions, including DNA methylation (Miller et al. 2008). With regard to nucleotide synthesis, folate, in the form of 5,10 methylenetetrahydropolyglutamic acid is involved in two pathways. In one pathway, the conversion of 5,10 methylene tetrahydropolyglutamic acid to dihydrofolate results in the formation of thymidylate. In another pathway, 5,10 methylenetetrahydromonomoglutamic acid, through a series of three reactions, is converted to dihydrofolate and purine (Sauer et al. 2009; Blom 2009; Miller et al. 2008).

Synthetic folate, in food or pill form, exists as pteroylmonoglutamic acid (folic acid) (Lucock 2000). In the intestine folic acid is reduced to tetrahydromonoglutamic acid in a two step process, with both reactions requiring dihydrofolate reductase (Troen et al. 2006; Sauer et al. 2009). Specifically, folic acid is reduced to dihydrofolate, which is subsequently reduced to tetrahydromonomoglutamic acid. Tetrahydromonoglutamic acid is methylated to 5-methyl-tetrahydromonoglutamic acid, enters the portal circulation where it is taken up by the liver and subsequently released into the systemic circulation (Wright et al. 2007). Unmetabolized folic acid has been found in the systemic circulation (Kelly et al. 1997). The mechanism by which this occurs may be related to the findings that dihydrofolate reductase in both the intestine and liver reduces folic acid at a significantly lower rate than it reduces dihydrofolate and that the activity of this enzyme in both the intestine and liver in humans may be low (Wright et al. 2007).
Unmetabolized folic acid in the plasma can be taken up by peripheral tissue where it is metabolized to reduced forms of folate (Troen et al. 2006).

**Figure 2.3 Folate Metabolism** (adapted from Sauer et al. 2009)

DHF = dihydrofolate; SAH = S-adenosyl homocysteine; 
SAM = S-adenosyl methionine; THF = tetrahydrofolate
2.3.2 Folic Acid Fortification of the Food Supply

In 1996, the Food and Drug Administration in the USA issued a final rule, effective January 1998, that required all dry enriched cereal-grain products (flour, rice, breads, rolls and buns, pasta, corn grits, corn meal, farina, macaroni, and noodle products) to be fortified with folic acid, at a dose of 140 µg/100g (Choumenkovitch et al. 2002). The purpose of this health strategy was to increase the folate intake of women of childbearing age in an effort to reduce the occurrence of neural tube defects in newborns (Food and Drug Administration 1993). Prior to the implementation of this strategy, simulated folic acid food fortification was used to estimate the change in observed folate intake (natural food folate and food folic acid from voluntary addition to ready to eat breakfast cereals) from before fortification to observed folate intake (natural food folate, food folic acid from voluntary addition to ready to eat breakfast cereals and food folic acid from mandatory addition to cereal-grain products) after fortification, in the general population (Food and Drug Administration 1993; Crane et al. 1995). The observed folate intake value at the 20\textsuperscript{th} percentile of the observed folate intake distributions before and after fortification suggested that, at the lower end, the estimated increase in folic acid intake would be 70 µg/d and 90 µg/d in females and male, respectively, ≥ 51 years old. The observed folate intake value at the 95\textsuperscript{th} percentile of the observed folate intake distributions before and after fortification suggested that, at the upper end, the estimated increase in folic acid intake would be 110 µg/d and 120 µg/d in females and male, respectively, ≥ 51 years old. Bailey et al. (2010) reported that, in the era of folic acid food fortification in the USA, mean usual folic acid intake from food was 148 ± 5.7 µg/d and 156 ± 6.0 µg/d in women 51 to 70 years old and ≥ 71 years old, respectively. Mean usual folic acid intake from food was 197 ± 7.6 µg/d and 200 ± 5.2 µg/d in males 51 to 70 years old and ≥ 71 years old, respectively.

Health Canada concurred with the USA decision to fortify flour with folic acid based both on the public health benefit and the benefit to Canadian industry of harmonizing with the USA (Canada Gazette 1998). Accordingly, Health Canada mandated folic acid fortification of all white flour and enriched pasta to 150 µg/100g and 200 µg/100g, respectively, effective November, 1998 (Canada Gazette 1998). Folate intake from food (natural and folic acid) was
found to be significantly higher after fortification compared to before fortification, in elderly women (≥ 65 years old) in Ontario (usual folate intake: 225.8 µgDFE; 95% CI: 217.3 to 234.5 vs. 326.5 µgDFE; 95% CI: 317.9 to 335.4) and in women of child bearing age in British Columbia (observed folate intake: 296 ± 153 µgDFE vs. 470 ± 200 µgDFE) (deWolfe 2007; French et al. 2003). Liu et al. (2004) measured folic acid intake from food fortification by elderly adults (≥ 65 years old), in the era of folic acid food fortification, living in Newfoundland, Canada, and reported the mean observed intake to be 74 µg/d. Recently, Shakur et al. (2010) analyzed national data and reported mean usual folic acid intake from food fortification in subjects ≥ 51 years old to be 78 ± 36 to 115 ± 54 µg/d, varying with age and gender.

Among the general population living in various parts of Canada, RBC folate, the storage form of folate, was found to be significantly higher after fortification compared to before fortification (Ray et al. 2002; Liu et al. 2004). Ray et al. (2002) conducted a retrospective study in Ontario using data of adult patients who had folate status measured based on clinical grounds. A radioassay was used to measure RBC folate, with a maximum reporting limit of 1450 nmol/L. Mean RBC folate was 680.3 nmol/L (95% CI: 668.8 to 691.9) before fortification (n=3257) and 851.6 nmol/L (95% CI: 841.2 to 862.0) after fortification (n=4171) (p< 0.001). Liu et al. (2004) prospectively studied elderly adults living in Newfoundland not taking folic acid supplements. A radioassay was used to measure RBC folate. Mean RBC folate was 625 nmol/L (95% CI: 601 to 649) before fortification (n=233) and 818 nmol/L (95% CI: 784 to 854) after fortification (n=204) (p<0.001). A similar picture has been noted with regard to unmetabolized folic acid in the plasma. That is, median (range) folic acid in the plasma of folate supplement nonusers was found to be significantly higher after fortification (0.25(0-15.18)) compared to before fortification (0.50(0-24.11)) (Kalmbach et al. 2008).

In Canada, folic acid food fortification has been effective, as De Wals et al. (2007) reported that the prevalence of neural tube defects significantly decreased after fortification (0.86 per 1000 births; 95% CI: 0.80 to 0.92) compared to before fortification (1.58 per 1000 births; 95% CI: 1.48 to 1.64), a 46% (95% CI: 40 to 51) reduction. Another positive consequence of fortification has been its effect on ptHcy in the general population. Pfeiffer et al. (2008) found that both ptHcy and the prevalence of hHcy, among those ≥ 20 years old in the general population in the USA, were significantly lower after fortification compared to before
fortification. For example, among males \( \geq 60 \) years, median ptHcy and the prevalence of hHcy were 10.7 (95% CI: 10.1 to 11.4) µmol/L and 31.8% (95% CI: 25.1 to 39.2) before fortification and 9.67 (95% CI: 9.34 to 10.0) µmol/L and 14.2% (95% CI: 10.5 to 19.0) after fortification. Also, fortification may have had a beneficial effect on CVD in Canada. Yang et al. (2006) showed that the annual percent change in stroke mortality rate averaged -1.0% (95% CI: -1.4 to -0.6) per year from 1990 to 1997 (before fortification) and accelerated to -5.4% (95% CI: -6.0 to -4.7) per year from 1998 to 2002 (after fortification) (p ≤ 0.0001).

Studies conducted in various CKD populations, in the era of folic acid food fortification, have reported that a majority of subjects have hHcy, including those receiving standard (94%; ptHcy > 12 µmol/L) or nocturnal hemodialysis (57%; ptHcy > 12 µmol/L), and those with a renal transplant (69.8%; ptHcy ≥ 12 µmol/L) (Friedman et al. 2002; Bostom et al. 1999). To the writer’s knowledge, the prevalence of hHcy, in the pCKD population, after fortification, has not been reported. Jamison et al. (2007) screened potential subjects with pCKD for the presence of hHcy (ptHcy ≥ 15 µmol/L), as a study inclusion criteria, during recruitment for a randomized control trial in the USA after fortification. The researchers reported that of the subjects screened (n=1619), three hundred and fourteen were excluded because of a ptHcy of <15 µmol/L (n=187). This suggests that the prevalence of hHcy may be approximately 88% in the pCKD population after fortification.

It is possible that the pCKD population still has a high prevalence of hHcy despite fortification, given that poor dietary folate intake may be present possibly due to poor overall food intake as a result of the presence of gastrointestinal symptoms of uremia. Uremia is a toxic syndrome caused by a decrease in kidney function and the subsequent retention of compounds that are toxic (Lindholm B et al. 2004). Anderstam et al. (1996) demonstrated that uremic toxins suppress food intake in animals. Uremic toxins may decrease food intake through anorexia which has been hypothesized to be related to an over production of brain serotonin (Murtagh et al. 2007; Aguilera et al. 2004). In support of this hypothesis have been the findings that, in uremia, there is an increase of cholecystokinin, cytokine TNF-α and the nitric oxide inhibitor asymmetrical dimethyl-L-arginine, a decrease in ghelin, and an altered plasma amino acid profile, each of which can increase serotonin (Aguilera et al. 2004). Anorexia has also been hypothesized to be related to a decrease in neuropeptide Y, as leptin has been found to be
increased in uremia (Murtagh et al. 2007; Aguilera et al. 2004; Aguilera et al. 2007; Carrero et al. 2008). Uremic toxins may also decrease food intake through inducing nausea, vomiting, early satiety, taste change and aversions to red meat, poultry and fish (Fernstrom et al. 1996; Dobell et al. 1993; Murtagh et al. 2007). Bossola et al. (2005) found that 38% of uremic patients reported at least one gastrointestinal symptom of uremia when questioned about these: nausea, vomiting, early satiety, taste and smell alterations, and meat aversion. Evidence that people with pCKD may have poor overall food intake secondary to gastrointestinal symptoms of uremia was provided by Bossola et al. (2005), who grouped subjects by observed energy intake and found that those with an observed intake of < 30 kcal/kg/day had significantly higher gastrointestinal uremic symptoms compared to those with an observed energy intake of ≥ 30 kcal/kg/d (p=0.045). If people with pCKD have low overall food intake secondary to gastrointestinal symptoms of uremia, they may have low dietary folate intake, despite folic acid food fortification and this may contribute to an ongoing high prevalence of hHcy. As previously mentioned, the prevalence of hHcy has not been described after initiation of folic acid food fortification in the pCKD population.

2.4 Intake and Status of Folate, Vitamin B\textsubscript{12}, Vitamin B\textsubscript{6}, Energy and Protein in Non Chronic Kidney Disease and Chronic Kidney Disease Populations

If people with pCKD have low overall food intake secondary to gastrointestinal symptoms of uremia, they may have low intake, from food, of all the nutrients that are determinants of ptHcy. As previously mentioned, nutritional determinants of ptHcy include folate, vitamin B\textsubscript{12}, vitamin B\textsubscript{6}, and energy-protein status, and protein intake.

Published studies that have described the average micronutrient intake of a group have reported this as either observed or usual intake. Observed intake of a group represents the average of individual observed intakes. Observed intake of a nutrient by an individual represents food intake data on a single day or the average of a small number of days (Food and Nutrition Board, Institute of Medicine 2001b; Food and Nutrition Board, Institute of Medicine 2001c; Barr et al. 2002). It does not represent the long-term usual intake by an individual. The usual intake of a group represents the average value of an observed intake distribution that has been statistically adjusted to account for within-person standard deviation (day-to-day variation in a
subject’s intake) and between-person standard deviation (subject-to-subject variation in requirement/intake). To obtain the within-person standard deviation, at least two days of observed food intake data is required for some or all individuals in the group.

To assess adequacy of micronutrient intake of a group the Institute of Medicine in the USA recommends using either the Probability Approach or a short-cut version of this approach called the Estimated Average Requirement cut-point method (Food and Nutrition Board, Institute of Medicine 2001b). Both methods require knowledge about an observed intake distribution that has been statistically adjusted to account for within-person variation in intake and between-person variation in requirement. With either of these methods the prevalence of inadequate/adequate usual intake of the group can be reported. It is important to recognize that published research reporting on the adequacy of micronutrient intake without adjusting for within-person variation in intake and between-person variation in requirement is reporting on the adequacy of observed intake, not usual intake, and has not used the probability approach.

In the general population in Canada, in the era of folic acid food fortification, median (25th, 75th percentile) usual folate intake from food (natural and folic acid) has been reported to be 471 (380,577) µg DFE/d and 403 (326,494) µg DFE/d by males 51 to 70 years old and > 70 years old, respectively (Statistics Canada 2004). The corresponding prevalence of inadequate usual folate intake from food was 11.5% and 21%. Median (25th, 75th percentile) usual folate intake from food (natural and folic acid) has been reported to be 390 (320,772) µg DFE/d and 328 (265,404) µg DFE/d by females 51 to 70 years old and > 70 years old, respectively. The corresponding prevalence of inadequate usual folate intake from food was 25% and 47%.

Fassett et al. (2007) studied a pCKD population in Australia, a country without folic acid food fortification, and reported observed folate intake from food and supplements to be 280.9 µg/d ± 108.4. It is possible that this reported value is an underestimate as the researchers did not express folate intake in dietary folate equivalents which would have accounted for the increased bioavailability of folic acid from supplements. In the era of folic acid food fortification, folate intake from food (natural and folic acid), or food (natural and folic acid) and supplements combined, and the proportion with inadequate intake, from food (natural and folic acid) alone, by the pCKD population, have not been published.
In the general population, after fortification, RBC folate was reported to be 851.6 nmol/L (95% CI: 841.2 to 862.0) and 818 nmol/L (95% CI: 784 to 854), among adults in Ontario (57.4 ± 21.1 years old) and in Newfoundland (> 65 years old), respectively (Ray et al. 2002; Liu et al. 2004). Importantly, after fortification, the prevalence of folate deficiency appears to be extremely low. Ray et al. (2002) reported the prevalence of deficient folate status, as assessed by RBC folate (< 215 nmol/L) to be 0.41% in Ontario; and Liu et al. (2004) reported the prevalence of deficient folate status, as assessed by RBC folate (< 373 nmol/L) to be 1.6% in Newfoundland. Recently, Colapinto et al. (2010) analyzed national data and reported the normal range of RBC folate concentration of the Canadian population to be 621 (95% CI: 544 to 699) to 2580 (95% CI: 2363 to 2797).

In the pCKD population, RBC folate has been reported in Sweden, a country without folic acid food fortification. Arnadottir et al. (1996) studied a pCKD population (n=19) with an eGFR 6 to 32 ml/min/1.73m². The average age of the population was 57 ± 17 years and no subject took a folic acid supplement. RBC folate concentration was measured using a radioassay, and was reported to be 302 ± 89 nmol/L. In the era of folic acid food fortification, RBC folate in the pCKD population has not been published.

In the general population in Canada, median (25th, 75th percentile) usual vitamin B₁₂ intake from food was reported to be 4.3 (3.3,5.9) µg/d and 3.7 (2.7,5.3) µg/d by males 51 to 70 years old and > 70 years old, respectively, and 3.3 (2.6,4.6) µg/d and 3.0 (2.3,4.2) µg/d by females 51 to 70 years old and > 70 years old, respectively (Statistics Canada 2004). Due to large sampling variability in these gender and age groups, there was no corresponding prevalence of inadequate usual intake with the exception of females > 70 years old, which was 15.3%. Vitamin B₁₂ intake from food, or food and supplement combined, and the proportion of people with inadequate dietary vitamin B₁₂ intake, from food alone, by the pCKD population, have not been published.

Ray et al. (2000) reported that 85% of the general population (n=692) in Ontario has a serum vitamin B₁₂ between 134.6 pmol/L to 663.7 pmol/L. In a later publication that included a larger sample size (n=4171), Ray et al. (2002) reported the mean serum vitamin B₁₂ to be 292.9 (95% CI: 288.3 to 297.6) pmol/L. The prevalence of vitamin B₁₂ deficiency has been described
in elderly subjects, not taking supplements, in various provinces in Canada. Garcia et al. (2008) studied a population (73 ± 4.9 years; n=281) in Ontario and found the prevalence of vitamin B\textsubscript{12} deficiency (serum vitamin B\textsubscript{12} < 165 pmol/L) to be 20%. Compared to this study, Liu et al (2004) used a lower cut-point to define vitamin B\textsubscript{12} deficiency (serum vitamin B\textsubscript{12} < 133 pmol/L) and reported the prevalence of deficient vitamin B\textsubscript{12} status to be 11.8% among an elderly (> 65 years old) population (n=186) in Newfoundland.

A prospective study (n=245) conducted in a pCKD population (eGFR: 13 to 24 ml/min/1.73m\textsuperscript{2}) in the USA, that did not report excluding supplement users, found median serum vitamin B\textsubscript{12} to be 326 pmol/L (Sarnak et al. 2002). Nerbass et al. (2006) studied a smaller (n = 66) pCKD population (29.8 ml/min/1.73m\textsuperscript{2}) in Brazil, not taking supplements, and reported a serum vitamin B\textsubscript{12} of 381 ± 174 pmol/L. In the same study, no subject was vitamin B\textsubscript{12} deficient (serum vitamin B\textsubscript{12} <147 pmol/L). Jungers et al. (1999) conducted a prospective vitamin intervention trial (n=78) in a pCKD population (28.4 ± 12.7 ml/min/1.73m\textsuperscript{2}) in France and reported a baseline serum vitamin B\textsubscript{12} (237 ± 119 pmol/L) that appears to be lower than the two studies mentioned above and a prevalence (28%) of vitamin B\textsubscript{12} deficiency (serum vitamin B\textsubscript{12} < 150 pmol/L) that appears to be higher than that reported by Nerbass et al. (2006). The two main causes of a vitamin B\textsubscript{12} deficiency include low vitamin B\textsubscript{12} intake and increased age (Food and Nutrition Board, Institute of Medicine 1998c; Allen 2009). Vitamin B\textsubscript{12} depletion can occur with aging because of malabsorption of food bound vitamin B\textsubscript{12} caused by atrophy of the gastric mucosa and the gradual loss of gastric acid, which releases the vitamin from food (Allen 2009). As such, it is possible that the lower serum vitamin B\textsubscript{12} and higher prevalence of vitamin B\textsubscript{12} deficiency found among the subjects in the study by Jungers et al. (1999) are due to a lower vitamin B\textsubscript{12} intake from food and supplements and/or older age of the participants.

Median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) usual vitamin B\textsubscript{6} intake from food in the general population in Canada was reported to be 2.03 (1.66,2.46) mg/d and 1.78 (1.4,2.19) mg/d by males 51 to 70 years old and > 70 years old was, respectively. The corresponding prevalence of inadequate usual vitamin B\textsubscript{6} intake from food was 10.9% and 23.1% (Statistics Canada 2004). In the same survey, median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) usual vitamin B\textsubscript{6} intake from food was 1.63 (1.37,1.93) mg/d and 1.50 (1.21,1.85) mg/d by females 51 to 70 years old and > 70 years old, respectively.
The corresponding prevalence of inadequate usual vitamin B$_6$ intake from food was 19.4% and 32.5%.

In a pCKD population in the USA mean observed vitamin B$_6$ intake from food was reported to be 2.0 ± 1.3 mg/d in those (n=6) with moderate kidney disease (serum creatinine 360 to 570 mg/dl) and 1.2 ± 0.5 mg/d in those (n=7) with severe kidney disease (620 to 1820 mg/dl) (Kopple et al. 1981). Of note, the sample size of these observed intakes was at most seven and thus, may not accurately reflect intake by the group. In addition, dietary reference intakes for adults (≥ 19 years old) for vitamin B$_6$ vary by life stage (age) and gender. Kopple et al. (1981) did not report observed intake by age and gender. In the pCKD population, vitamin B$_6$ intake from food alone has not been reported by age and gender. In addition, vitamin B$_6$ intake from food and supplements combined, and the proportion with inadequate intake, from food alone, by the pCKD population, has not been published.

Among supplement nonusers in the general population in the USA serum pyridoxal 5′-phosphate was 36 nmol/L (95% CI: 33 to 40) and 36 nmol/L (95% CI: 31 to 41) in adults 45 to 64 years old and in those ≥ 60 years old, respectively (Morris MS, 2008). In the same study, the prevalence of vitamin B$_6$ deficiency (pyridoxal 5′-phosphate < 20 nmol/L) in adults 45 to 64 years old and ≥ 60 years old, was 23% (95% CI: 20 to 27) and 24% (95% CI: 18 to 30), respectively. The mechanism(s) of vitamin B$_6$ deficiency in the general population is not clear but conditions reported to increase the risk of a vitamin B$_6$ deficiency include low vitamin B$_6$ intake, energy-protein undernutrition, smoking, elevated serum alkaline phosphatase, inflammation, a catabolic state, advanced age, kidney disease and high intake of protein, caffeine or alcohol (Spinneker et al. 2007; Groff JL et al. 1999; Leklem JE et al. 2001; Food and Nutrition Board, Institute of Medicine 1998a; Friso et al. 2001).

In a prospective study (n=245) conducted in a pCKD population (13 to 24 ml/min/1.73m$^2$) in the USA that did not report excluding supplement users, the median serum pyridoxal 5′-phosphate was found to be 38.8 nmol/L (Sarnak et al. 2002). In a cross-sectional study (n=19) of pCKD subjects (eGFR 6 to 32 ml/min/1.73m$^2$) not taking vitamin B$_6$ supplements a lower plasma pyridoxal 5′-phosphate was reported (23.7 ± 10.6 nmol/L) (Arnadottir et al. 1996).
Mean observed energy intake was found not to be significantly different between people with normal kidney function (24.7 ± 1.7 kcal/kg/d; n=15) and those with pCKD (23.8 ± 1.6 kcal/kg/d; n=15) in a prospective study conducted by O’Sullivan et al. (2002). In a larger study (n=487), Duenhas et al. (2003) retrospectively analyzed observed energy intake data that had been collected as part of a prospective nutritional protocol and found that observed energy intake spontaneously decreased as kidney function declined. Specifically, the researchers grouped participants by kidney function quartile and found that those in the lowest quartile (creatinine clearance < 19.9 ml/min/1.73 m²) had a significantly lower mean observed energy intake (23.5 ± 7.9 kcal/kg/d) compared to those in the highest quartile (creatinine clearance ≥ 43 ml/min/1.73 m²) (25.9 ± 9.2 kcal/kg/d). Numerous studies have measured average observed energy intake in pCKD populations, with a mean eGFR representing stage 4 CKD, and reported mean observed intake to range from 23.57 to 26.2 kcal/kg/d (O’Sullivan et al. 2002; Avesani et al. 2004; Huang et al. 2008; Carvalho et al. 2004). Avesani et al. (2005) studied a pCKD population (creatinine clearance 34.0 ± 18.2 ml/min/1.73 m²) in Brazil in which a majority of the subjects had been prescribed an energy intake consistent with the current practice guideline (30-35kcal/kg/d), and reported that 81% of the subjects had an observed intake below the guideline (National Kidney Foundation 2000). Similarly, Fassett et al. (2007) studied a pCKD population (creatinine clearance 40.8 ± 19.2 ml/min/1.73 m²) in Australia and found that 84% of the subjects had an observed energy intake below that recommended by the current practice guideline.

O’Sullivan et al. (2002) reported mean observed protein intake not to be significantly different between subjects with pCKD (creatinine clearance 28 ± 5 ml/min/1.73 m²) (protein 1.0 ± 0.1 g/kg/d; n=15) and those with normal kidney function (creatinine clearance 80 ± 6 ml/min/1.73 m²) (protein 1.1 ± 0.1 g/kg/d; n=15). In a larger study (n=487) conducted in a pCKD population, mean observed protein intake was found to be significantly lower in subjects in the lowest kidney function quartile (creatinine clearance < 19.9 ml/min/1.73 m²) (protein 0.88 ± 0.33 g/kg/d) compared to those in the highest quartile (creatinine clearance ≥ 43 ml/min/1.73 m²) (protein 1.04 ± 0.42 g/kg/d) (Duenhas et al. 2003). Numerous studies have measured average observed protein intake in pCKD populations with a mean eGFR representing stage 4 CKD, and reported mean observed intake to range from 0.8 to 1.04 g/kg/d. (O’Sullivan et al. 2002; Huang et al. 2008; Carvalho et al. 2004; Chauveau et al. 2003).
The current practice guideline recommends assessing energy-protein status in the pCKD population using SGA and serum albumin (National Kidney Foundation 2000). Energy-protein status has been measured in pCKD populations using SGA, and the prevalence of undernutrition was reported to be 20% by Campbell et al. (2007) and 28% by Lawson et al. (2001). Energy-protein status has been measured, objectively, in a pCKD population (eGFR: 18 ± 5 ml/min/1.73m²) using serum albumin, and the prevalence of undernutrition (< 38 g/L) was reported to be 0% (Carvalho et al. 2004). In a cross sectional study of a pCKD population (n=599) (eGFR: 21.5 ± 13 ml/min/1.73m²), serum albumin was found to be 39 ± 2.5 g/L (Huang et al. 2008).

In summary, if people with pCKD have low overall food intake, possibly as a result of the presence of gastrointestinal symptoms of uremia, they may have low intake, from food, of all the nutrients that are determinants of ptHcy. Nutritional determinants of ptHcy include folate, vitamin B₁₂, vitamin B₆, and energy-protein status, and protein intake. A review of the published research revealed that the following information, as it relates to the pCKD population, has not been reported: 1) Folate intake from food (natural and folic acid), or food (natural and folic acid) and supplements combined, and the proportion with inadequate intake, from food (natural and folic acid) alone, in the era of folic acid food fortification. 2) VitaminB₁₂ intake from food, or food and supplements combined, and the proportion with inadequate intake of these nutrients, from food alone. 2) Vitamin B₆ intake from food, by age and gender. 3) Vitamin B₆ intake from food and supplements combined, and the proportion with inadequate intake of this nutrient, from food alone.
Chapter 3

3 Rationale and Objectives

3.1 Rationale

An estimated 1.5 million Canadians have pCKD (Stigant et al. 2003). It is predicted that the incidence of CKD will increase in tandem with the expected increase in the incidence of type 2 diabetes, the leading cause of CKD (Atkins 2005). Patients with pCKD have a high prevalence of CVD, and mortality from CVD (Collins et al. 2003; Astor et al. 2008). Elevated plasma concentration of the amino acid homocysteine has been found to be associated with CVD, in pCKD (Jungers et al. 1997). Before folic acid food fortification, a large majority (89%) of people with pCKD were found to have an elevated ptHcy concentration (Nerbass et al. 2006). Studies conducted in pCKD populations living in countries without folic acid food fortification have reported folate status, as assessed by plasma or RBC folate, to independently predict ptHcy (Nerbass et al. 2006; Parsons et al. 2002). Health Canada mandated folic acid fortification of all white flour and enriched pasta effective November, 1998 (Canada Gazette 1998). In the general US population, the prevalence of hHcy, among those ≥20 years old was found to be significantly lower after fortification compared to before fortification (Pfeiffer et al. 2008). In the pCKD population the prevalence of hHcy has not been described after initiation of folic acid food fortification. What is the prevalence of hHcy in subjects with pCKD in the era of folic acid food fortification? It is possible that the pCKD population still has a high prevalence of hHcy despite fortification given that poor dietary folate intake may be present possibly due to poor overall food intake as a result of the presence of gastrointestinal symptoms of uremia. This study will determine the prevalence of hHcy, and its determinants, in this population, in the era of folic acid food fortification.

Published research indicates that the nutritional determinants of ptHcy include folate, vitamin B12, vitamin B6, and energy-protein status as well as protein intake. If people with pCKD have low overall food intake, possibly as a result of the presence of gastrointestinal symptoms of uremia, they may have low intake, from food, of all the nutrients that are determinants of ptHcy. A review of the published research revealed that the following
information, as it relates to the pCKD population, has not been reported: 1) Folate intake from food (natural and folic acid), or food (natural and folic acid) and supplements combined, and the proportion with inadequate intake, from food (natural and folic acid) alone, in the era of folic acid food fortification. 2) VitaminB$_{12}$ intake from food, or food and supplements combined, and the proportion with inadequate intake of these nutrients, from food alone. 2) Vitamin B$_{6}$ intake from food, by age and gender. 3) Vitamin B$_{6}$ intake from food and supplements combined, and the proportion with inadequate intake of this nutrient, from food alone. What is the intake of folate, vitamin B$_{12}$ and vitamin B$_{6}$ by subjects with pCKD? This study will determine intake and status of the known nutritional determinants of ptHcy, in subjects with pCKD, in the era of folic acid food fortification.

Currently, there are no practice guidelines for vitamin supplementation in the pCKD population. The lack of research regarding the proportion of this population that has inadequate dietary intake of the key vitamins involved in homocysteine metabolism, and the absence of practice guidelines for vitamin supplementation, has resulted in uncertainty among health care professionals as to the need for the supplementation of these vitamins in the pCKD population. This is a concern because elevated ptHcy concentration has been found to be associated with CVD, in pCKD (Selhub 1999; Jungers et al. 1997). This study will determine the proportion of pCKD patients with inadequate dietary intake of folate, vitamin B$_{12}$ and vitamin B$_{6}$. This information, considered in conjunction with vitamin status and ptHcy, will provide much needed evidence to guide clinical practice regarding the need for vitamin supplementation.
3.2 Objectives

In pCKD patients attending an ambulatory pCKD clinic, our objectives were to describe:

1. The prevalence of hHcy.

2. Intake of folate, vitamin B\textsubscript{12}, vitamin B\textsubscript{6}, energy and protein from food, food and supplements combined, and estimate the proportion that had inadequate intake of these nutrients from food alone.

3. Folate, vitamin B\textsubscript{12}, vitamin B\textsubscript{6}, and energy-protein status.

4. The association between ptHcy and the following:
   
   i. Kidney function.

   ii. Folate, vitamin B\textsubscript{12} and vitamin B\textsubscript{6} status.

   iii. Energy-protein status.

   iv. Intake of folate, vitamin B\textsubscript{12}, vitamin B\textsubscript{6}, energy and protein from food and supplements.

   v. Age, gender, coffee intake, alcohol intake, cigarette use, and use of medications that influence homocysteine metabolism.
Chapter 4

4 Methods

4.1 Study Design

This was a prospective cross-sectional study of subjects, with stages 3, 4 and 5 pCKD, designed to measure and describe: 1) Prevalence of hHcy. 2) Intake of folate, vitamins B\textsubscript{12} and B\textsubscript{6}, energy and protein from food, food and supplements combined, and the proportion that had inadequate intake of these individual nutrients from food alone. 3) Folate, vitamins B\textsubscript{12} and B\textsubscript{6}, energy-protein status. 4) Determinants of ptHcy.

4.2 Subjects

The study was approved by the University Health Network Research Ethics Board. All subjects provided written informed consent (Appendix B).

Patients newly referred to or already attending the ambulatory pCKD clinic at the Toronto General Hospital, University Health Network were considered for recruitment into the study. Exclusion criteria were: < 18 years old; eGFR \geq 60 \text{ ml/min/1.73m}^2; unable to read and understand English; unable to provide informed consent; documented vitamin B\textsubscript{12} deficiency or receiving vitamin B\textsubscript{12} injections; heart, liver or kidney transplant; human immunodeficiency virus; any metabolic disorder interfering with homocysteine metabolism; active cancer; liver dysfunction; involved with another study interfering with homocysteine metabolism; taking an antibiotic 7 days prior to blood sample draw; prescribed a vitamin supplement called Replavite\textsuperscript{®} (includes but is not limited to 1 mg folic acid, 6 \mu g vitamin B\textsubscript{12}, 10 mg vitamin B\textsubscript{6}), refusal to be approached by study investigator (Appendix C).
4.3 Study Procedure

There were two study visits. Study visit one occurred on the morning that participants were routinely asked to come in to the hospital for their blood sample one week prior to their regularly scheduled ambulatory pCKD clinic visit. At the first study visit, the participant met with the study investigator and the following occurred:

1. Review and signing of the consent form by the participant.
2. Blood samples were obtained by a laboratory technician.
3. The study investigator performed a SGA and measured participant’s height, weight, TSF, mid upper arm circumference and elbow breadth (Appendix D & E).
4. The study investigator interviewed the participant for a 24-hour dietary recall, gastrointestinal symptoms of uremia and use of micronutrient supplements in the past four months. The participant was also interviewed for the use of cigarettes, coffee, alcohol and medications (Appendix F to J)
5. The study investigator obtained demographic information from the participant and the participant’s medical chart (Appendix C).

Study visit two occurred at the participant’s regularly scheduled ambulatory pCKD clinic visit approximately one week after study visit one. At the second study visit, the study investigator interviewed the participant for a 24-hour dietary recall.

4.3.1 Collection and Analysis of Blood Sample

Subjects provided a venous blood sample (35mL) in ethylene diaminetetraacetic acid and non-ethylene diaminetetraacetic acid containing tubes for the determination of the following: ptHcy, RBC and plasma folate, hematocrit, and serum vitamin B12, pyridoxal 5’-phosphate, albumin, and creatinine. The sample was drawn in the morning, after an eight hour fast, because of the diurnal variation in ptHcy concentrations, with ptHcy being lowest in the first part of the day, and because a high protein meal may increase ptHcy concentration by 10 to 15% after 6 to 8 hours (Refsum et al. 2004).
4.3.1.1 Plasma Homocysteine

The blood sample for ptHcy was stored on ice and centrifuged within twenty minutes of sample collection because there is a time and temperature dependent increase in ptHcy, attributable to an ongoing release of homocysteine from red blood cells (Refsum H 2004). Plasma tHcy was analyzed by competitive immunoassay using the Immulite 2000® analyzer (Diagnostic Products Corporation) located in the medical laboratory at the University Health Network, Toronto, Ontario, Canada. Homocysteine was reduced and enzymatically converted to s-adenosylhomocysteine by dithiothreitol and s-adenosylhomocysteine hydrolase, respectively. S-adenosylhomocysteine was quantified. The coefficient of variation of this assay has been found to be 7.1 to 9.3% (La’ulu et al. 2008; Moller et al. 2002). Hyperhomocysteinemia was defined as a ptHcy > 13 µmol/L (Pfeiffer CM, 2008).

4.3.1.2 Red Blood Cell and Plasma Folate

The blood sample for RBC and plasma folate was kept on ice, protected from light and processed within two hours to prevent destruction of folate (Arcot et al. 2005). Red blood cell and plasma folate were analyzed in the laboratory of Dr. Deborah O’Connor at the Hospital For Sick Children, Toronto, Ontario, Canada. Red blood cell folate (nmol/mL) was calculated by subtracting plasma folate from whole blood folate and correcting for hematocrit (Scott et al. 1974). For whole blood folate, a 1% ascorbic acid solution (900µL) was mixed with whole blood (100 µL) to prevent folate oxidation. The mixture was incubated for 30 minutes at 37°C to facilitate the conversion of RBC folate polyglutamates to monoglutamates. For plasma folate, whole blood was centrifuged (1500g for 20 minutes at 4° C) and sodium ascorbate (1% wt:vol) was mixed with the isolated plasma to prevent folate oxidation. After processing whole blood and plasma folate, all samples were stored at -80°C. Whole blood and plasma folate were analyzed by microbiological assay using Lactobacillus rhamnosus (ATCC #7469, American Type Tissue Culture Collection, Manassas, VA) as the test organism (Molloy et al. 1997). The accuracy and reproducibility of the assay result was determined by the simultaneous analysis of a whole blood folate standard with a certified folate value of 29.5 nmol/L (Whole Blood 95/528; National Institute of Biological Standards and Control, Hertfordshire, United Kingdom). In this study, analysis of the whole blood folate standard yielded a folate content of 31.7 ± 1.0 nmol/L,
with an inter assay coefficient of variation of 4.6%. Folate deficiency was defined as a RBC folate < 317 nmol/L or a plasma folate < 6.8 nmol/L (Herbert 1990).

### 4.3.1.3 Serum Vitamin B$_{12}$

Determination of serum vitamin B$_{12}$ in six subjects was carried out using ADVIA Centaur® competitive immunoassay with (direct) chemiluminescent technology (Bayer Corporation), and in forty-six subjects using Architect Centaur® chemiluminescent microparticle immunoassay (Abbott Laboratories) in the medical laboratory at the University Health Network. The medical laboratory at the University Health Network changed the test used to measure serum vitamin B$_{12}$ during the study’s data collection and analysis period. The laboratory determined that the correlation coefficient between the two methods was 0.9530 and that the average bias, assessed using the “Bland Altman Plot” statistical method, was -32.0 pmol/L. With both methods sample vitamin B$_{12}$ was reduced by dithiothreitol to release it from endogenous binding proteins, and then quantified. The coefficient of variation was 7.1 to 8.0%. Vitamin B$_{12}$ deficiency was defined as a serum vitamin B$_{12}$ < 148 pmol/L (Carmel 1988).

### 4.3.1.4 Serum Vitamin B$_{6}$

Determination of serum vitamin B$_{6}$ (pyridoxal 5′-phosphate) occurred in the laboratory at St. Joseph’s Health Care in London, Ontario, Canada, using high performance liquid chromatography and sodium bisulfate derivation. The detection limit of this method has been found to be 10 nmol/L (Kimura et al. 1996). Results below the detection limit were automatically replaced with the default value of 10 nmol/L. The coefficient of variation was 3.3 to 7.3%. Vitamin B$_{6}$ deficiency was defined as a serum pyridoxal 5′-phosphate < 20 nmol/L (Lui et al. 1985).

### 4.3.1.5 Other Blood Samples

Standard analytical methods were used to determine serum albumin (bromocresol green solution), serum creatinine (Jaffe method) and hematocrit (electrical resistance corrected sodium method) in the medical laboratory at the University Health Network.
4.3.2 Measurement and Analysis of Energy-Protein Status

4.3.2.1 Subjective Global Assessment

Subjective global assessment is a validated method of measuring energy-protein status in CKD patients and is recommended for use by current practice guidelines (Steiber et al. 2007; Visser et al. 1999; National Kidney Foundation, 2000; Detsky et al. 1987). In this study, for SGA, historical information was collected and a physical exam conducted (Appendix D reprinted and used, with permission. Copyright Satellite Healthcare 2001).

Historical information included weight, weight change, dietary intake, gastrointestinal symptoms, functional capacity, and disease state/comorbidities as related to energy requirement. Physical exam included assessment of loss of subcutaneous fat, muscle wasting and the presence or absence of edema.

A 7 point scale was used to rate energy-protein status using SGA. Subsequently, participants were classified as energy-protein undernourished (rating of ≤ 5) or well-nourished (rating of 6 or 7). This classification was based on the finding that SGA reliably predicts well-nourished and undernourished status, but not degree of undernutrition (Cooper et al. 2002). It was also based on the finding that the mortality rate is significantly higher in pCKD patients classified as undernourished, compared to those classified as well-nourished, when assessed by SGA (Stenvinkel et al. 2002).

4.3.2.2 Anthropometrics

Anthropometric parameters used to measure energy-protein status were BMI, TSF and bone-free arm muscle area. For BMI, weight and height were measured. Body weight was measured using a scale that was calibrated (± 0.1 kg) and height was measured using a supine stadiometer that was fixed to the wall. For TSF and bone-free arm muscle area, TSF and mid arm circumference were measured using Lange calipers and a metal tape measure, respectively, following methods described by Frisancho (1990). It has been recommended that evaluation of anthropometric measurements include frame size (Frisancho, 1984). In the general population, elbow breadth has been found to exhibit the lowest correlation with skinfold thickness and as
such is recommended as a measure of frame size (Frisancho et al. 1983). Elbow breadth was measured with sliding calipers following methods described by Frisancho (1990).

Body mass index was calculated as \( \text{BMI} = \frac{\text{body weight}[\text{kg}]}{\text{height}[\text{m}]^2} \). A BMI \( \leq 20 \) represented an undernourished state, based on the finding that people with pCKD with a BMI of 15.3 to 20 kg/m\(^2\) have a significantly higher risk of mortality than those with a BMI of 20.1 to 25 kg/m\(^2\) (HR 1.96; 95% CI: 1.35 to 2.84) (Evans et al. 2005).

Bone-free arm muscle area was calculated from TSF and mid arm circumference using the following formulas (Frisancho1984; Heymsfield et al. 1982) where arm muscle area is AMA and mid arm circumference is MAC:

\[
\text{Male: Bone-free AMA (cm}^2\text{)} = \left(\frac{\text{MAC(cm)}-\pi \times \text{TSF(cm)}}{4\pi}\right)^2 - 10
\]

\[
\text{Female: Bone-free AMA (cm}^2\text{)} = \left(\frac{\text{MAC(cm)}-\pi \times \text{TSF(cm)}}{4\pi}\right)^2 - 6.5
\]

Reference data, using percentiles to reflect bone-free arm muscle area and TSF of the pCKD population, have not been published. Current practice guidelines recommend that anthropometric measurements in the CKD population treated with hemodialysis be compared to reference data from the general population (National Kidney Foundation, 2000; Frisancho, 1984). A similar guideline does not exist for the CKD population, not yet on dialysis. For this study, bone-free arm muscle area and TSF were compared to reference data from the general population using data published by Frisancho (1990), Frisancho (1984), and Kuczmarski et al. (2000) for subjects < 25 years old, 25 to 74 years old and > 74 years old, respectively. As recommended for the general population, a participant was considered undernourished if the value for the anthropometric measure was \( \leq \) the 15th percentile of the reference data (Frisancho 1984; American Dietetic Association 2000).

### 4.3.2.3 Serum Albumin

Serum albumin has not been validated as a marker of energy-protein status, however, it is recommended for use in the assessment of energy-protein status of patients with pCKD by the current practice guideline (National Kidney Foundation 2000). Subjects provided a fasting venous blood sample for serum albumin. A standard analytical method was used to determine
serum albumin (bromocresol green solution) in the laboratory in the University Health Network. A serum albumin $\leq 33$ g/L has been found to predict mortality in pCKD (Honda et al. 2006). As such, participants were classified as undernourished and well-nourished when serum albumin was $\leq 33$ g/L and $> 33$ g/L, respectively.

4.3.3 Measurement and Analysis of Intake of Folate, Vitamins B$_{12}$ and B$_{6}$, Energy, and Protein from Food and Supplements

To measure intake of folate, vitamins B$_{12}$ and B$_{6}$, energy and protein from food, participants were interviewed in person using the five-step multiple-pass 24-hour dietary recall method (Blanton et al. 2006). Two 24-hour dietary recalls were administered to participants during face-to-face interviews, approximately one week apart. Plastic food and 2 D models were used to assess portion size. This method is appropriate to use to report on the adequacy of micronutrient intake (Food and Nutrition Board, Institute of Medicine 2001b).

Nutrient analysis was performed using the 2007b Canadian Nutrient File (Health Canada). Mean intake of a nutrient from food for an individual was determined by averaging the two dietary recalls. Mean intake of folate, vitamin B$_{12}$ and vitamin B$_{6}$ are expressed as $\mu$g of dietary folate equivalents (DFE) per day, $\mu$g per day and mg dietary vitamin B$_{6}$ equivalents (DB$_{6}$E) per day, respectively. Mean intake (and estimated requirement) of energy and protein are expressed as kcal/kg/d and g protein/kg/d, respectively. As recommended by the current practice guideline, for participants who were undernourished or obese, their weight was adjusted and the adjusted weight was used to express mean observed intake and estimated requirement of energy and protein (National Kidney Foundation 2000. An edema-free body weight that was $< 95\%$ or $> 115\%$ of the median standard weight described by Frisancho (1984, 1990) and Kuczmarski (2000) was considered to be undernourished or obese, respectively. For participants who were undernourished or obese an adjusted edema-free body weight was calculated using the following equation: adjusted edema-free body weight (kg) = edema-free body weight (kg) + [(median standard body weight (kg) – edema-free body weight (kg)) x 0.25] (National Kidney Foundation, 2000).
To measure micronutrient supplement intake, participants were asked, on one occasion, using a frequency questionnaire, about supplement use in the past four months, which corresponds to the half-life of RBC folate.

Supplement use in the past four months, obtained by the frequency questionnaire, was converted to individual daily supplement intake data. Individual daily folic acid and vitamin B₆ supplement intake were multiplied by 1.7 and 1.27, respectively, to account for increased bioavailability of these vitamins in supplement form (Food and Nutrition Board, Institute of Medicine 1998a). It was assumed that folic acid supplement intake occurred with food intake. As described by Carriquiry (2003), individual daily supplement intake data was then combined with individual mean observed intake from food for a total intake of a nutrient for an individual.

4.3.4 Measurement and Analysis of Other Study Variables

Demographic data, including age, gender, etiology of pCKD, presence of CVD, presence of traditional risk factors for CVD as described by Menon et al. (2005), and number of months attending clinic were obtained from the participant’s medical chart.

The eGFR was used to express kidney function as recommended by the National Kidney Foundation and current practice guidelines (Eknoyan et al. 2003; National Kidney Foundation, 2002). The abbreviated Modification of Diet in Renal Disease study equation as recommended by the National Kidney Foundation and current practice guidelines, was used to estimate the glomerular filtration rate of each participant (Eknoyan et al. 2003; National Kidney Foundation, 2002) and is as follows: $\text{eGFR (ml/min/1.73m}^2) = 186 \times (\text{serum creatinine in mg/dL})^{-1.154} \times (\text{age in years})^{-0.203} \times (0.742 \text{ if female}) \times (1.210 \text{ if black})$. Serum creatinine in $\mu$mol/L was multiplied by 0.0113 to convert to serum creatinine in mg/dL.

Participants were interviewed in person to determine the presence or absence of anorexia, nausea, vomiting, early satiety, taste changes, and aversions to red meat, chicken or fish, as these are gastrointestinal symptoms of uremia described in the literature (Aguilera et al. 2004; Aguilera et al. 2007; Bossola et al. 2005; Fernstrom et al. 1996; Dobell et al. 1993; Murtagh et al. 2007; Carrero et al. 2008). Participants with $\geq 1$ gastrointestinal symptom of uremia were classified as having gastrointestinal symptoms of uremia. Participants were asked about the use of medications that influence ptHcy (Atar et al. 2005; Desouza et al. 2002; Jackson et al. 1998;
4.4 Statistical Analysis

A sample size of thirty-eight was derived from the following information and equation. Nerbass et al. (2006) reported a prevalence of hHcy of 89% in pCKD subjects classified as having an eGFR of 29.8 ± 14.3 ml/min/1.73m$^2$, living in a country without folic acid food fortification. Based on the fact that patients referred to the pCKD clinic at the University Health Network have a similar eGFR and the assumption that the prevalence of hHcy did not change after folic acid food fortification, we estimated the prevalence of hHcy in our study population to be 89%. This proportion (P), an alpha level of .05 ($z_\alpha = 1.96$) and a total width of confidence interval (W) of 20% were used in the following equation, $n = 4 \times z_\alpha^2 \times P \times (1 - P) \div W^2$, to obtain the sample size (Browner et al. 2001).

The prevalence of hHcy and the 95% confidence interval for this proportion are reported as a percent. A Shapiro-Wilk test confirmed that ptHcy data were not normally distributed and were therefore transformed to natural logarithms (Ln). Ln-transformed ptHcy data (Ln-ptHcy) were used to determine the mean ptHcy concentration and for parametric tests. The antilog of the mean (geometric mean) and the antilog of the 95% confidence interval are reported.

Pearson’s correlation coefficient (r) was used to determine bivariate relationships between ptHcy and independent variables. Independent variables were not Ln-transformed. Coefficient of determination ($r^2$) was used to explain the proportion (%) of variance in ptHcy contributed by an independent variable.

Backward multiple linear regression was used to determine the proportion of variance in ptHcy explained, jointly, by independent variables and to determine the variables that independently predicted ptHcy. A maximum of five independent variables could be introduced.
into the multiple linear regression analysis, which was determined by dividing the sample size by ten. The process for selecting the five independent variables was as follows: 1) Independent variables that had a bivariate relationship with ptHcy, with a p value of <0.200, were identified. 2) Using Pearson’s correlation coefficient (r), intercorrelation among the identified independent variables was determined. These variables were not Ln-transformed. Using the results of this analysis, the significant relationship (p<0.05) that had the highest correlation was identified, and the independent variable of that correlation having the lowest correlation with ptHcy was excluded. This process was repeated until five independent variables remained. 3) Presence of multicollinearity among the five independent variables used in multiple linear regression analysis was assessed using a variance inflation factor of > 5. Subsequent to the selection process, the five independent variables were entered simultaneously into the backward multiple linear regression model for analysis. During analysis, independent variables that maintained a p value < 0.200 remained in the model. For the proportion of variance in ptHcy, explained jointly by the independent variables remaining in the model, the adjusted coefficient of multiple determination (R²) is reported. An independent variable remaining in the model with a p value < 0.05 was considered to be an independent predictor of ptHcy. The magnitude of the contribution of each independent predictor to the variation in ptHcy is reported as a Beta (β).

The Institute of Medicine recommends reporting the average usual intake to describe the average micronutrient intake by a group (Food and Nutrition Board, Institute of Medicine 2001b). The average usual intake is determined by statistically adjusting the observed intake distribution of the group to account for within-person variation in intake and between-person variation in requirement/intake. Since our sample size was not large enough to be able to statistically adjust the observed intake distribution of the group we report the average observed intake by the group. Dietary reference intake for adults (≥ 19 years old) vary by life stage (age) and gender for vitamin B₆ but not for folate or vitamin B₁₂. As such, intake and adequacy of intake are reported by life stage (age) and gender for vitamin B₆ but not for folate or vitamin B₁₂.

To assess adequacy of micronutrient intake of a group the Institute of Medicine recommends using either the Probability Approach or a short-cut version of this approach called the Estimated Average Requirement cut-point method (Food and Nutrition Board, Institute of Medicine 2001b). Both methods require knowledge about an observed intake distribution that
has been statistically adjusted to account for within-person variation in intake and between-person variation in requirement. As previously mentioned, our sample size was not large enough to be able to statistically adjust the observed intake distribution of the group. As such, to assess adequacy of micronutrient intake from food of the group we first determined the adequacy of micronutrient intake from food of each individual subject using a method recommended by The Institute of Medicine (Food and Nutrition Board, Institute of Medicine 2001a). The result of this method was a z score which reflected the individual’s usual intake and the confidence level with which we could conclude that an individual’s usual intake was inadequate or adequate. A z score that provided a level of confidence of at least 85 percent was used to correctly conclude that an individual’s usual intake was inadequate or adequate. A z score that provided a level of confidence of less than 85 percent was used to conclude that the adequacy of an individual’s usual intake was uncertain. As such, an individual’s usual intake was classified as inadequate, adequacy uncertain or adequate. Subsequently, adequacy of micronutrient intake from food, by the group, was assessed by counting the number of subjects in each classification (inadequate, adequacy uncertain, adequate) and dividing the number of subjects in each classification by the total number of subjects in the group. Results are expressed as a percent. For each individual subject the specific process of this method was as follows: 1) Collected two days of observed intake data and calculated mean observed intake. 2) Calculated the difference (D) between mean observed intake and the estimated average requirement for the micronutrient. 3) Divided D by the standard deviation of D. The standard deviation of D was derived from the between-person standard deviation deviation (subject-to-subject variation in requirement/intake), within-person standard deviation (day-to-day variation in a subjects intake) and the number of days of observed intake for the individual. The between-person standard deviation was derived from the standard deviation of the requirement of the micronutrient. The within-person standard deviation was obtained from a pooled estimate of within-person standard deviation from a nationwide food consumption survey (Statistics Canada 2004). The result of this method was a z score.

The average energy and protein intake by the group are reported as observed intakes. Adequacy of energy and protein intake from food of each subject was determined by comparing the individual’s mean observed intake to recommended intake as outlined in the current practice guideline (National Kidney Foundation, 2000). A mean two day observed intake of an
individual below the guideline was classified as inadequate, and a mean observed intake of an individual that met or was above the guideline was classified as adequate. The prevalence of inadequate observed energy and protein intake by the group was assessed by counting the number of subjects in the group with an inadequate observed intake and dividing that number by the total number of subjects in the group. Results are expressed as a percent. Recommended intake of energy varies by age. As such, energy intake and adequacy of intake are reported by age.

The relationships between nutrient intake and continuous or dichotomous variables were analyzed by Pearson’s correlation coefficient or independent t-test, respectively. Recommended energy intake varies by age. Also, dietary reference intakes for adults (≥ 19 years old) for vitamin B₆ vary by life stage (age) and gender. As such, for energy and vitamin B₆, the relationship between intake and status were analyzed by age, and age and gender, respectively. The relationship between the presence, or absence, of gastrointestinal symptoms of uremia and continuous or dichotomous variables were analyzed by independent t-test and Chi-squared test, respectively. Dependent variables were Ln-transformed when data was not normally distributed.

Descriptive data are reported as a number, percent, median (25th, 75th percentile) or mean ± standard deviation.

All statistical analyses were performed using SPSS® for Windows™ software, Release 16.2 (Statistical Package for the Social Sciences, Inc., Chicago, IL) with the exception of the adequacy of usual micronutrient intake from food, which was calculated manually and is described above. A two tailed test of significance was used and a probability level of p<0.05 was considered statistically significant.
Chapter 5

5 Results

5.1 Subjects

Figure 5.1 describes the recruitment process which occurred from July, 2006 to July, 2008. Of patients newly referred to or already attending the ambulatory pCKD clinic, 76% were excluded from the study. Being unable to read and understand English was the primary reason for exclusion (26.0%), followed by having a heart, liver or kidney transplant (18.6%) (Table 5.1). Of patients approached, 50% agreed to participate in the study.

Data collection occurred from August, 2006 to May, 2008. Fifty-three subjects completed the study. One subject was removed from all data analysis because of the presence of a metabolic disorder (cystinosis) that interfered with homocysteine metabolism (n=52). Four subjects were removed from data analysis of ptHcy (n=48) because one did not fast for the blood sample, one had a ptHcy with a z score > 3, and two had cancer. One subject was removed from data analysis of RBC and plasma folate because an antibiotic was taken within 7 days of the blood sample draw (n=51). Two subjects had missing results for serum pyridoxal 5′-phosphate and these were coded as missing (n=50). Serum pyridoxal 5′-phosphate has a detection limit of 10 nmol/L. Three subjects had a result of < 10 nmol/L which were coded as 10 nmol/L. Two subjects had missing results for number of cigarettes used and these were coded as missing (n=50).
Patients newly referred to or already attending the ambulatory clinic during the recruitment period (July, 2006 to July, 2008).

\[ n = 440 \]

Patients excluded from the study.

\[ n = 334 \]

Patients approached to participate in the study.

\[ n = 106 \]

Patients who refused to participate in the study.

\[ n = 53 \]

Patients who agreed to participate in the study.

\[ n = 53 \]

Figure 5.1 Recruitment Process
Table 5.1 Reasons for Excluding Patients from the Study (n=334)

<table>
<thead>
<tr>
<th>Reason for Exclusion</th>
<th>n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unable to Read and Understand English</td>
<td>87(26.0)</td>
</tr>
<tr>
<td>Heart, Liver or Kidney Transplant</td>
<td>62(18.6)</td>
</tr>
<tr>
<td>Refused to be Approached by Study Investigator</td>
<td>59(17.7)</td>
</tr>
<tr>
<td>Prescribed a Vitamin Supplement Replavite®</td>
<td>38(11.4)</td>
</tr>
<tr>
<td>Active Cancer</td>
<td>33(9.9)</td>
</tr>
<tr>
<td>Unable to Provide Informed Consent</td>
<td>23(6.9)</td>
</tr>
<tr>
<td>Taking an Antibiotic Seven Days Prior to Blood Sample Draw</td>
<td>12(3.6)</td>
</tr>
<tr>
<td>Liver Dysfunction</td>
<td>9(2.7)</td>
</tr>
<tr>
<td>Documented Vitamin B₁₂ Deficiency or Receiving Vitamin B₁₂ Injections</td>
<td>7(2.1)</td>
</tr>
<tr>
<td>A Metabolic Disorder Interfering With Homocysteine Metabolism</td>
<td>2(0.6)</td>
</tr>
<tr>
<td>Human Immunodeficiency Virus</td>
<td>2(0.6)</td>
</tr>
</tbody>
</table>

Table 5.2 describes the characteristics of the study population. The mean age of the subjects was 64 ± 12 years, with most being male. The median (25th, 75th percentile) eGFR of the subjects was 20.2 (13.7 30.5) ml/min/1.73m², with most (44.2%) having stage 4 CKD. Diabetes was the primary cause of CKD (40.4%), followed by glomerulonephritis (23.1%). Fifty percent of the subjects had CVD and the primary risk factor for CVD was hypertension (92%), followed by dyslipidemia (84.6%). Seventy-five percent of subjects used medication(s) known to influence ptHcy. The four most commonly used medications known to influence ptHcy were aspirin (32.7%), insulin (26.9%), simvastatin (25%) and metoprolol (15.4%). A majority (57.7%) reported having at least one gastrointestinal symptom of uremia, with anorexia (38.5%) being the most common symptom reported.
Table 5.2 Characteristics of Adults with Predialysis Chronic Kidney Disease (n=52)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n(%)</th>
<th>Median(25th,75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>65(57,72)</td>
<td></td>
</tr>
<tr>
<td>Gender (male)</td>
<td>35(67.3)</td>
<td></td>
</tr>
<tr>
<td>eGFR(^a) (ml/min/1.73m(^2))</td>
<td>20.2(13.7 30.5)</td>
<td></td>
</tr>
<tr>
<td>eGFR(^a) (ml/min/1.73m(^2)) by Stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stage 3 (30-59 ml/min/1.73m(^2))</td>
<td>14(26.9)</td>
<td>34.0(31.1,36.0)</td>
</tr>
<tr>
<td>Stage 4 (15-29 ml/min/1.73m(^2))</td>
<td>23(44.2)</td>
<td>20.3(18.4, 22.4)</td>
</tr>
<tr>
<td>Stage 5 (&lt; 15 ml/min/1.73m(^2))</td>
<td>15(28.9)</td>
<td>11.3(8.9,13.1)</td>
</tr>
<tr>
<td>Etiology of Kidney Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>21(40.4)</td>
<td></td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>12(23.1)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>8(15.4)</td>
<td></td>
</tr>
<tr>
<td>Hypertension/Vascular Disease</td>
<td>7(13.4)</td>
<td></td>
</tr>
<tr>
<td>Congenital/Hereditary</td>
<td>3(5.8)</td>
<td></td>
</tr>
<tr>
<td>Systemic Vasculitis/Lupus</td>
<td>1(1.9)</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular Disease (yes)</td>
<td>26(50)</td>
<td></td>
</tr>
<tr>
<td>Risk Factors for CVD(^b) (yes)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>48(92.3)</td>
<td></td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>44(84.6)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>25(48.1)</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Estimated glomerular filtration rate.
\(^b\) Cardiovascular disease.
Table 5.2 continued: Characteristics of Adults with Predialysis Chronic Kidney Disease (n=52)

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n(%)</th>
<th>Median(25th,75th percentile)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Months Attending Clinic</td>
<td></td>
<td></td>
</tr>
<tr>
<td>New Referrals</td>
<td>30(57.7)</td>
<td>Not Applicable</td>
</tr>
<tr>
<td>Follow-ups</td>
<td>22(42.3)</td>
<td>19.0(7.75,25.5)</td>
</tr>
<tr>
<td>Vitamin Supplement Users</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Folic Acid</td>
<td>9(17.3)</td>
<td></td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>11(21.2)</td>
<td></td>
</tr>
<tr>
<td>Vitamin B6</td>
<td>11(21.2)</td>
<td></td>
</tr>
<tr>
<td>Taking Medication(s) Influencing Plasma Total Homocysteine (yes)</td>
<td>39(75.0)</td>
<td></td>
</tr>
<tr>
<td>Reported At Least One Gastrointestinal Symptom of Uremia</td>
<td>30(57.7)</td>
<td></td>
</tr>
<tr>
<td>Gastrointestinal Symptom Reported</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anorexia</td>
<td>20(38.5)</td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>14(26.9)</td>
<td></td>
</tr>
<tr>
<td>Early Satiety</td>
<td>14(26.9)</td>
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<tr>
<td>Vomiting</td>
<td>12(23.1)</td>
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</tr>
<tr>
<td>Taste Changes</td>
<td>9(17.3)</td>
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<tr>
<td>Red Meat Aversion</td>
<td>3(5.8)</td>
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</tr>
<tr>
<td>Chicken Aversion</td>
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<tr>
<td>Fish Aversion</td>
<td>0(0)</td>
<td></td>
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</tbody>
</table>
5.2 Prevalence of Hyperhomocysteinemia

The prevalence of hHcy was 93.8% (95% CI: 81.8 to 98.4). The geometric mean of ptHcy was 21.5 µmol/L (95% CI: 19.4 to 23.7), with a minimum and maximum value of 8.7 µmol/L and 44.7 µmol/L, respectively. When folic acid, vitamin B$_{12}$ and vitamin B$_{6}$ supplement users were removed from the data analysis the prevalence of hHcy was 94.7% (95% CI: 80.9 to 99.1).

5.3 Relationship between Plasma Total Homocysteine and Independent Variables

5.3.1 Bivariate Analysis

Table 5.3 describes relationships between ptHcy and independent variables in the subjects. Plasma total homocysteine was inversely associated with RBC folate ($r = -0.406$, $p=0.004$), plasma folate ($r = -0.295$, $p=0.042$) and TSF ($r = -0.317$, $p=0.028$) (figures 5.2, 5.3, 5.4). When folate supplement users were removed from data analysis, RBC folate remained inversely associated with ptHcy ($r = -0.364$, $p=0.019$; $n=41$). There was a significant association between ptHcy and energy-protein status (SGA) ($r = 0.357$, $p=0.013$) (figure 5.5). Mean ptHcy was 26.4 µmol/L (95% CI: 21.5 to 32.5) in those classified as undernourished ($n=12$) by SGA and 20.0 µmol/L (95% CI: 17.9 to 22.3) in those classified as well-nourished ($n=36$) ($p=0.013$). There was no significant association between ptHcy and any of the medications known to influence ptHcy (data not shown). Red blood cell folate, SGA, TSF and plasma folate contributed to 16.5%, 12.9%, 10.0% and 8.7% of the variance in ptHcy, respectively.

5.3.2 Multiple Linear Regression Analysis

Independent variables that had a bivariate relationship with ptHcy, with a p value of $<0.200$, were RBC folate, SGA, eGFR, gender, coffee intake, TSF, serum vitamin B$_{12}$, plasma folate, observed folate intake from food and supplements, and BMI. Among these independent variables, RBC folate, SGA, eGFR, gender, and coffee intake were identified as being the least intercorrelated (Table 5.4). Multicollinearity among these variables was absent and were
therefore used in multiple linear regression analysis (Table 5.5). Red blood cell folate, SGA and eGFR were, jointly, significantly associated with ptHcy ($F (3, 44) = 5.666, p=0.002$), explaining 22.9% of the variance in ptHcy. Independent predictors of ptHcy were RBC folate ($\beta = -0.327$, $p=0.017$) and SGA ($\beta = 0.305$, $p=0.024$). Estimated glomerular filtration rate did not independently predict ptHcy after adjusting for RBC folate and SGA ($\beta = -0.181$, $p=0.170$).
Table 5.3 Bivariate Analysis: Relationship between Plasma Total Homocysteine and Independent Variables in Adults with Predialysis Chronic Kidney Disease (n=48)

<table>
<thead>
<tr>
<th>Potential Predictor Variables</th>
<th>$r^2$</th>
<th>p-value $^d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed Kcalorie Intake (Kcal/kg/d)</td>
<td>-.013</td>
<td>.928</td>
</tr>
<tr>
<td>Observed Protein Intake (g protein/kg/d)</td>
<td>-.107</td>
<td>.470</td>
</tr>
<tr>
<td>Observed Folate Intake ($\mu$g/d of DFE)$^e$ from Food and Supplements</td>
<td>-.252</td>
<td>.085</td>
</tr>
<tr>
<td>Observed Vitamin B$_{12}$ Intake ($\mu$g/d) from Food and Supplements</td>
<td>-.106</td>
<td>.475</td>
</tr>
<tr>
<td>Observed Vitamin B$_6$ Intake (mg/d of DB$_6$E)$^g$ from Food and Supplements</td>
<td>-.066</td>
<td>.658</td>
</tr>
<tr>
<td>Coffee Intake (ml/d)</td>
<td>-.199</td>
<td>.174</td>
</tr>
<tr>
<td>Alcohol Intake (g/d)</td>
<td>-.036</td>
<td>.809</td>
</tr>
<tr>
<td>Red Blood Cell Folate (nmol/L)</td>
<td>-.406</td>
<td>.004</td>
</tr>
<tr>
<td>Plasma Folate (nmol/L)</td>
<td>-.295</td>
<td>.042</td>
</tr>
<tr>
<td>Serum Vitamin B$_{12}$ (pmol/L)</td>
<td>-.245</td>
<td>.093</td>
</tr>
<tr>
<td>Serum Pyridoxal 5′-Phosphate (nmol/L)</td>
<td>-.095</td>
<td>.531</td>
</tr>
<tr>
<td>Serum Albumin (g/L)</td>
<td>.159</td>
<td>.279</td>
</tr>
<tr>
<td>Body Mass Index (m$^2$)</td>
<td>-.250</td>
<td>.086</td>
</tr>
<tr>
<td>Tricep Skinfold Thickness (mm)</td>
<td>-.317</td>
<td>.028</td>
</tr>
<tr>
<td>Bone Free Arm Muscle Area (cm$^2$)</td>
<td>.018</td>
<td>.903</td>
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<tr>
<td>Subjective Global Assessment</td>
<td>.357</td>
<td>.013</td>
</tr>
<tr>
<td>Estimated Glomerular Filtration Rate (ml/min/1.73m$^2$)</td>
<td>-.205</td>
<td>.163</td>
</tr>
<tr>
<td>Age (years)</td>
<td>.021</td>
<td>.889</td>
</tr>
<tr>
<td>Gender</td>
<td>.228</td>
<td>.119</td>
</tr>
<tr>
<td>Cigarette Use (#/d)</td>
<td>.107</td>
<td>.481</td>
</tr>
</tbody>
</table>

$^a$ Transformed to its natural logarithm in $\mu$mol/L.

$^b$ Exception: serum Vitamin B$_6$ and cigarette use, n = 46.

$^c$ Pearson’s correlation coefficient.

$^d$ Significant = p<0.05.

$^e$ DFE = dietary folate equivalents. 1 $\mu$g natural food folate = 1 $\mu$g of DFE. 1 $\mu$g folic acid from food fortification or supplement = 1.7 $\mu$g of DFE.

$^f$ Natural and folic acid.

$^g$ DB$_6$E = dietary B$_6$ equivalents. 1 mg food vitamin B$_6$ = 1 mg DB$_6$E. 1 mg synthetic vitamin B$_6$ = 1.27 mg DB$_6$E.
Figure 5.2 The Relationship between Plasma Total Homocysteine and Red Blood Cell Folate in Adults with Predialysis Chronic Kidney Disease (n=48). The relationship between plasma total homocysteine transformed to its natural logarithm and red blood cell folate was significant ($r= -0.406$, $p=0.004$).

Figure 5.3 The Relationship between Plasma Total Homocysteine and Plasma Folate in Adults with Predialysis Chronic Kidney Disease (n=48). The relationship between plasma total homocysteine transformed to its natural logarithm and plasma folate was significant ($r= -0.295$, $p=0.042$).
Figure 5.4 The Relationship between Plasma Total Homocysteine and Tricep Skinfold Thickness in Adults with Predialysis Chronic Kidney Disease (n=48). The relationship between plasma total homocysteine transformed to its natural logarithm and tricep skinfold thickness was significant ($r=-0.317$, $p=0.028$).
Figure 5.5 Plasma Total Homocysteine Concentration in Adults with Predialysis Chronic Kidney Disease Classified as Energy-protein Undernourished and Well-Nourished, as assessed by Subjective Global Assessment. Plasma total homocysteine transformed to its natural logarithm was significantly higher in those undernourished (n=12) (26.4 µmol/L; 95% CI: 21.5 to 32.5) than in those well-nourished (n=36) (20.0 µmol/L; 95% CI: 17.9 to 22.3) (p=0.013).
Table 5.4 Intercorrelation Among Independent Variables that have an Individual Relationship with Plasma Total Homocysteine$^a$ in Adults with Predialysis Chronic Kidney Disease (n=48)

<table>
<thead>
<tr>
<th></th>
<th>RBC Folate$^b$</th>
<th>SGA</th>
<th>eGFR</th>
<th>Gender</th>
<th>TSF</th>
<th>Plasma Folate</th>
<th>Serum B$_{12}$</th>
<th>Folate Intake</th>
<th>BMI</th>
<th>Coffee Intake</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<tr>
<td>RBC Folate$^b$</td>
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<tr>
<td>r</td>
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<td>Gender</td>
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<td>.023</td>
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<td>Folate Intake$^i$</td>
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<tr>
<td>Coffee Intake$^k$</td>
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</tr>
</tbody>
</table>

$^a$μmol/L.

$^b$RBC = red blood cell Folate (nmol/L).

$^c$r = Pearson’s correlation coefficient.

$^d$SGA = subjective global assessment.

$^e$eGFR = estimated glomerular filtration rate (ml/min/1.73 m$^2$).

$^f$TSF = tricep skinfold thickness (mm).

$^g$Plasma folate = plasma folate (nmol/L).

$^h$Serum B$_{12}$ = serum vitamin B$_{12}$ (pmol/L).

$^i$Folate Intake = observed folate intake (µg/d of DFE) from food (natural and folic acid) and supplements.

$^j$BMI = body mass index (kg/m$^2$).

$^k$ml/d.
Table 5.5 Backward Multiple Linear Regression Analysis with Plasma Total Homocysteine\textsuperscript{a} as the Dependent Variable in Adults with Predialysis Chronic Kidney Disease (n=48)

<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Adjusted R\textsuperscript{2}</th>
<th>F</th>
<th>Beta Coefficient</th>
<th>p value\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables Included in the Model:</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red Blood Cell Folate</td>
<td>.229</td>
<td>5.666</td>
<td>-0.327</td>
<td>0.017</td>
</tr>
<tr>
<td>Subjective Global Assessment</td>
<td></td>
<td></td>
<td>0.305</td>
<td>0.024</td>
</tr>
<tr>
<td>Estimated Glomerular Filtration Rate</td>
<td></td>
<td></td>
<td>-0.181</td>
<td>0.170</td>
</tr>
<tr>
<td>Variables Excluded by the Model:</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coffee Intake</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

\textsuperscript{a} Transformed to its natural logarithm in µmol/L.

\textsuperscript{b} Significant = p<0.05.

5.4 Folate, Vitamin B\textsubscript{12} and Vitamin B\textsubscript{6} Intake and Status

Table 5.6 describes the median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) observed intake of natural folate from food, folic acid from food, and folic acid from supplements by the subjects. Observed intake of natural folate from food was 200 (151,287) µg DFE/d, observed intake of folic acid from food was 123 (67,196) µg DFE/d and observed intake of folate (natural and folic acid) from food was 338 (257,426) µg DFE/d. The proportion of subjects with inadequate usual folate intake from food (natural and folic acid) was 13.5\% (Table 5.7). Of the subjects (n=7) with inadequate usual folate intake from food (natural and folic acid), one subject took a folate supplement. The adequacy of usual folate intake from food (natural and folic acid) was uncertain in 61.5\% of the subjects.

Observed folate intake from food (natural and folic acid) did not differ significantly between those with vs without gastrointestinal symptoms of uremia (t (46) = 0.052, p=0.96).

No subject had an observed intake of folic acid from food alone that was greater than 1000 µg/d. Nine subjects used a folate supplement. Two (4\%) subjects had an observed folic acid intake from food and supplement that exceeded 1000 µg/d. In both subjects folic acid intake from supplement alone was 1000 µg.
Table 5.6 Observed Intake of Natural Folate from Food, Folic Acid from Food, and Folic Acid from Supplements by Adults with Predialysis Chronic Kidney Disease

<table>
<thead>
<tr>
<th></th>
<th>Supplement Users And Nonusers</th>
<th>Supplement Nonusers</th>
<th>Supplement Users</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=52</td>
<td>n=43</td>
<td>n=9</td>
</tr>
<tr>
<td>INTAKE (µg DFE/d)a</td>
<td>Median b</td>
<td>Median b</td>
<td>Median b</td>
</tr>
<tr>
<td>Natural Folate From Food c</td>
<td>200 (151,287)</td>
<td>197(150,306)</td>
<td>213(151,261)</td>
</tr>
<tr>
<td>Folic Acid From Food c</td>
<td>123 (67,196)</td>
<td>124(68,198)</td>
<td>122(49,193)</td>
</tr>
<tr>
<td>Folic Acid From Supplements d</td>
<td>- g</td>
<td>0(0,0)</td>
<td>1020(510,1529)</td>
</tr>
<tr>
<td>TOTAL (Food and Supplements)</td>
<td>389(282,640)</td>
<td>332(256,521)</td>
<td>1421(940,1833)</td>
</tr>
</tbody>
</table>

a DFE = dietary folate equivalents. 1 µg natural food folate = 1 µg of DFE. 1 µg folic acid from food fortification or supplement = 1.7 µg of DFE.

b Median(25th, 75th percentile).

c Mean of two 24-hour recalls.

d Frequency questionnaire.

e Data not shown.
Table 5.7 Estimated Average Requirement (EAR) of Folate, Vitamin B\textsubscript{12} and Vitamin B\textsubscript{6} by Age and Gender, and Adequacy of Usual Intake of these Nutrients from Food Alone by Adults with Predialysis Chronic Kidney Disease

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Age(years)</th>
<th>Gender</th>
<th>EAR</th>
<th>ADEQUACY</th>
<th>OF INTAKE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inadequate</td>
<td>Uncertain\textsuperscript{a}</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>n(%)\textsuperscript{b}</td>
<td>n(%)\textsuperscript{b}</td>
</tr>
<tr>
<td>Folate</td>
<td>&gt; 18</td>
<td>Female; Male</td>
<td>320 µg DFE\textsuperscript{c}</td>
<td>7(13.5)</td>
<td>32(61.5)</td>
</tr>
<tr>
<td>Vitamin B\textsubscript{12}</td>
<td>&gt; 18</td>
<td>Female; Male</td>
<td>2.0 µg</td>
<td>0(0)</td>
<td>43(82.7)</td>
</tr>
<tr>
<td>Vitamin B\textsubscript{6}</td>
<td>19-50</td>
<td>Female</td>
<td>1.1 mg DB\textsubscript{6}E\textsuperscript{d}</td>
<td>0(0)</td>
<td>2(100)</td>
</tr>
<tr>
<td>Vitamin B\textsubscript{6}</td>
<td>19-50</td>
<td>Male</td>
<td>1.1 mg DB\textsubscript{6}E\textsuperscript{d}</td>
<td>0(0)</td>
<td>1(25)</td>
</tr>
<tr>
<td>Vitamin B\textsubscript{6}</td>
<td>&gt;50</td>
<td>Female</td>
<td>1.3 mg DB\textsubscript{6}E\textsuperscript{d}</td>
<td>4(26.7)</td>
<td>9(60)</td>
</tr>
<tr>
<td>Vitamin B\textsubscript{6}</td>
<td>&gt;50</td>
<td>Male</td>
<td>1.4 mg DB\textsubscript{6}E\textsuperscript{d}</td>
<td>1(3.2)</td>
<td>24(77.4)</td>
</tr>
</tbody>
</table>

\textsuperscript{a} A level of confidence of at least 85% was used to correctly conclude that a subject’s intake was inadequate or adequate. A level of confidence 50 to 85% was used to conclude that the adequacy of a subject’s intake was uncertain.

\textsuperscript{b} (%) is n divided by the total n for each individual nutrient, by age and gender, expressed as a percent.

\textsuperscript{c} DFE = dietary folate equivalents. 1 µg natural food folate = 1 µg of DFE. 1 µg folic acid from food fortification or supplement = 1.7 µg of DFE.

\textsuperscript{d} DB\textsubscript{6}E = dietary B\textsubscript{6} equivalents. 1 mg food vitamin B\textsubscript{6} = 1 mg DB\textsubscript{6}E. 1 mg synthetic vitamin B\textsubscript{6} = 1.27 mg DB\textsubscript{6}E.
Table 5.8 describes the median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) observed intake of vitamin B\textsubscript{12} from food and from supplements by the subjects. Observed intake of vitamin B\textsubscript{12} from food was 2.9 (1.9, 4.1) \(\mu\)g/d. The proportion of subjects with an inadequate usual vitamin B\textsubscript{12} intake from food was 0%. The adequacy of usual vitamin B\textsubscript{12} intake from food was uncertain in 82.7% of the subjects (Table 5.7).

Table 5.9 describes the median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) observed intake of vitamin B\textsubscript{6} from food and from supplements, by the subjects, by age and gender. Five subjects had inadequate usual vitamin B\textsubscript{6} intake from food (Table 5.7). A majority of the subjects (n=3) who had inadequate usual vitamin B\textsubscript{6} intake from food (n=5) were not vitamin B\textsubscript{6} deficient, as measured by pyridoxal 5\textsuperscript{'}-phosphate, and did not take a vitamin B\textsubscript{6} supplement. The adequacy of usual vitamin B\textsubscript{6} intake from food was uncertain in 34 subjects.
Table 5.8 Observed Intake of Vitamin B$_{12}$ from Food, and Vitamin B$_{12}$ from Supplements by Adults with Predialysis Chronic Kidney Disease

<table>
<thead>
<tr>
<th>DAILY INTAKE (µg)</th>
<th>Supplement Users And Nonusers</th>
<th>Supplement Nonusers</th>
<th>Supplement Users</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=52</td>
<td>n=41</td>
<td>n=11</td>
</tr>
<tr>
<td>Vitamin B$_{12}$ From Food$^b$</td>
<td>Median$^a$</td>
<td>Median$^a$</td>
<td>Median$^a$</td>
</tr>
<tr>
<td></td>
<td>2.9(1.9,4.1)</td>
<td>3.0(1.9,4.1)</td>
<td>2.6(1.7,3.9)</td>
</tr>
<tr>
<td>Vitamin B$_{12}$ From Supplements$^c$</td>
<td>-$^d$</td>
<td>0(0,0)</td>
<td>6.0(3.0,25.0)</td>
</tr>
<tr>
<td>TOTAL (Food and Supplements)</td>
<td>3.6(2.2,6.4)</td>
<td>3.0(1.9,4.1)</td>
<td>9.8(5.4,28.0)</td>
</tr>
</tbody>
</table>

$^a$ Median(25th, 75th percentile).
$^b$ Mean of two 24-hour recalls.
$^c$ Frequency questionnaire.
$^d$ Data not shown.
Table 5.9 Observed Intake of Vitamin B₆ from Food, and Vitamin B₆ from Supplements by Adults with Predialysis Chronic Kidney Disease, by Age and Gender

<table>
<thead>
<tr>
<th></th>
<th>Supplement Users And Nonusers</th>
<th>Supplement Nonusers</th>
<th>Supplement Users</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=2</td>
<td>n=1</td>
<td>n=1</td>
</tr>
<tr>
<td>DAILY INTAKE (mg DB₆E)ᵃ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
</tr>
<tr>
<td>Vitamin B₆ From Foodᶜ</td>
<td>1.3 (-)</td>
<td>1.4(-)</td>
<td>1.2(-)</td>
</tr>
<tr>
<td>Vitamin B₆ From Supplementsᵈ</td>
<td>6.3(-)</td>
<td>0(0,0)</td>
<td>12.6(-)</td>
</tr>
<tr>
<td>TOTAL (Food and Supplements)</td>
<td>7.6(-)</td>
<td>1.4(-)</td>
<td>13.8(-)</td>
</tr>
<tr>
<td>MALES 19 to 50 Years</td>
<td>n=4</td>
<td>n=4</td>
<td>n=0</td>
</tr>
<tr>
<td>DAILY INTAKE (mg DB₆E)ᵃ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
</tr>
<tr>
<td>Vitamin B₆ From Foodᶜ</td>
<td>2.2 (1.2,2.8)</td>
<td>2.2 (1.2,2.8)</td>
<td>-</td>
</tr>
<tr>
<td>Vitamin B₆ From Supplementsᵈ</td>
<td>0(0,0)</td>
<td>0(0,0)</td>
<td>-</td>
</tr>
<tr>
<td>TOTAL (Food and Supplements)</td>
<td>2.2 (1.2,2.8)</td>
<td>2.2 (1.2,2.8)</td>
<td>-</td>
</tr>
<tr>
<td>FEMALES &gt; 50 Years</td>
<td>n=15</td>
<td>n=11</td>
<td>n=4</td>
</tr>
<tr>
<td>DAILY INTAKE (mg DB₆E)ᵃ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
</tr>
<tr>
<td>Vitamin B₆ From Foodᶜ</td>
<td>1.1 (0.7,1.4)</td>
<td>1.2(0.7,1.4)</td>
<td>1.1(0.8,1.5)</td>
</tr>
<tr>
<td>Vitamin B₆ From Supplementsᵈ</td>
<td>-</td>
<td>0(0,0)</td>
<td>11.4(10.2,31.8)</td>
</tr>
<tr>
<td>TOTAL (Food and Supplements)</td>
<td>1.3(0.8,11.3)</td>
<td>1.2(0.7,1.4)</td>
<td>12.8(11.4,26.3)</td>
</tr>
<tr>
<td>MALES &gt; 50 Years</td>
<td>n=31</td>
<td>n=25</td>
<td>n=6</td>
</tr>
<tr>
<td>DAILY INTAKE (mg DB₆E)ᵃ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
<td>Medianᵇ</td>
</tr>
<tr>
<td>Vitamin B₆ From Foodᶜ</td>
<td>1.5 (1.1,1.8)</td>
<td>1.4(1.1,1.7)</td>
<td>1.6(1.3,2.3)</td>
</tr>
<tr>
<td>Vitamin B₆ From Supplementsᵈ</td>
<td>-</td>
<td>0(0,0)</td>
<td>7.4(1.2,94.6)</td>
</tr>
<tr>
<td>TOTAL (Food and Supplements)</td>
<td>1.6(1.1,2.4)</td>
<td>1.4(1.1,1.7)</td>
<td>9.3(2.8,96.7)</td>
</tr>
</tbody>
</table>

ᵃ DB₆E = dietary B₆ equivalents. 1 mg food vitamin B₆ = 1 mg DB₆E. 1 mg synthetic vitamin B₆ = 1.27 mg DB₆E.
ᵇ Median(25th, 75th percentile).
ᶜ Mean of two 24-hour recalls.
ᵈ Frequency questionnaire.
ᵉ Data not shown.
Table 5.10 describes the folate, vitamin B\textsubscript{12} and vitamin B\textsubscript{6} status of the subjects. Plasma folate (n=51) was 44.3 (29.7, 85.2) nmol/L. Red blood cell folate (n=51) was 1601 (1226, 2435) nmol/L. None of the subjects were folate deficient as assessed by plasma or RBC folate concentration. Mean hemtocrit (n=51) was 36.4 ± 4.9 %.

There was a significant association between observed folate intake from food (natural and folic acid) and supplements, and RBC folate (r = 0.546, p=0.000; n=51) as well as plasma folate (r = 0.526, p=0.000; n=51) (figures 5.6, 5.8). When supplement users were removed from the analysis there was no association between observed folate intake from food (natural and folic acid) and RBC folate or plasma folate (figures 5.7, 5.9).

![Figure 5.6](image_url)

Figure 5.6 The Relationship between Red Blood Cell Folate, of Supplement Users and Nonusers, and Observed Folate Intake from Food (natural and folic acid) and Supplements in Adults with Predialysis Chronic Kidney Disease (n=51). The relationship between red blood cell folate, of supplement users and nonusers, transformed to its natural logarithm and observed folate intake from food (natural and folic acid) and supplements was significant (r = 0.546, p=0.000).
Figure 5.7 The Relationship between Red Blood Cell Folate, of Supplement Nonusers, and Observed Folate Intake from Food (natural and folic acid) in Adults with Predialysis Chronic Kidney Disease (n=42). The relationship between red blood cell folate, of supplement nonusers, transformed to its natural logarithm and observed folate intake from food (natural and folic acid) was not significant ($r = 0.156$, $p=0.325$).
Figure 5.8 The Relationship between Plasma Folate, of Supplement Users and Nonusers, and Observed Folate Intake from Food (natural and folic acid) and Supplements in Adults with Predialysis Chronic Kidney Disease (n=51). The relationship between plasma folate, of supplement users and nonusers, transformed to its natural logarithm and observed folate intake from food (natural and folic acid) and supplements was significant ($r = 0.526$, $p=0.000$).
Figure 5.9 The Relationship between Plasma Folate, of Supplement Nonusers, and Observed Folate Intake from Food (natural and folic acid) in Adults with Predialysis Chronic Kidney Disease (n=42). The relationship between plasma folate, of supplement nonusers, transformed to its natural logarithm and observed folate intake from food (natural and folic acid) was not significant (r = -0.075, p=0.637).

Serum vitamin B$_{12}$ (n=52) was 309 (215, 393) pmol/L. Five (9.6%) of the fifty-two subjects were vitamin B$_{12}$ deficient. Of the subjects who were vitamin B$_{12}$ deficient, none (0%) took a vitamin B$_{12}$ supplement.

There was no association between serum vitamin B$_{12}$ and observed vitamin B$_{12}$ intake, from food and supplements, or from food alone (figures 5.10, 5.11).
Figure 5.10 The Relationship between Serum Vitamin B\textsubscript{12}, of Supplement Users and Nonusers, and Observed Vitamin B\textsubscript{12} Intake from Food and Supplements in Adults with Predialysis Chronic Kidney Disease (n=52). The relationship between vitamin B\textsubscript{12}, of supplement users and nonusers, transformed to its natural logarithm and observed vitamin B\textsubscript{12} intake from food and supplements was not significant (r = 0.101, p=0.476).

Figure 5.11 The Relationship between Serum Vitamin B\textsubscript{12}, of Supplement Nonusers, and Observed Vitamin B\textsubscript{12} Intake from Food in Adults with Predialysis Chronic Kidney Disease (n=41). The relationship between vitamin B\textsubscript{12}, of supplement nonusers, transformed to its natural logarithm and observed vitamin B\textsubscript{12} intake from food was not significant (r = 0.105, p=0.513).
Serum pyridoxal 5′-phosphate (n=50) was 27 (19, 46) nmol/L. Thirteen (26%) of the fifty subjects were vitamin B₆ deficient. Of the subjects who were vitamin B₆ deficient, one (7.7%) reported taking a vitamin B₆ supplement.

Dietary reference intakes for adults (≥ 19 years old) for vitamin B₆ vary by life stage (age) and gender. As such, for vitamin B₆, the intention was to analyze the relationship between intake and status by age and gender. However, with the exception of males and females > 50 years old we were not able to analyze the data as the sample size was small. There was a significant association between observed vitamin B₆ intake, from food and supplements, and serum pyridoxal 5′-phosphate in both both males (r = 0.496, p=0.005; n=30) and females (r = 0.247, p=0.244; n=24), > 50 years old. However, when supplement users were removed from the data analysis the association was no longer significant (figures 5.12, 5.13, 5.14, 5.15).

![Figure 5.12](image-url)

Figure 5.12 The Relationship between Serum Vitamin B₆, of Supplement Users and Nonusers, and Observed Vitamin B₆ Intake from Food and Supplements in Males > 50 years old with Predialysis Chronic Kidney Disease (n=30). The relationship between vitamin B₆, of supplement users and nonusers, transformed to its natural logarithm and observed vitamin B₆ intake from food and supplements was significant (r = 0.496, p=0.005).
Figure 5.13 The Relationship between Serum Vitamin B₆, of Supplement Nonusers, and Observed Vitamin B₆ Intake from Food in Males > 50 years old with Predialysis Chronic Kidney Disease (n=24). The relationship between vitamin B₆, of supplement nonusers, transformed to its natural logarithm and observed vitamin B₆ intake from food was not significant ($r = 0.247$, $p=0.244$).

Figure 5.14 The Relationship between Serum Vitamin B₆, of Supplement Users and Nonusers, and Observed Vitamin B₆ Intake from Food and Supplements in Females > 50 years old with Predialysis Chronic Kidney Disease (n=14). The relationship between vitamin B₆, of supplement users and nonusers, transformed to its natural logarithm and observed vitamin B₆ intake from food and supplements was significant ($r = 0.834$, $p=0.000$).
Figure 5.15 The Relationship between Serum Vitamin B₆, of Supplement Nonusers, and Observed Vitamin B₆ Intake from Food in Females > 50 years old with Predialysis Chronic Kidney Disease (n=10). The relationship between vitamin B₆, of supplement nonusers, transformed to its natural logarithm and observed vitamin B₆ intake from food was not significant (r = 0.347, p=0.326).
Table 5.10 Folate and Vitamins B<sub>12</sub> and B<sub>6</sub> Status in Adults with Predialysis Chronic Kidney Disease

<table>
<thead>
<tr>
<th>Supplement Users and Nonusers</th>
<th>n&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Median(25&lt;sup&gt;th&lt;/sup&gt;,75&lt;sup&gt;th&lt;/sup&gt; percentile)</th>
<th>n(%)&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Plasma Folate (nmol/L)</strong></td>
<td>51</td>
<td>44.3(29.7,85.2)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Red Blood Cell Folate (nmol/L)</strong></td>
<td>51</td>
<td>1601(1226,2435)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Serum Vitamin B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)</strong></td>
<td>52</td>
<td>309(215,393)</td>
<td>5(9.6)</td>
</tr>
<tr>
<td><strong>Serum Pyridoxal 5′-Phosphate (nmol/L)</strong></td>
<td>50</td>
<td>27(19,46)</td>
<td>13(26)</td>
</tr>
<tr>
<td><strong>Supplement Nonusers</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Folate (nmol/L)</strong></td>
<td>42</td>
<td>38.5(28.0,62.9)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Red Blood Cell Folate (nmol/L)</strong></td>
<td>42</td>
<td>1527(1168,2089)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Serum Vitamin B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)</strong></td>
<td>41</td>
<td>281(185,373)</td>
<td>5(12.2)</td>
</tr>
<tr>
<td><strong>Serum Pyridoxal 5′-Phosphate (nmol/L)</strong></td>
<td>39</td>
<td>24(18,32)</td>
<td>12(30.8)</td>
</tr>
<tr>
<td><strong>Supplement Users</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Plasma Folate (nmol/L)</strong></td>
<td>9</td>
<td>96.0(84.0,135.0)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Red Blood Cell Folate (nmol/L)</strong></td>
<td>9</td>
<td>2811(2429,3900)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Serum Vitamin B&lt;sub&gt;12&lt;/sub&gt; (pmol/L)</strong></td>
<td>11</td>
<td>393(302,526)</td>
<td>0(0)</td>
</tr>
<tr>
<td><strong>Serum Pyridoxal 5′-Phosphate (nmol/L)</strong></td>
<td>11</td>
<td>129.0(27,216)</td>
<td>1(9.1)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasma folate < 6.8 nmol/L; Red Blood Cell folate of < 317 nmol/L (Herbert 1990). Serum vitamin B<sub>12</sub> < 148 pmol/L (Carmel 1988). Serum pyridoxal 5′-phosphate < 20 nmol/L (Lui et al. 1985).

<sup>b</sup> n is the total number of subjects.

<sup>c</sup> n is the number of subjects that have inadequate status and % is n<sup>c</sup> divided by n<sup>b</sup> x 100.
5.5 Energy and Protein Intake and Status

Table 5.11 describes the median (25th, 75th percentile) observed intake of energy and protein by the study population. In subjects < 60 years old, observed energy intake was 17 (13,25) kcal/kg/d and 84.2% had an intake below recommendation. In subject’s ≥ 60 years old, observed energy intake was 21 (16,27) kcal/kg/d and 81.8% had an intake below recommendation. Observed protein intake was 0.81 (0.68,1.10) g/kg/d and none of the subjects had an intake below the current guideline (National Kidney Foundation, 2000).

Observed energy intake did not differ significantly between those with vs without gastrointestinal symptoms of uremia among subjects < 60 years old (t (17) = 0.288, p=0.777) or among subjects ≥ 60 years old (t (14) = 1.866, p=0.083).

Table 5.12 describes the energy-protein status of the study population. Over a quarter (26.9%) of the subjects were energy-protein undernourished as assessed by SGA.

Of the subjects (n=14) who were energy-protein undernourished (SGA), one subject took a folate supplement. There was no significant association between the presence or absence of gastrointestinal symptoms of uremia and energy-protein status, as assessed by SGA or TSF (data not shown). Observed energy intake did not differ significantly between those who were energy-protein undernourished (SGA) and those who were energy-protein well-nourished (SGA), among subjects ≥ 60 years old (t (12.9) = 0.849, p=0.411). BMI was significantly and inversely associated with observed energy intake in subjects ≥ 60 years old (r = -0.530, p=0.002; n=33) and in subjects < 60 years old (r = 0.649, p=0.003; n=19).
Table 5.11 Recommended Intake\(^a\) of Energy and Protein, Observed Intake of Energy and Protein, and Prevalence of Observed Intake of Energy and Protein below Recommended Intake by Age in Adults with Predialysis Chronic Kidney Disease

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>Age(years)</th>
<th>Recommended Intake</th>
<th>(n^b)</th>
<th>Intake Median(25^{th},75^{th}) percentile</th>
<th>Below Recommended Intake (n(%)^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Energy</td>
<td>&lt;60</td>
<td>35 kcal/kg/d</td>
<td>19</td>
<td>17(13,25) kcal/kg/d</td>
<td>16(84.2)</td>
</tr>
<tr>
<td>Energy</td>
<td>(\geq60)</td>
<td>30 kcal/kg/d</td>
<td>33</td>
<td>21(16,27) kcal/kg/d</td>
<td>27(81.8)</td>
</tr>
<tr>
<td>Protein</td>
<td>&gt;18</td>
<td>0.60 g/kg/d</td>
<td>52</td>
<td>0.81(0.68,1.10) g/kg/d</td>
<td>0(0)</td>
</tr>
</tbody>
</table>

\(^a\) National Kidney Foundation 2000.  
\(^b\) Total number of subjects in nutrient and age category.  
\(^c\) \(n\) is the number of subjects in the nutrient and age category with inadequate intake.  \(\%\) is \(n\) divided by the total number of subjects in the nutrient and age category\(^b\) x 100.
<table>
<thead>
<tr>
<th>Reference for Energy-Protein Status</th>
<th>Reference Value for Low Energy-Protein Status</th>
<th>Median (25&lt;sup&gt;th&lt;/sup&gt; percentile, 75&lt;sup&gt;th&lt;/sup&gt; percentile)</th>
<th>Number and Prevalence (%) of Subjects with Energy-Protein Status Below Reference Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/L)</td>
<td>≤ 33&lt;sup&gt;a&lt;/sup&gt;</td>
<td>41(40,43)</td>
<td>1(1.9)</td>
</tr>
<tr>
<td>Body Mass Index (m&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>≤ 20&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26(24,32)</td>
<td>4(7.7)</td>
</tr>
<tr>
<td>Tricep Skinfold Thickness (mm)</td>
<td>&lt; 15&lt;sup&gt;th&lt;/sup&gt; percentile&lt;sup&gt;c&lt;/sup&gt;</td>
<td>23(15,31)</td>
<td>3(5.8)</td>
</tr>
<tr>
<td>Bone Free Arm Muscle Area (cm&lt;sup&gt;2&lt;/sup&gt;)</td>
<td>&lt; 15&lt;sup&gt;th&lt;/sup&gt; percentile&lt;sup&gt;c&lt;/sup&gt;</td>
<td>43(35,52)</td>
<td>10(19.2)</td>
</tr>
<tr>
<td>Subjective Global Assessment</td>
<td>Score ≤ 5&lt;sup&gt;d&lt;/sup&gt;</td>
<td>-</td>
<td>14(26.9)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Honda et al. (2006).
<sup>b</sup> Evans et al. (2005).
<sup>d</sup> Cooper et al. (2002).
Chapter 6

6 Discussion

6.1 Prevalence and Determinants of Hyperhomocysteinemia

A main finding of this study was that subjects with pCKD, in the era of folic acid food fortification, had a prevalence of hHcy that was high and a ptHcy concentration reflective of mild to moderate hHcy. The high prevalence of hHcy found in our subjects occurred despite the finding that observed folic acid intake from food fortification contributed to the observed total folate intake from food (natural and folic acid) and supplements by our subjects. It also occurred despite the finding that no subject was folate deficient, as assessed by RBC or plasma folate. This data, taken together, suggests that in the era of folic acid food fortification, total folate intake among our pCKD subjects was sufficient to prevent folate deficiency but not able to prevent a high prevalence of hHcy.

The high prevalence of hHcy found in this study is supported by Jamison et al. (2007) who screened potential subjects with pCKD for the presence of hHcy (ptHcy ≥ 15 µmol/L) as a study inclusion criterion during recruitment for a randomized control trial, in the USA after fortification. The researchers reported that of the subjects screened (n=1619), three hundred and fourteen were excluded secondary to a ptHcy <15 µmol/L (n=187), which suggests that the prevalence of hHcy was approximately 88%.

Folic acid food fortification seems to have had no effect on decreasing the prevalence of hHcy or concentration of ptHcy in the pCKD population with predominantly stage 4 and 5 CKD, as our study results are similar to the prevalence (89%) and concentration (median: 20.5 µmol/L) found among subjects with predominantly stage 4 and 5 pCKD living in a country without folic acid food fortification or before fortification (Nerbass et al. 2006; Sarnak et al. 2002).

In terms of the determinants of ptHcy in the era of folic acid food fortification, a key finding in this study was that, among subjects with pCKD, ptHcy was associated with folate status, as assessed by plasma or RBC folate, and energy-protein status, as assessed by SGA or
Importantly, folate status, as assessed by RBC folate, and undernutrition, as assessed by SGA, independently predicted ptHcy.

Kidney function (eGFR) was not associated with ptHcy in our study. This is in contrast to other studies that showed a strong relationship between kidney function and ptHcy. For example, kidney function, as assessed by serum creatinine, was found to be one of the strongest determinants of ptHcy in the general population (Ganji et al. 2009). As another example, kidney function, as assessed by eGFR, was found to be one of the strongest determinants of ptHcy in a pCKD population, that included subjects with a wide range of kidney function levels (eGFR: < 10 to 75 ml/min/1.73m²) (Parsons et al. 2002). It is likely we were not able to detect an association between ptHcy and kidney function because the variability in kidney function level, among our subjects, was small (eGFR range: 7.6 to 40.8 ml/min/1.73m²).

As previously mentioned, folate status, as assessed by RBC folate, independently predicted ptHcy among our subjects. Studies have found a similar relationship among subjects with pCKD, living in countries without folic acid food fortification. That is, studies have reported folate status, as assessed by plasma or RBC folate, to independently predict ptHcy (Nerbass et al. 2006; Parsons et al. 2002). Our result is also similar to that found in the general population, after fortification, with both RBC and serum folate found to independently predict ptHcy (Ganji et al. 2009). The mechanism by which folate status contributed to the variability in ptHcy in our study is related to folate’s role in the remethylation of homocysteine to methionine (Selhub 1999).

Among our pCKD subjects folate status was the strongest determinant of ptHcy. This finding is not consistent with published research, conducted in the general population or in subjects with healthy kidneys, reporting that folic acid supplementation or folic acid food fortification results in vitamin B₁₂ status being the dominant nutritional determinant of ptHcy (Quinlivan et al. 2002; Ganji et al. 2009). Quinlivan et al. (2002) conducted an intervention trial among healthy subjects who did not take food fortified with folic acid or personal B-vitamin supplements before or during the study. In this trial, subjects received increasing doses of folic acid supplement (0, 100, 200 and 400 µg/d) with each dose of folic acid taken for approximately 6 weeks. With each increase in dose of folic acid supplement the association between ptHcy and serum folate became weaker and the association between ptHcy and serum vitamin B₁₂ became
stronger. In an observational study conducted in the general population (n=16,254) after fortification, Ganji et al. (2009) found vitamin B_{12} status, as assessed by methylmalonic acid, to be the strongest predictor of ptHcy. In the study by Quinlivan et al. (2002) standardized doses of folic acid supplement would have decreased the variability in folate status among the subjects, which would have contributed to the diminished association found between ptHcy and serum folate. In our study, random use of foods fortified with folic acid and random use folic acid supplements may have contributed to the heterogeneity of folate status in our subjects, resulting in the strong relationship found between ptHcy and RBC folate. The difference in result found between our study and that of Ganji et al. (2009) is related to the omission of plasma methylmalonic acid as a potential determinant of ptHcy in our study. The reason for this omission is due to the fact that plasma methylmalonic acid is not a valid marker of vitamin B_{12} status in CKD (Vogiatzoglou et al. 2009). Specifically, plasma methylmalonic acid has been found to be positively associated with serum creatinine.

In our pCKD subjects, vitamin B_{12} status, as assessed by serum vitamin B_{12}, was not associated with ptHcy. Our result is inconsistent with that found in the general population, both pre and post folic acid food fortification, and in pCKD subjects living in countries without fortification. In the general population a meta-analysis of randomized controlled trials, conducted in countries without folic acid food fortification of the food supply, found that supplementation with 0.8 mg of folic acid, significantly, and maximally, lowered ptHcy by 23% (95% CI: 21 to 26) (Homocysteine Lowering Trialists’ Collaboration 2005). The addition of vitamin B_{12} (median dose: 0.4 mg) to folic acid significantly reduced ptHcy concentration by 7% (95% CI: 4 to 9) more than folic acid alone. In the era of folic acid food fortification, serum vitamin B_{12} in the general population (n=16,254) was found to independently predict ptHcy (Ganji et al. 2009). In a pCKD population (n=66), living in a country without fortification, serum vitamin B_{12} (382 ± 174) was found to contribute to 8.4% of the variability in ptHcy (Nerbass et al. 2006). The lack of an association found in our pCKD subjects in the era of folic acid food fortification is unlikely related to a limited variability in serum vitamin B_{12} (331 ± 181 pmol/L), but may be related to a small sample size (n=48).

Vitamin B_{6} status, as assessed by serum pyridoxal 5'-phosphate, was not a determinant of ptHcy in our pCKD subjects. Our result is consistent with an intervention trial and a meta-analysis of intervention trials that were not able to provide evidence that vitamin B_{6} intervention
lowers fasting ptHcy (Ubbink et al. 1996; Homocysteine Lowering Trialists’ Collaboration 2005). Vitamin B₆ is a cofactor for enzymes in the transsulfuration pathway, the elimination route of homocysteine. As such, the relationship between vitamin B₆ and ptHcy may be more pronounced in the non fasting state. Evidence for this comes from published research reporting that vitamin B₆ intervention, in subjects who were vitamin B₆ deficient (plasma pyridoxal 5'-phosphate < 32.0 nmol/L), resulted in a significant decrease in non fasting ptHcy (post methionine load test) and no significant difference in fasting ptHcy (Ubbink et al. 1996).

Observed protein intake was not associated with fasting ptHcy in our study. Menon et al. (2005) reported an inverse association between observed protein intake and ptHcy in a pCKD population, but that association was weak, with observed protein intake contributing to only 1.4% of the variability in ptHcy. In the same study, observed protein intake did not independently predict ptHcy.

As previously mentioned, energy-protein status, as assessed by SGA, independently predicted ptHcy, with undernourished subjects having a higher ptHcy concentration. In contrast to our finding, Suliman et al. (2002) found that compared to subjects who were well-nourished (SGA) subjects who were undernourished had a significantly lower ptHcy concentration. In a subsequent study, by Suliman et al. (2004), subjects were divided into three groups which included those who were well-nourished (SGA) and did not have inflammation (C-reactive protein), those who were undernourished and had inflammation, and lastly, a mix of those who were undernourished without inflammation and those who were well-nourished with inflammation. The researchers reported that low ptHcy was related to a combination of undernutrition and inflammation, not to undernutrition alone. In the same study, serum albumin was found to be significantly lower in the group of subjects who were both undernourished and inflamed (29 g/L; 95% CI: 28 to 31; n=47) compared to the subjects in other two groups. A majority of ptHcy is bound to albumin (Mansoor et al. 1992). As such, the mechanism of low ptHcy seen in subjects with both undernutrition and inflammation in the study by Suliman et al. (2004), may be through a decrease in serum albumin. The difference in the relationship between ptHcy and undernutrition (SGA) found between our study and that of Suliman et al. (2004) may be due to a difference in inflammation status and serum albumin concentration as a result of a difference in kidney function level between the two study populations. In our study, median eGFR was 20.2 ml/min/1.73m². Kidney function (median eGFR = 7.0 ml/min/1.73m²) in the
population studied by Suliman et al. (2004) appears to be lower than that found in our study population. Studies have shown that as kidney function declines, inflammation (C reactive protein) increases and serum albumin decreases (Zhang et al. 2009; Razeghi et al. 2008; Singh et al. 2007; Panichi et al. 2001; Parsons et al. 2002). As such, compared to the group of subjects who were both undernourished and inflamed in Suliman’s et al. (2004) study, our undernourished subjects may have had a higher serum albumin concentration (40.0 g/L; 95% CI: 38 to 41; n=12) and may have been less inflamed. This may have contributed to the difference in the relationship between ptHcy and undernutrition found between the studies.

Consistent with our finding that undernutrition, as assessed by SGA, is related to high ptHcy was our finding that TSF was inversely associated with ptHcy. There was also a trend in our study for energy-protein status, as assessed by BMI, to be inversely associated with ptHcy but this did not reach statistical significance. Studies conducted in pCKD populations with sample sizes larger than ours were able to detect a significant inverse association between BMI and ptHcy (n=804), and for BMI to independently predict ptHcy (n=197) (Menon et al. 2005; Parsons et al. 2002). Taken together, our data suggest that energy-protein undernutrition is related to hHcy in pCKD. This is a concern as more than one quarter of our subjects were energy-protein undernourished as measure by SGA.

The mechanism by which energy-protein status (SGA) contributed to hHcy in our study is not clear but insight into a potential mechanism comes from a study conducted in a non CKD population. Ingenbleek et al. (2002a) studied a community in an iodine-deprived goitrous area of Africa, with a limited food supply, especially animal protein. Subjects were divided into four groups to represent three stages of goitrous enlargement (I, II, III) and a healthy control. Protein status was assessed by transthyretin, a marker of total body nitrogen pool (Ingenbleek et al. 2002b). In all groups, transthyretin was inversely, and significantly, associated with ptHcy. Compared to healthy controls (268 ± 31mg/L), subjects with stage III goiter had a significantly lower plasma transthyretin concentration (146 ± 24 mg/L). Compared to healthy controls, subjects with stage III goiter had significantly lower plasma cystathionine, significantly higher ptHcy (27.3 ± 12.6µmol/L) and no significant difference in plasma methionine. Based on the premise that nitrogen deficiency depresses catabolizing enzymes and promotes the sparing of amino acids, the researchers suggested that, in protein undernutrition, the catabolizing enzymes in the transsulfuration pathway were down regulated, resulting in the upstream accumulation of
homocysteine, making homocysteine available for remethylation to methionine (Ingenbleek et al. 2002a; Ingenbleek et al. 2004). It is possible that, in our pCKD subjects, undernutrition contributed to hHcy by down regulating the transsulfuration pathway.

6.2 Intake and Status of Folate, Vitamin B<sub>12</sub>, Vitamin B<sub>6</sub>, Energy and Protein

Importantly, this was the first study to describe median observed intake of folate and vitamin B<sub>12</sub> from food, in the pCKD population. In addition, it was the first study to describe median observed intake of vitamin B<sub>6</sub> from food, by age and gender, in this population. These results were derived by the use of a standard survey method that has been validated in adults and the use of a food composition/nutrient database that contains the most current nutrient content of Canadian foods (Blanton et al. 2006; Health Canada).

The study results are also important because it was the first to describe the proportion of pCKD subjects with inadequate usual intake of folate, vitamin B<sub>12</sub> and vitamin B<sub>6</sub> from food. It is possible that the proportion of subjects with inadequate usual intake of these micronutrients from food is an underestimate, as the adequacy of usual intake of these micronutrients from food was uncertain in a majority of subjects. To increase the certainty with which we can classify an individual’s usual intake as inadequate or adequate we would need to increase the number of days of intake data for an individual. The number of days of intake data that would be required for an individual to obtain an 85% probability of correctly concluding that the individual’s usual intake is inadequate or adequate, are 6 to 12, 18 to 22, and 8 to 12 for folate, vitamin B<sub>12</sub> and vitamin B<sub>6</sub>, respectively, varying with age and gender (Gibson 2005).

Median (25<sup>th</sup>, 75<sup>th</sup> percentile) observed folate intake from food and supplements was 389 (282,640) µg DFE/d. This result appears to be higher than the mean observed folate intake (280.9 µg/d ± 108.4), from food and supplements, found in pCKD subjects (valid reporters and under reporters) in Australia, a country without folic acid food fortification (Fassett et al. 2007). The higher result found in our study may be due to folic acid fortification of the food supply in Canada, as folic acid from food fortification (123 (67,196) µg DFE/d) contributed to the total folate intake from food (natural and folic acid) and supplements by our subjects. The higher result may also be due to the use of dietary folate equivalents to express folate intake, which accounted for the increased bioavailability of folic acid from food and supplements. The lower result found by Fassett et al. (2007) may be related to their reported use of a database that was
not complete for all nutrients in all foods, especially for vitamin and minerals. Lastly, the different result may be related to a difference in the use of folate supplements.

In our study, median (25th, 75th percentile) observed folate intake from food (natural and folic acid) was 338 (257,426) µg DFE/d, a result which suggests that observed folate intake from food (natural and folic acid) in pCKD is similar to, or lower than, the median (25th, 75th percentile) usual intake of males aged 51 to 70 (471 (380, 577) µg DFE/d) and > 70 (403 (326, 494) µg DFE/d) and females aged 51 to 70 (390 (320, 772) µg DFE/d) and > 70 (328 (265, 404) µg DFE/d), in the Canadian general population (Statistics Canada 2004). Seven subjects, in our study, had inadequate usual folate intake from food (natural and folic acid). At the same time, none of the subjects in this study were folate deficient, as assessed by RBC folate. Of the subjects (n=7) with inadequate usual folate intake from food (natural and folic acid), only one subject took a folate supplement. As such, six subjects had inadequate usual folate intake from food (natural and folic acid), were not taking a folate supplement and still had a RBC folate concentration above the cut-point for deficiency. In addition, there was no significant association found between observed folate intake from food (natural and folic acid) and RBC folate, when supplement users were removed from the data analysis. The discrepancy between folate intake and folate status may be related to error in the measurement of folate intake, adequacy of usual folate intake and/or folate status. Potential sources of error related to the measurement of folate intake include the use of: 1) Food records that underestimated observed folate intake from food (natural and folic acid) due to underreporting (Fassett et al. 2007. 2) A food composition database (Canadian Nutrient File 2007b) that underreported the amount of folate in foods (Shakur et al. 2009). Potential sources of error related to the measurement of adequacy of usual folate intake from food (natural and folic acid) include the use of: 1) An estimated average requirement that was, itself, estimated from a variety of biomarkers not RBC folate alone, from studies that used different assays to measure RBC folate and from studies that used different methods to measure folate intake. 2) An estimated average requirement for folate for the healthy population, a between-person standard deviation (subject-to-subject variation in requirement/intake) for folate that was derived from the standard deviation of the requirement of the micronutrient for the healthy population, and a within-person standard deviation (day-to-day variation in a subjects intake) for folate that was obtained from a pooled estimate of within-person standard deviation from the healthy population, to calculate adequacy of usual intake by a
subject with predialysis chronic kidney disease. 3) Use of a small number (n=2) of days of intake data (Food and Nutrition Board, Institute of Medicine 1998b, 2001a, 2001c; Statistics Canada 2004). Potential sources of error related to the measurement of folate status include the use of: 1) Intake data reflective of a 7 day period while the half life of RBC folate is 121 days, indicating intake over a longer period of time (Herbert 1990). 2) Folate contamination of the microbiological assay (Scott et al. 1974). This last point, however, is an unlikely source of error, in our study, as the method used decreased the risk of, and tested for, folate contamination (Molloy et al. 1997).

An important finding, in this study, is that folic acid food fortification in Canada contributed to the total folate intake (food and supplements) by our pCKD subjects. Median (25th, 75th percentile) observed folic acid intake from food was 123 (67,196) µg DFE/d or 72 (40, 115) µg/d, a result consistent with the mean usual folic acid intake from food (74 µg/d) found among elderly adults (≥ 65 years old) in the general population living in Newfoundland, Canada, after fortification (Liu et al. 2004). Recently, Shakur et al. (2010) analyzed national Canadian data and reported mean usual folic acid intake from food fortification in subjects ≥ 51 years old to be 78 ± 36 to 115 ± 54 µg/d, varying with age and gender. Our subjects with pCKD may have a folic acid intake from food fortification close to that of the general Canadian population but it is possible that some of our subjects had a lower intake.

No subject had an observed intake of folic acid from food that was greater than 1000 µg/d, indicating that in the era of folic acid food fortification our pCKD subjects did not have a folic acid intake from food alone that exceeded the tolerable upper intake level for folic acid (Food and Nutrition Board, Institute of Medicine 1998b). This result is consistent with several studies conducted in the general population in Canada after fortification, that found adults ≥ 51 years old and women of child bearing age, not to exceed the tolerable upper intake level for folic acid from food alone (Liu et al. 2004; French et al. 2003; Shuaibi et al. 2008; Shakur et al. 2010).

Two subjects had an observed folic acid intake from food and supplement above the tolerable upper level for folic acid (1000 µg/d). Each of these subjects consumed 1000 µg folic acid from supplement. This result suggests that, in the era of folic acid food fortification, it is possible for a person with pCKD to exceed the tolerable upper level for folic acid if they are taking a folic acid supplement. In clinical practice a common vitamin supplement recommended
to patients with pCKD contains 1000 µg folic acid. Our data suggests that recommending a vitamin supplement containing 1000 µg folic acid will result in a patient exceeding the tolerable upper level for folic acid. This is a concern as excessive folic acid intake may have adverse effects, such as promoting cancer, atherosclerosis, progression of nephropathy and/or masking the hematological signs of a vitamin B<sub>12</sub> deficiency (Kim 2008; Sauer et al. 2009; Dong et al. 2002; House et al. 2010; Bender 2003).

An important finding, in this study, was that in the era of folic acid food fortification in Canada our pCKD subjects were not folate deficient as measured by RBC folate (< 317 nmol/L), the storage form of folate. In the general population in Ontario, the prevalence of folate deficiency, as assessed by RBC folate (< 215 nmol/L), after fortification also appears to be small (0.41%) (Ray et al. 2002).

Folate status, as assessed by RBC folate, was 1527 (1168,2089) nmol/L among supplement nonusers. This result appears to be higher than that found among pCKD subjects living in a country without folic acid food fortification. Arnadottir et al. (1996) reported mean RBC folate to be 302 nmol/L ± 89 nmol/L among pCKD subjects, living in Sweden, not taking folate supplements. The difference in our result may be related to folic acid food fortification. The difference may also be related to the different assays used to quantify RBC folate. We used a microbiological assay, while Arndottir et al. (1996) used a radioassay. Fazili et al. (2008) reported a lower whole blood folate concentration result (45%), by radioassay, compared to microbiological assay, possibility due to a decreased ability of the radioassay to detect 5-methlytetrahydrofolate, the predominant folate derivative in plasma and RBC folate.

Our RBC folate result also appears to be higher than that found in the general population, in the era of folic acid food fortification (Ray et al. 2002; Pfeiffer et al. 2007; Aufreiter et al. 2009; Choumenkovitch et al 2001; Colapinto et al. 2010).

Median (25<sup>th</sup>, 75<sup>th</sup> percentile) RBC folate concentration among supplement users and nonusers in our study was 1601 (1226,2435) nmol/L. Ray et al. (2002) reported a RBC folate concentration of 851.6 nmol/L (95% CI: 841.2 to 862.0) in the general population in Ontario. The lower result reported by Ray et al. (2002) may be related to the use of the radioassay, a maximum reporting limit (1450 nmol/L), a difference in folate supplement use and/or the use of subjects who had folate status tested based on clinical grounds. A cross-sectional study
conducted among adults (≥ 60 years old) in the general Canadian population, after fortification, found RBC folate concentration at the 5th, 50th and 97th percentiles to be 634 (95% CI: 481 to 787) nmol/L, 1409 (95% CI: 1260 to 1558) and 2887 (95% CI: 2678 to 3097) nmol/L, respectively (Colapinto et al. 2010). It seems that the median (25th, 75th percentile) RBC folate concentration (1601 (1226,2435) nmol/L) of the supplement users and nonusers in our study is higher than the RBC folate concentration found at the 50th percentile in the study conducted in the general Canadian population. The apparent lower result found by Colapinto et al. (2010) may be due to the use of the radioassay, a difference in folate supplement use and/or a larger sample size.

Median (25th, 75th percentile) RBC folate concentration (1527 (1168,2089) nmol/L) among supplement nonusers in our study seems to be higher than that reported by two studies conducted in the general population, after fortification, that used the same assay as our study to measure RBC folate (microbiological assay *Lactobacillus rhamnosus* ) and that excluded supplement users (Aufreiter et al. 2009; Choumenkovitch et al 2001). Aufreiter et al. (2009) measured RBC folate in a small number (n=6) of healthy adults in Toronto, Canada, and reported the mean concentration to be 1163 ± 196 nmol/L. Choumenkovitch et al. (2001) measured the RBC folate concentration of healthy adults in Framingham, Massachusetts, USA (n=354) and reported the mean to be 1019.7 nmol/L (95% CI: 975.7 to 1065.7). The mechanism by which RBC folate in pCKD subjects may be higher than that in the general population might be due to decreased kidney function as there is evidence that the healthy kidney regulates folate status (Ray et al. 2002; Pfeiffer et al. 2007; Aufreiter et al. 2009; Choumenkovitch et al 2001). Free folate is filtered in the glomeruli and reabsorbed by the proximal tubule by folate receptor mediated endocytosis, resulting in little urinary excretion of folate (Kownacki-Brown et al. 1993; Birn 2006; Scott et al. 1986a). As such, the normal physiological process is to conserve folate. Reabsorption of folate, however, is a saturable process, such that chronic daily oral intake of large doses of folic acid (2 mg), results in an increase in urine folate (Kownachki-Brown et al. 1993; Scott et al. 1986b). It seems possible that a decreased glomerular filtration rate could result in decreased filtration of folate, and hence, a decreased opportunity to excrete excessive folate in the urine. This idea could explain why no subject in the present study was folate deficient while 26.9% were energy-protein undernourished (SGA), with only one undernourished (SGA) subject taking a folate supplement. The possibility that kidney function alters folate
status is not supported by Parsons et al. (2002). These researchers studied pCKD subjects (n=197), not taking folate supplements, with a wide range of kidney function levels (eGFR < 10 to 75 ml/min/1.73m²) and did not find a significant difference in mean RBC folate concentration when participants were divided into five groups based on kidney function level. However, it is important to note that the study by Parsons et al. (2002) was conducted in a country without folic acid food fortification.

Other reasons why RBC folate in our pCKD subjects may appear higher than in the general population include: 1) RBC folate may not accurately reflect folate status in pCKD because red blood cell production is abnormal in CKD (Lankhorst et al. 2010). RBC folate represents the concentration of folate in one litre of red blood cell. Mean hemtocrit (36.4 ± 4.9 %; n=51) of our pCKD subjects appears to be lower than that of males (46.5 ± 2.9 %; n=1376) and females (41.1 ± 2.9 %; n=1007) in the general population (Amin et al. 2004). As such, compared to the general population, our pCKD subjects may have a higher folate concentration per one litre of red blood cell and, at the same time, a lower proportion of red blood cells in whole blood. 2) Growth the Lactobacillus rhamnosus reflective of the presence of folate and urea, not folate alone. Bacterial urease catalyses the hydrolysis of urea to ammonia and carbamate, with ammonia being the preferred nitrogen source for many bacteria (Collins et al. 1993). Brown et al. (1971) found that fecal bacterial urease activity increases as blood urea increases. In clinical practice, the blood urea nitrogen of people with pCKD can be elevated. However, that the growth of Lactobacillus rhamnosus reflects the presence of both folate and urea, in our study, is unlikely as Zotta et al. (2008) found that this strain of bacteria did not test positive for urease activity. In addition, median (25th, 75th percentile) plasma folate (38.5(28.0,62.9) nmol/L) among supplement nonusers in our study does not appear to be higher than that (48.1 ± 10.1 nmol/L) of subjects selected from the general population (Aufreiter et al. 2009). 3) Folate contamination of the microbiological assay (Scott et al. 1974). This, however, is unlikely, in our study, as the method used decreased the risk of, and tested for, folate contamination (Molloy et al. 1997).

Vitamin B₁₂ status of people (n=692) living in Ontario seems to be variable, with 85% of the population having a serum vitamin B₁₂ between 134.6 pmol/L to 663.7 pmol/L (Ray et al. 2000). In our study, median (25th, 75th percentile) serum vitamin B₁₂ concentration of supplement users and nonusers was 309 (215,393) pmol/L. This result appears to be similar to
that found among pCKD subjects in other published studies. Sarnak et al. (2002) studied a pCKD population (n=245) with stage 4 and 5 CKD (13 to 24 ml/min/1.73m²), in the USA, and found median serum vitamin B\textsubscript{12} to be 326 pmol/L. House et al. (2010) conducted a randomized control trial, in Canada, among subjects with stage 1, 2 and 3 diabetic nephropathy, and found baseline serum vitamin B\textsubscript{12} concentrations to be 348 ± 211pmol/L and 304 ± 142 pmol/L in the placebo and intervention group, respectively.

The prevalence (12.2%) of vitamin B\textsubscript{12} deficiency (serum vitamin B\textsubscript{12} < 148 pmol/L) found among supplement nonusers in our study, is higher than the prevalence (0%) (serum vitamin B\textsubscript{12} < 147 pmol/L) found by Nerbass et al. (2006) in pCKD subjects, living in Brazil. Conditions reported to increase the risk of a vitamin B\textsubscript{12} deficiency include low vitamin B\textsubscript{12} intake and increased age (Food and Nutrition Board, Institute of Medicine 1998c; Allen 2009). Vitamin B\textsubscript{12} depletion can occur with aging because of malabsorption of food bound vitamin B\textsubscript{12} caused by atrophy of the gastric mucosa and the gradual loss of gastric acid, which releases the vitamin from food (Allen 2009). The different result found between our study and that of Nerbass et al. (2006) is unlikely related to an age difference (62 ± 12 years vs. 58.6 ± 15.6 years) but may be due to a difference in vitamin B\textsubscript{12} intake. Nerbass et al. (2006) reported that pCKD clinic patients are routinely prescribed vitamin B\textsubscript{12} supplements. For their study, subjects were recruited from their clinic and classified as a supplement nonuser if they had missed taking the supplement any day in the month prior to the study. As such, some subjects may have been taking a vitamin B\textsubscript{12} supplement with the exception of one day. Vitamin B\textsubscript{12} can be stored and retained in the body for long periods of time, even years (Groff JL et al. 1999).

Among our pCKD subjects, median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) observed vitamin B\textsubscript{12} intake from food alone (2.9 (1.9,4.1) µg/d) appears to be similar to, or lower than the usual vitamin B\textsubscript{12} intake from food by males aged 51 to 70 (4.3 (3.3, 5.9) µg/d) and > 70 (3.7 (2.7, 5.3) µg/d) and females aged 51 to 70 (3.3 (2.6, 4.6) µg/d) and > 70 (3.0 (2.3, 4.2) µg/d), in the Canadian general population (Statistics Canada 2004). At the same time, median (25\textsuperscript{th}, 75\textsuperscript{th} percentile) serum vitamin B\textsubscript{12} concentration (309 (215,393) pmol/L) and the prevalence (9.6%) of deficient vitamin B\textsubscript{12} status (serum vitamin B\textsubscript{12} < 148 pmol/L) among our pCKD subjects (supplement users and nonusers) appears to be similar to that found among adults (57.0 ± 20.0 years) in the general population (n=4147) in Ontario (Ray et al. 2002). Specifically, Ray et al. (2002) reported a serum vitamin B\textsubscript{12} concentration of 292.9 pmol/L (95% CI: 288.3 to 297.6) and a prevalence of
deficient vitamin B\textsubscript{12} status of (serum vitamin B\textsubscript{12} < 150 pmol/L) of (7.8%). That the prevalence of vitamin B\textsubscript{12} deficiency in our pCKD subjects may be similar to that of the general population is not supported by a study conducted by Garcia et al. (2008) in Ontario. In our study the prevalence of deficient vitamin B\textsubscript{12} status was 12.2\% among supplement nonusers, while it was 20\% among subjects, not using supplements, in the the study by Garcia et al. (2008). The higher prevalence found in the study by Garcia et al. (2008) is due, in part, to a higher cut point used to define vitamin B\textsubscript{12} deficiency (serum vitamin B\textsubscript{12} < 165 pmol/L vs. < 148 pmol) and may be due to a difference in age, with their subjects appearing to be older (73 ± 4.9 years vs. 62 ± 12 years).

One might expect that vitamin B\textsubscript{12} status of our pCKD subjects would differ from that of the general population, or subjects with healthy kidneys, as there is evidence that the healthy kidney regulates vitamin B\textsubscript{12} status. Specifically, filtration of serum vitamin B\textsubscript{12}, bound to transcobalamin, occurs in the renal glomeruli (Groff JL et al. 1999; Birn 2006). Reabsorption of the vitamin occurs in the renal proximal tubule by receptor (megalin) mediated endocytosis, with urinary vitamin B\textsubscript{12} loss being minimal. As well, metabolism of vitamin B\textsubscript{12} may occur in the kidney, as the vitamin has been shown to accumulate in the renal proximal tubule (Birn H 2006). However, the possibility that kidney disease, itself, alters kidney function and thus, vitamin B\textsubscript{12} status, is not supported by studies that did not find a significant association between kidney function and serum vitamin B\textsubscript{12} concentration (Nakanishi et al. 2002; Nerbass et al. 2006; Parsons et al. 2002).

An important finding in this study is that, in the era of folic acid food fortification, almost one tenth of our subjects were vitamin B\textsubscript{12} deficient, while no subject was folate deficient. This may be a health concern in pCKD, as unmetabolized folic acid, in the presence of vitamin B\textsubscript{12} deficiency, may prevent megaloblastic anemia, masking the hematological sign of a vitamin B\textsubscript{12} deficiency and allowing for the progression of neurologic damage (Bender 2003; Dror et al. 2008). It is important to note, however, that the mechanistic hypotheses of the ability of unmetabolized folic acid to be able to drive DNA synthesis to prevent megaloblastic anemia despite a block in methionine synthase activity imposed by a vitamin B\textsubscript{12} deficiency has yet to be proven (Morris et al. 2010). Interestingly, a similar ratio of the prevalence of folate deficiency (RBC folate < 215 nmol/L) (0.41\%) to the prevalence of vitamin B\textsubscript{12} deficiency (serum vitamin B\textsubscript{12} ≤ 150 pmol/L) (7.8\%) was reported in the general population in Ontario after fortification (Ray et al. 2002).
Median (25th, 75th percentile) serum pyridoxal 5′-phosphate concentration among supplement non users in our pCKD subjects was 24 (18,32) nmol/L. This result appears to be similar to that of other pCKD subjects, not using supplements (Arnadottir et al. 1996). Arnadottir et al. (1996) studied pCKD subjects with an eGFR of 6 to 32 ml/min/1.73m², living in Sweden, and reported mean serum pyridoxal 5′-phosphate concentration to be 23.7 ± 10.6 nmol/L.

Median (25th, 75th percentile) observed vitamin B₆ intake (1.5 (1.1,1.8) mgDB₆E) from food by males > 50 years old in our study may be lower than the median (25th, 75th percentile) usual vitamin B₆ intake from food by males 51 to 70 years old (2.03 (1.66,2.46) mg/d) and > 70 years old (1.78 (1.4,2.19) mg/d), in the general population in Canada (Statistics Canada 2004). Similarly, the median (25th, 75th percentile) observed vitamin B₆ intake (1.1 (0.7,1.4) mgDB₆E) from food by females > 50 years old in our study, may be lower than the median (25th, 75th percentile) usual vitamin B₆ intake from food by females 51 to 70 years old (1.63 (1.37,1.93) mg/d) and > 70 years old (1.50 (1.21,1.85) mg/d), in the general population in Canada. It was also appears that median (25th, 75th percentile) serum pyridoxal 5′-phosphate concentration among supplement non users (24 (18,32) nmol/L) in our study may be lower than that found in the general population, not taking supplements (Morris et al. 2008). Morris et al. (2008) studied the general population in the USA and reported mean serum pyridoxal 5′-phosphate concentration to be 36 nmol/L (95% CI: 33 to 40) and 36 nmol/L (95% CI: 31 to 41) among adults 45 to 64 years old and ≥ 60 years old, respectively. That people with pCKD may have a lower vitamin B₆ status than the general population is supported by Lindner et al. (2002), who reported that mean plasma pyridoxal 5′-phosphate in subjects with pCKD (20.9 ± 19.8 nmol/L) was significantly lower than in subjects with healthy kidneys (55 ± 59 nmol/L). This finding occurred despite vitamin B₆ supplement use among some of the subjects with pCKD (35%), and no supplement use among those with healthy kidneys.

An important finding in this study is that the prevalence of vitamin B₆ deficiency was high. Among supplement users and nonusers it was 26%. When supplement users were removed from the data analysis the prevalence was 30.8%. This prevalence appears to be higher than that found in the general population in the USA among adults 45 to 64 years old (23%) and ≥ 60 years old (24%) not using supplements (Morris et al. 2008). The mechanism by which decreased kidney function might result in vitamin B₆ deficiency is not clear. Potential
mechanisms include: 1) Decreased vitamin B₆ intake from food secondary to gastrointestinal symptoms of uremia. A majority of our subjects reported having at least one gastrointestinal symptom of uremia. However, we did not find a significant association between vitamin B₆ intake from food, among supplement nonusers, and pyridoxal 5′-phosphate in male or female subjects, > 50 years old. 2) Increased urinary excretion of pyridoxal 5′-phosphate. In healthy adults, urine is the main elimination route for vitamin B₆, in the form of 4-pyridoxic acid, the catabolic end product of pyridoxal 5′-phosphate (Lui et al. 1985). Urine excretion of pyridoxal 5′-phosphate, itself, is small (9%), even in experimentally induced vitamin B₆ excess (Lui et al. 1985). In animals, it has been demonstrated that vitamin B₆ is reabsorbed by renal proximal tubular cells (Bowman et al. 1989). It might be that urine excretion of pyridoxal 5′-phosphate is increased in pCKD but to the writers knowledge this is not known. 3) Inflammation. A study conducted in the general population in the USA, found plasma pyridoxal 5′-phosphate to be significantly lower (36.5 nmol/L; 95% CI: 29.2 to 45.8) in subjects with a serum C-reactive protein ≥ 6 mg/L than (55.8 nmol/L; 95% CI: 52.6 to 59.2) in subjects with a serum C-reactive protein < 6 mg/L (Friso et al. 2001). Vitamin B₆ is required for nucleic acid synthesis which subsequently is required for protein biosynthesis, specifically proteins involved in the inflammation process (Rall et al. 1993; Friso et al. 2001). Numerous studies have shown that as kidney function declines, inflammation increases (Zhang et al. 2009; Razeghi et al. 2008; Singh et al. 2007; Panichi et al. 2001). As such, it is possible that our vitamin B₆ deficient subjects had inflammation with an increased utilization and thus, requirement for vitamin B₆. 4) Elevated alkaline phosphatase. Alkaline phosphatase activity can be increased in renal osteodystrophy (Bervoets et al. 2003). In the plasma, alkaline phosphatase hydrolyses pyridoxal 5′-phosphate to pyridoxal (Groff et al. 1999). As such, it is possible that alkaline phosphatase activity was increased in our vitamin B₆ deficient subjects with increased catabolism of pyridoxal 5′-phosphate to pyridoxal.

An overwhelming majority of subjects in our study had an observed energy intake below that recommended by the current practice guidelines for pCKD (National Kidney Foundation 2000). At the same time, 26.9% of our subjects, at most, were energy-protein undernourished (SGA). The discrepancy between the proportion of subjects with inadequate observed energy intake and the proportion with energy-protein undernutrition (SGA) may be related to error in the measurement of adequacy of energy intake and/or energy intake itself. The current
recommendations for energy and protein intake are based on evidence that a low protein diet may delay the progression of kidney disease and that an energy intake of 35 kcal/kg/d results in almost all subjects, on a low protein diet (0.60 g/kg/d), having a neutral or positive nitrogen balance (National Kidney Foundation 2000; Levey et al. 1996; Dukkipati et al. 2010; Kopple et al. 1986). As such, the current recommendations for energy, and protein, intake do not account for individual variation in requirement. As a result, in our study, when measuring adequacy of observed energy, and protein, intake in our study we were not able to use a statistical method that accounted for within-person variation in intake and between-person variation in requirement. Thus, the discrepancy between the proportion of subjects with inadequate observed energy intake and the proportion with energy-protein undernutrition (SGA) may be related to error in the measurement of adequacy of energy intake. With regard to error in the measurement of energy intake, itself, it is possible that our subjects underreported energy intake as we found an inverse association between observed energy intake and energy-protein status, as assessed by BMI, among subjects ≥ 60 years old and among subjects < 60 years. Support for this possibility comes from two studies conducted in pCKD populations, that each used an observed energy intake to resting energy expenditure ratio below 1.27 as indicative of underreporting. They found over 70% of the subjects underreported energy intake (Avesani et al. 2005; Fassett et al. 2007). Fassett et al. (2007) compared observed energy intake to energy requirement, as recommended by the current practice guidelines, and reported that the proportion of pCKD subjects with an observed intake below recommendation was 84% and 48% when underreporters were included in the data analysis and when they were excluded, respectively. In the same study the researchers found BMI to be significantly higher in underreporters compared to valid reporters.

No subject in our study had an observed protein intake below that recommended (0.6 g/kg/d) by the current practice guidelines for pCKD (National Kidney Foundation 2000). At the same time, 19.2% of our subjects were energy-protein undernourished, as assessed by bone-free arm muscle area, an indication of muscle protein wasting. The discrepancy between the proportion of subjects with inadequate observed protein intake and the proportion with energy-protein undernutrition (bone-free arm muscle area) may be related to error in the measurement of adequacy of protein intake. One source of error is, as previously mentioned, that the current recommendation for protein intake does not account for between-person variation in requirement. The result is that when measuring adequacy of protein intake we were not able to
use a statistical method that accounted for within-person standard deviation in intake and between-person standard deviation in requirement. Another source of error is that the current recommended protein intake for patients with pCKD may be an underestimate of protein requirement (National Kidney Foundation 2000). The origin of the current recommendation is based on a publication by Brenner in 1985 that reported a high protein intake in the presence of kidney disease contributes to renal hyperfiltration and hypertension. Hyperfiltration and hypertension are detrimental to glomerular structure and function, leading to renal failure (Brenner et al. 1992). The current practice guideline for protein intake (0.6 g/kg/d) in pCKD is also based on the recommendation, in 1985, by the World Health Organization, that the minimum daily protein requirement of healthy adults is 0.6 g protein/kg (Mandayam et al. 2006). This “minimum daily protein requirement” is in actual fact the estimated average requirement for healthy adults. A recent publication by Humayun et al. (2007) suggests that the estimated average requirement for protein in healthy adults is higher, at 0.93 g/kg/d. Finally, a source of error may be due to the use of an estimated average requirement for protein for healthy adults, for subjects with pCKD, as there is evidence that protein metabolism is altered in CKD (Du et al. 2005; Maratos-Flier 2008).

The prevalence of undernutrition was only 1.9 % when serum albumin, an objective measure, was used to assess energy-protein undernutrition (serum albumin ≤ 33 g/L). Carvalho et al. (2004) found a similar prevalence (0%) of undernutrition, when energy-protein undernutrition was defined as a serum albumin < 38 g/L, in pCKD subjects, living in Brazil. It is possible that serum albumin is not a sensitive indicator of energy-protein status in chronic disease, and evidence for this comes from a starvation study. The “Minnesota Experiment” (1944) found that healthy subjects following a semi-starvation diet for twenty-four weeks lost, on average, 24% body weight, with no significant change in serum albumin concentration (Keys et al. 1950).

In our study, the prevalence of undernutrition was high (26.9%) when SGA was used to assess energy-protein status. This is not a new finding as similar results were found by Lawson et al. (2001) and Campbell et al. (2007), who reported a prevalence of 28% and 20%, respectively, in subjects with pCKD. The mechanism of energy-protein undernutrition, as assessed by SGA, in our subjects is not clear. It may be, in part, related to low energy intake as a majority of subjects reported an observed energy intake below that recommended. However, in subjects ≥ 60 years old there was no significant difference in observed energy intake between
those who were energy-protein undernourished (SGA) and those who were energy-protein well-nourished (SGA). A review of the literature suggests that altered metabolism of fat and/or protein may have contributed to the low energy-protein status (SGA) in our subjects. Lipolysis has been shown to increase in the presence of elevated leptin concentrations, and leptin concentration has been found to be elevated in CKD (Yamamoto et al. 2009). Protein catabolism, through the ubiquitin-proteasome system, has been shown to be accelerated in animal models of CKD (Du et al. 2005). Activators of the ubiquitin-proteasome system, in uremia, include acidosis, elevated cytokines and insulin resistance (Papadoyannakis et al. 1984; Greiber et al. 1992; Carrero et al. 2009; Siew et al. 2007). Protein anabolism may be decreased in CKD, as growth hormone resistance has been demonstrated in uremia (Maratos-Flier 2008). The mechanism of energy-protein undernutrition (SGA) in our subjects may in fact be related to altered metabolism of protein as the prevalence of undernutrition was also high (19.2%) when bone-free arm muscle area was used to assess energy-protein status, suggesting that muscle protein wasting was prevalent in our subjects.
6.3 Limitations

Nutrient intake from food may have been underestimated. Evidence for this comes from the finding of an inverse association between observed energy intake and energy-protein status, as assessed by BMI, among subjects ≥ 60 years old and among subjects < 60 years. As such, our subjects may have underreported dietary intake. It is likely that folate intake from food was be an estimated. In this study nutrient analysis was performed using the 2007b Canadian Nutrient File. Shakur et al. (2009) recently found that the actual folate content of some food is, on average, 50% higher than that reported in the Canadian Nutrient File 2007b. This is probably due to over fortification of food with folic acid by food manufacturers in an effort to ensure that the amount of folic acid required by the regulations and declared on the labels will be present throughout the shelf-life of the product (Shakur et al. 2009; Choumenkovitch et al. 2002). Error in the measurement of adequacy of micronutrient intake may have occurred as a result of a small sample size, use of an estimated average requirement for a healthy population and the use of a limited number of days of intake data for each individual. Error in the measurement of adequacy of energy and protein intake may have occurred as a result of being unable to use a statistical method that accounted for within-person standard deviation in intake and between-person standard deviation in requirement. Our sample size was small. For this reason, the non significant association found between pHcy and some of the potential predictor variables may be related to a type 2 error. The results of this study may not be generalizable to all people with pCKD as our subjects predominantly had stage 4 and 5 pCKD. This study had a high exclusion rate (76%). As such, the results may not be generalizable to patients who are unable to read and understand English, or who have a heart, liver or kidney transplant, as these were the main reasons why a patient was excluded from the study.
6.4 Conclusions

This study shows that, in the era of folic acid food fortification, subjects with pCKD have a high prevalence of hHcy. This study also indicates that folate status and energy-protein undernutrition (SGA) independently predicted ptHcy in subjects with pCKD. Observed total folate intake appears to be sufficient to prevent folate deficiency but not able to prevent a high prevalence of hHcy. Lastly, adequacy of folate, vitamin B\textsubscript{12}, and vitamin B\textsubscript{6} intake from food was uncertain in a majority of subjects as a result of having an inadequate number of days of intake data for each subject.
7 Clinical Implications and Future Directions

A rationale for conducting this study was to be able to consider vitamin status in conjunction with ptHcy in order to provide much needed evidence to guide clinical practice regarding the need for vitamin supplementation in pCKD. An overwhelming majority of our subjects had an elevated ptHcy concentration. Folate status, as assessed by RBC folate, inversely, and independently predicted ptHcy. While published research has shown that intervention with mega doses of folic acid (5mg/d) significantly decreases ptHcy in pCKD this may not translate into a health benefit as there is no conclusive evidence that decreasing ptHcy reduces the risk of CVD (Bernasconi et al. 2006; Lonn et al. 2006; Bonaa et al. 2006; Zoungas et al. 2006; Mann et al. 2008; House et al. 2010). In addition, there is some evidence that excessive folic acid intake, itself, may have adverse health effects (Lindzon et al. 2009; Cole et al. 2007; Sauer et al. 2009; Dong et al. 2002; Venn et al. 2003). In our study, no subject was folate deficient, as assessed by RBC folate. This information, taken together, suggests that folate supplementation in pCKD to reduce ptHcy may not be indicated. Serum vitamin B\textsubscript{12} and serum pyridoxal 5’-phosphate were not associated with ptHcy in our study. As such, vitamin B\textsubscript{12} or vitamin B\textsubscript{6} supplementation to lower ptHcy in our pCKD patients may not be indicated.

In our study, energy-protein undernutrition, as measured by SGA, was associated with an elevated ptHcy. More than a quarter of our subjects were undernourished (SGA). As such, it is important to conduct research to determine if treatment of energy-protein undernutrition (SGA) in pCKD significantly decreases ptHcy.

The adequacy of usual intake of folate, vitamin B\textsubscript{12} and vitamin B\textsubscript{6} from food was uncertain in a majority of our subjects. The number of days of intake data that would be required, per individual, to obtain an 85% probability of correctly concluding that an individual’s mean usual intake is inadequate or adequate, ranges from 6 to 22, varying with the specific vitamin, age and gender (Gibson 2005). In clinical practice the human resources required to collect and analyse this amount of data is not available. As such, to assess the status of these
micronutrients in pCKD patients it would be appropriate to measure biochemical markers instead of dietary intake.

Median RBC folate in our pCKD patients appeared to be higher than that found in the general population in the era of folic acid food fortification. As published research has provided evidence that excessive folic acid intake may have adverse health effects it is important to conduct research to determine if RBC folate or unmetabolized folic acid in the plasma in pCKD are significantly higher than that of people with healthy kidneys and to determine if an elevated folate status or unmetaboized folic acid in the plasma have adverse health effects in pCKD (Lindzon et al. 2009; Cole et al. 2007; Sauer et al. 2009; Dong et al. 2002; House et al. 2010). In the era of folic acid food fortification, nearly one tenth of our subjects were vitamin B\textsubscript{12} deficient, while no subject was folate deficient. This may be a health concern as unmetabolized folic acid in the presence of vitamin B\textsubscript{12} deficiency, may prevent megaloblastic anemia, masking the hematological sign of a vitamin B\textsubscript{12} deficiency and allowing for the progression of neurologic damage (Bender 2003; Dror et al. 2008). More than one quarter of our subjects was vitamin B\textsubscript{6} deficient. This is a health concern because pathophysiological implications of a vitamin B\textsubscript{6} deficiency include anemia, impaired immune function, cancer, cognitive dysfunction, and CVD (Spinneker et al. 2007). The development of guidelines to routinely measure serum vitamin B\textsubscript{12} and serum pyridoxal 5′-phosphate are indicated.

In summary, folate, vitamin B\textsubscript{12} or vitamin B\textsubscript{6} supplementation to lower ptHcy in pCKD may not be indicated. To assess the status of these micronutrients in pCKD it would be appropriate to measure biochemical markers instead of dietary intake. In clinical practice, guidelines to routinely measure serum vitamin B\textsubscript{12} and serum pyridoxal 5′-phosphate are indicated. Future research should determine if treatment of energy-protein undernutrition (SGA) in pCKD significantly decreases ptHcy, if RBC folate in pCKD patients is significantly higher than that of people with healthy kidneys and if an elevated folate status has adverse health effects in pCKD.
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## Appendices

Appendix A: Medications that Influence Plasma Total Homocysteine Concentration

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<th>Medications That Affect Homocysteine Metabolism</th>
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Appendix B: Consent Form for Participation in a Research Study (4 pages)

(Informed) Consent Form For Participation In A Research Study

Title of Study

Prevalence of Hyperhomocysteinemia In Patients With Predialysis Chronic Kidney Disease After Folic Acid Food Fortification Of The Canadian Food Supply

The Study Investigators

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Co-Investigators
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Dr. Deborah O’Connor PhD, Assistant Professor, Department of Nutritional Sciences, University of Toronto. Director, Clinical Dietetics, The Hospital for Sick Children, 416-813-7844

You are being asked to take part in a research study. Before agreeing to participate in this study, it is important that you read and understand the following explanation of the proposed study procedures. The following information describes the purpose, procedures, benefits, discomforts, risks and precautions associated with this study. It also describes your right to refuse to participate or withdraw from the study at any time. In order to decide whether you wish to participate in this research study, you should understand enough about its’ risks and benefits to be able to make an informed decision. This is known as the informed consent process. Please ask the study staff to explain any words you don’t understand before signing this consent form. Make sure all your questions have been answered to your satisfaction before signing this document.

17-Feb-07
Background Information for this Study
Blood levels of the compound homocysteine increase as kidney function decreases in people with chronic kidney disease (CKD). This is a concern because research studies have found that a high blood level of homocysteine is a risk factor for cardiovascular disease. The reason why blood homocysteine levels increase in people with CKD is not clear. Research studies have found that some nutrients in the diet can influence blood levels of homocysteine and these nutrients are folate, vitamins B₁₂ and B₆, and protein. Specifically, low blood levels of folate, vitamins B₁₂ or B₆, and low dietary protein intake are all related to higher blood levels of homocysteine.

Canada started adding folic acid to our food supply in 1998. As mentioned above, folate can influence blood levels of homocysteine. In the general population, the proportion of people who have high blood levels of homocysteine decreased after the addition of folic acid. In people with CKD, there is no research describing the proportion of people who have high blood levels of homocysteine after the addition of folic acid.

As well, in people with CKD, there is no research describing the proportion of people who have low dietary intakes of the nutrients that influence blood levels of homocysteine.

Purpose of this Study
You have been asked to participate in this study because you are being followed in the Renal Management Clinic for the treatment of kidney disease. This study is designed to estimate the proportion of people with kidney disease who have high blood levels of homocysteine after the addition of folic acid to our food supply. In addition, this study is designed to assess the number of people who have inadequate intakes of the nutrients that influence blood levels of homocysteine.

Procedure for this Study
Approximately one to two hours of your time, within a one week period, will be required. One week before your usual clinic visit, you will come to the Toronto General Hospital. You will be reimbursed up to $19.00 for parking. You will meet with Jane Paterson who is a dietitian and the principal investigator (PI) of this study, for one to two hours, and who will:

1. Ask questions about what food you eat, what supplements and medications you use as well as other diet and health related questions.
2. Measure your body muscle and fat stores. For this you will wear a hospital gown.
3. Will escort you to the diagnostic test centre in the hospital where fasting blood samples will be obtained by a laboratory technician. For this study, a 35 ml blood sample will be drawn. You will need to fast overnight and so you will not be able to eat or drink anything (except water) after midnight. You will be reimbursed up to $6.00 for your breakfast. If you have diabetes and are taking medication to control their blood glucose, the sequence of the procedure during this study visit will be modified to permit fasting blood samples to be drawn first, diabetes medication taken and breakfast eaten prior to your interview with the dietitian.
4. Your blood sample for this study will be analyzed through the laboratory at the University Health Network. A portion of this blood sample will be transferred to a laboratory at the Hospital for Sick Children in Toronto, Ontario for analysis of serum and red blood cell folate. Leftover blood from your blood sample for this study will be: 1. transferred to a laboratory at St. Michael’s Hospital in Toronto, Ontario for storage and then analyzed for amino acids, sulfate and riboflavin for a future related study or 2. destroyed. You can choose if you would like your leftover blood to be stored or destroyed by indicating your choice on page 4 of this consent form.

Approximately one week later, at your usual clinic visit, the dietitian will meet with you for your usual nutrition care.

Potential Risks of this Study
Possible slight bruising from the needle site at the time the blood is collected.

Potential Benefits of this Study
You will not benefit directly from participating in this study. However, your participation in this study will help health professionals to know if high blood levels of homocysteine are still a concern in people with CKD after the addition of folic acid to our food supply. As well, your participation in this study will help health professionals to know what proportion of people have inadequate intakes of the vitamins that influence blood levels of homocysteine and this information may be used towards developing guidelines for vitamin supplementation in people with CKD.

Confidentiality
All information obtained during the study will be held in strict confidence. You will be identified with a study number only. No names or identifying information will be used in any publication or presentations. No information identifying you will be transferred outside the investigators in this study or this hospital.

Compensation
If you become ill or are physically injured as a result of participation in this study, medical treatment will be provided. The reasonable costs of such treatment will be covered by your health insurance for any injury or illness that is directly a result of participation in this trial. In no way does signing this consent form waive your legal rights nor does it relieve the investigators, sponsors or involved institutions from their legal and professional responsibilities.

Participation in this Study
Your participation in this study is voluntary. You can choose not to participate or you may withdraw at any time without affecting your medical or nutrition care.
Questions About this Study
1. If you have any general questions about the study please call the Principal Investigator, Jane Paterson, at 416-340-4800 ext. 8591.
2. If you have any questions about your rights as a research participant, please call Dr. R. Heslegrave, Chair of the University Health Network Research Ethics Board at 416-340-4557. This person is not involved with the research project in any way and calling him will not affect your participation in the study.

Consent

☐ I would like leftover blood from my blood sample for this study stored for a future related study.
☐ I would like leftover blood from my blood sample for this study destroyed.

I have had the opportunity to discuss this study and my questions have been answered to my satisfaction. I consent to take part in the study with the understanding I may withdraw at any time without affecting my medical or nutritional care. I have received a signed copy of this consent form. I voluntarily consent to participate in this study.

<table>
<thead>
<tr>
<th>Study Participant's Name (Please Print)</th>
<th>Study Participant's Signature</th>
<th>Date</th>
</tr>
</thead>
</table>

I confirm that I have explained the nature and purpose of the study to the subject named above. I have answered all questions.

<table>
<thead>
<tr>
<th>Name of Person Obtaining Consent</th>
<th>Signature of Person Obtaining Consent</th>
<th>Date</th>
</tr>
</thead>
</table>

17-Feb-07
# Appendix C: Screening (Inclusion/Exclusion Criteria) and Demographics Data Collection Form

**DATA COLLECTION FORM**

**Inclusion/Exclusion Criteria and Demographics**

<table>
<thead>
<tr>
<th>Study ID #</th>
<th>Date</th>
</tr>
</thead>
</table>

**Step #1:** A Study Investigator (SI) reviews patient’s chart for exclusion criteria.

<table>
<thead>
<tr>
<th>Exclusion</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prescribed a vitamin supplement called “Replavite”</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unable to understand or read English.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unable to provide informed consent.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt; 18 years old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Estimated glomerular filtration rate ≥ 60 mL/min/1.73m²</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receiving an antibiotic.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Active cancer, liver dysfunction, heart, liver or kidney transplant, HIV, a metabolic disorder that interferes with homocysteine metabolism</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Documented B12 deficiency or documentation of receiving B12 injections</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A serum potassium concentration upon referral to clinic of &lt; 3.0 or ≥ 6.0 mmol/L.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please exclude patient if any box was checked **YES.** **Excluded:** Yes No

**Step #2:** If patient is not excluded in Step #1 and the patient agrees to have an SI phone them about the study, the SI phones the patient to ensure they do not meet exclusion criteria.

<table>
<thead>
<tr>
<th>Exclusion</th>
<th>YES</th>
<th>NO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prescribed a vitamin supplement called “Replavite”.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unable to understand or read English.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unable to provide informed consent.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receiving vitamin B12 injections.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Receiving an antibiotic.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Involved with another study that interferes with homocysteine metabolism.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Please exclude patient if any box was checked **YES.** **Excluded:** Yes No

**Step #3:** If patient is not excluded SI collects demographic information from the medical chart.

**Demographics**

**Medical Chart Review**

**Past Medical History:**

1. chronic kidney disease secondary to ____________________________
2. cardiovascular disease                                     yes no
3. diabetes                                                  yes no
4. hypertension                                              yes no
5. dyslipidemia                                              yes no
6. smoker                                                   yes ex-smoker never (obtain at 1st study visit)
7. physical inactivity                                     yes no (obtain at 1st study visit)

**Age (years):** ________  **Gender:**  M  F

**Number Of Months Attending The Predialysis Clinic:** ________ (January 21, 2007)
Appendix D: Subjective Global Assessment Rating Form

<table>
<thead>
<tr>
<th>SUBJECTIVE GLOBAL ASSESSMENT RATING FORM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study ID #:</td>
</tr>
</tbody>
</table>

### HISTORY

<table>
<thead>
<tr>
<th>WEIGHT/WEIGHT CHANGE: (Included in K/D/O/OI SGA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1.</strong> Baseline Wt:</td>
</tr>
<tr>
<td>Current Wt:</td>
</tr>
<tr>
<td>Actual Wt loss/past 6 mo:</td>
</tr>
</tbody>
</table>

| 2. Weight change over past two weeks: | No change | Increase | Decrease |

### DIETARY INTAKE

| Rate 1-7 |
| No Change | Adequate | No Change | Inadequate |
| Change: | Sub optimal Intake: | Protein | Kcal | Duration |
| Full Liquid: | | Hypocaloric Liquid | Starvation |

### GASTROINTESTINAL SYMPTOMS (Included in K/D/O/OI SGA-anorexia or causes of anorexia)

<table>
<thead>
<tr>
<th>Symptom:</th>
<th>Frequency:*</th>
<th>Duration:*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anorexia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vomiting</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Never, daily, 2-3 times/wk, 1-2 times/wk > 2 weeks, < 2 weeks

### FUNCTIONAL CAPACITY

<table>
<thead>
<tr>
<th>Description</th>
<th>Duration:</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Dysfunction</td>
<td></td>
</tr>
<tr>
<td>Change in function</td>
<td></td>
</tr>
<tr>
<td>Difficulty with ambulation</td>
<td></td>
</tr>
<tr>
<td>Difficulty with activity (Patient specific “normal”)</td>
<td></td>
</tr>
<tr>
<td>Light activity</td>
<td></td>
</tr>
<tr>
<td>Bed/chair ridden with little or no activity</td>
<td></td>
</tr>
<tr>
<td>Improvement in function</td>
<td></td>
</tr>
</tbody>
</table>

### DISEASE STATE/COMORBIDITIES AS RELATED TO NUTRITIONAL NEEDS

<table>
<thead>
<tr>
<th>Primary Diagnosis</th>
<th>Comorbidities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal requirements</td>
<td>Increased requirements</td>
</tr>
</tbody>
</table>

Acute Metabolic Stress: | None | Low | Moderate | High |

### PHYSICAL EXAM

<table>
<thead>
<tr>
<th>Loss of subcutaneous fat (Below eye, triceps, biceps, chest) (Included in K/D/O/OI SGA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Some areas</td>
</tr>
<tr>
<td>Muscle wasting (Temple, clavicle, scapula, ribs, quadriceps, calf, knee, interosseous) (Included in K/D/O/OI SGA)</td>
</tr>
<tr>
<td>Some areas</td>
</tr>
<tr>
<td>Edema (Related to undernutrition-use to evaluate weight change)</td>
</tr>
</tbody>
</table>

### OVERALL SGA RATING

Very mild risk to well-nourished=6 or 7 most categories or significant, continued improvement.
Mild-moderate = 3, 4, or 5 ratings. No clear sign of normal status or severe malnutrition.
Severely Malnourished = 1 or 2 ratings in most categories/significant physical signs of malnutrition.

CANUSA study: A one unit increase on the seven point SGA scale was associated with a 25% decline in relative risk of death.
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Appendix E: Anthropometrics Data Collection Form

DATA COLLECTION FORM

Anthropometrics And Estimated Glomerular Filtration Rate

Study ID #: _____________
Date: ________________

Height: ________ cm. ________ m²

Weight: ________ kg.
Is participant edema free YES NO. If no, using clinical judgement what is
edema free weight ________ kg.
edema free BMI ________ m²

standard median weight ________ kg
% of standard median weight ________ kg
Adjusted edema free weight ________ kg

Weight x 6 months ago: ________ kg
% weight change x 6 months: ________ %

Weight x 2 weeks ago: ________ kg

elbow breadth ________ cm

Frame Size: Small Medium Large

Mid Upper Arm Circumference: ________ cm

Tricep Skinfold Thickness: ________ mm ________ cm; < 15th percentile: YES NO

≤ 74 years old: Arm Muscle Area: ________ cm²; < 15th percentile: YES NO
> 74 years old: Arm Muscle Circumference: ________ cm; < 15th percentile: YES NO

Gender: Female or Male
Racial Background: Black: Yes No
(October 30, 2006)
DATA COLLECTION FORM

24 Hr Food Recall

<table>
<thead>
<tr>
<th>Time</th>
<th>O</th>
<th>L</th>
<th>Food Description</th>
<th>Model # + Fraction of unit</th>
<th>Amount OR Actual Amount (eg. 125 ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>Time</td>
<td>O</td>
<td>L</td>
<td>Food Description</td>
<td>Amount</td>
<td></td>
</tr>
<tr>
<td>------</td>
<td>---</td>
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<td>------------------</td>
<td>--------</td>
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</tr>
</tbody>
</table>

**Occasion (O)**
- B = Breakfast
- L = Lunch
- BL = Brunch
- D = Dinner
- S = Snack

**Location of Where Food Was Eaten (L)**
- H = Home
- R = Restaurant/Fast Food
- W = Work
- O = Other

(October 29, 2006)
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Supplement Name</td>
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</table>

1. Yesterday, did you take any vitamin or mineral supplements? Yes No. If yes, what is the name and DIN of the supplement you took? How much did you take? During the last 4 months, did you take it every day? If no, how often?
2. In the last 4 months, did you take any other vitamin or mineral supplements? Yes No. If yes, what is the name and DIN of the supplement you took? How often did you take it? How many pills were taken on each occasion? Is this consistent?

* amount and # means: Amount of micronutrient per tab, pill, cap, tsp and # of tabs, pills, caps, tps taken.
**Last 4 Months means: During the last 4 months.
***Consistent means: Consistent Intake in the last 4 months. 

(October 29, 2006)
Appendix H: Cigarette, Coffee and Alcohol Use Frequency Questionnaire

DATA COLLECTION FORM

Use Of Coffee, Alcohol And Cigarettes

Study ID #: ____________________
Date: ________________________

<table>
<thead>
<tr>
<th></th>
<th>Per Day</th>
<th>Per Week</th>
<th>Per month</th>
<th>Total Coffee Intake Per Day (ml/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coffee Intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Per Day</th>
<th>Per Week</th>
<th>Per month</th>
<th>Total Alcohol Intake Per Day (ml/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alcohol Intake</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beer</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wine</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liquor</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Per Day</th>
<th>Per Week</th>
<th>Per month</th>
<th>Total Cigarettes Per Day</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cigarette Use</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Did you smoke?</td>
<td></td>
<td></td>
<td>YES</td>
<td>Ex-Smoker NO</td>
</tr>
</tbody>
</table>

Previous Nutrition Counseling

Have you have previous nutrition counseling? YES NO

If yes, was the counseling for the health of your kidney? YES NO

(January 21, 2007)
Appendix I: Gastrointestinal Symptoms of Uremia Data Collection Form

DATA COLLECTION FORM

Gastrointestinal Symptoms of Uremia Data Collection Form

Study ID #: ____________
Date: _______________

To the Patient: “I would like to ask you some questions about your appetite.”

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Question to Patient</th>
<th>No symptom</th>
<th>Fair</th>
<th>Poor</th>
<th>Symptom present</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Anorexia</td>
<td>How is your appetite?</td>
<td>Good</td>
<td>Fair</td>
<td>Poor</td>
<td></td>
</tr>
<tr>
<td>2. Nausea</td>
<td>Do you ever have nausea?</td>
<td>No</td>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>3. Vomiting</td>
<td>Do you ever vomit?</td>
<td>No</td>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>4. Early Satiety</td>
<td>When you sit down to have your meal, are you able to eat all the food on your plate?</td>
<td>Yes</td>
<td></td>
<td></td>
<td>No</td>
</tr>
<tr>
<td>5. Taste change</td>
<td>Have you noticed any change in the way food tastes or smells? (If yes, please explain)</td>
<td>No</td>
<td></td>
<td></td>
<td>Yes</td>
</tr>
<tr>
<td>6. Red Meat Aversion</td>
<td>How do you find the taste of red meat?</td>
<td>Good</td>
<td></td>
<td></td>
<td>Poor</td>
</tr>
<tr>
<td>7. Chicken Aversion</td>
<td>How do you find the taste of chicken?</td>
<td>Good</td>
<td></td>
<td></td>
<td>Poor</td>
</tr>
<tr>
<td>8. Fish Aversion</td>
<td>How do you find the taste of fish?</td>
<td>Good</td>
<td></td>
<td></td>
<td>Poor</td>
</tr>
</tbody>
</table>

(October 29, 2006)

NB.
If the patient responds “Poor” to symptom #6, 7 or 8, then
To the Patient: “Have you ever liked the taste of . . . .”
If the response is “NO”, then score the question as no symptom present.
Appendix J: Medication Use Data Collection Form

DATA COLLECTION FORM

Medications
(Prescribed By Physician)

Study ID #: _____________
Date: ________________

<table>
<thead>
<tr>
<th>medication</th>
<th>Dose</th>
<th>Start Date</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

(October 29, 2006)
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