The association urinary of vitamin D binding protein with kidney dysfunction and hypovitaminosis D in patients at risk for type 2 diabetes

By
Zhengnan (Windy) Wang

A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Nutritional Sciences
University of Toronto

© Copyright by Zhengnan (Windy) Wang 2018
The association of urinary vitamin D binding protein with kidney dysfunction and hypovitaminosis D in patients at risk for type 2 diabetes

Zhengnan (Windy) Wang
Masters of Science
Department of Nutritional Sciences
University of Toronto
2018

Abstract

One possible explanation for hypovitaminosis D in type 2 diabetes (T2DM) is that vitamin D metabolites may still be bound to the vitamin D binding protein (VDBP) as the protein is lost in urine. Given that VDBP may be linked to both kidney dysfunction and hypovitaminosis D, the objective is to investigate longitudinal associations of urinary VDBP on measures of kidney health and vitamin D status. Data from a cohort of adults at risk for T2DM was used (n=729). Albumin-to-creatinine ratio (ACR) and estimated glomerular filtration rate (eGFR) were measures of kidney function while 25-hydroxyvitamin D (25(OH)D) indicated vitamin D status. Adjusted generalized estimating equation models showed that uVDBP was associated with higher ACR over time, but was not associated with changes in eGFR or 25(OH)D. These results emphasize that although mild VDBP loss is not linked with hypovitaminosis D, urinary VDBP may be a useful biomarker for tubular damage.
Acknowledgments

I would like to extend my biggest thanks to my supervisor and mentor, Dr. Anthony Hanley, who has been a source of encouragement, wisdom, and wonderful advice over the last 4 years. He has inspired my love for research and taught me so much about science, academia, and being a better person. I could not have asked for a better mentor and supervisor, and I am positive that his guidance over the past years will help me through many future endeavors. Special thanks also go out to Dr. David Cole for sharing his ideas regarding the vitamin D binding protein and inspiring this thesis topic. He provided invaluable insight and feedback throughout my Master’s. I would like to thank my committee member, Dr. Ahmed El-Sohemy, for all his time, expertise, and providing a fresh and critical set of eyes on my research. Both have been instrumental in helping me grasp the underlying biological mechanisms of my analyses, and their feedback has been essential to the successful completion of this thesis.

The research nurses also deserve special recognition for all their work on the PROMISE cohort clinic visits, including Jan Neuman, Paula Van Nostrand, Nicole Rubio, Stella Kink, and Annette Barnie of the Leadership Sinai Centre for Diabetes at Mount Sinai Hospital, and Sheila Porter, Mauricio Marin, and Marnie Orcutt of the Centre for Studies in Family Medicine at the University of Western Ontario. Thank you to all the PROMISE participants for contributing to this study.

I would like to acknowledge the University of Toronto, the Government of Canada, and the Banting and Best Diabetes Centre for their generous scholarships and travel bursaries which has helped me throughout my research and allowed me the opportunity to present my findings at conferences.

Last but not least, I extend my warmest thanks to my fellow lab members, friends, and family for all their help and patience throughout these years. In particular, I want to thank Luke for taking the time to introduce me to and teach me about the wonderful world of data science and helping me understand endless error messages. To my mom and dad for their support and encouragement, and Nick for his wonderful humour and love which helped me through countless stressful nights. I am truly grateful to everyone I’ve met and everything I’ve experienced at the University of Toronto and am looking forward to taking all that I learned here into the next chapter of my life.
# Table of Contents

ACKNOWLEDGMENTS .................................................................................................................. III  
LIST OF ABBREVIATIONS ........................................................................................................ VI  
LIST OF TABLES ........................................................................................................................ IX  
LIST OF FIGURES ........................................................................................................................ X  
STATEMENT OF CONTRIBUTIONS ............................................................................................. XI  
CHAPTER 1 INTRODUCTION ....................................................................................................... 1  
CHAPTER 2 REVIEW OF THE LITERATURE ............................................................................... 3  
  2.1 PREVALENCE AND ECONOMIC BURDEN OF T2DM ......................................................... 3  
  2.2 T2DM PATHOPHYSIOLOGY ................................................................................................. 4  
  2.3 T2DM RISK FACTORS ...................................................................................................... 5  
      2.3.1 Non-modifiable risk factors ....................................................................................... 5  
      2.3.2 Modifiable risk factors ............................................................................................. 6  
  2.4 KIDNEY DYSFUNCTION ..................................................................................................... 8  
      2.4.1 Mechanisms of kidney dysfunction ... ......................................................................... 9  
      2.4.2 Albumin-to-creatinine ratio .................................................................................... 11  
      2.4.3 Glomerular filtration rate ......................................................................................... 11  
      2.4.4 Glomerular hyperfiltration ...................................................................................... 14  
      2.4.5 Risk factors for kidney disease ................................................................................ 18  
  2.5 VITAMIN D ......................................................................................................................... 22  
      2.5.1 Sources of vitamin D .............................................................................................. 24  
      2.5.2 Factors influencing vitamin D levels ....................................................................... 25  
      2.5.3 Vitamin D status and deficiency ............................................................................. 26  
      2.5.4 Vitamin D and type 2 diabetes ................................................................................ 29  
      2.5.5 Vitamin D and kidney disease ................................................................................ 30  
  2.6 VITAMIN D BINDING PROTEIN ......................................................................................... 34  
      2.6.1 Physiology ................................................................................................................ 34  
      2.6.2 Reabsorption of VDBP ............................................................................................. 36  
      2.6.3 Impact of VDBP loss on vitamin D status ................................................................. 38  
  2.7 KNOWLEDGE GAPS AND RATIONALE ............................................................................ 40  
CHAPTER 3 OBJECTIVES AND HYPOTHESES ....................................................................... 42  
  3.1 OBJECTIVES ....................................................................................................................... 42  
  3.2 HYPOTHESES .................................................................................................................... 42  
CHAPTER 4 THE UTILITY OF LONGITUDINAL URINARY VITAMIN D BINDING PROTEIN LOSS AS A MARKER FOR KIDNEY TUBULAR DYSFUNCTION ................................................. 44  
  4.1 ABSTRACT .......................................................................................................................... 44  
  4.2 INTRODUCTION ................................................................................................................. 45  
  4.3 METHODS .......................................................................................................................... 46  
      4.3.1 Study Population ...................................................................................................... 46  
      4.3.2 Anthropometric measures ....................................................................................... 47  
      4.3.3 Urinary measures ...................................................................................................... 48  
      4.3.4 Metabolic and blood measures ............................................................................... 49  
      4.3.5 Statistical analysis .................................................................................................... 50  
  4.4 RESULTS ............................................................................................................................ 52
## List of Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$1,25(\text{OH})_2\text{D}$</td>
<td>1,25-dihydroxyvitamin D$_3$</td>
</tr>
<tr>
<td>$25(\text{OH})\text{D}$</td>
<td>25-hydroxyvitamin D$_3$</td>
</tr>
<tr>
<td>ACCORD</td>
<td>Action to Control Cardiovascular Risk in Diabetes Trial</td>
</tr>
<tr>
<td>ACE</td>
<td>Angiotensin-converting enzyme</td>
</tr>
<tr>
<td>ACR</td>
<td>Albumin-to-creatinine ratio</td>
</tr>
<tr>
<td>AFM</td>
<td>Afamin</td>
</tr>
<tr>
<td>AFP</td>
<td>$\alpha$-fetoprotein</td>
</tr>
<tr>
<td>AGEs</td>
<td>Advanced glycosylated end-products</td>
</tr>
<tr>
<td>Ang II</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARB</td>
<td>Angiotensin-receptor blockers</td>
</tr>
<tr>
<td>ARIC</td>
<td>Atherosclerosis Risk in Communities Study</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>CKD</td>
<td>Chronic kidney disease</td>
</tr>
<tr>
<td>CKD-EPI</td>
<td>CKD-Epidemiology Collaboration group</td>
</tr>
<tr>
<td>CLIA</td>
<td>Chemiluminescent immunoassay</td>
</tr>
<tr>
<td>CREDIT</td>
<td>Chronic Renal Disease in Turkey study</td>
</tr>
<tr>
<td>CV</td>
<td>Coefficients of variation</td>
</tr>
<tr>
<td>CV$_i$</td>
<td>Intra-individual coefficient of variation</td>
</tr>
<tr>
<td>D2d</td>
<td>Vitamin D and Type 2 Diabetes Study</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DBP</td>
<td>Diastolic blood pressure</td>
</tr>
<tr>
<td>DCCT</td>
<td>Diabetes Control and Complications Trial</td>
</tr>
<tr>
<td>DEQAS</td>
<td>International External Quality Assessment Scheme for Vitamin D Metabolites</td>
</tr>
<tr>
<td>DM</td>
<td>Diabetes</td>
</tr>
<tr>
<td>DN</td>
<td>Diabetic nephropathy</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------</td>
<td>-------------------------------------------------</td>
</tr>
<tr>
<td>eGFR</td>
<td>Estimated glomerular filtration rate</td>
</tr>
<tr>
<td>ESRD</td>
<td>End-stage renal disease</td>
</tr>
<tr>
<td>FIND</td>
<td>Finnish Vitamin D Trial</td>
</tr>
<tr>
<td>GC</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>GEE</td>
<td>Generalized estimating equation</td>
</tr>
<tr>
<td>HbA1C</td>
<td>Glycated hemoglobin</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired fasting glucose</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired glucose tolerance</td>
</tr>
<tr>
<td>IL-18</td>
<td>Interleukin-18</td>
</tr>
<tr>
<td>ILGF-1</td>
<td>Insulin-like growth factor 1</td>
</tr>
<tr>
<td>IOM</td>
<td>Institute of Medicine</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>K/DOQI</td>
<td>National Kidney Foundation Kidney Disease Outcomes Quality Initiative</td>
</tr>
<tr>
<td>KIM-1</td>
<td>Kidney injury molecule 1</td>
</tr>
<tr>
<td>LC-MS</td>
<td>Liquid chromatography tandem mass spectroscopy</td>
</tr>
<tr>
<td>LOESS</td>
<td>Local polynomial regression</td>
</tr>
<tr>
<td>MAP</td>
<td>Mean arterial pressure</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MAQ</td>
<td>Modifiable Activity Questionnaire</td>
</tr>
<tr>
<td>MDRD</td>
<td>Modification of Diet in Renal Disease</td>
</tr>
<tr>
<td>MET</td>
<td>Metabolic equivalent of task</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>NEFA</td>
<td>Non-esterified fatty acid</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase-associated lipocalin</td>
</tr>
<tr>
<td>OGGT</td>
<td>Oral glucose tolerance tests</td>
</tr>
<tr>
<td>PD</td>
<td>Peritoneal dialysis</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>PTH</td>
<td>Parathyroid hormone</td>
</tr>
<tr>
<td>RAAS</td>
<td>Renin-angiotensin-aldosterone system</td>
</tr>
<tr>
<td>RAP</td>
<td>Endoplasmic reticulum-resident chaperone receptor-associated protein</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>rs4588</td>
<td>Lys/Lys at codon 420</td>
</tr>
<tr>
<td>rs7041</td>
<td>Glu/Glu codon at 416</td>
</tr>
<tr>
<td>SBP</td>
<td>Systolic blood pressure</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SNP</td>
<td>Single nucleotide polymorphism</td>
</tr>
<tr>
<td>SPF</td>
<td>Sun production factor</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element-binding protein</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 diabetes</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>UKPDS</td>
<td>UK Prospective Diabetes Study</td>
</tr>
<tr>
<td>uVDBP</td>
<td>Urinary vitamin D binding protein</td>
</tr>
<tr>
<td>uVDBP:cr</td>
<td>Urinary vitamin D binding protein adjusted for urinary creatinine</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VDBP</td>
<td>Vitamin D binding protein</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>VIDAL</td>
<td>Vitamin D and Longevity Trial</td>
</tr>
<tr>
<td>VITAL</td>
<td>Vitamin D and omega-3 trial</td>
</tr>
<tr>
<td>WC</td>
<td>Waist circumference</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WHR</td>
<td>Waist-to-hip ratio</td>
</tr>
</tbody>
</table>
List of Tables

**TABLE 4.1** Subject characteristics at baseline across uVDBP:cr tertiles................................. 53

**TABLE 5.1** Subject characteristics across vitamin D status at baseline........................................ 78

**TABLE 5.2** Subject characteristics across visit numbers for subjects with serum 25(OH)D measures........ 82

**TABLE 7.1** GEE results of uVDBP:cr over time and kidney outcomes (ACR and eGFR) over 6 years........... 138

**TABLE 7.2** GEE results of baseline uVDBP:cr and kidney outcomes (ACR and eGFR) over 6 years............... 139

**TABLE 7.3** GEE results of uVDBP over time and kidney outcomes (ACR and eGFR) over 6 years............... 141

**TABLE 7.4** GEE results of baseline uVDBP and kidney outcomes (ACR and eGFR) over 6 years............... 143

**TABLE 7.5** Subject characteristics at baseline and 3-year follow-up.............................................. 144

**TABLE 7.6** GEE results of uVDBP:cr over time and serum 25(OH)D over 6 years................................. 146

**TABLE 7.7** GEE results of baseline uVDBP:cr and serum 25(OH)D over 6 years................................. 147
List of Figures

FIGURE 2.1 Factors associated with glomerular hyperfiltration. Abbreviation used: RAAS (renin-angiotensin-aldosterone system). Adapted from Glomerular hyperfiltration: definitions, mechanisms and clinical implications (Helal et al. 2012)........................................................................................................16

FIGURE 2.2 Vitamin D metabolism........................................................................................................23

FIGURE 4.1 CONSORT diagram of sample size at each examination visit..................................................47

FIGURE 4.2 (A) Log baseline uVDBP:cr concentrations at different clinical stages of albuminuria. (B) Association between log uVDBP:cr concentrations and ACR at baseline.................................................................55

FIGURE 4.3 (A) Log baseline uVDBP:cr concentrations at different stages of kidney disease. (B) Association between log uVDBP:cr concentrations and eGFR at baseline.................................................................57

FIGURE 4.4 GEE models of uVDBP:cr over time and kidney outcomes (ACR and eGFR) over 6 years..........59

FIGURE 4.5 GEE models of baseline uVDBP:cr and kidney outcomes (ACR and eGFR) over 6 years.................60

FIGURE 5.1 CONSORT diagram of sample size at each examination visit..................................................70

FIGURE 5.2 Log baseline uVDBP:cr concentrations at different stages of vitamin D deficiency....................80

FIGURE 5.3 (A) Association between log uVDBP:cr concentrations and serum 25(OH)D at baseline. (B) LOESS shows a decrease in serum 25(OH)D at log uVDBP:cr values higher than 2.5 μg/mmol.........................................................81

FIGURE 5.4 GEE models of uVDBP:cr over time and serum 25(OH)D over 6 years.....................................84

FIGURE 5.5 GEE models of baseline uVDBP:cr and kidney outcomes (ACR and eGFR) over 6 years.................86

FIGURE 7.1 Log uVDBP:cr values for subjects with and without albuminuria at baseline...............................137

FIGURE 7.2 GEE models where the predictor (uVDBP) is not adjusted for urinary creatinine. Outcomes of the model were ACR and eGFR........................................................................................................140

FIGURE 7.3 GEE models where the predictor (baseline uVDBP) is not adjusted for urinary creatinine. Outcomes of the model were ACR and eGFR........................................................................................................142
Statement of Contributions

During my Master’s degree, I was responsible for constructing and refining the research questions and objectives, identifying the knowing gaps, conducting the literature review, cleaning, coding and trouble-shooting the uVDBP data from the lab, performing the statistical analysis, and interpreting the study results. My abstract was accepted into the journal *Diabetes*, and I also presented a poster of my findings at the American Diabetes Association conference. I have also helped with cleaning and managing the data set used in this project (PROMISE). My contributions include:

- Inputting dietary information from food frequency questionnaires (DHQ)
- Adding new variables (e.g. urinary vitamin D binding protein, albumin-to-creatinine ratio, estimated glomerular filtration rate) to the master data set

The code for my statistical analysis can be found at [https://github.com/windyzn/urinaryDBP](https://github.com/windyzn/urinaryDBP) as reference for future students.
CHAPTER 1
Introduction

In 2013, there were 382 million people diagnosed with diabetes across 130 countries, with prevalence expected to increase to 592 million by 2035 (1). In Canada, the prevalence of diabetes was estimated at 3.5 million and this number is expected to almost double over the next decade (2). The World Health Organization (WHO) reported that diabetes was directly responsible for 1.5 million deaths in 2014, with another 2.2 million deaths attributable to higher fasting blood glucose (3). Type 2 diabetes (T2DM) affects approximately 90% of the global diabetes burden (2). Among the numerous complications associated with T2DM, approximately 50% of patients develop kidney damage in their lifetime, and 10-40% will eventually suffer from kidney failure (4). Diabetic nephropathy (DN) is the chronic loss of kidney function occurring in those with diabetes and is one of the most significant causes of morbidity and mortality for those with the disease. Protein loss in the urine due to damage to the glomeruli may result in the nephrotic syndrome, and the estimated glomerular filtration rate (eGFR) may progressively fall from its normal value of over 90 ml/min/1.73m$^2$. When eGFR reaches less than 15 ml/min/1.73m$^2$, the patient is said to have end-stage renal disease (ESRD), which can only be treated by kidney transplant or dialysis (5,6).

Recently, emerging evidence has suggested a role for suboptimal vitamin D status in the etiology of T2DM (7–11). Prospective studies have reported consistent associations of low 25-hydroxyvitamin D$_3$ (25(OH)D) with incident diabetes (12–14), but results of randomized control trials involving vitamin D supplementation have been inconsistent to date (10,15,16). There are various mechanisms which link vitamin D to diabetes pathology. Rat models have demonstrated that pancreatic insulin secretion is inhibited by vitamin D deficiency (17). Injection of 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D) significantly increases β-cell cytosolic Ca$^{2+}$ levels, which promotes the exocytosis of insulin from islet cells (18). Vitamin D can also bind to intracellular vitamin D receptors to regulate the body’s response to glucose by altering transcription of insulin receptor genes (19). As well, vitamin D has been found to improve insulin sensitivity in peripheral tissues (20,21).

Additional aspects of vitamin D metabolism have been previously investigated. 7-dehydrocholesterol from sunlight and cholecalciferol from the diet are the precursors to
active vitamin D (22). One hydroxyl group is added by 25-hydroxylase in the liver to form 25(OH)D, the storage form of vitamin D. Another hydroxyl group is added in the kidneys by 1α-hydroxylase to produce active 1,25(OH)₂D (22,23). Since both 25(OH)D and 1,25(OH)₂D are fat-soluble vitamins, they are bound to carriers in the circulation (23). In addition to allowing vitamin D metabolites to be transported in blood, these carrier proteins help regulate levels of free 25(OH)D and 1,25(OH)₂D in the body (24,25). The vitamin D binding protein (VDBP) has high affinity and high capacity to bind its metabolites, and it carries 85-90% of 25(OH)D and 1,25(OH)₂D in circulation (25–27).

In recent years, vitamin D deficiency has been recognized as a prominent feature of chronic kidney disease (CKD), and there is evidence to suggest that vitamin D deficiency, in turn, accelerates the progression of kidney disease (9). Vitamin D deficiency is a common feature of chronic kidney disease even in its early stages (28). The kidney is the major organ involved in the synthesis of the vitamin D metabolites; the proximal tubule transporter, megalin, is crucial to the reabsorption mechanism of 25(OH)D and the synthesis of 1,25(OH)₂D. After the 25(OH)D + VDBP complex is endocytosed into the kidney proximal cell via the megalin receptor, 25(OH)D is hydroxylated into 1,25(OH)₂D and the vitamin D binding protein is recycled back into the body (29). Studies have revealed an important renoprotective role that the vitamin D hormone and its analogs play against renal injury, and therapeutic efficacy has been reported for low-calcemic vitamin D analogs in various kidney disease models as well as in clinical trials (30–33).

While many studies have focused on low vitamin D levels as a risk factor for the development of diabetes, it is conceivable that hypovitaminosis D may also be modulated by diabetes pathology itself. There are a number of potential pathways through which diabetes may influence poor vitamin D status; for example, available vitamin D is used to reduce systemic inflammation associated with chronic diseases such as diabetes (28,34,35). Independent of diabetes, hypovitaminosis D is prevalent in patients with kidney disease (28,32,36). In addition, urine proteomic studies have reported elevated levels of VDBP in patients with diabetes and nephropathy (37). Hence, in the diabetic kidney, increased filtration of 25(OH)D + VDBP due to damage to the glomerular basement membrane of the nephron combined with compromised reabsorption by the renal proximal tubule may lead to excessive urinary loss of 25(OH)D. However, the effect of uVDBP loss on kidney function and 25(OH)D statuses have not been studied prospectively, and the utility of uVDBP to predict loss of kidney function and vitamin D status over time has not been observed. Therefore, given these gaps in the literature, the objectives of this thesis are to examine the prospective associations between vitamin D binding protein, kidney dysfunction, and serum levels of vitamin D in a population at high risk for type 2 diabetes.
CHAPTER 2
Review of the Literature

2.1 Prevalence and economic burden of T2DM

Diabetes is recognized as a major public health problem that has reached epidemic proportions worldwide, with type 2 diabetes (T2DM) accounting for approximately 90% of cases (38). Diabetes and its complications bring about a substantial economic loss to patients and their families, and to health systems and national economies through direct medical costs and loss of work and wages. In 2013, the number of people with diabetes across 130 countries was found to be 382 million, with prevalence expected to increase to 592 million by 2035 (1). Most people with diabetes live in low- and middle-income countries and these places will experience the greatest increase in cases of diabetes over the next 22 years (1). Prevalence in Canada is similarly high, with 7.9% of the adult population living with diabetes (39). Using administrative data, Hux et al. reported the prevalence of T2DM in Ontario to be approximately 4.6% – 9.6% (40). More recent data from Health Canada reported Ontario to have the third highest prevalence of diabetes in Canada, with an age-standardized prevalence of 6.0% (39).

The economic burden of diabetes is substantial, with the global cost estimated to be $1.31 trillion USD (41). The cost to manage the disease was estimated to be $9 billion per year in Canada (39). Notably, indirect costs (defined as productivity or production losses associated with morbidity and premature mortality) accounted for 34.7% of the total burden (41,42). Additionally, economic costs are predicted to increase by 75% in the next ten years, mirroring the predicted increase in the prevalence of diabetes (39).
2.2 T2DM pathophysiology

Diabetes is a chronic disease that occurs either when the pancreas does not produce enough insulin (type 1 diabetes) or when the body cannot effectively use the insulin it produces (type 2 diabetes). Type 2 diabetes (T2DM) is characterized by raised blood sugar or hyperglycemia, which arises from two underlying pathophysiological disorders: insulin resistance and pancreatic β-cell dysfunction (43). Insulin resistance is characterized by the impaired ability of insulin to transport glucose into cells, while β-cell dysfunction refers to the suboptimal production of insulin from pancreatic β-cells (43). Under normal physiological conditions, insulin is secreted from β-cells in the pancreas in response to an increase in blood glucose. However, in an insulin-resistant state, potential defects in insulin receptors and/or the insulin signaling pathway lead to impaired insulin action. As a result, less glucose is transported into target cells. The primary role of the β-cell is to maintain glucose homeostasis by synthesizing, storing, and releasing the hormone insulin (44). Healthy β-cells respond to elevated blood glucose by immediately secreting insulin (44). In an insulin-resistant state, β-cells secrete larger amounts of insulin to compensate for the deteriorating sensitivity (45). Over time, the increased demand on the β-cells leads to declines in insulin secretion and β-cell dysfunction. Once impaired, β-cells are unable to overcome insulin resistance, resulting in elevated blood glucose and concentrations (45). Although both insulin resistance and β-cell dysfunction contribute to the development of T2DM, diabetes occurs only at the point when there is a substantial impairment of insulin secretion from the β-cells (45,46).

Over time, hyperglycemia may lead to serious damage to the heart, blood vessels, eyes, kidneys, and nerves (47). In epidemiological studies, insulin resistance and β-cell dysfunction have been shown to predict the development of T2DM independent of other risk factors (48,49). Impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) are intermediate conditions in the transition between normal blood glucose levels and T2DM; people with IGT or IFG are at increased risk of heart attacks and strokes (47).
2.3 T2DM risk factors

The risk of type 2 diabetes is determined by an interplay of non-modifiable factors—such as genetics, ethnicity, and age—and modifiable metabolic factors—such as being overweight or obese, consuming an unhealthy diet, achieving insufficient physical activity, and smoking.

2.3.1 Non-modifiable risk factors

Genetics

It is well-established that there is a genetic component to T2DM risk. Individuals having first-degree relatives with T2DM are at a higher risk for developing T2DM themselves (50). Many studies have also examined the concordance rates of T2DM in monozygotic (genetically identical) and dizygotic (fraternal) twins. Medici et al. reported that 76% of monozygotic twins who were initially discordant for T2DM became concordant after 15 years (51). Specific populations have also been shown to possess different frequencies of risk alleles for T2DM (52). Since the development of methods for genome-wide association studies, many genetic variants (single nucleotide polymorphisms) have been identified as risk factors for T2DM (51,53–55). To date, over 60 genetic loci have been identified, with the majority of loci associated with β-cell growth and development (46). There are on-going studies based on the whole exome and genome sequencing that help identify some of the low-frequency, high-impact genetic variants contributing to the development of T2DM (56).

Age

Older age is a well-known risk factor for the development of T2DM. Glucose metabolism begins to progressively decline in the third and fourth decade of life, and decreased glucose tolerance is associated with advancing age (57). Additionally, aging has been found to have a direct effect on tissue sensitivity to insulin (57).

Ethnicity

The burden of T2DM is typically increased for African-Americans, Hispanics, Asians, and Indigenous groups compared to Caucasians (58). A combination of genetic factors and environmental changes associated with assimilating to a western culture have been suggested to explain differences in risk for T2DM across ethnic groups (58). It has also been established that certain ethnic groups are at higher risk for the development of T2DM. Assimilating to a western environment has been associated with changes in diet and exercise. Epidemiological data from African-American cohorts have shown a 12 times greater prevalence of T2DM compared to their native African counterparts (59–61). Additionally, Mexican-Americans living in California showed a higher prevalence of T2DM compared to Hispanics living in Mexico (62).
2.3.2 Modifiable risk factors

*Obesity and body composition*

Among the modifiable risk factors for T2DM, obesity is the most extensively studied and best elucidated. Traditionally, body mass index (BMI) has been used to determine the prevalence of overall obesity (63). However, in recent years, there has been increased interest in assessing body fat distribution as a result of the increased appreciation of the metabolic properties of abdominal adiposity. In this context, alternative measures such as waist circumference (WC) and waist-to-hip ratio (WHR) have been used as proxy measures for abdominal adiposity. Higher BMI and WC are associated with increased risk of type 2 diabetes, but the relationship varies in different populations (64). For example, South-East Asians have been found to develop diabetes at a lower BMI compared to populations of European origin (65). Abdominal obesity has been widely reported as a risk factor for T2DM and has been increasingly associated with a poor metabolic profile (66). The relationship is supported by results from several large prospective epidemiological studies which found that measurements of WC and WHR were associated with diabetes incidence (67–70). Over the past two decades, numerous studies have looked at the differences in measurements among BMI, WC, and WHR in predicting T2DM. A meta-analysis of 32 cohort studies found that BMI, WC, and WHR all had similar associations with incident diabetes (64). However, a more recent meta-analysis of over 300,000 individuals from diverse populations around the world found that WHR was a better predictor compared to WC and BMI for diabetes risk (71).

The mechanism for the association between increased abdominal obesity and the development of T2DM is partially explained by the release of non-esterified fatty acid (NEFA) from the abdominal fat depot (72,73). Higher NEFA levels are linked to insulin resistance through the inhibition of glucose uptake (which occurs in a dose-dependent manner) (74), hepatic gluconeogenesis (75), and a reduction of glucose clearance (76). Recent evidence has implicated a role of other agents, including pro-inflammatory cytokines and hormones (77,78). The production of these products is concurrently increased with abdominal obesity (66).

*Diet*

Several dietary practices are linked to unhealthy body weight and/or risk of T2DM, including high intake of saturated fatty acids, high total fat intake, and inadequate consumption of dietary fibre (79–81).

Some dietary factors—such as coffee, fibre, and whole grains, and dairy products—have been shown to have a protective effect on T2DM (82–85). However, other dietary factors like sugar-sweetened beverages and high consumption of dietary fat have been associated
with an increased risk for obesity and the development of T2DM (86,87,87–91). High intake of sugar-sweetened beverages also increases the likelihood of being overweight or obese, especially among children (92,93). In addition, specific foods and nutrients, such as vitamin D, have been investigated for their protective effects against the development of T2DM. The WHO states that insulin sensitivity and glucose uptake can be improved by including sufficient fibre and replacing saturated fatty acids with polyunsaturated fatty acids in the diet (94). Dietary recommendations by WHO and the Food and Agriculture Organization for the prevention of T2DM include limiting saturated fatty acid intake to <10% of total energy intake, achieving a minimum daily intake of 20g of fibre, and reducing free sugar intake to <10% of total energy (81,92).

Early childhood nutrition affects the risk of T2DM later in life. Factors that appear to increase the risk of disease include poor fetal growth, low birth weight followed by rapid postnatal catch-up growth, and high birth weight (81,95–100). Diabetes in pregnancy and gestational diabetes increase the risk of future obesity and T2DM development in offspring (47).

Physical activity
Regular physical activity reduces the risk of diabetes and raised blood glucose, and is an important contributor to overall health, energy balance, weight control, and obesity prevention – all of which are risk factors linked to future diabetes prevalence (94). A sedentary lifestyle is a significant risk factor for the development of T2DM (86,101). Population-based data from Cuba show a fall in T2DM during a period of economic crisis when the population experienced a reduction in calorie intake and a simultaneous increase in physical activity (102), suggesting that changes in diet and physical activity do affect the prevalence of T2DM. Physical activity has been well-established to improve control of blood glucose and is a key factor in the prevention and management of T2DM (103). Exercise has also been associated with a substantially reduced risk for T2DM in large prospective cohort studies, including the Nurses’ Health Study and the Physicians’ Health Study (104,105). Moderately intense activities—such as walking and gardening—are the most common forms of physical activity (106). A recent meta-analysis of 10 prospective studies by Jeon et al found that moderate intensity physical activity, such as brisk walking, had a significant negative association with the risk of T2DM independent of BMI (107). It is recommended that children and youth aged 5–17 years should have at least 60 minutes of moderate- to vigorous-intensity physical activity daily and that adults aged 18–64 years should have at least 150 minutes of moderate-intensity aerobic physical activity spread throughout the week (108). For older adults, the same amount of physical activity is recommended, but should also include balance and muscle strengthening activities tailored to ability and circumstances.
Smoking
Many studies have shown an association between cigarette smoking and T2DM. Smoking increases the risk of developing T2DM, with the highest risk found among heavy smokers (109). The increased risk remains elevated for approximately 10 years after smoking cessation, falling more quickly for lighter smokers (110). In a meta-analysis of 25 prospective cohort studies, cigarette smoking has been shown to have a pooled adjusted relative risk of 1.44 (95% CI 1.31 – 1.58) with the risk of T2DM (109). The risk of T2DM was greater in those who smoked more than 20 cigarettes per day compared to those who smoked less than 20 cigarettes a day. The risk was also lower for former smokers compared with currently active smokers, reflecting a dose-dependent association between smoking and incident T2DM (109).

2.4 Kidney dysfunction
According to Diabetes Canada, diabetes is the leading cause of kidney disease in Canada. Chronic kidney disease (CKD) has a worldwide prevalence affecting 7.2% of the global adult population, with the number drastically increasing to 23.4 – 35.8% in those over the age of 65 (111). Although there are numerous causes—including glomerular kidney disease, tubular and interstitial kidney disease, obstructive uropathy, pre-renal and vascular disorders, and hypertension—diabetes is the most common reason for CKD worldwide (112). Approximately 10-40% of individuals with T2DM suffer from kidney damage, and 50% of those will eventually develop kidney failure (113).

CKD is defined as a progressive, irreversible loss of kidney function. The National Kidney Foundation Kidney Disease Outcomes Quality Initiative defined CKD as lasting ≥3 months with either kidney damage (structural or functional abnormalities of the kidney) or a glomerular filtration rate of less than 60 ml/min/1.73m² (111). CKD is associated with an increased risk of cardiovascular disease, mortality, and end-stage renal disease. Unfortunately, therapies are limited and mainly focus on treatment of associated causes, including diabetes and hypertension, slowing the progression of the disease, treatment of complications, and preparation for renal replacement therapy (112).

As the kidneys fail, blood urea nitrogen (BUN) and creatinine levels will rise in circulation (114). End-stage renal disease (ESRD) occurs when the kidneys function at 10-15% or less and are no longer able to support physiological function (115). At this stage, hemodialysis, peritoneal dialysis (PD), or kidney transplantation is needed. Hemodialysis is a treatment that removes waste and extra fluid from the blood. During hemodialysis, blood is pumped into a
dialysis machine where it goes through a dialyzer. As blood is filtered, it is returned to the bloodstream. In peritoneal dialysis, the blood is cleaned inside the body using the peritoneum (lining of the abdomen) as a natural filter. Dialysate flows into the abdomen through a PD catheter, which is placed using a minor surgical procedure. Waste passes from the blood into the dialysate, which can be drained after. Kidney transplant replaces a patient’s damaged kidney with a healthy kidney from another individual. However, a kidney transplant is still classified as a treatment rather than a cure; after a kidney transplant, the person with a kidney transplant still has kidney disease and may still need to take some of the medication and precautions they took prior to the surgery.

2.4.1 Mechanisms of kidney dysfunction

Under normal physiological conditions, the kidneys filter blood to remove waste through the urine. Additionally, the kidneys also regulate the amount of fluid and salts in the body and play a large role in controlling blood pressure. Over time, high blood glucose levels that characterize diabetes damage small capillaries in the body. When blood vessels in the kidneys are injured, the organ’s ability to filter blood is impaired. As a result, waste materials may build up in the blood and the body may retain more sodium, leading to weight gain and ankle swelling. As well, proteins may leak through the damaged walls of the blood vessels and appear in the urine. Diabetes may also cause nerve damage, resulting in difficulty in emptying the bladder. The pressure resulting from a full bladder can back up and injure the kidneys. Urine that remains in the bladder for extended periods of time increase the risk of infection from the rapid bacterial growth in urine that has high glucose levels (116).

There are various etiologies involved in the pathogenesis of diabetic CKD. The initial mechanism of damage involves adaptive hyperfiltration, which leads to long-term damage of the nephrons (112). Additional mechanisms of the disease include advanced glycosylated end-products (AGEs), vascular endothelial growth factor, pro-renin and the renin-angiotensin system, cytokines, nephrin expression, mesangial hypertrophy, glomerular basement thickening, and impaired podocyte and endothelial function (112).

Advanced glycosylated end-products are covalently glycosylated proteins whose synthesis increases with hyperglycemia (117). AGEs accumulate in the extracellular matrix and the glomerular basement membrane, resulting in altered elasticity, ionic charge, and thickness (118). These products bind with cell surface receptors and initiate cellular signaling cascades associated with increased vascular cell adhesion molecule-1 (VCAM-1) expression, causing vascular injury, mesangial cell growth, enhanced expression of growth factors, extracellular membrane proteins, reactive oxygen species (ROS) and activation of protein kinase C (PKC), and release of cytokines and growth factors (112).
Hyperglycemia activates isoforms of PKC through diacylglycerol (DAG), which activates mitogen-activated protein kinases (MAPK) and vasotropic substances such as angiotensin II (ang II), endothelin, and prostanoids causing glomerular hyperfiltration (112). PKC activation increases ROS and the actions of fibrotic factors such as TGF-β and connective tissue growth factor, resulting in glomerular hypertrophy and mesangial expansion (112). ROS increases cytokines and extracellular membrane proteins type IV collagen, leading to glomerulosclerosis and renal failure (118).

Vascular endothelial growth factor expression is upregulated in podocytes by hyperglycemia, which increases vascular permeability in the nephron (119). Nephrin, a protein found in podocyte, plays a crucial role in maintaining an intact filtration barrier. Lower renal expression of nephrin in kidney biopsies of patients with diabetes has been previously reported (120).

Mammalian target of rapamycin (mTOR) is a serine/threonine protein kinase which integrates multiple signals—including insulin, energy balance, and oxidative stress—to regulate cell growth and survival (121). This protein kinase may play a role in glomerular hypertrophy and podocyte enlargement (112). In the glomeruli of patients with diabetic nephropathy, mTOR upregulates target genes such as vascular endothelial growth factor (VEGF), sterol regulatory element-binding protein (SREBP), mitochondria genes, and mTOR messenger ribonucleic acid (mRNA) levels (122).

The renin-angiotensin-aldosterone system (RAAS) plays an important role in regulating blood volume and systemic vascular resistance. As the name implies, there are three important components to this system: 1) renin, 2) angiotensin, and 3) aldosterone. Renin, which is released primarily by the kidneys, stimulates the formation of angiotensin in blood and tissues, which in turn stimulates the release of aldosterone from the adrenal cortex. Ang II stimulates synthesis of matrix proteins, increases VEGF, oxidative stress, and decreases nitric oxide production and loss of endothelial integrity (112). As well, ang II causes efferent arteriolar vasoconstriction and increased intraglomerular pressure, leading to renal hyperfusion (123,124). Aldosterone is known to be mitogenic and increases renal fibrosis through pro-fibrotic transforming growth factor beta (TGF-β).

Elevated levels of inflammatory markers have been shown to be proportional to the degree of albuminuria (123). In patients with T2DM and persistent microalbuminuria, elevated levels of biomarkers of inflammation and endothelial dysfunction predicted the development of nephropathy over a 2-year follow-up period (125). Inflammatory factors lead to the
accumulation of macrophages in the tubular interstitium, producing free radicals, inflammatory cytokines, and proteases that induce tubular damage (126,127).

2.4.2 Albumin-to-creatinine ratio

The earliest sign of diabetic kidney disease is often an increased excretion of albumin in the urine (128). The detection of very small amounts of protein in the urine (2 – 20 mg/mmol) is known as a condition called microalbuminuria. As kidney disease progresses, more protein is found in the urine and macroalbuminuria (>20 mg/mmol) occurs. Albuminuria is typically greater in smokers with diabetes compared to non-smokers (129).

Microalbuminuria has been found to predict progression to clinical proteinuria and decreased renal function in patients with T2DM (128,130,131). The rate of progression to microalbuminuria from normoalbuminuria in the UK Prospective Diabetes Study (UKPDS) was 2% per year; from microalbuminuria to macroalbuminuria was 2.8% per year, and macroalbuminuria to elevated creatinine or renal replacement therapy was about 2.3% per year (6). Patients without microalbuminuria at diagnosis remained free of nephropathy for a median period of 19 years, while those who had microalbuminuria progressed to macroalbuminuria in 11 years (6). Infrequently, microalbuminuria may regress to normoalbuminuria in T2DM. A study by Parving et al found 21% of subjects recovered their kidney function over 2 years (132). Thus, although microalbuminuria precedes proteinuria and decreased renal function, not all microalbuminuria cases will progress. The challenge is to identify those with microalbuminuria who are likely to progress from those who will not. Glomerular filtration rate may also decline in the absence of any microalbuminuria (130,133).

2.4.3 Glomerular filtration rate

The glomerulus is the filtering unit of the kidney and is made up of a specialized bundle of capillaries that are uniquely situated between two resistance vessels (134). The main function of the glomerulus is to filter plasma to produce glomerular filtrate, which passes down the length of the nephron tubule to form urine. In addition to testing for ACR in the urine, the glomerular filtration rate (GFR) can be estimated as an indication of kidney function at the level of the glomerulus.

The gold standard for measuring GFR is the constant intravenous infusion technique using inulin, a compound which is freely filtered in the glomerulus and neither reabsorbed nor secreted thereafter. However, inulin clearances are rarely performed even in the research setting. Other methods estimating GFR include creatinine clearance, which overestimates GFR due to the tubular secretion of creatinine, particularly at low levels of GFR (135).
There are also several equations that are commonly used to estimate GFR, but they tend to be inaccurate in normal and high ranges of GFR, and usually underestimate true GFR (136). However, the advantage of these methods is that GFR can be estimated using blood samples. Traditionally, a single measure of plasma creatinine has been used to determine glomerular function. For any individual, plasma creatinine values are tightly distributed around a homeostatic set-point, with an intra-individual variation (CV) of 5.3% (137). The consequence of this is that an individual may show a significant increase in plasma creatinine with deterioration in renal function, yet still have a result which falls within the reference interval. Furthermore, plasma concentrations of creatinine are affected by other factors including muscle mass, diet, gender, age, and ethnicity. Calibration bias and measurement imprecision for plasma creatinine have a large impact on the uncertainty in eGFR, especially with lower plasma creatinine values (corresponding to better renal function) (138). Another compound used to estimate GFR is cystatin C. Many studies have compared cystatin C concentrations or cystatin C-derived equations with gold standard methods. Most studies have found cystatin C or the reciprocal of cystatin C to be superior, or at least equivalent to serum creatinine for the detection of decreased GFR (139,140). However, the lack of an international standardized calibrator currently limits the use of cystatin C equations. More importantly and to justify its additional cost, its use will depend on evidence that it significantly improves clinical outcomes.

In 1976, Cockcroft and Gault published an equation to predict creatinine clearance based on age, weight, height and plasma creatinine, together with correction factors (141). Although helpful, it has many inherent limitations, having been derived mostly from hospitalised men (with only nine females in the cohort), all of whom had CKD. The requirement for weight and height to be provided also restricted its ability to be reported by the laboratory.

In 2005, the recommended method for estimating GFR was the Modification of the Diet in Renal Disease (MDRD) equation, which is validated in patients with T2DM, non-diabetic kidney disease, and in transplant recipients (142). The study was a multi-centre trial to evaluate the effect of dietary protein restriction and blood pressure control on progression of renal disease in 1628 patients with CKD, with the added objective of developing an equation that could improve the prediction of GFR from plasma creatinine (143). A 6-variable equation was derived, and subsequently, a simplified 4-variable version which included age, gender, plasma creatinine value, and white or black race differentiation was published. Results were expressed as per 1.73m² of body surface area (143). It has been validated in the US and European Caucasian populations, as well as African-Americans, but not with other racial and ethnic groups (142).
One advantage of the MDRD equation over Cockcroft and Gault’s is the lack of requirement for either body weight or height to be supplied. Given that the MDRD equation was originally derived from a group of CKD patients, its utility for healthy individuals remains unclear, and strictly it has not been validated in children under 18 years of age, in pregnant women, in patients above 70 years of age, and in ethnic groups other than African-American (137). The poorer performance of the MDRD formula has been reported at low plasma creatinine concentrations (144).

Comparative performance of the MDRD and the Cockcroft-Gault formulae has been assessed in numerous studies. Generally, the MDRD has been shown to perform somewhat better than Cockcroft-Gault in most studies, with less bias and a higher proportion of results in agreement with a radionuclide gold standard (145,146). However, the Cockcroft-Gault formula has been reported to be more accurate when plasma creatinine is within the reference interval (147). Recalibration of creatinine assays to align with IDMS has been shown to improve the performance of the MDRD equation (148).

Given that the MDRD equation was developed in a population with sub-optimal kidney function, its accuracy in predicting GFR is best reflected in those with mild kidney impairment. It is recognised that MDRD tends to underestimate renal function in those with a normal eGFR >90 mL/min/1.73m². In response to these concerns, the CKD-Epidemiology Collaboration group (CKD-EPI) developed and validated a new equation in 2009 designed to match the accuracy of the MDRD equation at GFR <60 mL/min/1.73m² and to offer greater accuracy at higher GFR, minimizing the over-diagnosis of CKD with the MDRD equation (149). The new CKD-EPI equation was developed from 8254 data points from six studies and four clinical populations, with original serum creatinine values recalibrated to the Roche enzymatic method (149). The variables in the CKD-EPI equation with creatinine include serum creatinine, sex, race, and age on the natural scale. This equation was shown to be as accurate as MDRD in the subgroup with eGFR <60 ml/min/1.73m² and substantially more accurate in the subgroup with eGFR >60 ml/min/1.73m², and confers less underestimation of GFR in subjects with normal renal function (149,150).

Improved accuracy of the CKD-EPI equation could have important implications for public health as well as in clinical practice. Application of the CKD-EPI equation in the Australian, Diabetes, Obesity and Lifestyle Study yielded a lower estimated prevalence of CKD compared with the MDRD equation, namely 11.5% compared with 13.4% (151).
2.4.4 Glomerular hyperfiltration

Glomerular hyperfiltration is a phenomenon that can occur in various clinical conditions, including kidney disease. In general, hyperfiltration is an observed absolute increase in glomerular filtration rate, but no formal definition has been agreed upon, and the pathophysiological mechanisms, which are likely to vary with the underlying disease, are not well-explored. Glomerular hyperfiltration can either be defined as an abnormally high whole-kidney glomerular filtration rate (GFR), increased filtration fraction, or as increased filtration per nephron (135).

Conventionally, whole-kidney elevated GFR hyperfiltration has been defined as GFR of more than two standard deviations above the mean GFR of healthy individuals (152). In some studies, the threshold for glomerular hyperfiltration has ranged from 125 ml/min/1.73m$^2$ to 175 ml/min/1.73m$^2$ (153–156). However, there are some limitations of this definition of hyperfiltration: it does not consider age-related declines in GFR and it ignores the fact hyperfiltration can take place in a single nephron with absolute decreased GFR. Therefore, some studies define glomerular hyperfiltration as an increased filtration fraction (157). The filtration fraction is the ratio of GFR to the effective renal plasma flow; the filtration fraction increases if effective renal plasma flow decreases more than GFR. A healthy filtration fraction in young adults is approximately 18.7±3.2% (157).

Differences in the definition of glomerular hyperfiltration are compounded by the use of various methods for measuring GFR and the difficulty of obtaining accurate filtration measurements. The majority of studies assessing hyperfiltration measured GFR using filtration markers such as iohexol or isotopically labeled iothalamate—ethylenediaminetetraacetic acid and diethylenetriaminepentaacetic acid—using a single-injection technique (135). The determination of filtration fraction requires simultaneous measurements of GFR and renal plasma flow using radiolabeled p-aminohippurate (PAH), which is removed from the circulation almost entirely using a single pass through the kidney by glomerular filtration and proximal tubular secretion (157). PAH clearance is, therefore, representative of renal blood flow but is inaccurate if tubular function is compromised.

Hyperfiltration can be caused by afferent arteriolar vasodilation, as seen in patients with diabetes or after a high-protein meal, and/or by efferent arteriolar vasoconstriction owing to the activation of the renin-angiotensin-aldosterone system, thus leading to glomerular hypertension (135). The increase in GFR is mediated in part by vascular endothelial growth factors (VEGF), transforming growth factor-beta (TGF-β), increased generation of nitric oxide, as well as an increased production of renal kallikrein and other vasoactive kinins (158–161). The mechanisms of glomerular hyperfiltration at the single-nephron and whole-kidney level
may differ (162,163). GFR is determined by renal plasma flow—defined as the hydraulic pressure gradient across the kidney membrane—and the ultrafiltration coefficient; these parameters can be altered in patients with hyperfiltration (135). Renal hemodynamic changes that result in glomerular hyperfiltration are likely to have a crucial role in the initiation and progression of CKD (135). Increased filtration per nephron occurs as an adaptive response to nephron loss and leads to glomerular hypertension and subsequent glomerulosclerosis with progressive renal function decline (135).

The ability to increase GFR after protein loading is known as the renal functional reserve, and loss of this reserve is characteristic of CKD (164). Hyperfiltration might also directly contribute to the progression of CKD. A study conducted by Brenner et al. hypothesised that progressive deterioration of kidney function was a result of compensatory glomerular hemodynamic changes occurring in response to nephron loss (162,165,166). Using an animal model of renal mass reduction, the remaining nephrons underwent hypertrophy, which was associated with reduced arteriolar resistance and increased glomerular blood flow (162). Afferent arteriolar resistance decreases more than efferent arteriolar resistance with the progression of kidney disease (162). As a consequence, intraglomerular hyperfiltration occurs.
Hyperfiltration has been shown in experimental studies to mediate progressive kidney damage following various injuries (163, 167–170). In these models of CKD, glomerular hyperfiltration occurs at the single-nephron level and is associated with normal or reduced total GFR. However, in patients with T2DM, an absolute increase in GFR is often observed, representing hyperfiltration at the whole-kidney level (135). Increased GFR can occur as an early manifestation of diabetes, but it remains to be established whether glomerular hyperfiltration is a precursor of CKD (135). Ultimately, the mechanisms of glomerular hyperfiltration in disease conditions are variable and not entirely clear. Longitudinal studies are needed to examine whether treatment of hyperfiltration will slow the progression of CKD.

**Hyperfiltration in diabetes**

Studies of newly diagnosed patients with T2DM using filtration markers and PAH have shown that glomerular hyperfiltration occurs early in the course of T2DM in about 50% of patients (171–173). The mechanisms leading to the development of hyperfiltration in patients with diabetes are not fully understood, and several hypotheses have been proposed. In several human studies of early diabetes and some animal models, primary abnormalities in vascular control lead to renal vasodilation and increased renal blood flow (171–175). Animal studies have shown that afferent glomerular arterioles dilate more than efferent arterioles, leading
CHAPTER 2 REVIEW OF THE LITERATURE

17

to increases not only to GFR but also intraglomerular pressure and the filtration fraction (162). These hemodynamic changes have been proposed to mediate diabetic glomerulopathy in streptozotocin-induced diabetic Munich-Wistar rats (176). Increased GFR and renal plasma blood flow have also been reported in patients with newly diagnosed diabetes, which is indicative of afferent renal vasodilation. In addition, the filtration fraction was elevated in these studies, indicating glomerular hypertension (171–174). The mechanism leading to afferent glomerular vasodilation is unclear, but elevated levels of insulin, insulin-like growth factor 1 (ILGF-1), atrial natriuretic peptide, advanced glycation end products, and intrarenal nitric oxide signaling have been previously suggested (172,174,177–181). Whole-kidney hyperfiltration at the onset of diabetes typically reverses after insulin therapy is given, implying that elevated plasma glucose levels play a role (174,182). Similarly, an elevated GFR seems to be correlated with poor diabetic control (174,182).

Some papers suggest that glomerular hyperfiltration in diabetes is a result of increased proximal tubular reabsorption of glucose and sodium, which causes vasodilation secondary to suppressed tubuloglomerular feedback (183,184). Studies in patients with type 1 and type 2 diabetes further support the hypothesis that increases in proximal tubular sodium reabsorption lead to glomerular hyperfiltration through decreased tubuloglomerular feedback by decreasing sodium chloride delivery to the macula densa (185,186). At the cellular level, the high concentration of glucose in the glomerular filtrate of patients with diabetes promotes a coupled sodium and glucose reabsorption in the proximal tubule (135).

However, diabetes-induced glomerular hyperfiltration has been shown to occur in adenosine receptor A1-deficient mice, which lack the tubuloglomerular feedback mechanism (187). Further research is needed to determine whether the mechanisms proposed in the etiology of hyperfiltration in animal models can be applied to human patients with diabetes. Interestingly, increased proximal tubular sodium reabsorption has been demonstrated in obese individuals without diabetes, but was thought to be a consequence rather than a cause of hyperfiltration because the increased filtration fraction increases post-glomerular oncotic pressure, and therefore favours sodium reabsorption (188,189).

Renal injury associated with hyperfiltration may be mediated by activation of the RAAS, which leads to maladaptive renal and systemic hemodynamic responses, increased arterial stiffness, and endothelial dysfunction (190,191). Therefore, the most effective intervention may be targeting hyperfiltration and the RAAS. The increase in glomerular pressure could be attenuated by blocking the vasoconstrictive effect of angiotensin II on the glomerular efferent arteriole. The beneficial effect would be independent of the anti-hypertensive properties of RAAS inhibitors (135). Animal data suggest that chronic therapy with ACE inhibitors or angiotensin-receptor blockers (ARB) promotes the regression of
glomerulosclerosis, even in later phases of the disease (192). Randomized control trials in humans confirm that ACE inhibitors and ARBs can slow the progression of diabetic nephropathy (193–195). However, ACE inhibitors and ARBs are limited to the extent to which they inhibit the activity of RAAS. Angiotensin II and aldosterone may be the mechanism involved in limiting the effects of ACE inhibitors and ARBs (196).

Rosiglitazone is an insulin-sensitizing drug that is known to improve endothelial dysfunction. It has been shown to ameliorate hyperfiltration in patients with early T2DM, improve nitric oxide bioavailability, and decrease renal end-organ damage in subjects with T2DM and microalbuminuria (197). Renal nerves might play an important role in mediating glomerular hyperfiltration in experimental diabetes (135). In this regard, chronic renal denervation also prevents hyperfiltration in diabetic rats (198). Aldosterone antagonists have been shown to attenuate obesity-induced hypertension and hyperfiltration (199). Short-term use of continuous positive airway pressure may improve glomerular hyperfiltration in patients with obstructive sleep apnea (200). Finally, maintaining a healthy weight, particularly in patients with underlying kidney disease, will remove a synergistic factor for glomerular injury, thus delaying the progression to ESRD (201–203).

Glomerular hyperfiltration in patients with diabetes has been associated with the development of diabetic nephropathy (DN) and a poor prognosis of the disease (135). However, the poor prognosis might relate to poor metabolic control rather than to glomerular hyperfiltration itself. A recent study with 426 patients from the Joslin Diabetes Centre was unable to detect an association between hyperfiltration and subsequent development of microalbuminuria during 15 years of follow-up (204). A meta-analysis of 10 cohort studies involving 780 subjects followed for a median of 11 years suggested a modestly increased risk of progression to DN in patients with early glomerular hyperfiltration compared to those with normal GFR (182). However, interpretation of these observational studies is hampered by the use of different methods to measure GFR and numerous confounding variables, such as diabetes duration, quality of metabolic and blood pressure control, duration of follow-up, as well as publication bias (182). Whether early hyperfiltration is an independent risk factor the development of DN remains uncertain; even in animal models, the association has not been consistent (172). Further prospective and controlled studies are needed to explore the potentially lasting harmful effects of glomerular hyperfiltration in patients with diabetes.

2.4.5 Risk factors for kidney disease
There are several non-modifiable and modifiable risk factors for the development of kidney disease. Major non-modifiable risk factors include duration of diabetes, ethnicity, and
genetics. Fortunately, most risk factors for kidney disease are modifiable, including smoking, poor diet, blood pressure, inactivity, and overweight and obesity.

Non-modifiable risk factors
The duration of diabetes was associated with the progression of nephropathy in a long-term follow-up study in Saudis with duration of diabetes greater than 10 years (205). The odds ratio of nephropathy was 4.6-fold higher in urban African-Americans with duration of diabetes greater than 5 years compared to those who had diabetes less than 1 year (206).

Ethnicity also appears to play a role in the risk of kidney disease. The prevalence of CKD is highest among the African-American and Native American populations, and lowest among the Caucasian and Asian populations, which parallels the rate of diabetes in these populations (115,207). The trend of increasing prevalence is expected to continue to grow as the elderly population continues to experience life expectancy (112).

As CKD has a heritable component, Köttgen et al. have conducted genome-wide association studies to identify susceptibility loci for lower GFR in subjects of European ancestry (208). It was found that mutations of uromodulin, which encodes Tamm–Horsfall protein in the urine, were associated with differences in renal function (208). Another identified mutation is related to APOL1 (209). An autosomal recessive pattern of inheritance was demonstrated and associated with a substantially higher risk of ESRD. Interestingly, APOL1 mutations are found exclusively among individuals of African descent, which may be one explanation the increased risk of CKD among that population (209). An insertion or deletion polymorphism of the angiotensin-converting enzyme (ACE) gene predicted severe structural kidney changes in patients with microalbuminuria (210). However, some studies show that there are no associations between these genetic factors and kidney function (211). Identifying the genetic risk of diabetic nephropathy requires further study.

Having a co-morbidity such as heart disease and family history of kidney failure increases the risk of kidney disease (212). Song et al. asked dialysis patients in the United States to complete a voluntary questionnaire on family history of ESRD (213). After the exclusion of patients with ESRD due to hereditary disorders and urologic causes, nearly 23% of incident dialysis patients had close relatives with ESRD (213).

Advancing age and obesity are also risk factors for kidney dysfunction (214). Renal function decreases with age in both men and women (215). Among the elderly population, more than one-half of the subjects screened had CKD stages 3–5 (GFR<60ml/min/1.73m²) according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (K/DOQI) guidelines (152). In the Chronic Renal Disease in Turkey (CREDIT) study, the odds ratios of
CKD ranged from 1.45 to 2.18 for every 10-year increase in age among subjects older than 30 years of age in Turkey (216).

**Modifiable risk factors**

A diet low in protein is recommended for reduced risk of kidney disease. In the early stages of CKD, protein intake should not exceed 0.8 – 1.0 g/kg body weight per day, while at later stages of CKD, protein should be limited to 0.8 g/kg body weight per day (152). Dietary restriction can help improve renal function by decreasing urinary albumin excretion and thus, attenuating declines in eGFR (152,217). Additionally, a diet consisting of <130g of carbohydrate per day has been advised to decrease the toxic effects of hyperglycemia on the kidneys (217).

Many studies have shown that hyperglycemia is strongly associated with the development and progression of CKD (218). In the Diabetes Control and Complications Trial (DCCT) of subjects with type 1 diabetes and the UKPDS trial of subjects with type 2 diabetes, it was shown that improved glycemia resulted in significantly lower progression rates for albuminuria (219). The Atherosclerosis Risk in Communities Study (ARIC) study found an increased hazard ratio for CKD with increasing HbA1C; compared to an HbA1C of <6%, the hazard ratio was 1.37, 2.49, and 3.67 at HbA1C values of 6-7%, 7-8%, and >8%, respectively. Furthermore, each 1% increase in HbA1C was associated with a 31% higher risk for CKD (220). Subjects who developed either albuminuria or retinopathy early in the study were at much higher risk of CKD compared to those with neither morbidity. Of individuals who developed CKD, only 25% had established retinopathy, 13% had albuminuria, 29% had both, and 33% had neither (220). Another large study, a trial by the ADVANCE collaborative group, with T2DM patients showed a 21% relative risk reduction in nephropathy with intensive glycaemic regulation (target HbA1C was set to be ≤6.5%) compared to conventional treatment (mean HbA1C 7.3%) (221). When intensive glucose control was coupled with good blood pressure control, there was an additive benefit in overall clinical outcomes; there was a 33% risk reduction in new or worsening nephropathy, a 54% reduction in new-onset macroalbuminuria, and a 26% reduction in new-onset microalbuminuria (222). The Action to Control Cardiovascular Risk in Diabetes (ACCORD) trial demonstrated a 21% reduction in microalbuminuria in the intensively treated subjects and a decrease in macroalbuminuria (223). Diabetes and nephrology professional organisations recommend an overall HbA1C goal of 7.0% in order to best preserve renal function (152,217).

Maintaining a healthy blood pressure is also important for the management of kidney dysfunction in diabetes. One of the earliest trials which demonstrated the benefit of maintaining lower blood pressure in subjects with T2DM free of nephropathy was the
Normotensive ABCD study (224). 480 normotensive subjects were randomized into intensive control (mean blood pressure 128/75mmHg) and moderate control (mean blood pressure 137/81mmHg) groups. After a 5-year follow-up, there was a significant increase in the rate of urinary albumin excretion in the moderate versus intensive treatment group. Overall renal function remained stable in both normoalbuminuric and microalbuminuric groups. The progression from normoalbuminuria to microalbuminuria, or microalbuminuria to macroalbuminuria, was found to be slower in the intensive group compared to those in the moderate blood pressure group. This was not the case in patients who had already displayed overt macroalbuminuria at baseline, as their renal function continued to decline regardless of which blood pressure group they were assigned (224).

Ravid et al. conducted a 5-year randomized control trial of 94 T2DM subjects with microalbuminuria but normal blood pressure and renal function (214). They found that there was a benefit of tighter blood pressure control on renal function in all patient groups, including normotensive subjects. In patients taking enalapril (a drug used to treat high blood pressure), the initial microalbumin of 143mg/24h decreased to a mean of 123mg/24h but rose to 140mg/24h after 5 years. In contrast, the placebo group began with a mean microalbumin level of 123mg/24h with an increase in microalbuminuria to a mean of 310mg/24h (225). In addition, there were significant differences in kidney function which decreased by 13% in the placebo group over the 5-year follow-up, while the enalapril group remained stable. These findings lead to the conclusion that intensive blood pressure control is beneficial for renal protection, especially when initiated early.

Elevated systolic blood pressure has been linked to accelerated diabetic nephropathy (214,226,227). The development of impaired renal function was associated with higher mean blood pressures, even in patients who were normotensive with and without proteinuria in a long-term follow-up of patients with T2DM (214). The guideline for blood pressure control in diabetes is currently <130mmHg for systolic blood pressure and <80mmHg for diastolic blood pressure (217). According to the American Society of Hypertension, patients with hypertension and eGFR <50ml/min/1.73m² should be started on anti-hypertension medications (228).

Smoking can increase CKD risk through inflammation, oxidative stress, prothrombotic shift, endothelial dysfunction, glomerulosclerosis and tubular atrophy (229). In a study of 7476 nondiabetic participants, smoking >20 cigarettes per day increased the risk of CKD (230). In another study, each additional five cigarettes smoked per day were associated with an increase in serum creatinine >0.3mg/dl by 31% (231).
2.5 Vitamin D

Vitamin D is a group of fat-soluble secosteroids responsible for the intestinal absorption of calcium and phosphate. The two main forms of vitamin D are cholecalciferol (vitamin D$_3$) and ergocalciferol (vitamin D$_2$) (232). Vitamin D$_3$ is mainly synthesized in the skin upon exposure to UV-B rays or obtained from nutritional sources like fatty fish, while vitamin D$_2$ is obtained by irradiation of ergosterol, a plant sterol found in the cell membrane of yeast and fungus (232). Enzyme 25-hydroxylase in the liver converts vitamin D$_3$ into 25-hydroxyvitamin D (25(OH)D), which is the storage form of vitamin D. Subsequently, 25(OH)D moves to the kidneys and is either catabolized by 25-hydroxyvitamin D$_3$-24-hydroxylase into 24,25-dihydroxyvitamin D (233) or hydroxylated into 1,25-dihydroxyvitamin D (1,25(OH)$_2$D), the active vitamin D hormone which stimulates calcium reabsorption from the bone, gut, and kidneys (234). Vitamin D is essential for the regulation of calcium. Without vitamin D, only 10 – 15% of dietary calcium is absorbed (235). In addition, the vitamin D endocrine system is involved in the induction of cell differentiation, inhibition of cell growth, immunomodulation, and control of other hormonal systems (236).
After 1,25(OH)\(_2\)D enters a target cell, it has a wide range of biological actions, including inhibition of cellular proliferation and induction of terminal differentiation, inhibition of angiogenesis, stimulation of insulin production, inhibition of renin production, and stimulation of macrophage cathelicidin production (237–240). Vitamin D activates its cellular
receptor—the vitamin D receptor—which alters the transcription rates of target genes responsible for the biological responses (241).

The production of 1,25(OH)$_2$D is stimulated by parathyroid hormone (PTH), which becomes activated by lower serum calcium levels (242). 1,25(OH)$_2$D increases serum calcium levels through various mechanisms, including activation of the vitamin D-dependent transport system in the small intestine for increased absorption of calcium, mobilizing calcium from bone storage, and increasing calcium reabsorption at the kidneys (242). There is a negative feedback through calcium, which decreases PTH, and a direct negative feedback from 1,25(OH)$_2$D to PTH (243). The threshold of serum 25(OH)D where PTH begins to rise is approximately 75 nmol/L (232).

2.5.1 Sources of vitamin D
Under normal physiological conditions, vitamin D is synthesized in the skin when exposed to UV-B radiation (between 290 – 315 nm) from the sun (244). However, synthesis is affected by numerous factors, including season, age, skin pigmentation, latitude, use of sunscreen, clothing, and skin exposure (245).

Other ways in which vitamin D can be obtained include ingestion from the diet or in the form of supplements. Dietary sources of vitamin D include cod liver oil (from whole fish), fatty fish (e.g. wild salmon, mackerel, herring, sardines, trout, and eel), egg yolks, and UV irradiated shiitake mushrooms (246). Fortified foods—such as milk, soy milk, orange juice, and margarine—are also common dietary sources of vitamin D (246,247). Originally, milk was fortified with vitamin D by feeding the cows irradiated yeast (247). This technique was replaced in the 1940s by the simpler and more effective method of adding vitamin D concentrate to milk (247).

Currently, both Canada and the United States require mandatory vitamin D fortification of some dairy products and food items (i.e. milk, margarine, orange juice, and yeast-leavened bakery products); however, Canada has far fewer fortified products compared to the United States (246). In the USA, vitamin D fortification is strictly regulated pertaining to the categories of foods, the functional use and level of use, thus limiting over-fortification (247). Similar to USA regulations, in Canada, the law mandates fortification of milk (180 IU/250 mL), milk alternatives and margarine (530 IU/100 gm), but fortification level is limited (247).

There has also been controversy regarding the effectiveness of the consumption vitamin D$_2$ versus vitamin D$_3$ (248,249). A recent meta-analysis of 7 randomized controlled trials by Tripkovic et al. found that vitamin D$_3$ is more effective at raising serum 25(OH)D levels than vitamin D$_2$, but more research is needed in this area (250).
2.5.2 Factors influencing vitamin D levels

2.5.2.1 Non-modifiable factors

Melanin pigmentation, which acts as a natural sunscreen and absorbs solar UV radiation, competes with 7-dehydrocholesterol, meaning individuals with darker skin experience less absorption of UV-B rays and subsequently have less cutaneous vitamin D synthesis due to their high melanin levels.

Specific genes are thought to influence the variability in vitamin D concentrations (251,252). For instance, single nucleotide polymorphisms (SNPs) for genes encoding the vitamin D binding protein (VDBP) have been shown to influence 25(OH)D concentrations (253). The rs4588 and rs7041 SNPs have been shown to cause changes in the VDBP structure that result in protein variants of VDBP: Gc1f, Gc1s, and Gc2 (253). These common variants affect the binding affinity of 25(OH)D to VDBP and the amount of 25(OH)D that can be used by cells and tissues (253). Higher frequency of the Gc1f variant in darker-skinned individuals may explain the greater affinity of VDBP in these individuals, and consequently, augmented transport of vitamin D metabolites. The Gc1f and Gc2 allele are both more commonly found among African-Americans, while the Gc1s allele is more commonly found among people with lighter skin (254).

Decreased concentrations of 7-dehydrocholesterol are observed with increasing age (250). Older age is also associated with decreased formation of 1,25(OH)2D (255). As renal function declines with age, there is a decrease in the activity of the renal enzyme 1α-hydroxylase that converts 25(OH)D into 1,25(OH)2D (255). A glomerular function rate of ≤ 50mL/min has been found to affect the production rate of 1,25(OH)2D production (256). As older age is associated with lower GFR and decreased the production of 1,25(OH)2D in an elderly population is common (255). Production of 1,25(OH)2D was found to be reduced by 50% as a result of an age-related decline in renal function, although serum 1,25(OH)2D levels are maintained in part by secondary hyperparathyroidism (257).

Another reason for the decrease in vitamin D synthesis is the geographical shift in the solar zenith angle. The solar zenith angle is the angle measured from directly overhead to the centre of the sun’s disc. This angle is a function of the time of year (season), time of day, and latitude (258). There is greater UV-B radiation reaching the earth’s surface at the smallest zenith angle which occurs during the summer. In contrast, the solar zenith angle is the largest during the winter, resulting in less UV-B radiation.

Co-morbidities may influence the amount of vitamin D in the body. Patients with malabsorption disorders such as cystic fibrosis, celiac disease, and Crohn’s disease (259) or
those who have had bariatric or gastric procedures (260,261) are often unable to absorb this fat-soluble vitamin. Vitamin D metabolism is also impaired in individuals with liver and kidney disease, resulting in lower levels of both vitamin D metabolites (262,263). Patients with nephrotic syndrome have been shown to lose 25(OH)D bound to the vitamin D binding protein (VDBP) in the urine (237). Individuals with chronic granuloma-forming disorders, some lymphomas, and primary hyperparathyroidism are also at high risk for vitamin D deficiency (264).

2.5.2.2 Modifiable factors

A key factor modulating cutaneous production of vitamin D is the amount of exposed skin, which is dependent on the amount of body covered with clothing and the use of sunscreen. Wearing sunscreen with sun production factor (SPF) of 30 reduces vitamin D synthesis in the skin by more than 95% (265). It is well-established that vitamin D levels are lower in winter months compared to summer months. This can be partially explained by less frequent outdoor time and more clothing in winter.

Obesity and body composition also influence vitamin D status. The literature has consistently shown that those with higher body mass index (BMI) have lower serum 25(OH)D levels, but the exact underlying mechanism has not been established (266–269). The inverse association between 25(OH)D and adipose tissue has been suggested to be a result of the sequestering of 25(OH)D in adipose tissue thereby decreasing its bioavailability (269–273). A recent study also suggests that volumetric dilution as a function of body weight explains low serum 25(OH)D levels in overweight and obese subjects (274).

In addition, vitamin D status is affected by certain medications, including anticonvulsant drugs, glucocorticoids, anti-estrogens, anti-retroviral drugs, and medications to treat AIDS/HIV. These drugs enhance the catabolism of both 25(OH)D and 1,25(OH)₂D (275). In contrast, oral contraceptives have been consistently shown to increase circulating levels of 25(OH)D (276–280).

2.5.3 Vitamin D status and deficiency

A normal vitamin D status is essential for human health. With the identification of 25(OH)D and 1,25(OH)₂D, methods were developed to measure these metabolites in the circulation. Plasma 25(OH)D concentration is the most commonly used measure of vitamin D status. Although 1,25(OH)₂D is the active vitamin D metabolite, it is not a good marker of vitamin D status due to its low plasma concentrations, short half-life, and tightly regulated production based on calcium requirements (281). Compared to the 7-hour half-life of 1,25(OH)₂D, the
serum half-life of 25(OH)D is approximately 25 days, which yields a more accurate indication of vitamin D stores over a longer period of time (282,283).

Currently, the most accurate method of measuring 25(OH)D is using isotope dilution ultra-high performance liquid chromatography (HPLC) and atmospheric pressure chemical ionization-mass spectrometry (284,285). This assay includes a lipid extraction of the serum followed by preparative chromatography; the 25(OH)D fraction is applied to HPLC and the UV absorption of 25(OH)D is used to measure its concentration. HPLC is considered to be the gold standard but was a very cumbersome assay, and, thus, is not routinely used by reference laboratories for clinical samples.

Though less accurate, cheaper and simpler assays for 25(OH)D are available. The first assays for 25(OH)D used the competitive protein binding format with VDBP as the binder (286). The advantage of this assay is that VDBP recognizes both 25(OH)D₂ and 25(OH)D₃, but disadvantage is that other vitamin D metabolites (i.e. 24, 25-dihydroxyvitamin D) are also detected and thus the assay is unable to separately quantify the two forms of 25(OH)D (vitamin D₂ and vitamin D₃) (287). In 1985, a radioimmunoassay (RIA) was developed for 25(OH)D (288). This assay (Diasorin) recognized both 25(OH)D₂ and 25(OH)D₃, but like the VDBP competitive protein binding assay, the RIA for 25(OH)D also recognized 24,25(OH)₂D and other polar metabolites to the same extent (288). Thus, both the VDBP and the RIA assays typically overestimate 25(OH)D levels by approximately 10-20% (286). A new RIA assay was developed which has a 100% specificity for 25(OH)D₃ and only 75% specificity for 25(OH)D₂ (286). Chemiluminescent immunoassay (CLIA) was also developed for identifying vitamin D metabolites. CLIA is an immunoassay technique where the label is a luminescent molecule (289). Chemiluminescent methods can be direct—using luminophore markers—or indirect—using enzyme markers (289). One CLIA—the Roche Elecsys Vitamin D Total assay—is a competitive chemiluminescence binding assay which uses ruthenium-labelled VDBP as the capture agent (290). One advantage of CLIA over other types of immunoassays is that this technique is can detect small amounts of vitamin D metabolites because light absorption is used as the detection method. However, CLIA also captures other vitamin D metabolites and tends to overestimate 25(OH)D (291). Recently, liquid chromatography tandem mass spectroscopy (LC-MS) was applied for the direct measurement of 25(OH)D in human serum. This assay quantitatively measured both 25(OH)D₂ and 25(OH)D₃ (292).

There is no absolute consensus as to what a normal range for 25(OH)D should be (237). Part of the difficulty is how a normal interval is determined; it is typically established by obtaining blood samples from several hundred volunteers and deeming them to be free of conditions that affect vitamin D status, perform the measurement of the analyte, and calculate a
distribution with a mean ± 2SD as the normal range (237). Several studies have observed that PTH levels began to plateau when 25(OH)D levels were between 75 – 100 nmol/L, suggesting that this may be the optimal interval for 25(OH)D (293–295).

Malabanan et al. conducted provocative tests by giving 50,000 IU of vitamin D once a week to healthy adults who had 25(OH)D values between 27.5 – 62.5 nmol/L for 8 weeks (296). At the end of 8 weeks, it was observed that 25(OH)D levels increased on average by more than 100%. An analysis of the change in PTH levels for each of the subjects revealed that the mean decrease of PTH declined by 55% in subjects who had 25(OH)D between 27.5 – 37.5 nmol/L and declined by 35% compared to those with 25(OH)D levels between 40.0 – 47.5 nmol/L. Subjects who had 25(OH)D >50 nmol/L had no significant change in their PTH level (296). Thus, based on these results, it was suggested that vitamin D deficiency should be defined as 25(OH)D above 50 nmol/L. Heaney et al. measured the efficiency of intestinal calcium absorption in women who had a mean 25(OH)D value of 50 nmol/L before and after taking 25(OH)D supplements to raise their blood level to a mean of 80 nmol/L (297). They reported a 45-65% increase in the efficiency of intestinal calcium transport when subjects had 25(OH)D of >80 nmol/L (297). Collectively, these results have lead most experts to agree that 25(OH)D of <50 nmol/L is considered to be vitamin D deficient whereas a 25(OH)D of 50 – 75 nmol/L is considered to be insufficient. The preferred level for 25(OH)D is now recommended by many experts to be >75 nmol/L (298–300).

The upper limit of normal has also been studied by different groups (299,301). The early established upper limit being 137 nmol/L seemed to be inadequate because lifeguards who are exposed to a lot of sunlight typically reported 25(OH)D levels of 250 – 312 nmol/L, but did not experience vitamin D intoxication (299,301). Based on the literature, it appears that vitamin D intoxication does not occur until blood levels are above 375 – 500 nmol/L (302,303). Currently, vitamin D intoxication is defined as a 25(OH)D >375 nmol/L, which is associated with hypercalcemia, hypercalciuria and often hyperphosphatemia.

Very few foods naturally contain or are fortified with vitamin D, so the major cause of vitamin D deficiency is inadequate exposure to sunlight (304–307). Vitamin D deficiency results in abnormalities in calcium, phosphate, and bone metabolism (235). Specifically, a deficiency in vitamin D causes a decrease in the efficiency of interstitial absorption of dietary calcium and phosphate, leading to an increase of PTH (237,308–310). Secondary hyperparathyroidism maintains serum calcium in the normal range, but at the expense of mobilizing calcium from bone and increasing phosphate wasting in the kidneys (235). The PTH-mediated increase in osteoclastic activity results in bone weakness and causes a general decrease in bone mineral density, which leads to osteopenia and osteoporosis. Phosphaturia
caused by secondary hyperparathyroidism results in lower phosphate levels, which causes a mineralization defect in the skeleton (237,311). In younger children who have little mineral in their skeleton, the mineralization defect results in rickets (299,312). In adults, the epiphyseal plates are closed and there is enough mineral in the skeleton to prevent skeletal deformities; as a result, the mineralization defect is known as osteomalacia often goes undetected (235). However, osteomalacia causes a decrease in bone mineral density and is associated with aches and pains in the bones and muscles (296,313). Vitamin D deficiency also causes muscle weakness. Affected children have difficulties standing and walking (298,312), whereas elderly patients have increased sway and more frequent falls, thereby increasing their risk of fracture (314,315).

Although the most well-known health benefit of vitamin D is for bone health, emerging evidence has suggested that vitamin D plays a role in a multitude of non-skeletal health outcomes, including cancer, autoimmune disorders, cardiovascular diseases, type 2 diabetes, and kidney disease (29,31,316). Unfortunately, the literature on these non-skeletal outcomes with vitamin D remains controversial regarding causation. Based on the review of data available, the Institute of Medicine (IOM) concluded that the only health outcome that was causally associated with vitamin D was bone health and that the evidence for vitamin D on non-skeletal health outcomes was not compelling. Currently, there are three large randomized controlled trials (VITAL, FIND, VIDAL) underway that are studying the effect of vitamin D supplementation on health outcomes such as cancer, heart disease, and diabetes (317–319).

### 2.5.4 Vitamin D and type 2 diabetes

In addition to the well-established effects of vitamin D on bone health, a wealth of evidence has suggested a potential role for vitamin D in several non-skeletal health outcomes such as autoimmune disorders, cardiovascular disease, cancer, and T2DM. In particular, emerging evidence has demonstrated a consistent association between vitamin D status and T2DM.

The exact mechanisms through which vitamin D may be associated with T2DM are still uncertain, although several possible mechanisms have been intensively investigated. Rat models have demonstrated that pancreatic insulin secretion is inhibited by vitamin D deficiency (17). Injection of 1,25(OH)2D significantly increases β-cell cytosolic Ca2+ levels, which promotes the exocytosis of insulin from islet cells (18). There is a direct effect of vitamin D on increasing β-cell function, and thus, suggesting an inverse effect on insulin resistance (320,321). Vitamin D can also bind to intracellular vitamin D receptors to regulate the body’s response to glucose by altering transcription of insulin receptor genes (19). The active vitamin D metabolite, 1,25(OH)2D, has been shown to bind to the intracellular vitamin
D receptor in the β-cell (320). Activation of vitamin D may also occur locally within the β-cell as 1α-hydroxylase is expressed inside β-cells (320). The vitamin D response element has been found in the promoter region of the insulin receptor gene and is thought to stimulate the expression of insulin receptors (321). Vitamin D may also have indirect effects on insulin resistance through the regulation of insulin-mediated intracellular processes by its regulation of the intracellular calcium pool (321). As well, vitamin D has been found to improve insulin sensitivity in peripheral tissues (20,21).

Studies using animal models have linked vitamin D deficiency with impaired insulin secretion (17,241,322). Consistent with these findings, ecological evidence suggests that high rates of metabolic disorders, such as T2DM, are found in areas further from the equator (323,324). Similarly, seasonal variation in glucose levels, insulin concentrations, and T2DM diagnoses have been reported; there are increased diagnosis and poorer control of glycemia in patients with T2DM in the winter months compared to the summer months (325,326). Several cross-sectional studies have reported a significant negative association between serum 25(OH)D and T2DM (327,328). In addition, prospective studies have consistently linked low serum 25(OH)D levels to incident diabetes (329–335).

However, despite the consistent findings from observational studies, results from randomized control trials (RCTs) involving vitamin D supplementation have been inconclusive to date. A pooled analysis of seven clinical trials of vitamin D supplementation did not show any beneficial effect on reducing incident diabetes (323). A recent systematic review and meta-analysis by George et al. concluded that supplementation does not affect glucose tolerance in those with normal glycemia, but there have been slight improvements to fasting glucose levels and insulin sensitivity in subjects with impaired glucose tolerance and diabetes (336). Unfortunately, most existing trials prescribed high doses of vitamin D in supplement form, are short in duration, may be at risk for selection bias, have incomplete reporting, and only included a small number of subjects. Nevertheless, trials which are designed specifically to investigate the effect of vitamin D supplementation on the risk of T2DM are still in progress (337).

**2.5.5 Vitamin D and kidney disease**

There are several mechanisms which link vitamin D to kidney dysfunction. It is well-established that there is reduced 1α-hydroxylase activity in patients with CKD; the 1α-hydroxylase enzyme is the last step in the conversion of 25(OH)D into the active vitamin D metabolite, 1,25(OH)₂D (263).
Emerging evidence suggests that the progression of chronic kidney disease (CKD) may be linked to low vitamin D levels and that patients with CKD have an exceptionally high rate of vitamin D deficiency compared to the general population (338). In addition, vitamin D also plays a part in the regulation of the renin-angiotensin-aldosterone system (339,340) and the nuclear factor κB pathway (340). These non-classical effects of vitamin D may play a relevant role in the mortality and morbidity of patients with CKD, specifically affecting the possible progression of renal disease and existing vascular diseases, both of which are major causes of death in this population (339,340).

New studies have established that not only does vitamin D maintain calcium and phosphate homeostasis, it also plays a role in cell differentiation and acts as an anti-proliferative factor with actions on the renal, cardiovascular, and immune systems (237,240,340–342). In CKD, the conversion from 25(OH)D to 1,25(OH)2D becomes impaired due to the loss of 1α-hydroxylase activity (343). As a result, the ability to maintain adequate serum calcium is impaired and may lead to secondary hyperparathyroidism, increased bone turnover, and risk of metabolic bone disease. Decreased clearance of phosphate in the urine with the consequent increase in serum phosphate further complicates this picture by stimulating PTH and boosting production of fibroblast growth factor 23, which further suppresses renal 1α-hydroxylation of 25(OH)D (344).

Given the impaired 1α-hydroxylase activity associated with CKD, most of the research on vitamin D replacement has focused on the 1,25(OH)2D form of this vitamin. However, the deficiency of 25(OH)D has been linked to complications in ESRD (345). Thus, the nutritional form, 25(OH)D, may be important in the CKD population as well. Deficiency of 25(OH)D is common in the CKD population, and deficiency appears to be independently associated with an increased risk of mortality in retrospective studies (346,347). Treatment with vitamin D2, the nutritional form of vitamin D, has been shown to be an effective initial therapy for hyperparathyroidism in stage 3 CKD patients, but the benefits for this treatment in stage 4 CKD are not clear (30). As well, the discovery of 1α-hydroxylase outside of the renal system raises the possibility of 25(OH)D providing additional value in the management of CKD (348).

In animal models, there are emerging studies to suggest that supplementation of active vitamin D could play a role in slowing CKD progression. Using rats that have undergone subtotal nephrectomy, supplementation with vitamin D and its analogues was found to prevent glomerulosclerosis (349,350). In the same rat model, vitamin D treatment decreased podocyte injury, loss, and hypertrophy, resulting in decreased albuminuria (351). Vitamin D receptor knockout mice developed more severe albuminuria and glomerulosclerosis compared to wild-type in a streptozotocin-induced model of diabetes (352). Therapy with
1,25(OH)\(_2\)D has been shown to decrease albuminuria in numerous animal models (349,352–355).

Common complications of CKD—such as atherosclerosis and vascular calcification—have also been studied in animal models. An early model of atherosclerosis found that rats developed atherosclerosis when given an extremely high dose of vitamin D\(_2\) (356). Interestingly, there is potentially an important difference in action depending on the dose of the vitamin D analog. In a mouse model of kidney disease, low levels of vitamin D were protective against vascular calcification, whereas higher doses were associated with more calcification (357).

In humans, multiple observational studies have shown low levels of both vitamin D metabolites in patients with CKD and ESRD (347,358). Low 25(OH)D in patients with CKD and ESRD has been associated with a higher risk of all-cause mortality and a faster progression of kidney disease (347,359–361). However, observational studies of vitamin D levels may be potentially confounded by patients having low vitamin D levels due to less sun exposure or poor nutrition.

Fortunately, there are several clinical trials assessing vitamin D supplementation on kidney outcomes. Adequate supplementation of vitamin D in deficient CKD populations has been found to potentially reduce premature morbidity and mortality (339,340). In a study of 242 patients with end-stage renal disease (ESRD), Shoji et al. found better survival in subjects who received oral alfacalcidol (1-hydroxyvitamin D, which is converted to 1,25(OH)\(_2\)D by the liver) (362). In a separate study using ESRD patients, there was a 26% reduction in mortality associated with intravenous vitamin D treatment (363). Tentori et al. conducted a study with nearly 15000 patients on hemodialysis and found a similar result associated with vitamin D analogue therapy (paricalcitol, calcitriol, or doxercalciferol) compared to those receiving no treatment (364). Kalantar-Zadeh et al. conducted a large study with more than 58000 hemodialysis patients and found that paricalcitol administration was associated with improved survival compared to those who received no vitamin D (365). An association was found between oral calcitriol and improvement of survival in 520 pre-dialysis patients with CKD stages 3 – 5, suggesting that the protective effect of vitamin D may extend to patients with more moderate kidney disease (32). A meta-analysis by Palmer et al. examined data from 76 randomized control trials of vitamin D compounds in CKD where the outcome was an improvement of hyperparathyroidism (33). Overall, they found that vitamin D compounds did not show a consistent reduction in PTH levels and that for suppression of PTH, intravenous administration was superior to oral vitamin D, but this may be due to higher doses used for intravenous methods (33). It should be noted that the meta-analysis
combined outcomes from both pre-dialysis and dialysis patients, as well as both adult and pediatric populations; heterogeneity in the effects of vitamin D on the different populations could lead to a failure to detect important influences in the outcomes. These findings emphasize the importance of well-controlled trials that examine clinical outcomes, rather than potential surrogates that may not accurately predict mortality and morbidity. Although observational studies appear to be consistent, results from randomized trials with clinical outcomes are inconclusive. The biology of vitamin D’s effects on CKD progression, however, are strongly supportive.

Several small, randomized control trials have recently evaluated the effect of active vitamin D therapy on albuminuria. One study using 61 subjects found lower urinary protein-to-creatinine ratios and lower PTH levels in patients taking paricalcitol compared to those in the placebo group (366). The results were supported by another study, which showed lower C-reactive protein levels and lower rates of 24-hour albumin excretion in subjects taking paricalcitol compared to placebo (36). Another large, placebo-controlled, double-blind, randomized control trial (VITAL study) in 281 subjects with T2DM concluded with similar findings (367). The results showed that there was a significant reduction in the urinary albumin-to-creatinine ratio (ACR) in subjects taking 2 μg of paricalcitol compared to placebo. This was associated with a lowering of eGFR calculated from serum creatinine. The dose-dependent response of paricalcitol on eGFR was observed even after 12 weeks: mean eGFR was 2 ml/min/1.73m² lower in subjects who received the 1 μg dose and 4 ml/min/1.73m² lower in subjects on the 2 μg dose. The authors hypothesized that the lowering of eGFR (increase in serum creatinine) is caused by an effect of paricalcitol on creatinine metabolism (367). Interestingly, a recent study showed that in a small group of participants with kidney disease, paricalcitol increased serum creatinine levels without affecting iothalamate GFR estimates (368). However, it is still unclear whether the improvement in albuminuria for those taking paricalcitol translates to better clinical outcomes, such as slower progression to dialysis.
2.6 Vitamin D Binding Protein

Steroid hormones such as vitamin D metabolites are lipids, meaning that rather than being transported by lipoproteins such as cholesterol, these cholesterol derivatives are bound to specific carrier proteins in circulation. These carrier proteins are thought to keep steroids in a biologically inactive form and regulate the amount of free hormone that would enter the target cells by passive diffusion (369).

The vitamin D binding protein (VDBP), also known as GC-globulin, is a multifunctional protein known for its role in the transport of vitamin D metabolites. In addition to vitamin D metabolites, VDBP also binds fatty acids and actin monomers, preventing their polymerization that could be detrimental to the circulatory system (370).

With the recent increased attention regarding the benefits of vitamin D (bone health and immunological regulation), there has been a resurgence of interest in VDBP. Since VDBP is the primary transporter of vitamin D metabolites, it has a role in maintaining the total levels of vitamin D and in regulating the amounts of free (unbound) vitamin D available for specific tissues and cell types to use.

2.6.1 Physiology

In the circulation, VDBP—which has a very high affinity for 25(OH)D, and somewhat lower affinity for 24,25(OH)₂D, and 1,25(OH)₂D—carries 85-90% of vitamin D metabolites (27). VDBP has a high homology to albumin (232). The vitamin D binding protein acts as the main plasma carrier for vitamin D metabolites. This 50 kDa protein has the highest affinity for 25(OH)D and is present in 100-fold molar excess in the plasma, meaning that virtually all 25(OH)D is bound to its carrier protein (371). Since VDBP is the major transporter of vitamin D metabolites, it plays an important role in maintaining total levels of vitamin D and in regulating the amount of free (unbound) vitamin D available. It was originally thought that VDBP regulates the bioavailability of vitamin D metabolites, protecting the organism against excessive amounts of the free vitamin (372). However, this concept was challenged by the findings in VDBP knockout mice (373). Rather than having higher levels of 25(OH)D and 1,25(OH)₂D as expected, these animals have significantly reduced plasma levels of vitamin D metabolites (373). On a vitamin D depleted diet, the animals suffered from vitamin D deficiency and bone formation defects. Additionally, 25(OH)D was inappropriately delivered to the liver, where it was catabolized, or was lost in the urine because of the lack of uptake by the kidney. The crucial role of this carrier molecule for targeting the activation of 25(OH)D was further supported by the observation that VDBP knockout mice are protected, rather than sensitized, to vitamin D intoxication (373).
The human VDBP gene consists of 13 exons and 12 introns residing on chromosome 4 at 4q11-q1340 near a cluster of genes coding for related albumin proteins: albumin, α-fetoprotein (AFP), and afamin (AFM) (374). A clone containing a 105kb human VDBP genome fragment was able to confer tissue-specific, high-level expression, suggesting that VDBP's regulatory sequences resided in this stretch of the genome and was independent of the regulatory regions of albumin-related protein genes 1.5Mb away (375). Analysis of the proximal promoter of the VDBP gene revealed three hepatocyte nuclear factor-1 (HNF-1) binding sites that serve as targets for HNF-1α and HNF-1β transcription factors (376). VDBP is post-translationally modified by cleavage of the N-terminal 16-amino-acid leader and glycosylation, resulting in a protein with a molecular weight of 52 – 59kDa (377). The three major polymorphic forms—GC1F, GC1S, and GC2—are composed of identical amino acid sequences except at positions 416 and 420 (378,379); relative to GC1F, GC1S has glutamic acid instead of aspartic acid at residue 416, and GC2 has lysine instead of tyrosine at amino acid 420 (370). Polymerase chain reaction has been used to detect VDBP mRNA in various organs; VDBP is highly expressed in the liver, and at much lower levels in the kidneys, yolk sac, testis, and abdominal fat (380).

Similar to albumin, the crystal structure of VDBP has numerous α-helical structures that form three structurally similar domains (381,382). However, the overall 3-dimensional arrangement of the domains yields a structure different from albumin. The first domain contains 10 α-helices. Helices 1 – 6 of this domain form a surface vitamin D binding cleft (383). The second domain is similar, except that a coil-fold replaces helix 7 (383). The third domain is truncated at the C-terminal end, and contain only four α-helices (383).

In studies using transgenic knockout mice that were either heterozygous (carried one functional copy) or homozygous (had no functional copies) for the VDBP allele, researchers found that total 25(OH)D and 1,25(OH)2D levels were dependent on the number of functional VDBP alleles (373). Specifically, they measured 25(OH)D levels and found a dose-dependent response with VDBP allele number; wild-type mice, heterozygous, and total knockout mice had 25(OH)D levels of 85nM, 52nM, and 6.2nM, respectively (373). A similar response was observed for 1,25(OH)2D as well, with wild-type, heterozygous, and total knockout mice showing 58pM, 41pM, and 7pM of 1,25(OH)2D, respectively (373). Interestingly, in humans, no VDBP null individuals have been identified, although one allele has been found to have a low expression but a normal affinity for ligands (384).

The measured affinity constant for 25(OH)D binding to VDBP was found to be 7 x 10^8 M^-1, which is many folds greater than 25(OH)D’s binding affinity for albumin (6 x 10^5 M^-1) (385). However, because albumin is more abundant than VDBP, even at a much lower affinity,
about 10% of total 25(OH)D is bound to albumin (385). VDBP affinity for 1,25(OH)₂D is also much greater than albumin’s affinity, at 4 x 10⁷ M⁻¹ and 5.4 x 10⁵ M⁻¹, respectively (386). Thus, only minute quantities of 25(OH)D and 1,25(OH)₂D exist in the free, unbound form, and because VDBP is in vast excess compared to vitamin D ligands, most VDBP circulates without any vitamin D metabolites attached (253). Other ligands that bind to VDBP include actin and fatty acids (387).

2.6.2 Reabsorption of VDBP

The final step in the activation of vitamin D is the conversion of 25(OH)D to 1,25(OH)₂D in renal proximal tubules. The renal proximal tubule possesses an extensive apical endocytic apparatus involved in the reabsorption of molecules filtered in the glomeruli. Several key receptors appear to be involved in this function, which serves not only to conserve protein but also reabsorb different vitamins in complex with their binding proteins (371). Recent studies have shown that 25(OH)D is specifically targeted to the proximal tubule through its carrier protein, the VDBP and that this complex is actively internalized by the endocytic apparatus of the proximal tubule that is involved in the reabsorption of molecules filtered by the glomeruli (388).

Megalin is a single-spanning transmembrane protein belonging to the low-density lipoprotein receptor family that is able to internalize many ligands such as vitamin-binding proteins, lipoproteins, hormones, albumin, sex hormone-binding globulin, drugs, and thyroglobulin (389–394). This endocytic receptor is expressed in the proximal tubule cells of the kidney. VDBP, a megalin ligand, is freely filtered across the glomerulus. In the proximal tubule, reabsorption of the VDBP + 25(OH)D complex by megalin endocytosis facilitates the hydroxylation of 1,25(OH)₂D via 1α-hydroxylase (371).

The role of the endocytic apparatus as the retrieval mechanism for the VDBP + 25(OH)D complex by the proximal tubule cells was suggested in mice carrying a disruption of the megalin gene (395). Megalin knockout mice developed vitamin D deficiency and bone disease owing to an inability of the proximal tubules to capture the VDBP + 25(OH)D complexes from the glomerular filtrate, which then become lost in the urine (395). These animals had a decrease of >70% in plasma levels of 25(OH)D and 1,25(OH)₂D (395). Almost all of the megalin-deficient mice died perinatally from developmental defects of the forebrain and/or from respiratory failure; only 1 out of 50 of these megalin knockout mice survived to adulthood (396). A mouse model with a kidney-specific megalin gene defect has recently been produced, generated using the Cre/loxP system (397). The kidney-specific megalin knockout mice were viable and fertile, and the renal expression of megalin was decreased by as much as 90% (397). In these kidney-specific megalin knockout mice, severe
plasma vitamin D deficiency, hypocalcemia, and severe bone disease were observed. The megalin-mediated uptake mechanism also appears to be functional in breast epithelial cells, where fluorescently tagged VDBP uptake has been observed by microscopy and blocked by receptor-associated protein as a competitive inhibitor for megalin binding (398). Megalin-mediated uptake of 25(OH)D-bound VDBP has also been suggested to occur in bone cells, although megalin expression was demonstrated by PCR only (399). The importance of megalin as the capture molecule for VDBP was further confirmed in other animal models of reduced receptor function. Rats treated with maleate, a substance that causes shedding of megalin, had an increased excretion of VDBP (400).

Cubilin is another receptor identified in the proximal tubule endocytic apparatus. Structurally, it is a very different peripheral membrane protein with no obvious transmembrane domain. The molecule is dominated by 27 CUB domains, which are involved in ligand binding (394). Since cubilin lacks transmembrane or cytoplasmic domains required for endocytosis, this receptor associates with megalin to recycle and internalize its ligands (401). The role of cubilin in the renal uptake of VDBP was identified when VDBP affinity chromatography was applied to identify membrane proteins in the kidney that could be involved in the uptake of VDBP + 25(OH)D complexes (402).

The endoplasmic reticulum-resident chaperone receptor-associated protein (RAP) is important for normal processing of megalin in kidney proximal tubules (403). In RAP knockout mice, expression of megalin is reduced by 50%, and this reduction is correlated with increased urinary excretion of VDBP (404). Interestingly, while RAP blocked VDBP binding to megalin, binding to cubilin remained unperturbed (401). To investigate the relative contribution of cubilin and megalin in the uptake process, the internalization of VDBP + 25(OH)D by BN/MSV cells in the presence of antibodies specific for each of the two receptors was tested. The addition of anti-cubilin antibodies inhibited the cellular uptake of VDBP + 25(OH)D by up to 70% while the control immunoglobulin G (IgG) had no effect. Anti-megalin antibodies produced a similar reduction in VDBP + 25(OH)D endocytosis. When both antibodies were applied, VDBP + 25(OH)D was only slightly more impaired (around 80%), suggesting that cubilin and megalin function through the same endocytic pathway (402).

Since VDBP knockout mice did not exhibit the same drastic phenotype as the megalin knockout mice, it is probable that in VDBP knockout mice, mechanisms independent of the 25(OH)D-VDBP-megalin uptake—such as 25(OH)D-albumin-megalin uptake or megalin-independent processes—can recover adequate amount of vitamin D to compensate (27). In addition, there appears to be a non-megalin VDBP entry mechanism as several studies have reported VDBP entry into cells that are unlikely to express megalin (405–409). Although not
yet confirmed, it is hypothesized that VDBP interaction with actin on membranes may play a role in this type of entry (407,409).

In summary, megalin and cubilin have similar endocytic pathways and are essential proteins for the normal reabsorption of VDBP. However, there may be uptake pathways for 25(OH)D independent of VDBP which allows for adequate recovery of vitamin D when VDBP function is compromised.

2.6.3 Impact of VDBP loss on vitamin D status
Since the majority of circulating 25(OH)D and 1,25(OH)₂D is tightly bound to VDBP and albumin, with less than 1% circulating in an unbound form, factors affecting VDBP alter the status of vitamin D metabolites. Clinically, increased urinary loss of VDBP has been associated with tubular dysfunction in patients with nephrotic syndrome (410), renal Fanconi syndrome (411), and Itai-itai disease (412).

There are supportive animal data which have focused on low vitamin D status as a result of renal reabsorption problems in diabetic rats (413). Excessive urinary excretion of the 25(OH)D + VDBP complex has been found in rats with both T1DM and T2DM (414–416). A digestion-resistant starch diet prevented urinary excretion of VDBP in rats with T1DM, indicating a potential pathway between glucose and VDBP elimination (416). Further study by Koh et al. investigated whether feeding resistant starch that stopped urinary excretion of VDBP could similarly prevent vitamin D loss in diabetic rats (415). It was found that rats fed the control diet had 89% and 97% higher urinary excretion of 25(OH)D and 1,25(OH)₂D, respectively. Serum 25(OH)D levels were also 31% lower in the control group. Histopathologic examinations of the kidneys revealed that the resistant starch diet attenuated diabetes-mediated damage by 21%, suggesting that loss of VDBP plays a role in lower vitamin D status and kidney damage.

Research has found that patients with nephrotic syndrome and renal failure have low serum concentrations of 25(OH)D (417). It is possible that the downstream effect of the low 25(OH)D substrate results in lower plasma levels of other vitamin D metabolites, such as 1,25(OH)₂D and 24,25(OH)₂D; a deficiency of these compounds causes defective intestinal absorption of calcium and resistance to the calcemic actions of PTH, leading to hypocalcemia. An experiment was conducted in 12 patients with nephrotic syndrome compared to participants with normal renal function. Blood levels of 25(OH)D, 1,25(OH)₂D, and 24,25(OH)₂D were all significantly lower in those with nephrotic syndrome compared to subjects with normal renal function (p<0.001). In addition, the calcemic response to PTH in nephrotic syndrome subjects was significantly lower than normal. The results indicate that a
deficient state of all studied vitamin D metabolites exists in patients with nephrotic syndrome and this abnormality underlies the resistance to the calcemic response to PTH (417).

A strong negative correlation has been observed between serum concentrations of total and free 1,25(OH)\(_2\)D with worsening renal function and urinary VDBP loss (418). Patients on peritoneal dialysis and nephrotic patients had significantly lower levels of serum VDBP and a higher percentage of free 1,25(OH)\(_2\)D compared to normal subjects (418). A retrospective, cross-sectional study using 472 individuals by Blanton et al. examined whether serum levels or genotypes of VDBP associate with vitamin D status in subjects with T1DM (14). Single nucleotide polymorphism (SNP) typing for VDBP polymorphisms (SNP rs4588 and rs7041) was performed on this cohort. They found that serum VDBP levels were highest in healthy control subjects, intermediate in first-degree relatives with type 1 diabetes, and lowest in type 1 diabetic patients (14). However, VDBP levels did not associate with serum vitamin D levels, age, or disease duration. No differences in genotype frequencies of the VDBP polymorphisms were associated with serum VDBP levels or between type 1 diabetic patients and control subjects (14). Another cross-sectional study evaluated 25(OH)D and 1,25(OH)\(_2\)D status in relation to VDBP in patients with CKD and long-term hemodialysis (HD) patients (419). Plasma 25(OH)D was determined using chromatography and serum and urine VDBP concentrations were assayed using ELISA. They found that plasma 25(OH)D levels were lower in subjects with CKD and HD compared to healthy controls. Serum VDBP was significantly higher in those with CKD compared to other groups. There was no correlation found between serum vitamin D and VDBP, but the urinary concentration of VDBP in CKD was correlated with proteinuria (419). However, one limitation of the study was that 25(OH)D was assessed in plasma while VDBP was measured in the serum.

Regarding VDBP in T2DM, a recent study by Rahman et al. investigated the association of serum vitamin D levels and vitamin D binding protein gene polymorphisms with the onset of T2DM (420). In agreement with past studies, they reported a significantly lower level of vitamin D in type 2 diabetic patients compared to control patients. There was also a negative correlation between vitamin D levels and fasting blood glucose among those with T2DM. The Glu/Glu codon at 416 (rs7041) and Lys/Lys at codon 420 (rs4588) variants of the VDBP gene were significantly more common in T2DM subjects compared to controls. The subjects with Glu/Glu and Lys/Lys genotypes respectively at codon 416 and 420 were at high risk of developing type 2 diabetes, suggesting that there may be an association of vitamin D and VDBP gene polymorphisms with the occurrence of T2DM (420). In an urban Pakistani population, a case-control study was conducted on 330 subjects with T2DM and their age- and gender-matched controls to assess if genotypes/diplootypes of vitamin D binding protein have any association with T2DM (421). Mean serum concentration of 25(OH)D was
significantly higher among the patients compared to the controls, but not significantly different by genotypes or diplotypes. However, multiple conditional logistic regression revealed an association of group-specific 1-2 genotype of the VDBP gene with the risk of T2DM (421). A meta-analysis of 6 studies by Wang et al. examining the relationship between VDBP polymorphisms and risk of T2DM found that there were no significant associations between codon 416 and codon 420 polymorphisms of the VDBP gene and the risk of T2DM in the overall analyses (422). In stratified analysis, significant associations between the codon 420 polymorphism and T2DM were found in Asians but not in Caucasians (422). For the codon 416, the significant association with T2DM was also detected in Asians but not in Caucasians (422).

Thrailkill et al. investigated potential mechanisms contributing to proteinuria in patients with nephropathy using a proteomics approach (423). The urine proteome of 12 healthy non-diabetic subjects was compared with subjects with type 1 diabetes. They found an abundance of megalin and cubilin in subjects with T1DM and microalbuminuria compared to those without diabetes and proteinuria. The findings suggest that the increased loss of megalin and cubilin could contribute to increased protein spillage in diabetes and to deficient states of important vitamins and hormones. In a later study by the same group, researchers found that urinary concentrations of VDBP were increased in subjects with T1DM compared to healthy controls (12). Multivariate regression modeling revealed that urinary VDBP (uVDBP) correlated with serum 1,25(OH)2D concentrations (β=0.607, p=0.037) (12). Vitamin D deficiency and insufficiency were also slightly more prevalent in T1DM subjects with albuminuria, suggesting that the loss of VDBP may be related to lower vitamin D status in this population (12). In a later study conducted by Tian et al., urine samples from 160 healthy and T2DM subjects showed that urinary expression of VDBP was significantly elevated in patients with T2DM, and that renal VDBP loss increased with the severity of nephropathy (13). However, it should be noted that the study only included Han Chinese individuals and results may not be generalisable to other population groups. Taken together, these findings suggest that uVDBP may be a potential biomarker for the early detection of diabetic nephropathy, but further studies are required to examine the pathogenic mechanisms of elevated VDBP levels and their role in kidney dysfunction.

2.7 Knowledge gaps and rationale

Although it has been well-established that a strong connection exists between low vitamin D levels, kidney dysfunction, and type 2 diabetes, it is unclear whether hypovitaminosis D is a
cause or consequence of the disease. Results from randomized control trials regarding vitamin D supplementation among individuals with T2DM have been inconsistent to date, potentially due to the complex disease mechanisms, differences between ethnicities, small sample sizes, short duration of trials, variable doses, and/or differences in outcomes (15). Many studies have investigated the hypothesis that lower vitamin D levels may lead to the development of T2DM (20,22,420–422), but lower vitamin D status may also be a consequence of T2DM. Systemic inflammation in chronic diseases like diabetes tends to lower serum levels of vitamin D (424,425). In addition, it is likely that the vitamin D binding protein may play a role in low vitamin D status in diabetic subjects with kidney dysfunction. The paper published by Koh et al. found that when T1DM rats were unable to spill VDBP in the urine, they had a reduced renal loss of both vitamin D metabolites and higher serum 25(OH)D (415). In humans, Thraikill et al. reported higher urinary VDBP loss in T1DM compared to healthy controls, and Tian et al. later conducted a study using subjects with T2DM which showed that uVDBP loss increases with dysglycaemia, but also increases with the severity of nephropathy (13,423). Together, these results suggest that it is likely that 25(OH)D is still bound to the VDBP and the resulting complex is lost in renal excretion when kidney problems arise in patients with diabetes.

However, there is still only limited research available on the renal loss of VDBP in humans. The existing studies are short in duration and involve a small number of subjects. Few studies have examined the relationship between urinary VDBP loss and kidney damage markers, and it is unclear if VDBP loss is contributing to lower serum 25(OH)D levels cross-sectionally or longitudinally. In addition, no studies to date have examined the association of urinary VDBP with eGFR and albuminuria as measures of kidney function. Most importantly, the utility of urinary vitamin D binding protein to predict declines in kidney and vitamin D outcomes over a long period of time has not been established.
CHAPTER 3
Objectives and Hypotheses

3.1 Objectives

1. **A)** To examine longitudinal associations between urinary vitamin D binding protein and kidney dysfunction as assessed by albumin-to-creatinine ratio (ACR) and estimated glomerular filtration rate (eGFR).
   **B)** To assess if baseline urinary vitamin D binding protein concentrations are associated with declines in kidney function over time.

2. **A)** To study longitudinal associations between urinary vitamin D binding protein concentrations and serum 25(OH)D levels.
   **B)** To evaluate if baseline urinary vitamin D binding protein concentrations are associated with changes in vitamin D status prospectively.

3.2 Hypotheses

1. **A)** High urinary vitamin D binding protein levels will be associated with a higher ACR and lower eGFR over time.
   **B)** Higher baseline urinary vitamin D binding protein concentrations will be associated with worse kidney outcomes as assessed by these measures prospectively.

2. **A)** Subjects with high urinary vitamin D binding protein will have lower serum 25(OH)D concentrations longitudinally.
   **B)** Urinary vitamin D binding protein loss at baseline will be associated with worse vitamin D status over time.
CHAPTER 4

The Utility of Longitudinal Urinary Vitamin D Binding Protein Loss as a Marker for Kidney Tubular Dysfunction
CHAPTER 4
THE UTILITY OF LONGITUDINAL URINARY VITAMIN D BINDING PROTEIN LOSS AS A MARKER FOR KIDNEY TUBULAR DYSFUNCTION

4.1 Abstract

**Background:** Recent studies have reported elevated urinary vitamin D binding protein (uVDBP) concentrations in patients with diabetic kidney disease, although the utility of uVDBP to predict declines in kidney function over time has not been examined. Our objective was to assess the association of uVDBP with longitudinal declines in kidney function.

**Methods:** Data were derived from PROMISE, a cohort of adults at risk for type 2 diabetes. We used data from 3 clinical visits over 6 years (n = 729). Urinary albumin-to-creatinine ratio (ACR) and eGFR were used as measures of kidney function. Measurements of uVDBP were performed with ELISA and normalized to urine creatinine. Generalized estimating equations (GEE) evaluated longitudinal associations of uVDBP with measures of kidney function. Covariates included visit number, baseline age, sex, ethnicity, and glycemic status.

**Results:** Renal VDBP loss increased with ACR severity at baseline. Subjects with normoalbuminuria, microalbuminuria, and macroalbuminuria had median log uVDBP concentrations of 1.62 μg/mmol, 2.63 μg/mmol, and 2.48 μg/mmol, respectively (all p<0.001), and ACR positively correlated with uVDBP concentrations (r=0.38, p<0.001). There was no significant association between uVDBP and eGFR at baseline (p=0.67). Adjusted GEE models indicated that each SD difference in uVDBP was associated with higher ACR (β=32.21, p<0.001). Baseline uVDBP was also associated with higher ACR over 6 years (β=29.90, p<0.001). However, uVDBP was not associated with changes in eGFR longitudinally.

**Significance:** These results suggest that loss of uVDBP over time may be a useful marker for predicting renal tubular damage in subjects at risk for diabetes.
4.2 Introduction

Chronic kidney disease (CKD) is a risk factor for progression to end-stage renal disease, adverse cardiovascular events, and overall mortality (426–429). Although there are numerous determinants of CKD, diabetes is the most common cause worldwide (112). Among the numerous complications associated with diabetes, approximately 50% of patients develop kidney damage in their lifetime, and 10 – 40% of those will eventually suffer from kidney failure. Unfortunately, kidney disease usually progresses silently, with substantial declines in function prior to clinical detection (430,431). Therefore, early detection is important in lessening the burden of kidney damage in this population.

Although microalbuminuria is a widely used indicator of kidney dysfunction, its diagnostic accuracy is limited by the fact that structural damage might precede albumin excretion (432). Studies have shown that advanced structural alterations in the glomerular basement membrane may already have occurred by the time albuminuria becomes clinically evident (433,434). Tubular damage also plays a major role in the development of nephropathy, therefore, sensitive and specific biomarkers that can detect the severity of kidney dysfunction are needed (435). In addition to testing for albumin in the urine, the estimated glomerular filtration rate (eGFR) is widely used clinically as an indication of kidney function at the level of the glomerulus (134).

Recently, a number of papers have reported elevated urinary concentrations of vitamin D binding protein (VDBP) in animal models and patients with diabetes (413,436,437). These observations support the hypothesis that urinary VDBP may be a useful early biomarker for the detection of kidney dysfunction in diabetic nephropathy (435,436,438–441). In particular, previous literature has shown that loss of VDBP tends to increase with the severity of kidney dysfunction in humans (12,13,439). Thrailkill et al. found higher urinary VDBP concentrations in subjects with type 1 diabetes compared to healthy controls (12). Both Tian et al. and Khodeir et al. reported that urinary VDBP levels were significantly elevated in patients with T2DM with microalbuminuria and T2DM with macroalbuminuria compared to controls with T2DM and normoalbuminuria (13,439). However, these studies have been limited by cross-sectional designs and small sample sizes. In addition, the utility of uVDBP to predict declines in kidney function over time has not been examined. Thus, the objective of this paper was to examine the association of renal vitamin D binding protein loss with declines in kidney function over 6 years of follow-up.
4.3 Methods

4.3.1 Study Population
The study used data from the Prospective Metabolism and Islet Cell Evaluation (PROMISE) cohort study (442,443). PROMISE is a longitudinal observational study of participants with one or more risk factors for type 2 diabetes mellitus, including obesity, hypertension, family history of diabetes and/or a history of gestational diabetes or birth of a macrosomic infant. Between May 2004 and December 2006, participants aged 30 years and older were recruited from the general population into the PROMISE cohort (n=736). Fasting blood samples were collected and 75 g oral glucose tolerance tests (OGTT) were conducted with additional blood samples collected at 30 and 120 minutes for glucose and insulin measurements.

After excluding subjects with hemolysed urine samples (n=6), 729 subjects with uVDBP measures remained at baseline. Among these, 558 returned for the 3-year follow-up and 486 for the 6-year follow-up (FIGURE 4.1). Annual telephone contact is maintained with participants and follow-up clinical visits occur every three years. At each clinic visit, participants underwent extensive metabolic characterization, anthropometric measurements, blood and spot urine sample collection, and structured questionnaires which assessed ethnicity, smoking history, family history of diabetes, and other important covariates such as socioeconomic status.

Research ethics approval was obtained from Mount Sinai Hospital and the University of Western Ontario. Research nurses at the respective institutions were centrally trained on the standardized procedures for conducting the characterizations.
4.3.2 Anthropometric measures

Anthropometric measurements were determined twice, and averages were used in the analyses. Height, weight, and waist circumference (WC) were measured at all clinic visits using standard procedures. WC was measured at the natural waist, identified as the narrowest part of the torso between the umbilicus and the xiphoid process. Height was measured using a stadiometer, without shoes, and back straight against the wall with the head positioned in the Frankfurt plane. Weight was measured on a medical balance beam scale in light clothing and with shoes off. Body-mass index was calculated using the formula:

\[ BMI = \frac{\text{weight (kg)}}{\text{height (m)}^2} \]
Blood pressure was measured twice on the right arm with the participant seated after 5 minutes of resting using an automated sphygmomanometer. Mean arterial pressure (MAP) was calculated from systolic blood pressure (SBP) and diastolic blood pressure (DBP) using the formula:

$$MAP = \frac{SBP + 2(DBP)}{3}$$

### 4.3.3 Urinary measures

A morning spot urine sample was collected for the determination of albumin, creatinine, estimated glomerular filtration rate (eGFR), and VDBP. Urinary albumin was measured using the Roach/Hitachi MODULAR P analyzer. The lower detection limit of 54 μmol/L was calculated as the value lying 3 standard deviations (SD) above that of the lowest standard. Urinary albumin and creatinine were used to calculate the albumin-to-creatinine ratio (ACR):

$$ACR = \frac{\text{urine albumin (mg/L)}}{\text{urine creatinine (mmol/L)}}$$

ACR was used to determine the severity of albuminuria based on guidelines from Diabetes Canada (4). Clinical categories included normoalbuminuria (ACR <2 mg/mmol), microalbuminuria (ACR 2-20 mg/mmol), and macroalbuminuria (ACR >20 mg/mmol).

Estimated GFR was calculated using the CKD-Epi equation and serum creatinine (444). Clinical cut-offs of eGFR (normal, mild kidney disease, and moderate kidney disease) were based on guidelines from Diabetes Canada (4); normal GFR was defined as >90 ml/min/1.73², mild kidney disease was eGFR between 60 – 89 ml/min/1.73², and moderate kidney disease included subject whose eGFR was <60 ml/min/1.73². The R package nephro was used to calculate eGFR following the CKD-Epi formulae (445):

If female and serum creatinine ≤0.7 mg/dl:

$$144 \times \left(\frac{\text{creatinine}}{0.7}\right)^{-0.329} \times 0.993^{Age} \times 1.159 \text{ if black}$$

If female and serum creatinine >0.7 mg/dl:

$$144 \times \left(\frac{\text{creatinine}}{0.7}\right)^{-1.209} \times 0.993^{Age} \times 1.159 \text{ if black}$$

If male and serum creatinine ≤0.9 mg/dl:
Urinary VDBP was measured at Sunnybrook Research Institute. Measurements of uVDBP were assayed by manual enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik, catalog # K2314, Bensheim, Germany) following manufacturer instructions. For urine samples, the obtained VDBP value is multiplied by a dilution factor of 10 (446). Samples with concentrations above the measurement range were further diluted and re-assayed. The upper limit of the measurement range was calculated as the highest concentration of the standard curve x sample dilution factor (60 ng/mL). The lower limit of the measurement range was calculated as analytical sensitivity x sample dilution factor used (1.23 ng/mL) (446). The coefficients of variation (CV) for within-run and between-run are respectively 5.01% and 6.02%.

4.3.4 Metabolic and blood measures

Blood samples were drawn after an 8 – 12 hour overnight fast at each clinic visit to measure nutritional, liver, adipose, inflammatory, and kidney biomarkers. Following the fasting blood sample collection, a 75 g OGTT was conducted, with additional blood samples being drawn at 30 minutes and 2-hours post-glucose load. OGTT blood samples were immediately processed for the determination of serum glucose, and remaining samples were processed and frozen at -70°C for the determination of blood biomarkers. Glucose was determined using an enzymatic hexokinase method on the Roche Modular platform (Roche Modular, Roche Diagnostics) with a detection range of 0.11 (2 mg/dL) to 41.6 mmol/L.

Impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and diabetes (DM) were categorized using 2006 WHO criteria (447). Participants were categorized as having IFG if their fasting blood glucose was between 6.1 – 6.9 mmol/L and as having IGT if their fasting glucose was <7.0 mmol/L and their 2-hour OGTT blood glucose was <11.1 but ≥7.8 mmol/L. Participants were considered to have diabetes if their fasting blood glucose was ≥7.0 mmol/L and/or if their 2-hour glucose was ≥11.1 mmol/L.

4.3.5 Statistical analysis

All statistical analysis was performed using R 3.4.3 statistical computing environment (448) with the consideration of two-sided $p<0.05$ to be statistically significant. Packages used for
computation include \textit{ggplot2} 2.2.1 for graphics (449) and \textit{mason} 0.2.5 for longitudinal analysis (450). Distributions of continuous variables were assessed for normality, and natural log transformations of skewed variables were used in statistical analyses and plots. The code to generate the results show in this manuscript can be found at \url{https://github.com/windyzn/urinaryDBP}.

Means and standard deviations were calculated for all normally distributed continuous variables while medians and interquartile ranges were presented for non-normally distributed variables. The independent variables for cross-sectional analyses were ACR and eGFR, while the dependent variable was urinary VDBP concentration adjusted for urinary creatinine to account for differences in urine volume between samples (uVDBP:cr) (451–453). Analysis of variance (ANOVA) with Tukey’s Honest Significant Difference (HSD) test examined the mean differences for continuous dependent variables across categorical independent variables, while chi-square tests were conducted to assess differences across categorical variables. Spearman rank correlation analyses were conducted to assess univariate associations between non-normally distributed continuous variables. Multiple linear regression was used to assess the association between kidney outcomes and uVDBP concentrations cross-sectionally at baseline while adjusting for age, sex, ethnicity, and glycemic status.

For the primary analysis, generalized estimating equation (GEE) models were used to determine the longitudinal association between the outcome variables and the predictor variables (454). An autoregressive of order 1 working correlation matrix was specified for GEE models given the longitudinal design, though other correlation matrices (e.g. exchangeable) had similar model fits when evaluated (data not shown). GEE is well suited to longitudinal data from cohort studies, as it is flexible to missed visits. The predictor variables were scaled (mean-centered and standardized) to compare across test results. In the first set of GEE models, the independent predictor variable was urinary vitamin D binding protein adjusted for urinary creatinine concentrations (uVDBP:cr). In the second set of models, baseline uVDBP:cr was set as a time-independent predictor variable (held constant over time). Both models had the same outcome variables and covariates. The outcome variables—ACR and eGFR—were time-dependent as they were measured at each clinical follow-up. No imputation was conducted on missing values. Covariates included in the GEE models were identified using previous literature and postulated biological mechanisms underlying the hypothesized associations. The final GEE model we selected had follow-up duration, baseline age, sex, ethnicity, and glycemic status as covariates. Continuous covariates (excluding time) were scaled and the resulting GEE $\beta$ coefficients were exponentiated; scaling, log-transforming, and exponentiating allowed interpretation of the
GEE β coefficient as an expected percent change in the outcome variable for each SD increase in the predictor variable given the that the covariates were held constant. Subjects with macroalbuminuria (n=5) and eGFR < 60 ml/min/1.73m² (n=12) at baseline were excluded from analysis. Interaction with time was also assessed.

Sensitivity analyses were conducted to assess the impact of excluding subjects with urinary VDBP values below the detection limit of the assay (<1.23 ng/mL) (n=132). Due to the small number of subjects in PROMISE who had macroalbuminuria and eGFR < 60 ml/min/1.73m² at baseline, sensitivity analyses were run for the cross-sectional analyses with the categories combined (i.e. microalbuminuria and macroalbuminuria; eGFR < 90 ml/min/1.73m²). Another sensitivity analysis was carried out to determine the impact of replacing BMI with waist circumference (WC) as a covariate during analyses.
4.4 Results

4.4.1 Baseline Results
Baseline characteristics across tertiles of urinary vitamin D binding protein to urinary creatinine ratio (uVDBP:cr) were available for 729 individuals in the PROMISE cohort (TABLE 4.1). There were no differences in mean age across uVDBP:cr tertiles \( (p=0.18) \). Compared to the proportion of males in the lower tertiles, there was a significantly lower percentage of males in the highest tertile of uVDBP loss \( (p<0.001) \). Overall, there was a significant difference in the distribution of ethnicity between the uVDBP:cr tertiles \( (p=0.03) \). The mean body mass index \( (p=0.088) \) and waist circumference \( (p=0.714) \) were not significantly different between tertiles. There were no significant differences in glycemic status distribution across uVDBP:cr tertiles at baseline \( (p=0.466) \). With the exception of eGFR, urinary measures of interest varied between uVDBP:cr categories. Albumin-to-creatinine ratio (ACR) and urinary microalbumin values were highest in the 3\(^{rd} \) tertile of uVDBP:cr \( (p<0.001) \). In contrast, urinary creatinine \( (p=0.033) \) and urinary calcium \( (p<0.001) \) decreased slightly as uVDBP:cr increased. There were significant differences in systolic blood pressure \( (p=0.011) \), diastolic blood pressure \( (p=0.002) \), and mean arterial pressure \( (p=0.002) \) across uVDBP:cr tertiles at baseline. Subjects with higher uVDBP:cr loss tended to have higher blood pressure.
### TABLE 4.1 Subject characteristics at baseline across uVDBP:cr tertiles.

<table>
<thead>
<tr>
<th></th>
<th>1st tertile (n=219)</th>
<th>2nd tertile (n=282)</th>
<th>3rd tertile (n=226)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.07 (10.21)</td>
<td>49.55 (9.75)</td>
<td>50.77 (10.20)</td>
<td>0.177</td>
</tr>
<tr>
<td>Sex (males)</td>
<td>83 (37.9)</td>
<td>107 (37.9)</td>
<td>42 (18.6)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td>0.034</td>
</tr>
<tr>
<td>European</td>
<td>130 (59.4)</td>
<td>188 (66.7)</td>
<td>155 (68.6)</td>
<td></td>
</tr>
<tr>
<td>Latino/a</td>
<td>47 (21.5)</td>
<td>32 (11.3)</td>
<td>29 (12.8)</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>15 (6.8)</td>
<td>28 (9.9)</td>
<td>14 (6.2)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>27 (12.3)</td>
<td>34 (12.1)</td>
<td>28 (12.4)</td>
<td></td>
</tr>
<tr>
<td>Glycemic Status</td>
<td></td>
<td></td>
<td></td>
<td>0.466</td>
</tr>
<tr>
<td>Normal glyceremia</td>
<td>187 (85.4)</td>
<td>234 (83.0)</td>
<td>178 (78.8)</td>
<td></td>
</tr>
<tr>
<td>Pre-diabetes</td>
<td>9 (4.1)</td>
<td>14 (5.0)</td>
<td>15 (6.6)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>23 (10.5)</td>
<td>34 (12.1)</td>
<td>33 (14.6)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>31.18 (6.51)</td>
<td>30.57 (5.87)</td>
<td>31.79 (6.16)</td>
<td>0.088</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>99.81 (15.24)</td>
<td>99.01 (15.62)</td>
<td>98.65 (14.84)</td>
<td>0.714</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>94.98 (14.81)</td>
<td>93.63 (14.61)</td>
<td>96.71 (14.70)</td>
<td>0.064</td>
</tr>
<tr>
<td>ACR (mg/mmol)</td>
<td>0.48 [0.26, 0.71]</td>
<td>0.47 [0.32, 0.67]</td>
<td>0.84 [0.53, 1.51]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary creatinine (mmol/L)</td>
<td>12.67 (5.99)</td>
<td>11.79 (7.06)</td>
<td>11.07 (6.05)</td>
<td>0.033</td>
</tr>
<tr>
<td>Urinary microalbumin (mg/L)</td>
<td>5.00 [2.45, 9.05]</td>
<td>4.00 [2.00, 8.95]</td>
<td>8.00 [4.00, 19.00]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary calcium (mmol/L)</td>
<td>2.77 (1.89)</td>
<td>2.18 (1.70)</td>
<td>2.06 (1.55)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary VDBP (ng/ml)</td>
<td>6.10 [1.15, 16.66]</td>
<td>51.73 [30.65, 79.65]</td>
<td>109.33 [70.91, 169.89]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Urinary VDBP:cr</td>
<td>1.06 (1.08)</td>
<td>5.09 (0.99)</td>
<td>15.18 (22.45)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>123.75 (14.70)</td>
<td>126.37 (16.33)</td>
<td>128.31 (16.48)</td>
<td>0.011</td>
</tr>
</tbody>
</table>
Values reported as mean ± standard deviation for normal continuous variables, median (95% CI) for non-normal continuous variables, and n (%) for categorical variables. Significance for continuous and discrete variables was assessed using analysis of variance (ANOVA) and chi-squared test of independence, respectively.

<table>
<thead>
<tr>
<th></th>
<th>78.50 (9.16)</th>
<th>79.98 (10.20)</th>
<th>81.90 (11.28)</th>
<th>0.002</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diastolic blood pressure</strong></td>
<td>93.58 (10.28)</td>
<td>95.44 (11.42)</td>
<td>97.37 (12.12)</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Mean arterial pressure</strong></td>
<td>78.50 (9.16)</td>
<td>79.98 (10.20)</td>
<td>81.90 (11.28)</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Concentrations of log $uVDBP:cr$ increased with increasing ACR (FIGURE 4.2A). The median log value of $uVDBP:cr$ for subjects with normoalbuminuria (n=671) was 1.61 (IQR 0.81 – 1.96). Across albuminuria categories of microalbuminuria (n=51) and macroalbuminuria (n=5), the median log $uVDBP:cr$ value increased to 2.51 (IQR 1.87 – 3.07) and 4.61 (IQR 3.05 – 5.24) respectively. Overall, there was a significant difference in $uVDBP$ loss between albuminuria categories ($p<0.001$). Post-hoc analysis using Tukey’s HSD found differences between normoalbuminuria and microalbuminuria ($p<0.001$) and normoalbuminuria and macroalbuminuria ($p<0.001$) groups, but no significant difference was found between subjects with microalbuminuria and macroalbuminuria ($p=0.11$).

Due to the small number of subjects in the microalbuminuria and macroalbuminuria categories at baseline, sensitivity analyses were conducted. ANOVA was used to assess if there was a difference in urinary VDBP concentration in subjects with albuminuria (either microalbuminuria or macroalbuminuria) compared to those without proteinuria (FIGURE 7.1). The median log $uVDBP:cr$ for the proteinuria group was 2.60 μg/mmol (IQR 1.92 – 3.33) while the median log value was 1.61 μg/mmol (IQR 0.81 – 1.96) for the normoalbuminuria group, and this difference was statistically significant ($p<0.001$). We also found that the significant positive association between ACR and $uVDBP$ was maintained even when VDBP was not adjusted for urinary creatinine ($p<0.001$).

ACR was significantly associated with $uVDBP:cr$ at baseline using linear regression ($p<0.001$) (FIGURE 4.2B). The unadjusted $\beta$ coefficient (95% CI) was 0.35 (0.33, 0.37), $p<0.001$. The significant association remained when the model included adjustments for age, sex, ethnicity, and glycemic status ($\beta=0.36$ (0.33, 0.38), $p<0.001$). The positive correlation between $uVDBP:cr$ and ACR was also significant ($r=0.37$, $p<0.001$).
Urinary VDBP:cr concentrations did not differ between eGFR groups (FIGURE 4.3A). The median value of log uVDBP:cr for subjects with normal eGFR (n=471) was 1.68 (IQR 0.95 –
2.07). Median log uVDBP:cr concentrations decreased slightly across eGFR categories: subjects with mild kidney disease had a median value of 1.57 (IQR 0.90 – 1.94) and subjects classified as having moderate kidney disease had a median uVDBP:cr value of 1.29 (IQR 0.79 – 2.00). Overall, there was no significant difference in uVDBP loss between clinical eGFR categories ($p=0.665$). There was also no association between continuous eGFR and log uVDBP:cr at baseline (FIGURE 4.3B). Linear regression found the unadjusted $\beta$ coefficient (95% CI) to be -0.03 (-0.10, 0.05), $p=0.52$. When adjusted for age, sex, ethnicity, and glycemic status, the $\beta$ coefficient and 95% CI were -0.02 (-0.09, 0.04), $p=0.43$. The correlation coefficient, assessed using Spearman’s rank correlation, was non-significant ($r=0.05$, $p=0.171$).
FIGURE 4.3 (A) Log baseline uVDBP:cr concentrations at different stages of kidney disease. (B) Association between log uVDBP:cr concentrations and eGFR at baseline.
4.4.2 Longitudinal Results

Generalized estimating equations (GEE) were used to assess longitudinal associations between uVDBP:cr and kidney outcomes over 6 years. Interaction with time was assessed using both visit number and follow-up duration; no significant interactions were found.

We found that after adjusting for follow-up duration (in years), baseline age, sex, ethnicity, and glycemic status, each standard deviation (SD) difference in uVDBP:cr was associated with higher ACR consistently over 6 years, with an estimate of 32.21 (95% CI 16.9 – 49.52, $p<0.001$) (FIGURE 4.4). Follow-up duration and sex were also significantly associated with ACR in the model, with estimates of 3.82 (95% CI 2.49 – 5.18, $p<0.001$) and -24.34 (95% CI -32.31 – -15.43, $p<0.001$), respectively (FIGURE 4.4). No other covariates were associated with changes in the outcome.

Urinary VDBP concentration was not associated with changes in eGFR longitudinally while adjusting for follow-up duration (in years), baseline age, sex, ethnicity, and glycemic status ($\beta = 0.29$, 95% CI -0.23 – 0.80, $p=0.28$) (FIGURE 4.4). In this model, follow-up duration, baseline age, and ethnicity were significantly associated with changes in eGFR over time. Follow-up duration and age at baseline were both negatively associated with the outcome ($p<0.001$). Subjects of European descent tended to have a 4.12% lower eGFR (95% CI -6.03 – -2.18, $p<0.001$) compared to non-Europeans. Sex and glycemic status were not significantly associated with eGFR over 6 years.
Adjusted GEE models were also used to assess whether baseline urinary VDBP concentrations were associated with declines in kidney function over 6 years (FIGURE 4.5). One SD increase in baseline uVDBP:cr was associated with 29.05% increase (95% CI 23.26 – 35.13) in the concentration of ACR ($p<0.001$) (FIGURE 4.5). Again, follow-up duration and sex significantly contributed to the model with estimates of 4.13 (95% CI 2.63 – 5.66, $p<0.001$) and -22.22 (95% CI -29.96 – -13.62, $p<0.001$), respectively. No other covariates in the model were associated with the outcome.

When the outcome was eGFR, baseline uVDBP:cr was not significantly associated with changes over 6 years ($\beta = 0.81$, 95% CI -0.23 – 1.85, $p=0.13$) after adjusting for covariates (FIGURE 4.5). However, follow-up duration ($\beta = -0.60$, 95% CI -0.83 – -0.38) and baseline age ($\beta = -8.04$, 95% CI -8.86 – -7.21) were, again, negatively associated with a change in eGFR over time (FIGURE 4.5). As well, those of European origin had 4.16% lower filtration rate compared to other ethnicities in the cohort (95% CI -6.06 – -2.22) (FIGURE 4.5). No significant associations were found with other covariates.
FIGURE 4.5 GEE models of baseline uVDBP:cr and kidney outcomes (ACR and eGFR) over 6 years.

Tabular data can be found in Table 7.2. Sensitivity analysis using uVDBP unadjusted for urinary creatinine can be found in Figure 7.3.
4.5 Discussion

In this study, subjects with microalbuminuria and macroalbuminuria at baseline had higher values of renal VDBP excretion increased compared to those with normoalbuminuria. Urinary VDBP loss worsened as more albumin was spilled into the urine at baseline and over time. It was also found that a 1 standard deviation increase in baseline uVDBP:cr was associated with a 29.07% higher ACR over 6 years. In contrast, no significant differences in uVDBP concentration were found between eGFR categories at baseline and over a 6-year follow-up.

There was a strong association between urinary ACR and vitamin D binding protein concentrations at baseline and throughout the 6-year follow-up. Previous studies have also observed a positive association between albuminuria status and loss of VDBP. Thrailkill et al. found that in subjects with type 1 diabetes, urinary VDBP:cr was significantly higher in those with diabetes and proteinuria (ACR >30 mg/g) compared to those with diabetes and normoalbuminuria and healthy age-matched controls; the healthy subjects had median uVDBP:cr of 1949 ng/g while those with normoalbuminuria had concentrations of 7043 ng/g and 68844 ng/g, respectively (all p<0.001) (12). The same association was observed in the PROMISE cohort at baseline; subjects with normoalbuminuria had the lowest median uVDBP:cr concentration while subjects with macroalbuminuria had the highest urinary loss of VDBP (p<0.001). The significant positive association between ACR and uVDBP was maintained even when VDBP was not adjusted for urinary creatinine, similar to findings from this project. Interestingly, Thrailkill et al. did not find a significant association between plasma VDBP and worsening albuminuria status, suggesting that the body may be able to compensate for urinary VDBP loss by increasing synthesis of VDBP in the liver (12). However, Thrailkill et al. did find a significant positive association between uVDBP:cr and ACR using Spearman’s rank correlation (r=0.537, p<0.001) (12), which is comparable to the correlation found in this cohort at baseline (r=0.37, p<0.001). In Tian et al.’s study using subjects with T2DM and varying severity of proteinuria, it was also shown that the expression of uVDBP was significantly higher in subjects with T2DM + microalbuminuria and T2DM + macroalbuminuria compared to subjects with only T2DM (p<0.001) (13). As well, those with T2DM + macroalbuminuria had a significantly higher expression of uVDBP (p<0.001) (13). The Spearman’s rank correlation between uVDBP and ACR was r=0.707 (13). The results from earlier studies support the cross-sectional findings from this project. No previous papers have examined the association between kidney markers and uVDBP prospectively. Our study supports the notion that the strong positive association between uVDBP:cr and ACR is maintained over time, and that a higher concentration of uVDBP:cr at baseline is significantly associated with worse ACR over 6 years.
While there was a strong association between ACR and uVDBP:cr, the relationship between eGFR and uVDBP:cr was less clear. We found no significant associations between estimated glomerular filtration and uVDBP:cr at baseline or over 6 years. Non-significant associations in eGFR may be attributed to the fact that a large number subjects in PROMISE were relatively healthy at baseline. At the time of recruitment, subjects with clinically diagnosed kidney disease were excluded from the cohort. It is also conceivable that some subjects displayed characteristics of hyperfiltration, a hypothesized precursor of intraglomerular hypertension that is commonly observed in patients at early stages of diabetes (455). This phenomenon may have obscured the associations between uVDBP and eGFR.

Findings from the current study were similar to previous papers which have examined uVDBP with kidney markers. In a study of children with steroid-resistant nephrotic syndrome (mean eGFR was 119 ± 11.4 ml/min/1.73m²) and steroid-sensitive nephrotic syndrome (mean eGFR was 135 ± 6.1 ml/min/1.73m²), Bennett et al. found that, overall, uVDBP in patients was negatively correlated with eGFR (r= -0.76, p=0.03), but there was a slight positive association observed between glomerular filtration and uVDBP at eGFR >100 mL/min/1.73m² (456). Our longitudinal analysis shows that although the association between eGFR and uVDBP:cr was not significant, the direction of association was positive (FIGURE 4.4 and FIGURE 4.5). Subjects in PROMISE were likely in the earliest stages of kidney dysfunction at the 6-year follow-up and most had eGFR values greater than 90 ml/min/1.73m² (n=471). In addition, any potential negative association between eGFR and uVDBP:cr in PROMISE may be masked by hyperfiltration in some subjects. Mirković et al. also examined the relationship between uVDBP and established markers of proximal tubular damage and relation inflammation. They found no association between uVDBP excretion and eGFR in subjects with normoalbuminuria and microalbuminuria (441).

The exact reasons underlying the enhanced excretion of urinary VDBP in patients with diabetic nephropathy (DN) remain unclear. One possible explanation is that elevated urinary VDBP levels may be associated with renal tubular damage in DN patients (410,411). Renal tubular epithelial cell damage becomes increasingly severe as DN develops. In a previous study, increased excretion of urinary VDBP was observed following long-term cadmium exposure, and it was suggested that the marked loss of VDBP in the urine may be linked to renal tubular dysfunction and bone lesions in the inhabitants of cadmium-polluted areas (457).

Although albumin and vitamin D binding protein share the megalin/cubilin-coupled receptor for reabsorption purposes, the reabsorption mechanism of albumin and VDBP is not identical (441). It has been argued that the capacity of the megalin/cubilin-mediated mechanism for
tubular uptake of albumin is low. Studies have found that the urinary albumin excretion in megalin-defective mice and cubilin-defective dogs is only slightly increased, representing merely a small increase in the excretion of non-degraded albumin (458). Albumin has been observed to have alternate binding sites on isolated proximal tubule segments and several receptors for tubular uptake of albumin, such as the Fc-receptor, have been identified (459–462). In addition, several unidentified renal albumin receptors have been recognised by albumin-affinity chromatography and localized by immunohistochemistry (463). However, the exact localization and functional importance of these receptors remain to be established. Alternate mechanisms of albumin reabsorption have been suggested, including fast, high-capacity retrieval pathway for non-degraded albumin located distal to the glomerular basement membrane (458,464). A study by Eppel et al. determined that the reabsorption process is located in the glomerulus or early tubular system, as micropuncture of the proximal tubule failed to change the degree of albumin spillage (464). Thus, although albuminuria is a good indicator for the progression of renal disease and is used as an early marker for kidney damage, there are several limitations to this biomarker (465–467). Microalbuminuria has been shown to revert to normoalbuminuria in some cases, meaning that although microalbuminuria precedes a decrease in renal function, not all cases will progress (132). In clinical practice, ACR is only able to indicate the magnitude of proteinuria and not the origin of loss (glomerular, tubular, or both) due to its multiple reabsorption pathways. As such, while ACR is a good marker of overall kidney dysfunction, uVDBP:cr may be a better indicator of renal proximal tubule injury.

In contrast, the loss of VDBP in the urine of megalin-deficient mice highlights the important role of tubular uptake of VDBP from glomerular filtrates (395,468,469). A possible explanation for the null association between uVDBP and eGFR in this study is that injury at the proximal tubule (changes in megalin/cubilin expression) plays a larger role in the renal loss of VDBP compared to damage at the glomeruli. Decreased megalin expression in proximal tubule cells has been observed in the early diabetic stages of experimental animals (470). Megalin function is also believed to be impaired in the early stages of diabetic nephropathy (471). Kaseda et al. found that the expression of megalin in cultured kidney proximal tubule cells was upregulated following treatment with insulin or highly concentrated glucose (472). Together, these findings suggest the mechanism for loss of VDBP in the urine may be due to the decrease in megalin expression in the proximal tubules, leading to lower reabsorption of the carrier protein. Cubilin function has also been found to be impaired in early diabetic nephropathy as urinary excretion of transferrin—an endocytic ligand of cubilin—is significantly increased in patients with the disease (473). As such, the null association between eGFR and uVDBP may be due to the different underlying mechanism in which uVDBP is lost in the urine.
Overall, there has been a very limited number of studies which have examined the relationship between urinary VDBP with established kidney biomarkers. In particular, studies with human subjects have used cross-sectional designs and have involved very few subjects. The present study—to the best of our knowledge—examines for the first time the associations between uVDBP, ACR, and eGFR in a large prospective cohort at multiple time points over 6 years.

Strengths of the current study include the use of validated and established measures of kidney function. Unlike most previous studies which used the MDRD equation to estimate GFR, we used the CKD-Epi equation which offers greater accuracy at higher filtration rates (137). In addition, the current study is the first to examine the longitudinal relationship between urinary VDBP loss and kidney markers in a large, well-characterized, multi-ethnic sample. The prospective study allowed for adjustment of multiple covariates in multivariate models, and there is the potential for better understanding of the association between uVDBP and kidney damage with longer clinical follow-up in the future. Lastly, the use of GEE models in the current study was beneficial as this statistical method offers flexibility for changing sample sizes in longitudinal studies and accounts for the lack of independence between the repeated measurements at clinic visits from the same participant.

An important limitation of the study in understanding CKD progression is the relatively healthy study population in the cohort at early years of follow-up. Normoglycemic subjects with risk factors for the development of T2DM were recruited, and subjects with established kidney disease were screened out of the population. Consequently, there was a small number of subjects in more advanced kidney dysfunction categories (i.e. macroalbuminuria and eGFR <60 mL/min/1.73m²). However, since we are continuing to follow this cohort, more subjects are expected to progress toward more severe kidney categories over time. As well, we were unable to compare with other markers of acute kidney injury—such as kidney injury molecule 1 (KIM-1), interleukin-18 (IL-18), and neutrophil gelatinase-associated lipocalin (NGAL)—as these were not determined in the PROMISE cohort. Therefore, further studies with varying degrees of kidney dysfunction and additional markers of renal damage are required to determine the specificity of uVDBP as an early detection tool for renal disease.

In conclusion, we found that there was a strong association between ACR and uVDBP:cr both cross-sectionally and prospectively, though no association was observed for eGFR. Results from this study deepen our understanding of vitamin D binding protein in the urine, as well as the relationship between uVDBP and kidney dysfunction. Although further research is needed, uVDBP may be a potential marker that can be used in conjunction with existing
biomarkers for a complete assessment of proteinuria and drastically lessen the damage of kidney disease in this population.
CHAPTER 5

Association of Urinary Vitamin D Binding Protein with Vitamin D Status
CHAPTER 5
Association of Urinary Vitamin D Binding Protein with Vitamin D Status

5.1 Abstract

**Background:** While many studies have documented associations between low vitamin D and risk of incident diabetes, it has also been proposed that hypovitaminosis D may also be a consequence of diabetes pathology. One possible mechanism for hypovitaminosis D in type 2 diabetes (T2DM) is that vitamin D metabolites may still be bound to their carrier protein when it is lost in urine during the progression of kidney disease. As only a few studies have investigated this possibility, our objective was to assess the association of urinary vitamin D binding protein (uVDBP) concentrations with longitudinal changes in serum 25(OH)D levels.

**Methods:** Data were derived from PROMISE, a longitudinal cohort study of adults at risk for T2DM. We used data from 3 clinical visits over 6 years (n=682). Serum 25-hydroxyvitamin D (25(OH)D) was used as an indication of vitamin D status. Measurements of uVDBP were performed with ELISA and normalized to urine creatinine. Generalized estimating equation (GEE) models evaluated longitudinal associations of uVDBP with 25(OH)D; covariates included visit number, baseline age, sex, ethnicity, BMI, physical activity, and glycemic status.

**Results:** Urinary VDBP concentration differed with vitamin D status at baseline. Subjects with deficient, insufficient, and sufficient vitamin D levels had median log uVDBP concentrations of 1.62μg/mmol, 1.68μg/mmol, and 1.52μg/mmol, respectively (p=0.026). At baseline, 25(OH)D was not associated with uVDBP:cr (β=0.32, p=0.46). Adjusted GEE models indicated that SD differences in uVDBP:cr (β=0.38, p=0.58) or baseline uVDBP:cr (β=-0.69, p=0.35) were not associated with changes in 25(OH)D over 6 years.

**Significance:** Moderate urinary loss of uVDBP over 6 years does not impact vitamin D status in subjects at risk for diabetes.
5.2 Introduction

Approximately 30%-50% of people are recognized to have poor vitamin D status, and insufficiency and deficiency of vitamin D have been documented worldwide (28). Although it is well-established that the presence of hypovitaminosis D increases the risk of rickets and fractures, emerging evidence also suggests that vitamin D deficiency may be associated with onset and progression of type 2 diabetes (T2DM) (16,20,22,316,323,328,474). It is well recognized from observational studies that low 25-hydroxyvitamin D (25(OH)D) is common in patients with T2DM and prospective studies have yielded consistent associations between low 25(OH)D and risk of incident diabetes (7–11,16,35). However, evidence from controlled trials involving vitamin D supplementation has been contradictory to date (10,15,16).

The exact pathways through which vitamin D may be associated with T2DM are still uncertain, but several possible mechanisms—such as low vitamin D inhibiting pancreatic insulin secretion (17), 1,25-dihydroxyvitamin D (1,25(OH)₂D) increasing β-cell secretion of insulin (18), regulation of insulin receptor genes by vitamin D (19), and vitamin D improving insulin sensitivity in peripheral tissues (20,21)—have been proposed. Consistent with these findings, ecological evidence suggests that high rates of metabolic disorders, such as T2DM, are found in areas further from the equator (323,324).

While many studies have documented associations of low vitamin D concentrations with risk for the development of T2DM, it has also been proposed that hypovitaminosis D may also be a consequence of diabetes pathology itself. In circulation, 85-90% of vitamin D metabolites are carried by the vitamin D binding protein (VDBP) (25–27). Recently, a number of papers have reported elevated urinary concentrations of the vitamin D binding protein in animals and patients with diabetes (413,436,437). It is possible that vitamin D metabolites may still be bound to VDBP as the protein is spilled. These observations suggest the possibility that that urinary loss of VDBP could contribute to hypovitaminosis D in individuals with diabetes and kidney disease (12,13).

However, previous studies on vitamin D and uVDBP have been limited by cross-sectional designs, small sample sizes, lack of adequate covariate adjustment (e.g. season), and homogeneity of study populations (26,475,476). In addition, no previous study has assessed if urinary VDBP concentrations may be linked to lower serum 25(OH)D longitudinally (12,419,476). Our objective, therefore, was to assess the association of 25(OH)D with urinary vitamin D binding protein in a large multi-ethnic sample of subjects at risk for T2DM over 6 years.
5.3 Methods

5.3.1 Study Population
The PROMISE cohort has been previously described in chapter 4.3.1. In brief, PROMISE is a longitudinal observational study of subjects aged ≥ 30 years recruited from London and Toronto, Ontario who were at high risk for T2DM (442,443,477). Annual telephone contact is maintained with participants and follow-up clinical visits occur every three years. At each clinic visit, participants undergo extensive metabolic characterization, anthropometric measurements, blood and spot urine sample collection, and structured questionnaires which assessed ethnicity, smoking history, family history of diabetes, and other important covariates such as socioeconomic status.

Fasting blood samples were collected and 75 g oral glucose tolerance tests (OGTT) were conducted with additional blood samples collected at 30 and 120 minutes for glucose and insulin measurements (n=739). After excluding subjects without serum 25(OH)D measures at baseline (n=57), 682 subjects remained at baseline. Among these, 447 (66%) had measurements for 25(OH)D at the 3-year follow-up and 114 (17%) at the 6-year follow-up (FIGURE 5.1).

Research ethics approval was obtained from Mount Sinai Hospital and the University of Western Ontario. Research nurses at the respective institutions were centrally trained on the standardized procedures for conducting the characterizations.
5.3.2 Anthropometric measurements

Anthropometrics were measured twice and the average was used as the final value in the analyses. Height, weight, and waist circumference (WC) were measured at all clinic visits using standard procedures. WC was measured at the natural waist, identified as the narrowest part of the torso between the umbilicus and the xiphoid process. Height was measured using a stadiometer, without shoes, and back straight against the wall with the head positioned in the Frankfurt plane. Weight was measured on a medical balance beam scale with shoes off and in light clothing. Body-mass index was calculated using the formula:
Blood pressure was measured twice on the right arm with the participant seated after 5 minutes rest using an automated sphygmomanometer. Mean arterial pressure (MAP) was calculated from these two values using the formula:

\[
MAP = \frac{SBP + 2(DBP)}{3}
\]

5.3.3 Serum measures

A blood sample was drawn after an 8 – 12 hour overnight fast at each clinic visit to measure nutritional, liver, adipose, inflammatory, and kidney biomarkers. OGTT blood samples were immediately processed for the determination of serum glucose, and remaining samples were processed and frozen at -70°C for the determination of blood biomarkers. Glucose was determined using an enzymatic hexokinase method on the Roche Modular platform (Roche Modular, Roche Diagnostics) with a detection range of 0.11 (2 mg/dL) to 41.6 mmol/L.

Impaired fasting glucose (IFG), impaired glucose tolerance (IGT), and diabetes (DM) were categorized using the 2006 WHO criteria (447). Participants were categorized as having IFG if their fasting blood glucose was between 6.1 – 6.9 mmol/L and as having IGT if their fasting glucose was < 7.0 mmol/L and their 2-hour OGTT blood glucose was < 11.1 but ≥ 7.8 mmol/L. Participants were considered to have diabetes if their fasting blood glucose was ≥ 7.0 mmol/L and/or if their 2-hour glucose was ≥ 11.1 mmol/L.

Parathyroid hormone (PTH) was measured using an electrochemiluminescence immunoassay on the Roche Modular E170 analyser (Laval, QC), which has a detection range from 0.127 – 530 pmol/L. Serum creatinine was measured using standard laboratory procedures.

Vitamin D status, specifically 25-hydroxyvitamin D, was measured in serum using DiaSorin’s 25-OH vitamin D TOTAL competitive chemiluminescence immunoassay on an automated LIAISON analyzer (Stillwater, MN). This assay has 100% specificity for both 25(OH)D$_2$ and 25(OH)D$_3$ and has a detection limit of 10 nmol/L. There was an intra-assay coefficient variation (CV) of 6.7% and an inter-assay CV of 11.6%, based on the measurements in the baseline blood samples. The 25(OH)D TOTAL method has been validated against the DiaSorin radioimmunoassay ($r=0.92$), which was the first test approved for clinical diagnosis by the Food and Drug Administration and also is the most widely used method (478). In addition, the laboratory in which this assay was conducted participates in the International External
Quality Assessment Scheme for Vitamin D Metabolites (DEQAS, Northwest Thames, U.K.), and it has been reported that the 25(OH)D results from this laboratory were consistently within one standard deviation of the group mean in the international DEQAS proficiency surveys (478). Aliquots of serum 25(OH)D were stored at -80°C for an average of seven years from the time they were initially drawn and processed. Under these conditions, 25(OH)D has been shown to be stable in serum or plasma (479,480). Vitamin D status was classified as per Endocrine Society guidelines as vitamin D deficient (25(OH)D < 50 nmol/L; n = 291), vitamin D insufficient (25(OH)D ≥ 50 nmol/L and < 75 nmol/L; n = 263), and vitamin D sufficient (25(OH)D ≥ 75 nmol/L; n = 128) (235,237). Endocrine Society cut-offs were chosen over the lower cut-offs defined by the Institute of Medicine (IOM) due to the kidney-related outcomes in our study. While IOM defined vitamin D categories based on bone health outcomes, Endocrine Society cut-offs were derived using serum PTH response to vitamin D supplementation (235). As parathyroid hormone acts on the conversion from 25(OH)D to 1,25(OH)₂D in the kidneys, we believe that the PTH-derived categories for vitamin D status are more suitable for this project. The season of blood sample collection was also documented and was categorized as follows: summer (May-October) and winter (November-April).

5.3.4 Urine measures
A morning spot urine sample was collected for the determination of albumin, creatinine, estimated glomerular filtration rate (eGFR), and VDBP. Urinary albumin was measured using the Roach/Hitachi MODULAR P analyzer. The lower detection limit of 54 μmol/L was calculated as the value lying 3 standard deviations (SD) above that of the lowest standard.

Estimated GFR was calculated using the CKD-Epi equation and serum creatinine (444). The R package nephro was used to calculate eGFR following the CKD-Epi formulae (445):

If female and serum creatinine ≤ 0.7 mg/dl:

$$144 \times \left( \frac{\text{creatinine}}{0.7} \right)^{-0.329} \times 0.993^{\text{Age}} \times 1.159 \text{ if black}$$

If female and serum creatinine > 0.7 mg/dl:

$$144 \times \left( \frac{\text{creatinine}}{0.7} \right)^{-1.209} \times 0.993^{\text{Age}} \times 1.159 \text{ if black}$$

If male and serum creatinine ≤ 0.9 mg/dl:
Urinary VDBP was measured at Sunnybrook Research Institute. Measurements of uVDBP were assayed by manual enzyme-linked immunosorbent assay (ELISA) (Immundiagnostik, catalog # K2314, Bensheim, Germany) following manufacturer instructions. For urine samples, the obtained VDBP value is multiplied by a dilution factor of 10 (446). Samples with concentrations above the measurement range were further diluted and re-assayed. The upper limit of the measurement range was calculated as the highest concentration of the standard curve x sample dilution factor (60 ng/mL). The lower limit of the measurement range was calculated as analytical sensitivity x sample dilution factor used (1.23 ng/mL) (446). The coefficients of variation (CV) for within-run and between-run are respectively 5.01% and 6.02%.

5.3.5 Questionnaires

Sociodemographic and other covariates were assessed using structured standardized questionnaires at each clinic visit. Questionnaires collected information on medical history (i.e. presence of other chronic diseases such as hypertension, cancer, peripheral arterial disease, stroke, myocardial infarction, high cholesterol, or kidney or thyroid disease), education, occupation, income, ethnicity, sex, age, family history of type 2 diabetes, self-reported weight at 18 years (in kg), and self-reported birthweight.

In the lifestyle questionnaire, physical activity was determined using a version of the Modifiable Activity Questionnaire (MAQ) (481). The MAQ collects information on both leisure and occupational activity—including intensity, frequency, and duration—over the past year. The MAQ has been shown to have good reliability and validity (482). Each reported activity from the MAQ was weighted by its relative metabolic intensity, referred to as the metabolic equivalent of task (MET). MET is a physiological measure expressing the energy cost of physical activities and is defined as the ratio of metabolic rate during a specific physical activity to a reference metabolic rate, set by convention to 3.5 mL O₂·kg⁻¹·min⁻¹ or approximately:

\[
144 \times \left( \frac{\text{creatinine}}{0.7} \right)^{-0.411} \times 0.993^{\text{Age}} \times [1.159 \text{ if black}]
\]

If male and serum creatinine >0.9 mg/dl:

\[
144 \times \left( \frac{\text{creatinine}}{0.7} \right)^{-1.209} \times 0.993^{\text{Age}} \times [1.159 \text{ if black}]
\]
1 MET = \frac{kcal}{kg \times h} = 4.184 \frac{kJ}{kg \times h} = 1.162 \frac{W}{kg}

MET can be thought of as an index of the intensity and energy expenditure of activities in a way that is comparable among people of different weight. The MAQ allowed for the derivation of MET-hours per week (MET·h·wk\(^{-1}\)) as the final unit of expression.
5.3.6 Statistical analysis

All statistical analysis was performed using R 3.4.3 statistical computing environment (448) with the consideration of two-sided $p<0.05$ to be statistically significant. Packages used for computation include \textit{ggplot2 2.2.1} for graphics (449) and \textit{mason 0.2.5} for longitudinal analysis (450). Distributions of continuous variables were assessed for normality, and natural log transformations of skewed variables were used in statistical analyses and plots. The code to generate the results show in this manuscript can be found at \url{https://github.com/windyzn/urinaryDBP}.

Means and standard deviations were calculated for all normally distributed continuous variables while medians and interquartile ranges were presented for non-normally distributed variables. The independent variables for cross-sectional analyses were vitamin D status and serum 25(OH)D levels. The dependent variable was urinary VDBP concentration adjusted for urinary creatinine to account for differences in urine volume between samples (uVDBP:cr) (451–453). Analysis of variance (ANOVA) with Tukey’s Honest Significant Difference (HSD) test examined the mean differences for continuous dependent variables across categorical independent variables, while chi-square tests were conducted to assess differences across categorical variables. Spearman rank correlation analyses were conducted to assess univariate associations between non-normally distributed continuous variables. Multiple linear regression was used to assess the association between 25(OH)D and uVDBP concentrations cross-sectionally at baseline while adjusting for age, sex, ethnicity, MET, BMI, seasonality, and glycemic status.

For the primary analysis, generalized estimating equation (GEE) models were used to determine the longitudinal association between the outcome variables and the predictor variables (454). An autoregressive of order 1 working correlation matrix was specified for GEE models given the longitudinal design, though other correlation matrices (e.g. exchangeable) had similar model fits when evaluated (data not shown). GEE is well suited to longitudinal data from cohort studies, as it is flexible to missed visits. The predictor variables were scaled (mean-centered and standardized) to compare across test results. The independent predictor variable in the first set of GEE models was urinary vitamin D binding protein adjusted for urinary creatinine concentrations (uVDBP:cr). In the second set of models, baseline uVDBP:cr was set as a time-independent predictor variable (held constant over time). Both models had the same outcome variables and covariates. The outcome variable, serum 25(OH)D, was time-dependent as it was measured at each clinical follow-up. No imputation was conducted on missing values. Covariates included in the GEE models were identified using previous literature and biological mechanisms underlying the hypothesized associations. The final GEE model we selected had follow-up duration, baseline age, sex,
ethnicity, MET, BMI, and glycemic status as covariates. Continuous covariates (excluding time) were scaled and the resulting GEE β coefficients were exponentiated; scaling, log-transforming, and exponentiating allowed interpretation of the GEE β coefficient as an expected percent change in the outcome variable for each SD increase in the predictor variable given that the covariates were held constant. Subjects with vitamin D deficiency at baseline were excluded from analysis. Interaction with time was also assessed.

Two additional sensitivity analyses were conducted. One analysis assessed the impact of excluding subjects with urinary VDBP values below the detection limit of the assay (<1.23 ng/mL) (n=132). Additionally, due to the smaller number of subjects with serum 25(OH)D measures at the 6-year follow-up, another analysis was done to determine if results changed when all follow-up visits were combined (i.e. 3-year and 6-year visits).
5.4 Results

5.4.1 Baseline results

Baseline characteristics across vitamin D status were available for 682 individuals in the PROMISE cohort (TABLE 5.1). The mean age was higher in those with insufficient (51.1 years) and sufficient (51.4 years) vitamin D status groups compared to the deficient group (47.86 years) ($p<0.001$). Overall, there was no significant difference in the percentage of males in different vitamin D categories ($p=0.57$). Overall, there was a significant difference in the distribution of ethnicity between the vitamin D categories ($p<0.001$). Both BMI and waist circumference were higher as vitamin D status worsened (all $p<0.001$). There was no significant difference in physical activity level (as assessed by MET scores) in the different vitamin D categories ($p=0.095$). Most urinary measures were not different across vitamin D status groups. However, there was a significant difference in estimated GFR and urinary microalbumin. Subjects who had deficient vitamin D tended to have the highest eGFR value (97.74 mL/min/1.73 m$^2$). Those in the insufficient and sufficient groups had relatively similar values at 92.60 mL/min/1.73 m$^2$ and 92.95 mL/min/1.73 m$^2$, respectively ($p<0.001$). The median urinary microalbumin value was highest in the deficient category (6.3 mg/L) and lowest in the sufficient category (4.40 mg/L) ($p=0.028$). Urinary VDBP with and without creatinine adjustment were both not significantly different across vitamin D groups ($p=0.85$ and $p=0.616$, respectively). Parathyroid hormone was also highest in the deficient category and lowest in the sufficient category ($p<0.001$). There were no significant differences in blood pressure measures (systolic, diastolic, and mean arterial pressure) across vitamin D groups. Of the 682 subjects at baseline with serum 25(OH)D data, the proportion of subjects with normal glucose tolerance was higher in the sufficient vitamin D status group (87.5%) compared to the deficient (81.8%) and insufficient (83.3%) groups. In contrast, there was the highest percentage of subjects with diabetes in the deficient group (15.5%) and lowest in the sufficient group (6.2%). Those classified as having pre-diabetes had the highest proportion of subjects in the insufficient group (6.5%). Overall, there were significant differences in glycemic status distribution across vitamin D status at baseline ($p=0.016$).
### TABLE 5.1 Subject characteristics across vitamin D status at baseline.

<table>
<thead>
<tr>
<th></th>
<th>Deficient (n=291)</th>
<th>Insufficient (n=263)</th>
<th>Sufficient (n=128)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>47.86 (9.37)</td>
<td>51.11 (10.31)</td>
<td>51.35 (10.32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (% males)</td>
<td>98 (33.7)</td>
<td>78 (29.7)</td>
<td>39 (30.5)</td>
<td>0.573</td>
</tr>
<tr>
<td>Ethnicity</td>
<td></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>European</td>
<td>148 (50.9)</td>
<td>195 (74.1)</td>
<td>101 (78.9)</td>
<td></td>
</tr>
<tr>
<td>Latino/a</td>
<td>58 (19.9)</td>
<td>31 (11.8)</td>
<td>9 (7.0)</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>34 (11.7)</td>
<td>16 (6.1)</td>
<td>4 (3.1)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>51 (17.5)</td>
<td>21 (8.0)</td>
<td>14 (10.9)</td>
<td></td>
</tr>
<tr>
<td>Glycemic Status</td>
<td></td>
<td></td>
<td></td>
<td>0.016</td>
</tr>
<tr>
<td>Normal glycemia</td>
<td>238 (81.8)</td>
<td>219 (83.3)</td>
<td>112 (87.5)</td>
<td></td>
</tr>
<tr>
<td>Pre-diabetes</td>
<td>8 (2.7)</td>
<td>17 (6.5)</td>
<td>8 (6.2)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>45 (15.5)</td>
<td>27 (10.3)</td>
<td>8 (6.2)</td>
<td></td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>32.97 (6.76)</td>
<td>30.07 (5.28)</td>
<td>29.53 (5.59)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Waist circumference (cm)</td>
<td>102.85 (15.44)</td>
<td>96.77 (14.89)</td>
<td>95.69 (14.32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MET (kcal/kg/h)</td>
<td>43.66 (66.01)</td>
<td>53.95 (64.24)</td>
<td>55.71 (63.46)</td>
<td>0.095</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>97.74 (14.64)</td>
<td>92.60 (14.61)</td>
<td>92.95 (14.27)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACR (mg/mmol)</td>
<td>0.56 [0.35, 0.95]</td>
<td>0.57 [0.35, 0.93]</td>
<td>0.46 [0.29, 0.77]</td>
<td>0.188</td>
</tr>
<tr>
<td>Urinary creatinine (mmol/L)</td>
<td>12.56 (6.42)</td>
<td>11.39 (6.47)</td>
<td>11.38 (6.49)</td>
<td>0.064</td>
</tr>
<tr>
<td>Urinary microalbumin (mg/L)</td>
<td>6.30 [3.00, 11.35]</td>
<td>5.00 [2.10, 11.45]</td>
<td>4.40 [2.07, 9.25]</td>
<td>0.028</td>
</tr>
<tr>
<td>Urinary calcium (mmol/L)</td>
<td>2.36 (1.72)</td>
<td>2.29 (1.83)</td>
<td>2.25 (1.45)</td>
<td>0.797</td>
</tr>
<tr>
<td>Urinary VDBP (ng/ml)</td>
<td>53.50 [10.45, 95.38]</td>
<td>46.95 [16.84, 94.68]</td>
<td>46.30 [19.69, 81.24]</td>
<td>0.805</td>
</tr>
</tbody>
</table>
Concentrations of log uVDBP:cr differed across vitamin D status categories at baseline (FIGURE 5.2). The median log value of uVDBP:cr for subjects with vitamin D deficiency (n=291) was 1.62 μg/mmol (IQR 0.32 – 2.03). Subjects with insufficient vitamin D status (n=262) had median log uVDBP:cr concentration of 1.68 μg/mmol (IQR 1.13 – 2.03), and those with sufficient vitamin D levels (n=128) had median log uVDBP:cr of 1.52 μg/mmol (IQR 1.10 – 2.09). Overall, there was a significant difference in uVDBP:cr loss between vitamin D categories (p=0.026). Post-hoc analysis using Tukey’s HSD found a significant difference between vitamin D deficient and insufficient groups (p=0.026), but no significant difference between any other groups.
As a continuous variable, serum 25(OH)D was positively correlated with uVDBP:cr at baseline (FIGURE 5.3A). Linear regression found the unadjusted β coefficient (95% CI) to be 0.96 (0.03, 1.88), $p=0.04$. When adjusted for age, sex, ethnicity, BMI, MET, and glycemic status, the association was no longer significant; the β coefficient and 95% CI was 0.32 (-0.53, 1.17), $p=0.46$. The Spearman’s rank correlation coefficient was $r = 0.03$ ($p=0.42$). Further analysis using local polynomial regression (LOESS) curves shows that there was a decrease in serum 25(OH)D at log uVDBP:cr values higher than 2.5 μg/mmol (FIGURE 5.3B).
CHAPTER 5 UVDBP AND VITAMIN D

FIGURE 5.3 (A) Association between log uVDBP:cr concentrations and serum 25(OH)D at baseline. (B) LOESS shows a decrease in serum 25(OH)D at log uVDBP:cr values higher than 2.5 μg/mmol.
5.4.2 Prospective results

There were 689 subjects at baseline, 445 subjects at the 3-year follow-up, and 114 subjects at the 6-year follow-up with serum 25(OH)D measures (TABLE 5.2). As expected, the mean age increased from 49.77 years to 55.82 years throughout the study ($p<0.001$). Compared to baseline, there was a smaller percentage of subjects with normal glycemic status at subsequent clinical visits. In contrast, the proportion of subjects with pre-diabetes and diabetes increased at follow-up visits. BMI decreased from 31.19 kg/m$^2$ to 29.37 kg/m$^2$ at the 6-year follow-up ($p<0.001$), but there was no significant change in waist circumference. Indicators of kidney function—eGFR and ACR—were worse at follow-up visits compared to baseline. Mean serum 25(OH)D concentrations were higher at the follow-up visits compared to baseline ($p<0.001$). Corresponding to the increase in 25(OH)D, PTH values were smaller at 6-year compared to the baseline measurement. In order to assess if there was still a significant increase in 25(OH)D levels, a sensitivity analysis was conducted where only 3-year follow-up was analysed due to the limited number of subjects with 25(OH)D values at the 6-year follow-up visit (see TABLE 7.5); all significant differences shown in TABLE 5.2 remained significant.

TABLE 5.2 Subject characteristics across visit numbers for subjects with serum 25(OH)D measures.

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=682)</th>
<th>3-year (n=445)</th>
<th>6-year (n=114)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>49.77 (10.04)</td>
<td>53.16 (9.66)</td>
<td>55.82 (9.62)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Sex (% males)</td>
<td>215 (31.5)</td>
<td>122 (27.4)</td>
<td>37 (32.5)</td>
<td>0.29</td>
</tr>
<tr>
<td>Ethnicty</td>
<td></td>
<td></td>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>European</td>
<td>444 (65.1)</td>
<td>314 (70.6)</td>
<td>83 (72.8)</td>
<td></td>
</tr>
<tr>
<td>Latino/a</td>
<td>98 (14.4)</td>
<td>53 (11.9)</td>
<td>7 (6.1)</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>54 (7.9)</td>
<td>31 (7.0)</td>
<td>14 (12.3)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>86 (12.6)</td>
<td>47 (10.6)</td>
<td>10 (8.8)</td>
<td></td>
</tr>
<tr>
<td>Glycemic Status</td>
<td></td>
<td></td>
<td></td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td>Normal glucose tolerance</td>
<td>569 (83.4)</td>
<td>326 (73.3)</td>
<td>82 (71.9)</td>
<td></td>
</tr>
<tr>
<td>Pre-diabetes</td>
<td>33 (4.8)</td>
<td>99 (22.2)</td>
<td>31 (27.2)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>80 (11.7)</td>
<td>20 (4.5)</td>
<td>1 (0.9)</td>
<td></td>
</tr>
</tbody>
</table>
Generalized estimating equations (GEE) were used to assess longitudinal associations between uVDBP:cr and vitamin D status over 6 years. The outcome of the model was serum 25(OH)D concentration and the main predictor was uVDBP:cr at all time points. There were no significant interactions with time (assessed using follow-up duration and visit number) (data not presented). We found that after adjusting for follow-up duration (in years), baseline age, sex, ethnicity, BMI, physical activity, and glycemic status, there were no significant associations between standard deviation (SD) differences in uVDBP:cr and change in 25(OH)D concentrations longitudinally (β = 0.38 [95% CI -0.98, 1.76]) (p=0.58) (FIGURE 5.4). In this model, follow-up duration, baseline age, ethnicity, BMI, and season were significantly associated with changes in 25(OH)D over time. One SD change in follow-up duration amounted to 4.60% (3.7, 5.5) increase in 25(OH)D concentration (p<0.001). Age at baseline was also positively associated with the outcome (β = 2.46 [95% CI 0.25, 4.73] (p=0.03).
Subjects of European descent had a 9.39% (3.76, 15.32) higher 25(OH)D concentration compared to non-Europeans \((p<0.001)\). BMI was associated with a -5.84% (-8.16, -3.47) decrease in 25(OH)D over time \((p<0.001)\). As well, serum 25(OH)D concentrations were higher in summer months compared to winter months \((\beta = 5.58 [95\% \text{ CI } 1.98, 9.3]) (p<0.001)\). Sex, physical activity levels, and glycemic status were not significantly associated with 25(OH)D over 6 years (FIGURE 5.4).

**FIGURE 5.4** GEE models of uVDBP:cr over time and serum 25(OH)D over 6 years.

Tabular data can be found in Table 7.6.

Adjusted GEE models were also used to assess if baseline urinary VDBP:cr concentrations are associated with changes in vitamin D status over 6 years (FIGURE 5.5). Again, the model excluded subjects who were vitamin D deficient at baseline. The outcome assessed was serum 25(OH)D concentration at all time points while the predictor in the model was uVDBP:cr at baseline. Covariates of the model included follow-up duration (in years), baseline age, sex, ethnicity, physical activity, BMI, and glycemic status. Interaction with time
was assessed using both visit number and follow-up duration; no significant interactions were found (data not shown). Like the previous model, a 1 SD increase in baseline uVDBP:cr was not significantly associated with changes in 25(OH)D over 6 years ($\beta = -0.69$ [95% CI -2.13, 0.77]) ($p=0.35$) after adjusting for covariates (FIGURE 5.5). Several covariates in the model were significantly associated with the outcome. Follow-up duration ($\beta = 4.59$ [95% CI 3.7, 5.49]) ($p<0.001$) and baseline age ($\beta = 2.41$ [95% CI 0.2, 4.67]) ($p=0.03$) were both positively associated with an increase in 25(OH)D over time. Subjects of European origin had 9.39% (3.77, 15.31) higher 25(OH)D levels compared to subjects of other ethnicities ($p<0.001$). Serum 25(OH)D was lower when subjects had higher BMI ($\beta = -5.92$ [-8.25, -3.54]) ($p<0.001$). Similar to the model where the predictor was uVDBP:cr at all time points, serum 25(OH)D was a 5.54% (1.95, 9.26) higher in the summer compared to winter months ($p<0.001$). No significant associations were found with other covariates.
FIGURE 5.5 GEE models of baseline uVDBP:cr and kidney outcomes (ACR and eGFR) over 6 years.

Tabular data can be found in Table 7.7.
5.5 Discussion

This study demonstrated that, at baseline, urinary vitamin D binding protein concentration differed with vitamin D status, but 25(OH)D was not associated with uVDBP:cr after accounting for covariates. Using adjusted generalized estimating equation models, it was found that there was no association between urinary VDBP concentration and serum 25(OH)D concentration. Baseline uVDBP:cr was also not associated with any change in 25(OH)D over 6 years. This was the first study to examine the prospective association of serum 25(OH)D with urinary vitamin D binding protein concentrations.

Only a limited number of studies have investigated the relationship between urinary VDBP and 25(OH)D levels (12,26,475,476). Previous studies in animal models have demonstrated a potential mechanism which links urinary loss of VDBP to vitamin D status. Zucker fatty rats—a model of type 2 diabetes—showed reduced 25(OH)D and 1,25(OH)_{2}D serum levels which were associated with elevated urinary levels of these vitamin D metabolites and VDBP (413). In a study conducted in diabetic nephropathy-prone mice, urinary 25(OH)D excretion increased along with increasing uVDBP concentrations (414). Smazal et al. have also demonstrated that type 2 diabetic rats exhibit vitamin D deficiency due to aberrant megalin-mediated endocytosis and excessive urinary excretion of 25(OH)D and VDBP (416). Another study using Zucker fatty rats found that when rats were fed a diet which prevented urinary excretion of VDBP, urinary loss of 25(OH)D and 1,25(OH)_{2}D was lower and serum 25(OH)D was higher when compared to the control group (415). This digestion-resistant starch was also able to prevent elevated 25(OH)D and VDBP in the urine in rats with type 1 diabetes. In contrast, most previous studies in humans have not been able to establish a correlation between urinary VDBP and serum 25(OH)D (14,476,483–485).

In albuminuric patients with type 1 diabetes, urinary megalin and cubilin excretion were increased along with uVDBP (423). Thrailkill et al. reported that uVDBP excretion in T1DM patients was higher than that in the control group, and suggested a possible relationship between uVDBP concentrations and vitamin D deficiency, which was also observed in the study population (12). However, most follow-up studies investigating the association between uVDBP and vitamin D status in human subjects reported no link between the two variables (14,476,483–485); one genetic study in infants and toddlers reported a positive correlation (486). A recent paper by Kim et al. demonstrated that serum VDBP and urinary VDBP:cr concentrations were not different between pediatric patients with type 1 diabetes compared to a healthy control group (476). Additionally, serum 25(OH)D levels did not correlate with uVDBP:cr (476). Other papers have examined possible associations between serum and urinary VDBP but reported that urinary VDBP loss, serum VDBP concentration,
and vitamin D status were all independent of each other (419,487). Similar to these findings, uVDBP:cr did not associate with serum 25(OH)D levels in our study after adjustment for covariates. However, LOESS curves showed a decrease in serum 25(OH)D concentrations at log uVDBP:cr values higher than 2.5 μg/mmol, suggesting that higher concentrations of uVDBP loss may be needed for serum 25(OH)D levels to be affected (FIGURE 5.3B).

Unlike previously published studies which used cross-sectional designs, we measured the association between 25(OH)D and uVDBP over a 6-year follow-up period. This longitudinal analysis provided us further information regarding how the loss of this carrier protein affects vitamin D status over time. Results from our adjusted GEE model found that baseline levels or changes in uVDBP:cr over time were not significantly associated with any changes in serum 25(OH)D over 6 years. This may be attributed to the fact that VDBP circulates at a concentration (5 x 10^-6 mol/L) approximately 100-fold higher than that of its ligand 25(OH)D (5 x 10^-8 mol/L) (253). Only a severe and long-term loss of VDBP, as observed in nephrotic syndrome with continuous proteinuria, could lead to decreases in the 25(OH)D level (475). As well, the lack of association between serum VDBP and 25(OH)D from previous studies supports the conclusion that VDBP has little effect on concentrations of vitamin D metabolites (419,487). However, 25(OH)D was significantly associated with baseline age, ethnicity, BMI, and season in the GEE model (FIGURE 5.4 and FIGURE 5.5). The age-dependency of vitamin D status has been previously described by others (250,255,257,484,488). There are several proposed mechanisms which link lower 25(OH)D to higher BMI, including lower dietary intake (489,490), reduced interstitial absorption (260,261) and sequestering of 25(OH)D in adipose tissue (269–271). Studies have also shown that ethnicity plays a role in vitamin D status; geographical latitude affects the amount of sunlight received and higher melanin pigmentation competes with 7-dehydrocholesterol and acts as a natural sunscreen to absorb solar UV radiation (328,491). Lastly, the dependency of serum 25(OH)D on season—which indirectly measures sunlight exposure—is well-established in the literature (252,328,488,492).

Exaggerated urinary loss of VDBP in diabetes, particularly in persons with kidney dysfunction, could contribute mechanistically to vitamin D deficiency in this disease. Our baseline analysis demonstrated a correlation between estimated glomerular filtration rate (eGFR) and vitamin D status (FIGURE 4.3). Ordinarily, the VDBP + 25(OH)D complex within the glomerular filtrate is endocytosed in the proximal tubule by megalin and cubilin. Previous studies have also established that vitamin D receptor agonists prevent kidney damage and GFR loss in animal models (340). These agonists suppress renin secretion and downstream activation of the renin-angiotensin-aldosterone system, which plays a key role in the pathogenesis of kidney disease by increasing blood pressure and stimulating fibrosis within the kidney. Problems
with the proximal tubule reabsorption process, such as loss of megalin function, is also likely involved in the mechanism of vitamin D deficiency in animal models of diabetes (12,423,493,494).

Strengths of this study include multiple measurements of serum 25(OH)D in the same subjects over time. We used a large, multi-ethnic cohort, and were able to adjust for many relevant covariates such as season and physical activity. In addition, the use of GEE models in the current study was beneficial as this statistical method offers flexibility for fluctuating sample sizes in longitudinal studies and accounts for the lack of independence between the repeated measurements at clinic visits from the same participant. This was the first study to examine urinary vitamin D binding protein concentrations with serum measures of 25(OH)D longitudinally.

This study, however, also has a number of limitations. First, the sample size of subjects with 25(OH)D measurements at later follow-up visits are somewhat small (n=114 at the last clinical follow-up) and therefore insufficient power may have reduced the ability to detect significant effects. It should be noted that our sensitivity analysis showed that excluding the 6-year follow-up data from analysis did not impact any variables of interest, and thus the direction of our associations was unlikely to have been impacted (TABLE 7.5). However, the strength of association may be stronger with more subjects, and thus, longitudinal analyses may have yielded significant findings. Second, although many variables related to VDBP and vitamin D were assessed in the study, information on vitamin D dietary intake and participant’s sun exposure behaviour were only measured in the 3rd follow-up clinic visit and therefore we were unable to examine how changes in these factors over the follow-up period contributed to changes in 25(OH)D. We were also unable to account for supplementation of vitamin D. Third, we focused only on total vitamin D serum level and did not consider its free/bioavailable fraction; with respect to vitamin D physiology, a recent study found that lower VDBP resulted in higher vitamin D bioavailability (495). Lastly, serum vitamin D binding protein was not assayed in the PROMISE cohort, and as a result, we could not evaluate any possible associations between urinary VDBP concentrations and serum VDBP concentrations.

In conclusion, our results showed no association between serum 25(OH)D and uVDBP in subjects who were at risk for T2DM over a 6-year follow-up after adjustment for covariates. Further studies involving subjects with more severe kidney damage and longer follow-up are needed to assess whether uVDBP concentrations are a useful marker of risk of future vitamin D deficiency.
CHAPTER 6

Overall Discussion

6.1 Objectives and summary of findings

1) To examine longitudinal associations between urinary vitamin D binding protein and kidney dysfunction as assessed by albumin-to-creatinine ratio (ACR) and estimated glomerular filtration rate (eGFR).

In this study, urinary VDBP loss was consistently associated with higher albumin (ACR) loss in the urine, but there was no significant association with change in eGFR. Our GEE models showed that for 1 standard deviation increase in uVDBP:cr over time, there was a 32.21% (CI 16.9, 49.52) increase in ACR. One standard deviation increase in uVDBP:cr over time did not result in any significant change in eGFR after adjusting for covariates (p=0.29).

2) To assess if baseline urinary vitamin D binding protein concentrations are associated with declines in kidney function over time.

In terms of the association between baseline uVDBP:cr and kidney outcomes, it was found that an increase in urinary VDBP loss was associated with higher ACR but no change in eGFR at later time points. One standard deviation increase in baseline uVDBP:cr was associated with a 29.07% (CI 23.26, 35.16) higher ACR over 6 years. In contrast, no significant differences in uVDBP concentration were found between eGFR categories at baseline and over a 6-year follow-up (p=0.13).

3) To study longitudinal associations between urinary vitamin D binding protein concentrations and serum 25(OH)D levels.

Using adjusted generalized estimating equation models, it was found that there was no association between longitudinal urinary VDBP concentration and serum 25(OH)D concentration.
4) To evaluate if baseline urinary vitamin D binding protein concentrations are associated with changes in vitamin D status prospectively.

Baseline uVDBP:cr was not associated with any change in 25(OH)D over 6 years after adjusting for the relevant covariates in the GEE model. The association between uVDBP:cr and 25(OH)D may be different at higher urinary VDBP concentrations.

6.2 Overall summary

Recent studies have reported elevated urinary vitamin D binding protein in patients with diabetes and nephropathy, suggesting a possible mechanism for hypovitaminosis D in individuals with diabetes and kidney dysfunction (12,411). However, the effects of uVDBP loss on kidney function and 25(OH)D status have not been studied prospectively. Therefore, the first objective of the thesis project was to examine the longitudinal association of urinary vitamin D binding protein concentrations with markers of kidney disease, specifically albuminuria and low GFR. The second objective was to investigate the potential association of urinary vitamin D binding protein with vitamin D status in subjects at risk for T2DM.

In chapter 4, the association of uVDBP:cr with longitudinal changes in kidney function was assessed in a cohort of adults at risk for T2DM. The analysis indicated that urinary VDBP concentrations increased with ACR severity at baseline, but there were no significant associations between uVDBP:cr and eGFR. There was a positive association between uVDBP:cr concentration and ACR over 6 years even after the adjustment of covariates. Specifically, each SD difference in uVDBP:cr was associated with a 32.21% increase in urinary ACR concentration. Baseline uVDBP:cr was also associated with higher ACR over 6 years of follow-up. However, baseline urinary VDBP:cr concentration or uVDBP:cr over time was not associated with changes in eGFR longitudinally after adjusting for follow-up duration (in years), baseline age, sex, ethnicity, and glycemic status ($p=0.28$ and $p=0.13$, respectively).

The albumin-to-creatine ratio is widely used as an indicator of the severity of kidney disease and is one of the earliest signs of diabetic nephropathy (128). Microalbuminuria has been found to predict progression to clinical proteinuria and decreased renal function in patients with T2DM (128,130,131). Albuminuria has also been found to be strongly associated with end-stage renal disease (pooled HR 3.04) and cardiovascular events after adjusting for risk factors and eGFR in a collaborative meta-analysis (496,497). The association between ACR and eGFR has been less extensively studied, but there is a slight negative association between the two kidney outcomes at the population level. Hoefield et al. found that in subjects with
diabetes and macroalbuminuria, eGFR declined at 5.7% per year while the eGFR of those with microalbuminuria or without albuminuria declined at 1.5% and 0.3% per year, respectively, independently of age \( (p<0.001) \) (498). In general, the progression of kidney disease in the diabetic population without albuminuria is relatively benign compared to those with albuminuria.

Previous studies have identified a positive association between albuminuria status and loss of VDBP in humans (12,13). Thrailkill et al. found that in subjects with type 1 diabetes, urinary VDBP:cr was significantly higher in those with diabetes and proteinuria (ACR >30 mg/g) compared to those with diabetes and normoalbuminuria and healthy age-matched controls (12). Later, Tian et al. and Khodeir et al. reported that the severity of uVDBP loss increased with the degree of albuminuria; there was a higher concentration of uVDBP in subjects with T2DM + microalbuminuria and T2DM + macroalbuminuria compared to subjects with only T2DM (13). Although albumin and vitamin D binding protein share the megalin/cubilin-coupled receptor for reabsorption purposes, the reabsorption mechanism of albumin and VDBP is not identical (441). It has been argued that the capacity of the megalin/cubilin-mediated mechanism for tubular uptake of albumin is low (458). As well, albumin has been observed to have alternate binding sites on isolated proximal tubule segments and several receptors for tubular uptake of albumin, such as the Fc-receptor, have been identified. A previous study suggested that the reabsorption of albumin is mainly located in the glomerulus or early tubular system, as micropuncture of the proximal tubule failed to change the degree of albumin spillage (464). In contrast, the loss of VDBP in the urine of megalin-deficient mice highlights the important role of tubular uptake of VDBP from glomerular filtrates (395,468,469).

While there was a strong association between ACR and uVDBP:cr, the relationship between eGFR and uVDBP:cr was less clear. The lack of a significant association between uVDBP and eGFR may be attributable to the large number of relatively healthy subjects in PROMISE at baseline (only 12 subjects with eGFR < 60 ml/min/1.73m\(^2\)) and, perhaps, characteristics of hyperfiltration (455). Another possible explanation for the null association between uVDBP and eGFR in this study is that injury at the proximal tubule (changes in megalin/cubilin expression) plays a larger role in the renal loss of VDBP compared to damage at the glomeruli (470–473). Fewer previous papers have studied the association between uVDBP and eGFR and results have been inconsistent, likely due to differences in population characteristics and underlying severity of kidney dysfunction (439,441,456).

The pathophysiological mechanisms underlying the enhanced excretion of urinary VDBP in patients with diabetic nephropathy (DN) remain unclear. One possible explanation is that
elevated urinary VDBP levels may be associated with renal tubular damage in DN patients (410,411,441). Although albumin and vitamin D binding protein have similar molecular weights and share the megalin/cubilin-coupled receptor for reabsorption purposes, alternate mechanisms of albumin reabsorption have been suggested, including fast, high-capacity retrieval pathway for non-degraded albumin located in the glomerulus or early tubular system (458,464). In contrast, the loss of VDBP in the urine of megalin-deficient mice highlights the important role of tubular uptake of VDBP from glomerular filtrates (395,468,469). Although albuminuria is the clinical standard for assessing the progression of renal disease, it is only able to indicate the magnitude of proteinuria and not the origin of loss (glomerular or tubular) due to its multiple reabsorption pathways. In contrast, reabsorption of VDBP is localized to the megalin/cubilin receptors at the proximal tubules, suggesting uVDBP as a good biomarker for renal tubular injury.

To the best of our knowledge, no previous studies have examined the association between established kidney markers and uVDBP in humans prospectively. Our work supports our hypothesis that the strong positive association between uVDBP:cr and ACR is maintained over time, and that a higher concentration of uVDBP:cr at baseline is significantly associated with increased ACR over 6 years. Although further research is needed, the strong association observed between ACR and uVDBP both cross-sectionally and prospectively points to uVDBP as a potential early marker that may be used in conjunction with ACR for a complete assessment of proteinuria and drastically lessen the risk of kidney failure in this population.

In chapter 5, we looked at the association of urinary vitamin D binding protein concentration with longitudinal changes in vitamin D status assessed using serum 25(OH)D levels. While many papers have documented the association of low vitamin D with the risk the development of T2DM, it has also proposed that hypovitaminosis D is a consequence of diabetes pathology itself. One mechanism for low 25(OH)D levels in patients with diabetes may be the loss of the VDBP + 25(OH)D complex in the urine. We found that while urinary VDBP:cr concentrations differed with vitamin D status at baseline, there were no significant associations between uVDBP:cr and serum 25(OH)D after adjusting for sex, ethnicity, BMI, physical activity, and glycemic status. As well, changes in uVDBP:cr longitudinally or uVDBP:cr concentrations at baseline were not associated with any changes in 25(OH)D over 6 years of follow-up when assessed using generalized estimating equation models.

In both diabetic animals and humans, increased urinary megalin and cubilin excretion have been reported along with urinary VDBP, which suggested a possible relationship between uVDBP and vitamin D deficiency, which was also observed in the study population (12,416,423). However, although animal models have linked low 25(OH)D levels to elevated
uVDBP concentrations, most studies in human subjects do not show a significant correlation between uVDBP and serum 25(OH)D (14,476,483–485). VDBP circulates at a concentration approximately 100-fold higher than 25(OH)D, which suggests that only a severe and long-term loss of VDBP could lead to decreases in serum 25(OH)D levels (253,475). In our study, there was a drop in 25(OH)D concentration only at very high uVDBP:cr concentrations, supporting the hypothesis that more severe losses of VDBP in the urine may be needed before vitamin D status is affected.

Previous studies have also established a link between vitamin D and kidney health. Vitamin D receptor agonists have been shown to prevent kidney damage, GFR loss, and suppress the activation of the renin-angiotensin-aldosterone system (12,423,493,494). The majority of subjects in the PROMISE cohort had relatively normal kidney function as assessed by ACR and eGFR at baseline and at the 6-year follow-up visit, which may contribute to the lack of association between uVDBP and serum 25(OH)D in this population.

Taken together, the findings of my MSc research highlight the longitudinal association of urinary vitamin D binding protein with kidney dysfunction markers and provide insight regarding hypovitaminosis D in subjects at risk for T2DM. Urinary VDBP concentrations were strongly associated with ACR both cross-sectionally and longitudinally, but no associations were observed for eGFR and serum 25(OH)D. Damage to the post-glomerular components of the renal system (i.e. the megalin and cubilin receptors in the proximal tubule) may explain the association observed between uVDBP and albumin but not eGFR. Since the circulating concentration of VDBP is much higher than 25(OH)D, the lack of association observed between uVDBP and serum 25(OH)D may be due to the relatively mild amount of VDBP found in the urine because few subjects had severe kidney dysfunction in the PROMISE cohort even at the 6-year follow-up.

### 6.3 Strengths and limitations

#### 6.3.1 Strengths

Strengths of the current study include the use of a longitudinal cohort design, which allows for analysis of temporal associations between urinary vitamin D binding protein concentrations and outcomes such as kidney disease measures and hypovitaminosis D; neither of these relationships had been previously studied longitudinally. The cohort also contained validated and established measures of kidney function, in addition to other questionnaire, demographic, metabolic, and anthropometric variables. Given the complex
data available in PROMISE, statistical models specifically suited for longitudinal studies were used. GEE models, in particular, offer flexibility for fluctuating sample sizes in longitudinal studies while accounting for the lack of independence between repeated measures at follow-up visits from the same subject. The code used for analysis is publicly available, providing a level of rigor, transparency, and strength to our findings presented here (499,500).

6.3.2 Limitations
This study has several limitations that should be considered when interpreting the results. Since PROMISE is a longitudinal observational cohort, there may be some residual confounding due to lack of adjustment for confounders or suboptimal measurement of the covariates included in the GEE models used. It is also possible that confounding pathways exist which have not yet been described. As well, the majority of subjects in the cohort were female and of European ancestry, and as a result, results may not be generalizable to other demographic groups. Since the PROMISE cohort was designed to study individuals at risk for T2DM, subjects with clinically recognized kidney disease at baseline were excluded from the study. Thus, an important limitation of the project is that the cohort was still relatively healthy and most subjects did not have severe complications during the early years of follow-up. As such, perhaps associations were not observed between uVDBP and 25(OH)D and eGFR because the VDBP loss was not severe enough to influence serum levels of its ligand. However, since it is still an ongoing study, subjects might be expected to develop more severe kidney dysfunction over time. Another limitation of the study is that the number of subjects with serum 25(OH)D measurements at the third follow-up visit are much smaller than the initial baseline and first follow-up measurement and therefore we might not have had sufficient power to detect associations with 25(OH)D. It should be noted that our sensitivity analysis showed that excluding the 6-year follow-up data from analysis did not impact any variables of interest, and thus the direction of our associations were unlikely to have been impacted (TABLE 7.5). However, the strength of association may be stronger with more subjects, and thus, longitudinal analyses may have yielded significant findings. Lastly, there were several variables that were not available in the PROMISE dataset—such as KIM-1, NGAL, dietary vitamin D, and serum VDBP—that might have yielded more sensitive measurements regarding kidney damage and etiological pathways behind hypovitaminosis D in this population.
6.4 Implications

Interest in vitamin D has markedly increased over the last decade. Moreover, the theory that low vitamin D status is a contributing factor to the incidence of chronic disease is at the heart of an ongoing debate about recommendations for vitamin D intake and supplementation. Although there is little known about why vitamin D status is suboptimal in individuals with diabetes, data suggests that it may be in part due to compromised kidney function (344,361,413,423). Findings from this project improve our understanding of urinary VDBP in general, as well as the relationship between urinary VDBP and markers of kidney dysfunction. We also assessed the longitudinal impact of uVDBP loss in terms of vitamin D status in this large, multi-ethnic cohort. Although we found that the loss of this protein does not appear to affect vitamin D status over 6 years, results may be different for studies of longer duration involving subjects with worse kidney health at baseline. Further research in this area is needed to assess the potential for uVDBP as a biomarker for detecting early proximal tubular damage and determining risk stratification with regard to kidney function, which will allow for targeted prevention and better clinical management approaches.
6.5 Future directions

There are several directions for future research based on the findings from this thesis project. First, there are limited data available regarding urinary vitamin D binding protein in humans, and to the best of our knowledge, PROMISE is the first cohort with longitudinal uVDBP concentration measures. It would also be helpful to include serum VDBP in future studies to determine if renal VDBP loss impacts serum levels of the protein.

In terms of evaluating the utility of uVDBP as a predictor of kidney damage, studies using longer follow-up periods would be of value as a greater proportion of subjects would likely have a sharper decline in kidney function at later time-points. Secondly, a previously published paper has described uVDBP responding to renoprotective therapies in CKD patients (441); it would be interesting to see if these medications act as effect modifiers in the relationship between uVDBP and existing kidney markers. As well, additional investigations could be conducted examining possible associations of uVDBP with markers of acute kidney injury that are purported to be more sensitive such as KIM-1, IL-18, and NGAL. One previous study has described an association between uVDBP and tubular damage markers KIM-1 and NGAL independently of albuminuria, suggesting that uVDBP may be a novel biomarker of tubulointerstitial damage (438). Taken together, additional studies in this area are needed in order to determine the precision and accuracy of uVDBP as a marker for overall kidney dysfunction and proximal tubular injury.

Due to the limited number of subjects in our study with macroalbuminuria and eGFR < 60 ml/min/1.73m² during the first 6 years of follow-up, we were unable to conduct stratified analyses regarding the association between uVDBP:cr and 25(OH)D. As more subjects progress to chronic kidney disease, future research with the PROMISE cohort will be able to assess the association between serum 25(OH)D and uVDBP while accounting for the severity of kidney damage in subjects. Although many variables related to vitamin D were adjusted for in this study, further research is required to assess the association of uVDBP:cr and 25(OH)D while taking into account dietary intake, supplementation, and sun exposure. With respect to vitamin D physiology, a recent study found that lower VDBP resulted in higher bioavailable/free vitamin D (495); further research may investigate the relationship between urinary VDBP and the free/bioavailable fraction of vitamin D. Overall, future studies should include longer follow-ups with more repeat measurements of 25(OH)D to adequately study long-term vitamin D status, particularly in subjects with severe kidney damage. The continued epidemiological investigation into the role of urinary VDBP is warranted, as these studies shed light on potential etiological pathways underlying hypovitaminosis D in diabetes and kidney disease.
6.6 Conclusion

In conclusion, we found that urinary VDBP concentrations were strongly associated with ACR both cross-sectionally and longitudinally, but no associations were observed for eGFR and serum 25(OH)D. Although the overall association was not significant, we observed a decrease in serum 25(OH)D levels at higher concentrations of uVDBP, suggesting that uVDBP may be involved in the mechanism of hypovitaminosis D but only at higher concentrations. Results from this thesis deepen our understanding of trajectories of VDBP in the urine, as well as the longitudinal relationships between uVDBP, kidney damage markers, and vitamin D status. Findings from this thesis provide future avenues of research into the potential for uVDBP to be an early marker of tubular damage that can be used in conjunction with ACR for a more comprehensive assessment of kidney damage.
References


152. NKF KDOQI Guidelines [Internet]. [cited 2018 Feb 12]. Available from: http://www2.kidney.org/professionals/kdoqi/guidelines_ckd/p4_class_g1.htm


318. Vitamin D and Longevity (VIDAL) Trial [Internet]. [cited 2018 Feb 20]. Available from: https://vidal.lshtm.ac.uk/home/


337. Vitamin D and Type 2 Diabetes Study (D2d) [Internet]. [cited 2018 Feb 20]. Available from: https://clinicaltrials.gov/ct2/show/NCT01942694


446. Immunodiagnostik. Vitamin D binding Protein (VDBP) ELISA [Internet]. [cited 2018 Apr 23]. Available from: http://www.immundiagnostik.com/en/home/products/kits-assays/skeletal-system.html?tx_mokom01immunprodukte_pi1%5Ban%5D=K%202314&tx_mokom01immunprodukte_pi1%5Bag%5D=401&cHash=b4d2363473


7.1 Supplementary figures and tables from chapter 4

**FIGURE 7.1** Log uVDBP:cr values for subjects with and without albuminuria at baseline.

*Albuminuria is classified as subjects with ACR $\geq 2$ mg/mmol (n=56).*
### TABLE 7.1 GEE results of uVDBP:cr over time and kidney outcomes (ACR and eGFR) over 6 years.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Predictor</th>
<th>Covariates</th>
<th>β (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>uVDBP:cr (μg/mL)</td>
<td></td>
<td>32.21 (16.9, 49.52)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ACR (mg/mmol)</td>
<td>Follow-up duration (months)</td>
<td></td>
<td>9.59 (6.17, 13.12)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>3.01 (-1.97, 8.23)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>-24.34 (-32.31, -15.43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>-4.61 (-13.9, 5.67)</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>5 (-4.9, 15.94)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>10.52 (-7.31, 31.78)</td>
<td>0.27</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>uVDBP:cr (μg/mL)</td>
<td></td>
<td>0.28 (-0.23, 0.79)</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td></td>
<td>-1.48 (-2.03, -0.93)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>-8.06 (-8.88, -7.23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>-0.39 (-2.46, 1.73)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>-4.09 (-6, -2.15)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>-0.46 (-2.4, 1.53)</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>1.34 (-3.19, 6.09)</td>
<td>0.57</td>
</tr>
</tbody>
</table>

*Estimates are represented as percent difference with 95% CI for each SD increase in predictor/covariate. Subjects with macroalbuminuria and eGFR <60 ml/min/1.73m² at baseline were excluded from analysis. Sensitivity analysis using uVDBP unadjusted for urinary creatinine can be found in Figure 7.2.*
### TABLE 7.2 GEE results of baseline uVDBP:cr and kidney outcomes (ACR and eGFR) over 6 years.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Predictor</th>
<th>Covariates</th>
<th>$\beta$ (95% CI)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ACR (mg/mmol)</strong></td>
<td>Baseline uVDBP:cr (μg/mL)</td>
<td></td>
<td>29.07 (23.26, 35.16)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td></td>
<td>10.39 (6.52, 14.41)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>4.02 (-0.95, 9.25)</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>-22.12 (-29.88, -13.5)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>-5.89 (-14.89, 4.06)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>3.95 (-5.85, 14.76)</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>12.39 (-7.31, 36.28)</td>
<td>0.23</td>
</tr>
<tr>
<td><strong>eGFR (ml/min/1.73m$^2$)</strong></td>
<td>Baseline uVDBP:cr (μg/mL)</td>
<td></td>
<td>0.81 (-0.22, 1.86)</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td></td>
<td>-1.48 (-2.03, -0.93)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>-8.05 (-8.88, -7.23)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>-0.2 (-2.27, 1.92)</td>
<td>0.85</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>-4.13 (-6.03, -2.19)</td>
<td>$&lt;0.001$</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>-0.47 (-2.4, 1.51)</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>1.38 (-3.16, 6.12)</td>
<td>0.56</td>
</tr>
</tbody>
</table>

*Estimates are represented as percent difference with 95% CI for each SD increase in predictor/covariate. Subjects with macroalbuminuria and eGFR <60 ml/min/1.73m$^2$ at baseline were excluded from analysis. Sensitivity analysis using uVDBP unadjusted for urinary creatinine can be found in Figure 7.3.*
**FIGURE 7.2** GEE models where the predictor (uVDBP) is not adjusted for urinary creatinine. Outcomes of the model were ACR and eGFR.

Tabular data can be found in Table 7.3.
### TABLE 7.3 GEE results of uVDBP over time and kidney outcomes (ACR and eGFR) over 6 years.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Predictor</th>
<th>Covariates</th>
<th>$\beta$ (95% CI)</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR (mg/mmol)</td>
<td>uVDBP (μg/mL)</td>
<td></td>
<td>16.55 (5.93, 28.23)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td></td>
<td>10.33 (6.61, 14.17)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>3.86 (-1.44, 9.45)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>-28 (-35.48, -19.64)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>-4.5 (-14.4, 6.54)</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>4.79 (-5.44, 16.14)</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>10.54 (-8.86, 34.05)</td>
<td>0.31</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73m²)</td>
<td>uVDBP (μg/mL)</td>
<td></td>
<td>-0.59 (-1.04, -0.14)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td></td>
<td>-1.48 (-2.03, -0.93)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>-8.03 (-8.85, -7.19)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>-0.45 (-2.52, 1.66)</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>-4.09 (-6, -2.14)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>-0.47 (-2.41, 1.5)</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>1.43 (-3.14, 6.22)</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Estimates are represented as percent difference with 95% CI for each SD increase in predictor/covariate. Subjects with macroalbuminuria and eGFR <60 ml/min/1.73m² at baseline were excluded from analysis.
FIGURE 7.3 GEE models where the predictor (baseline uVDBP) is not adjusted for urinary creatinine. Outcomes of the model were ACR and eGFR.

Tabular data can be found in Table 7.4.
### TABLE 7.4 GEE results of baseline uVDBP and kidney outcomes (ACR and eGFR) over 6 years.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Predictor</th>
<th>Covariates</th>
<th>β (95% CI)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACR (mg/mmol)</td>
<td>Baseline uVDBP (μg/mL)</td>
<td>18.16 (11.8, 24.88)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td>10.55 (6.66, 14.57)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td>4.43 (-0.89, 10.03)</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td>-27.74 (-35.12, -19.52)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td>-5.71 (-15.38, 5.05)</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td>3 (-6.92, 13.98)</td>
<td>0.57</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td>11.47 (-8.56, 35.89)</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td></td>
<td>eGFR (ml/min/1.73m²)</td>
<td>Baseline uVDBP (μg/mL)</td>
<td>0.05 (-0.97, 1.09)</td>
<td>0.92</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (months)</td>
<td>-1.48 (-2.03, -0.93)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td>-8.04 (-8.87, -7.21)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td>-0.45 (-2.51, 1.66)</td>
<td>0.67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td>-4.09 (-6, -2.15)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td>-0.45 (-2.39, 1.53)</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td>1.37 (-3.17, 6.13)</td>
<td>0.56</td>
<td></td>
</tr>
</tbody>
</table>

*Estimates are represented as percent difference with 95% CI for each SD increase in predictor/covariate. Subjects with macroalbuminuria and eGFR <60 ml/min/1.73m² at baseline were excluded from analysis.*
### 7.2 Supplementary figures and tables from chapter 5

**TABLE 7.5 Subject characteristics at baseline and 3-year follow-up.**

<table>
<thead>
<tr>
<th></th>
<th>Baseline (n=729)</th>
<th>3-year (n=636)</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>49.77 (10.04)</td>
<td>53.16 (9.66)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Sex (% males)</strong></td>
<td>215 (31.5)</td>
<td>122 (27.4)</td>
<td>0.16</td>
</tr>
<tr>
<td><strong>Ethnicity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>European</td>
<td>444 (65.1)</td>
<td>314 (70.6)</td>
<td>0.30</td>
</tr>
<tr>
<td>Latino/a</td>
<td>98 (14.4)</td>
<td>53 (11.9)</td>
<td></td>
</tr>
<tr>
<td>South Asian</td>
<td>54 (7.9)</td>
<td>31 (7.0)</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td>86 (12.6)</td>
<td>47 (10.6)</td>
<td></td>
</tr>
<tr>
<td><strong>Glycemic Status</strong></td>
<td></td>
<td></td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Normal glucose tolerance</td>
<td>569 (83.4)</td>
<td>326 (73.3)</td>
<td></td>
</tr>
<tr>
<td>Pre-diabetes</td>
<td>33 (4.8)</td>
<td>99 (22.2)</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>80 (11.7)</td>
<td>20 (4.5)</td>
<td></td>
</tr>
<tr>
<td><strong>BMI (kg/m²)</strong></td>
<td>31.19 (6.19)</td>
<td>31.38 (6.47)</td>
<td>0.63</td>
</tr>
<tr>
<td><strong>Waist circumference (cm)</strong></td>
<td>99.14 (15.34)</td>
<td>99.23 (15.44)</td>
<td>0.93</td>
</tr>
<tr>
<td><strong>MET (kcal/kg/h)</strong></td>
<td>49.91 (64.98)</td>
<td>49.24 (61.75)</td>
<td>0.86</td>
</tr>
<tr>
<td><strong>eGFR (ml/min/1.73m²)</strong></td>
<td>94.86 (14.75)</td>
<td>89.32 (15.86)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>ACR (mg/mmol)</strong></td>
<td>0.54 [0.35, 0.93]</td>
<td>0.61 [0.42, 0.95]</td>
<td>0.002</td>
</tr>
<tr>
<td><strong>Urinary VDBP (ng/ml)</strong></td>
<td>48.16 [15.32, 93.01]</td>
<td>45.15 [12.59, 95.15]</td>
<td>0.58</td>
</tr>
<tr>
<td><strong>Urinary VDBP:cr</strong></td>
<td>5.12 [2.51, 7.72]</td>
<td>5.35 [1.66, 7.99]</td>
<td>0.81</td>
</tr>
<tr>
<td><strong>Serum 25(OH)D (nmol/L)</strong></td>
<td>55.42 (22.96)</td>
<td>73.98 (26.43)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Parathyroid Hormone (pmol/L)</strong></td>
<td>4.57 (1.71)</td>
<td>4.99 (1.83)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>
### Systolic blood pressure (mmHg)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>126.32 (16.02)</td>
</tr>
<tr>
<td></td>
<td>126.16 (15.20)</td>
</tr>
<tr>
<td>p</td>
<td>0.86</td>
</tr>
</tbody>
</table>

### Diastolic blood pressure (mmHg)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>80.22 (10.34)</td>
</tr>
<tr>
<td></td>
<td>80.32 (10.21)</td>
</tr>
<tr>
<td>p</td>
<td>0.88</td>
</tr>
</tbody>
</table>

### Mean arterial pressure (mmHg)

<table>
<thead>
<tr>
<th></th>
<th>Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Value</td>
<td>95.59 (11.41)</td>
</tr>
<tr>
<td></td>
<td>95.60 (11.05)</td>
</tr>
<tr>
<td>p</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Values reported as mean ± standard deviation for normal continuous variables, median (95% CI) for non-normal continuous variables, and n (%) for categorical variables. Significance for continuous and discrete variables was assessed using analysis of variance (ANOVA) and chi-squared test of independence, respectively.
### TABLE 7.6 GEE results of uVDBP:cr over time and serum 25(OH)D over 6 years.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Predictor</th>
<th>Covariates</th>
<th>β (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum 25(OH)D (nmol/L)</td>
<td>uVDBP:cr (μg/mL)</td>
<td></td>
<td>0.38 (-0.98, 1.76)</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>Follow-up duration (years)</td>
<td></td>
<td>4.6 (3.7, 5.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Baseline age (years)</td>
<td></td>
<td>2.46 (0.25, 4.73)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Sex (male)</td>
<td></td>
<td>1.85 (-2.68, 6.58)</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>Ethnicity (European)</td>
<td></td>
<td>9.39 (3.76, 15.32)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>BMI (kg/m²)</td>
<td></td>
<td>-5.84 (-8.16, -3.47)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>MET (kcal/kg/h)</td>
<td></td>
<td>-0.94 (-2.55, 0.69)</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td></td>
<td>0.6 (-4.41, 5.87)</td>
<td>0.82</td>
</tr>
<tr>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td></td>
<td>-2.26 (-9.49, 5.54)</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Season (summer)</td>
<td></td>
<td>5.58 (1.98, 9.3)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

 Estimates are represented as percent difference with 95% CI for each SD increase in predictor/covariate. Subjects with deficient vitamin D status at baseline were excluded from analysis.
### TABLE 7.7 GEE results of baseline uVDBP:cr and serum 25(OH)D over 6 years.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Predictor</th>
<th>Covariates</th>
<th>β (95% CI)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Serum 25(OH)D (nmol/L)</td>
<td>Baseline uVDBP:cr (μg/mL)</td>
<td>-0.69 (-2.13, 0.77)</td>
<td>0.35</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Follow-up duration (years)</td>
<td>4.59 (3.7, 5.49)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Baseline age (years)</td>
<td>2.41 (0.2, 4.67)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Sex (male)</td>
<td>1.63 (-2.9, 6.37)</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethnicity (European)</td>
<td>9.39 (3.77, 15.31)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BMI (kg/m²)</td>
<td>-5.92 (-8.25, -3.54)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>MET (kcal/kg/h)</td>
<td>-0.96 (-2.56, 0.66)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dysglycaemia (prediabetes)</td>
<td>0.63 (-4.36, 5.88)</td>
<td>0.81</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dysglycaemia (diabetes)</td>
<td>-2.24 (-9.51, 5.61)</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Season (summer)</td>
<td>5.54 (1.95, 9.26)</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Estimates are represented as percent difference with 95% CI for each SD increase in predictor/covariate. Subjects with deficient vitamin D status at baseline were excluded from analysis.