

Diabetic Kidney Disease with a focus on Renal Hyperfiltration

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By

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Summary

Diabetic Kidney Disease is a significant health care issue. Based on 2019 data from the Australia and New Zealand dialysis and transplant registry, 39% of new patients receiving renal dialysis in Australia are due to diabetes [1]. Despite advances in preventing and managing diabetic kidney disease, the prevalence of people with newly diagnosed end stage kidney disease is projected to increase [2]. Therefore, it is important to understand processes that may contribute to the development of diabetic kidney disease, and to discover methods that enable earlier detection of those at risk of progression in diabetic kidney disease. This will facilitate early targeted intervention and ultimately translate to effective prevention.

This thesis includes seven studies based on the unified concept of evaluating diabetic kidney disease with a focus on determining the significance of renal hyperfiltration in diabetes, as well as evaluating renal biomarkers and functional magnetic resonance imaging that may provide an insight into detecting those at risk of diabetic kidney disease. We studied renal hyperfiltration in diabetes as well as renal hyperfiltration in pregnancy, in women with and without diabetes. We also investigated pregnancy outcomes in women with type 1 and type 2 diabetes, with an emphasis on renal indices and diabetic kidney disease. This thesis is structured in the following way:

Chapter one is a review of the literature on the current predictors of diabetic kidney disease. I will discuss the role of estimated and measured glomerular filtration rate, as well as albuminuria in predicting the development and progression of kidney disease in patients with diabetes.

Chapter two is a literature review of the presumed pathological renal hyperfiltration in diabetes, as well as the physiological renal hyperfiltration observed in pregnancy.

Chapter three is a review of selected candidate renal biomarkers that were assessed in this thesis. This chapter will focus on the published associations of these markers with diabetic kidney disease, as well as renal hyperfiltration in diabetes.

Chapter four is an extensive review of the novel technique of renal functional magnetic resonance imaging and the current data in diabetic kidney disease.

Chapter five is a review of the literature on measuring renal function, and assessment of hyperfiltration in pregnancies affected by type 1 and type 2 diabetes. Current literature on the pregnancy outcomes, particularly those affected by diabetic kidney disease will also be presented.

Chapter six outlines the first study of this thesis which is the evaluation of renal function in diabetes and pregnancy with inulin plasma clearance.

Chapter seven is a retrospective study analyzing pregnancy related renal function trajectory in women with type 1 and type 2 diabetes. In this study, we aimed to investigate the short and mid-term changes in renal function in women with and without type 1 and type 2 diabetes.

Chapter eight is an exploratory prospective study comparing renal hyperfiltration in women with and without type 1 and type 2 diabetes in pregnancy, as well as elucidating the relationship between renal biomarkers and hyperfiltration in diabetes.

Chapter nine is a retrospective study assessing risk factors for pregnancy outcomes in type 1 and type 2 diabetes.

Chapter ten is a retrospective study assessing the association between maternal renal function and pregnancy outcomes in type 1 and type 2 diabetes.

Chapter eleven is a prospective feasibility study investigating functional magnetic resonance imaging in predicting diabetic kidney disease in type 1 diabetes.

Chapter twelve is a prospective feasibility study investigating renal biomarkers in type 1 diabetes and renal hyperfiltration.

Chapter thirteen presents an overall summary and conclusion for the thesis.

Abstract

Currently, it is not known if the process of renal hyperfiltration is adaptive as a compensatory effect akin to pregnancy or is maladaptive and can result in non-reversible renal damage and the development of diabetic kidney disease. The overall aim of my study was to investigate renal hyperfiltration in diabetes, by comparing this to renal hyperfiltration in pregnancy, assessing renal biomarkers and utilizing functional magnetic resonance imaging.

Estimated glomerular filtration rate based on The Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula was used as the main surrogate measurement of renal function in this thesis. Where possible, measured glomerular filtration rate using renal Diethylenetriamine Penta-acetic Acid (DTPA) and plasma inulin clearance were employed. The threshold of renal hyperfiltration in this thesis is set at an estimated or measured glomerular filtration of $>120\text{ml/min/1.73m}^2$. Candidate inflammatory and oxidative stress serum and urinary renal biomarkers were selected based on current evidence in diabetic kidney disease. 3-Tesla magnetic resonance imaging was used to acquire images on renal tract, with data providing information on renal hypoxia and microstructural changes.

The main findings from assessing women with type 1 and type 2 diabetes during pregnancy were that women with type 1 diabetes may have an attenuated ability to hyperfiltrate in pregnancy, which is possibly related to pre-existing impaired renal function. However, in general, women with type 1 and type 2 diabetes, including those with mild to moderate diabetic kidney disease are not at risk of accelerated renal function decline from pregnancy. Both type 1 and type 2 diabetes carry a similar high risk for poorer maternal and fetal outcomes compared to healthy pregnancies. For these outcomes, a lower estimated glomerular filtration rate was associated with pre-term birth and neonatal intensive care admission independent from albuminuria. Understanding how pregnancies in type 1 and type 2 diabetes can affect maternal renal function; and on the other hand, how maternal renal function can affect pregnancy outcomes are important in guiding clinicians to provide optimal care this high-risk antenatal cohort.

Secondly, with regards to functional magnetic resonance imaging, we demonstrated that there is no evidence that renal hypoxia or micro-structural changes occur in those with renal hyperfiltration and type 1 diabetes. Magnetic resonance imaging parameters also do not reflect level of renal function determined with a direct measurement of glomerular filtration rate, nor do they strongly correlate with selected inflammatory and oxidative stress markers in type 1 diabetes.

Finally, we did not observe any difference in the concentration of renal biomarkers in those who were hyperfiltering, compared to those who were not hyperfiltering in type 1 diabetes. Elevation of renal biomarkers during peak hyperfiltration in the second trimester of pregnancy were comparable in women with and without diabetes. Our findings of a lack in rise of inflammatory and oxidative stress biomarkers do not support the hypothesis that inflammation is present in renal hyperfiltration in diabetes.

In conclusion, findings from this thesis suggest that hyperfiltration in diabetes may not necessarily reflect a maladaptive stage of diabetic kidney disease. Due to the exploratory nature of the studies, along with the limitation of estimated glomerular filtration rate, future work involving larger cohort of participants and measured glomerular filtration rate may provide more in-depth understanding of renal hyperfiltration in diabetes. Understanding the significance of renal hyperfiltration is important, particularly as this process occurs in the early stages of diabetes and would be an avenue of targeted therapy to prevent not only the progression, but development of diabetic kidney disease.

Declaration

This is to certify that

- i) This thesis comprises only my original work towards the PhD except where indicated in the Preface
- ii) Due acknowledgment has been made in the text to all other material used
- iii) This thesis is less than 100,000 words in length, exclusive of tables, figure, bibliographies and appendices

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Preface

The studies comprised in this thesis were designed with the assistance and guidance of Associate Professor Elif Ekinici, Associate Professor Ruth Lim, Professor Richard MacIsaac and Professor George Jerums. Ethics approval were obtained from the Austin Health Human Research Ethics Committee and Mercy Hospital for Women's Human Research Ethics Committee. I was involved in conducting all stages of the research protocol, including study design, ethics approval, participant recruitment, data collection and entry, radiology, laboratory and statistical analysis.

I collaborated with researchers in the Department of Radiology, Austin Health, Department of Obstetric Medicine, Mercy Hospital for Women's, and the Department of Clinical Research at The Florey Institute of Neuroscience and Mental Health.

Material from Chapter 9 has published online ahead of print in Internal Medicine Journal (Appendix One). Material from Chapter 10 has been accepted and pending publication in Journal of Diabetes Research and Complication (Appendix Two). I wrote the publication with editorial assistance rendered by A/Prof Elif Ekinici.

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I have been very fortunate to have a team of helpful colleagues whom I am very thankful for. Michele Milne guided and assisted me in laboratory analysis; Dr. Elissa Botterrill, Dr. Ning Mao Kam and Gay Fleming assisted in participant recruitment; Shawna Farquharson aided in acquisition of magnetic resonance imaging at the Florey Institute; Dr. Cara Tanner and Dr. Sofia Nahavandi assisted in data collection; Associate Professor Martha Lappas and Dr. Alexis Shub for their laboratory and clinical collaboration at the Mercy Hospital for Women's.

Finally, most importantly, without my family, especially my loving parents Hood Pau and Kam Heng, and the unwavering support and continual encouragement of my husband, Wei Ming, I would not have been able to complete my PhD in a relatively timely manner

Publications and Presentations relating to this thesis

Chapter Seven:

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J Seah, Kam NM, Wong L, Jerums G, Permezel M, Churilov L, Shub A, Houlihan C, Ekinici E. A Single Centre 10-year Retrospective Study on Pregnancy outcomes in Type 1 and Type 2 Diabetes. Australia Diabetes Society, Adelaide. 2015. Poster Presentation. (Abstract number 132)

J Seah, Kam NM, Wong L, Jerums G, Permezel M, Churilov L, Shub A, Houlihan C, Ekinici E. Maternal Characteristics of Pregnant Women with Type 1 and Type 2 Diabetes: A Single Center Study. Australasian Diabetes in Pregnancy Society, Adelaide. 2015. Short listed for Poster Award. (Abstract number 117)

Chapter Ten:

J Seah, NM Kam, L Wong, C Tanner, RJ MacIsaac, A Shub, C Houlihan, EI Ekinici. The Association Between Maternal Renal Function and Pregnancy Outcomes in Type 1 and Type 2 Diabetes. Accepted for publication in Diabetes Research and Clinical Practice, Manuscript ID: DIAB-2019-1394)

J Seah, Kam NM, Jerums G, Shub A, Houlihan C, Ekinici E. Association Between Maternal Renal Function and Fetal Outcomes in Type 1 And Type 2 Diabetes. International Society of Nephrology World Congress of Nephrology, 2019.VIC. Poster Presentation. (WCN19-0707)

J Seah, Kam NM, Jerums G, Shub A, Houlihan C, Ekinici E. Obstetric Outcomes in Pregnancies Complicated by Pre-existing Maternal Diabetes According to Indicators of Diabetic Kidney Disease. Australia Diabetes Society, Adelaide.2015 and Australasian Diabetes in Pregnancy Society, Adelaide. 2015. Oral Presentation. (Abstract Number ADS:203 and ADIPS:14)

Chapter Eleven:

J Seah, Botterill E, Jerums G, MacIsaac R, Lim R, Ekinici EI. Functional MRI in predicting Diabetic Kidney Disease. International Society of Nephrology World Congress of Nephrology, 2019.VIC. Poster Presentation. (WCN19-0704)

J Seah, Botterill E, Jerums G, MacIsaac R, Lim R, Ekinici EI. Functional MRI changes in Type 1 Diabetes with and without Renal Hyperfiltration. Australian Diabetes Society Annual Scientific Meeting, 2016. Gold Coast. Awarded Clinical Science Poster Award. (Abstract number 221)

Chapter Twelve:

J Seah, Milne M, Obeyesekere V, Lim R, Jerums G, MacIsaac RJ, Ekinici EI. Tumour Necrosis Factor-alpha Receptor 1 and 2 are Reduced in patients with Type 1 Diabetes and Hyperfiltration. Australian Diabetes Society Annual Scientific Meeting, 2016. Gold Coast. Oral Presentation. (Abstract number 34)

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Other publication as co-author arising during this candidature related to the works of diabetic kidney disease and pregnancies affected by type 1 and type 2 diabetes:

Navahandi,S. *JM Seah.*, A,Shub., C.Houlihan., E.Ekinci. Biomarkers for Macrosomia Prediction in Pregnancies Affected by Diabetes. *Frontiers in Endocrinology*. 2018 July; 31(9): 407.

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Abbreviations

ACR	Albumin creatinine ratio
ADC	Apparent diffusion coefficient
AER	Albumin excretion rate
AGEs	Advanced glycation end products
BOLD	Blood oxygen dependent
eGFR	Estimated glomerular filtration rate
CKD	Chronic kidney disease
CKD-EPI	Chronic Kidney Disease Epidemiology Collaboration
CRP	C-reactive protein
DKD	Diabetic kidney disease
DTI	Diffusion tensor imaging
ESKD	End stage kidney disease
FA	Fraction anisotropy
KIM-1	kidney injury molecule
MCP1	monocyte chemoattractant protein 1
mGFR	Measured glomerular filtration rate
MRI	Magnetic resonance imaging
NGAL	neutrophil gelatinase-associated lipocalin
NLR	Neutrophil to lymphocyte ratio
NS	Non-significant
RAAS	Renin angiotensin aldosterone system
R2*	Resonance transverse relaxation rate
sRAGE	soluble receptor for advanced glycation end products
sIL2R	soluble Interleukin-2 Receptor
sTNFR1	soluble Tumour Necrosis Factor alpha-receptors 1
sTNFR2	soluble Tumour Necrosis Factor alpha-receptors 2
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

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Review of the Literature

Chapter One

Current Predictors of Diabetic Kidney Disease

Establishing precise, predictive clinical and biochemical markers to identify patients who are at risk of diabetic kidney disease (DKD) is necessary for clinical decision-making. Studies on the development and progression of DKD generally include albuminuria status and rate of GFR decline. There are, however, concerns with the assessment of both albuminuria and GFR status, using estimates of GFR (eGFR) as neither has the sensitivity and specificity in accurately predicting the development or rate of progression of DKD in patients with diabetes.

1.2 Glomerular Filtration Rate -Direct measurement and estimation of GFR

Glomerular filtration rate (GFR) is the filtering capacity of the kidneys and provides a good index of chronic kidney disease (CKD). However, GFR cannot be measured directly. The ideal method to measure GFR is inulin clearance, as it is freely filtered through the glomerulus and is neither resorbed nor secreted[3]. Due to the technicalities of performing inulin clearance, other clearance markers and methods have been used, selected based on availability and institution expertise. These include clearance of chromium 51–labeled ethylenediaminetetraacetic acid (^{51}Cr -EDTA), $^{99\text{m}}$ -technetium- diethylene-triamine-penta-acetic acid ($^{99\text{m}}\text{Tc}$ -DTPA), iohexol, and iothalamate. Timed (24-hour) creatinine clearance has also been traditionally used to estimate GFR, as an improved alternative to serum creatinine alone.

However, these methods are time consuming and labour intensive. Additionally, even methods of directly measuring GFR with clearance markers have varying degrees of precision and bias when compared to the gold standard of inulin[4]. Consequently, equations used to estimate GFR based on patient's age, gender, ethnicity and serum creatinine have gained widespread acceptance. The Cockcroft-Gault (C-G), and Modification of Diet in Renal Disease (MDRD) are equations that were commonly used prior to the improved Chronic Kidney Disease Epidemiology Collaboration (CKDEPI) formula, which is the current recommended method of estimating GFR based on the National Kidney Guidelines Foundation. In the general population, compared to MDRD, CKD-EPI formula retained its accuracy at $\text{GFR} < 60 \text{ ml/min/1.73m}^2$, and had improved precision and is less bias at $\text{GFR} > 60 \text{ ml/min/1.73m}^2$ [5]. Estimated GFR (eGFR) as the basis of Kidney Disease: Improving Global Outcomes (KDIGO) chronic

kidney disease classification is applied not only in clinical settings, but also increasingly used in clinical trials. Recent large multicenter diabetes trials including Diabetes Control and Complications Trial (DCCT)/Epidemiology of Diabetes Interventions and Complications (EDIC) study in type 1 diabetes [6] and Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation (ADVANCE) [7] incorporated eGFR as a renal outcome. Over the last decade, cystatin-c, an endogenous peptide has also been proposed as an alternative surrogate marker for GFR.

Many formulas estimating GFR have been devised with gradual improvement over the years, but their performances remain contested. Overall, GFR estimating equations based on serum creatinine values have poor performance in diabetes. In type 2 diabetes, both the MDRD and CKD-EPI equation systemically underestimates measured GFR, with accuracy (percentage of estimate within 30% of measured GFR) around mid-60% [8, 9]. eGFR at higher levels are also associated with higher variability. Our group has previously demonstrated in diabetes (N=199, 75% type 2 diabetes) that both CKD-EPI and MDRD significantly underestimate GFR to a similar extent by a mean of -12 ± 1.4 ml/min/1.73 m² and -11 ± 2.1 ml/min/1.73 m² respectively[10]. This could easily lead to misclassification of hyperfiltration, and was highlighted in a Finn-Diane study on type 1 diabetes, which established different rates of hyperfiltration depending on the eGFR formula utilised (CKD-EPI, MDRD, and C-G)[11]. The cross-sectional studies above, however, do not allow for interpretation of how eGFR will perform longitudinally in diabetes. To address this, Gaspari et al [12] evaluated the agreement between eGFR (based on 15 different creatinine-based formulas) and measured GFR iohexol plasma clearance multiple time-points based on two previous large prospective trials on a cohort of type 2 diabetes individuals (N=600, median follow up 4 years) with normo- or microalbuminuria. The investigators found that no formula, including the CKD-EPI and MDRD accurately estimate GFR and GFR decline overall, particularly in the hyperfiltering group.

Creatinine based eGFR equations have higher variability at extreme ends. Factors that can affect tubular creatinine handling will result in over or under estimation of GFR, depending on tubular creatinine resorption and secretion, with hyperfiltration as a prime example. In progressive renal failure, creatinine undergoes tubular secretion in addition to glomerular filtration, and extra-renal elimination (mainly gastro-intestinal) of

creatinine occurs in advanced renal failure[3], which can result in overestimation of GFR. This can potentially underestimate GFR loss, particularly in significant renal impairment. In addition, hyperglycaemia itself has been reported to increase serum creatinine, and as a result underestimation of GFR[12]. Interestingly, serum creatinine levels are higher (82.2 ± 19.4 vs. 73.4 ± 13.3 mmol/L, $p = 0.006$, adjusted for glucose levels and body mass index) in type 2 diabetes patients compared to healthy controls despite similar measured GFR (based $^{51}\text{Cr-EDTA}$) [9]. Imprecision may also be partially explained by the inherent shortcomings including extreme ends of age, body mass index and nutrition, which can affect muscle mass[8], as well as medication use that affects creatinine handling[13]. These are not uncommon cofounders in diabetes. Creatinine affecting estimation of GFR also affect the performance of timed urinary creatinine clearance. Notably, as creatinine is freely filtered, and actively secreted and resorbed by the tubules, creatinine clearance typically overestimates GFR by 10-15%[13]. One common technical difficulty of precise creatinine clearance measurement would be inaccurate or incomplete 24 h urine collection.

Finally, in the last decade, cystatin-c, a low molecular mass protein was of interest as a surrogate measurement of GFR. This was mainly due to renal function being the main determinant in serum cystatin-c concentration, which was independent from factors including body mass composition, age and acute phase reaction[14]. Cystatin-c-derived GFR performance appears to be similar or even better than creatinine based eGFR for both type 1 diabetes and type 2 diabetes, but with a limitation of a high intra-individual variability [15-17]. As a method that does not appear to be significantly superior in accuracy or precision but much more expensive, cystatin-c has limited practicality and implications.

It is important to acknowledge that there are limitations with eGFR, as prediction formulae may lack accuracy, and caution has to be exercised when interpreting studies with eGFR as a renal endpoint. However, there is value in large quality diabetes trials which incorporate eGFR, as this can create clinical applicability of study findings, where eGFR is frequently used in clinical medicine. Achieving this requires understanding the confounding physiology and methodological considerations of GFR estimation, coupled with careful interpretation of GFR as an index of renal function.

1.2 GFR as a predictor of DKD progression

In the general population, the combination of eGFR (using the CKD-EPI equation), and albuminuria effectively predicts the progression to end stage kidney disease (ESKD). Interestingly, neither diabetes, nor known renal risk factors including hypertension and cardiovascular disease added any predictive value to projecting end stage kidney disease as established by a large prospective epidemiology study (N=65,589, in which 124 participants developed ESKD, with a follow up of 10.3 years) [18]. Similarly, in diabetes, there are emerging large-scale studies demonstrating the risk factors for progression to ESKD. These trials generally have either measured or estimated GFR, along with albuminuria both as a predictor of renal decline, and as an index of renal function.

Earlier data prior to the introduction of sodium glucose co-transporter inhibitors have demonstrated that renal function decline defined as a GFR loss of $>3.3\%$ /year occurs in approximately 9%, 31% and 68% of individuals with type 1 diabetes, and 32%, 42% and 74% of individuals with type 2 diabetes; in those with baseline normo-, micro- and macroalbuminuria respectively[19, 20]. Specifically, GFR was measured (^{51}Cr -EDTA) serially over four years to determine renal function decline in those with type 2 diabetes. The cumulative incidence of EDKD at 10 years were significantly higher at 41% in those with renal function decline, compared to 15% to those without renal function decline in the initial 4 years [19]. This arbitrary definition of annual GFR loss is based on the concept that a decline in renal function in excess of those attributable to normal aging, based on a threshold of the 97.5 percentile distribution of decline in creatinine clearance established in a previous longitudinal study on a cohort of individuals, without diabetes or hypertension [21]. However, defining rate of decline in renal function based on a fixed annual GFR loss percentage can potentially skew results in longitudinal observations, especially when rate of progression in GFR may vary irrespective of the baseline GFR. Notably, measurable age-related GFR decline without diabetes does not occur prior to 40-50 years [22], and age-related annual decrease in GFR of $1 \text{ ml/min}/1.73 \text{ m}^2$ occurs after the age of 40 years in the non-diabetic population[23]. There has also been a variable rate of decline in GFR that has been reported in diabetes, up to 12 ml/min/year [24]. On a prospective study over a median of 6.5 years, in 227 type 2 diabetes individuals, the mean rate of measured GFR (^{51}Cr -

EDTA) loss was recognized at 5.2(4.1) ml/min/year. Adjusting to other clinical co-morbidities, although a higher baseline albuminuria and lower GFR was associated with a shorter time to doubling of serum creatinine or ESKD; only albuminuria was associated with mortality[25].

Increasingly, due to the challenges in performing measured GFR, as well as the widespread use of eGFR clinically; there are large scaled studies that perform analysis based on eGFR and not measured GFR. 1682 individuals with type 2 diabetes and predominantly normo-albuminuria with a mean baseline eGFR of 79.6(12) ml/min/1.73m² were followed for 10 years. 15% were rapid decliners as defined as an eGFR decline of >4.0%/ year. Both lower baseline GFR and a high albuminuria rate were independent markers of eGFR decline. Macroalbuminuria was the strongest predictor at an annual rate of eGFR loss at -2.7(0.4) ml/min/ 1.73m, compared to -0.9(2.9) ml/min/1.73m² of the overall cohort[26]. The ADVANCE study of 11,140 type 2 diabetes participants who mainly had normo-albuminuria at baseline with a mean eGFR of 74.6(17.6)ml/min/1.73m² demonstrated baseline low eGFR and albuminuria as strong risk factors of developing doubling of serum creatinine, renal replacement or renal death over a 5 year period[27]. In type 1 diabetes, macroalbuminuria has also been established as a strong predictor of developing progressive renal decline to an eGFR<60ml/min/1.73m² in the DCCT/EDIC trial spanning a mean of 19 years. Of those with sustained eGFR<60ml/min/1.73m², baseline mean eGFR was relatively preserved at 108(33)ml/min/1.73m², and many of those that subsequently developed an eGFR<60ml/min/1.73m² would have been missed solely on screening of a single albumin excretion rate[28]. Similarly, in a recent study following three thousand participants with type 1 or type 2 diabetes and eGFR<60ml/min/1.73m² over a 10-year period up to 2017, it was found that those with albuminuria had a more rapid decline in eGFR[29]. In those with normoalbuminuria, there was a mean annual eGFR decline of 1.9ml/min/2.73m² in both type 1 and type 2 diabetes. Additionally, it was found that an accelerated eGFR decline occurred in those with less intervention with lipid lowering and anti-hypertensive treatments including renin angiotensin system blockers.

1.3 The role of Albuminuria in the progression of DKD

Albuminuria has traditionally been accepted as an independent marker to evaluate DKD. As evident from studies above, there is robust evidence that albuminuria is associated with GFR decline. However, the presence of macro or micro-albuminuria is usually, but not always specific for chronic kidney disease [30]. In addition, approximately two-thirds of patients with DKD will have macro- or micro-albuminuria, with the rest having normo-albuminuria [30, 31]. The reliability of microalbuminuria is also questionable, as levels may not only stabilize with no progression to overt proteinuria, but may even regress and normalize [32-35]. Albuminuria has also been a therapeutic target in diabetes over the last two decades, with evidence of the protective effect of albuminuria reduction. Two large randomized, prospective studies in type 2 diabetes demonstrated renin angiotensin aldosterone system (RAAS) inhibition has a 23-28% risk reduction in development of end stage renal failure. The effect was mainly due to reduction in albuminuria, with protection exceeded that of changes in blood pressure [36, 37]. In type 1 diabetes, RAAS inhibition has similarly shown reduction in albuminuria and delaying rate of GFR decline independent of blood pressure [38, 39]. This highlights both the significance of albuminuria in disease progression, but also the ability of albuminuria to regress, making it an imprecise marker in DKD progression.

Despite the potential inaccuracy of albuminuria status in predicting DKD progression, it is still important and remains highly relevant in current clinical practice. This is particularly so with macroalbuminuria, once developed, is likely to indicate irreversible glomerular damage leading to ESKD. This was illustrated by Pavkov et al demonstrating that GFR loss often occurs prior to the onset of macroalbuminuria, but the progression to ESKD at 10 years is strongly dependent on the development of macroalbuminuria in type 2 diabetes [19]. In addition, the relationship between albuminuria and type 2 diabetes related cardiovascular mortality has also been established [40]. It is therefore important to consider both albuminuria status and GFR as complimentary, but not necessarily obligatory manifestation of DKD.

1.4 Other Clinical Renal Risk Factors

Apart from albuminuria and reduced eGFR as outlined above, there are other clinical risk factors that play an important role in progression of DKD. For example, in type 1 diabetes, the UKDPDS outcome equation included the following variables: age, duration of diabetes, sex, ethnicity, current smoking status, systolic blood pressure, HbA1c, BMI, heart rate, atrial fibrillation, peripheral vascular disease, high density and low density lipid profile, and white cell count[41]. Risk factors including gender, lipid profile, body mass index and smoking have strong influences on cardiovascular outcomes in diabetes, but their relationship on the progression on DKD have not been consistently established. The association of diabetic retinopathy and albuminuria and low eGFR has been reported[42, 43]. Although the association between diabetic retinopathy and DKD is well established, it is not clear if retinopathy is a risk factor for the development and progression of DKD, or whether the presence of albuminuria is a marker of retinopathy risk until recently. A type 1 diabetes study by Gordin et al. demonstrated a discrepancy between retinopathy and DKD risk[44]. This study established that the degree of nephropathy was no different in those with and without diabetic retinopathy (N=12, based on histology classification including glomerular basement membrane thickness, sclerosis or arterial hyalinization on postmortem kidneys). In addition, in this study which included cohort from both the Joslin (N=762) and Finn-Diane Study (N=675), a lower prevalence of cardiovascular disease were found in those with stage 3b DKD but without diabetic retinopathy, suggesting that there may be potential different tissue specific vascular effect in both DKD and diabetic retinopathy.

The two risk factors in the progression of DKD are glucose and blood pressure management. In type 1 diabetes, in the DCCT/EDIC trial, the cumulative incidence of macroalbuminuria was 6% and 17% in the intensive and convention glucose control arms respectively [45]. Hyperglycaemia is associated with accelerated annual eGFR decline in normo-albuminuria type 2 diabetes individuals, over a 3 year follow up period[46]. Systolic and mean arterial, but not diastolic blood pressure has been linked to DKD. A recent meta-analysis from 41 random controlled trials established maintaining a systolic blood pressure at 140mmHg results in significant ESKD reduction in diabetes, but with a very small blood pressure margin, with no further

benefit on renal outcomes below 130mmHg[47]. While interpreting these studies, it is important to note that despite multivariate analysis, controlling for these factors in clinical trials can be difficult, due to the visit-to-visit variability particularly for blood pressure.

Utilizing risk prediction model can be a way of identifying people with diabetes who may develop diabetic kidney disease or progress to end stage kidney disease. Available diabetic kidney disease risk models which are constructed based on testing the different combination of risk factors are mostly based on cross-sectional studies or post-hoc randomised controlled trials [48-50]. Jiang et al. recently established a model for predicting diabetic kidney disease (defined as eGFR <60ml/min/1.73m² and/or urinary albumin-creatinine ratio $\geq$ 30mg/g) based on a meta-analysis of twenty cohort of patients with type 2 diabetes (N=41271), with an external validation cohort of patients with type 2 diabetes (N=380, median follow up of 2.9 years) [51]. At baseline, all patients had an eGFR $\geq$ 60ml/min/1.73m² and a urinary albumin-creatinine ratio of <30mg/g at baseline. The following risk factors were significantly associated with the onset of diabetic kidney disease, with the respective pooled relative ratio as follow: age (1.09), body mass index (1.07), diabetic retinopathy (1.72), smoking (1.49), HbA1c (1.17), systolic blood pressure (1.03), high density lipoprotein cholesterol (0.75), triglycerides (1.15), urinary albumin creatinine ratio (1.25), and eGFR (2.20). As demonstrated by the above study, as these traditional risk factors are so closely linked to diabetic kidney disease, it is highly difficult to ascertain the residual risk of diabetic kidney disease *after* accounting for these factors. Additionally, these factors typically have significant heterogeneity at the onset of diabetes, which are major limitations of diabetic kidney disease prediction models. Hence, integrating the traditional risk factors with proteomics and genomics may be a way moving forward in increasing the accuracy of predicting the onset and progression of diabetic kidney disease.

1.5 Summary of current clinical predictors of DKD

In conclusion, studies predicting DKD progression are limited by methodological concerns including sample size, the heterogeneity of the diabetic population, the lack of access to measured GFR, incomplete statistical analysis and the extensive duration

of observation that is required to firmly establish renal function decline. Partly due to these factors, it is difficult to accurately predict the progression of DKD centered on baseline low GFR/eGFR, high albuminuria grade, together with other clinical risk markers. Henceforth, there is a need for improved study methodology, and to appraise new markers in an attempt to enhance the prediction of the development and progression of DKD.

Review of the Literature

Chapter Two

Renal Hyperfiltration in Diabetes and in Pregnancy

Renal hyperfiltration has been arbitrarily defined in the literature as a measured Glomerular Filtration Rate (mGFR) of $>125\text{-}140\text{ml/min/1.73m}^2$, or 2 standard deviations above the mean of healthy controls[52]. Direct measurement of GFR with infusion of plasma markers (e.g. inulin clearance) is the most accurate method of determining hyperfiltration. Currently, there are no available creatinine-based eGFR that reflects true measured GFR in hyperfiltration accurately[12, 53].

Many conditions including renal diseases (focal segmental glomerulosclerosis, polycystic kidney disease), high protein diet, obesity, sleep apnoea, malignancies and blood disorders can potentially result in hyperfiltration [54]. Intriguingly, the underlying mechanism and clinical significance of hyperfiltration is both unclear and varies between different conditions. For the purpose of this literature review, we will focus on the topic of hyperfiltration in both diabetes and in pregnancy.

It is important to note that in the pre-existing literature, there is a wide range of what is defined as hyperfiltration from 90.7 to $175\text{ ml/min/1.73m}^2$, which can be gender and age dependent [55]. For the purpose of this PhD, renal hyperfiltration was defined at a threshold of estimated and measured GFR of $\geq 120\text{ml/min/1.73m}^2$ consistently throughout the different studies. This was based on firstly, no healthy controls that were recruited prospectively ($N=24$), had an $\text{eGFR} \geq 120\text{ml/min/1.73m}^2$ (in the non-pregnant state). Secondly, we infer that if any positive findings were elucidated on a lower eGFR threshold, this would minimize false positive results.

2.1 Hyperfiltration in Diabetes

2.1.1 Pathophysiology of Hyperfiltration in Diabetes

Hyperfiltration is not necessarily present in all cases of diabetes, with a wide prevalence of hyperfiltration being approximately 25-75% in type 1 diabetes and 0-40% in type 2 diabetes [52]. In diabetes, the potential mediators of hyperfiltration include hyperglycaemia, insulin resistance, dyslipidaemia and obesity. A study in type 1 diabetes patients established that measured GFR using $^{51}\text{Cr-EDTA}$ rose significantly by 11.9% in the hyperfiltering group, but not in those who were normofiltering when

plasma glucose was maintained at 13.5nmol/L via glucose clamping [56]. Comparing patients with a mean duration of 10 years in type 1 diabetes over a year, an earlier study by the same authors demonstrated improvement in glucose control and resolution of hyperfiltration with intensive insulin pump therapy compared to convention therapy. A month after cessation of pump therapy, glycaemic management deteriorated and GFR returned to hyperfiltration range [57]. It was not established if intensive insulin administration, or improvement in glucose management resulted in the GFR changes. Interestingly, with the opposite physiological roles of glucagon and insulin in maintaining glucose homeostasis, there is evidence that glucagon, along with vasopressin and urea can result in hyperfiltration by affecting tubular fluid delivery to the macula densa and inhibiting tubulo-glomerular feedback[58].

Obesity, which is prevalent in diabetes, may also contribute to GFR rise and have been associated with hyperfiltration. In a small cohort of eight patients with a body mass index (BMI) $>38\text{kg/m}^2$ (mean BMI $48\pm 2\text{ kg/m}^2$) compared to nine with a BMI $<38\text{kg/m}^2$ (mean BMI $22\pm 1\text{ kg/m}^2$). Results on oral glucose tolerance test were significantly higher in the BMI $>38\text{ kg/m}^2$ group, but the diagnosis of diabetes were not specified although none were actively treated for diabetes. There was a significant difference in baseline measured GFR 145 ± 14 and $90\pm 5\text{ ml/min/1.73m}^2$ in those with the unhealthy and healthy weight range respectively [59]. However, it was not clear if GFR as measured by inulin clearance was corrected for body surface area (BSA), which can underestimate GFR in severe obesity. This study also demonstrated that between 12 -17 months post bariatric surgery, GFR reduced by 24%, but this change in GFR did not correlate with the change in BMI. Resolution of hyperfiltration with the reduction in BMI with bariatric surgery was also demonstrated in more than half of the participants in larger study (n=138, 32% with diabetes). 90% of the cohort with baseline hyperfiltration (defined based on eGFR above the 95th centile of matched gender and age) [60]. Nevertheless, a reduction of body mass index and adiposity itself do not explain the reduction in GFR, as not all participants had resolution of hyperfiltration. Unknown factors including inflammation and oxidative stress may potentially influenced GFR reduction. This is particularly likely, as obesity is an independent risk factor of chronic kidney disease related to kidney fibrosis and inflammation[61]. The above bariatric study adjusted for age, as notably, age also influences hyperfiltration. Using measured GFR, our group has previously studied 662 patients with type 2

diabetes and found hyperfiltration to be more common in the younger cohort, with prevalence of hyperfiltration reducing from 16.6% to 7.4% when adjusted for age [62].

Hyperglycaemia has been identified as a precipitant, but the exact underlying pathophysiology of hyperfiltration in diabetes has not been clearly elucidated. There are two mechanisms that have been proposed (Figure 2.1). Both are closely related to renal blood flow (RBF) that maintain a constant renal flow, intra-glomerular pressure and GFR[63]. Firstly, the “haemodynamic vascular effect” is a consequence of imbalance in vaso-adaptive response from vasoactive humoral factors (nitric oxide, kallikrein-kinins, atrial natriuretic peptide, angiotensin II) [64]. The net result is dilatation of the renal afferent arteriole and constriction of the efferent arteriole within the glomeruli [65]. The alternative hypothesis is the inhibition of the “tubular-glomerular feedback” demonstrated in experimental mice models [66, 67]. Auto-regulatory tubular-glomerular feedback is a protective mechanism that detects the increase in sodium-chloride concentration in tubular fluid reaching the macula densa cells. This increases afferent arteriole resistance, with a net result of a decrease in the diameter of the glomerular afferent arteriole. Hyperglycaemia upregulates sodium-glucose-co-transporters 2 (SGLT2), which enables glucose resorption (together with sodium) in the proximal tubules. This, in turn reduces sodium delivery to the macula densa, and inhibits tubuloglomerular feedback and results in dilatation of the afferent arteriole [68] that subsequently leads to a raised GFR. Furthermore, tubuloglomerular feedback response has also recently been found to be blunted through stimulation of nitric oxide via sodium-glucose-co-transporters 1 (SGLT1) sensing an increase in luminal glucose[69]

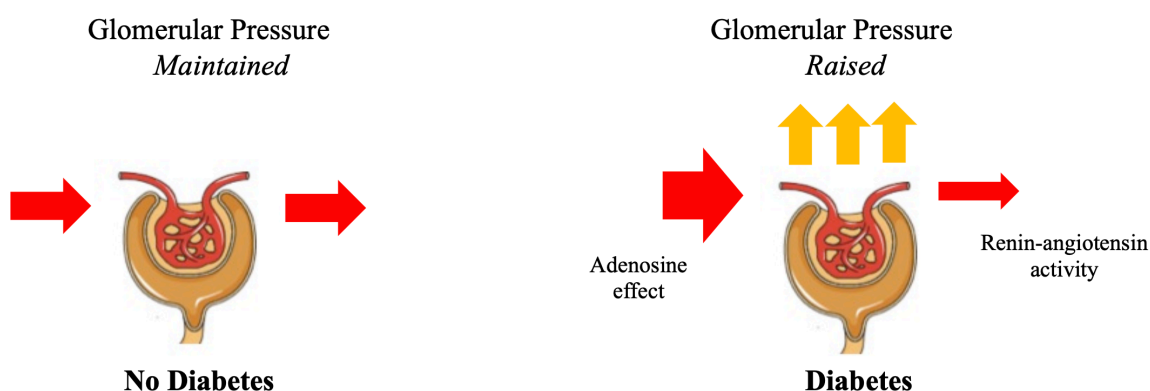
The tubular hypothesis is further supported by Cherney et al [70] who studied the effect of SGLT2 inhibitor (empagliflozin 25mg) on twenty-seven participants with type 1 diabetes and hyperfiltration (mean baseline measured GFR $172 \pm 23 \text{ ml/min/1.73m}^2$) compared to thirteen participants with type 1 diabetes and normofiltration (mean baseline GFR $117 \pm 11 \text{ ml/min/1.73m}^2$) over an eight week period. In a clamped euglycaemic state, administration of a SGLT2 inhibitor attenuated hyperfiltration by a significant GFR reduction of $33 \text{ ml/min/1.73m}^2$, $p < 0.01$, with similar findings in the clamped hyperglycaemia state. Other significant haemodynamic changes that suggest the reduction in GFR is likely related to pre-glomerular vasoconstriction was the

accompanying reduction in renal blood flow, but a rise in renovascular resistance and filtration fraction based on inulin and para-aminohippurate (PAH) clearance. There was no observed difference in GFR at baseline and end of study in participants with normofiltration. Furthermore, there was an accompanying rise in adenosine which is thought to mediate the tubulo-glomerular feedback, as well as an increase circulating renin-angiotensin- aldosterone (RAAS) mediators.

Of note, RAAS inhibitors ameliorate glomerular hyperfiltration by preferentially dilating the efferent renal artery. This effect of post glomerular dilatation in SGLT2 inhibitors was first observed in a very recent study aimed to assess the haemodynamic effect of SGLT2 inhibitors (dapagliflozin), in twenty-four participants with type 2 diabetes, compared to gliclazide which is thought to be haemodynamic neutral [71]. Apart from the significant reduction of GFR, Van Bommel et al demonstrated with 12 weeks of SGLT2 inhibition, instead of a rise in renovascular resistance as established by Cherney et al in type 1 diabetes, there was an accompanying reduction in renovascular resistance and filtration fraction instead. It was postulated that prostaglandin release, which was measured, is responsible for this novel finding of the efferent arteriole dilatation being responsible for the GFR reduction with the administration of SGLT2 inhibitors. The surprising mechanistic difference in GFR reduction in both studies is likely multifactorial. Apart from being older, having a shorter duration of diabetes and a higher body mass index, the participants with type 2 diabetes had a much lower baseline measured GFR (euglycaemic clamp: 110 ± 27 ml/min/1.37m² vs. 172 ± 23 ml/min/1.37m²). Interestingly, whilst there was a considerable reduction in GFR at the end of the study in the type 2 diabetes cohort, there was a lack of GFR decline in the type 1 diabetes normo-filtering group, despite similar baseline GFR. Other notable differences include a significantly much higher renovascular resistance in the type 2 diabetes group (135 mm/Hg/L vs. 0.052 mm/Hg/L), suggesting much narrower afferent arteriole and limiting the possibility of further constriction. In addition, whilst almost 70% of participants in the type 2 diabetes group were on a RAAS inhibitor, being on this class of medication was an exclusion criterion in the type 1 diabetes study.

At this point, the exact mechanism underpinning hyperfiltration in diabetes remains elusive. Based on the current literature, we propose a mechanism depicted below in Figure 2.1.

Figure 2.1 Proposed mechanism of hyperfiltration in diabetes based on literature



In the normal glomerulus without diabetes (left), the balance between afferent and efferent arteriole determines intra-glomerular pressure. The tubular delivery of solute at the macula densa regulates tubulo-glomerular feedback and the subsequent the dilation and constriction of the arterioles. In diabetes (right), due to the high filtered load of glucose, as well as the increase in sodium-glucose co-transporters in the proximal tubule results in increased solute reabsorption, with reduced sodium chloride delivery to the macula densa. Overall, the afferent arteriole dilates in response the tubulo-glomerular feedback with efferent arteriole constriction in response to local renin-angiotensin activity.

The above-described mechanisms with glomerular hypertension playing a central role, however, are only part of the complex hyperfiltration physiology. Despite identifying metabolic factors (hyperglycaemia, insulin resistance and obesity) as primary catalysts, what has not been clearly defined are the cytokine pathways that drive the haemodynamic and tubular feedback alterations. Studies that demonstrate inflammation and oxidative stress in hyperfiltration in diabetes may provide further mechanistic insight. This is further outlines in Chapter 3: Review of the Literature- Renal Biomarkers in Diabetic Kidney Disease and Renal Hyperfiltration in Diabetes.

Notwithstanding that the proposed mechanisms of hyperfiltration in diabetes involve numerous cytokine pathways, it is beyond our current knowledge if these renal changes are adaptive as a compensatory effect to avoid further damage; or are maladaptive and will result in non-reversible renal consequences.

2.1.2 Hyperfiltration in Diabetes as a predictor of DKD

To determine its clinical significance, investigators dating back to the 1980s have attempted to assess renal outcomes in diabetes and hyperfiltration. However, till date studies have not consistently demonstrated that hyperfiltration in diabetes predisposes to progression of DKD, either with albuminuria or decline in GFR.

In 2009, Magee et al. conducted a meta-analysis following 10 cohort studies in type 1 diabetes. In those with baseline hyperfiltration, the pooled odds of progression of at least to microalbuminuria was 2.7(95CI 1.2-6.1) times that of patients with normofiltration at baseline[72]. However, there was moderate heterogeneity, with different hyperfiltration thresholds, with a relatively small sample size (total N=780). The results may have also been influenced by a couple of earlier small studies, which had odds of progression nearly doubling that of the closest results (33.1 and 16.1 vs. 10.0)[73-75]. The Finn Diane group published a study subsequently, on 2318 type 1 diabetes patients who were followed up for a median of 5.2 years [11]. The risk of developing microalbuminuria, in the 10% of patients who had hyperfiltration ($eGFR > 125 \text{ ml/min/1.73m}^2$) and normoalbuminuria did not differ from those without hyperfiltration. In addition, the Joslin Kidney Study on 426 patients with type 1 diabetes [76] demonstrated events of microalbuminuria in 24% of the cohort with hyperfiltration (cystatin C $eGFR > 97.5^{\text{th}}$ of the sex-specific distribution of a similar aged, non-diabetic population) did not differ from normofiltration, at multiple time points in the 15 years of follow up. Absolute end of study $eGFR$ and albuminuria values were not reported. The results of these two later studies may potentially reflect modern medical practices, influenced potentially by RAAS inhibition and better glycaemic management, with the median glycosylated haemoglobin A-1C (HbA1c) of both studies $< 9\%$, in contrast to approximately 8.5-12% in earlier studies. Nonetheless, these negative findings on microalbuminuria risk may not truly reflect DKD involvement, as early $eGFR$ decline can precede the onset of microalbuminuria in type 1 diabetes[77]. The most recent type 1 diabetes study on hyperfiltration assessed rapid $eGFR$ decline (defined as $> 3 \text{ ml/min/1.73m}^2/\text{year}$) over 6 years in 664 type 1 diabetes patients [78]. After multivariate adjustment, the odds of rapid $eGFR$ decline were greater in the hyperfiltration group ($eGFR > 120 \text{ ml/min/1.73m}^2$) compared to those who were not hyperfiltering (OR 5.00, 95CI 3.03-8.25, $p < 0.0001$). $eGFR$ of the rapid decliners were

also significantly higher (121 ± 20 vs. $98 \pm 25 \text{ ml/min/1.73m}^2$). However, rates of hyperfiltration may not be accurately reflected in these later studies that did not utilise measured GFR.

In type 2 diabetes, majority of evidence established a lack of effect of hyperfiltration in predicting progression of renal failure[79-81]. However, longitudinal studies that attempted to assess factors of rapid GFR decliners were not specifically aimed to assess hyperfiltration, with the median baseline measured or estimated GFR ranging 84-122ml/min/1.73m². On the other hand, an observational 4-year cohort study on 1000 type 2 diabetes patients found higher baseline eGFR independently correlated with eGFR decline[82]. Again, there were limitations with the median baseline eGFR using a Japanese population specific eGFR formula was quite low at 77.8ml/min/1.73m² (65.8 to 90.9). The article also did not document data on eGFR at end of follow up, or the slope of eGFR decline. Another study found that overall mGFR decline in type 2 diabetes was 3-4 times faster than that of the general population at 3ml/min/1.73m² using serial iohexol measured GFR over a median of 4 years. It was demonstrated that in those who were persistently hyperfiltering at six months, progression to the next stage of albuminuria (normo- to microalbuminuria, and micro- to macroalbuminuria) doubled that of those whom had hyperfiltration ameliorated at 6 months, or to those who were normofiltering at baseline even after adjusting for RAAS inhibition, HbA1c and blood pressure (HR 2.26 (1.1-4.5) $p < 0.02$)[83].

The inconsistent results in the above studies on the relationship of hyperfiltration to renal decline are likely due to methodological differences including insufficient follow up duration to detect a decline in renal function; the diverse methods of measuring renal function and use of heterogenous renal endpoints. Other confounding systemic factors include BMI, diet, blood pressure and age, which can affect hyperfiltration. Of importance as well, is the concept of renal functional reserve. There may be increased nephron endowment in early diabetes, resulting in global hyperfiltration. However, with any nephron loss, there is also a compensatory rise in remaining nephrons' GFR to maintain total GFR[84]. Hence, studies may underestimate the existence of hyperfiltration using arbitrary defined GFR cut-offs, particularly in later stages of DKD when compensatory single nephron hyperfiltration occurs to achieve maximal GFR.

Apart from longitudinal clinical studies, structural renal changes in hyperfiltration and diabetes have also been studied to determine their pathological significance. Renal enlargement and hyperfiltration both occur early on in diabetes. A few early studies in type 1 diabetes which have shown reduction in hyperfiltration range GFR did not demonstrate any reduction in kidney volume over 6-12 months [57, 85]. However, the short-term follow up in these studies may not be sufficient to detect any significant anatomical changes. Using regression models, morphological glomerular changes in type 1 diabetes have been detected at an approximate measured GFR range of 110ml/min/1.73m² [86, 87], indicating that structural changes and renal injury may occur while hyperfiltration was taking place, or during the process of GFR normalization. In a much larger 5 year study on 170 type 1 diabetes participants with baseline hyperfiltration (GFR>130/ml/min/1.73m²) and normoalbuminuria, the group with persistent microalbuminuria had the highest GFR and glomerular basement membrane width, with borderline significance after adjusting for HbA1c (p=0.048)[88]. The authors did acknowledge that it remains unidentified if these structural changes are related to diabetes, or hyperfiltration independently.

In comparison to the above, the Diabetes Control and Complications Trial / Epidemiology of Diabetes Interventions and Complications (DCCT/ EDIC) group [89] published a recent study based on 446 type 1 diabetes participants over 30 years, using iothalamate GFR. It was found that there was no association with hyperfiltration with the risk of developing advanced kidney disease. The major strengths of this study were utilizing measured GFR following up a large cohort over a significant time frame.

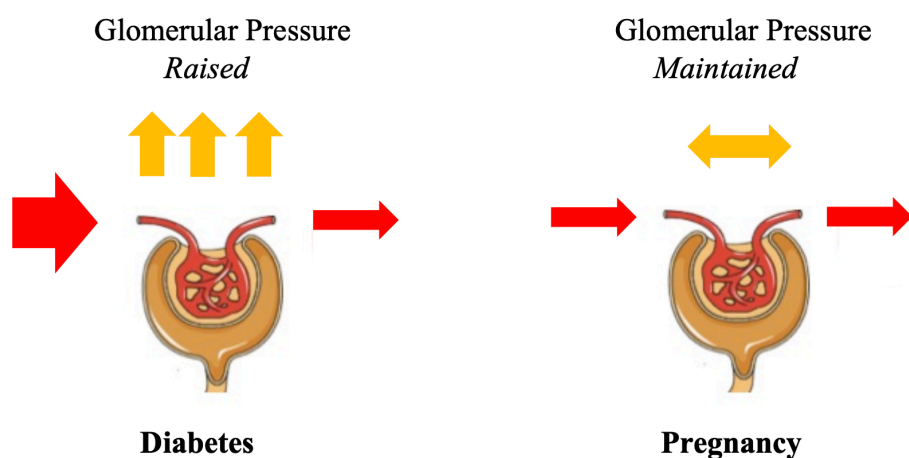
2.2 Hyperfiltration in pregnancy

2.2.1 Changes in Renal function in Pregnancy

Glomerular hyperfiltration is a well-recognized physiological response in pregnancy. This haemodynamic adaptation occurs within the first few weeks of gestation, with an average peak increase in mGFR of approximately 50-80% in the second trimester, remains constant thereafter, and returns to baseline function by 3 months post-partum [90-92]. It has been established in murine models that there is a lack of glomerular hypertension in pregnancy, likely due to matched dilatation in both afferent and efferent arteries[93], and interestingly even with ablation of renal mass[94].

The parallel reductions in renal arterial resistance are in contrast to hyperfiltration in diabetes (Figure 2.2). The etiology underlying this protective adaptation of reduced renal vascular resistance in pregnancy is incompletely understood, with angiotensin-renin pathways, relaxin, and endothelial factors (Nitric Oxide and Prostaglandin) being proposed[95]. Other haemodynamic adaptations include a rise in renal plasma flow (RPF) of up to 70%, blood pressure reduction despite volume expansion, tubular handling of protein, glucose, and uric acid also modifying GFR [96]. Accompanying this are anatomical alterations with increase in renal size and dilatation of the collecting system[92].

Figure 2.2 Hyperfiltration in diabetes and pregnancy



In contrast to the proposed mechanism in diabetes with glomerular hypertension as a result of unbalanced arteriole pressure, there is parallel reductions in both glomerular afferent and efferent arteriole resistance in pregnancy.

2.2.2 Impact of Pregnancy (Hyperfiltration) on Maternal Renal Function

Hyperfiltration in healthy pregnancies has generally been proposed to not have any detrimental effects on renal function. A study on 500 women reported no association between higher parity and decline in eGFR estimated using the Modification of Diet in Renal Disease (MDRD) equation[97]. Another study assessing parity concluded that increasing parity was associated with a reduction in risk of chronic renal failure related death [98]. These studies support the notion that hyperfiltration occurring in women with multiple pregnancies does not appear to affect subsequent renal function. This protective effect of hyperfiltration may be related to the absence of increased intra-glomerular pressure during pregnancy in contrast to diabetes. Overall, it is generally accepted that the frequency of pregnancies does not have a negative impact on renal function not only in healthy women, but in people with mild renal disease, without significant hypertension or pre-eclampsia[99]. However, there is evidence of accelerated deterioration of postpartum renal function in women with a history of moderate-severe renal insufficiency (defined as a serum creatinine $\geq 124\mu\text{mol/L}$) [100, 101].

In diabetes, to determine if pregnancy affects the course of DKD, Miodiovnik et al [102] retrospectively studied 182 women with type 1 diabetes, comparing those with and without DKD, at conception. DKD was as defined as total 24-hour protein excretion of $\geq 500\text{ mg/d}$ or persistent positive dipstick for proteinuria at baseline before 16 weeks of gestation. After a median follow up of 9.1 years, it was concluded that there was no association between pregnancy and the risk of developing new or worsening DKD based on creatinine clearance. In addition, the study found no association between parity and the risk of developing DKD in women with type 1 diabetes. However, the study lacked a healthy control comparison group. Additionally, the criteria and the definition for DKD which did not consider the physiological effects of pregnancy on proteinuria is somewhat questionable

There is strong evidence that in diabetes, pregnancy can result in markedly increased proteinuria that resolves post-partum as demonstrated by an ancillary study of the Diabetes Control and Complications (DCCT) study group capturing 270 pregnancies in women with type 1 diabetes compared to data from 500 women who did not conceive

[103]. Over a median of 6.5 years, it was established that pregnancy carried a transient risk of worsening in retinopathy and albuminuria, which resolved by the end of the study. Within the intensive treatment group (adjusted to baseline albumin excretion rate (AER) and HbA1c), pregnancy was associated with statistically significantly higher AER, and the difference persisted up to a year post-delivery. Overall, the study demonstrate that pregnancy does not carry a higher risk of developing DKD in the long term. However, exclusion of women with a baseline AER >40mg/24hours also does not provide insight regarding women affected by severe DKD. The European Diabetes Aetiology of Childhood Diabetes On An Epidemiological Basis (EURODIAB) Prospective Complication study [34] similarly examined progression of microvascular complications in pregnancies affected by type 1 diabetes. Over an average of 7 years, there was no significant difference in albumin excretion rates between the 163 women who conceived, and those who didn't. With only data on urinary albuminuria status but no information on renal function, both studies concluding that pregnancy does not have an impact on long term progression of renal complications may be misleading. This is because the lack of albuminuria does not necessarily preclude progression of DKD, as a decline in GFR can occur in the context of normoalbuminuria [30, 104, 105].

Apart from the above two multicenter trials, current evidence on the effect of pregnancy on the progression of renal decline in diabetes are largely based on small case series and case studies [106-110]. These are generally retrospective studies, with many of the studies not adjusting for potential confounding risk factors of renal function decline. Majority of the studies also define diabetes related nephropathy based on proteinuria or albuminuria as an end point, and not on renal function. Some studies included first trimester urinary albumin creatinine ratio or albumin excretion rate values as baseline readings. This can result in misclassification of DKD, particularly as women with diabetes can experience transient rise urinary albumin excretion in pregnancy[111].

One of the longer (median 16 years) follow up observational studies from the Steno Diabetes Center of 26 women with type 1 diabetes and mild DKD found no significant difference in annual creatine clearance decline (3.2[3.4] vs 3.2[5.1]) ml/min/year compared to 67 non-pregnant healthy women[112]. These women had comparable baseline risk factors. The diagnosis of DKD was based on persistent albuminuria (>300mg/dl), and the absence of diabetic retinopathy, of which it is appreciated in

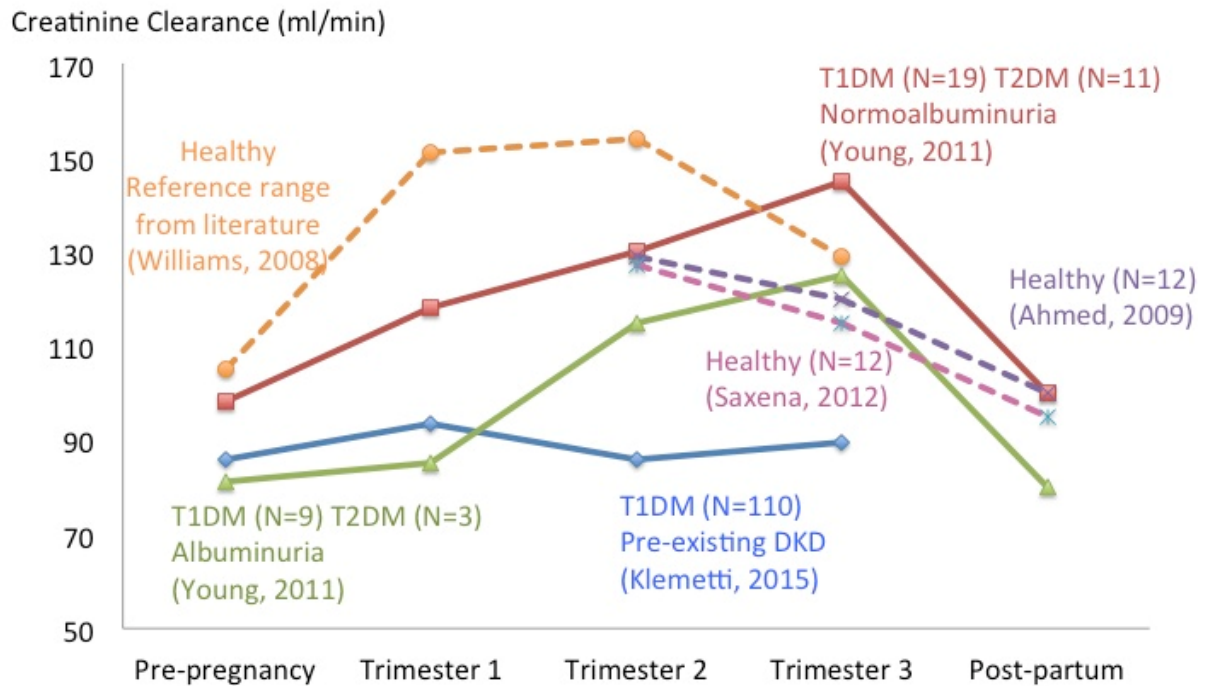
recent times that DKD and retinopathy may not be mutually exclusive. All women in this study also had tight blood pressure management. Earlier findings on 11 women with mild to moderate DKD (mean Serum Creatinine 115 $\mu\text{mol/L}$) based on 4 years of data prior to and post pregnancy by Reece et al [107] also found no deterioration in estimated GFR. This study was limited by the lack of data with most women having only one pre-conception serum creatinine.

On the other hand, for moderate- severe DKD, the evidence regarding the impact of pregnancy on maternal renal health is even less conclusive. This is partly due to the very limited number of studies, as women of child-bearing age tend to have less severe diabetes related complications and may not be available for follow up. Authors following 17 women with moderate DKD (serum creatinine 120-125 $\mu\text{mol/L}$) for up to 8 years post-pregnancy concluded that pregnancy does not adversely affect maternal renal function based on the overall mean decline in eGFR (Cockcroft-Gault formula) of 0.55 mL/min/month [113]. However, this annual decline of 6.6ml/min in eGFR is quite substantial, as it doubles the decline compared to a recent study in non-pregnant individuals with stage 3 DKD ($<60\text{ml/min/1.73m}^2$) associated with macroalbuminuria. [29]. This 16-year study by Vistisen et al followed-up participants with type 1 diabetes ($n=935$, median follow up of 5.1 years) and type 2 diabetes ($N=1984$, median follow up of 3.7 years) with stage 3 DKD. Based on CKD-EPI eGFR, for each category of albuminuria (normo-, micro- and macroalbuminuria), the annual decline was 1.9, 2.3 and 3.3ml/min/1.73m for type 1 diabetes and 1.9, 2.1 and 3.0ml/min/1.73m for type 2 diabetes.

Another two studies reported progression of moderate-severe DKD with pregnancies affected by type 1 diabetes. In a retrospective study on eleven women with a baseline serum creatinine $>124\mu\text{mol/L}$, permanent rise in serum creatinine with the need to initiate dialysis was observed in almost half of the women at 12 months post-partum[114]. This study utilized reciprocal of serum creatinine to determine GFR with considerable interindividual variability, but with a predominantly linear slope of decline. Another study quantified a creatine clearance decline of 1.4ml/min/ month in five women with a baseline creatinine clearance of $<80\text{ml/min}$ and proteinuria of $>2\text{g/24 hours}$. All women required renal dialysis at a median of 30 months post-partum[115].

There are no studies that we are aware of that compares renal function trajectory either with measured, or estimated method of GFR within a single prospective cohort of women with, and without diabetes during pregnancy. Majority of literature in pregnancy utilize serum creatinine as a measurement of renal function, and this is likely due to the lack of an established pregnancy-range reference for mGFR and eGFR. Studies on women with and without diabetes assessing urinary creatinine clearance, demonstrates variable results as depicted in Figure 2.3 adapted from existing literature [96, 110, 116-118]. Although it is not completely appropriate to compare these studies directly, peak creatinine clearance in type 1 and type 2 diabetes does not appear to be significantly different from healthy women as demonstrate. On the other hand, women with diabetes and renal impairment, based on different albumin excretion criteria at baseline, appear to have a blunted peak creatinine clearance in pregnancy. Whether GFR rise in pregnancy is blunted in women with diabetes compared to those without is not known. Hence, it is difficult to ascertain whether the observed renal function trajectory is the consequence of the potentially maladaptive sustained elevation of GFR during pregnancy, or a result of the other many factors during pregnancy. Specifically, hypertension and pre-eclampsia can predispose women with CKD and DKD to developing worsening renal function. On the other hand, modern clinical protective measurements including intensive glycaemia and blood pressure management during pregnancy may contribute to maternal renal protection. Another limitation of these studies that must be considered is the heterogeneity in renal function assessment, of which many of these surrogate measures are not validated particularly in the context of pregnancy.

Figure 2.3 Comparison of creatinine clearance in pregnancies with and without diabetes



The above figure adapted from various studies depict urinary creatinine clearance representing renal function in women with, and without diabetes pre, during and post pregnancy. Although there are established pregnancy reference serum creatinine, currently there are no clear guidelines on healthy reference ranges of pregnancy-adjusted creatinine clearance, estimated or measured glomerular filtration rate.

Women with type 1 and type 2 diabetes with pre-existing albuminuria or diabetic kidney disease appears to have a lower degree of physiological hyperfiltration compared to women without diabetes.

DKD: Diabetic Kidney Disease, T1DM: type 1 diabetes, T2DM: type 2 diabetes.

2.2.3 Maternal-Fetal Outcomes in Pregnancy related Hyperfiltration

Alterations in renal haemodynamic in pregnancies are essential to accommodate growth and maintain fetal wellbeing. However, the value of hyperfiltration as a predictor of pregnancy outcomes has not been studied widely, particularly in pregnancies affected by diabetes. Independent of diabetes, Picolli et al observed from two separate cohorts that a baseline eGFR of $>90\text{ml/min/1.73m}^2$ (using CKD-EPI formula) were associated with adverse pregnancy outcomes [119]. This association is quite concerning because an eGFR of $>90\text{ml/min/1.73m}^2$ is generally presumed as clinically acceptable renal function. However, as the upper limit of eGFR was not specified, the association of outcomes with hyperfiltration can't be ascertained. A different study by the same Italian group using an eGFR with a cut-off of both 120 and 130ml/min/1.73m^2 defined as hyperfiltration, no differences were observed between those that were hyperfiltering compared to those who were not hyperfiltering [120]. A more recent study by a Korean group found a U-shape relationship between second trimester eGFR and adverse composite pregnancy outcomes of pre-eclampsia, low birth weight and pre-term births. Unexpectedly, it was highlighted in the study that an eGFR of $120\text{-}150\text{ml/min/1.73m}^2$ was associated with the lower pregnancy risk, in comparison to groups with an eGFR $90\text{-}120\text{ml/min/1.73m}^2$ and $\text{eGFR}>150\text{ml/min/1.73m}^2$ [121]. The reason behind a higher pregnancy risk with an $\text{eGFR}>150\text{ml/min/1.73m}^2$ is not clear and may be related to the glomerular capacity to hyperfiltrate.

2.3 Summary of Hyperfiltration in Diabetes

Due to the complex nature of DKD, the pathophysiologic role of hyperfiltration in diabetes remains elusive. At this juncture, there are conflicting evidence on the impact of hyperfiltration in diabetes. Elucidating whether hyperfiltration in diabetes is a potential factor in contributing to the progression of renal failure is important. If we establish the pathological significance of hyperfiltration, instituting measures at this early stage of DKD will likely be of benefit in preventing the progression of DKD. In addition, understanding if inflammation or oxidative stress is associated with renal hyperfiltration in diabetes could also provide invaluable insight to the molecular mechanistic pathway of DKD.

To the best of our knowledge, we know of no studies comparing hyperfiltration in pregnancy to hyperfiltration in diabetes. Comparing the presumed pathological state in diabetes to the physiological response in pregnancy will facilitate our understanding on the significance of hyperfiltration in diabetes.

Review of the Literature

Chapter Three

Renal Biomarkers in Diabetic Kidney Disease and Renal Hyperfiltration in Diabetes

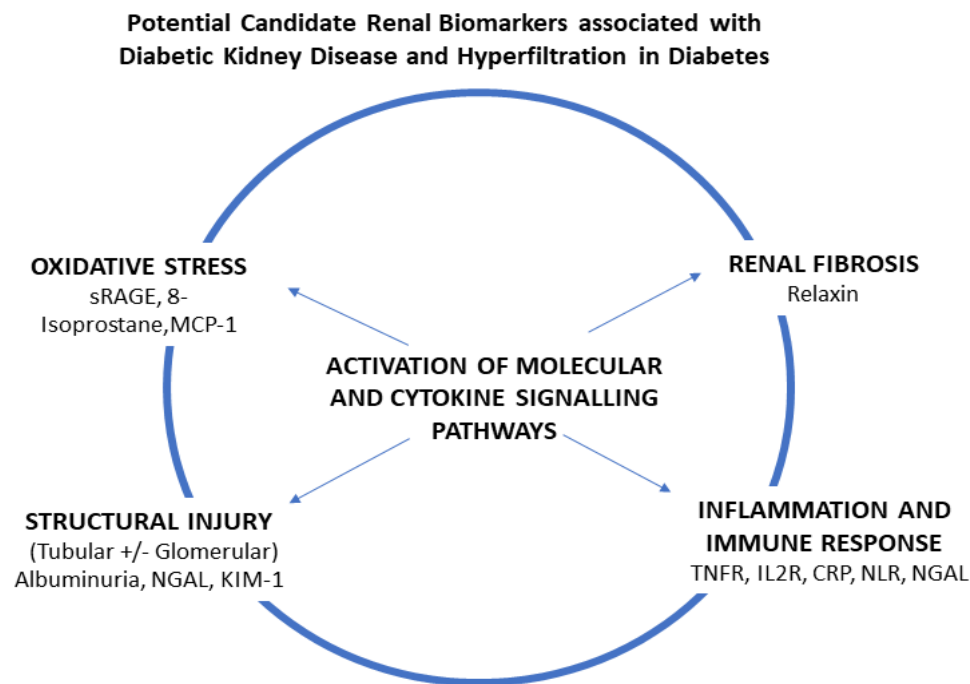
3.1 Renal Biomarkers

Biomarkers are utilized in the investigation of diabetes complications. Understanding the role of biomarkers are important not only as an attempt to investigate the mechanistic link in the development of diabetic kidney disease for potential therapeutic purpose, but also to investigate the utility of these markers as predictors of DKD, so that effective interventions can potentially be put in place. Traditionally, single candidate or a small panel of markers are analyzed and selected based on in-vitro and pre-clinical studies, either as a potential link to the cause of DKD, or to the site of nephron injury. Increasingly however, the multi-omics approach is gaining momentum in this field. Nevertheless, at this stage, ideal biomarkers to detect the onset or progression of DKD which are easily accessible and measurable, with high applicability, sensitivity and specificity are not yet available.

Pro-inflammatory cytokines, reactive oxidative stress and advanced glycation end products have been found to play a role in diabetes, DKD and pregnancy, individually and in combination. The interaction between these biomarkers and these disease states are highly complex, with the exact relationship not yet fully understood. As there are currently no specific markers for identifying and predicting DKD, the following section will focus on biomarkers with potential association between glycation, oxidative stress and inflammation in DKD (Figure 3.1) given the emerging role of the glycation and inflammatory axis in the development and progression of DKD.

Selected biomarkers that will be discussed in detail are soluble receptor for advanced glycation end products (sRAGE), soluble Tumour Necrosis Factor alpha-receptors 1 and 2 (TNFR1/2), soluble Interleukin-2 Receptor (sIL2R), monocyte chemoattractant protein 1(MCP1/ CCL2), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule (KIM-1), 8-Isoprostane, relaxin, C-reactive protein (CRP) and Neutrophil to lymphocyte ratio (NLR).

Figure 3.1 Proposed candidate biomarkers associated with diabetic kidney disease and hyperfiltration in diabetes



The above diagram summarises the candidate renal biomarkers assessed in this thesis. Candidate markers were selected based on the potential inflammatory and oxidative stress association in diabetic kidney disease, as well as being markers of renal structural injury.

sRAGE: Advanced glycation end products; TNFR1/2: soluble Tumour Necrosis Factor alpha-receptors 1 and 2; sIL2R: soluble Interleukin-2 Receptor; MCP1: monocyte chemoattractant protein 1; NGAL: neutrophil gelatinase-associated lipocalin; KIM1: kidney injury molecule; 8IP: 8-Isoprostane; CRP: C-reactive protein ; NLR: Neutrophil to lymphocyte ratio

3.1.1 Soluble Advanced Glycation Endo Product Receptor

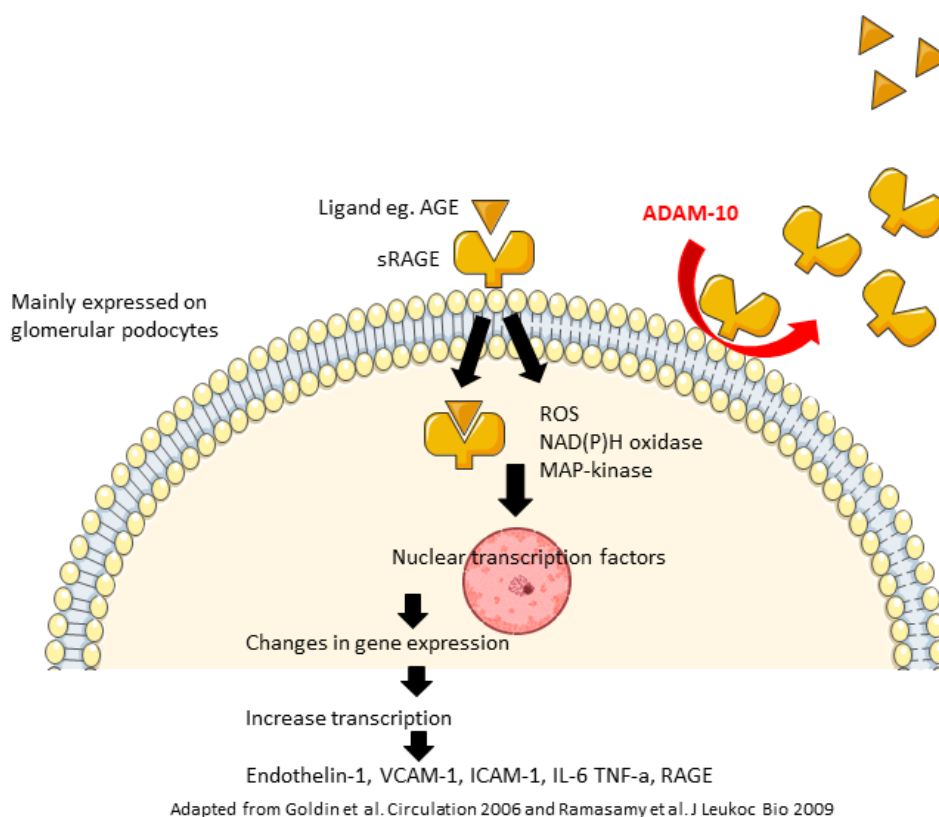
Non-enzymatic glycation and oxidation of lipids and amino acids form advanced glycation end products (AGEs). AGEs exert its' effect by binding through their cellular receptor (RAGE). Soluble circulating RAGE (sRAGE) can be measured in peripheral blood, and is a secretory splice isoform of RAGE that lacks a trans-membrane domain and circulates in the plasma acting as a decoy receptor for AGEs by sequestering RAGE ligands or competing with RAGE for ligand binding[122]. This cyto-protective effect of AGE-RAGE interaction in turn reduces inflammation and oxidation.

With regards to the associations between sRAGE and inflammation in type 2 diabetes, sRAGE has been reported to correlate with markers of inflammation including soluble Vascular Cell Adhesion Molecule-1 (sVCAM-1), Monocyte chemotactic Protein-1 (MCP-1), Tumour Necrosis Factor-alpha (TNF-a), CRP and IL6 [123-125]. Furthermore, based on murine models, it has also been demonstrated that hyperglycaemia activates podocyte RAGE. This up-regulates VCAM-1 and enhances activation of inflammatory cells which drives mesangial expansion and glomerulosclerosis resulting in the development and progression of DKD [126].

In DKD, correlations between sRAGE and albuminuria have been repeatedly demonstrated in type 2 diabetes. sRAGE levels have been demonstrated to be higher in patients with type 2 diabetes and proteinuria, along with titer of AGEs increasing in proportion to the degree of albuminuria in patients with type 2 diabetes [127]. In a study of 90 patients with type 2 diabetes, despite there being no associations with eGFR, sRAGE was reported to correlate independently to albuminuria in the presence of normal eGFR[128]. The same authors also reproduced similar results on a different study on type 2 diabetes, with sRAGE correlating with albuminuria, but not with eGFR, and notably not with either HbA1c or with fasting glucose [129]. The positive association with albuminuria, but lack of correlation of sRAGE with eGFR in type 2 diabetes suggests that not only sRAGE may be a potential early marker of DKD before the decline in eGFR, but also contests the concept that sRAGE levels are increased due to decrease in renal function as previously reported [130]. However, it has been demonstrated in a large study of 4197 patients with type 2 diabetes in the Action in Diabetes and Vascular Disease: Preterax and Diamicron MR Controlled Evaluation

(ADVANCE) trial that not only the group with the highest tertile of sRAGE had a lower mean eGFR, but that sRAGE were also associated with new or worsening DKD over a 5 year period. Circulating AGEs were also independently associated with DKD [131]. However, although it appears that the AGE-sRAGE axis is implicated in the development of DKD in observational studies, the exact mechanism is not fully understood, as depicted in Figure 3.2 adapted from Goldin and Ramasay et al. [132, 133].

Figure 3.2 Proposed mechanism of action of sRAGE



The exact AGE-sRAGE pathway is not known. sRAGE activity has been proposed to be from cleavage from cell surface by ADAM-10, or through ligand binding of AGE to sRAGE on membrane surfaces. This results in either direct or indirect action on the nuclear transcription pathway resulting in changes in gene expression and transcriptions of various cytokines. This may potentially lead to upregulation of inflammatory cells and result in glomerular or tubular damage. AGE: Advanced glycation end products; sRAGE: soluble circulating receptor of advanced glycation end products; ADAM10: A Disintegrin and metalloproteinase domain-containing protein 10; ROS: reactive oxidative stress; NAD(P)H: nicotinamide adenine dinucleotide phosphate; MAP-kinase: mitogen activated protein-kinase; VCAM1: vascular cell adhesion molecule 1; ICAM1: intercellular adhesion molecular 1.

There are limited studies exploring the relationships between sRAGE and type 1 diabetes. There are no studies that have reported the relationship between sRAGE and DKD in type 1 diabetes, or with hyperfiltration. In a study on patients with type 1 diabetes without DKD (as defined by a urinary albumin excretion of $>30\text{mg}/24\text{hours}$) or macrovascular disease, Challier et al found sRAGE levels were elevated in patients with type 1 diabetes compared to healthy controls[134]. No differences in sRAGE levels were observed in those with (N=9) and without retinopathy (N=37).

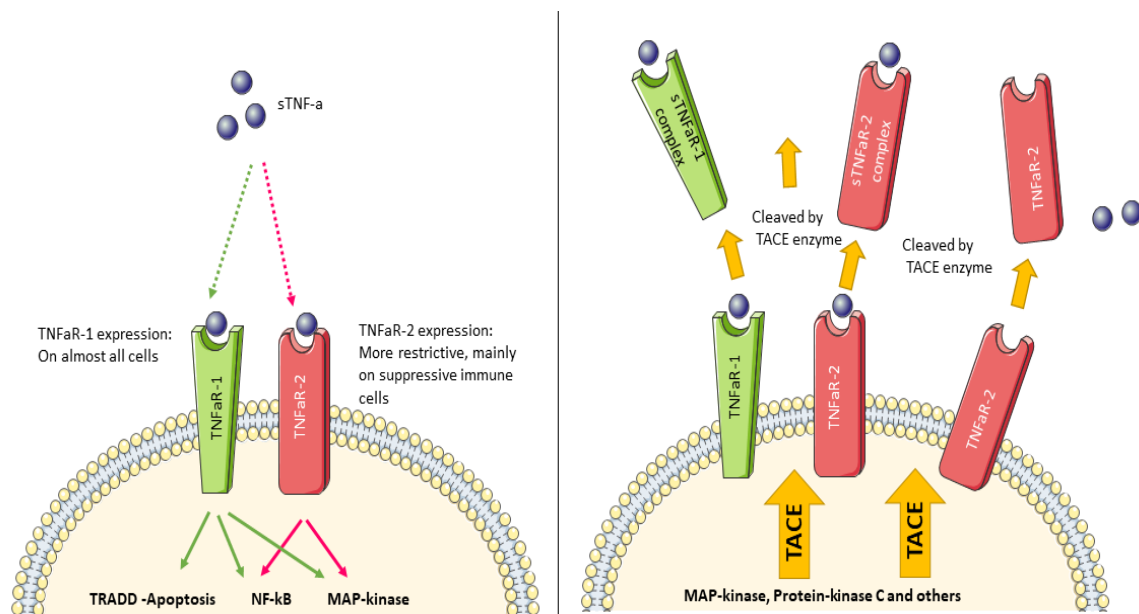
Finally, care must be taken while interpreting results. Although most studies report sRAGE, it should be noted that measurement of total sRAGE includes sRAGE, and a splice variant, endogenous secretory RAGE (esRAGE) [132]. Whilst sRAGE was elevated, esRAGE was not, in the above cited study by Humpert et al. investigating the relationship with albuminuria in type 2 diabetes[129]. In addition, levels of sRAGE can be elevated or reduced depending on different disease state. The reason for this is unclear, but it has been proposed that diseases including diabetes and renal impairment has elevated levels of AGEs, which increases the expression of RAGE[135].

Pharmacological agents also appear to affect sRAGE levels. sRAGE are increased in patients taking medications including perindopril, atorvastatin, rosiglitazone, and reduced with the use of telmisartan[132]. This is particularly confusing as all the above medications have previous reports on its anti-inflammatory effect. There is no explanation for this to date, whether these agents upregulate sRAGE as a cause or effect of circulating AGE is not known. Additionally, both perindopril and telmisartan target have opposing effect on sRAGE levels, potentially suggesting that the effect of angiotensin-converting enzyme-inhibitors or angiotensin receptor blockade on sRAGE may not be directly through the renin-angiotensin pathway. Nevertheless, these findings have been inconsistent. Perindopril, an Angiotensin Converting Enzyme-Inhibitors (ACE-i) increased sRAGE levels in a crossover study of patients of 24 months with type 1 diabetes in comparison to placebo or nifedipine, a calcium channel blocker. In addition, the evidence was further supported with murine models in the same paper [136]. On the other hand, sRAGE levels were found to be no different in participants with type 2 diabetes receiving ACE-I or Angiotensin II Receptor antagonists compared to those who did not in subanalysis in a different study on patients with type 2 diabetes [127].

3.1.2 Serum and urinary soluble Tumour Necrosis Factor alpha-receptors 1 and 2

Tumour Necrosis Factor alpha (TNF α) and its' receptors are present in renal glomerular and tubular cells [137] and have been implicated in the pathogenesis of DKD. The TNF-alpha family is a complex cytokine system, with the physiological role and mechanism of action that remains incompletely understood. The principle role of TNF is the regulation of immune cells. Abberant activity is even more elusive, as it is not known which factors increase the amount of circulating soluble (TNF), expression of sTFR1/2 on membrane surfaces, the binding of sTNF to the receptors or the cleavage of sTNFR into soluble receptors or complexes. The TNFR1/2 pathway as illustrated in Figure 3.3 adapted from Goldin and Campbell et al [133, 138] involves activation of membrane bound TNFR 1/2 on target cells by TNF. TNFR 1 is expressed on most cells, but TNFR2 expression is more restrictive and mainly on immune cells including the T-lymphocytes and myeloid lineages including macrophages. Once bound and activated, TNFR sheds from the cellular membrane surface into the circulation as soluble TNFR (sTNFR) by tumour necrosis factor-alpha converting enzyme (TACE). The regulation of TACE also remains poorly understood[139]. Activation also result in downstream signalling of cascade cytokine pathway including apoptosis, nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-kB) and mitogen-activated protein kinase (MAP-kinase). At low concentrations, sTNFR stabilize TNF and enhances its activities. Whereas at high concentrations sTNFR antagonise TNF and prevents further TNF binding to membrane TNFR [140, 141].

Figure 3.3 Tumour necrosis factor receptor 1 and 2 pathways



Adapted from Goldin et al, Circulation 2006, Campbell et al, Immunol Cell Biol 2003

Ligands including sTNF-α binds to TNFR1 that is expressed on most cells, and TNFR2 which is preferentially expressed on immune cells including lymphocytes and monocytes/ macrophages. Once bound, activation of various pathways including TRADD- apoptosis, NF-κB or MAP-kinase occurs resulting in a cascade of molecular signaling. Activation of receptors also result in cleavage by TACE from the membrane surface and sheds into the circulation.

sTNFα: soluble tumour necrosis factor-alpha; sTNFR1/2: soluble tumour necrosis factor receptor 1 and 2; TRADD: *Tumor necrosis factor* receptor type 1-associated DEATH domain protein; NF-κB: nuclear factor kappa-light-chain-enhancer of activated B-cells; MAP-kinase: mitogen-activated protein kinase; TACE: tumour necrosis factor-alpha converting enzyme

One of the earliest studies on (TNF-α) activity and DKD was demonstrated histologically on glomerular basement membrane in experimental diabetic models by Hesegawa et al [142]. Since then, it has been systemically demonstrated that TNFα activity are implicated in both structural and renal function decline in patients with diabetes. The most robust evidence with the progression of DKD with TNF system activity has been with serum soluble TNFR1/2 levels, which predicts development of ESKD in both type 1 diabetes and type 2 diabetes [143-146]. Unlike the receptors, findings of sTNF and DKD has been equivocal. sTNF levels do not appear to predict ESKD in diabetes base on renal function decline [143, 144, 147] and histology [148], which suggests that TNFR1/2, but not sTNF are associated with the early glomerular

structural changes. On the other hand, it has been demonstrated that elevated sTNF levels are associated with albuminuria [149, 150].

As discussed by Pavkov et al, without clear experimental model evidence, it is *assumed* that sTNF binding to its' receptors mediates the effect of vasoconstriction and GFR decline, the disruption of glomerular barrier and subsequent albumin permeability, as well as recruitment of inflammatory cells into the kidneys [147]. Although the exact mechanism of the TNF system is unknown and highly complex, the discrepancy in associations with DKD with sTNF and sTNFR may perhaps be due to the simplistic quantification of circulating levels may not be an accurate reflection of either marker concentration, or activation of the pathway. There may be also factors that are currently not known. For example, whether there are other signaling molecules that we do not know of that can activate sTNFR1/2; or potentially once activated, there may be other inherent neutralizing factors that attempt to dampen downstream signaling.

In type 2 diabetes, baseline serum TNFR levels can reliably predict not only mortality [151, 152], but also clinical progression of DKD, and glomerular structural changes in DKD. Circulating TNFR1 (but not TNFR2) correlated with albuminuria and renal histological changes of mesangial expansion in 22 patients with type 2 diabetes [153] which is considered a structural hallmark of GFR reduction [154]. A more recent study extended from a 6 year randomized control trial on losartan with 83 American-Indian patients with type 2 diabetes with a median duration of disease of 14 years had similar histology outcomes of TNFR1/2 levels correlating with early glomerular structural changes including reduced percentage of normally fenestrated endothelium and with increased mesangial fractional volume [148]. The Finn-Diane Study group confirmed findings of TNFR1 predicting ESKD in a cohort of 429 patients with type 1 diabetes and overt nephropathy over 9 years[145]. The Joslin Diabetes Group have demonstrated elevated TNFR 1/2 strongly predicts ESKD in Caucasian and Pima Indian patients with type 2 diabetes predominantly with an eGFR > 60mls/min/1.73m² [144, 147] over a decade of follow up. The same group [148] demonstrated a lower mGFR iothalamate clearance was associated with higher serum TNFR levels.

In mice models, administration of a TNFR fusion protein appears to reduce urinary TNF, which contributes to sodium retention and renal hypertrophy in the early stages

of diabetes[155]. Although it is not known which is cause and effect, renal hypertrophy and hyperfiltration are causally linked. Therefore, there may be a link between TNFR and hyperfiltration that is yet to be understood.

Studies in type 1 diabetes have been undertaken in patients with relatively well-preserved renal function and albuminuria. The Joslin Group demonstrated TNFR1/2 predict the loss of renal function and progression to DKD stage 3 and above over 12 years of follow up [143]. One of the largest studies in type 1 diabetes was from the DCCT/EDIC trial demonstrating TNFR1/2 correlating with a 30-40% increased odds of developing macroalbuminuria [146]. Baseline TNFR1/2 was not reported. Overall, the current evidence supports the concept that serum TNFR1/2 levels is inversely associated with GFR in DKD.

On the other hand, there is limited information regarding whether serum and urinary TNFR levels parallel each other, that is when TNFR is increased, urinary excretion increases accordingly. It is not known if urinary TNFR is affected by filtration in a similar fashion to creatinine, whereby a lower serum creatinine and higher urinary creatinine excretion accompanies a higher GFR. Until recently, this has not been studied in detail. Niewczas et al demonstrated for the first time in selected type 1 and type 2 diabetes individuals at risk of end stage kidney disease who had elevated serum TNFR superfamily, urinary levels were similarly elevated using the slow off-rate modified aptamer (Soma) Scan Platform[156]. This suggest systemic overproduction, rather than impaired renal handling as the aetiology of elevated TNF receptors in serum. The group also demonstrated some, but not significant correlation between circulating levels of both TNFR1 and 2 with glomerular expression of the genes expressing these proteins, along with lack of mRNA expression on renal biopsy, suggesting that the source of these markers is likely not from the kidney.

In glomerulonephritis, a significant negative correlation between urinary TNFR1 and estimated creatinine clearance has been demonstrated[157]. Despite the lack of measurement of serum TNFR concentration, the authors proposed that TNFR1 excretion reflects increased TNFR shedding from the cell membranes, and a marker of increased TNFa expression on renal cells, akin to one of the proposed mechanism in a type 1 diabetes study by the Joslin group[158]. Findings of the negative relationship

between both serum and urinary TNFR1/2 with eGFR were further supported in a subsequent study in IgA Nephropathy. Interestingly, based on the equivocal relationship between serum and urine levels of TNFR1/2 with the expression of tubulointerstitial and glomerular expression of these receptors, the study concluded that serum and urinary TNFR1/2 are likely from an external site other than the kidneys [159]. TNFR1/2 are both expressed on various cell surfaces, with TNFR2 predominantly on immune cells. Therefore, to understand how TNFR is exactly expressed by immune cells in the kidneys, we need prior understanding of the pathophysiology of tissue-resident lymphocyte and mononuclear phagocyte system (including monocytes and macrophages) that are present in the kidneys [160, 161]. However, till date little is known regarding the expression and migration of these cells in the presence of DKD from current experimental studies. A recent study in eighty-eight renal autopsy samples of histologically proven DKD demonstrate that specific anti-inflammatory staining of macrophages (CD163+) are present in the glomeruli and interstitium compared to controls [162]. Interestingly, the number of macrophages were similar in those with and without DKD. By extension, if TNFR1/2 are expressed on kidney-specific macrophages, local expression of these markers in those with diabetes will differ in comparison to those without DKD. Nevertheless, this is a basic hypothesis, as it is clear that the immune system is highly complex and the TNF pathway till date has not been fully explored.

At present, the exact source of TNFR still remains unclear, with the causal role of TNFR on the pathogenesis of DKD also unknown. It has been demonstrated that TNF pathway has a link with DKD injury via upregulating monocyte chemoattractant protein-1 (MCP-1) in renal mesangial cells [163] and podocytes [164] which results in accumulation of macrophages. Whether TNFR has a direct role in renal injury, or reflects TNFR shedding from cell membrane and acts as a decoy for elevated TNF- α in renal injury and activates downstream pro-inflammatory mediators remains to be investigated.

3.1.3 Serum and Urinary Interleukin 2 Receptor

Binding of soluble Interleukin 2 (sIL2) to its' receptor IL2R, results in the release of sIL2R, which has been shown to be a useful marker of T-cell activation[165] and plays a part in inflammation and immunologic responses. Currently, there are very few studies investigating sIL2 and sIL2R in diabetes. The association of sIL2R with progression of coronary artery calcification was demonstrated in type 1 diabetes in a case control study. The proportion of DKD was not known but serum creatinine did not differ between groups[166].

In a small study comparing diabetes patients with and without DKD, plasma sIL2R levels correlated positively with proteinuria, and the levels were higher in the DKD group[167]. Another study [168] on various glomerular diseases including in a subset of 14 patients with DKD demonstrated elevated serum sIL2R compared to healthy controls. sIL2R levels were not only elevated in renal impairment but had a strong negative correlation with creatinine clearance. Interestingly, there was a positive correlation between serum and urinary IL2R. As urinary and serum levels appear to parallel each other, this may potentially suggest that excretion of sIL2R may not be filtration dependent. However, this needs to be further investigated, as in the study, no association between urinary IL2R with either renal function, or glomerular disease was observed.

At present, the very limited literature on both sIL2 and sIL2R in DKD makes it difficult to draw any firm inference and more studies are warranted. This is of particular interest, as IL2 exert its effect through T-cell activation, and although the relationship of T-cells and DKD is poorly understood, there have been reports of the association of regulatory T-cells in DKD and albuminuria [169, 170]. In fact, Zhang et al demonstrated that in type 2 diabetes, not only certain T-helper cells were elevated in DKD, but that sIL2 were increased in those with DKD and positively correlated with albuminuria[170]. But of opposing findings, was an experimental model of type 2 diabetes and *depleted* regulatory T-cells. These mouse models exhibited albuminuria and glomerular hyperfiltration, with the authors concluding the potential therapeutic value of regulatory T-cells[171].

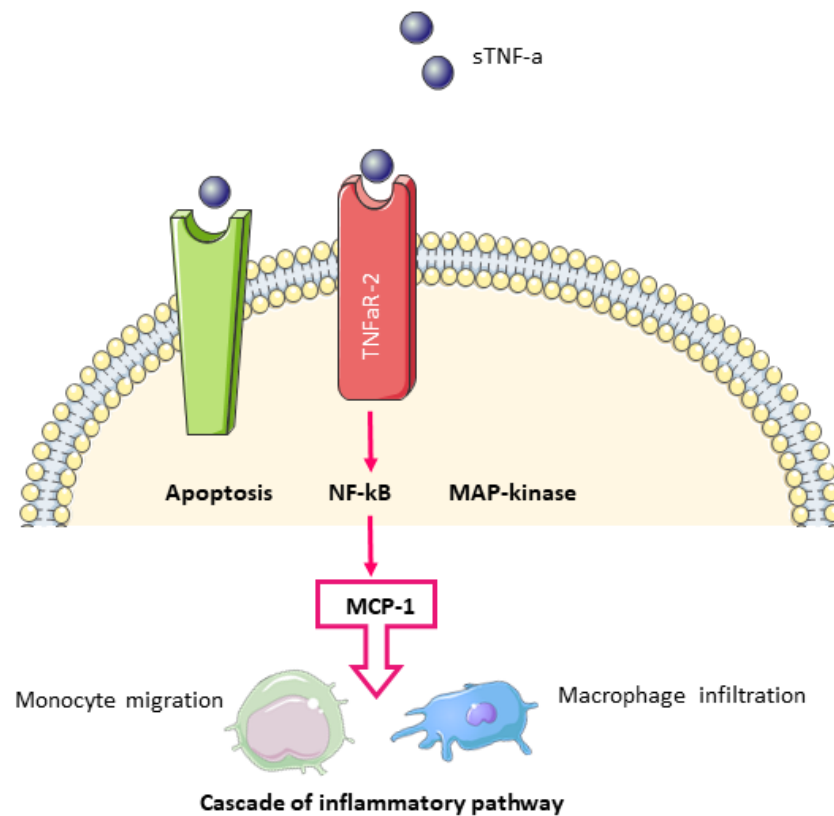
3.1.4 Monocyte chemo-attractant protein-1

Macrophage infiltration of the glomeruli and tubular interstitium in diabetes has been demonstrated along with progressive nephropathy [172]. Monocyte chemo-attractant protein-1 (MCP1) that is expressed on both renal glomeruli and tubular cells belongs to the family of inflammatory chemokine that is responsible for monocyte and macrophage recruitment [173], and may be a potential surrogate marker of macrophages.

The relationship between DKD and serum/ urinary MCP1 has been contradictory. Elevated urinary, but not serum MCP1 with supporting tubulointerstitial macrophage expression on histology has been observed in type 2 diabetes and albuminuria [174]. On the other hand, other type 2 diabetes clinical studies have demonstrated that serum MCP1, but not urinary levels are elevated in DKD [175], with its' source of renal injury potentially of tubular origin based on correlation with tubular markers [176]. Similarly, elevated serum MCP1 mediated by oxidative stress was also observed in type 1 diabetes patients with microalbuminuria [177]. In another study in individuals with type 1 diabetes, although low urinary levels of MCP1 were detected in microalbuminuria, there was a lack of relationship between urinary MCP1 in the progression of microalbuminuria over a 2-year period[178]. This suggest that MCP1 may potentially be involved early in the development of DKD. However, it has also been demonstrated that urinary MCP1 levels have an inverse relationship with eGFR in type 2 diabetes, with titers increasing with worsening DKD [179, 180].

Overall, it seems that MCP1 may be relevant in the progression of DKD, but the current evidence remains ambiguous and warrants further investigation. Finally, one other notable observation on MCP1 is that the signaling of MCP1 expression occurs via TNFR2, which is increasingly relevant in DKD as depicted in Figure 3.4 adapted from Chung and Goldin et al [133, 164]. In the same study, it was also found that urinary, plasma as well as local podocyte MCP1 concentrations correlated with albuminuria in type 2 diabetic db/db mice.

Figure 3.4 Association between MCP1 and TNFR2



Adapted from Chung et al, Nephron Extra 2015; Goldin et al, Circulation 2006

Increased levels of sTNFa on podocytes induces MCP1 production via activation of TNFR2 (but not TNFR1). Subsequent activation is via the NF-kB axis.

MCP-1: Monocyte chemo-attraction protein 1; sTNFa: soluble tumour necrosis factor-alpha; sTNFR1/2: soluble tumour necrosis factor receptor 1 and 2; NF-kB: nuclear factor kappa-light-chain-enhancer of activated B-cells; MAP-kinase:mitogen-activated protein kinase

3.1.5 Serum and Urinary Kidney injury molecule-1 and Neutrophil gelatinase-associated lipocalin

Kidney injury molecule-1 (KIM1) is a transmembrane protein that is expressed on the luminal surface of proximal tubules, with expression upregulated during renal injury. Neutrophil gelatinase-associated lipocalin (NGAL) is an iron transporting protein that is filtered by the glomerulus and resorbed by tubules in the normal kidney[181, 182]. Both markers are frequently studied together as established markers of renal tubular injury.

A recent meta-analysis [183] based on ten prospective studies of various renal diseases has identified urinary NGAL, but not KIM1 as an independent predictor of ESKD (RR 1.4(1.2-1.6), $p<0.01$) as well as a predictor of overall mortality. Similarly, specifically in type 2 diabetes, whilst a weak correlation was found for urinary NGAL with ESKD, no association was found with urinary KIM1 and ESKD[182]. This contrasts with another type 2 diabetes study, which found that higher baseline titers of urinary KIM1, but not urinary NGAL was associated with greater eGFR decline over a median of 3.5 years. However, neither urinary KIM1 or NGAL predicted progression of albuminuria in the relatively short period of observation[184]. On the other hand, rapid decliners (defined as a reduction in baseline eGFR $>25\%$ annually) in a cohort of type 2 diabetes had higher baseline urinary NGAL and KIM1, compared to non-rapid decliners[185]. A major limitation, however, was the short follow up period (median of 12.4 months).

In a very well powered type 1 diabetes study part of the Finnish Diabetic Nephropathy (Finn-Diane) Study, it was observed that urinary KIM1 did not predict progression to ESKD defined as requiring renal dialysis [186]. However, the study was followed up for only a median of 6 years, with more than half of the participants with normo-albuminuria with relatively well-preserved renal function (median eGFR of 88ml/min/1.73m²). In addition, only 10% of the cohort developed progression in DKD defined as passage from of albuminuria status (normo- to micro- or micro to macro-albuminuria) or to requiring dialysis. Despite not predicting ESKD, the study identified a causal association between increased urinary KIM1 and low GFR, independent of glycaemia management or albumin excretion rate using Mendelian Randomization analysis and Genomic Wide Associations (GWAS). This finding is in contrast to a

couple of smaller type 1 diabetes studies using more conventional statistical analysis [187, 188], one of which did not find any additional prognostic value of either urinary KIM1 or NGAL on the decline in GFR. In fact, although urinary KIM1 was shown not to predict early progressive eGFR decline, plasma KIM1 did [188]. This highlights that the biology of corresponding plasma and urinary cytokine/ chemokine can differ and may reflect distinctive pathologies.

Based on the above, there seems to be an association with NGAL and KIM1 as a marker of DKD. However, they do not appear to be a predictor of later stages of diabetic kidney disease, although existing studies are relatively short in duration. The prognostic value of these markers in determining progression of DKD is not clear.

3.1.6 Relaxin

Pro-fibrotic factors, especially Transforming Growth Factor-beta1 (TGF-beta) has been widely studied and linked to glomerulosclerosis and tubular-interstitial fibrosis in DKD[189]. On the other hand, there is increasing evidence on the reno-protective effect of endogenous anti-fibrotic factors. This includes the effects of the anti-fibrotic factor relaxin which is present in three forms in humans (H1, H2 and H3 relaxin) [190]. H2 is the human relaxin of interest, as it is the only form that is present in the circulation. It is also the main hormone that facilitates female reproductive function, as well as cardiovascular vasodilatory effects. H2-relaxin binds to its' receptor relaxin peptide receptor 1 (RXFP1) and exert its' anti-fibrotic effects through TGF-beta, and its' vasodilatory effects through the nitric oxide pathway[191]. There are current phase III studies on relaxin in heart failure and emerging pre-clinical trials in pro-fibrotic conditions[192].

Studies administering exogenous H2-relaxin that is biologically active in rodents have shown variable renal outcomes. H2-relaxin appears to preserve renal mass and reverse some structural damage in less than a month on mice models [193, 194]. Notably, relaxin's effect is independent from blood pressure regulation, and as it improves predicted creatinine clearance, the reno-protection observed with relaxin is possibly related to increased renal blood flow [190]. However, no positive reno-protective findings were uncovered using relaxin in type 1 diabetes mice induced with early DKD [195, 196]. It is pertinent to consider, however that relaxin biology is different in rats and that these findings do not necessary translate to human studies, which are lacking in the literature.

In human studies, endogenous plasma H2-relaxin levels are lower in diabetes compared to controls [197]. At this point, little is known regarding the association of relaxin with DKD. In a study assessing end stage kidney disease with approximately a quarter of the patients with DKD, relaxin was an independent predictor of mortality in males but not females [198]. Gender differences in mortality related to relaxin from the study were consistent in mice on cardiac parameters[199]. Whether the gender impact and effect of relaxin is related to its role on female reproductive cycle needs to be further appraised. Although there are no established association between relaxin and DKD as

yet, its' role in fibrosis and vasodilation in the pathogenesis in DKD is potentially an area worth exploring.

3.1.7 Serum and Urinary 8-Isoprostane

Oxidative stress in the glomeruli and tubules has been identified on renal tissues in DKD [200]. 8-Isoprostaglandin F_{2α}, or 8-Isoprostane is an end-product of lipid peroxidation, and is a useful marker of quantifying oxidative stress, particularly directed against phospholipids [201]. The source of renal 8-Isoprostane is unknown. There are contradicting findings of whether plasma and urinary 8-Isoprostane are elevated in type 1 diabetes compared to healthy controls [202, 203]. Relationship with renal function was not determined in either study. In diabetic rats, incubation of glomerular cells of diabetic rats with 8-Isoprostane stimulated production of TGF-beta, a pro-fibrotic factor of DKD. Plasma and urinary levels of 8-Isoprostane along with TGF-beta was also found to decrease with treatment with vitamin E. [204]. Despite the potential role of oxidative stress in DKD, urinary 8-Isoprostane was reported to correlate positively with eGFR in a type 2 diabetes study[205]. The authors postulated that this might be a reflection of oxidative stress being implicated early on the course with initiation, rather than the progression of DKD.

Importantly, 8-Isoprostane should not be confused with 8-hydroxy-2'-deoxyguanosine (8-OHdG), which is also a marker of oxidative stress but targeting cellular DNA oxidation. Elevated 8-OHdG has been reported in diabetes and proteinuria compared to those without proteinuria[206], as well as a predictor of progression of DKD in type 2 diabetes[207]. Diabetic mice studies assessing the effect of candesartan has established a correlation of both urinary 8-isoprostane and 8-OHdG with albuminuria, but the relationship of these markers to each other were not examined [200]. Positive effect of Angiotensin II receptor blockade on the levels of 8-Isoprostane and 8-OHdG in this study was echoed in a type 2 diabetes DKD clinical study[208], suggesting a potential role of the angiotensin pathway mediating oxidative stress in DKD.

3.1.8 C-reactive protein and Neutrophil-Lymphocyte Ratio

C-reactive protein (CRP) is an acute phase protein that reflects response of the innate immune system. There are many studies that have established the presence of elevated CRP levels in diabetes, particularly so in those with complications[209]. In terms of DKD, CRP levels correlates with albuminuria status in mainly observational studies on both type 1 diabetes [210, 211]and type 2 diabetes[212, 213].

Leucocytes infiltrate kidneys at a cellular level, and induce functional changes including albuminuria and eGFR decline in diabetes[214, 215]. Neutrophil and lymphocytes are readily accessible measurements of leucocyte production. However, as these individual markers are increased in a wide range of inflammatory response, there is an emerging interest in the role of neutrophil-lymphocyte ratio (NLR) in DKD, which may be more specific. In type 2 diabetes, the higher the urinary albumin excretion is, the higher the neutrophil levels are; but lymphocyte levels reduce accordingly[216]. It remains to be investigated why circulating lymphocytes levels are inversely associated with albuminuria, particularly when lymphocyte knockout diabetic mice exhibited lymphocyte deficiency, reducing albuminuria and preventing glomerular changes in keeping with DKD [215]. It has been shown however, that although the mechanism remains unidentified, diabetes induces apoptosis in lymphocytes [217]. Taken together, one could postulate that although lymphocytes are associated with renal damage, in poorly controlled diabetes including in DKD, reduction of peripheral lymphocyte count from apoptosis may reflect impaired immunity. From the limited available studies, NLR levels appear to correlate with albuminuria and lower eGFR, along with predicting eGFR loss in type 2 diabetes [218-220].

Although CRP and NLR has a positive relationship with albuminuria and eGFR decline, the prognostic value of these markers in the detection and progression of DKD remain to be explored, especially when both markers are elevated in various inflammatory disease.

3.2 Biomarkers in Diabetes related Hyperfiltration

There are currently limited studies on inflammatory and oxidative biomarkers in hyperfiltration. The most widely studied marker is Nitric oxide (NO). Experimental studies suggest NO plays a role in the preferential dilation of afferent arterioles and functional glomerular hyperfiltration [221]. In a study comparing type 1 diabetes patients with and without microalbuminuria with healthy controls, serum nitrite and nitrate (as index of NO production) was found to be elevated in the group with microalbuminuria, which had a significantly higher mean DPTA mGFR of 173 ± 14 ml/min/1.73m² [222]. Similarly, serum and urinary nitrite and nitrate were found to be elevated in type 2 diabetes and hyperfiltration, regardless of albuminuria status[223]. In another type 2 diabetes study, patients with hyperfiltration had a higher urinary nitric oxide synthase production and expression on renal biopsies than non-hyperfiltration or healthy controls. In this cohort, 3 out of 5 kidney biopsies affected by microalbuminuria and hyperfiltration showed expression of endothelial cell NO staining [224], further supporting that NO metabolites are likely of renal origin. Hyperfiltration in the above two instance, however, was defined using an eGFR > 135ml/min/1.73m² using the Cockcroft and Gault formula which can overestimated mGFR; and on repeated 24 hour urinary creatinine clearance which was lower than expected hyperfiltration range, between a mean of 98.3-107ml/min/1.48 m². Another study that found a positive relationship between NO with hyperfiltration in type 2 diabetes, also measured malondialdehyde (MDA), a marker of lipid peroxidation, but did not analyse its relationship with renal function[223]. 8-Isoprostane has not been studied in hyperfiltration.

Following these early studies on endothelial dysfunction, inflammatory cytokines in hyperfiltration has recently been investigated in type 1 diabetes. Clamp induced hyperglycaemia which resulted in hyperfiltration was associated with increased levels of various urinary cytokines and chemokines [225]. However, it was not ascertained if the elevated markers were associated with hyperfiltration independently. A panel of diverse urinary inflammatory markers was also found to be elevated in type 1 diabetes and hyperfiltration (measured GFR > 135ml/min/1.73m²), compared to healthy controls and type 1 diabetes without hyperfiltration [226]. The same group noted similar findings for serum and urinary cytokines in normotensive adolescents with type 1

diabetes and normoalbuminuria. Elevated urinary MCP1, IL-12, IFN12, sCD40L, FGF2, TNFB, MIP1A and IL2 levels were found to be elevated in the hyperfiltration group compared to either healthy controls or type 1 diabetes patients who were not hyperfiltering. However, all apart from IL2 lost significance after adjusting for plasma glucose at time of collection. [227]. There is currently no data on serum or urinary IL2R levels in hyperfiltration.

In type 2 diabetes, tubular markers were specifically evaluated in hyperfiltration (defined as an eGFR exceeding sex-specific 97.5th percentile of 224 healthy controls and based on cystatin-C levels conversion). hyperfiltration patients (eGFR 135 ± 13.5 ml/min/1.73m²) had higher urinary NGAL and KIM1, but lower serum NGAL compared to those who were normofiltering (eGFR 93.3 ± 12.7 ml/min/1.73m²) and healthy controls (eGFR 99.1 ± 13.8 ml/min/1.73m²)[228]. It is not clear if the lower serum levels and higher urinary levels of NGAL is related to increased urinary excretion due to the hyperfiltration state, or potentially suggesting tubular damage. The authors concluded that this is direct evidence that hyperfiltration is deleterious in DKD. In addition, lower urinary NGAL and KIM1 concentration at baseline is associated with microalbuminuria regression independently over a 2 year period in type 1 diabetes [178]. The findings of these two studies support that tubular injury may be involved very early on in the development of DKD, and perhaps may be related to hyperfiltration.

The only clinical study examining the relationship of TNFR and hyperfiltration in diabetes stratified patients with type 2 diabetes into three different percentile group based on circulating TNFR1/2. The median mGFR in the lowest (25th percentile) group was within hyperfiltration range (median 136 ml/min/1.73m²; IQR 117, 160, N=19) and (median 134 ml/min/1.73m²; IQR 117, 158, N=21) for both TNFR1/2 [148]. There are currently no studies examining TNFR 1/2 in the presence of hyperfiltration in patients with type 1 diabetes. sRAGE and AGE has not been directly studied in hyperfiltration. It also should be noted that it remains unclear if sRAGE are elevated due to renal impairment, or up-regulated as a protective response to effects of RAGE ligands[229].

In addition to its' anti-fibrotic effects, relaxin is of interest as it stimulates vasodilation and hyperfiltration in small arteries of pregnant and non-pregnant experimental

models[230]. The same group also found that removal of circulating relaxin in pregnant rats has shown to abolish the rise of GFR and renal plasma flow (RPF)[231]. On the other hand, despite an increase in RPF, administration of relaxin in a small group of non-pregnant women and men did not result in a rise in GFR[232]. The discrepant results may imply that rats and humans do not respond similarly to relaxin. What caused the lack of GFR rise is unknown but do indicate that relaxin might be one of the key factors underlying the different mechanism in hyperfiltration for both diabetes and pregnancy. At this stage, the role of relaxin in DKD related hyperfiltration is not known. Notably, in pregnancy, relaxin-2 levels particularly in early pregnancy has been found to be higher in gestational DM [233] as well as in type 1 diabetes compared to healthy pregnancies[234]. Whether this is related to the need for an augmented renodilatory effect by relaxin in diabetes remains to be investigated, particularly as plasma levels of relaxin-2 are in contrast lower in diabetes compared to controls in non-pregnant women and men[197]. Renal function, which may provide some insight, was not measured in either study.

A study on pre-eclampsia followed up 127 women at the 6 weeks post-pregnancy mark, and found elevated CRP levels as well as the presence of hyperfiltration (mean eGFR $150 \pm 42 \text{ ml/min/1.73m}^2$)[235]. However, inference of the relationship between CRP and hyperfiltration could not be drawn as analysis on the correlation between CRP and renal function was not conducted. In a large sample of non-diabetic population, elevated CRP was associated (OR 1.7; 95%CI 1.2-2.5) with hyperfiltration, defined as exceeding the 95% confidence interval of the mean creatinine clearance of the population. However, after accounting for body mass index, the relationship was no longer significant[236]. Whether this is the case with CRP and diabetes related hyperfiltration is not known, as it should be emphasized that the process of hyperfiltration in diabetes, obesity and pregnancy are distinct to each other.

Albeit limited, with the relationship between inflammation and hyperfiltration in diabetes remaining elusive, emerging evidence supports that inflammation is potentially implicated in diabetes related hyperfiltration.

3.3 Consideration of Candidate Biomarkers

As evident from the literature, studies have not demonstrated consistent results on the associations of the selected candidate biomarkers and DKD. Although concentrations of these biomarkers have generally been demonstrated to be elevated in DKD, it may not predict progression of DKD. This may be a result from various limitations. Firstly, majority of data in this field are from observational cross-sectional studies with relatively small sample sizes. Moreover, consideration has to be taken that factors including advanced age, smoking, diet, elevated body mass index, concomitant comorbidities and medications are potential limiting aspects in assessing markers of inflammatory pathways. These confounding elements are particularly common patients with diabetes, and despite adjusting for covariates in most studies discussed above; it is highly difficult to ascertain complete independent relationships of biomarkers from these factors. In addition, the heterogeneity of studies does not allow for precise interpretation. This includes the use of various laboratory and analytical techniques, different methods of defining renal function and broad definition of surrogate renal endpoints.

In addition, majority of studies generally capture biomarkers at a single time point. The reliance of a single measurement of any marker limits interpretation, as levels may vary within individuals over time, particularly as it is difficult to account for potential bias including other pre-existing or acute medical conditions that may predispose to a systemic inflammatory state and contribute to fluctuation in concentrations. Hence, although measurement of a biomarker at the start of a prospective study is valid, it is not clear if serial measurements will have a role in improving prognostication of DKD progression.

3.4 Summary of Biomarkers

Despite the promising role in predicting DKD, biomarkers are unlikely to be utilised alone, but as an adjunct to improve upon the predictive utility of other readily available, traditional clinical and biochemical information. It is important to note that the purpose of evaluating renal biomarkers in this thesis is not to predict the development or progression of DKD, but to consider their association in DKD and investigate their potential role in hyperfiltration.

To identify clinically useful biomarkers in our study will involve limiting assessment of known and promising markers; or experimenting on novel markers not linked to DKD and/or hyperfiltration. As our study involved a relatively small sample size, and included both diabetes and pregnancy state, we had to strike a balance to reduce the risk of negative discovery rate, but also to minimize measuring multiple analytes simultaneously to avoid bias in seeking positive associations of the selected biomarker with DKD and hyperfiltration. In this present study, we evaluated selected markers with their antecedent rationale that there are some experimental and clinical evidence that these markers have an association with DKD and hyperfiltration in diabetes. In addition, although glomerular and tubulointerstitial damage are established factors in the progression of DKD; the initiating pathophysiological site of hyperfiltration is not entirely known. Hence, these selected biomarkers may possibly indicate the degree of either glomerular or tubular involvement as the site of renal injury in hyperfiltration.

This thesis will focus on the afore-mentioned candidate markers summarized in Table 3.1; however, these are by no means a comprehensive list of inflammatory markers that have been investigated on either the prediction of the development, or progression of DKD. As the majority of urinary proteome originates from the renal system[237], urine is a valuable source of renal disease biomarkers. Measuring corresponding serum and urinary levels can provide insight into the relationship markers with renal function and renal excretion, particularly as plasma and urine levels do not necessarily correlate or are interchangeable. Importantly, for biomarkers that have been observed to correlate negatively with renal function in DKD, it may be anticipated that excretion of these markers may follow the fashion of creatinine excretion, dependent on glomerular filtration with concentration declining in hyperfiltration physiologically. However, the

premise of this thesis is that hyperfiltration is a pathological state of DKD. Consequently, if glycation and inflammatory markers are elevated in hyperfiltration, it supports the notion that inflammation is potentially involved in the process of diabetes related hyperfiltration and may be one of the initiating phases of the development of DKD. It is, however, a simplistic view that the ability to detect a particular biomarker in DKD or hyperfiltration is adequate to measure inflammation or oxidative stress and to reflect the underlying pathophysiology of DKD.

Notably as well, the Joslin group has recently published significant advancement in the area of renal markers in DKD after the commencement of this thesis. The researchers identified specific inflammatory markers that are associated with end stage kidney disease in type 1 and type 2 diabetes, known as Kidney Risk Inflammatory Signature (KRIS) using global proteomic profiling approach in three different cohort of participants. Importantly, urinary proteomic data from the group suggest that systemic overproduction rather than dysfunctional renal handling is likely the source of elevated markers. It was also identified that the effect of majority of the proteins on renal function decline was independent from albuminuria[156]. Apart from TNFR1 and 2, none of the none of the other 15 KRIS proteins that were identified were measured in our study.

Lastly, as a final consideration, as hyperfiltration state in pregnancy is used as a comparative hyperfiltration process in diabetes for this thesis, the relationship of these biomarkers in pregnancies should be noted. This is especially so when elevated pro-inflammatory cytokines are found in women with healthy, uncomplicated pregnancies[238]. Importantly, it is not the aim of the study to assess the effect pregnancy has on the circulating and urinary excretion of the candidate biomarkers. This is summarized in Table 3.1.

Table 3.1 Summary of candidate biomarkers

	<i>Type 1 Diabetes DKD</i>	<i>Type 2 Diabetes DKD</i>	<i>Diabetes related Renal Hyperfiltration</i>	<i>Healthy Pregnancies</i>	<i>Notes</i>
sRAGE	Not known	↑	Not known	↓*[239]	
Serum TNFR1/2	↑	↑	↓	↑*[240, 241]	Inverse relationship with renal function
Urinary TNFR1/2	Not known	Not known	Not known	↑*[240, 241]	
Serum sIL2R	↑	↑	Not known	↓*[242] [243]	Lymphocyte activation
Urinary sIL2R	No association	No association	Not known	Not known	Lymphocyte activation Inverse relationship with renal function
Serum MCP1	↑	No association	Not known	↑*[244, 245]	? Tubular marker
Urinary MCP1	Not known	↑	↑	Not known	Inverse relationship with renal function
Serum NGAL	↑	↑	↓	↑*[246, 247]	Tubular marker
Urinary NGAL	↑	↑	↑	Not known	
Serum KIM1	Not known	↑	Not known	Not known	Tubular marker
Urinary KIM1	↑	↑	↑	↑*[248]	
Serum 8-Isoprostane	Not known	Not known	Not known	↑[249]	
Urinary 8-Isoprostane	Not known	↑	Not known	↑*[250, 251]	Positive relationship with renal function ? Stimulate fibrosis
Relaxin	Not known	Not known	Not known	↑	
CRP	↑	↑	Not known	↑*[252]	
NLR	Not known	↑	Not known	↑*[253]	

*Levels increase in pregnancies with complications

soluble advanced glycation end products (sRAGE), soluble Tumour Necrosis Factor alpha-receptors 1 and 2 (TNFR1/2), soluble Interleukin-2 Receptor (sIL2R), monocyte chemoattractant protein 1(MCP1/ CCL2), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule (KIM-1), 8-Isoprostane, relaxin, C-reactive protein (CRP) and Neutrophil to lymphocyte ratio (NLR)

Review of the Literature

Chapter Four

Functional Magnetic Resonance Imaging in Predicting Diabetic Kidney Disease

4.1 Hypoxia in DKD

In the healthy renal system, the proximal tubules within the cortex consume 80% of the oxygen delivered to the kidneys[254]. The renal medulla is in a constant state of relative hypoxia due to the low vascular density, arterial-venous shunting and high oxygen consumption required for the active sodium-chloride co-transporters which are located in the distal convoluted tubule [255].

Although renal hypoxia have been noted to be associated with the development of chronic kidney disease (CKD) [256], it is a highly complex process with the underlying mechanism of renal perfusion injury and ischaemia leading to kidney disease yet to be elucidated. Computer and mathematical models using cell tissues, parameters of oxygen diffusion, oxygen consumption, capillary and tubular flows have been developed in an attempt to understand oxygen dynamics [257, 258]. It has been demonstrated in renal biopsies taken for clinical reasons that reduced capillary density, as a surrogate marker of hypoxia, can be associated with tubulointerstitial damage [259]. In non-diabetic rat models, using dinitrophenol, a mitochondria uncoupler to induce renal hypoxia, it has been shown that reduced oxygenation can trigger nephropathy independent from oxidative stress, hyperglycaemia, glomerular filtration and renal blood flow on histology [260].

In diabetes, sustained hyperglycaemia resulting in hyperfiltration and increased blood flow can result in an increased arterial-venous oxygen gradient and subsequent inefficient shunting to the peri-tubular capillaries[261]. Hyperfiltration can also lead to increased glucose filtration with enhanced sodium-glucose resorption in the cortical proximal tubules[262]. In combination, this may result in increased oxygen demand and potentially tubulo-interstitial hypoxia. In terms of therapeutic targets for hypoxia in DKD, it has been hypothesized that the SGLT2-inhibitors ameliorate hyperfiltration, increase erythropoietin production and hematocrit levels, which is associated with improvement of oxidative stress, hypoxia, and can potentially reverse renal remodeling [263]. Considering the above, it appears that hyperfiltration may play an important role in the development of hypoxia in DKD.

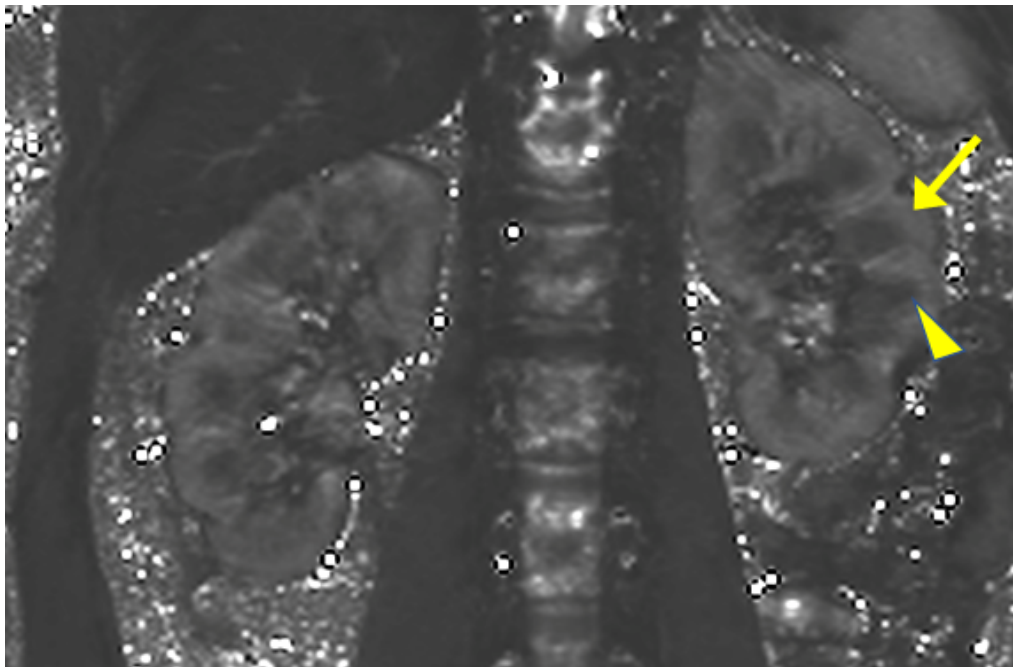
Given the role of hypoxia in DKD and the emergence of SGLT2-inhibitors with their potential reno-protective effects, measuring intra-renal oxygenation is of interest. Magnetic Resonance Imaging (MRI), which has been long established to investigate anatomical and structural changes in renal health, is a relatively new method to assess DKD. The fundamental principle of functional MRI in assessing renal hypoxia and structural function is based on the ability to quantitatively measure intrarenal oxygenation, as well as structural changes centered on water motion. In comparison to its utility in neuroimaging, functional MRI is in its infancy in the area of investigating renal diseases.

4.2 Principles of Blood Oxygen Dependent (BOLD) MRI: Measuring Intrarenal Oxygenation

Blood oxygen dependent (BOLD) MRI is a non-invasive functional MRI technique based on the magnetic properties of haemoglobin, with deoxygenated haemoglobin acting as an endogenous contrast agent. Deoxyhaemoglobin in the red blood cells causes perturbations in microscopic field gradients within red blood cells and their surrounding vessels, leading to signal attenuation on T2*-weighted MR images. The rate of magnetic spin dephasing, resonance transverse relaxation rate ($R2^*$), is a tissue relaxation constant derived from BOLD imaging ($1/T2^*$) and reflects oxygen consumption and metabolic activity within tissues. An increase in $R2^*$ reflects poor oxygen content in tissues, and therefore a decrease in intra-renal oxygenation [264, 265]. Figure 4.1 is an example of a T2* map where $R2^*$ values are derived.

Importantly, there are challenges with BOLD imaging, whereby patient preparation, MRI acquisition, analysis and limitations need to be considered to provide consistent and accurate results [266]. These potential challenges with BOLD imaging will be discussed in the section below “*Considerations in functional MRI (BOLD and DTI)*”.

Figure 4.1 Example of T2* map representing BOLD imaging



This figure demonstrates T2* map of which R2* values are derived and representing BOLD. This is an example of a 50-year-old healthy control participant in my thesis with an eGFR 83ml/min/1.73m². Cortex (arrow) and medulla (arrowhead) can be readily differentiated. Lower medullary signal indicates greater hypoxia (lower T2* and higher R2*). In this case, the R2* values were left cortex:14.8 (1/s) and left medulla: 30.7(1/s).

4.2.2 Blood Oxygen Dependent (BOLD) MRI: Measuring Intrarenal Oxygenation in Diabetes and Hyperfiltration

Studies attempting to assess the hypoxia model for CKD disease progression using BOLD MRI have demonstrated inconsistent results. Whilst there are studies that report lack of renal hypoxia in CKD [267, 268], studies that do observe renal hypoxia in CKD have established different degrees of hypoxia in different conditions and degrees of renal function impairment [269, 270]. One of the more recent studies which strongly supports the hypoxia theory in DKD followed up participants with CKD (N=112) for three years, and demonstrated that higher cortical R2* (reflecting lower oxygenation) correlated with more rapid annual eGFR decline and higher need for renal replacement therapy [271].

Animal models using BOLD-MRI have demonstrated that diabetes is associated with renal hypoxia as demonstrated by higher R2* readings [272-274]. These associations, however, were observed in early stages of renal impairment. Consistent with this was a study comparing participants with type 2 diabetes to healthy controls, in which more pronounced hypoxia in the renal medulla was seen compared to the cortex in the earlier stages of diabetic kidney disease (median eGFR $88 \pm 22 \text{ ml/min/1.73m}^2$) [275]. In fact, it was also demonstrated that whilst medullary oxygenation improved, cortical hypoxia deteriorated with more advanced stages of diabetic kidney disease (in both stage 4 and 5 DKD according to the Morgensen criteria, with a median eGFR of (56 ± 17) and $(18 \pm 10) \text{ ml/min/1.73m}^2$ respectively). Similar findings were echoed by Wang et al, who observed more pronounced hypoxia in earlier stages (mean eGFR $67 \text{ ml/min/1.73m}^2$) of diabetic kidney disease compared to later stages (mean eGFR $37 \text{ ml/min/1.73m}^2$) of renal impairment in type 2 diabetes [276].

There has been no prior publication of BOLD data in type 1 diabetes. The only known clinical study on BOLD MRI in type 1 diabetes was by Thewall et al. [277] who described equivocal findings, without publishing their R2* data. In this study, 17 participants with type 1 diabetes and 10 healthy controls underwent BOLD MRI assessment along with renal volume, renal vascular resistance and renal artery flow. The authors reported that a difference in cortical and medullary R2* were noted, with no observed changes in R2* with diuresis in five healthy controls. It appears that as

there was no difference in volunteers in BOLD MRI results pre- and post-water loading, where an improved medullary oxygenation would be expected, further analyses in type 1 diabetes participants were not conducted.

Water loading, as performed in the type 1 study above, along with frusemide diuresis has been shown to improve medullary oxygenation as measured with BOLD MRI. These are provocative manoeuvres which could provide further information on the mechanisms resulting in change in oxygenation. Because although it provides an estimate of renal oxygenation level, BOLD imaging does not differentiate and quantify the changes in oxygen delivery or demand. The effect of frusemide inducing an improvement in medullary oxygenation may possibly reflect acute reduction in medullary oxygen consumption as a result of blocking active sodium transport in the loop of Henle [271]. Similarly, a water-load effect (which has opposite clinical effect of frusemide diuretics) has also been documented to improve medullary oxygenation [278]. The exact mechanism of how alteration in oxygenation occur by these measures are not known. Perhaps, water loading expands plasma volume which increases renal blood flow that can result in improved oxygenation. Indirectly, this may imply that hypoxia as reflected by BOLD MRI can be a result of renal ischaemia and perfusion injury. Importantly, improved medullary oxygenation induced with water diuresis in healthy participants, is blunted in type 2 diabetes as observed with BOLD MRI pre and post water diuresis [279, 280]. Of note, baseline BOLD MRI readings in these studies did not establish any differences in renal oxygenation between healthy controls and T2DM.

Further investigating water diuresis and medullary oxygenation using BOLD MRI, was a study by Prasad et al using cyclooxygenase inhibition in 18 healthy controls [278]. It was demonstrated that water diuresis not only improved medullary oxygenation, but also increased prostaglandin E2, a known vasodilator. With cyclooxygenase inhibition, the effect on prostaglandin E2 production as well as medullary oxygenation reduced. This observation suggests that an increase in prostaglandin E2 production from water diuresis may contribute to medullary vasodilation, increase blood flow and subsequently attenuate medullary hypoxia. Another physiologic challenge is the inhibition of the angiotensin-aldosterone system. Interestingly, in a study on 12 participants with type 2 diabetes with a mean eGFR of

62±22ml/min/1.73m² [281], it was demonstrated that inhibiting the angiotensin-aldosterone system, which can preserve renal decline in DKD, does not improve renal oxygenation in type 2 diabetes. In addition, higher blood pressure and increased urinary sodium excretion was found to result in lower medullary oxygenation. These findings emphasize the importance of tubular sodium handling, which can be affected by hyperfiltration, in medullary oxygenation.

In terms of hyperfiltration, there is no publication that specifically assess BOLD MRI in those with diabetes and renal hyperfiltration. Whether the phenomenon of hypoxia detected in the early stages of DKD in the aforementioned type 2 diabetes studies above [275, 276] are potentially related to glomerular hyperfiltration that occurs early on in diabetes is not known. Studies are necessary to investigate this further, because if hyperfiltration is associated with hypoxia, this suggests that hyperfiltration may have a pathogenic role in the development of diabetic kidney disease. We hypothesize that one of the potential mechanisms of hyperfiltration aggravating hypoxia is by increasing urinary erythropoietin loss. This is because although degradation and removal of erythropoietin occurs mainly through endocytosis of their corresponding receptor, it can also be cleared through glomerular filtration when erythropoietin receptors are saturated [282, 283]. Moreover, diabetes has been linked to functional erythropoietin deficiency[284], despite anaemia not being apparent until renal function decline occurs.

In addition, if there are hypoxic changes within the medulla in individuals with hyperfiltration compared to those without hyperfiltration, this could potentially support the tubular theory of hyperfiltration, as well as the hypoxic tubular hypothesis of diabetic kidney disease, as the majority of the tubules are predominantly within the medulla. We postulate that maladaptation in medullary vasodilation contributes to the development of DKD, starting from the early stage of hyperfiltration. However, it is important to note that the medulla is in a constant state of relative hypoxia as previously discussed, with the cortex potentially not affected as it is better oxygenated and does not exhibit hypoxic features until it reaches a point of significant physiological stress. It has also been postulated that a small change in oxygenation in the cortex may not affect haemoglobin saturation to the same degree as the medulla due to its oxygen tension and dissociation, and may not be detected by BOLD MRI[276].

4.3 Principles of Diffusion Tensor Imaging (DTI) MRI: Measuring Intrarenal Water Diffusion

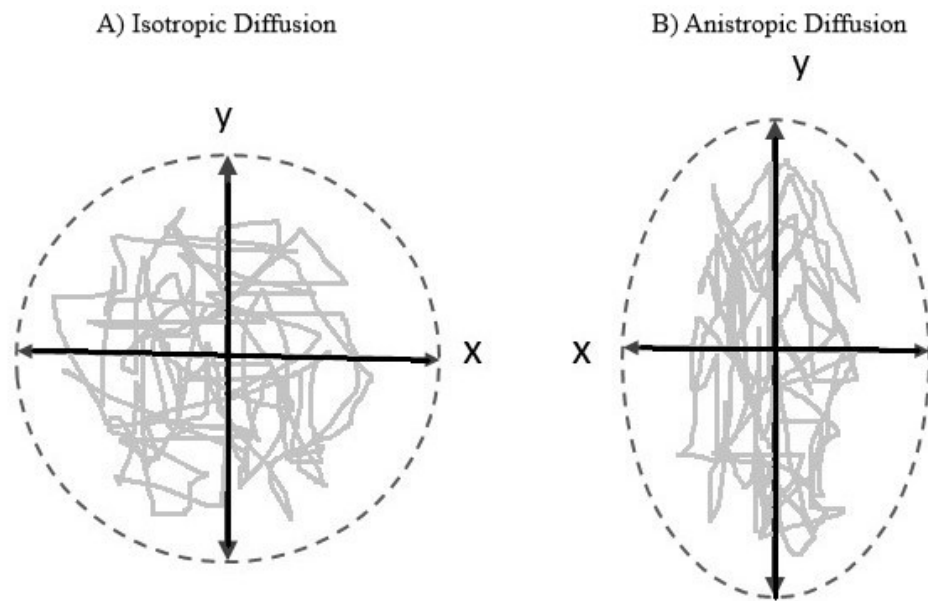
Evaluation of diffusion tensor imaging (DTI) imaging can be concurrently undertaken when performing a BOLD MRI study during the same examination. Rather than assessing hypoxia, it is based on the principle of utilizing diffusion of water to characterize the structural changes within tissues and may potentially act as a marker for water diffusion. The diffusion characteristics of water molecules within the kidney can be dependent on numerous factors, including microcirculation, blood flow perfusion, glomerular filtration, tubular resorption and secretion [285].

DTI provides an assessment of microstructural characteristics of biological tissue via two different measurements expressed as:

- (i) apparent diffusion coefficient (ADC), indicating the magnitude of water motion, reflecting capillary perfusion and diffusion and
- (ii) fractional anisotropy (FA), representing the directionality of water motion, a reflection of the structural arrangement of the kidney parenchyma.

For healthy participants, imaged at 3.0 Tesla magnetic field strength, it has been reported that the average FA values of the renal cortex are 0.15 ± 0.03 and 0.49 ± 0.04 for the renal medulla. Values for FA ranges from 0-1, with 0 being isotropic without directionality, whilst 1 represents completely anisotropic diffusion in one direction (Figure 4.2) [286]. The more structured the organ or tissue is, the higher the FA will be. The renal medulla, which is highly structured, with more homogeneity compared to the cortex will have a higher FA, as water movement is more ordered and flows in a single direction [287]. FA will therefore potentially aid in identifying microstructural renal lesions.

Figure 4.2 Explanatory diagram on isotropic anistropic diffusion



Adapted from Rosenbloom et al 2004

Water molecules in renal tissues are constantly moving.

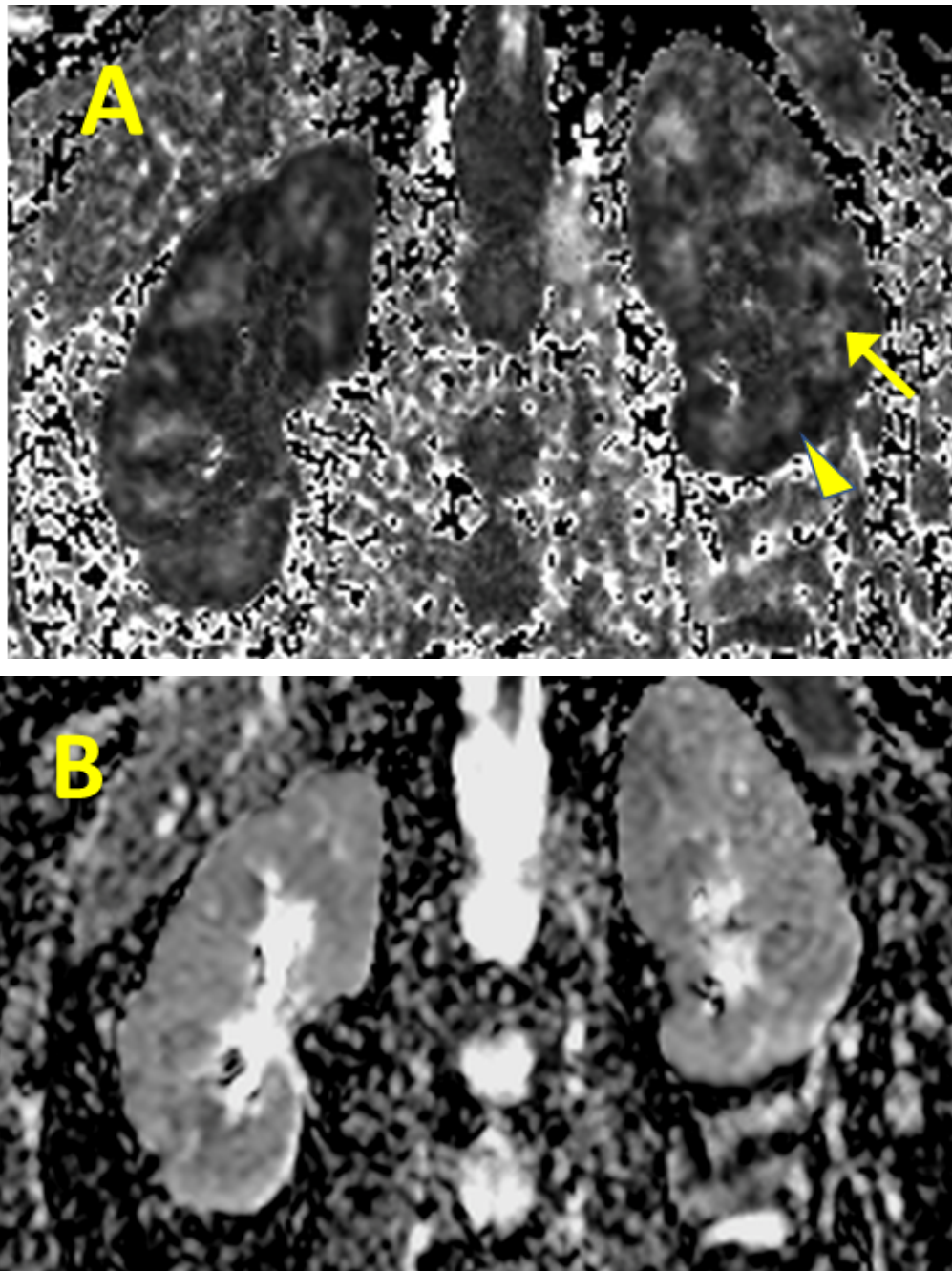
- A) When motion is unconstrained, diffusion is isotropic- with motion occurring equally and randomly in all directions. (As reflected by a lower FA value)
 - B) When motion is constrained, diffusion is anisotropic- with motion more oriented more in one direction than another, for eg. along the y-axis, or x-axis alone. (As reflected by a higher FA value)
-

Depending on the system, protocol and parameters selected, ADC values can vary. Similar to BOLD imaging, the reproducibility of DTI is variable, and challenges for comparison between studies are difficult due to the diverse participant characteristics. Hydration status is also a potential confounder, with conflicting results demonstrating the influence of water loading and diuresis[288]. For reference, reported ADC values in a study in healthy participants are $2.46 \times 10^{-3} \pm 0.09 \text{ mm}^2/\text{s}$ and $2.08 \times 10^{-3} \pm 0.08 \text{ mm}^2/\text{s}$ for renal cortex and medulla respectively [289]. A high ADC suggests a large amount of water movement, whilst a low ADC suggests restricted water motion. For example, in renal fibrosis and altered renal structure, a low ADC and low FA may be anticipated. Histopathology and DTI parameters have been studied in 75 patients with non-diabetes specific chronic kidney disease with glomerulosclerosis, and it was

established that both cortical and medullary ADC and FA correlated with eGFR, with measurements lower in more advanced glomerulosclerosis and tubulointerstitial fibrosis [290]. On the other hand, despite similar findings of a lower ADC with more severe glomerulosclerosis established on histology, Li et al did not find that ADC values correlated with mGFR[291]. However, the association of DTI parameters and the underlying pathophysiology in renal disease remains to be determined with histopathology and pre-clinical studies, with potential causes ranging from structural glomerular or tubular damage, vascular disease, to altered perfusion. Figure 4.3 demonstrates example of FA and ADC maps in a healthy control.

It should be noted that intravoxel incoherent motion (IVIM) is another alternative advanced novel technique based on diffusion weighted imaging (DWI) allowing assessment of tissue characteristics. This imaging subtype is beyond the scope of this review and was not performed in our study. Notably, vascular perfusion and tubular flow, which is not directly evaluated with ADC and FA can be assessed with the IVIM technique [292].

Figure 4.3 Example of FA and ADC maps



This figure demonstrates A) FA map B) ADC map on a 50-year-old healthy control with eGFR 83ml/min/1.73m². Cortex (arrow) and medulla (arrowhead) can be readily differentiated with both techniques.

On A) higher medullary signal compared to cortex reflects higher fractional anisotropy (Left cortex: 0.11 and Left medulla 0.45)

On B), it is more difficult to differentiate between cortex and medulla, with relatively similar ADC values (Left cortex: 2.69 and Left medulla: 2.62(10³ mm²/s))

4.3.2 Diffusion Tensor Imaging (DTI) MRI: Measuring Intrarenal Water Diffusion in Diabetes and Hyperfiltration

Similar to BOLD MRI, there are limited studies of DTI in DKD. A recent study has found lower FA but higher ADC in the renal cortex in patients with type 2 diabetes and variable renal function compared to healthy controls of comparable age, sex and BMI [293] suggesting that there may be potential structural changes that may result in restricted water motion in diabetes. Both FA and ADC of the renal cortex also correlated with albuminuria, N-acetyl-beta-D-glucosaminidase (NAG), and Transforming-growth-factor (TGF- β 1). With no histopathology, it is difficult to determine what these correlations truly represent. Nevertheless, albuminuria and NAG can indicate renal tubular damage; and TGF- β 1 has been linked in renal parenchyma disease. Hence, DTI associations with these biomarkers may in turn characterize architectural changes within the renal tubules and parenchyma. As the primary aim of the study was to assess renal cortex changes, medullary parameters were not measured. In addition, although the diagnosis of diabetes was according to the American Diabetes Association, it was unclear and not specified if the cohort included patients with type 1 or type 2 diabetes. This is in line with findings of an earlier pilot study, which reported lower cortical FA in patients with more advanced DKD (eGFR <60ml/min/1.73m²), but not in DKD with an eGFR >60ml/min/1.73m² when compared to healthy controls. Medullary ADC and FA was also lower in the diabetes group with a lower eGFR compared to healthy controls [287].

Similarly, in a larger type 2 diabetes study assessing only ADC value in different stages of DKD (Stage 1- 5, with 10-18 participants in each group) using urinary creatinine clearance of GFR [294], it was found that more advanced DKD stage is accompanied by lower mean renal ADC, with significant differences noted in the group with an eGFR < 60mls/min/1.73m². Notably, in participants with stage 1 DKD, which was considered hyperfiltration by the authors and defined as an eGFR >100ml/min/1.73m², mean ADC was not significantly different compared to healthy controls. Besides this difference in ADC in those with varying stages of DKD, ADC has also been demonstrated to correlate with eGFR ($r^2=0.56$, $p<0.01$) in another study on 43 participants with type 2 diabetes (mean eGFR 45.8 ± 30.1 ml/min/1.73m², urinary

protein 980 ± 2229 mg/g of urinary Creatinine, HbA1c 7.0 ± 1.2 %). Of note, BOLD MRI parameters did not correlate with eGFR in this study [295].

In a study assessing DTI in early DKD in 30 patients with type 2 diabetes and normal renal function (as determined by serum creatinine and cystatin C) but albuminuria [285], a downward trend for ADC (which is consistent with changes noted with progressive renal disease) was detected with worsening albuminuria in type 2 diabetes. Instead of the anticipated lower FA in renal disease, it was observed that medullary FA was significantly elevated in the microalbuminuria group compared to healthy controls. The authors suggested this was consistent with findings in type 2 diabetes by et al. [287], with early stages of DKD (< stage 3 DKD, eGFR 87 ± 16 ml/min/1.73m²) that may indicate a compensatory effect with intact tubular microstructures early on. Only with advancing DKD with more dynamic renal tubule changes, FA will progressively decline.

4.4 Considerations in functional MRI (BOLD and DTI)

As BOLD MRI and DTI are relatively new approaches in assessing renal function, this technology remains a research tool and further evaluation needs to be conducted for it to be integrated into clinical practice. However, being non-invasive and not requiring any exogenous contrast agent, BOLD and DTI-MRI are very promising for detecting and monitoring kidney disease in diabetes. The ability to obtain microstructural information through DTI without the invasive means of histology can be significant in contributing to our understanding of the mechanism underlying DKD. Furthermore, investigating the hypoxia hypothesis underlying DKD with BOLD MRI is very valuable particularly with potential therapeutics that have the ability to provide renal protection via renal oxygen handling. The other aspect is that functional MRI is a relatively efficient method that could be potentially applied to assessing DKD, with both BOLD imaging and DTI producing reliable repeated measurements with a coefficient of variation <10% in both healthy subjects and patients with chronic kidney disease [296-298]. Of note, the total scan time to acquire BOLD images (with breath hold) as well as DTI images (with bellows/ respiratory navigator ~10 min or less) is under 15 minutes.

It is difficult to explain the discordant results of the studies in DKD. In part, there are no standardization of studies[266], with challenges of widespread, reproducible clinical studies stemming from the many potential confounding factors that are summarized in Table 4.1 below.

Table 4.1 Factors affecting MRI BOLD and DTI

Patient-related	Scanner-related	Analysis-related
• Drugs • Anaemia • Hydration • Sodium intake • Bowel gas • Respiratory motion • Bulk motion • Renal adiposity on DWI • Implanted metal, e.g. surgical clips, stents	• Poor magnetic field homogeneity • Distortion artefacts • Unsuccessful respiratory motion compensation • Insufficient spatial resolution to accurately resolve cortex and medulla • Poor signal to noise ratio	• Reader experience • Method of analysis (e.g. manual placement of regions of interest, twelve-layer concentric objects and fractional hypoxia technique) • Difficulties separating cortex from medulla • Inconsistent post processing

Importantly, to date, functional MRI studies in DKD include wide and heterogenous groups of participants in limited numbers. A major patient related factor that accounts for disparities is medications. Both angiotensin-renin-aldosterone system inhibitors, and frusemide that are commonly prescribed for patients with diabetes can affect BOLD MRI results by increasing renal oxygenation [264]. Whether this has long term beneficial reno-protective effect still remains to be investigated. Of note, sodium/glucose co-transporters that have been shown to decrease oxygen consumption in diabetic rats via arterial and venous blood samples [299] have not been studied in clinical BOLD imaging or DTI. There is a study protocol without published results to date, which aims to explore the effects of empagliflozin on renal tissue oxygenation using BOLD-MRI[300].

Similarly, sodium intake and hydration status that directly affect renal solute handling are also known factors that affects MRI signal [278, 301] . Anaemia that could increase

deoxy-haemoglobin levels clearly impacts BOLD signals. In addition, even without the presence of anaemia, with the normal range of haemoglobin so diverse (e.g. 130-180 g/L in a healthy male), the degree of how much 1g/L of haemoglobin affects BOLD $R2^*$ measurements are not known. Moreover, there are functional or anatomical artifacts that are unpredictable, difficult to control, and can only be detected after imaging has taken place. For example, bowel gas can affect magnetic field homogeneity, while respiratory or bulk motion impacts image quality. Excessive renal fat has also been shown to underestimate ADC [302] .

Technical interference is another aspect that contributes to results disparity by hindering the detection of renal pathology. This includes insufficient spatial resolution to accurately resolve cortex and medulla, poor magnetic homogeneity of the scanner leading to image distortion, insufficient signal to noise ratio that may for example occur with longer TE imaging in BOLD MRI, and lack of/ or unsuccessful respiratory motion compensation, for example inaccuracies of a respiratory navigator used for DTI imaging, as DTI acquisitions are longer than a breath hold. Furthermore, MRI technology has evolved with different analytical methods utilized. Reports in the literature on MRI BOLD and DTI incorporate two different MRI field strengths: 1.5 and 3.0 Tesla. Earlier studies also used diffusion weighted imaging (DWI), which is a more basic technique that only assesses ADC and not FA.

Finally, analysis related factors from different studies make it difficult to interpret results. This includes the various methods of analyzing images, where some studies draw the classical regions of interest (as we have), others divide the renal parenchyma into twelve layers of equal thickness and some utilize the fractional hypoxia technique, which counts the percentage of voxels with an $R2^*$ value $> 30 \text{ seconds}^{-1}$ [303] Reader experience also play a part as it is challenging to separate cortex from medulla for BOLD MRI and DTI measurements, especially with advancing CKD. Inconsistent post processing is also factor. For example, the latest guidelines for DWI/DTI suggest that images should undergo motion correction and removal of poor quality images from raw data prior to making the FA and ADC maps[304], however, it does not appear that most published studies to date have done so.

Despite its limitations, functional MRI clearly has many advantages and can definitely provide valuable insights in DKD. It is therefore pertinent for future studies to have a unified standard method to further evaluate functional MRI in DKD to overcome the potential confounding factors and translate its promising research utility to be clinically applicable.

4.5 Summary of BOLD and DTI MRI

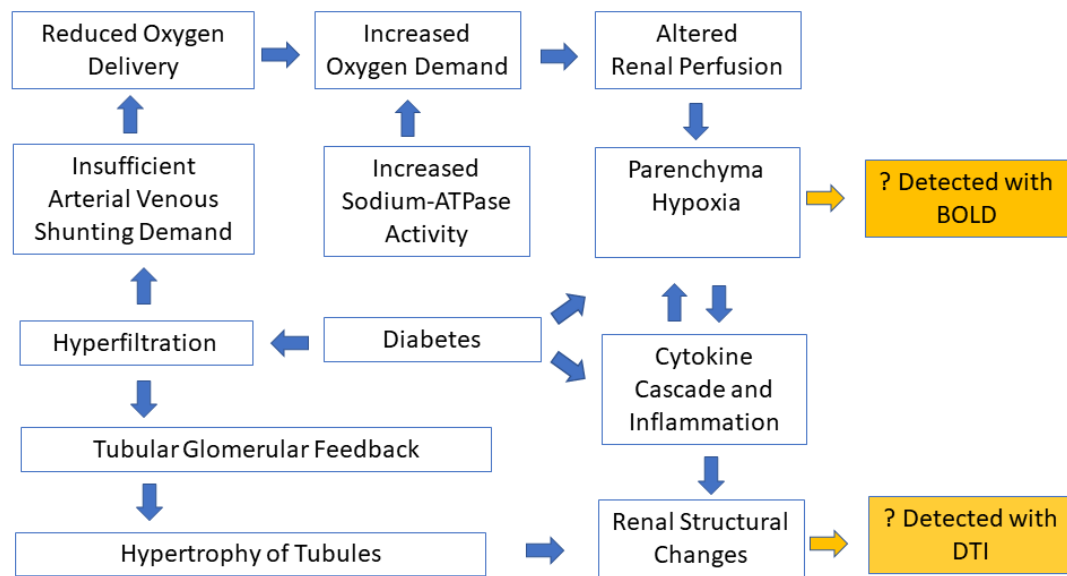
Overall, although undoubtedly more studies are warranted, functional renal BOLD and DTI MRI is a promising clinically viable non-invasive approach in the detection and monitoring of DKD. A summary of available studies in diabetes is presented in Table 4.2.

Despite the discrepant findings and difficulty in drawing a firm conclusion, it appears that BOLD imaging detects hypoxia at earlier stages of DKD than DTI. Based on the collective of small studies thus far, decreased FA and ADC levels in diabetes compared to healthy controls are most pronounced below an eGFR threshold of $<60\text{mls/min/1.73m}^2$, suggesting structural changes that can be detected by DTI may occur at later stages of DKD. However, there are also evidence of early microstructural changes in type 2 diabetes.

Scrutinizing the individual utility of functional MRI, it is not well defined if BOLD-detected hypoxic changes are related to increased oxygen demand, including hyperfiltration; or due to reduced oxygen delivery due to ischaemia. It is also not clear which particular renal structural changes DTI parameters detect without concurrent histology. Nevertheless, taken together, BOLD MRI and DTI parameters may be able to provide insights into renal changes in diabetes by evaluating oxygenation, and structural changes of the kidneys without the requirement for tissue sampling.

The potential utility of functional MRI in the proposed hyperfiltration-hypoxic model of DKD can be considered in the following diagram in Figure 4.4.

Figure 4.4 Proposed detection of oxygenation status and microstructural changes in diabetic kidney disease with MRI BOLD and DTI



In summary, the two approaches of MRI BOLD and DTI allow for functional and structural correlates to be derived from MRI to better understand the pathogenesis of kidney disease in diabetes. In combination, functional MRI and current established biochemical methods may allow for more accurate identification of patients at-risk for developing DKD. By recognizing early stages of DKD, prevention with current medical management including intensive blood pressure and glycaemic control could be administered before irreversible renal injury occurs.

Table 4.2 Current clinical studies using MRI BOLD and DTI comparing participants with type 2 diabetes and healthy controls

Author/Year	N	Correlation with eGFR	Renal Status	Cortical BOLD R2*		Medullary BOLD R2*	
Yin ^[275] 2012	46	Cortical R2* : Negative Medullary R2* : Positive	-	↑		↑	
Wang ^[276] 2011	20	Cortical R2* : NS Medullary R2* : Negative	<i>eGFR (ml/min/1.73m²)</i> ≥ 60 <60	NS NS		↓ ↓	
Economides ^[279] 2004	38	-	-	NS		NS	
Epstein ^[280] 2002	9	-	-	NS		NS	
Pruijm ^[281] 2013	12	-	-	NS		NS	
Feng ^[305] 2020	30	Cortical R2* : NS Medullary R2* : NS	<i>Albuminuria Status</i> Normoalbuminuria Microalbuminuria	↑ NS		NS NS	
				Cortical DTI ADC FA		Medullary DTI ADC FA	
Feng ^[305] 2020	30	Cortical R2* : NS Medullary R2* : NS	<i>Albuminuria Status</i> Normoalbuminuria Microalbuminuria	NS NS	NS NS	NS NS	NS ↓
Razek ^[293] 2017	42	-	In Diabetes, higher cortical FA and lower ADC was detected in macroalbuminuria compared to microalbuminuria	↓	↑	-	-

Lu ^[287] 2011	15	-	<i>eGFR (ml/min/1.73m²)</i> ≥ 60 <60	NS ↓	NS NS	- -	↓ ↓
Combined Cortical and Medullary DTI							
				ADC	FA		
Cakmak ^[294] 2014	78	-	<i>eGFR (ml/min/1.73m²)</i> > 90 ≥ 15 < 15	NS ↓ ↓	- - -		
Chen ^[285] 2014	30	-	<i>Albuminuria status</i> Macroalbuminuria Microalbuminuria	↓ ↓	NS ↑		

N= Number of participants with Type 2 Diabetes. NS: Nonsignificant results. “-“ Data analysis not performed.

Review of the Literature

Chapter Five

Pregnancies Affected by Type 1 and Type 2 Diabetes

Based on the National Health Survey data, the prevalence of type 1 diabetes and type 2 diabetes accounts for approximately 0.4% and 1.0% of women of child-bearing age respectively; with 0.6% of pregnancies affected by diabetes each year in Australia[306]. The trend of increasing maternal age, along with the rising prevalence of type 2 diabetes also underscores the importance of a shifting focus on pregnancies affected by type 2 diabetes. Adverse maternal fetal outcomes in type 2 diabetes have been demonstrated to be no less than type 1 diabetes[307]. Despite the increasing recognition of the high-risk state of pregnancies affected by diabetes, there is a significant gap in pregnancy outcomes between women with, and without pre-existing diabetes. Adverse maternal and neonatal outcomes including perinatal mortality, congenital malformation and large for gestational age have been reported up to nine times higher than that of pregnancies not affected by diabetes [308, 309]. The risk of adverse pregnancy outcomes also further increases in diabetes kidney disease[109, 310].

5.1 Evaluating renal function in Pregnancy

Identifying changes and deterioration in maternal renal function is necessary during pregnancy, as this can have consequences on both maternal and fetal outcomes. Therefore, it is important to appreciate the current approaches on determining renal function. The unsurpassed method of quantifying renal function in pregnancy remains inulin clearance. Although it crosses the placenta, inulin is physiologically inert with no reports of increasing teratogenicity risk and has been safely used in pregnancy [311-313]. Due to its' radioactivity and iodine content, measuring clearance of radioisotopes and radio-contrast agents (iohexol, iothalamate, technetium-99m-DTPA) are not suitable in pregnancy. These techniques are also time consuming, and expensive and at this point in time, rarely used in clinical settings. There is undeniably a need for a similarly accurate, but more accessible method of evaluating renal function. However, till date, there has been varying degree of success in estimating renal function in pregnancy, most of which do not reflect true measured GFR.

Serum creatinine can be affected by dietary protein, muscle mass, medications, gender and racial diversity. Another consideration is that serum creatinine is prone to assay interference. Out of the two most common analytical methods, the Jaffe method has

greater precision than the enzymatic method, but is subject to bias from interfering substance, and risk of misclassification for CKD-based eGFR at the limit of 60ml/min/1.73² [314]. Despite its limitation, and the need for varying reference range throughout pregnancy (between 80-90mmol/l) [315], serum creatinine is still considered one of the most reliable and easily accessible surrogate markers of renal function in pregnancy. In pregnancy, the steady and unaltered creatinine production, hyperfiltration and plasma expansion results in an expected decline in serum creatinine, which values >0.8mg/dl (70.7mmol/L) is generally considered out of normal range in healthy pregnancies [316].

Creatinine based equations for GFR is considered more accurate and precise than measurement of serum creatinine alone in the general population. However, these formulae tend to underestimate true measured GFR in pregnancy, particularly at the higher end of GFR. With the hyperfiltration state in pregnancy, both CKD-EPI and MDRD formula has been shown to be dis-concordant with measured GFR to a similar degree, with a bias of around 40ml/min/1.73m² [317-319]. Serum cystatin-C, a peptide compound which may be of some use in the non-pregnancy cohort, has been studied and does not correlate with inulin clearance in pregnancy, and is therefore not suitable for renal function assessment in pregnancy as well[118].

Creatinine clearance can over-estimate GFR. As creatinine is freely filtered, but not resorbed with around 10% secreted by the proximal tubule, the amount of creatinine excreted is therefore the composite of both filtered and secreted creatinine[320, 321]. Therefore, urinary creatinine-based renal assessment can over-estimated GFR. In pregnancy, urethral dilatation resulting in urinary retention with inclusion of urine which entered the ureters prior to collection period can also potentially contribute to overestimation in GFR. On the other hand, pregnancy limiting mobility, as well as issues of nocturia and polyuria may result in incomplete collection, which can have an opposite effect on GFR. It has been demonstrated under controlled clinical trial environment, that 24- hour urinary creatinine clearance can underestimate GFR measured directly with inulin in twelve healthy, normotensive pregnant women [117]. Notably, these tests were performed in a research setting with fixed salt setting, in which may be difficult to replicate in a routine clinical setting. Nevertheless, despite

the biased estimate of GFR, endogenous creatinine clearance over a 24-hour period remains the most precise published method of predicting GFR in pregnancy.

It is evident that there are clear limitations in renal function assessment in pregnancy, as well as its assessment in diabetes. Currently, there are no published validation studies on surrogate measurements for GFR in pregnancies affected by diabetes. Accordingly, results from studies with no access to measured GFR should always be interpreted with caution, considering these surrogate measurements may not represent true renal function. Notably, studies on DKD in pregnancies invariably use either serum creatinine or albuminuria to define the presence of kidney disease due to the lack of a reliable surrogate measure for GFR. The definition of DKD in these studies varies, with some using timed albumin excretion rate, while others spot albumin creatinine ratio, and even proteinuria. These measurements yield different classification of albuminuria state and decreases in accuracy and sensitivity in order. Albuminuria, as a risk marker also poses limitations including the difficulty in excluding urinary tract infections, which are common in pregnancies.

5.2 Maternal-Fetal Outcomes in Diabetes and Diabetic Kidney Disease

In pre-gestational type 1 and type 2 diabetes, one of the major concerns of pregnancy is the effect it has on accelerating diabetes related complications. The impact of pregnancy on maternal renal function has largely been reviewed in “Chapter 2: Review of the literature- Renal hyperfiltration in diabetes and in pregnancy”. Effectively, the evidence at present is strongly focused on type 1 diabetes and is suggestive that overall pregnancy does not appear to have a deleterious effect on the progression of pre-existing mild DKD; while the evidence on moderate-severe cases is less conclusive, with some studies suggesting a future decline in renal function over time. There is also a gap in the literature regarding pregnancy effects on renal function in women with pre-existing type 2 diabetes. There are no current studies that we know of that compare pregnancy related renal trajectory in women with type 1 and type 2 diabetes to healthy controls.

Apart from diabetic kidney disease, another concern is potential acceleration of diabetic retinopathy during pregnancy. In an ancillary study of the Diabetes Control and

Complications (DCCT) study on pregnant women with type 1 diabetes, the progression of retinopathy developed in 17% and 5.7% in the convention and intensive group respectively [322]. The findings of development and worsening of retinopathy particularly in the second trimester that regresses after delivery is consistent with findings in other studies [323-325]. Higher blood pressure, the presence of pre-existing retinopathy and prolonged diabetes duration has been identified as risk factors in pregnancy related progression of retinopathy[326]. Improvement in glycaemia management has a paradoxical relationship with diabetic retinopathy in pregnancy. While poor glycaemia management control is a risk factor of developing retinopathy, it has also been recognized that rapid improvement of glycaemia management which often occurs in pregnancy aimed to reduce pregnancy complications can worsen pre-existing retinopathy[327]. Concerningly, rapid optimization of glycaemia management in pregnancy also generates the additional complication of hypoglycaemia, in which severe cases can result in maternal neurocognitive debility.

Other uncommon, but significant conditions in diabetes that can complicate pregnancies include the presence of autonomic neuropathy, gastroparesis, coronary artery and cerebrovascular disease. Notably as well, due to the rising prevalence of obesity, and trend in advancing maternal age, macro-vascular complications which has generally not been a concern in the younger childbearing population needs to be increasingly considered in clinical practice given the co-existence of atherosclerosis in type 2 diabetes and the increasing prevalence of type 2 diabetes.

5.3 Obstetric outcomes in Diabetes and Diabetic Kidney Disease

Pregnancies affected by diabetes are accompanied by increased adverse obstetrics outcomes, including large for gestational age, pre-term birth and perinatal death as emphasized by a recent Australian study comparing pregnancy outcomes in pre-gestational diabetes to a large cohort of women without diabetes over 2010-2013[328, 329]. Certain risks, particularly pre-eclampsia are further amplified with the presence of DKD, mostly defined with the presence albuminuria by 8-30 fold increase in the risk of preeclampsia [330]. Serum creatinine >176mmol/l, severe hypertension and nephrotic range proteinuria (>3g/24hour), has been observed as predictors of adverse maternal and fetal outcomes[331]. This highlights the potential adverse effects of renal

impairment on diabetic pregnancies. The current studies on pregnancy outcomes in DKD are mainly on type 1 diabetes, however, there is an increasing focus on type 2 diabetes in recent years. The comparison control groups in these studies are at best women with diabetes and normoalbuminuria, but not healthy women without diabetes. Due to the heterogeneity of studies, it is not possible to directly compare results on pregnancy outcomes. Nevertheless, Table 5.1 summarizes the frequency of common obstetrics outcomes from selected studies from the literature to provide some perspective of the risk in pregnancies affected by DKD.

Table 5.1 Frequency (in percentage) of selected pregnancy outcomes in type 1 and type 2 diabetes

	<i>N</i>	<i>Pre-eclampsia</i>	<i>Pre-term birth <34 weeks</i>	<i>Pre-term birth <37 weeks</i>	<i>NICU</i>	<i>SGA</i>	<i>LGA</i>	<i>Perinatal Death</i>
No Diabetes ^{#[328]}	27,075	2	NA	8	3	15	8	2
Type 1 Diabetes ^{# [328]}	138	5	NA	39	11	7	44	7
Overall Cohort								
Type 2 Diabetes ^{#[329]}	107	9	NA	23	7	16	20	2
Overall Cohort								
Type 1 Diabetes ^[116]	43	42	20.9	77	41	23	28	4
Proteinuria								
Type 1 Diabetes ^{* [332]}	15	20	7	47	33	7	53	0
Microalbuminuria								
Type 1 Diabetes ^{* [332]}	11	36	27	82	64	36	0	0
Macroalbuminuria								
Type 2 Diabetes ^{* [332]}	10	10	10	30	44	20	30	0
Microalbuminuria								
Type 2 Diabetes ^{* [332]}	5	40	40	60	60	40	18	0
Macroalbuminuria								
Type 1 Diabetes ^{+ [310]}	203	6	6	35	-	-	-	-
Normoalbuminuria								
Type 1 Diabetes ^{+ [310]}	26	42	23	62	-	-	-	-
Microalbuminuria								
Type 1 Diabetes ^{+ [310]}	11	64	45	91	-	-	-	-
Macroalbuminuria								

Data expressed as % for frequency of outcomes (rounded to closest decimal point)

Diabetic Kidney Disease=DKD; NICU= Neonatal Intensive Care Unit; SGA=Small for gestational age; LGA=Large for gestational age; “-“ Not reported

Study cohort included comparison between: # Non-diabetic population with T1DM and separately with T2DM ; * T1DM and T2DM individuals with micro- and macroalbuminuria; + T1DM individuals with normo-, micro-, and macroalbuminuria

With the definition of DKD varying across studies, the approximate prevalence of DKD in pregnancies ranges between 5-25% in type 1 diabetes [333]. The literature on DKD in type 2 diabetes is sparse, but has been reported to affect around 2.0% of pregnancies [332]. Observing the development and trends of pregnancies in DKD over time, a Finnish study evaluated obstetric outcomes in 145 type 1 diabetes pregnancies affected by nephropathy (defined as protein excretion $>0.3\text{g}/24\text{hours}$ or positive protein on urinary midstream dipstick) over two separate decades (1988-1999 and 2000-2011)[116]. Not surprising, a higher maternal age, longer duration of diabetes, and higher anti-hypertensive use prior to pregnancy were reported in the latter group. But pre-conception and pregnancy HbA1c levels and pregnancy blood pressure were no different between both groups. Across all outcomes observed, apart from neonatal intensive care admission that was higher in 2000-2011, at 48.8% compared to 26.2% ($p=0.02$), there was no significant difference in outcome across both decades. This trend may reflect modern clinical practice of either improvement in recognizing the clinical need, or the increase capacity to provide intensive care.

One of the large earlier studies evaluated women with type 1 diabetes between 1993-1999 without antihypertensive treatment. Among the 846 women, 10% were affected by microalbuminuria, and this is associated with an adjusted four-fold increase in pre-eclampsia. Pre-eclampsia was also related to the three-fold risk of pre-term delivery <34 weeks in those with microalbuminuria[334]. Ekbom et al compared 206 type 1 diabetes pregnancies between 1996-2000, with baseline normoalbuminuria to those with albuminuria[310]. The presence of microalbuminuria significantly increases the frequency of small for gestational age and pre-eclampsia, which resulted in a higher rate of pre-term delivery. The risk of pre-eclampsia is further augmented with the presence of macroalbuminuria. Similar outcomes of pre-eclampsia, pre-term delivery and small for gestational age was reported by a study from the center by Damm et al. on women with type 1 diabetes and type 2 diabetes affected by microalbuminuria (urinary albumin-creatinine ratio (ACR) $30\text{-}299\text{mg/g}$) and nephropathy defined as $\text{ACR} \geq 300\text{mg/g}$ between 2007-2012 [332]. The frequency of these outcomes in the type 1 diabetes cohort for this study, however, was half of that compared to Ekbom et al. Notably HbA1c at the initial visit was lower in the study by Damm et al., which may have impacted on the lower rates of adverse events.

These findings of the associations between the presence of DKD and higher incidence of pre-eclampsia and pre-term delivery are in line with previous earlier studies[102, 335]. However, in clinical practice, it may be difficult to discriminate between pre-eclampsia, worsening diabetic kidney disease and hypertension. In addition to preeclampsia, babies of mothers affected by DKD have a higher incidence of small for gestational age and intra-uterine growth restriction despite the background risk of macrosomia in diabetes[102, 109, 110, 336]. Finally with perinatal death, as one of most severe pregnancy consequences, although it's frequency is higher in diabetes compared to the general population, DKD do not appear to increase the risk further[333].

5.4 Risk factors affecting Pregnancy Outcomes in Diabetes

It is crucial to understand the factors in pregnancy that predisposes women with diabetes and their babies to adverse outcomes. Observing studies over time, there is a tendency of increasing maternal age in parallel to the aging population. As age itself predispose to higher pregnancy risks[337], this may explain the persistent rates of pregnancy complications in DKD despite advancing medical management. In addition, pregnancy complications in this cohort of women may also be due to the trend of increasing body mass index in the general population, and the rising prevalence of type 2 diabetes that is associated with obesity. A United Kingdom population based cohort study on 24241 women found a steady increase in body mass index over the period of 1976 to 2005, and that higher body mass index is an independent risk of pre-eclampsia, gestational hypertension, macrosomia and induction of labour[338].

Two other very important modifiable risks are glycaemic control and blood pressure. Preconception and early glycaemic control is vital, with the current Australasian Diabetes In Pregnancy Society guidelines recommending a pre-conception HbA1c of at least <7%, and ideally within the normal reference range[339]. The risk of fetal malformation in type 1 diabetes is 4.2%, compared to 1.2% in the general population [340]. A population based United Kingdom study also reported the risk of fetal malformation in diabetes (no difference between type 1 and type 2 diabetes), is three times to that of women without diabetes. The presence of diabetic nephropathy

increased the risk independently, however the definition of nephropathy was not cited in the study. The odds of fetal malformation increased by 30% with every percentage increase in pre-conception HbA1c independently, with no risk benefit below 6.3%[341].

One of the most recognized outcomes in pregnancies affected by diabetes is large for gestational age babies, which carries significant morbidity and is directly related to glucose control. A study on 29 type 1 diabetes pregnant women with glucose profile measured with continuous glucose monitoring system has found that glucose control in the second trimester is of particular significance to birth weight centiles [342]. Furthermore, every percentage increment in mean HbA1c during pregnancy is also associated with increased odds of pre-term birth in type 1 diabetes and gestational hypertension in type 2 diabetes, along with perinatal death in both type 1 diabetes and type 2 diabetes [328, 329]. These studies highlight the benefits of improving pregnancy HbA1c levels, but also illustrate the recent emphasize in research on HbA1c, despite its' role in determining pregnancy glycaemia management remains unclear. This is mainly due to the physiological changes of reduced fasting plasma glucose in early pregnancy, plasma volume expansion, and increased erythrocyte turnover [343]. Nevertheless, although HbA1c may not capture the glucose variability in pregnancy accurately, this should not negate the potential utility of HbA1c as a potential marker for glucose control to a certain degree. Additionally, it has been demonstrated that the use of continuous glucose monitoring during pregnancy in women with type 1 diabetes is associated with improved neonatal outcomes, including lower incidence of large for gestational age and neonatal intensive care admission [344]. This is attributed to reduced exposure to maternal hyperglycaemia, reflected by a small but significant difference in HbA1c compared to group without continuous glucose monitoring, but also more time in target. Of interest, since 2019, the Australian government subsidizes continuous glucose monitoring for women with type 1 diabetes during pregnancy.

Poor glycaemia management is also associated with pre-eclampsia[345], which is closely linked to blood pressure management in pregnancy. Pre-eclampsia in pregnancies affected by DKD has been proposed to be as result of combination of endothelial dysfunction, impaired vasodilatory capacity, early activation of the renin-angiotensin system and cardiac overload [331]. From the same research group (Lyons

et al.) using the same cohort of pregnant women with type 1 diabetes who were followed up prospectively (N=151), circulating angiogenic factors (higher soluble fms-like tyrosine kinase 1-sFlt1)[346], cholesterol-rich lipoprotein particles[347] and inflammatory markers (C-reactive protein, soluble E-selectin, interleukin-1 receptor antagonist, eotaxin) were all shown to be associated with preeclampsia[348]. In a study by Clausen et al, Endothelial adhesion markers ICAM-1 and VCAM-1 were found to be significantly elevated in early pregnancy of type 1 diabetes women who developed pre-eclampsia compared to those who did not, along with higher HbA1c (8.2% vs. 7.2%), urinary albumin excretion (194mg/24hours vs. 7mg/24hours) and blood pressure (122/75mmHg vs 111/69mmHg)[349].

However, similar to the papers by Lyons et al, measurements of serum creatinine or other surrogate markers of GFR were not performed. It is not known if endothelial markers, which are involved in the inflammatory pathways, have an association with pre-existing renal impairment in pregnancies affected by diabetes. The study findings do support evidence of early pregnancy related elevated blood pressure and albuminuria as a risk or predictor of developing pre-eclampsia. This is in line with findings of another study by the same group, who performed careful blood pressure monitoring with serial office and 24-hour ambulatory blood pressures on 74 women with type 1 diabetes. 14 women who developed pre-eclampsia had significantly higher mean systolic and diastolic blood pressure, as well as urinary albumin excretion before and throughout every stage of pregnancy[345]. At present, blood pressure target of <140/70 is recommended for pregnancy in diabetes [350], with data derived from non-gravid women with diabetes, and pregnant women without diabetes. However, although mean baseline systolic blood pressure in the aforementioned type 1 diabetes study was significantly higher in the group that developed pre-eclampsia, the systolic blood pressure was within the recommended target range at 131 ± 41 mmHg[345]. This highlights our current lack of knowledge on the optimal range of blood pressure in diabetes to prevent progression of renal function, or to achieve optimal maternal fetal outcome in DKD. Nielsen et al. conducted two separate studies in women with type 1 diabetes. For the microalbuminuria cohort in 2006 (N=20) and 2009 (N=10) the investigators aimed for a blood pressure of 140/90mmHg and 135/85mmHg in each study respectively[336, 351]. Despite the higher initial mean HbA1c ($7.3 \pm 1.5\%$ vs. $6.8 \pm 0.5\%$); and urinary albumin excretion $91 \pm (30-198)$ mg/24hours vs. $74 \pm (30-$

287)mg/24hours in the study with lower blood pressure targets; lower frequencies of pre-eclampsia, and pre-term delivery was also reported in the latter study which had earlier blood pressure intervention and a 5mmHg overall reduction of blood pressure.

Based on these small observational studies, current evidence support early blood pressure intervention in DKD may translate to more favourable pregnancy outcomes. However, large-scale studies have not demonstrated the beneficial effect of tighter blood pressure management in pregnancy from the recommended target of 140/70mmHg. A multicenter trial on 1030 non-diabetes specific cohort of women with pre-existing hypertension randomly assigned women to either tight or less-tight control, with a significant mean difference of 5.8mmHg (138.8 ± 0.5 mmHg vs. 133.1 ± 0.5 mmHg), and 4.6mmHg (89.9 ± 0.3 mmHg vs. 85.3 ± 0.3 mmHg) in systolic and blood pressure respectively in pregnancy. Despite the overall lower rates of gestational hypertension, there was no difference in pregnancy outcomes with tighter blood pressure management including pre-eclampsia[352]. The lack of benefit in pregnancy outcomes with tighter blood pressure was also reflected in a Cochrane review on 46 studies. Moreover, there are previous reports of small for gestational age and intrauterine growth restriction with lower blood pressure which has to be taken into account[353].

Finally, to achieve acceptable blood pressure management, the first line treatment in diabetes is RAAS inhibitors, which also regresses albuminuria. In non-gravid women with diabetes with hypertension and/or DKD planning for pregnancies, RAAS-inhibitors can have a carry-on effect and contribute to positive pregnancy outcomes especially when preconception albuminuria is a risk factor associated with pre-eclampsia. However, due to its association with fetal abnormalities [330], RAAS-inhibitors should be ceased immediately prior to, or once conception is known.

5.5 Summary and Consideration of Studies on Pregnancies affected by Diabetes

Strikingly, there are varying rates of obstetric complications in diabetes studies and this may reflect local obstetric and clinical practices. However, there are also methodological considerations that must be taken into account. As with most studies in pregnancy, there is a lack of large prospective and randomized controlled trials in

pregnancies affected by diabetes. There are also inconsistencies in many aspects of research in this area that makes data pooling and interpretation difficult, including the lack of universal definitions of obstetric outcomes. Additionally, there is a trend of increasing maternal age, as well as evolving clinical management that may potentially have an impact on risk factors including glycaemia and blood pressure management, in which not all studies adjust for these factors. Specifically, for diabetic kidney disease, current studies lack the absence of comparison healthy group of pregnant women without diabetes. In addition, existing literature incorporates broad stages and definition of kidney disease. Moreover, albuminuria statuses, renal function, variables including blood pressure and HbA1c are also measured at different time points in pregnancy from study to study. Taking these into consideration, it is not possible to draw definite conclusions based on present evidence. Nevertheless, despite the variable study settings and study cohorts, careful review of these studies indicates the high-risk state of pregnancies affected by diabetes and particularly more so in DKD. Specifically, there appears to be convincing evidence that the high morbidity in DKD is primarily through pre-eclampsia.

Study One

Chapter Six

Evaluating renal function in diabetes and pregnancy with plasma inulin clearance

6.1 Introduction

Assessment and measurement of renal function is an important aspect in this PhD. We measured glomerular filtration rate (GFR) using two different methods in the two prospective studies in Chapter 8 (Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy) and Chapter 11 (Functional MRI in predicting diabetic kidney disease). Measured GFR were obtained using ^{99m}Tc -technetium- diethylene-triamine-penta-acetic acid (^{99m}Tc -DTPA) clearance in Chapter 11 in participants with type 1 diabetes. In Chapter 8, as ^{99m}Tc -DTPA is not considered safe in pregnancy, plasma clearance of inulin was considered the ideal method for measuring renal function in pregnant women. It is important to note that unfortunately, due to adverse events coinciding with inulin infusion in our study, we had to withhold any further administration of inulin in view of the ethical concerns. The study protocol was amended to exclude inulin infusion, and we continued evaluation of renal function using 24-hour urinary creatinine clearance and Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula based estimated GFR.

This chapter will summarise the study methodology and adverse events in relation to inulin infusion. Results and discussion pertaining to available data on plasma clearance of inulin will also be outlined in this chapter, with the primary aim of comparing inulin clearance to CKD-EPI based estimated GFR and urinary creatinine clearance.

Outcome Measures:

The outcome measures were to correlate the following three different methods of renal function assessment in pregnant women with and without diabetes: (i) measured GFR based on inulin clearance, (ii) estimated GFR based on the CKD-EPI equation, and (iii) 24-hour urinary creatinine clearance.

Specific Testable Hypotheses:

Measured GFR (Inulin) correlates with estimated GFR based on the CKD-EPI equation and 24- hour urinary creatinine clearance in pregnancy.

6.2 Methods

Study Design

Detailed study methodology is outlined in Chapter Eight: Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy. To summarise, healthy control women as well as women with type 1 and type 2 diabetes were recruited and followed up longitudinally at four visit time points (trimester 1, trimester 2, trimester 3 and post-partum). Clinical history, examination, serum and 24-hour urinary collection were obtained during all four time points.

Initial study protocol required a bolus of intravenous inulin to obtain measured GFR in trimester 2 and at post-partum. Technique of a single bolus injection of inulin and plasma measurement was based on Jung et al [354]. The authors proposed a practical approach for determining GFR following a single injection of inulin in patients with a wide variety of diagnoses, including diabetes. Using a one-compartment model, and GFR by ^{99m}Tc -DTPA clearance as comparison, Jung et al have shown that GFR can be accurately calculated after only two plasma samples, taken at 20 and 240 minutes after injection of the inulin bolus. This technique of plasma clearance of inulin as a marker of glomerular filtration rate in type 2 diabetes has also been previously assessed and presented by our research group[355]. In 26 non-pregnant participants with type 2 diabetes, calculating GFR using samples at 20 and 120 minutes after inulin injection gave comparable results with that of ^{99m}Tc -DTPA-GFR with an accuracy of 72% and bias of 2.4ml/min/1.73m².

Intravenous inulin (20mls 25% inutest equivalent to 5g sinistrin) via an intravenous catheter was administered following a blank blood sample. A 10ml bolus injection of normal saline flush was subsequently administered. Blood samples were drawn at 30, 60, 90 and 120 minutes. Compared to Jung et al, we increased the frequency of blood sampling. Total duration was also reduced to 120 minutes, as the hyperfiltration state of pregnancy would have likely resulted in undetectable levels by 240 minutes. Participants were required to fast for at least eight hours due to the potential glucose interference on inulin clearance, as inulin is a form of polysaccharide.

Inulin concentration

Inulin concentration was determined with an enzymatic method using fructose estimation after hydrolysis of inulin with inulinase. The total analytical variations coefficient of variation was 2.0-2.9%.

Calculation of measured GFR with Inulin Plasma Clearance

Plasma clearance was calculated based on the 4 blood samples following the inulin bolus.

The plasma concentration-time curve of inulin (Slope-intercept GFR) was calculated from the following formula [354] :

Slope-intercept GFR (mL/min) = $k \times \text{inulin dose } (\mu\text{g}) / C_0 (\mu\text{g/mL})$ where k is the slope of the semilog plot of plasma inulin concentration versus time (plotted using 4 points: 30 minutes, 60 minutes, 90 minutes and 120 minutes), and C_0 is the calculated inulin concentration at time zero (intercept). Inulin concentration was also calculated by subtracting blank values to account for endogenous contributions.

This value was multiplied by the body surface area calculated as $((0.007184 \times \text{Height (cm)})^{0.725} \times \text{Weight (kg)})^{0.425}$ and divided by 1.73 in order to express the GFR as mL/min/1.73m².

The BSA-slope intercept GFR value (mL/min/1.73m²) was corrected by the Brochner-Mortensen correction factor = $(0.990778 \times \text{GFR}) - (0.001218 \times \text{GFR}^2)$ [356].

Calculation of estimated GFR based on CKD-EPI formula

Estimated Glomerular Filtration Rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formulation for females as below[357]. To convert from absolute values to values normalized to 1.73 m² body surface area (BSA), results were multiplied by 1.73/BSA[358].

If serum creatinine ≤ 62 mol/L: $144 \times (\text{serum creatinine} \times 0.0113 / 0.7)^{0.329} \times 0.993^{\text{age}}$

If serum creatinine > 62 mol/L: $144 \times (\text{serum creatinine} \times 0.0113 / 0.7)^{1.209} \times 0.993^{\text{age}}$

Calculation of Creatinine Clearance

Creatinine clearance was measured using plasma and 24-hour urine collection:

$$\text{Creatinine Clearance (mL/min)} = (\text{U Cr} \times \text{Vol} \times 16.7) / (\text{Pl Cr} \times \text{time})$$

To ensure accurately timed urine collection, all participants were provided with detailed verbal and written instructions on how to undertake 24-hour urine collections. Urinary volume and creatinine for each collection were reviewed to ensure adequate collection.

Statistical Analysis

Descriptive statistics and concordance correlation coefficient of measured GFR (inulin), estimated GFR, and Creatinine Clearance in the second trimester of pregnancy were undertaken.

6.3 Adverse Events in Relation to Inulin

Nineteen participants completed inulin infusion in the second trimester of pregnancy, between week 18 and 22. No participants had inulin infusion at their post-partum visit. There were 3 definite, and 2 potential reactions out of 19 episodes. The three definite reactions occurred during May, July and August 2014.

- i. One participant developed hay fever like symptoms of watery eyes, nasal congestions, peri-oral paraesthesia, pruritus and mild dyspnea 20 minutes post administration with complete resolution by 90 minutes.
- ii. One participant developed nausea, cutaneous rash, chest constriction and light headedness 5 minutes post inulin injection. Phenergan 10mg administered and symptoms resolved by 120 minutes.
- iii. One participant developed immediate hay-fever like symptoms and full body urticarial rash.

Two additional potential hypersensitivity reactions were initially deemed clinically to be unlikely to be an inulin reaction.

- i. One participant developed mild exacerbation of asthma requiring bronchodilator (> 12 hours post inulin administration).
- ii. One participant developed pruritis for 20 minutes post inulin administration.

None of the participants had airway compromise. Vital signs and blood glucose levels were monitored, with observations up to four hours post reaction. Participants did not experience hypoglycaemia or were haemodynamically unstable. We had no other reactions using the current batch of inulin in another 11 cases. These reactions occurred with the same batch of inulin, but from vials of different boxes. Expiry date was February 2015. Inulin was stored under manufacturer's recommended temperature control guidelines.

There have been no other reported similar reactions documented or known to the company- Frenius Kabi, Austria with the current batch of inulin administered. The appropriate notifications were lodged and reported to Mercy Health Ethics Committee, Austin Health Ethics Committee, Therapeutic Goods Australia and the manufacturing company. No further action was conducted to investigate the product contents. The study was aborted due to the possibility of future potential risk to the participants. In 2018 (three years from aborting the study), Inutest 25% was recall was listed on the Austrian Federal Office for Safety in Health Care website [359]. The Austrian Federal Office for Safety confirmed through correspondence that increased anaphylactic reactions were reported in France after administration of the product. The exact nature of the recall and exact details are not known, as the company did not respond to attempts for clarification.

Inulin Safety

In the current existing literature, there are no reported adverse effects of inulin in the literature, except for two cases reporting anaphylaxis from oral ingestion of vegetables and processed foods containing inulin [360, 361]. Previously two anaphylaxis cases has been reported in 2002 and 2004 from intravenous inulin administration [362, 363].

There has been three documented minor reaction of pruritis, respiratory symptoms and swollen eyes reported by Chandra et al. using Inutest from the same manufacturing company in 2002[362]. In the existing Inutest product information, the risk of allergic or anaphylactic reaction has been described to be uncommon at a risk of $\geq 1/1,000$ to $< 1/100$. Based on a database in France involving three nephrology department from the period of Jan 2003 to Dec 2006, inulin hypersensitivity reaction is rare. The rate of

adverse reaction to inulin was between 0.43% and 0.49%, with no life-threatening complications observed [364].

Safety in Pregnancy

Inulin clearance for GFR estimation has been used successfully in previous publications with no reported adverse effects to mother or fetus [311-313]. Even though inulin has been found to cross the placenta, based on its chemical and physiological properties, no toxic long-term effect can be expected with regards to teratogenicity, or peri and post-natal development.

Outcome of adverse events related to Inulin

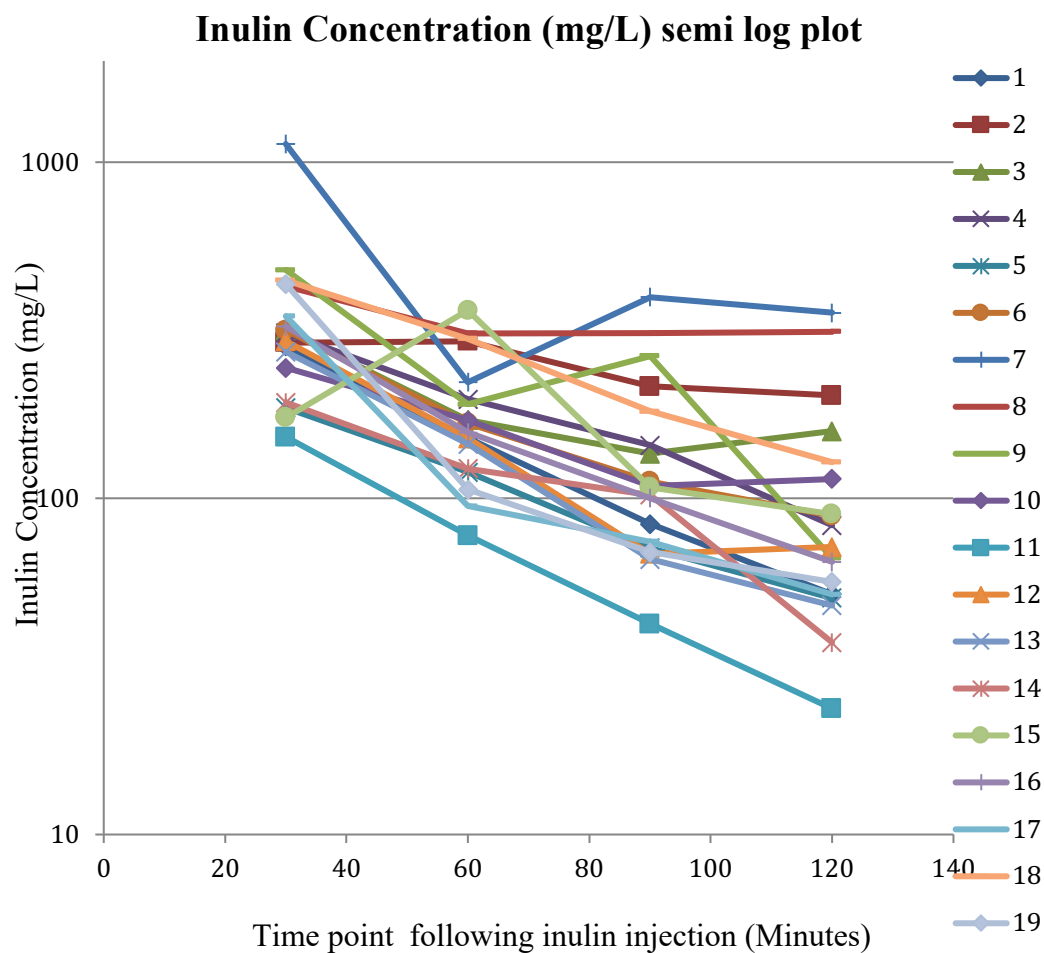
Although the reported adverse events of inulin are rare in the literature, in view of the unusual succession of potential hypersensitivity events encountered, inulin administration was deemed unsafe and unethical especially in the setting of pregnancy. We altered the study protocol and continued to assess renal function with only CKD-EPI-eGFR and 24-hour urinary creatinine clearance.

6.4 Results

19 participants completed inulin clearance study during the second trimester (N=9 with type 1 diabetes, N=5 with type 2 diabetes and N=5 healthy controls). The overall cohort median age was 32 (30,34) years old, BMI was 26 (25, 34) kg/m², systolic blood pressure was 109 (101, 122) mmHg, and diastolic blood pressure was 66 (60,80) mmHg. The median HbA1c for the participants with diabetes were 6.3 (5.8, 6.9) %.

Individual inulin concentration (mg/L) plot for each of the 19 individuals are presented in Figure 6.1.

Figure 6.1 Individual inulin concentration semi log plot



Measured GFR (inulin), estimated GFR and creatinine clearance are demonstrated in Table 6.1.

Median measured GFR (inulin) was 160 (121, 162) ml/min/1.73m² in healthy controls, 116 (58, 160) ml/min/1.73m² in women with type 1 diabetes and 191 (178,239) ml/min/1.73m² in women with type 2 diabetes.

Median eGFR was 131 (127, 131) ml/min/1.73m² in healthy controls, 126 (112,130) ml/min/1.73m² in type 1 diabetes, and 127 (124,131) ml/min/1.73m² in type 2 diabetes.

Median creatinine clearance was 151 (116,161) ml/min in healthy controls, 114 (85, 156) ml/min in type 1 diabetes and 156 (146, 176) ml/min in women with type 2 diabetes.

Table 6.1 Measured GFR, estimated GFR and creatinine clearance in trimester 2 of pregnancy

	Type 1 Diabetes N=9	Type 2 Diabetes N = 5	Healthy Controls N =5
Measured GFR (inulin) ml/min/1.73m²	116 (58, 160)	191 (178,239)	160 (121, 162)
Estimated GFR ml/min/1.73m²	126(112,130)	125 (124, 131)	131 (127,131)
Creatinine Clearance ml/min	114 (85, 156)	156 (146, 176)	151 (116,161)
Data expressed as median (IQR)			

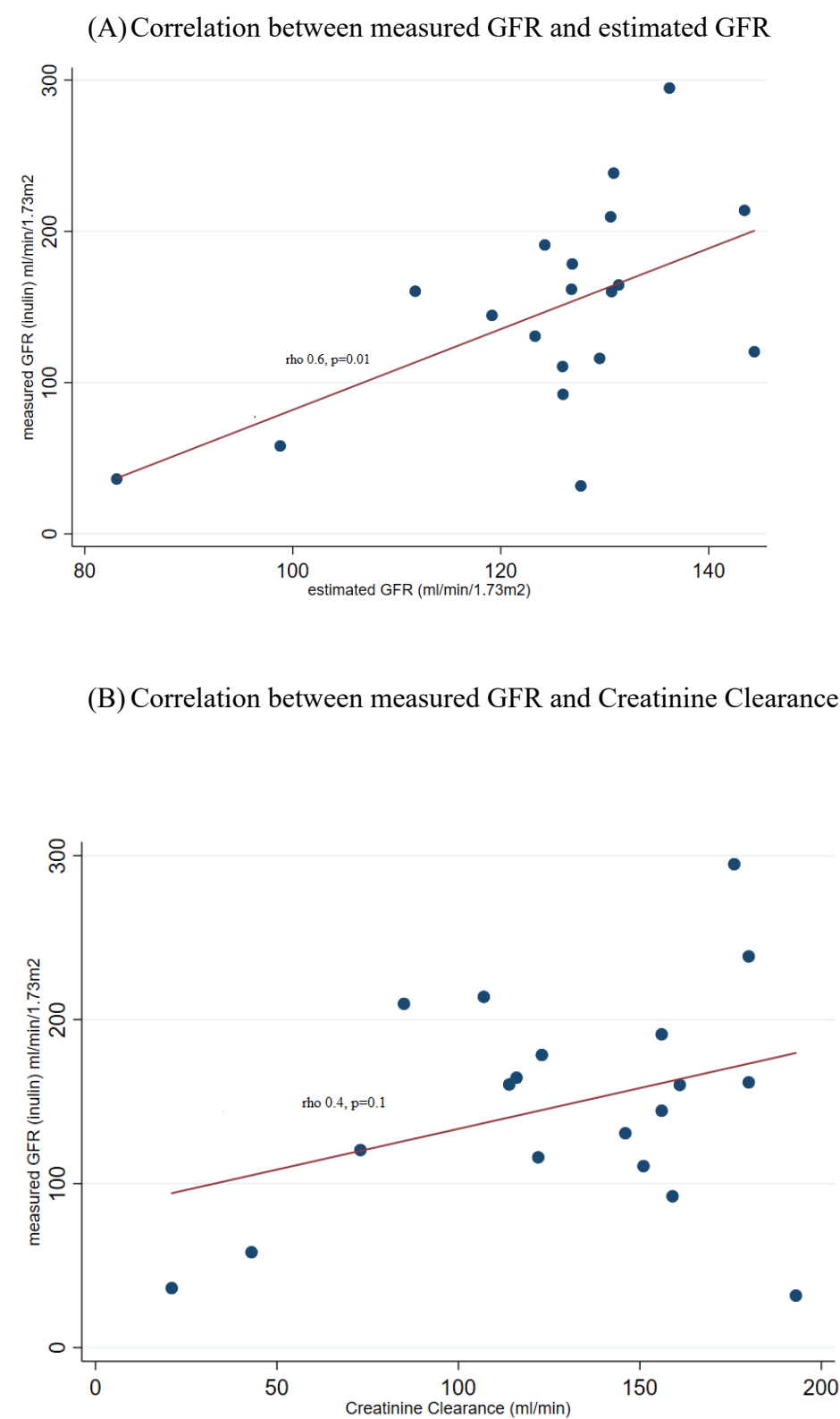
Correlation between measured GFR, estimated GFR and Creatinine Clearance

For the whole cohort, correlation of measured GFR, estimated GFR and Creatinine Clearance are demonstrated in Figure 6.2. A positive correlation was demonstrated between measured GFR and eGFR (ρ 0.6, $p=0.01$), and between eGFR and creatinine clearance (ρ 0.5, $p=0.03$). No relationship was observed between inulin GFR and creatinine clearance (ρ 0.4, $p=0.1$). Removing the three outliers (number 7,9 and 15 on Figure 6.1) with fluctuating inulin concentration levels did not improve overall correlation.

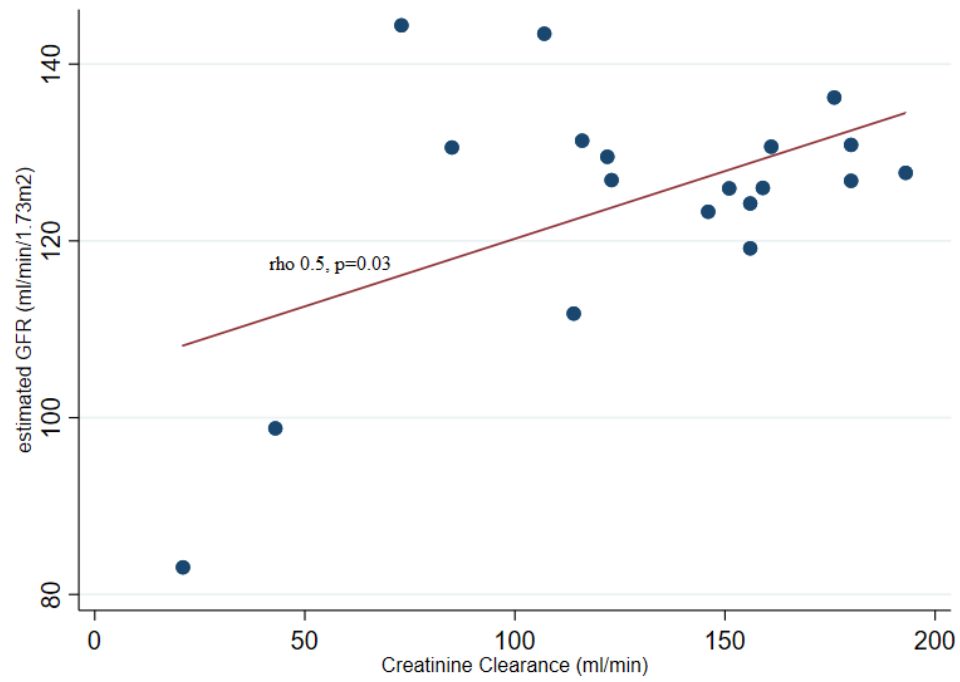
In the combined Type 1 and Type 2 Diabetes group, there was similarly a positive correlation between measured GFR and eGFR (ρ 0.7 $p=0.01$), and eGFR with creatinine clearance (ρ 0.7, $p=0.01$). There was a lack of correlation between measured GFR and creatinine clearance (ρ 0.3 $p=0.2$ rho).

In healthy controls, there was a lack of correlation between measured GFR and eGFR (ρ 0.3, $p=0.6$), and measured GFR with creatinine clearance (ρ -0.4, $p=0.5$). There was negative correlation between eGFR and creatinine clearance (ρ -0.9, $p=0.04$).

Figure 6.2 Relationship between measured GFR, estimated GFR and creatinine clearance in the second trimester of pregnancy (in the whole cohort i.e. women with and without diabetes)



(C) Correlation between estimated GFR and Creatinine Clearance



6.5 Discussion

Accurate assessment of renal function is very important in managing those affected by renal disease. The clinical significance of determining the accuracy and relationship between estimated and measured GFR is vital, as this has implications not only for monitoring disease progression, but also for drug dosing purposes, where pharmacokinetics and pharmacodynamic studies are usually referenced to measured GFR. At this point of time, there is no single equation estimating creatinine clearance or renal function in general, that works for all populations. For example, creatinine-based estimation of GFR tend to have poor performance, both in diabetes and pregnancy separately. Furthermore, the accuracy of creatinine-based equations to estimate renal function in pregnancies affected by type 1 and type 2 diabetes is not known. There is a need for accurate assessment of renal function to enable clinicians to detect any deterioration in women with type 1 and type 2 diabetes, especially in those with pre-existing diabetic kidney disease. Currently, serum creatinine, which can be affected by dietary protein, muscle mass, and analytical interference is the main clinical method of assessing renal function in pregnancy.

The ideal method of assessing renal function in healthy or disease states, including in diabetes and in pregnancy remains *renal clearance of an infused GFR marker* (inulin or iohexol). Estimated GFR based on the CKD-EPI formula which is widely reported in clinical practice tends to underestimate true GFR in both diabetes and pregnancy [8, 319]. We know of no validation studies evaluating inulin clearance with either the 24-hour urinary creatinine clearance or estimated GFR based on the CKD-EPI equation in pregnancies affected by type 1 and type 2 diabetes.

This cross-sectional study was designed to determine the accuracy of the eGFR based on the CKD-EPI equation, and urinary creatinine clearance, and how well these two methods predicted the ‘gold standard’ inulin clearance in women with and without diabetes during, and post pregnancy. As the target recruitment of N =60 was not met, and no participant completed the infusion post-pregnancy, we could not compare how accurate CKD-EPI based eGFR and creatinine clearance predicted inulin clearance. With the small number of participants, we conducted concordance correlation coefficient analysis instead.

The most pertinent finding was that there was an overall positive correlation between mGFR and eGFR in the second trimester of pregnancy. The correlation improved slightly (from rho 0.6 to rho 0.7) by studying only women with type 1 and type 2 Diabetes, excluding those without diabetes.

However, there was a lack of correlation between inulin clearance and urinary creatinine clearance in our cohort. This is in contrast to a study comparing the different methods of renal function estimation on twelve pregnant, normotensive healthy women [117]. This study demonstrated that despite the tendency of creatinine clearance to underestimate true GFR, there is good precision between inulin clearance and 24-hour urinary creatinine clearance. Specifically, for comparison to our results, the precision for trimester 2 was $R=0.7$, $p=0.01$ (inulin-GFR 154 ± 6 ml/min, creatinine clearance 129 ± 11 ml/min). The lack of any relationship observed with urinary creatinine clearance within our cohort may indicate the most common technical pitfall, which is incomplete urine collection despite provision of instructions.

Furthermore, for healthy controls, in addition to a lack of relationship between measured GFR with both eGFR and creatinine clearance, there was an unexpected finding of eGFR correlating negatively with creatinine clearance. However, with only five participants in the control group, with the variability in creatinine generation and excretion that is dependent of age, weight, and diet could also account for the discrepant results.

In addition, inulin clearance results from this sample must be interpreted with caution. Firstly, the underlying aetiology of the relatively high rate (26%) of possible hypersensitivity reaction is not known. Despite all our efforts in transport, storing and administering the inulin as per manufacturer's guidelines, it is not known if there have been any external circumstances that has altered the inulin and product contents. We could not ascertain if it was a faulty batch of inulin. Hence, as an extension, it is unknown if results from the plasma clearance are accurate.

Secondly, analysing plasma clearance of a single bolus of inulin has limitations itself. Plasma clearance using blood samples of a bolus of inert tracer (including inulin) tends

to underestimate true GFR values in healthy participants of normal renal function [365]. Pregnancy adds another layer of complexity when measuring plasma clearance. Pregnancy affects volume of distribution, and subsequently the ‘time for equilibration’ of inulin. We attempted to rectify this by reducing endpoint collection at 120 minutes, as higher elimination rate in pregnancy meant that by 240 minutes as documented by the Jung approach [354] would result in undetectable levels. The time points used in our calculations were based on the most accurate indication of the terminal elimination on the semi-log plot of plasma concentration versus time. In addition, fluid retention in pregnancy may also prolong distribution of plasma to extracellular fluid, resulting in overestimation of true GFR. Moreover, in circumstances when true GFR is low, a longer sampling interval is required to accurately measure the plasma clearance of inulin. Failure to do so may result in under-estimation of GFR as well [366]. In our cohort, we did not adjust the sampling interval in women with pre-existing diabetic kidney disease (for e.g. those with lower estimated GFR). This may explain the inconsistent results that we obtained, whereby the median measured GFR is higher than median estimated GFR for both type 2 diabetes and healthy controls. In contrast, median measured GFR was lower than median estimated GFR in type 1 diabetes.

Of note, measurement of the levels of the end-product of inulin, hydroxymethylfuraldehyde can be influenced by non-inulin sources. However, we attempted to minimise this by limiting oral intake with at least eight hours of fasting, as well as subtracting blank concentration of inulin that accounts for endogenous source.

Finally, noting the above potential limitations and precautions, our study results nevertheless suggest that there is a correlation between measured GFR and CKD-EPI eGFR in pregnant women with diabetes. This is an encouraging finding for future exploration of the prediction value of pregnancy eGFR in diabetes, as there are no published correlation studies of measured GFR and CKD-EPI eGFR in pregnancies affected by type 1 and type 2 diabetes. There are however many challenges in performing such a study. For example, the need to be conducted under strict research setting with restricted protein and salt diet, as well as having to consider other potential confounding factors including blood pressure, and the use of interfering medications.

6.6 Conclusion

Our results of a relatively good correlation between mGFR using inulin clearance and CKD-EPI-based eGFR in pregnancies affected by diabetes must be interpreted with caution due to the limitations of both small numbers of participants. There was no correlation between 24-hour urinary creatinine clearance and measured GFR, likely reflecting inaccuracy of 24h urine collections in this cohort and inherent creatinine excretion discrepancies between individuals. Ultimately, no estimating equation is optimal, and CKD-EPI-based eGFR has its' limitation in assessing renal function in both diabetes and pregnancy.

The following chapters of this theses, chapter seven, eight, nine, and ten all utilised eGFR as the primary method of evaluating renal function. In these chapters, renal function is an essential aspect of the studies, however, the aim of these studies was not to predict the accuracy of the various methods of renal function assessment. As CKD-EPI-based eGFR is a readily accessible, relatively accurate and widely used method to assess renal function and trajectories of renal function, we believe results from these chapters could potentially be translated to clinical practice.

Study Two

Chapter Seven

Pregnancy related renal function trajectories in women with type 1 and type 2 diabetes

7.1 Introduction

Understanding the trajectory of renal function during and post pregnancy is essential in guiding health care professionals caring for the high-risk pregnancy group of women affected by type 1 and type 2 diabetes. In type 1 diabetes, the presence of pre-existing kidney disease prior to pregnancy can influence subsequent renal function. Observational studies have demonstrated that pregnancy affects renal function in moderate to severe diabetic kidney disease (DKD), but not in those with normal or mild renal impairment [102, 110, 113, 115, 367]. On the other hand, the effects of pregnancy on renal function in women with type 2 diabetes are less well documented. There are also limited studies comparing women with type 1 and type 2 diabetes to healthy controls. It is also not well documented if the degree of physiological renal hyperfiltration that occurs during pregnancy differs between healthy pregnancies and pregnancies affected by type 1 and type 2 diabetes. This is important because there may be implications in maternal renal physiology and renal compensation in pregnancy. Furthermore, although renal function can return to pre-pregnancy levels within a few months following pregnancy, it is not clear how quickly renal function normalizes back to baseline in women with pre-existing diabetes compared to women without diabetes.

This study aimed to investigate the short and medium-term changes in renal function as reflected by CKD-EPI based eGFR, associated with pregnancy in women with type 1 and type 2 diabetes when compared to healthy women without diabetes.

Outcome Measures:

The primary outcome measures were eGFR at 24 months pre-, during, and up to 24 months post pregnancy in women with and without pre-gestational diabetes.

Secondary outcome measures were eGFR changes in a subset of women with pre-pregnancy diabetes related hyperfiltration to those who were not hyperfiltering.

Specific Testable Hypotheses:

1. Compared to women without diabetes, women with type 1 and type 2 diabetes
 - (i) have lower degree of physiological hyperfiltration during pregnancy
 - (ii) have slower recovery of renal function post pregnancy

2. Women with pre-pregnancy diabetes related renal hyperfiltration have reduced capacity to achieve physiological pregnancy hyperfiltration compared to women without pre-pregnancy renal hyperfiltration.

7.2 Methods

This was an observational study of women who delivered at the Mercy Hospital for Women, Victoria. We obtained ethics approval through the Human Research Ethics committee at Mercy Health.

Participants

All singleton pregnancies in women with pre-gestational type 1 and type 2 diabetes mellitus who delivered at the Mercy Hospital for Women, which is a tertiary maternity hospital between July 2004 and June 2014 were included. Antenatal care records, hospital clinical database (Birthing Outcome Systems, BOS) and pathology records containing glycosylated haemoglobin (HbA1c) results were used to identify women with diabetes. Data from the comparator group of healthy women without diabetes were obtained from a longitudinal study (period of 2006-2011) at the same center from the [368]. Exclusion criteria for healthy controls included a past history of thyroid disease, diabetes mellitus, intravenous drug use or presence of major systemic illness.

We excluded the following in the final analysis:

- i. Women with twin pregnancies or miscarriage prior to 20 weeks,
- ii. Women without at least two documented biochemistry measurements (to calculate eGFR) at each time point (pre-pregnancy, each trimester of pregnancy and post pregnancy)
- iii. Data from subsequent pregnancies for the same participant (only data from the initial pregnancy within the study period was included)

Biochemistry data were collected based on the following time points:

- i. Pre-pregnancy – data within 24 months preceding pregnancy
Considered as *baseline*
- ii. During pregnancy- data at all three trimesters
Trimester 1: Before 12 weeks
Trimester 2: Before 22 weeks
Trimester 3: Before 32 weeks
- iii. Post-pregnancy- data up to 24 months post pregnancy

Clinical and demographic data

Clinical anthropometric and demographics of participants were obtained from hospital records. Diagnosis of type 1 and type 2 diabetes obtained from hospital database were vetted and cross-checked with hospital clinician records. Data on blood pressure from each antenatal visit were collected from hospital records and the average blood pressure in each trimester was recorded. Obstetrics and neonatal outcomes were obtained through the hospital's Birthing Outcome Systems (BOS) clinical database, a database outlining pregnancy related outcome for both the mother and the neonate.

Biochemistry

Biochemistry results including, serum urea, serum creatinine, HbA1c, urinary albumin-to-creatinine ratio (ACR), urinary albumin excretion rate (AER), urinary creatinine clearance was obtained through five major state-wide pathology services across Victoria. Assays were laboratory dependent and are shown in Table 7.1. All available measures during were recorded and stored as time series data: 24 months pre-pregnancy, three trimesters of the pregnancy, and 24 months post-pregnancy.

Estimated glomerular filtration rate (eGFR) was derived from serum creatinine using the Chronic Kidney Disease Epidemiology (CKD-EPI) equation. Renal indices of serum urea, creatinine and eGFR were documented for women with and without diabetes. 24-hour urinary creatinine clearance, albumin excretion rate, and albumin creatinine ratio were only available for women with type 1 and type 2 diabetes, but not for women without diabetes.

Hyperfiltration were defined as an eGFR of $>120\text{ml/min.1.73m}^2$.

Table 7.1 Assays used by pathology labs for major biochemical measures

Pathology lab	Test	Method	Clinical Analyser
Laboratory 1	HbA1c	Antibody immunoassay	Cobas Integra 800, Roche, Switzerland
	Serum creatinine	Jaffe method	Cobas C8000, Roche Switzerland
	Serum urea	Enzymatic method	Cobas C8000, Roche Switzerland
	Urinary creatinine	Jaffe method	Cobas C8000, Roche Switzerland
	Urinary albumin	Immunoturbidimetry	Turbidimeter, Dade Behring, Germany
Laboratory 2	HbA1c	Cation-exchange HPLC*	D100, Bio-Rad, United States
	Serum creatinine	Jaffe method	Advia 2400, Siemens, Germany Cobas Integra 800, Roche, Switzerland
	Serum urea	Enzymatic method	Advia 2400, Siemens, Germany
	Urinary creatinine	Jaffe method	Advia 2400, Siemens, Germany
	Urinary albumin	Immunoturbidimetry	Advia 2400, Siemens, Germany
Laboratory 3	HbA1c	Cation-exchange HPLC*	D100, Bio-Rad, United States
	Serum creatinine	Jaffe and enzymatic method±	Cobas Integra 800, Roche, Switzerland
	Serum urea	Kinetic method	Cobas Integra 800, Roche, Switzerland
	Urinary creatinine	Jaffe method	Cobas Integra 800, Roche, Switzerland
	Urinary albumin	Immunoturbidimetry	Cobas Integra 800, Roche, Switzerland
Laboratory 4	HbA1c	Antibody immunoassay	Cobas Integra 800, Roche, Switzerland
	Serum creatinine	Jaffe method +/- enzymatic method^	Advia 2400, Siemens, Germany Advia XPT, Siemens, Germany
	Serum urea	Enzymatic	Advia 2400, Siemens, Germany

		Advia	XPT,	Siemens,
		Germany		
	Urinary creatinine	Jaffe method	Advia	XPT,
			Germany	Siemens,
	Urinary albumin	Immunoturbidimetry	Advia	2400,
			Germany	Siemens,
			Advia	XPT,
			Germany	Siemens,
Laboratory				
5				
	HbA1c	Cation-exchange HPLC*	ADAMS	A1c, HA-8180,
			Arkray, Japan	
	Serum creatinine	Jaffe method	CX3,	Beckman Coulter,
			United States	
	Serum urea	Enzymatic method	CX3,	Beckman Coulter,
			United States	
	Urinary creatinine	Jaffe method	CX3,	Beckman Coulter,
			United States	
	Urinary albumin	Immunoturbidimetry	CX3,	Beckman Coulter,
			United States	
Listed laboratory method, unless otherwise stated, were current as of July 2016.				
±Enzymatic method used from August 2015 onwards; Jaffe method used prior to this date.				
^Enzymatic method sometimes utilised when an abnormal serum creatinine measurement is detected.				
Abbreviations: HbA1c, glycosylated haemoglobin; HPLC, high-performance liquid chromatography				

Statistical Analysis

Group comparisons pre- during and post-pregnancy were performed using Chi-Square tests and Fisher exact tests for categorical variables, and Mann-Whitney U tests and Kruskal-Wallis rank tests for continuous variables. Results for median eGFR are expressed as median and interquartile range.

Quantile regression with data clustering (to cluster data of each individual) was used to investigate pregnancy-associated changes in biochemical parameters from baseline across each trimester of pregnancy and up to 24 months post-pregnancy in each group and sub-group. Results for change in eGFR based on quantile regression are expressed as co-efficient variation and 95% confidence interval.

Age and age at diabetes diagnosis were included in the model. Body mass index and blood pressure measurements were only available during pregnancy and could not be

included as independent variable in the analysis. Albumin excretion rate was excluded from the analysis due to the limited number samples.

Analysis for post-pregnancy eGFR includes readings immediately post to 24 months unless stated otherwise. The analysis of eGFR returning to baseline accepts an eGFR range of (+/-) 5ml/min/1.73m². For example, a post-pregnancy eGFR of 95-105 ml/min/m² were considered within range for a baseline eGFR of 100ml/min/m².

7.3 Results

Participants characteristics

We identified 316 consecutive singleton pregnancies (91 with type 1 diabetes, 106 with type 2 diabetes and 119 healthy participants without diabetes). We included women with at least 2 documented biochemistry results to allow calculation of eGFR, and to increase data accuracy. A total of 127 women (49 with type 1 diabetes, 49 with type 2 diabetes and 29 control participants without diabetes) were included in the final analysis. Only results from the first documented pregnancy was included in the analysis.

Median length of time for baseline/ pre-pregnancy eGFR measurements was 23 weeks prior to gestation for type 1 diabetes, 15 weeks prior to gestation for type 2 diabetes and 47 weeks prior to gestation for women without diabetes.

The median post-delivery time period of which eGFR was calculated to have returned to baseline was 38 weeks post gestation in type 1 diabetes, 32 weeks post gestation for type 2 diabetes and 14 weeks post gestation in women without diabetes.

Maternal characteristics in first trimester is documented in Table 7.2. Women without diabetes were younger, and had a lower BMI compared to women with type 1 and type 2 diabetes. There were significant differences in baseline characteristics between women with type 1 diabetes and type 2 diabetes (Table 2). These include a shorter duration of diabetes 3(1,4) years vs. 14 (4,19) years, higher BMI 34 (30,38) kg/m², vs. 27(23,34) kg/m², a lower HbA1c 6.6 (6.0,7.9) % vs. 7.1 (6.5, 8.7)%, higher systolic 120(120,130)mmHg vs. 115 (110,120)mmHg and diastolic 70(75,80)mmHg vs. 70 (70,80)mmHg blood pressure, and a higher incidence of pre-existing hypertension 15(30)% vs. 3 (6)% in women with type 2 diabetes (Table 7.2). There was no significant difference in smoking status, pre-existing diabetic kidney disease, renin angiotensin-aldosterone system inhibitors, statin or aspirin therapy.

Table 7.2 Maternal characteristics in women at trimester one of pregnancy

	Type 1 Diabetes	Type 2 Diabetes	Controls	p- value
N	49	49	29	
Age (years)	30 (28, 32)	36 (31, 39)	32 (29, 34)	<0.01
Body Mass Index (kg/m ²)	27 (23,34)	34 (30, 38)	26 (23, 34)	<0.01
Smoking	5 (10)	7 (14)	2(7)	0.7
Duration of Diabetes	14 (4, 19)	3(1,4)	-	<0.01
Systolic Blood Pressure (mmhg)	115 (110,120)	120(120,130)	-	<0.01
Diastolic Blood Pressure (mmhg)	70 (70,80)	70(75,80)	-	0.02
HbA1c (%)	7.1 (6.5, 8.7)	6.6 (6.0, 7.9)	-	<0.01
HbA1c (mmol/mol)	54 (48,72)	49 (42,63)	-	<0.01
Pre-existing Diabetic Kidney Disease	7 (14)	4 (10)	-	0.2
Pe-existing hypertension	3 (6)	15 (30)	-	<0.01
RAAS inhibitors	4 (8)	7 (14)	-	0.4
Statins therapy	2 (4)	6 (12)	-	0.2
Aspirin therapy	9 (19)	17 (35)	-	0.1

Data are expressed as median (25th, 75th percentile) or n (%). Group comparisons were performed using Chi-Square tests and Fisher exact tests for categorical variables, and Mann-Whitney U tests and Kruskal-Wallis equality-of-populations rank tests for continuous variables.

*RAAS (renin-angiotensin-aldosterone system) inhibitors includes angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blockers (ARBs).

Pregnancy associated changes in eGFR

The following data on pregnancy associated changes in eGFR at different time points are presented as:

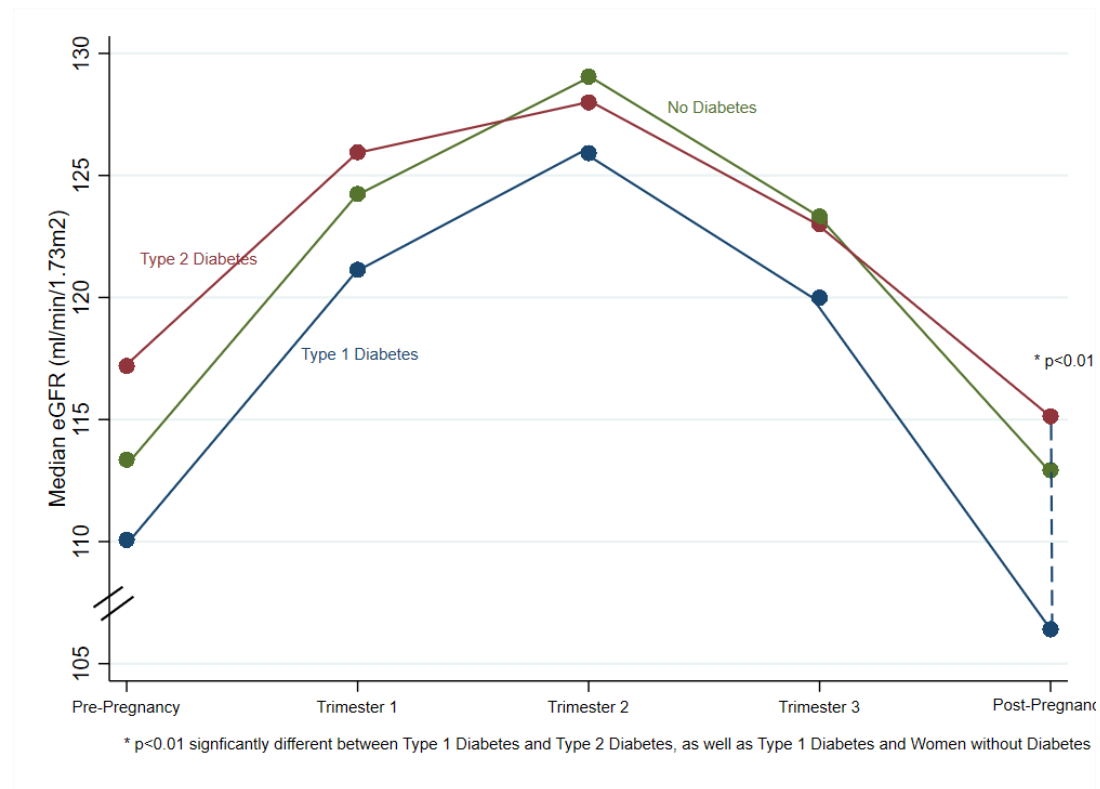
- (i) The eGFR for each group (median, interquartile range)
- (ii) The degree of eGFR changes within each group (co-efficient variation, 95% confidence interval)

Median eGFR pre-, during and post-pregnancy in women with and without diabetes are tabulated in Table 7.3. Figure 7.3 depicts the changes in eGFR for women with and without diabetes.

Table 7.3 Estimated GFR pre-, during, and post-pregnancy in women with and without diabetes

CKD-EPI eGFR (ml/min/1.73m²)	Type 1 diabetes	Type 2 diabetes	No Diabetes	p- value
Pre-Pregnancy	110(102,120)	117(98,122)	113(103,119)	0.7
Trimester 1	121(117,129)	126(120,130)	124(120,130)	0.4
Trimester 2	126(117,135)	128(119,135)	129(123,132)	0.7
Trimester 3	120(111,131)	123(116,129)	123(122,127)	0.6
Post-Pregnancy at 6 months	109 (89,119)	114 (98, 121)	111 (96, 119)	0.3
Post-Pregnancy at 24 months	106(79,117)	115(105,122)	113(100,120)	<0.01
Results expressed as median (interquartile range). Group comparisons were performed using Mann-Whitney U tests.				

Figure 7.1 Pregnancy related estimated GFR changes in women with and without diabetes



At the pre-pregnancy time point, there were no significant differences in median eGFR between women with type 1 diabetes, type 2 diabetes and healthy controls.

In pregnancy, overall, there were no observed differences in median eGFR between type 1 diabetes, type 2 diabetes and healthy controls in all three trimesters.

eGFR peaked in the second trimester and significantly increased from baseline in all three groups. In type 1 diabetes, the eGFR increased by 14.3 mL/min/1.73m² (co-efficient: 14.3; 95CI [7.6, 21.0] mL/min/1.73m²; $p<0.01$) compared to baseline. In type 2 diabetes, eGFR increased by 12.6 mL/min/1.73m² (co-efficient: 12.6; 95CI [7.9, 17.2] mL/min/1.73m²; $p<0.001$). In healthy controls, eGFR increased by 18.0 mL/min/1. (co-efficient: 18.0; 95CI [12.0, 24.1] mL/min/1.73m²; $p<0.01$).

Compared to healthy controls, the median increase in eGFR from baseline was not statistically significant between type 1 diabetes and type 2 diabetes, 1.2 mL/min/1.73m²

(co-efficient: 1.2 (-3.7, -6.1), $p=0.6$), more in type 1 diabetes and 1.1 ml/min/1.73m² (co-efficient-1.1 (-6.8, -4.6), $p=0.7$) less in type 2 diabetes. Median eGFR increase from baseline to peak was not different in type 1 and type 2 diabetes (co-efficient: 5.1 (-0.2, -10.5), $p=0.06$).

At 24 months post pregnancy, the median eGFR was significantly lower in type 1 diabetes 106 (79,117) ml/min/1.73m² compared to healthy controls 113 (100,120) ml/min/1.73m², and type 2 diabetes 115 (105,122) ml/min/1.73m² ($p<0.01$). Compared to pre-pregnancy levels, median eGFR at 24 months post-pregnancy was significantly lower in women with type 1 diabetes ($p=0.002$), but not in women with type 2 diabetes ($p=0.8$) and healthy controls ($p=0.6$) (Table 7.4).

For sub-analysis at 6 months post-pregnancy, there were no significant differences in median eGFR between the three groups. For all three groups, levels returned to baseline by six months post-delivery with no significant differences in eGFR pre- and 6 months post pregnancy (Table 7.4).

Table 7.4 Comparing post-pregnancy estimated GFR to pre-pregnancy estimated GFR

CKD-EPI eGFR (ml/min/1.73m²)	Pre- pregnancy	Post-Pregnancy 6 months	Post-Pregnancy 24 months
Type 1 Diabetes	110(102,120)	109 (89,119), $p=-0.2$	106 (79,117), $p=0.002$
Type 2 Diabetes	117(98,122)	114 (98, 121), $p=0.2$	115 (105,122), $p=0.8$
Healthy Controls	113(103,119)	111 (96, 119), $p=0.4$	113 (100,120), $p=0.6$

Results expressed as median (interquartile range). Group comparisons were performed using Mann-Whitney U tests.

The rate of eGFR returning to baseline

The following data on the rate of eGFR returning to baseline is presented as:

- (i) *Event rate* reflects the probability that an individual would experience the event of having eGFR returning to pre-pregnancy levels. This is reported as Hazard ratio and 95% confidence interval.
- (ii) *Rate of return to baseline from peak* (within each group, and when comparing one group to other), reflects how many ml/min/1.73m² eGFR returns to baseline per day.

This is reported as co-efficient variation and 95% confidence interval.

Compared to type 1 diabetes, there was no difference in event rate of eGFR returning to baseline in women with type 2 diabetes (Hazard ratio: 1.4, 95CI [0.9 2.4], $p=0.2$) and those without diabetes (Hazard ratio: 1.2, 95CI[0.6,2.4], $p=0.5$). Age (Hazard ratio:1.0,95CI[1.0,1.1], $p=0.2$), duration of diabetes (Hazard ratio:1.0,95CI[1.0,1.1], $p=0.4$) and baseline eGFR (Hazard ratio:1.0, 95CI[1.0,1.0], $p=0.3$) did not affect the event rate of eGFR returning to baseline.

Overall, eGFR returned to baseline from peak (trimester 2 of pregnancy) at the rate of 0.05 ml/min/1.73m² per day in type 1 diabetes (co-efficient: 0.05, 95CI [0.03, 0.06], $p<0.01$); 0.04 ml/min/1.73m² per day in women with type 2 diabetes (co-efficient: 0.04, 95CI [0.02, 0.06], $p<0.01$); and 0.02 ml/min/1.73m² per day in healthy controls (co-efficient: 0.02, 95CI [0.00, 0.03], $p=0.02$).

The above rate of return of eGFR back to baseline was not different between the groups. Compared to healthy controls, the rate of return of median eGFR per day to baseline from peak, was not different in type 1 diabetes (co-efficient: -0.003, 95CI [-0.04, 0.04], $p=0.9$), and type 2 diabetes (co-efficient: 0.02, 95CI [-0.02, -0.06], $p=0.3$). Median rate of return of eGFR to baseline were also not different between type 1 and type 2 diabetes (co-efficient: -0.03, 95CI [-0.7, -0.02], $p=0.2$). Baseline eGFR and age did not affect the rate of eGFR returning to baseline.

Exploratory analysis-Is there a difference in achieving peak pregnancy eGFR in women with diabetes and pre-existing hyperfiltration to those without hyperfiltration?

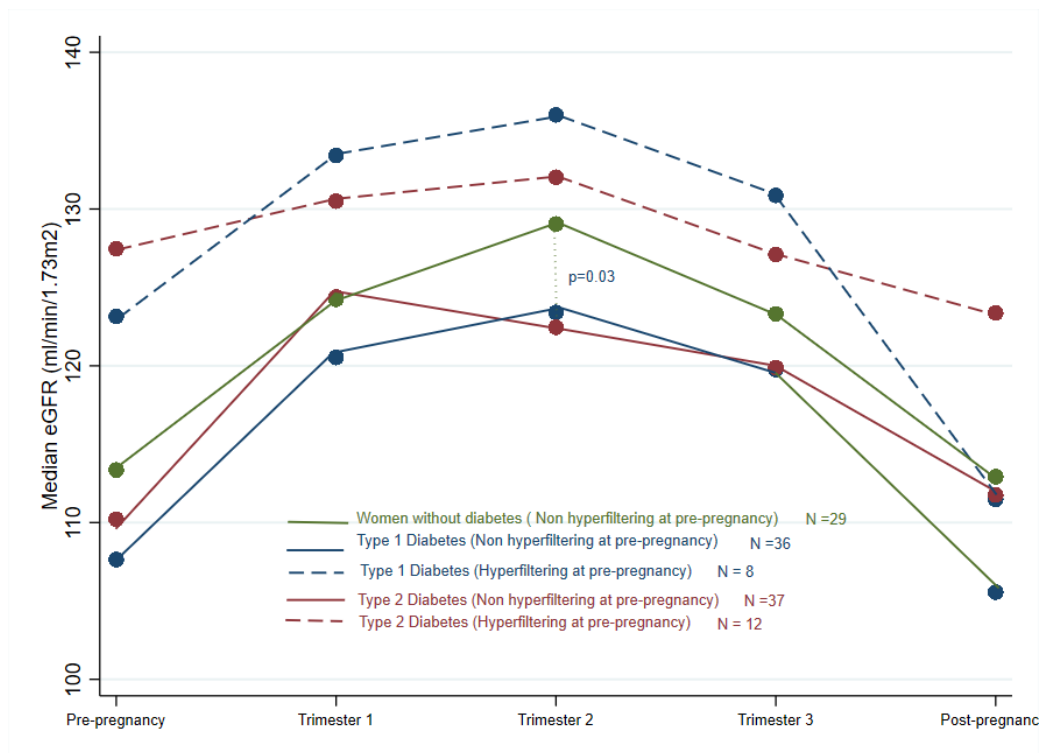
In exploratory analysis, we examined whether the presence of hyperfiltration defined arbitrarily with a cut-off of CKD-EPI eGFR $> 120\text{ml/min/1.73m}^2$ was associated with peak renal function during pregnancy. 8 women with type 1 diabetes, and 12 with type 2 diabetes had an eGFR $>120\text{ml/min/1.73m}^2$ at the pre-pregnancy time point. None of the women without diabetes had an eGFR $>120\text{ml/min/1.73m}^2$ at baseline. The trajectory of eGFR pre, during and post pregnancy in healthy women, women with type 1 and type 2 diabetes who were hyperfiltering (eGFR $>120\text{ml/min/1.73m}^2$) and those who were not (eGFR $<120\text{ml/min/1.73m}^2$) at baseline are presented in Figure 7.2.

At baseline, the median eGFR for women who were hyperfiltering compared to those who were not were 123 (121,127) ml/min/1.73m² vs. 107 (100,116) ml/min/1.73m² in type 1 diabetes ($p<0.01$); and 127 (123,131) ml/min/1.73m² vs. 110 (96,118) ml/min/1.73m² in type 2 diabetes ($p<0.01$).

In Trimester 2, the peak median eGFR was 136 (134, 137) ml/min/1.73m² vs. 123 (117,127) ml/min/1.73m² in type 1 diabetes ($p<0.01$); and 132(118,136) ml/min/1.73m² vs. 122(118,135) ml/min/1.73m² in type 2 diabetes ($p=0.07$), for those who were hyperfiltering to those who were not, respectively.

For those with type 1 and type 2 diabetes who were hyperfiltering at baseline compared to healthy controls, there was no difference in peak eGFR in Trimester 2. However, within the non-hyperfiltering group, women with type 1 diabetes had a significantly lower peak eGFR at Trimester 2 ($p=0.03$) compared to healthy controls. There was no significant difference in Trimester 2 eGFR between women with type 2 diabetes (that was non-hyperfiltering) and healthy controls ($p=0.2$).

Figure 7.2 Pregnancy related estimated GFR changes in healthy controls, and women with and without pre-existing hyperfiltration in diabetes



Exploratory analysis in women with baseline reduced renal function defined as an eGFR <90 ml/min/1.73m²

5 women with type 1 diabetes, and 4 women with type 2 diabetes had a baseline eGFR <90 ml/min/1.73m². No women had a baseline GFR lower than 60mls/min/1.73m². Compared to those with a baseline eGFR ≥90 ml/min/1.73m², those with a baseline eGFR <90 ml/min/1.73m² had (Table 7.5):

1. a significantly lower median eGFR at baseline and post-pregnancy in both type 1 and type 2 diabetes.
2. a pregnancy peak eGFR that was significantly lower in type 1 diabetes
3. no difference in pregnancy peak eGFR in in the type 2 diabetes group.

The women with type 1 diabetes and a baseline eGFR<90 ml/min/1.73m² had a significantly lower eGFR at the post-pregnancy period compared to baseline (p=0.01). Women with type 1 diabetes and a baseline eGFR<90 ml/min/1.73m² (p=0.9), as well as both groups of women with type 2 diabetes (p=-0.9 and p=0.8), had a post-pregnancy median eGFR similar to baseline.

Table 7.5 Pregnancy related estimated GFR in women with type 1 and type 2 diabetes comparing those with baseline eGFR ≥90 ml/min/1.73m² to those with eGFR <90 ml/min/1.73m²

	eGFR <90 ml/min/1.73m ²	eGFR > 90 ml/min/1.73m ²	p-value
	Type 1 Diabetes	Type 1 Diabetes	
N	5	44	
Pre-pregnancy	78 (77,82)	113 (107, 121)	<0.001
Trimester Two	104 (74, 119)	127 (123, 137)	0.01
Post-Pregnancy	82 (63, 93)	109 (85, 118)	0.001
	Type 2 Diabetes	Type 2 Diabetes	
N	4	45	
Pre-pregnancy	84 (80,87)	118 (104, 123)	0.001
Trimester Two	124 (114, 131)	129 (120,136)	0.4
Post-Pregnancy	80 (65,113)	116 (108,123)	0.001

Pregnancy related urinary creatinine clearance and albumin creatinine ratio in Type 1 and Type 2 Diabetes.

Urinary creatinine clearance and ACR levels pre-, during and post are tabulated in Table 7.6

Table 7.6 Creatinine clearance and albumin creatinine ratio pre-, during, and post pregnancy

Creatine Clearance (ml/min)	Type 1 diabetes	Type 2 diabetes	p-value
Pre-Pregnancy	111 (69,130)	113 (93,214)	0.6
Trimester 1	156(137,171)	230(201,260)	0.1
Trimester 2	125(115,148)	212(209,215)	0.2
Trimester 3	136(133,145)	188(123,252)	0.7
Post-Pregnancy at 6 months	121 (81,131)	130 (97, 159)	0.5
Post-Pregnancy at 24 months	117(92,137)	114(101,159)	0.5

ACR (mg/mmol)			
Pre-Pregnancy	0.9(0.6,2.0)	1.2 (0.9,3.9)	0.3
Trimester 1	0.7 (0.5,1.7)	0.9(0.5,1.5)	0.6
Trimester 2	0.9 (0.6,2.8)	1.5 (0.8,1.8)	0.4
Trimester 3	0.8(0.4,4.3)	1.4(0.6,15.0)	0.3
Post-Pregnancy at 6 months	1.3(0.5, 11.0)	6.2 (2.4, 12.7)	0.1
Post-Pregnancy at 24 months	1.8(0.7,12.4)	1.9(1.0,6.9)	0.8

For both the pre-pregnancy and post pregnancy timepoint, creatinine clearance and ACR for women with type 1 diabetes was not statistically different to women with type 2 diabetes.

During pregnancy, there was a trend of rising ACR with a peak in the second trimester, but this was not significantly different to pre-pregnancy levels (p=0.8). Creatinine clearance peaked in the first trimester.

When we compared pre-pregnancy to 6- and 24-month post-pregnancy levels creatinine clearance was not significantly different in both type 1 and type 2 diabetes

(Table 7.7). There was a non-significant rise in 6 and 24-months post pregnancy ACR in both type 1 and type 2 diabetes.

Table 7.7 Comparing post-pregnancy creatinine clearance to pre-pregnancy creatinine clearance

Creatinine Clearance (ml/min)	Pre-pregnancy	Post-Pregnancy 6 months	Post-Pregnancy 24 months
Type 1 Diabetes	111 (69,130)	121 (81,131), $p=0.6$	117(92,137), $p=0.7$
Type 2 Diabetes	113 (93,214)	130 (97, 159), $p=0.8$	114(101,159), $p=0.8$
ACR (mg/mmol)			
Type 1 Diabetes	0.9 (0.6,2.0)	1.3(0.5, 11.0), $p=0.3$	6.2 (2.4, 12.7), $p=0.1$
Type 2 Diabetes	1.2 (0.9,3.9)	1.8(0.7,12.4), $p=0.06$	1.9(1.0,6.9), $p=0.9$

Pregnancy related serum urea and creatinine changes

Serum urea and creatinine decreased significantly ($p<0.01$) throughout all trimesters, with the largest reduction in second trimester, likely in keeping with physiological hyperfiltration. Levels returned to baseline by six months post-delivery (Table 7.8).

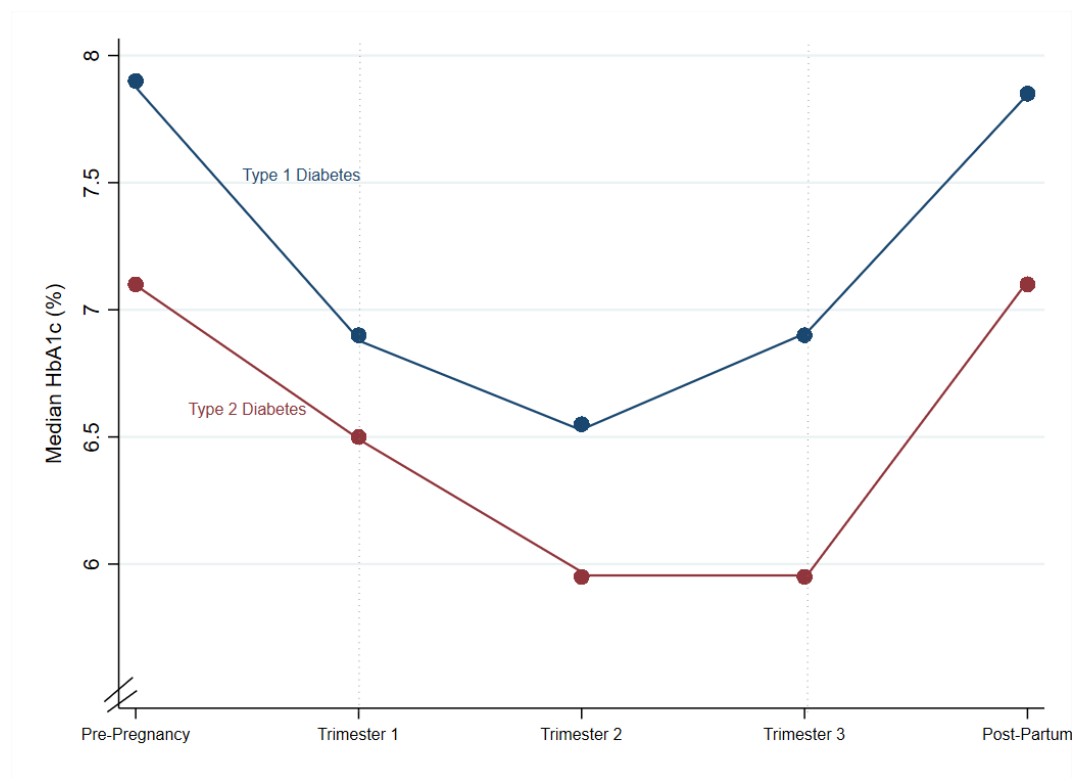
Table 7.8 Serum urea and creatinine pre, during and post-pregnancy

	Type 1 diabetes	Type 2 diabetes	No Diabetes	p- value
Serum urea (mmol/L)				
Pre-Pregnancy	4.3(3.3,5.3)	3.9(3.1,5.3)	4.1(3.4,4.7)	0.7
Trimester 1	3.4 (2.8,4.2)	2.9 (2.3,3.6)	3.1 (2.9,3.7)	0.1
Trimester 2	3.1(2.7,4.1)	2.3(1.8,3.2)	2.8(2.6,3.1)	<0.01
Trimester 3	3.1(2.4,4.3)	2.9(2.4,3.7)	2.8(2.4,2.4)	0.6
Post-Pregnancy at 6 months	4.8 (3.5, 6.1)	4.3 (3.3, 5.7)	4.6(3.4,5.6)	0.5
Post-Pregnancy at 24 months	5.3(3.9,6.7)	4.3(3.3,5.2)	4.3(3.5,5.2)	<0.01
Serum creatinine (mmol/L)				
Pre-Pregnancy	65(59,70)	58(49,69)	65(56,67)	0.1
Trimester 1	55 (48,61)	46 (41,52)	50 (47, 53)	0.03
Trimester 2	51(42,56)	43(40,49)	46(42,51)	0.04
Trimester 3	56(47,62)	47(44,55)	50(45,53)	0.1
Post-Pregnancy at 6 months	65 (58,77)	59 (52, 69)	65 (56,71)	0.01
Post-Pregnancy at 24 months	67(60,83)	57(51,65)	63(55,70)	<0.01

Pregnancy related HbA1c changes in type 1 and type 2 diabetes

A significant decrease in HbA1c was observed in both women with type 1 and type 2 diabetes during pregnancy (Figure 7.3). The median decrease in HbA1c was 1.1 % in women with type 1 diabetes (95CI [-1.72, -0.52]; $p < 0.001$) and 0.9 % in women with type 2 diabetes (95 CI [-1.38, -0.39]; $p < 0.001$). HbA1c returned to pre-pregnancy levels within the 12 months following pregnancy in both groups.

Figure 7.3 Pregnancy related HbA1c changes in type 1 and type 2 diabetes



7.4 Discussion

The most pertinent findings in this cohort study were:

- i) The physiological pregnancy peak in eGFR, and the rate of recovery in eGFR back to baseline did not differ between women with and without diabetes.
- ii) Women with type 1 diabetes and who did not have pre-pregnancy diabetes related hyperfiltration had a significantly lower peak of eGFR compared to healthy controls.

While some studies have demonstrated that pregnancy has a negative effect on renal function in diabetes, others have not. These contradicting findings are partially explained by the small case series and cross-sectional studies which have used heterogenous methodology when assessing renal function. In addition, as diabetes management has improved significantly over the years, adverse effects of pregnancy on renal function that has been recently reported and earlier studies may not be applicable with current advances in diabetes care.

We know of no recent study that utilises CKD-EPI based eGFR to compare renal trajectory in women with, and without diabetes with a focus on the rate of recovery in eGFR post-delivery.

The effect of pregnancy on eGFR

As expected, in pregnancy, all three groups demonstrated a physiological rise in eGFR that peaked in the second trimester. Changes in eGFR in women without diabetes were not significantly different compared to women with type 1 or type 2 diabetes as a group. However, it was found that in women with type 1 diabetes who did not have pre-existing hyperfiltration, there was significant lower physiological peak in pregnancy. Possibly as eGFR is at a lower “starting” point for this group, the peak eGFR that could be achieved is naturally lower. An alternate explanation is that within this group, there are women with diabetic kidney disease with a reduced capacity to hyperfiltrate in pregnancy. This is in line with small case series which has reported physiological changes in renal function (as defined predominantly serum creatinine) is impaired in

mild to moderate DKD[106, 107, 109]. The definition of DKD and monitoring of renal function in pregnancy in these earlier studies however are based predominantly on changes in serum creatinine and not GFR.

The underlying mechanism of this reduced ability to achieve physiological renal hyperfiltration is not known. In pregnancy, the reduction in afferent and efferent arteriole resistance is thought to result in increased blood flow and subsequently rise in GFR[54]. In addition, in a non-pregnant cohort, it has been demonstrated that arterial stiffness is significantly higher in 60 participants with diabetes compared to 16 healthy controls[369]. In this study, arterial stiffness was reflected by renal resistance index measured with intrarenal arterial doppler sonography. Hence, we postulate that it is possible that in DKD, stiffening of the arteriole vessels may affect the ability of these vessels to relax. However, although the 5 women with type 1 diabetes and a baseline $eGFR < 90 \text{ ml/min/1.73m}^2$ had a much lower peak $eGFR$ in pregnancy, there was no difference in achieving the physiological rise in the type 2 diabetes cohort. Inference of the results is also limited by the small number of women with DKD. Moreover, baseline $eGFR$ within the type 1 diabetes group who were not hyperfiltering were not particularly low at 107 (100,116) ml/min/1.73m^2 . Hence, as this group of women have what is generally considered normal, healthy baseline $eGFR \geq 90 \text{ ml/min/1.73m}^2$, there is a need for studies to investigate the underlying mechanism that could potentially account for the significant discrepancy in achieving peak physiological pregnancy $eGFR$. It should be noted as well that as $eGFR$ tends to underestimate true GFR at the high-normal range particularly during pregnancy, and the true GFR gap between the two groups may be even wider.

In this study we demonstrated that in general, in accordance to existing studies [103, 110, 115, 367, 370] pregnancy associated changes in renal indices are transient in women with diabetes. We observed that changes in $eGFR$, as well as serum urea, creatinine and ACR in women with and without diabetes normalises within the 6 months following delivery. The rate of $eGFR$ normalisation post-delivery were also similar between the groups of women.

However, one of the notable observations was that although there was no difference in median $eGFR$ at 6 months post-delivery, at the 24 months mark, there was a significant

decline in median eGFR in women with type 1 diabetes compared to pre-pregnancy levels. Median eGFR for women with type 1 diabetes was also much lower than healthy controls. Of interest, in both women with type 1 and type 2 diabetes, those with a baseline $\text{eGFR} \geq 90 \text{ ml/min/1.73m}^2$ did not have a reduction in their post-pregnancy renal function. In contrast, the subset of women with an $\text{eGFR} < 90 \text{ ml/min/1.73m}^2$ at baseline had significantly lower post-pregnancy eGFR. As this finding is based on exploratory analysis with our study not powered for further sub-analysis, it is not known if those with a greater degree of pre-existing renal impairment may have skewed our results. Nevertheless, this suggest that pregnancy may have a long-term impact on renal function in women with diabetes and a lower renal function reserve, which is consistent with a few small case cohort studies[114, 115].

To assess potential confounding factors that may affect renal function returning to baseline, we studied the effect of age, duration of diabetes and HbA1c. There was a lack of post-pregnancy data on body mass index and blood pressure to include these factors into the model. Although age and duration of diabetes does not appear to affect the event rates of eGFR returning to baseline, there are many unaccounted factors that could influence the decline in renal function, including HbA1c which was observed to rise accordingly post-delivery. Interestingly, compared to women with type 2 diabetes, the rate of pre-existing hypertension and blood pressure at baseline was lower in type 1 diabetes. Perhaps these women with type 1 diabetes received better blood pressure care post-delivery as a protective mechanism to eGFR decline. Nonetheless, diabetes is a progressive disease even in the absence of pregnancy. The interactions in the risk of worsening renal function in women with diabetes is complex and the lack of data on confounding factors do not allow us to assess the interaction with eGFR. Our observation of renal function decline at 24 months post-pregnancy contrasts with one of the major type 1 diabetes trials (The Diabetes and Complication Trial, DCCT) which prospectively followed up 270 pregnancies on an average of 6.5 years[103]. The DCCT trial did not observe any changes in nephropathy rates, however, nephropathy was defined by the presence of albuminuria, and not based on GFR changes. The strict research setting where women received intensive treatment for glycaemia and blood pressure management even prior to conception as part of pregnancy planning care would have potentially a protective effect on subsequent renal function

The effect of pregnancy on other renal indices

With regards to other renal indices, consistent with the known physiology of pregnancy, serum creatinine decreased significantly during pregnancy but returned to baseline shortly post-delivery. Urinary creatinine clearance rose accordingly in pregnancy and returned to baseline by 6 months. However, creatinine clearance was noted to peak in the first and not second trimester of pregnancy. Although urinary creatinine clearance is one of the most reliable method of measuring renal function, drawbacks including incomplete timed collection that can lead to potentially inaccuracy in observations.

Interestingly, ACR did not significantly increase in pregnancy in either type 1 or type 2 diabetes. In this small exploratory case series, there were five cases of pre-eclampsia in type 1 diabetes and ten cases in type 2 diabetes. Associated with this was a modest rise in ACR in type 2 diabetes up to 5-fold up to 6 months post-delivery, but ACR remained within normal limits for most women. This is in contrast with previous reports on women with insulin dependent diabetes, in which significant increase in urinary albumin excretion occurs not only in those with pre-existing albuminuria, but also in women with normo-albuminuria, up to 4-fold in pregnancy. Our study demonstrating no long-term effect on albuminuria is in keeping with the Diabetes Control and Complication Trial (DCCT) of 180 women followed up for 6.5 years [103]. Notably, these women were under strict trial protocol and were assigned to intensive therapy even prior to conception which could affect renal outcomes. Other studies have also found that albuminuria tend to normalise by 6-12 weeks post-delivery [111, 371]. However, the incidence of pre-eclampsia, which can affect urinary albumin concentration was not documented in these studies. Our findings were also limited by the small number of women who had albumin to creatinine ratio measured. Presumably, those who did have albuminuria status monitored had a component or are at higher risk of diabetic kidney disease. Apart from descriptive statistics, albumin excretion rate (AER) values were excluded from the analysis due to insufficient number of observations. The huge increase in median AER up to 164 (2, 981) $\mu\text{g}/\text{min}$ observed the second trimester in type 1 diabetes was mainly due to results from a single participant with pre-existing diabetic kidney disease who developed pre-eclampsia in pregnancy.

Study Limitations

Apart from the above-mentioned limitations, as with the existing literature, accurate measurement of renal function in pregnancy and diabetes was a limitation. Although CKD-EPI has been shown to underestimate true GFR in pregnancy and in diabetes [8, 117, 317-319], it remains one of the most widely used prediction equation clinically. Hence, despite the potential of not reflecting true GFR, our findings may have some applicability clinically. Biochemistry obtained from different pathology services may also contain some degree of inter-assay variability. Nevertheless, we selected only the five major pathology service within the state, and inter-laboratory calibration is tightly regulated by state-wide pathology accreditation.

Women from our cohort were selected from a single tertiary obstetric hospital, and notably no women had a baseline eGFR < 60 ml/min/1.73m², hence results are not likely applicable to those with more severe DKD. Selection bias may also exist by excluding women with two or less post-pregnancy eGFR readings. By doing so, we reduced our sample size from 316 to 127, and may have inadvertently collected results from women who had underlying medical issues warranting frequent biochemistry monitoring. However, inclusion of multiple eGFR readings (as a time series variable) was important to enable accurate data collection, rather than relying on a single datapoint. In addition, due to the retrospective nature of this study, we did not have access to potential confounding factors including blood pressure and medications. We were however able to cross check biochemistry with urinary microscopy and culture to ensure that renal indices were not affected by acute urinary tract infection.

Overall, despite the limitations, this is one of the largest observational studies to investigate the effects of pregnancy on renal function in women with both type 1 and type 2 diabetes, and the only known study to compare renal function in women with and without diabetes during and post- pregnancy.

7.5 Final Considerations and Implications

Our study demonstrates that in general, women with type 1 and type 2 diabetes including those with mild to moderate DKD are not at risk of accelerated renal function decline from pregnancy. Being aware that eGFR does not decline, and the rate of eGFR recovery post-delivery in women with diabetes is no different than those without diabetes provides additional evidence for clinicians to guide diabetes care.

This study also highlights the attenuation of the physiological rise in pregnancy related hyperfiltration in women with type 1 diabetes and lower baseline renal function. It is not known if inflammation or oxidative stress in diabetes could potentially contribute the lack of eGFR rise. This warrants further investigation, particularly when the underlying mechanism of hyperfiltration in pregnancy and diabetes is not clearly understood.

Based on the findings of this retrospective study, we conducted a prospective study detailed in the following chapter (Chapter 8-Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy) , to gain more insight into the renal trajectory and associated renal biomarkers in pregnancies affected by type 1 and type 2 diabetes.

Study Three

Chapter Eight

Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy

8.1 Introduction

Renal hyperfiltration occurs both in early diabetes, as well as normal pregnancies. Whilst the potential pathological significance of renal hyperfiltration leading to raised glomerular pressure in diabetes remains debatable; hyperfiltration in pregnancy is considered a physiological adaptation. Currently, it is not known whether pregnancy have an added effect on the initial increased GFR that is seen in diabetes, or if there is a limit to the kidneys' ability to hyperfiltrate. We are unaware of any studies that have measured glomerular filtration rate during pregnancy in women with diabetes, with and without pre-existing hyperfiltration. Comparison of the presumed pathological state in diabetes to the physiological response in pregnancy may facilitate our understanding of the significance of hyperfiltration in diabetes.

This was an exploratory prospective study which aimed to compare hyperfiltration in pregnancy to hyperfiltration in diabetes in women with and without pre-existing hyperfiltration; and to elucidate the relationship between inflammatory biomarkers and hyperfiltration in pregnant women with and without diabetes. Of note, renal function was initially determined by plasma inulin clearance, but this approach was halted prematurely due to allergic reactions to renal function. Renal function was therefore determined by eGFR.

Outcome Measures:

The primary outcome measure was renal function as quantified with eGFR based on the Chronic Kidney Disease Epidemiology (CKD-EPI) formula during pregnancy.

Secondary outcome measures assessed the relationship between clinical, biochemistry and serum/urinary inflammatory renal biomarkers and eGFR during pregnancy.

Specific Testable Hypotheses:

Compared to healthy control pregnant women, pregnant women with type 1 or type 2 diabetes will have less capacity to achieve peak pregnancy eGFR. Hyperfiltration is associated with an increased inflammatory and oxidative stress as measured by serum and urinary renal biomarkers.

8.2 Methods

Study Design

This is an exploratory, prospective feasibility study with longitudinal follow up of pregnant women with and without type 1 and type 2 diabetes.

Inclusion criteria for healthy controls:

1. Well-controlled blood pressure on study entry, defined as a systolic blood pressure below 140mmHg and diastolic blood pressure below 90mmHg
2. Age less than 40 years old
3. Normal renal function based on estimated GFR (i.e. $eGFR \geq 90 \text{ mL/min/1.73m}^2$ and no pre-existing albuminuria)

Inclusion criteria for participants with diabetes:

1. Well-controlled blood pressure on study entry, defined as a systolic blood pressure below 140mmHg and diastolic blood pressure below 90mmHg
2. Age less than 40years old
3. Type 1 or type 2 diabetes mellitus based on clinical diagnosis and medical history.

Exclusion criteria:

1. Major systemic illness
2. Twin or triplet pregnancies
3. Miscarriage
4. Allergy to inulin
5. Gestational diabetes

Detailed clinical parameters, serum and 24-hour urinary collection were obtained at the following four time points (three during pregnancy and post-partum):

Trimester 1: 10-12 weeks

Trimester 2: 18-22 weeks

Trimester 3: 32-34 weeks

Post-pregnancy: 3- 9 months

For each time point, participants fasted from midnight, until completion of blood sample collections in the morning. Support and advice regarding management of oral hypoglycaemic agents and insulin administration during the period of fasting were provided by the PhD candidate who specializes in clinical diabetes management. Fasting blood glucose levels were obtained to confirm diabetes status of the participants. If the fasting blood glucose level was equal to or below 5.5mmol/L and patients had no known history of diabetes, these patients were recruited as healthy controls. If the fasting blood glucose were above 5.5mmol/L, and these patients were not known to have diabetes, they were excluded from the study and appropriate clinical follow up appointments were made.

This study was approved by Austin Health and Mercy Hospital for Women's Human Research Ethics Committee and individuals provided written informed consent.

Clinical Anthropometric Data

Clinical examination including weight, height and manual lying blood pressure were obtained at each visit were by me (a single investigator). Clinical data including age, medical co-morbidities, medication, duration of diabetes, obstetric complications, maternal and fetal outcomes were collected. Medical records were also manually reviewed during each visit to cross check clinical data.

Biochemical Data

Serum and urine collection were processed on the day of collection. Pathology tests including full blood examination, serum urea and electrolytes, and urinary electrolytes were performed at a single laboratory- Austin Health Pathology Services.

HbA1c

HbA1c was measured using a turbidimetric inhibition immunoassay on Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany), standardised to the International Federation of Clinical Chemistry reference method. The between-run CV was 2.5% and 1.5% for HbA1c values of 5.6% and 9.7%, respectively. Glucose was measured using the Glucose HK Gen.3 system (Roche Diagnostics, Mannheim, Germany) on

the Cobas c702 analyser (Roache Diagnostics, Mannheim, Germany). The intra-assay CV was 0.8%.

Renal Function Measurement

Renal function was measured with creatinine clearance and eGFR. Creatinine clearance was calculated based on 24-hour urine collection. Estimated GFR was calculated using the CKD-EPI formula. Detailed calculation of estimated GFR based on the CKD-EPI formula and 24-hour creatinine calculation is detailed in Chapter 6: Evaluating renal function in diabetes and pregnancy with inulin plasma clearance.

As it was not feasible and practical to obtain pre-pregnancy results with only two participants recruited over a three-year period, *post-partum renal function* arbitrarily served as the surrogate pre-pregnancy renal function. Of note, as per findings in Chapter 7: Pregnancy related renal function trajectory in women in type 1 and type 2 diabetes, we demonstrated no significant differences in eGFR pre- and 6 months post-partum in women with and without diabetes.

Therefore, in the current study, the *degree of hyperfiltration, or change in peak filtration* was calculated as follows

= Post-partum renal function -Trimester 2 renal function.

Pre-pregnancy renal hyperfiltration in diabetes is arbitrarily defined as an eGFR > 120ml/min/1.73m².

Biomarkers Assays

A total of seventeen markers were assayed in the serum and urine. Biomarker assays were performed on stored samples. All collected serum samples were centrifuged at 3500xg for 10min at 4°C. Serum and plasma were divided into aliquots of at least 1ml and frozen in 2ml cryostorage vials at -80°C. For urine, a 10ml aliquot from a 24hr urine collection had 50uL 5M NaOH added to better preserve albumin for long term storage, before dividing into 2x 5ml aliquots & freezing at -80°.

The following markers were assessed in serum: C-reactive protein (CRP), Neutrophil to Lymphocyte ratio (NLR), soluble receptor for advanced glycation end products (sRAGE), soluble tumour necrosis factor receptor type 1 / 2 (sTNFR1/2), monocyte chemoattractant protein 1(MCP1/ CCL2), total 8-Isoprostane (8IP), neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule (KIM-1), soluble Interleukin-2 Receptor (sIL2R) and relaxin.

The following markers were assessed in urine: Soluble tumour necrosis factor receptor type 1 / 2 (sTNFR1/2), monocyte chemoattractant protein 1(MCP1/ CCL2), total 8-Isoprostane (8IP), neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule (KIM-1).

C-reactive protein (CRP)

CRP measurements were undertaken on the Roche Cobas C8000 and measured using an immunoturbidimetric assay, with sensitivity very close to high sensitivity assays.

Neutrophil to Lymphocyte ratio (NLR)

Flow cytometry was performed using a semi-conductor laser and hydrodynamic focusing to determine neutrophil and lymphocyte counts to obtain NLR.

The following kits were utilized to measure *serum* analytes:

Merck Millipore Luminex HSCRMAG-32K-4plex: sTNFR 1 and 2, sRAGE, sIL2R

R&D systems LXSAM-03: MCP1, KIM1

Enzo Life Sciences ADI-900-091: 8-Isoprostane

R&D systems DLN20: NGAL

The following kits were utilized to measure *urinary* analytes:

R&D systems Quantikine ELISA assay (DRT100 and 200): TNFR1 and 2

R&D systems DCP00: MCP1

R&D systems LHK000, LHK1757, LHK1750: KIM1, NGAL

Enzo Life Sciences ADI-900-010: 8-Isoprostane

Invitrogen BMS212INST: sIL2R

Assays were performed according to manufacturer's guidelines. The intra- and inter-assay coefficient variation for the panels above were all $\leq 5-15\%$

Statistical Analysis and Power

This is an exploratory feasibility study. A total of 50 pregnant women were recruited into the study: 15 healthy women, 35 women with Type 1 and type 2 diabetes. The power of this study was based on mean coefficient of variation for total body clearance with the single injection method of inulin clearance is 3.9% [372]. For unpaired data (comparison of diabetes versus controls groups), we estimate that a difference of 1.25 standard deviations will be clinically significant. Therefore, to attain 80% statistical power at 5% probability, 24 subjects needed to be studied [373]. For paired data (comparison of intra-pregnancy and post-pregnancy data in same subjects), we estimate that a difference of 1.0 standard deviation will be clinically significant. Therefore, to attain 80% statistical power at 1% probability, 13 subjects will need to be studied per group.

Group comparisons at different time points were performed using Chi-Square tests and Fisher exact tests for categorical variables, and Mann-Whitney U tests and Kruskal-Wallis rank tests for continuous variables. Quantile regression was used to assess the degree of hyperfiltration or change in peak eGFR between groups. Post-pregnancy eGFR was included in the model. Other confounding risk factors were not included due to co-linearity of the data. Inclusion of age, body mass index, HbA1c and eGFR (at the second trimester and post-pregnancy time-point) into the statistical model yielded a mean variance inflation of factor of 7.3, and a condition number 91.6, suggesting high co-linearity.

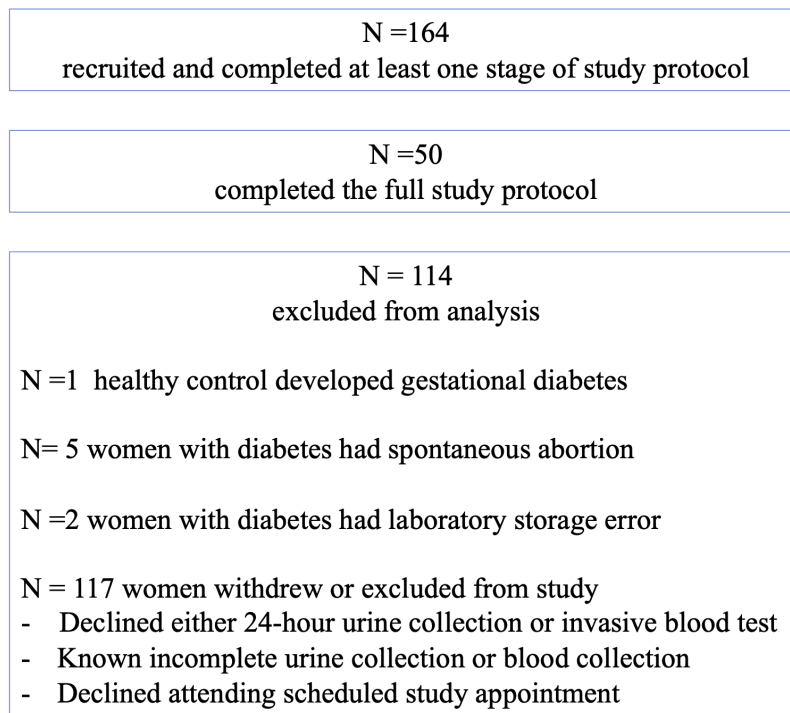
Urinary marker concentrations were corrected for urinary excretion rate using urinary volume over a 24-hour timed collection. All markers (apart from NLR) were log-transformed for statistical analysis. The relationship between renal function with renal biomarkers were assessed with Spearman rank-order correlation. For analysis of serum and urinary biomarkers, a p-value of < 0.003 was considered significant based Bonferroni corrections.

Recruitment

Over the three-year period of active recruitment, all obstetrics referrals to the Mercy Hospital for Women (MHW) were screened to recruit healthy controls. On an average, on a weekly basis, five to ten patients met the study eligible criteria and were invited to participate. Participants with type 1 and type 2 diabetes were recruited via the high-risk antenatal diabetes clinic at the Mercy Hospital for Women.

164 women were successfully recruited and commenced the study protocol (Figure 8.1). 50 participants completed all of the procedures within the protocol and were included in the final analysis. 114 participants who completed at least one stage of the study at a single time point (including clinical assessment, blood and urine collection) were excluded from the analysis. Out of these, one healthy control developed gestational diabetes on routine third trimester oral glucose tolerance test, five participants with pre-gestational diabetes had spontaneous abortion, two participant's blood and urine sample from the second trimester were not stored for analysis due to laboratory technical error. The remaining participants either withdrew from the study or had incomplete blood or urine collection. The main reason that accounted for withdrawal and incomplete sample collection was the need for invasive blood test and 24-hour urine collection which participants found cumbersome. In addition, many participants reported difficulty in attending the scheduled study appointments due to the lack of transport, being the only caregiver for young children and the lack of motivation at the later stages of pregnancy in participating in research studies.

Figure 8.1 Participant recruitment and withdrawals



To minimize withdrawal rate and incomplete sample collection, enrolled participants were contacted at two weeks, one week and again two days prior to the trimester specific clinical assessment and sample collection. Follow up contact and re-scheduling were made at least on three occasions if participants failed to attend the study appointment. In addition, I collected the completed 24 hour-urine collection bottle and conducted clinical assessment where it was most convenient for the participant if they were unable to attend the scheduled study appointment at the research center. However, blood sampling was not ethically approved to be conducted outside of the research/pathology collection center.

Amendment of Study Protocol

Two notable revisions of the study protocol were made. Firstly, the decision to use eGFR as a measurement of renal function instead of measured GFR based on plasma inulin clearance, which had to be terminated due to allergic reactions. This is discussed in Chapter 6: Evaluating renal function in diabetes and pregnancy with inulin plasma clearance.

Secondly, the initial protocol included pre-pregnancy data collection. However, the number of successful recruitments with pre-pregnancy data was very low (N=2 over three years). This was despite the attempts of recruiting participants via the following processes:

- i) General practitioners in the region of North East Valley Division of General Practice were advised about the study. Physical visits were made to medical practices to discuss the study in detail to encourage women to participate.
- ii) Patients who have had a previous pregnancy at the MHW, or those who attended the Austin Health diabetes clinic and meet the inclusion criteria were contacted via mail from a database to invite them to participate in the study, should they contemplate future pregnancies. Follow-up phone calls were made to improve the rate of participation.
- iii) Posters were advertised via hospital and community forums (including local Banyule council, local libraries, web-based companies)

Due to lack of feasibility of data collection, it was determined futile to continue the study to incorporate data from the pre-pregnancy time point. Hence, the protocol was amended to include women with a starting data point from the first trimester of pregnancy.

8.3 Results

Baseline characteristics and Maternal Outcomes (Table 8.1)

50 participants were included in the final study analysis. There were 36 participants with type 1 (n=21) or type 2 (n=15) diabetes, and 14 healthy controls. Apart from women with diabetes having a higher BMI (26 vs 24, $p=0.01$), there were no significant differences in baseline characteristics for women with diabetes compared to controls. Specifically, there were no statistically significant difference in age, blood pressure, and aspirin use between the control group and the diabetes group. Use of renin-angiotensin receptor inhibitors and statin were low in diabetes and as expected, there were no pregnant women on these medications.

There were no significant differences between healthy controls and women with diabetes for the four main obstetrics maternal outcomes of pre-eclampsia, pregnancy induced hypertension, postpartum hemorrhage and emergency cesarean delivery.

Table 8.1 Maternal Characteristics of women with and without diabetes during trimester 1

	Type 1 and Type 2 Diabetes	Healthy Control	<i>P</i> value
<i>n</i>	36	14	
<i>n</i> (type 1 vs. type 2 diabetes)	21 vs.15		
Age (years)	34 (29, 37)	33 (30, 38)	0.7
BMI (kg/m ²)	26 (24, 31)	24 (22, 25)	0.01
Smoking at conception	7 (19.4)	1 (7.1)	0.3
Duration of diabetes (years)	8 (2, 15)	NA	
HbA _{1c} (%)	6.7 (5.9, 8.1)	NA	
HbA _{1c} (mmol/mol)	50 (41, 65)	NA	
Systolic blood pressure	110 (104, 120)	120 (100, 108)	0.3
Diastolic blood pressure (mmHg)	65 (60, 70)	67 (60, 70)	0.8
Serum Urea (mmol/l)	3.0 (2.8,4.7)	3.3 (2.6,3.6)	0.2
Serum creatinine (μmol/l)	51 (47, 59)	50 (46, 58)	0.6
CKD-EPI eGFR	123 (113, 127)	125 (120, 128)	0.3
Urinary creatine	143 (114,174)	(129,159)	1.0
Urinary albumin creatinine ratio	0.8 (0.6,4.1)	0.7 (0.4,0.9)	0.2
Pre-existing hypertension	3 (8.3)	0 (0)	0.3
Renin angiotensin aldosterone System Inhibition at conception	3 (8.3)	0 (0)	0.3
Statins therapy at conception	0 (0)	0 (0)	-
Metformin therapy at	4 (11.1)	0 (0)	0.2
Aspirin therapy at	2 (5.6)	0 (0)	0.4
Microvascular complications	4 (11.1)	0 (0)	0.2
Macrovascular complications	0 (0)	0 (0)	-
Data expressed median (interquartile range) or n (%)			
Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables			
Body mass index = BMI; glycosylated haemoglobin = HbA _{1c} ; Chronic Kidney Disease			
Microvascular complications includes diabetic kidney disease, diabetic retinopathy, peripheral neuropathy and peripheral vascular disease			
Macrovascular complications include ischaemic heart disease and cerebrovascular accidents			
Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR			

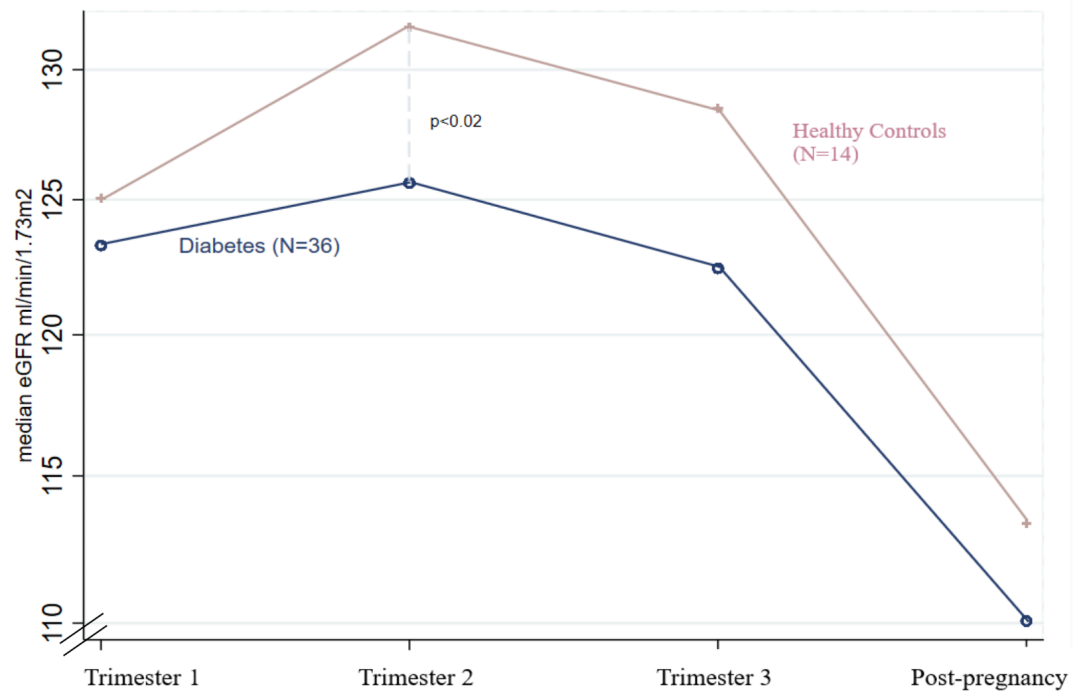
Renal Trajectory pre-, during and post pregnancy (Table 8.2 and Figure 8.2)

Median eGFR peaked in the second trimester of pregnancy for women with and without diabetes. The peak median eGFR was significantly lower ($p = 0.02$) in women with diabetes 126 (118,132) ml/min/1.73m² compared to those without diabetes 132 (128, 137) ml/min/1.73m².

Table 8.2 Estimated eGFR in women with and without diabetes

	Diabetes	No Diabetes	p- value
CKD-EPI eGFR (ml/min/1.73m²)			
Trimester 1	123 (113,127)	125 (120,128)	0.3
Trimester 2	126 (118, 132)	132 (128,137)	0.02
Trimester 3	122 (117,130)	128 (121,139)	0.06
Post-Pregnancy	110 (97,123)	113 (93, 118)	0.9
Data expressed median (interquartile range). Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR			

Figure 8.2 Renal Trajectories (estimated GFR) during and post pregnancy in women with and without diabetes.

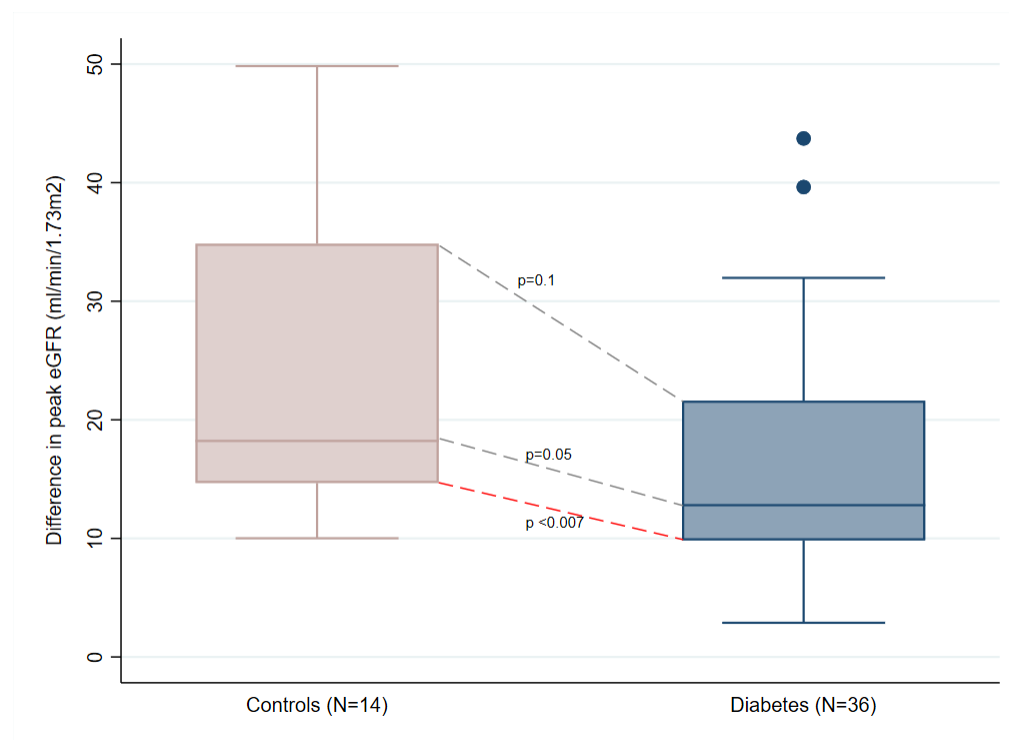


Change in peak eGFR in pregnant women with and without diabetes compared to baseline (post-partum) values

To determine the change in peak eGFR (*post-partum – trimester 2*), post-partum renal function was factored into quantile regression analysis.

Change in peak eGFR, (Figure 8.3) was significantly different between diabetes and healthy controls at the 25th centile (co-efficient -6.25, 95CI -12.47, -0.25, $p=0.049$), i.e. women with diabetes had a 6.25 ml/min/1.73m² less increase in eGFR compared to healthy women. At the 50th centile (median), there was no statistical difference between the healthy controls and diabetes group (co-efficient -4.84, 9CI -13.6, 3.89, $p=0.3$). Similarly, in the 75th centile, although there appeared to be a greater difference in eGFR change (co-efficient: -9.26, 95CI -24.11, 5.59, $p=0.2$), this was not significant due to the large confidence intervals around this difference.

Figure 8.3 Change in estimated GFR from baseline to second trimester in women with and without diabetes



Renal Biomarkers

Renal biomarkers concentration in women with and without diabetes during and post pregnancy

For the diabetes cohort, when comparing basic characteristics in the second trimester to post-pregnancy, HbA1c was lower at 6.0 (5.5, 6.8) % in the second trimester compared to 6.7 (5.9, 8.8) % in the post-pregnancy period. Blood pressure was not statistically different in the second trimester compared to post-pregnancy.

In terms of renal biomarkers, CRP, NLR, ssIL2R, sTNFR1, sTNFR2, uTNFR1, uTNFR2, uMCP1, and sKIM1 were significantly elevated in second trimester compared to post-pregnancy (Table 8.3). In contrast, the only marker that was elevated in the second trimester compared to post-pregnancy was sMCP1 (Figure 8.4).

For healthy controls, (Table 8.4) when comparing basic characteristics in the second trimester to post-pregnancy, BMI was higher in the second trimester at 25.1(23.0, 26.5) kg/m² compared to post-pregnancy BMI of 23.5(22.2,25.6) kg/m². There was no difference in blood pressure.

In terms of renal biomarkers, NLR value, sTNFR2, and sKIM1 concentration were elevated in the second trimester compared to post-pregnancy. Paired serum and urinary MCP1 concentrations were higher in the post-pregnancy period compared to second trimester of pregnancy (Figure 8.4)

When comparing women with and without diabetes, post-pregnancy sKIM1 concentration doubled in women with diabetes 42(22,61) pg/ml compared to healthy controls 22 (12,33) pg/ml; p=0.002 (Figure 8.5). There were no significant differences between women with and without diabetes for the rest of the renal biomarkers in the second trimester of pregnancy or at the post-pregnancy period.

Table 8.3 Levels of renal biomarkers in women with diabetes

	<i>Trimester 2</i>	<i>Post-Partum</i>	<i>P value</i>
<i>CRP</i>	4.4 (3.0,10.0)	2.0 (0.7,4.20)	<0.001
<i>NLR</i>	3.6 (3.1, 4.3)	1.7 (1.3,2.3)	<0.001
<i>sRAGE(pg/ml)</i>	78 (37, 82)	37 (29, 82)	0.2
<i>ssIL2R(pg/ml)</i>	662 (511, 906)	576 (425, 826.9)	0.01
<i>sTNFR1(pg/ml)</i>	1600 (1342, 1922)	1303 (990, 1837)	0.002
<i>uTNFR1(ng/24hr)</i>	3563(2162,4408)	775(502,1991)	<0.001
<i>sTNFR2(pg/ml)</i>	10002 (8294, 11509)	74934 (6319, 9759)	<0.001
<i>uTNFR2(ng/24hr)</i>	5234 (3783,7250)	3333 (2174,5026)	<0.001
<i>s8IP(ng/ml)</i>	121 (102, 150)	111 (86, 132)	0.004
<i>u8IP(ng/24hr)</i>	6142(4344,11492)	5527(4271,8564)	0.5
<i>sNGAL (ng/ml)</i>	99 (66, 127)	75 (56, 126)	0.06
<i>uNGAL (ng/24hr)</i>	13103(8667,28362)	14352(6789,22495)	0.01
<i>sMCP1(pg/ml)</i>	21 (19, 29)	37 (28, 43)	<0.001
<i>uMCP1(ng/24hr)</i>	286(229,422)	166(136,211)	<0.001
<i>sKIM1(pg/ml)</i>	55 (36,74)	42 (22, 61)	<0.001
<i>uKIM1(ng/24hr)</i>	819(120,1355)	757(484,1542)	0.9
<i>Relaxin (pg/ml)</i>	180 (134,259)	Undetectable	

Data expressed median (interquartile range). Bonferroni corrected p-value significant at < 0.003.

Group comparisons were performed using Mann-Whitney U tests

CRP: C-reactive protein, NLR: Neutrophil to Lymphocyte ratio, sRAGE: soluble receptor for advanced glycation end products, sIL2R: serum soluble Interleukin-2 Receptor, sTNFR1/2: serum soluble tumour necrosis factor receptor type 1 / 2; uTNFR1/2: urinary soluble tumour necrosis factor receptor type 1 / 2, s8IP: serum total 8-Isoprostane, u8IP: urinary total 8-Isoprostane, sNGAL: serum neutrophil gelatinase-associated lipocalin, uNGAL: urinary neutrophil gelatinase-associated lipocalin; sMCP1: serum monocyte chemoattractant protein 1, uMCP1: urinary monocyte chemoattractant protein 1, and kidney injury molecule (KIM-1), Relaxin: serum relaxin.

Table 8.4 Levels of renal biomarkers in women without diabetes

	<i>Trimester 2</i>	<i>Post-Partum</i>	<i>P value</i>
<i>CRP</i>	3.6 (2.5,4.6)	1.3 (0.5, 2.6)	0.3
<i>NLR</i>	3.2 (3.3, 4.2)	1.7 (1.7,2.0)	0.002
<i>sRAGE(pg/ml)</i>	37.2 (28, 72)	67.4 (37, 82)	0.04
<i>ssIL2R(pg/ml)</i>	485 (386, 540)	373 (281, 699)	0.1
<i>sTNFR1(pg/ml)</i>	1366 (1292, 1582)	1196 (1072, 1393)	0.03
<i>uTNFR1(ng/24hr)</i>	2872(2041,3354)	534 (190,1156)	0.005
<i>sTNFR2(pg/ml)</i>	8711 (7862, 9451)	6736 (5896, 8475)	0.001
<i>uTNFR2(ng/24hr)</i>	4653 (3826,5555)	2020 (1688, 2970)	0.008
<i>s8IP(ng/ml)</i>	118 (87, 133)	87 (82, 110)	0.01
<i>u8IP(ng/24hr)</i>	6309(4462,11978)	4364(2532,7617)	0.3
<i>sngal (ng/ml)</i>	104 (63, 143)	130 (87, 159)	0.6
<i>ungal (ng/24hr)</i>	17821(16763,23733)	7561(6535,8931)	0.003
<i>sMcp1(pg/ml)</i>	21 (17, 25)	33 (28, 44)	0.001
<i>uMcp1(ng/24hr)</i>	284(229,417)	178(163,207)	0.002
<i>sKIM1(pg/ml)</i>	38 (27,48)	22(12,33)	0.001
<i>uKIM1(ng/24hr)</i>	842(696,936)	942(757,1016)	0.1
<i>Srelaxin(pg/ml)</i>	288 (170,380)	Undetectable	-

Data expressed median (interquartile range). Bonferroni corrected p-value significant at < 0.003

.Group comparisons were performed using Mann-Whitney U tests

CRP: C-reactive protein, NLR: Neutrophil to Lymphocyte ratio, sRAGE: soluble receptor for advanced glycation end products, sIL2R: serum soluble Interleukin-2 Receptor, sTNFR1/2: serum soluble tumour necrosis factor receptor type 1 / 2; uTNFR1/2: urinary soluble tumour necrosis factor receptor type 1 / 2, s8IP: serum total 8-Isoprostane, u8IP: urinary total 8-Isoprostane, sNGAL: serum neutrophil gelatinase-associated lipocalin, uNGAL: urinary neutrophil gelatinase-associated lipocalin; sMCP1: serum monocyte chemoattractant protein 1, uMCP1: urinary monocyte chemoattractant protein 1, and kidney injury molecule (KIM-1), Relaxin: serum relaxin

Figure 8.4 Serum and urinary MCP1 concentration in women with and without diabetes

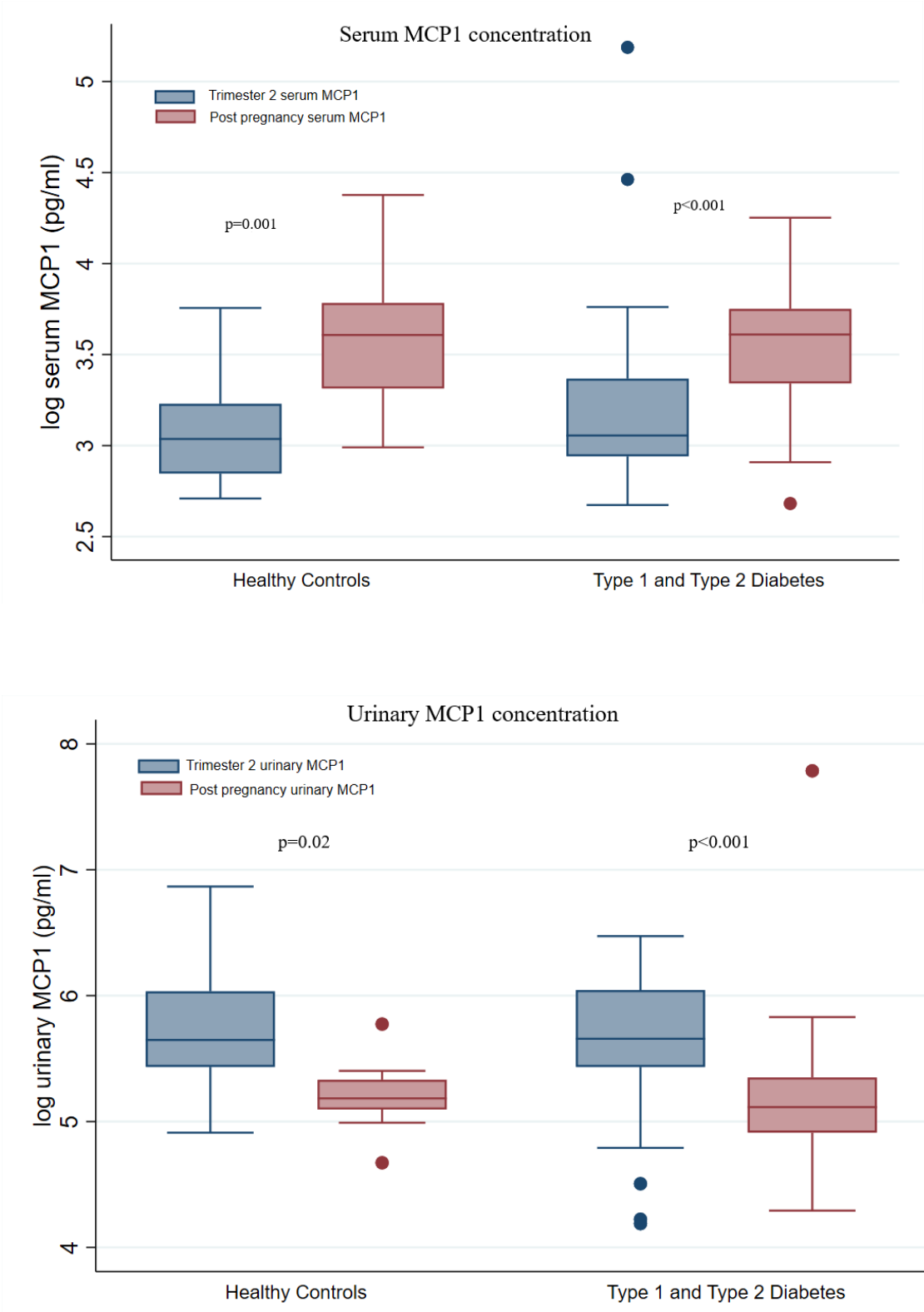
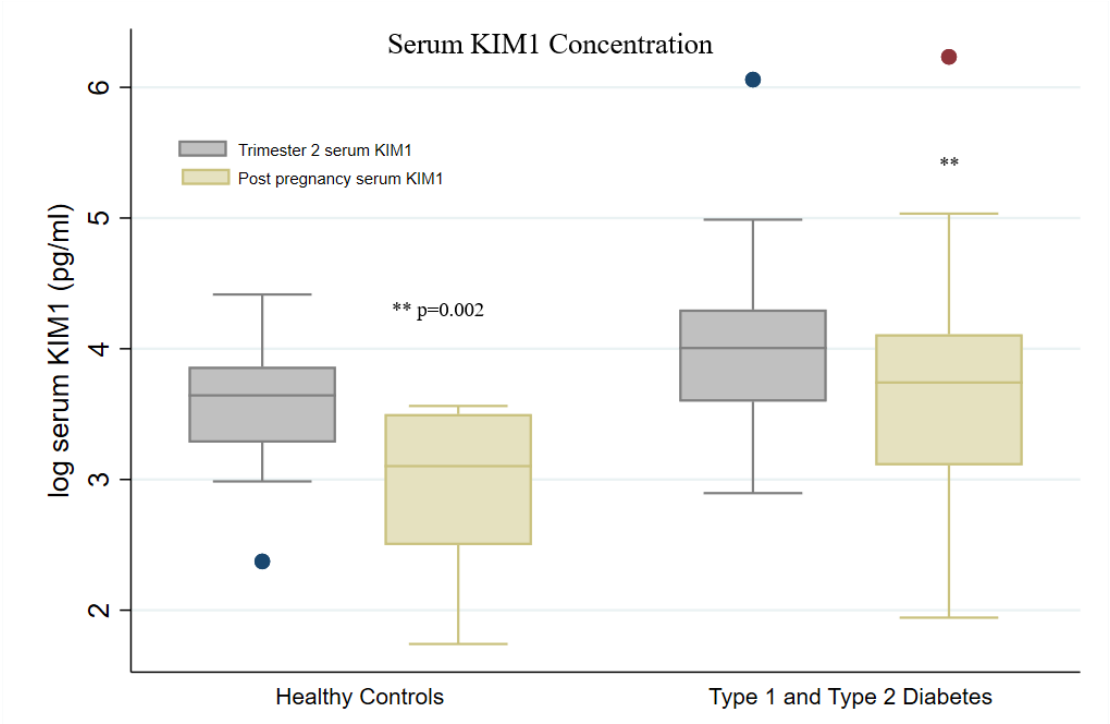


Figure 8.5 Serum KIM1 concentration in women with and without diabetes trimester 2 and post-pregnancy



Relationship between renal biomarkers and renal function (Table 8.5)

No relationship was observed between eGFR with any of the candidate biomarkers.

Table 8.5 Spearman correlation between estimated GFR and renal biomarkers

<i>Relationship with eGFR</i>	<i>Trimester 2</i>	<i>Post-Pregnancy</i>
<i>CRP</i>	0.1 (0.7)	-0.1 (0.5)
<i>NLR</i>	-0.1 (0.4)	-0.3 (0.05)
<i>sRAGE(pg/ml)</i>	-0.2 (0.3)	-0.01 (0.9)
<i>ssIL2R(pg/ml)</i>	-0.3 (0.05)	-0.2 (0.1)
<i>sTNFR1(pg/ml)</i>	-0.1 (0.5)	-0.02 (0.9)
<i>uTNFR1(ng/24hr)</i>	-0.4 (0.01)	0.1 (0.6)
<i>sTNFR2(pg/ml)</i>	-0.1 (0.7)	-0.2 (0.2)
<i>uTNFR2(ng/24hr)</i>	-0.3 (0.05)	0.1 (0.5)
<i>s8IP(ng/ml)</i>	0.1 (0.7)	-0.1 (0.6)
<i>u8IP(ng/24hr)</i>	0.02 (0.9)	0.2 (0.3)
<i>sNGAL (ng/ml)</i>	0.01 (1.0)	0.02 (0.9)
<i>uNGAL (ng/24hr)</i>	-0.03 (0.9)	-0.002 (1.0)
<i>sMCP1(pg/ml)</i>	-0.03 (0.9)	-0.2 (0.1)
<i>uMCP1(ng/24hr)</i>	-0.2 (0.2)	0.04 (0.8)
<i>sKIM1(pg/ml)</i>	-0.3 (0.06)	0.1 (0.5)
<i>uKIM1(ng/24hr)</i>	0.06 (0.7)	0.2 (0.1)
<i>Relaxin (pg/ml)</i>	-0.1 (0.5)	-

Spearman correlation with data expressed as rho (p value). Bonferroni corrected p-value significant at < 0.003. CRP: C-reactive protein, NLR: Neutrophil to Lymphocyte ratio, sRAGE: soluble receptor for advanced glycation end products, sIL2R: serum soluble Interleukin-2 Receptor, sTNFR1/2: serum soluble tumour necrosis factor receptor type 1 / 2; uTNFR1/2: urinary soluble tumour necrosis factor receptor type 1 / 2, s8IP: serum total 8-Isoprostane, u8IP: urinary total 8-Isoprostane, sNGAL: serum neutrophil gelatinase-associated lipocalin, uNGAL: urinary neutrophil gelatinase-associated lipocalin; sMCP1: serum monocyte chemoattractant protein 1, uMCP1: urinary monocyte chemoattractant protein 1, and kidney injury molecule (KIM-1), Relaxin: serum relaxin.

Subset Analysis within the Diabetes Cohort

Comparing women with pre-pregnancy diabetes and hyperfiltration to those without hyperfiltration. (Table 8.6, Figure 8.6)

For the diabetes cohort (N=36), subset analyses were conducted comparing those who had non-pregnancy (baseline) related hyperfiltration, to those who did not hyperfiltrate. Post-partum eGFR were considered surrogate pre-pregnancy/ baseline renal function. Hyperfiltration was arbitrarily defined as an eGFR >120 ml/min/1.73m².

10 participants had a baseline eGFR > 120ml/min/1.73m². The median eGFR was 125 (124,128) ml/min/1.73m² in those who were hyperfiltering, compared to 107 (86,112) ml/min/1.73m² in those who were not hyper-filtering (p<0.001). Apart from a younger age 28(23, 35) vs. 34 (31, 37) years p=0.02 in the hyperfiltering group, there were no significant differences in baseline characteristics (including BMI, systolic and diastolic blood pressure, smoking status, renin-angiotensin system inhibitors, aspirin, or statin use, as well as HbA1c and duration of diabetes) between those with and without hyperfiltration.

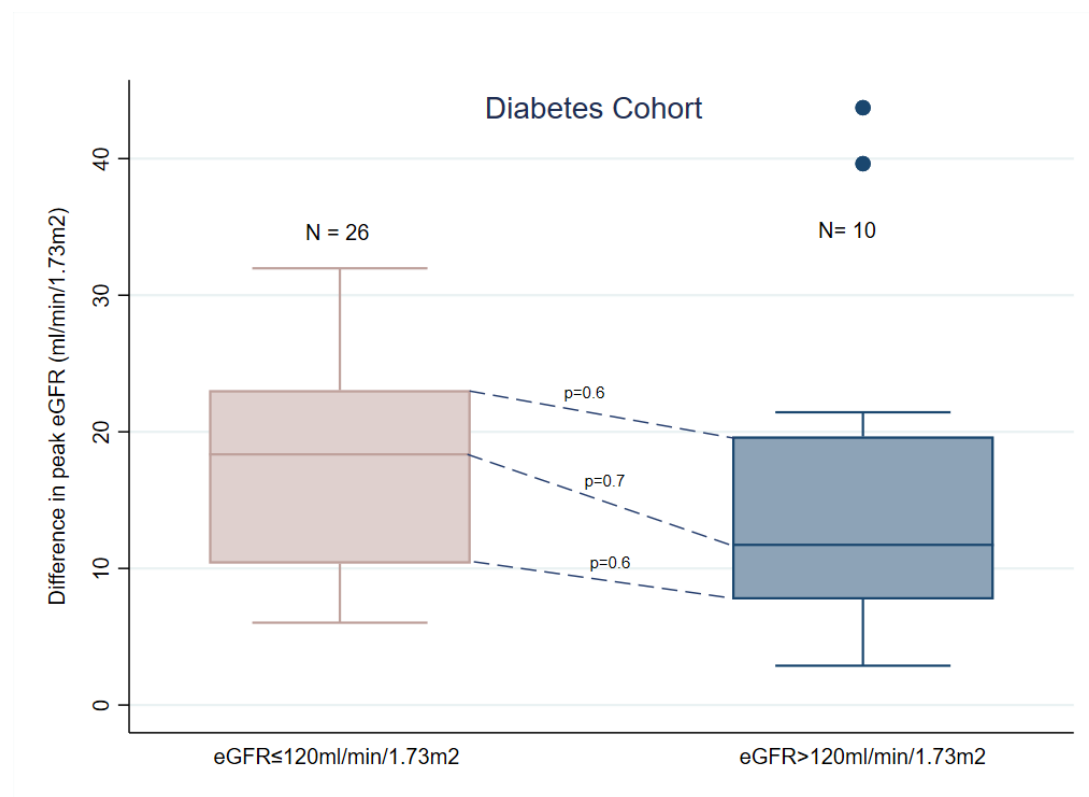
The peak median eGFR achieved was significantly lower 123(114,127) vs. 133 (131, 147) ml/min/1.73m² in the non-hyperfiltering group (Table 8.6).

Table 8.6 Median estimated GFR in those with and without pre-pregnancy hyperfiltration

	Diabetes eGFR ≤120 ml/min/1.73m²	Diabetes eGFR > 120 ml/min/1.73m²	P value
N	26	10	
First Trimester	116(108,123)	132(127,137)	<0.001
Second Trimester	123(114,127)	133(131,147)	<0.001
Third Trimester	119(113,124)	135(130,138)	<0.001
Post-Pregnancy	107 (86, 112)	125(123, 128)	<0.001
Data expressed as median (interquartile range)			

There were no significant differences in the *change in peak eGFR* achieved between both groups (Figure 8.6). Compared to those who were hyperfiltering, those who were not hyperfiltering had a non-significant 0.8ml/min/1.73m² less increment in eGFR within the 25th centile group (co-efficient: -0.8, 95CI [-4.9,3.4], p=0.7). In addition, those within the 50th and 75th centile had a non-significant eGFR increment in the hyperfiltering group by 2.1ml/min/1.73m² (co-efficient: 2.1, 95CI [-7.9, 12.1], p=0.7) and 4.0ml/min/1.73m² (co-efficient: 4.0, 95CI [-13.5,21.4], p=0.6) respectively.

Figure 8.6 Change in estimated GFR from baseline to second trimester in women with diabetes, for those with and without pre-existing hyperfiltration.



With regards renal biomarkers, Trimester 2 NLR values (3.2[2.9, 3.4] vs. 3.8[3.4,4.4], p=0.02) and post-partum sMCP1 values (29.2[22.8,38.5] vs. 38.7[29.3,44.5] pg/ml, p=0.04) were lower in the hyperfiltering group. There were no other significant differences in the other selected biomarkers between both groups.

Comparing women with diabetes and pre-pregnancy eGFR<90 ml/min/1.73m² to those with an eGFR≥ 90 ml/min/1.73m²

Further sub-analysis on women with a pre-pregnancy eGFR <90ml/min/1.73m² were performed. There were five women with diabetes (four with type 1 and one with type 2 diabetes) with a baseline eGFR < 90ml/min/1.73m². These women had longer diabetes duration 23(17,26) years vs. 7(2,12) years, p=0.03, and higher use of aspirin 40% vs. 3% (p<0.001) and RAAS inhibitors 40% vs 0% (p=0.01). There were otherwise no differences in baseline characteristics including age, BMI, systolic/diastolic blood pressure, HbA1c, smoking status and statin use between both groups.

Women in the lower eGFR group had lower median eGFR throughout the whole pregnancy and achieved a significantly lower median eGFR peak median in second trimester (Table 8.7). However, there were no significant statistical differences between the two groups in the *change in peak eGFR* (Figure 8.7). For the women with an eGFR<90 min/1.73m², the eGFR increment during pregnancy was non statistically less than those with an eGFR>90ml/min/1.73m² by 4.2ml/ min/1.73m² (co-efficient -4.2, 95CI [-1.8,9.6], p=0.9), 7.8ml/ min/1.73m² (co-efficient -7.8, 95CI [-23.2, 7.5], p=0.3) and 0.7 ml/min/1.73m² (co-efficient -0.7, 95CI [-21.3, 19.9], p=0.5) at the 25th, 50th and 75th centile respectively.

There were some differences in renal biomarker concentration, with the following markers all being significantly *higher* in the group with an eGFR<90ml/min/1.73m² (Figure 8.8). This includes, in the second trimester, serum sIL2R (6.8[6.8,6.9] vs. 6.2[5.6,6.5] pg/ml, p =0.01), serum sTNFR1 (7.5 [7.2,7.8] vs. 7.2[6.1, 7.4] pg/m, p=0.03), serum sTNFR2 (9.4[9.3,9.5] vs.9.0[8.6,9.2]pg/ml, p=0.003); and post-pregnancy serum sIL2R (6.7[6.5,./9] vs.6.0[5.1,6.3]pg/ml, p=0.007) and serum sTNFR2 (9.3[9.2,9.4] vs. 8.6[8.5,8.9]pg/ml, p=0.001).

Table 8.7 Median estimated GFR in women with diabetes stratified by eGFR < and ≥ 90 ml/min/1.73m²

	Diabetes eGFR <90 ml/min/1.73m ²	Diabetes eGFR ≥ 90 ml/min/1.73m ²	P value
N	5	31	
First Trimester	97 (93, 98)	123 (117, 128)	0.006
Second Trimester	99 (94,112)	127 (108, 127)	<0.001
Third Trimester	80(67, 104)	124 (119,130)	<0.001
Post-Pregnancy	78(74, 82)	113 (83, 113)	<0.001

Data expressed as median (interquartile range)

Figure 8.7 Change in estimated GFR from baseline to second trimester in women with diabetes stratified by pre-pregnancy eGFR < and ≥ 90 ml/min/1.73m²

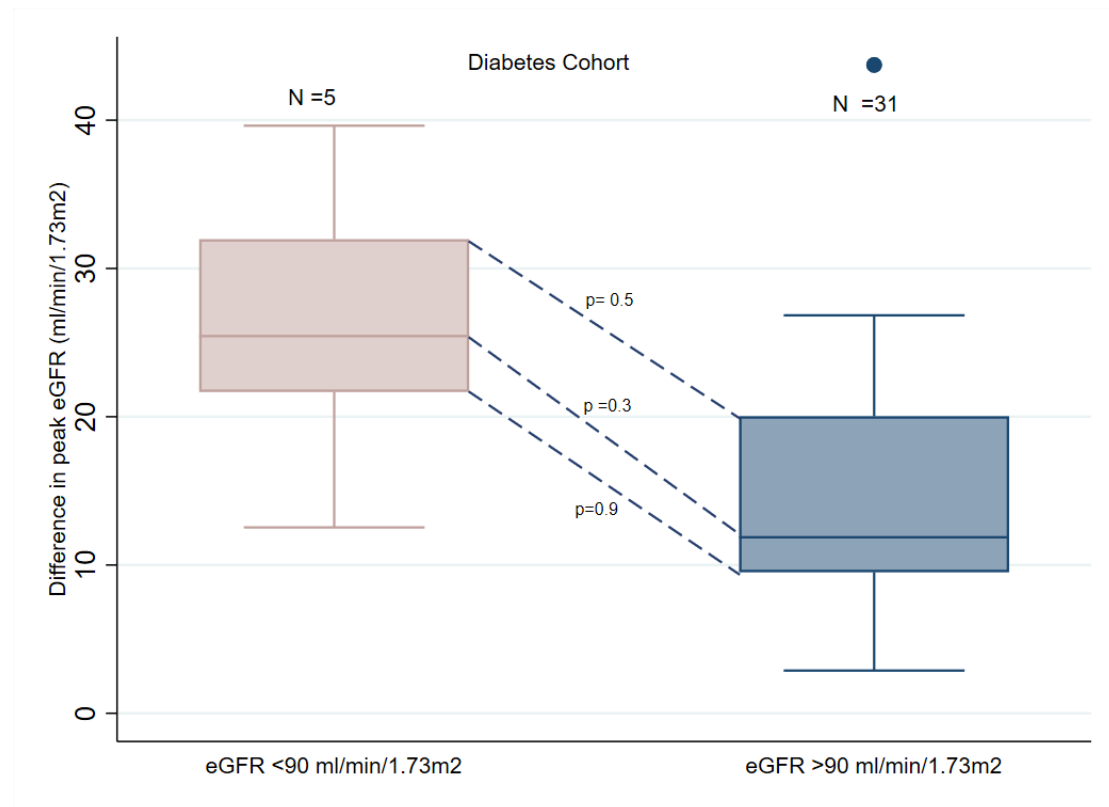
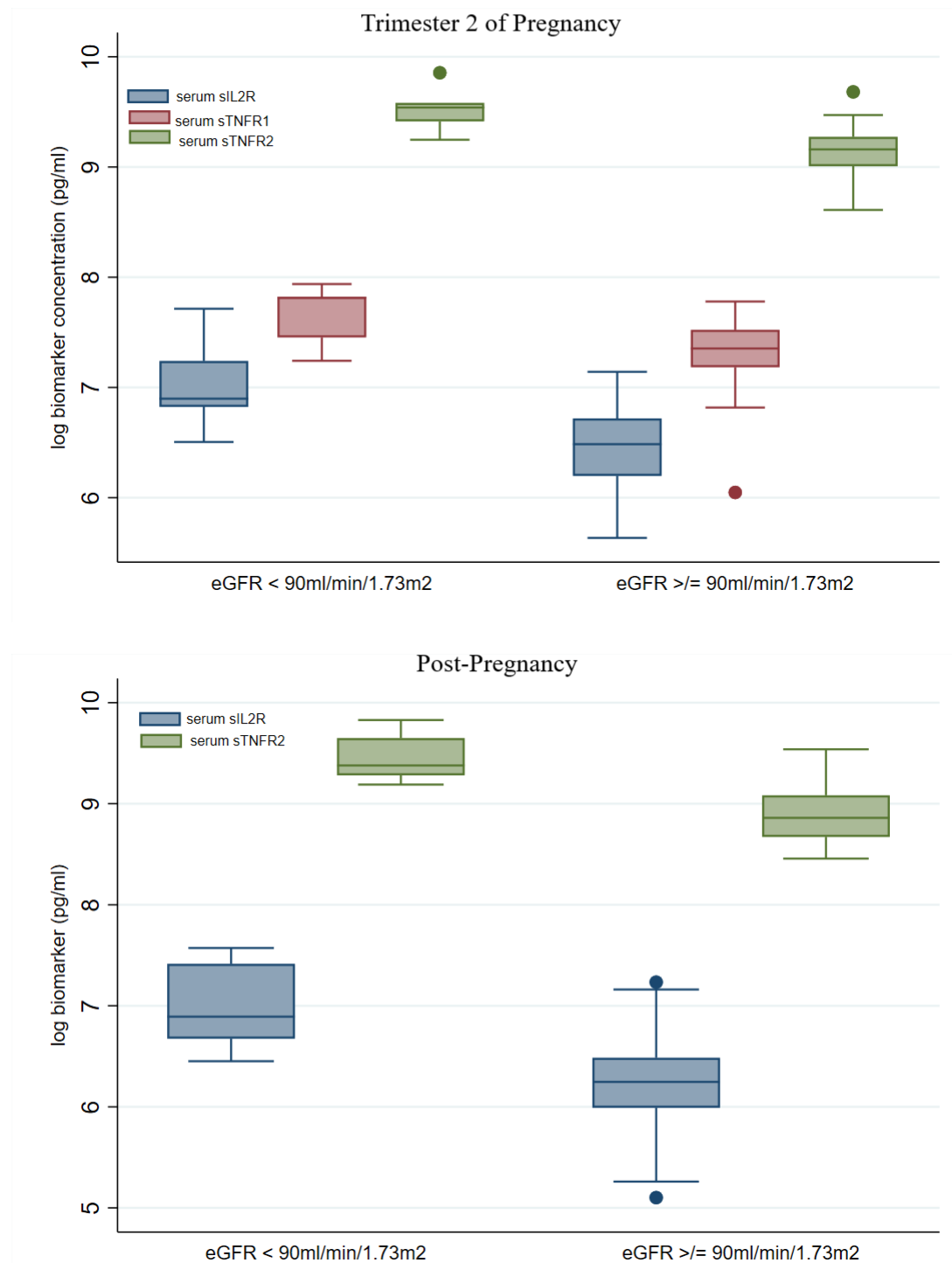


Figure 8.8 Selected renal biomarkers which demonstrated higher concentrations in women with diabetes eGFR < 90 ml/min/1.73m² compared to those with eGFR ≥ 90ml/min/1.73m²



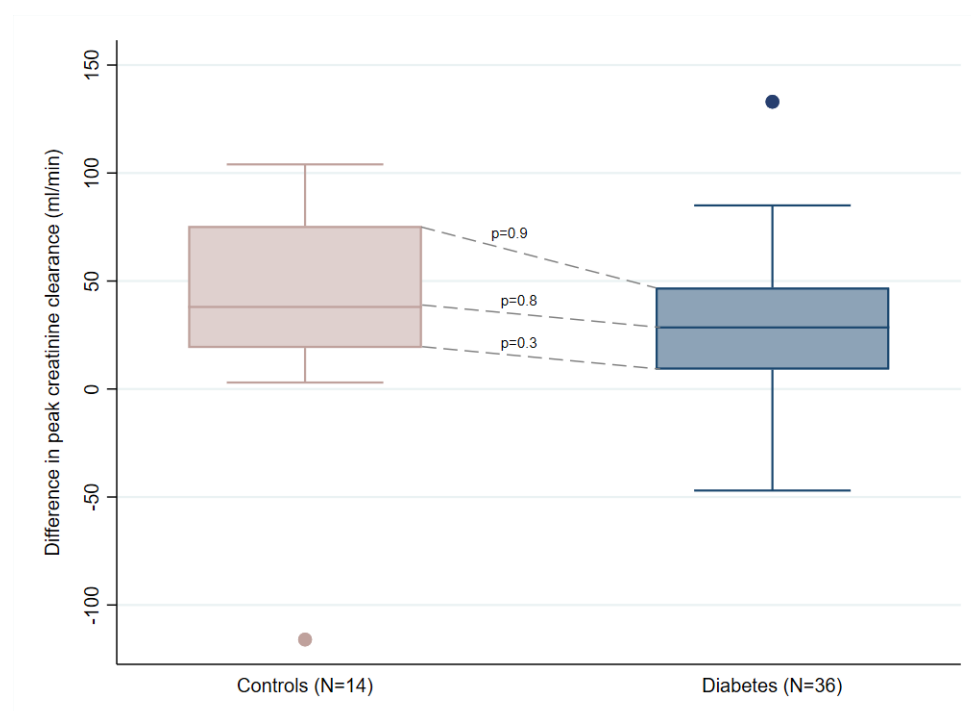
Additional Analyses using Creatinine Clearance as a surrogate marker of renal function during pregnancy

There were no significant differences in 24-hour urinary creatinine clearance between women with and without diabetes during and post pregnancy. The peak creatine clearance was achieved in the second trimester for women with 146 (112, 179) ml/min and without diabetes 151 (133, 180) ml/min.

For the change in peak creatine clearance, there were no significant statistical differences between healthy controls and diabetes cohort. The results were as follow: median (co-efficient -4.6, 95CI-36.6, 27.3, $p=0.8$); 25th centile (co-efficient-4.1, 95CI - 57.5, 49.4, $p=0.9$); and 75th centile (co-efficient -22.6, 95CI -67.5, 22.3, $p=0.3$). (Figure 8.9)

No relationship was observed between eGFR or creatinine clearance with any of the candidate biomarkers. Subset analysis on the diabetes cohort stratifying women based on creatinine clearance (hyperfiltration to those without hyperfiltration; creatinine clearance $\leq$ 90ml/min) were not conducted.

Figure 8.9 Change in creatinine clearance from baseline to second trimester in women with and without diabetes



8.4 Discussion

In this exploratory prospective feasibility study, the most pertinent findings of this study were:

1. Women with diabetes had reduced capacity to hyperfiltrate in pregnancy compared to healthy controls
2. The state of diabetic hyperfiltration was not associated with elevated levels of circulating markers of inflammation, suggesting that hyperfiltration may be physiological rather than pathological.

Whereas hyperfiltration in pregnancy is considered a normal physiological phenomenon, the significance of hyperfiltration in diabetes as a predictor of future development and acceleration of renal decline remains unclear. This study aimed to further explore the differences of hyperfiltration between diabetes and pregnancy, particularly to assess renal biomarkers with an aim to provide a better understanding into the mechanisms underlying the hyperfiltration in each case.

Although some case series have reported that women with diabetes may not demonstrate normal physiological rise in renal function during pregnancy [106, 107, 374], we know of no other studies that have investigated and directly compare eGFR in women with and without diabetes in pregnancy. However, as highlighted in our literature review, it should be noted that there are potential issues of utilizing eGFR in pregnancy.

Pregnancy related Renal Hyperfiltration

There was no difference in post-partum (baseline) median eGFR between women with, and without diabetes. As expected, eGFR peaked during the second trimester of pregnancy in both pregnant women with and without diabetes. We have demonstrated in our retrospective observational study that the median peak eGFR in the second trimester of pregnancy was no different in women with and without diabetes. This contrasts with this present study findings of the median peak eGFR being significantly lower in women with diabetes compared to healthy controls. It is not clear what contributes to this difference between both studies. One of the possible explanations is

that eGFR data that were included in the retrospective study may include intentional testing for a clinical indication. Hence, it may be possible that eGFR readings may have been monitored in high risk women with lower renal reserve, or from complications including pre-eclampsia which may reduce the overall median eGFR in the retrospective cohort. Secondly, although direct comparison between the two studies is not possible, the baseline median eGFR (110 vs. 114 ml/min/1.73m²) in women with diabetes was slightly lower in this study, with a similar median eGFR of 113 ml/min/1.73m² in the control group for both studies. It is possible that when women with diabetes start off with a lower eGFR, the peak eGFR achieved in pregnancy may be lower.

Another consideration when interpreting our data is the limitation of the CKD-EPI formula in estimating GFR. CKD-EPI eGFR has been demonstrated to underestimate GFR in type 2 diabetes, by a mean of around 12 ml/min/1.73m² in those with a measured eGFR > 90 ml/min/1.73m² [10]. By utilizing CKD-EPI eGFR, particularly at the higher renal threshold of hyperfiltration in pregnancy, we may be under-estimating true GFR in women with diabetes. Therefore, it is possible that the true peak GFR achieved in the women with diabetes in our study may be higher than the estimated value, and as a result there may not be a difference in median GFR compared to healthy controls. However, we would like to highlight that CKD-EPI formula also underestimates GFR in healthy pregnancies as well [319]. At present, there is no direct evaluation of the accuracy and bias of the CKD-EPI formula in pregnancies affected by diabetes. It is not known if the degree of GFR bias and precision in pregnant women with and without diabetes is similar. As outlined in Chapter 6: Measuring renal function in diabetes during pregnancy with plasma inulin clearance, we attempted to assess the accuracy of the equation in women with diabetes during pregnancy by comparing CKD-EPI eGFR to measured GFR with inulin. However, the study had to be terminated due to allergic reactions to inulin.

Nevertheless, we found that compared to healthy women, women with diabetes had a significantly less eGFR increment from baseline to peak eGFR in the second trimester of pregnancy by a total of 6.25 ml/min/1.73m². However, this was selectively only in the group with the least eGFR changes (25th centile) from baseline to the second trimester of pregnancy. On the other hand, in those who demonstrated a larger eGFR

change from baseline to second trimester (i.e. those within the median and 75th centile), there was no significant differences in the total eGFR increment comparing those with and without diabetes.

Our results suggest that women with diabetes have an attenuated ability to achieve peak eGFR in pregnancy, possibly due to pre-existing DKD and lower renal function to start off with. This suggestion is supported by findings from our retrospective study, where the subset of women with type 1 diabetes and no pre-existing hyperfiltering having a significantly lower median eGFR in the second trimester compared to those with type 1 diabetes and pre-existing hyperfiltration (123 versus 136 ml/min/1.73m²). Similarly, in this study, women with diabetes and no pre-existing hyperfiltration achieved a lower eGFR peak (123 versus 133 ml/min/1.73m²) compared to those without pre-existing hyperfiltration. In addition, women with diabetes and a lower baseline eGFR (<90ml/min/1.73m²) also had a significantly lower median eGFR throughout all three trimesters of pregnancy. Yet, although median eGFR was significantly different in the second trimester, when we analyzed the *change in peak eGFR*, there was no difference between the groups (eGFR<90 vs. ≥ 90 ml/min/1.73m²; and pre-existing hyperfiltration vs. no pre-existing hyperfiltration). In addition, as baseline eGFR was factored into quantile regression analysis, it does not support the hypotheses that peak eGFR may be dependent of baseline eGFR.

To investigate hyperfiltration and this subtle difference in renal trajectory during pregnancy, we measured relaxin levels. Relaxin is thought to result in reno-dilation and subsequent physiological hyperfiltration in pregnancy [375-377]. We found that relaxin was not detectable in the post-pregnancy phase. During the second trimester, concentration of relaxin was not significantly different between those with and without diabetes, nor did it differ in the diabetes cohort stratified by eGFR. In addition, there was no measurable differences in any of the candidate renal biomarkers between those with and without diabetes during pregnancy to potentially account for the difference in the attenuated ability of achieving peak physiological hyperfiltration in pregnancies affected by diabetes. We also found no significant associations between eGFR and any of the renal biomarkers (in those with and without diabetes) that could account for the potential renal changes in pregnancy.

Overall, in both the diabetes and healthy controls cohort, renal biomarkers tend to be higher in pregnancy compared to the non-pregnant state, in keeping with the view that pregnancy may be an inflammatory state [238]. Although the following four markers, NLR, sTNFR2, sKIM1, and urinary MCP1 were elevated in pregnancy for both cohorts, it is unlikely that any of the elevated markers in pregnancy would have contributed to physiological renal hyperfiltration though we can't infer causality as this is an observational study. It is likely that this elevation is consistent with the pro-inflammatory state of pregnancy. In a large longitudinal cohort study of 290 women, a large panel of seventy pro- and anti-inflammatory markers were assessed, and it was demonstrated there is a reduction of majority of markers in the post-partum period compared to third trimester in pregnancy [378]. However, similar to our study, the associations with pregnancy related adverse outcomes that can have a pro-inflammatory effect including pre-eclampsia, hypertension and medications were not analyzed. In both the diabetes and healthy control group, BMI was higher in pregnancy, and this could have potentially contributed to elevated levels as well.

Diabetes related Renal Hyperfiltration

We observed no changes to suggest that diabetes related hyperfiltration is a pro-inflammatory state. In fact, those with pre-existing hyperfiltration were found to have lower NLR in pregnancy and lower sMCP1 post-pregnancy. This is in contrast with the current limited studies examining renal markers in diabetes related hyperfiltration in type 1 [225, 227] and type 2 diabetes[228].

Examining inflammatory markers in hyperfiltration is of interest, as the pathological effect of hyperfiltration in the development of DKD is not clear. The current literature on diabetes related hyperfiltration have been largely based on type 1 diabetes with conflicting findings on the effect of developing albuminuria [11, 73, 75, 76], which itself does not necessary precludes decline in renal function. Other studies which demonstrate rapid decline in eGFR [78, 83, 379, 380] in those with hyperfiltration is limited by interpretation if the decline is a true decline in eGFR, or a regression to the mean. Nonetheless, since the commencement of this study, a recent well conducted non-pregnancy publication from the DCCT/EDIC that included a large cohort of type 1 diabetes participants (N=446 with 106 with hyperfiltration defined as measured GFR

in three different groups of >130 , > 140 and 150ml/min/1.73m^2) over 30 years using iothalamate GFR demonstrated that early hyperfiltration was not associated with subsequent risk of developing an $\text{eGFR}<60\text{ml/min/1.73m}^2$ [89].

As an increase in the urinary clearance may influence serum levels of inflammatory biomarkers in hyperfiltration, we assessed paired serum and urinary levels of TNFR1, TNFR2, 8-Isoprostane, NGAL, MCP1 and KIM1. Although we did not establish any positive correlation between any of the increasing urinary excretion rates and serum levels for any of the paired markers, we also found no significant differences in the excretion rate of the urinary biomarkers between those who were hyperfiltering and those who were not, both during and post pregnancy. Parallel to this, urinary proteomic data has also shown that overproduction, rather than dysfunctional renal handling may be responsible for the increase circulating inflammatory markers in advanced DKD [381].

Despite the lack of correlation demonstrated between eGFR and renal markers, we noted significantly higher renal biomarkers in the subgroup of women with diabetes and an $\text{eGFR}<90\text{ml/min/1.73m}^2$. This is consistent with our current understanding that systemic inflammation occurs in DKD. Additionally, we found that paired serum and urinary MCP1 had an opposite effect during and post pregnancy in both the diabetes and control group (Figure 8.3). Whereby, during pregnancy, serum MCP1 was significantly lower, and urinary MCP significantly higher. In contrast, post pregnancy, serum MCP 1 was significantly higher, whilst urinary MCP was significant lower. Although this pattern suggests that pregnancy hyperfiltration could potentially result in a higher excretion of MCP1 resulting in a higher urinary concentration but corresponding lower serum level, the lack of correlation between both serum and urinary MCP1 with eGFR do not support this. In addition, this finding is in contrast to a study which found higher serum MCP1 levels in pregnancy compared to a non-pregnant cohort[244].

Finally, of interest with renal biomarkers, the only marker that significantly differs between the diabetes and control cohort was serum KIM 1 in the post-partum state (Figure 8.5). Notably as well, in those with and without diabetes, serum KIM1 levels were significantly elevated during the second trimester compared to the post-partum

state. Taken together, this may imply that in women with diabetes, post-pregnancy serum KIM1 levels that doubled that of women without diabetes may reflect tubular injury in pregnancy that persisted post-delivery with those with diabetes.

Study Limitations

We acknowledge the importance and potential effect of glucose level, body mass index and age in renal hyperfiltration. However, we could not include any other potential confounding factors in our analysis as this pilot study was not powered sufficiently to allow for statistical adjustments. The threshold of hyperfiltration in the literature ranges significantly and we used an arbitrary cut off. In addition, the study protocol had to be adjusted to terminate plasma inulin clearance which would provide measured GFR. Therefore, analysis was based on eGFR as the primary measurement of renal function, which has its' limitation, particularly in pregnancy and diabetes. Supplementary analyses with urinary creatinine clearance were conducted, and no significant different findings were noted compared to eGFR.

Inference of this study was also limited by the lack of pre-conception data. Despite attempting to, we did not obtain sufficient numbers for pre-pregnancy data. This study demonstrates some of the difficulties in recruiting pregnant women with diabetes in clinical studies. Therefore, eGFR functioned as a surrogate for initial renal function. It should be noted that despite the significant renal hemodynamic changes that occurs during pregnancy, it is generally accepted that renal function reverts back to pre-pregnancy levels by two months post-partum [92, 382]. This was further supported by our data in Chapter 7: Pregnancy related renal function trajectory in women in type 1 and type 2 diabetes. Whilst acknowledging the limitations from our retrospective study, our data likewise confirms that by six months, eGFR returns to pre-pregnancy levels at a similar rate for both women with and without diabetes.

8.5 Final Consideration and Implications

In conclusion, in this exploratory study, some women with diabetes may have reduced capacity to hyperfiltrate in pregnancy compared to healthy controls, particularly those with a lower pre-pregnancy eGFR. Hyperfiltration was not associated with higher oxidative and inflammatory circulating renal biomarkers. The underlying etiology of hyperfiltration remains to be fully elucidated but is likely a physiological phenomenon in diabetes. Further validation of these findings with larger sample size, and measured GFR would be important to explore the mechanism and contributing factors that could have resulted in the lack of eGFR rise in these women.

In addition, we did not demonstrate any renal biomarker variations that were associated with oxidative stress, inflammation or structural injury in diabetes related hyperfiltration. The lack of elevation the candidate biomarkers do not support the notion that hyperfiltration is a marker of DKD.

Study Four

Chapter Nine

Risk factors for pregnancy outcomes in type 1 and type 2 diabetes

9.1 Introduction

Adverse maternal and neonatal outcomes have been reported up to nine times higher in women affected by pre-gestational diabetes compared to those without. [383, 384] Despite the objective of the St Vincent's Declaration in 1989 to improve pregnancy outcomes in type 1 diabetes, there remains a significant gap. [385] Pregnancy outcomes in women with type 1 diabetes have generally been a focus in the literature. However, the incidence of pregnancies affected by type 2 diabetes has surpassed that of type 1 diabetes in Australia. [386-388] This predominance of type 2 diabetes, to almost four times that of type 1 diabetes has also been shown in a large diabetes-pregnancy epidemiology study. [389] The rising prevalence of type 2 diabetes in younger women, apart from increasing rates of obesity, is also likely due to increased identification pre-conception or early pregnancy; a recognised window of opportunity to minimise risk for future pregnancies. The appreciation of both the pregnancy risks of gestational diabetes and high future risk of post gestational type 2 diabetes, [390] has generated considerable interest in identifying pregestational diabetes. [391-394]

Studies have demonstrated robust links between congenital malformation and spontaneous abortion with poor pre-conception glycaemia management in women with both type 1 and type 2 diabetes. [395] Suboptimal glycaemia management, particularly in the third trimester is associated with preeclampsia in women with type 1 diabetes. [396] Such a relationship has not been established in pregnancies of women with type 2 diabetes and the presence of pre-existing hypertension in this cohort complicates the picture. Furthermore, a major challenge in interpreting current literature in this field is the lack of homogeneity in outcome measures and determination of risk factors throughout pregnancy.

Aim:

To explore differences in pregnancy outcomes between women with type 1 and type 2 diabetes and to examine the relationships between potential adverse risk factors and pregnancy outcomes in this cohort of women.

Specific Testable Hypotheses:

We hypothesized that women affected by type 1 and type 2 diabetes will have greater risk of adverse pregnancy outcomes compared to women without diabetes.

9.2 Methods

Women with type 1 and type 2 diabetes, who delivered at the Mercy Hospital for Women, a tertiary obstetric medical centre in Heidelberg, Melbourne, Australia, over a 10-year period from July 2004 to July 2014 were identified via Mercy Hospital for Women Birthing Outcome Systems database. Diagnosis of type 1 and type 2 diabetes was confirmed on obstetricians' or endocrinologists' clinical entry. Patients with unclear diabetes diagnosis, gestational diabetes, and inconsistent or lack of baseline measures of glycaemia were excluded. One hundred and nineteen healthy women as an extension from a separate study were included in the current study as the control group [397, 398]. The prospective cohort were healthy pregnant women presenting at or prior to 13 weeks of gestation at the same tertiary obstetric hospital between 2006-2011. Exclusion criteria included women with known type 1 and type 2 diabetes, history of thyroid disease and positive thyroid peroxidase antibodies, thyroid hormone replacement therapy, history of intravenous drug abuse or the presence of major systemic illness

We excluded women with multiple-pregnancies, spontaneous or elective abortion within the first 20 weeks of pregnancy. Study investigators reviewed all medical record and pathology entries from the diagnosis of pregnancy until discharge following delivery. Mercy Health Human Research Ethics Committee approved this study.

Study participants were reviewed on a regular basis, throughout pregnancy and managed by a multidisciplinary team of obstetricians, endocrinologists, dieticians and diabetes nurse educators as per recommended guidelines. Glycaemia and blood pressure targets were based on the Australasian Diabetes in Pregnancy guidelines. [399] Insulin was used as first line therapy for management of type 2 diabetes in pregnancy, however, concomitant pre-pregnancy metformin use also occurred in approximately 56% of women with type 2 diabetes.

Glycosylated hemoglobin (HbA_{1c}) was measured at baseline and approximately every 6 weeks throughout gestation using a turbidimetric inhibition immunoassay on Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany), standardized to the International Federation of Clinical Chemistry reference method. Other biochemistry including serum creatinine, albumin creatinine ratio (ACR) or albumin excretion rate were performed at baseline, and as clinically indicated.

Women were generally delivered at approximately 38 weeks or earlier if clinically indicated.

Anthropometric and Pathology Data

We collected anthropometric data throughout pregnancy, starting from the first antenatal visit. The initial visit entries, generally in the first trimester, were considered as baseline data. Data were obtained by manual reviews of the patient medical record. Presence of pre-existing hypertension was based on clinician entry and use of anti-hypertensive agents. Women who were on anti-hypertensive agents solely for the purpose of reno-protection without hypertension were not classified as having pre-existing hypertension. Macrovascular complications included ischaemic heart disease, cerebrovascular disease and peripheral vascular disease. Microvascular complications incorporated retinopathy, nephropathy, and peripheral neuropathy.

Blood pressure and all biochemical parameters from each trimester during routine, stable clinical assessments were averaged for each individual. Clinical and biochemical parameters from every trimester were included as means for each covariable, as the cumulative exposures throughout pregnancy were considered important for pregnancy outcomes. Biochemical and clinical data obtained during acute and intra-partum admissions were excluded. HbA_{1c} was used to represent glycaemic management. Renal function was measured by serum creatinine. Due to the lack of an alternative, simple, routine method of measuring glomerular filtration rate, estimated glomerular filtration rate (eGFR) was calculated based on the Chronic Kidney Disease Epidemiology Cohort (CKD-EPI) formula. [400]

Overall, the following risk factors were included in analyses: maternal age, body mass index (BMI), type of diabetes, mean HbA_{1c}, mean eGFR, and mean systolic and diastolic blood pressure, when applicable. Rather than trimester specific values, the mean HbA_{1c}, blood pressure and eGFR of the whole pregnancy were utilised in the analytical model to take into consideration the overall levels of these risk factors throughout pregnancy.

Outcome Measures

The major obstetric outcome measures for this study were: preeclampsia, preterm birth < 37 weeks and < 32 weeks, large for gestational age (LGA), small for gestational age (SGA) and neonatal intensive care (NICU) admission.

Maternal and perinatal outcomes were defined using The Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) recommended guidelines[401] and clinician diagnosis and entry. The diagnosis of pre-existing hypertension was based on clinician entry. Pregnancy induced hypertension was based on the absence of a pre-existing diagnosis of hypertension, as well as systolic blood pressure > 140 mmHg with or without diastolic blood pressure > 90 mmHg, on three or more measurements. Preeclampsia was defined as a development of systolic blood pressure > 140 mmHg with or without diastolic blood pressure > 90 mmHg, and proteinuria (> 0.3 g/24h or ≥ 30 mg/mmol) after 20 weeks' gestation. Primary postpartum haemorrhage was defined as a blood loss of either > 500 ml post vaginal delivery or > 1000 ml post caesarean delivery within 24 hours of delivery.

'Preterm birth < 37 weeks' was categorised as delivery from 32 completed weeks to prior to 37 completed weeks, and 'very preterm birth < 32 weeks' as delivery prior to 32 completed weeks (with no separation made between spontaneous and iatrogenic). Infant birthweight was measured at delivery using standard weighing scales. Birthweight percentiles and z-scores corrected for gestational age were calculated based on a reference population at the same hospital. [402] The z-score was calculated as: (observed birthweight – mean birthweight for gestational age)/ standard deviation of the mean birthweight. LGA and SGA, were defined as birthweights > 90th centile and < 10th centile, respectively. Neonatal jaundice was defined as hyperbilirubinemia

requiring phototherapy. Major congenital malformations were those responsible for death, those causing a significant future disability, or those requiring major surgery requiring correction. Neonatal hypoglycaemia was defined as blood glucose of < 2.6 mmol/l in the early neonatal period. Fetal death in utero was defined as fetal death later than 20 weeks' gestation.

Statistical Analysis

Univariate analysis was performed with Wilcoxon rank sum test for continuous variables, and Chi-square tests or Fisher's exact tests for categorical variables. Comparisons were made between type 1 and type 2 diabetes pregnancies (Table 9.1 and Table 9.3), and healthy controls with the combined diabetes group (Table 9.2 and Table 9.4).

We undertook univariate logistic analyses to assess associations with established risk factors (including age, blood pressure, BMI, renal function and HbA1c) and four major outcomes of preeclampsia, preterm birth < 32 and 37 weeks, and neonatal intensive care admission in women with and without pregestational diabetes (combined type 1 and type 2 diabetes). Sub-group analysis was also performed comparing outcomes in women with type 1 diabetes to type 2 diabetes.

The co-linearity of the model prohibited appropriate inclusion of potential risk factors into a multivariable analysis. We have considered the following potential risk factors to include in our model for adjustment: age, smoking, BMI, HbA1c, systolic blood pressure, diastolic blood pressure and eGFR. Collinearity analysis for the whole cohort demonstrated a VIF range between 1.03-2.97. All variables had a VIF > 1 , and the condition number was significantly high at 65.0, suggesting high collinearity.

Due to the multiple outcome measures, we corrected for multiplicity testing using the Bonferroni correction with a threshold of significance at $P = 0.03$.

9.3 Results

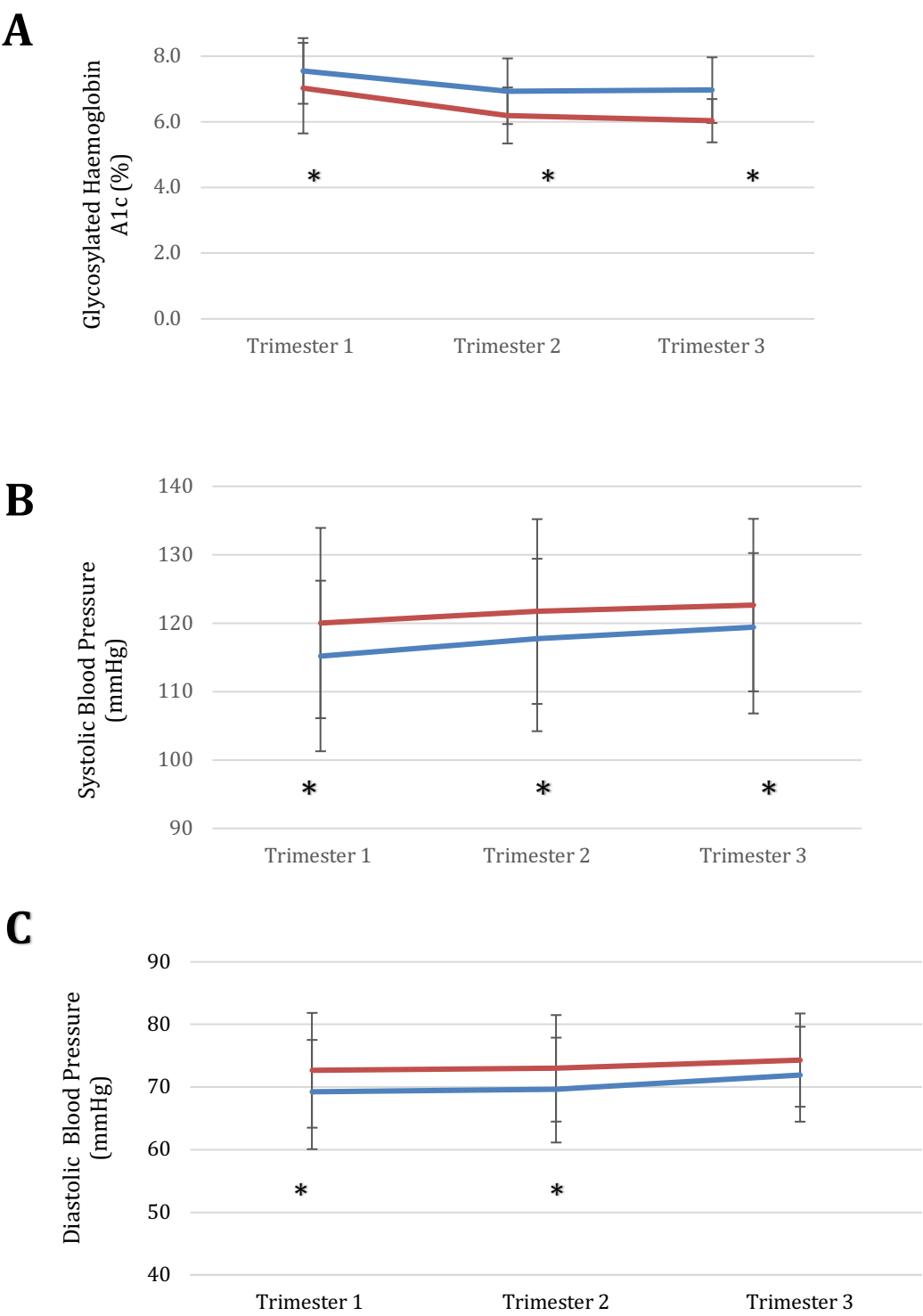
Maternal Characteristics

Over the ten-year period, 307 singleton pregnancies were included in the analysis: 119 healthy women without diabetes; 92 women with type 1 diabetes; and 106 women with type 2 diabetes (Table 9.1 and Figure 9.1).

Women with type 2 diabetes had more advanced age and higher BMI. Women with type 1 diabetes had statistically significant longer duration of diabetes, marginally higher HbA_{1c}, and higher prevalence of microvascular complications. Lower renal function, as reflected by lower eGFR and higher serum creatinine, was evident in the type 1 diabetes cohort (Table 9.1).

Longitudinal data across all three trimesters (Figure 9.1), demonstrated that women with type 2 diabetes had a significantly lower HbA_{1c} (Figure 1a) but a higher blood pressure (Figure 1b and 1c) compared to women with type 1 diabetes. Women without pre-gestational diabetes were younger and had a lower BMI compared to women with type 1 and type 2 diabetes (Table 9.2)).

Figure 9.1 Comparison of (A) systolic blood pressure and (B) diastolic blood pressure and (C) glycosylated haemoglobin (HbA1c) in women with type 1 diabetes and type 2 diabetes during pregnancy



Data performed expressed as mean + standard error of the mean. * p <0.05.
 Blue line: Type 1 Diabetes, Orange Line: Type 2 Diabetes

Table 9.1 Maternal characteristics in women with type 1 diabetes and type 2 diabetes at baseline

	Type 1 Diabetes	Type 2 Diabetes	<i>P</i> value
<i>n</i>	92	106	
Age (years)	31.0 (27.5, 34.3)	34.5 (31.0, 37.4)	< 0.001
BMI (kg/m ²)	27.2 (23.0, 31.0)	33.0 (29.0, 38.0)	< 0.001
Parity	1.0 (0.0,1.0)	1.0 (0.0,1.0)	0.1
Smoking status at conception			0.9
Current smoker	7 (9.8)	6 (5.7)	
Ex-smoker	9 (7.6)	11 (10.4)	
Non-smoker	76 (82.6)	89 (83.9)	
Duration of diabetes (years)	12.0 (5.0, 19.0)	3.0 (2.0, 5.0)	< 0.001
HbA _{1c} (%)	7.0 (6.5, 8.2)	6.9 (6.0, 7.8)	0.03
HbA _{1c} (mmol/mol)	53.0 (47.5, 66.1)	51.9 (42.1, 61.7)	0.03
Systolic blood pressure (mmHg)	115.0 (110.0, 120.0)	120.0 (110.0, 125.0)	0.02
Diastolic blood pressure (mmHg)	70.0 (65.0, 70.0)	70.0 (70.0, 80.0)	0.02
Serum creatinine (μmol/l)	54.0 (47.0, 59.0)	46.0 (40.5, 51.5)	< 0.001
CKD-EPI eGFR (ml/min/1.73m ²)	120.8 (116.7, 128.5)	124.8 (121.6, 130.6)	0.02
Pre-existing hypertension	10 (10.9)	23 (21.7)	0.04
Renin angiotensin aldosterone System Inhibition at conception	8 (8.7)	8 (7.6)	0.8
Statins therapy at conception	4 (4.4)	9 (8.5)	0.2
Metformin therapy at conception	0 (0)	55 (51.9)	< 0.01
Microvascular complications	21 (22.6)	8 (7.5)	< 0.01
Macrovascular complications	2 (2.2)	0 (0)	0.1

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

Body mass index = BMI; glycosylated hemoglobin = HbA_{1c}; Chronic Kidney Disease Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR

Table 9.2 Selected maternal characteristics in women with and without diabetes at baseline

	Diabetes (Type 1 & Type 2)		Healthy Controls		P- value
N	198		119		
Age (years)	33.0	(28.9, 36.2)	31.0	(28.0, 35.0)	0.04
BMI (kg/m ²)	30.0	(26.0, 35.5)	25.0	(23.0, 32.0)	<0.01
Serum creatinine (μmol/l)	49.5	(43.0, 57.0)	49.0	(44.0, 54.0)	0.8
CKD-EPI eGFR (ml/min/1.73m ²)	123.6	(118.2,129.4)	125.3	(119.6, 130.6)	0.2
Albumin-to-creatinine ratio (mg/g)	7.1	(4.4, 21.2)	-		
Data expressed median (interquartile range) or n (%) Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables Body mass index = BMI; Chronic Kidney Disease Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR					

Maternal and Neonatal Outcomes

Pregnancy and neonatal outcomes are demonstrated comparing type 1 and type 2 diabetes (Table 3) individually. Furthermore, to illustrate the differences between healthy and pregnancies affected by diabetes, we combined type 1 and type 2 diabetes; comparing the collective diabetes cohort to controls (Table 4). Birth weight gestational centile (median 97.4% vs. 72.4%; $P < 0.001$) and LGA rates (63.4% vs. 35.8%; $P < 0.001$) were higher in women with type 1 diabetes compared to type 2 diabetes. Women with type 2 diabetes had an approximate doubling of SGA compared to type 1 diabetes (15.1% vs. 5.4%; $P = 0.02$). There was no statistically significant difference in other major perinatal and obstetrics outcomes between women with type 1 and type 2 diabetes (Table 9.3). Sixteen percent of women with diabetes (type 1 and type 2 combined) developed preeclampsia compared to four percent in the control group ($P = 0.003$).

Compared to healthy pregnancies (Table 9.4) the odds of preeclampsia (odds ratio [OR], 4.4; 95% confidence interval [CI], 1.7-11.6; $P = 0.003$), emergency caesarean delivery (OR, 2.7; 95% CI, 1.5-4.9; $P = 0.001$), LGA (OR, 2.8; 95% CI, 1.7-4.7; $P < 0.001$), Apgar score less than 7 (OR, 5.8; 95% CI, 1.3-25.7 $P = 0.02$), neonatal intensive care admission (OR, 5.2; 95% CI, 2.0-13.7; $P = 0.001$), preterm birth at less than 32 (OR, 12.5; 95% CI, 1.7-94.8; $p = 0.01$) and 37 weeks (OR, 6.3; 95% CI, 3.0-13.1; $P < 0.001$) were all significantly higher in women with diabetes. Only SGA and neonatal congenital malformation was not significantly different between the two groups.

Table 9.3 Comparison of obstetric and perinatal outcomes in women with type 1 and type 2 diabetes

	Type 1 diabetes	Type 2 diabetes	Odds ratio (95% CI)	P-value
<i>n</i>	92	106		
Obstetric outcomes				
Preeclampsia	12 (12.9)	20 (18.9)	0.6 (0.3, 1.4)	0.3
Pregnancy-induced hypertension	4 (4.3)	11 (10.4)	0.4 (0.1, 1.3)	0.1
Emergency caesarean delivery	32 (34.4)	33 (31.1)	1.2 (0.7, 2.3)	0.5
Postpartum haemorrhage	13 (14)	10 (9.4)	1.6 (0.7, 3.8)	0.3
Perinatal outcomes				
Gestational age (weeks)	37.4 (36.0, 38.1)	37.3b (38.3, 40.4)	-0.1 (-1.0, 0.8)	0.8
Preterm birth < 37 weeks' gestation	31 (33.3)	36 (34)	1.0 (0.5, 1.8)	1.0
Preterm birth < 32 weeks' gestation	10 (10.8)	9 (8.5)	1.3 (0.5, 3.4)	0.6
Birthweight gestational centile (%)	97.4 (79.2, 99.8)	72.4 (32.4, 94.4)	20.2 (11.7, 28.8)	<0.001
Large for gestational age	59 (63.4)	38 (35.8)	3.2 (1.8, 5.7)	<0.001
Small for gestational age	5 (5.4)	16 (15.1)	0.3 (0.1, 0.9)	0.03
Major congenital malformation	3 (3.2)	3 (2.8)	1.2 (0.2, 5.9)	0.9
Fetal death in utero/ in labour	2 (2.2)	3 (2.8)	0.8 (0.1, 4.7)	0.8
Neonatal jaundice	9.0 (9.7)	17.0 (16.0)	0.6 (0.2, 1.3)	0.2
Apgar score < 7 at 5 minutes	8 (8.6)	10 (9.4)	0.9 (0.3, 2.4)	0.9
NICU Admission	18.0 (19.4)	19.0 (17.9)	1.1 (0.5, 2.3)	0.8
Neonatal hypoglycaemia	25 (26.9)	24.0 (22.6)	1.3 (0.7, 2.4)	0.5

Data expressed median (interquartile range) or n (%) Group comparisons were performed using logistic regression. Data expressed as Odds ratio for categorical variables and Co-efficient variation for continuous variable. Neonatal intensive care unit = NICU

Table 9.4 Comparison of obstetric and perinatal outcomes in women with and without diabetes

	Diabetes (Type 1 & Type 2)	Healthy controls	Odds ratio (95% CI)	P-value
<i>n</i>	198	119		
Obstetric outcomes				
Preeclampsia	32 (16.2)	5 (4.2)	4.4 (1.6, 11.6)	0.003
Pregnancy-induced hypertension	15 (7.6)	3 (2.5)	3.2 (0.9, 11.2)	0.07
Emergency caesarean delivery	65 (32.8)	18 (15.1)	2.7 (1.5, 4.9)	0.001
Postpartum haemorrhage	23(11.6)	11(9.2)	1.3 (0.6, 2.8)	0.5
Perinatal outcomes				
Gestational age (weeks)	37.3 (36, 38.1)	39.4(38.3, 40.4)	-2.8 (-3.4, -2.1)	< 0.001
Preterm birth < 37 weeks' gestation	67 (33.8)	9 (7.6)	6.3 (3.0, 13.1)	< 0.001
Preterm birth < 32 weeks' gestation	19 (9.6)	11 (0.8)	12.5 (1.7, 94.8)	0.01
Birthweight gestational centile (%)	89.5 (61.7, 98.7)	69.7 (41.0, 48.9)	10.8 (3.7, 18.0)	0.003
Large for gestational age	97.0 (49.5)	30 (25.2)	2.8 (1.7, 4.7)	< 0.001
Small for gestational age	21.0 (10.6)	9.0 (7.6)	1.5 (0.6, 3.3)	0.4
Major congenital malformation	6 (3)	4 (3.4)	0.9 (0.2, 3.3)	0.9
Fetal death in utero/ in labour	5(2.5)	0 (0)	NA	NA
Neonatal jaundice	26 (13.1)	4 (3.4)	4.3 (1.5, 12.6)	0.008
Apgar score < 7 at 5 minutes	18 (9.1)	2 (1.7)	5.9 (1.3, 25.7)	0.02

NICU Admission	37 (18.7)	5 (4.2)	5.2 (2.0, 13.7)	0.001
Neonatal hypoglycaemia	49 (24.7)	4 (3.4)	9.5 (3.3, 27.0)	< 0.001

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using logistic regression.

Data expressed as Odds ratio for categorical variables and Co-efficient variation for continuous variable.

Neonatal intensive care unit = NICU

Predictors of adverse pregnancy outcomes in women with pre-gestational diabetes

HbA_{1c} was not associated with major outcomes. A one mmHg increase in systolic and diastolic blood pressure was associated with higher odds of developing preeclampsia (OR of 1.1 [95% CI, 1.0-1.1; $P = 0.001$] and 1.1 [95% CI, 1.1-1.2; $P < 0.001$], respectively), and preterm birth at 37 weeks (OR of 1.1 [95% CI, 1.0-1.1; $P < 0.001$] and 1.1 [95% CI, 1.0-1.1; $P = 0.005$], respectively)

Higher BMI was associated with higher odds of developing pregnancy-induced hypertension (OR, 1.1; 95% CI, 1.0-1.1; $P = 0.01$) and preeclampsia (OR, 1.0; 95% CI, 1.0-1.1; $P = 0.05$). Increasing age (per year) was also associated with higher odds of developing preterm birth < 32 weeks (OR, 1.1; 95% CI, 1.0-1.2; $P = 0.01$). Each ml/min/1.73m² decrease in eGFR ($P < 0.001$) was associated with higher odds of developing preeclampsia (OR, 1.1; 95% CI, 1.0-1.1), preterm birth 32 (OR, 1.1; 95% CI, 1.0-1.1) and 37 weeks (OR, 1.0; 95% CI, 1.0-1.1) and neonatal intensive care admission (OR, 1.1; 95% CI, 1.0-1.1).

Smoking was not associated with any major outcomes apart from the higher odds of developing pre-eclampsia (OR, 1.9; 95%CI, 1.1, 3.1).

As limited by the co-linearity of the model, multivariable analyses for the above risk factors were not conducted.

9.4 Discussion

The most important findings in this study were that the rates of poor outcomes were comparable between type 1 and type 2 diabetes. The measures of poorer outcomes in pre-existing maternal diabetes were present across most (thirteen out of sixteen) of outcome measures, and consistent with current literature. [403] In addition, we identified maternal blood pressure, BMI, and renal function to be important predictors of risk for poor outcomes.

Given that the participant numbers in both groups were small, we kept the diabetes group together. To avoid multiplicity of testing of the dataset leading to false positive findings, we did not perform formal analysis comparing type 1 diabetes and type 2 diabetes separately to those without diabetes. However, exploratory sub-analysis comparing outcomes in type 1 diabetes as well as type 2 diabetes individually alone to healthy controls demonstrated similar differences (with the combined type 1 and type 2 diabetes cohort). The only exception was that incidence of neonatal jaundice was not higher in type 1 diabetes compared to healthy controls. Additionally, birthweight gestational centile, and hence incidence of large for gestational age did not differ when comparing healthy controls to type 2 diabetes alone.

Comparison to Previous Studies

We found similar pregnancy outcomes in type 1 and type 2 diabetes. This is in contrast with some studies [403-406] suggesting more adverse consequences in pregnancies affected by type 2 diabetes. A study by Hillman and colleagues[407] attempting to address this concluded that pregnancy outcomes were better in type 2 diabetes when intensified glycaemic management, as gauged by HbA_{1c}, were as rigorous as in women with type 1 diabetes. In our study, we did not find better outcomes in women with type 2 diabetes despite lower HbA_{1c}. The glycaemic pattern throughout pregnancy from the study by Clausen et al. was not dissimilar to our study, along with others which failed to find any differences in outcomes between type 1 and type 2 diabetes. [404] Of note however, timely intensive glucose therapy was shown to reduce spontaneous abortion and congenital malformation in the diabetes in Complication and Control Trial (DCCT) in women with type 1 diabetes. [408] It should be noted that in our study, we averaged

HbA_{1c} throughout the three trimesters of pregnancy, as we felt that cumulative exposure, rather than a single time point glycaemic management was considered more important.

Findings of LGA rates being significantly higher in pre-gestational diabetes compared to healthy women, supports glucose as a major mediator of growth. [409] Exaggeration of the physiological insulin resistance in pregnancy that occurs in maternal diabetes can promote hyperglycaemia. Independent association of HbA_{1c} with LGA, has been found in previous type 1 diabetes studies. [410, 411] However a mean HbA_{1c} of 6.5% (48 mmol/mol), which was found to be associated with other poor outcomes in previous studies including preterm birth before 37 weeks, composite neonatal outcomes and preeclampsia, [410] was not found in our cohort. A systemic review of 13 studies in relation to HbA_{1c} and outcomes identified overall poorer, but unpredictable and diverse outcomes from study to study. [412] Of note, in the presence of an acceptable HbA_{1c}, higher median glucose readings especially in the second trimester are closely related to LGA in type 1 diabetes. [411] These inconsistencies linking HbA_{1c} to adverse outcomes illustrate the limitation of using HbA_{1c} as an indicator of fetal glucose exposure during pregnancy. Targeting near normal glycaemia throughout the entire pregnancy is desirable [413, 414] and may be more enabled by further advances in technology of insulin delivery and intensive real time glucose monitoring. In fact, a recent multicentre randomized controlled trial [415] demonstrated improved neonatal outcomes for mothers with type 1 diabetes using continuous glucose monitoring during pregnancy, with lower incidence of large for gestational age, fewer neonatal intensive care admissions and fewer instances of neonatal hypoglycaemia.

Obesity has been linked with intra-uterine growth restriction, [416] which may partially explain the lower birthweight centile in infants born to type 2 diabetes women in our cohort, with a median BMI within the obese range. The effect of obesity and risk of SGA may be related to the inadequate weight gain of women [417] with the introduction of gestational weight gain guidelines which recommends a lower weight gain pregnancy threshold for women with elevated BMIs. [418] In contrast, a recent study has shown a different pattern, whereby there appears to be an association with maternal obesity and accelerated fetal growth, especially when combined with gestational diabetes. [419] In addition, a recent Australian study established a higher

BMI in women with both type 1 and type 2 diabetes with infants more likely to be LGA compared to healthy controls. [328, 329] Whether BMI explains the significantly lower birth weight and smaller gestational age in our type 2 diabetes cohort will need to be further investigated, especially with the lack of documented gestational weight changes in our cohort of women, as well as being confounded by the increasing co-morbidities that accompany this, including hypertension. [420]

Hypertension has a combined additive effect with diabetes on SGA, [421] which may partly explain the higher rates of SGA in our type 2 diabetes cohort. Intensive blood pressure management in diabetic kidney disease has been shown to benefit outcomes in this group of high-risk pregnancy. [336, 422]

Supplementing current Australian Pre-gestational Outcome Data

The similar proportion of both type 1 and type 2 women with diabetes in our study may still reflect the under-recognition of the need to direct specialist care for these high-risk women. This is further highlighted with 25% of all type 2 diabetes and 3% of type 1 diabetes patients registered with the National Diabetes Service Scheme were from childbearing age of 21-39 years. [423, 424] From a study published in 2005, involving ten teaching hospitals in Australia [388] with comparable numbers (n = 180), the mean age of conception in our study was higher, particularly in type 2 diabetes reflecting current societal trends of deferred pregnancies. The incidence (4.6% vs. 8.1%) of major congenital malformations was also less, however, our study excluded women with spontaneous abortion of pregnancy before 20 weeks and elective terminations. Taking into consideration, findings of a New Zealand study, [406] which demonstrated higher perinatal mortality in type 2 diabetes and gestational diabetes compared to our study, exclusion of gestational diabetes in our study may potentially exclude very small number of undiagnosed late type 2 diabetes cases that may contribute to neonatal deaths.

In contrast to our findings, a 2010 report based on the National Hospital Morbidity Database [386] found that mothers with type 1 diabetes had higher rate of hypertension. We believe that manually obtaining blood pressure readings, as performed in our study, provided better overall representation of the presence of hypertension than that of a

single documentation of self- or clinician reported diagnosis. The findings of higher preterm birth and caesarean section in type 1 diabetes compare to type 2 diabetes were not replicated by findings of our study. This contrast may reflect different population groups, as our study was conducted at a tertiary centre, and improvements in clinical practice over time.

The most recent Australian study reporting pregnancy outcomes in type 1 [328] and type 2 diabetes [329] compared to the same cohort of healthy controls highlighted the adverse outcomes in this high risk group, underscoring the importance of HbA_{1c} and glycaemic management. Our study complements the literature by examining the association of outcomes with additional variables of blood pressure, eGFR and other clinical and biochemical data. Risk factors for patients with diabetes were also pooled in our study when compared to healthy controls, which may explain some of the discrepancies in our respective study findings.

Strengths and Limitations

This was an observational, retrospective study and we recognize that there may be incomplete data collection. For example, we excluded women with inconsistent and absent baseline glycaemia measurement. This may result in missing some cases of pre-gestational diabetes, but on the other hand allow for a low false positive rate on the diagnosis of type 2 diabetes. The cause of preterm birth was also not determined, which would have allowed insight into future management of these cases.

The applicability of findings outside of our institution may also be limited. This includes the relatively well-controlled blood pressure and reasonable glycaemic management which may reflect practices within a tertiary obstetric hospital and affect the overall outcomes. However, as the focus on the importance of obstetric blood pressure and glycaemic management is increasing, our findings may be applicable more broadly with time.

Finally, as this is one of the larger observational studies in Australia in recent years, our findings may be helpful in counselling women with diabetes regarding pregnancy outcomes in contemporary Australian practice.

9.5 Conclusion

Both type 1 and type 2 diabetes carry a similar high risk for poorer maternal and fetal outcomes compared to healthy pregnancies. Although estimates of associations between the maternal risk factors and adverse outcomes should be regarded as exploratory in a cohort of this sample size, age, higher BMI, higher systolic and diastolic blood pressure, and lower eGFR predicted poorer outcomes.

Study Five

Chapter Ten

The association between maternal renal function and pregnancy outcomes in type 1 and type 2 diabetes

10.1 Introduction

In women without diabetes, pre-existing chronic kidney disease (CKD), has been linked to high obstetric risk. [425] Previous research has demonstrated that the risk of adverse pregnancy outcomes, including preterm birth < 37 weeks, neonatal intensive care unit admission and small for gestational age, is up to threefold higher in pregnancies affected by CKD. Furthermore, this risk was observed even in women with what is generally accepted as “normal” renal function, with an estimated Glomerular Filtration Rate (eGFR) > 90 mL/min/1.73m², categorized as stage 1 CKD using the Kidney Disease Improving Global Outcomes (KDIGO) guidelines. [119] In women with type 1 [116, 310] and type 2 diabetes [332] pregnancies affected by diabetic kidney disease have a significantly higher obstetric and perinatal risk compared to those without diabetic kidney disease. Specifically, women with type 1 diabetes who have either micro- or macro-albuminuria are at a five to eight times increased risk of developing preeclampsia compared with women with type 1 diabetes and normal urinary albumin excretion. [310]

Aim:

To investigate the prognostic value of eGFR and albumin-to-creatinine ratio (ACR) in determining four major obstetric outcomes: preeclampsia; preterm birth at < 32 weeks; preterm birth at < 37 weeks; and neonatal intensive care unit admission.

Specific Testable Hypotheses:

We hypothesize that in women with pre-existing diabetes, lower eGFR and higher albuminuria are independent predictors of poorer maternal and fetal outcomes.

10.2 Methods

This is a retrospective study with detailed methods outlined in the previous chapter, Chapter 9: Risk Factors for Pregnancy Outcomes in type 1 and type 2 diabetes.

In summary, we identified women with type 1 and type 2 diabetes who delivered in a single tertiary obstetric hospital between July 2004 to July 2014. Healthy women who were prospectively recruited in a separate study were included in the current study as the comparator control group. We collected anthropometric data throughout pregnancy, starting from the first antenatal visit. Clinical and biochemical parameters from every trimester were included. Biochemistry data that were included in this study comprised of all available results on either the hospital pathology database, or clinical records during the pregnancy. Acute and intra-partum admission biochemical and clinical data were excluded.

Rather than trimester specific values, the mean HbA_{1c}, blood pressure, eGFR and ACR across the entire pregnancy were utilised in the analytical model to take into consideration the overall levels of these risk factors throughout pregnancy.

Outcome Measures

For the purpose of this study, we identified four major outcomes based on the literature to avoid multiplicity of testing. The four outcome measures were: preeclampsia, preterm birth < 32 weeks and < 37 weeks, and neonatal intensive care unit admission.

We defined stages of diabetic kidney disease according to the Kidney Disease Improving Global Outcome (KDIGO) guidelines [426] with the following eGFR (ml/min/1.73m²):

- Stage 1: 90-120
- Stage 2: 60-89
- Stage 3: 30-59
- Stage 4: 15-29
- Stage 5: <15

Hyperfiltration was arbitrarily defined as an eGFR > 120 mL/min/1.73m². Microalbuminuria was defined as urinary albumin excretion of 30-300 mg/day or urinary ACR of 31-310 mg/g.

Statistical Analysis

Univariate analysis was performed with Wilcoxon Rank Sum test for continuous variables, and Chi-square tests or Fisher's exact tests for categorical variables. Comparisons were made between healthy controls with the combined type 1 and type 2 diabetes group (Table 10.1 and Table 10.3) and type 1 and type 2 diabetes pregnancies (Table 10.2)

We undertook univariate and multivariable logistic analyses to assess the risk of four major outcomes of preeclampsia, preterm birth < 32 and 37 weeks and neonatal intensive care unit admission in women with type 1 and type 2 diabetes. Presence of albuminuria and CKD-EPI eGFR < 120 mL/min/1.73m² were included in the multivariable regression models as categorical and continuous variables.

The co-linearity of the model prohibited appropriate inclusion of other established risk factors in the multivariable analysis. We have considered the following potential risk factors to include in our model for adjustment: age, BMI, HbA1c, systolic blood pressure, diastolic blood pressure and duration of diabetes. Collinearity analysis for the whole cohort demonstrated a VIF > 1 for all variables, and the condition number was significantly high at 40, suggesting high collinearity. This is most likely due to the small sample size.

10.3 Results

Maternal Characteristics

Over the 10-year period, 307 singleton pregnancies were included in the analysis: 119 healthy women without diabetes, 93 with type 1 diabetes, and 106 women with type 2 diabetes. Compared to healthy controls, women with diabetes (combined type 1 & 2 diabetes groups) had higher BMI, but similar eGFR readings (Table 10.1 and Figure 10.1).

When the maternal characteristics of the diabetes cohorts were compared (Table 10.2), those with maternal type 2 diabetes had more advanced age, higher BMI, and a higher rate of pre-existing hypertension. Women with type 1 diabetes had longer duration of diabetes, marginally higher HbA_{1c} and higher prevalence of microvascular complications. Rates of albuminuria were not significantly different between type 1 and type 2 diabetes. However, renal function, as measured by CKD-EPI eGFR [121 (117, 128)ml/min/1.73m² vs 125(122, 131)ml/min.1,73m², p =0.02] and serum creatinine values [0.61 (0.51,0.67)mg/dl vs. 0.52(0.46,0.59)mg/dl, p=0.002], were lower in the type 1 diabetes cohort. Longitudinal data across all three trimesters demonstrated that women with type 2 diabetes had a significantly lower HbA_{1c} and higher systolic and diastolic blood pressure compared to women with type 1 diabetes.

Table 10.1 Selected maternal characteristics in women at baseline (diabetes vs. healthy control)

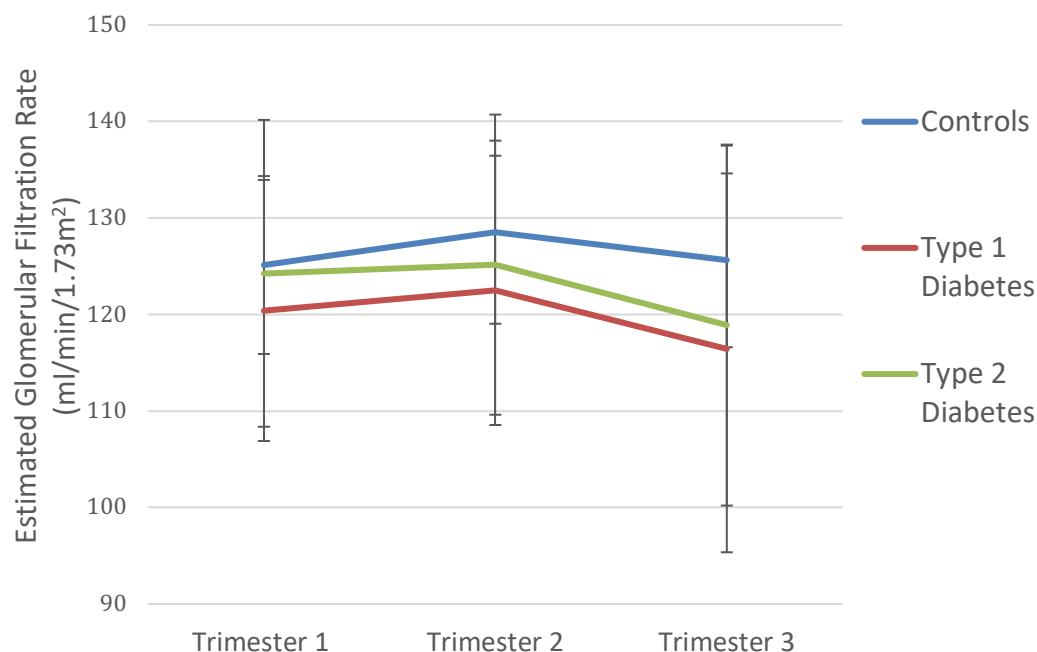
	Type 1 and Type 2 Diabetes	Healthy Controls	<i>P</i> -value
N	198	119	
Age (years)	33 (29, 36)	31(28, 35)	0.04
BMI (kg/m ²)	30 (26, 36)	25 (23, 32)	< 0.001
Serum creatinine (mg/dL)	0.59 (0.49, 0.64)	49 (44, 54)	0.8
CKD-EPI eGFR (mL/min/1.73m ²)	124 (118, 129)	125 (120, 131)	0.2
Albumin-to-creatinine ratio (mg/g)	7.1 (4.4, 21.2)	-	

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

Body mass index = BMI; Chronic Kidney Disease Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR

Figure 10.1 Comparison of estimated GFR in women with type 1 diabetes, type 2 diabetes and healthy controls



Data performed with Mann-Whitney U-test and expressed as mean + standard error of the mean

Table 10.2 Maternal characteristics in women with type 1 diabetes and type 2 diabetes at baseline

	Type 1 Diabetes	Type 2 Diabetes	<i>P</i> value
N	92	106	
Age (years)	31 (27, 34)	35 (31, 37)	< 0.001
BMI (kg/m ²)	27 (23, 31)	33(29, 38)	< 0.001
Smoking status at recruitment			0.9
Current smoker	7 (9.8)	6 (5.7)	
Ex-smoker	9 (7.6)	11 (10.4)	
Non-smoker	76 (82.6)	89 (83.9)	
Duration of diabetes (years)	12 (5, 19)	3 (2, 5)	< 0.001
HbA _{1c} (%)	7.0 (6.5, 8.2)	6.9 (6.0, 7.8)	0.03
HbA _{1c} (mmol/mol)	53 (48, 66)	52 (42, 62)	0.03
Systolic blood pressure (mmHg)	115 (110, 120)	120 (110, 125)	0.02
Diastolic blood pressure (mmHg)	70 (65, 70)	70 (70, 80)	0.02
Serum creatinine (mg/dL)	0.61 (0.51, 0.67)	0.52 (0.46, 0.59)	0.002
CKD-EPI eGFR (mL/min/1.73m ²)	121 (117, 128)	125 (122, 131)	0.02
Albumin-to-creatinine ratio (mg/g)*	7.1 (4.4, 18.6)	11.5 (7.1, 26.6)	0.2
Normoalbuminuria	63 (76.8)	49 (77.8)	
Microalbuminuria	13.0 (15.9)	10.0 (15.9)	
Macroalbuminuria	6.0 (7.3)	4.0 (6.4)	
Pre-existing hypertension	10.0 (10.9)	23.0 (21.7)	0.04

Renin angiotensin aldosterone System Inhibition at conception	8.0 (8.7)	8 (7.6)	0.8
Statins therapy at conception	4.0 (4.4)	9.0 (8.5)	0.2
Metformin therapy at conception	0 (0)	55.0 (51.9)	< 0.01
Microvascular complications	21.0 (22.6)	8.0 (7.5)	< 0.01
Macrovascular complications	2.0 (2.2)	0 (0)	0.1

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

*First trimester albumin-to-creatinine ratio were available for 53/92 (58%) women with type 1 and 40/106 (38%) women with type 2 diabetes

Body mass index = BMI; glycosylated hemoglobin = HbA_{1c}; Chronic Kidney Disease

Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI-GFR

Albumin-to-Creatinine Ratio and CKD–EPI eGFR as Risk Factors

All women in the control group had an eGFR > 90 mL/min/1.73m². Albuminuria status for healthy controls was not available. According to indices of renal status for the diabetes cohort (Table 10.1), the median ($\pm$ interquartile range) eGFR and ACR were 124 (118, 129) mL/min/1.73m² and 7.1 (4.4, 21.2) mg/g, respectively.

The following results on multivariable logistic analysis were based on data in women with type 1 and type 2 diabetes. Analysis on albuminuria and eGFR were based on results that were available for the 144 and 186 women with type 1 and type 2 diabetes.

With the selected four predefined outcomes, in women with type 1 and type 2 diabetes, compared to those who were hyperfiltering, women with both stage 1 and stage 2+ diabetic kidney disease had higher odds of developing preeclampsia (odds ratio [OR] of 4.5 [95% confidence interval [CI], 1.6-12.3; $P = 0.04$] and 3.9 [95% CI, 1.7-8.9; $P = 0.001$], respectively) and of having a preterm birth < 37 weeks (OR 2.3 [95% CI, 1.2-4.6; $P = 0.01$] and 4.9 [95% CI, 1.7-14.5; $P = 0.04$], respectively). There was also an additional risk of neonatal intensive care admission (OR, 4.9; 95% CI, 2.0-11.6; $P < 0.001$) and preterm birth < 32 weeks (OR, 5.0; 95% CI, 1.7-14.5; $P < 0.001$) in the Stage 2+ diabetic kidney disease women (Table 10.3).

Compared to women with normoalbuminuria, women with microalbuminuria and macroalbuminuria had a higher odds of developing preeclampsia (OR 5.7 [95% CI, 1.8-17.8; $P = 0.03$] and 5.5 [95% CI, 2.5-11.8; $P < 0.001$], respectively) and a higher odds of having a preterm birth < 37 weeks (OR 3.9 [95% CI, 1.5-9.9; $P = 0.04$] and 3.5 [95% CI, 1.6-7.7; $P = 0.02$], respectively). In addition, women with macroalbuminuria had higher odds of having a very preterm birth (< 32 weeks) (OR, 4.2; 95% CI, 1.9-9.5; $P < 0.01$) and of their newborn being admitted to a neonatal intensive care unit (OR, 2.4; 95% CI, 1.2-4.6; $P = 0.01$).

Furthermore, when analyzed as a continuous variable, both eGFR and albuminuria levels were associated with higher risk of all four adverse outcomes. Using multivariable analysis, when adjusted for each other, associations between eGFR and albuminuria with adverse outcomes persisted. Both eGFR (OR, 1.03; 95% CI, 1.00-

1.06; $P = 0.04$) and albuminuria status (OR, 1.95; 95% CI, 1.14-3.35; $P = 0.02$) were associated with preterm birth < 37 weeks. Specifically, with every 1 mL/min/1.73m² decline in eGFR, there was 4% higher odds of developing preterm birth < 32 weeks (OR, 1.04; 95% CI, 1.00-1.09; $P = 0.02$) and 3% higher odds of requiring admission to neonatal intensive care unit (OR, 1.03; 95% CI, 1.00-1.06; $P = 0.02$), but not preeclampsia; whilst, increased albuminuria was associated with a significantly higher odds of developing preeclampsia (OR, 2.7; 95% CI, 1.42-4.99; $P = 0.002$).

We acknowledge the importance of including HbA_{1c}, systolic and diastolic blood pressure, BMI, age and duration of diabetes in analysis. However, due to the collinearity of the model, multivariate analyses for potential confounding risk factors were not conducted.

10.4 Discussion

To the best of our knowledge, there is limited literature studying the utility of eGFR, using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation in predicting adverse obstetric outcomes in type 1 and type 2 diabetes. As CKD-EPI is the current routine method of assessing eGFR in the Australian clinical setting, the aim of this study was to investigate its' prognostic value in determining major obstetric outcomes.

The most pertinent findings of this study were that with every 1 mL/min/1.73m² decline in eGFR, there was 4% higher odds of developing preterm birth < 32 weeks. Furthermore, independent of eGFR, increased albuminuria was associated with preeclampsia. Moreover, lower eGFR was associated with preterm birth < 32 weeks and neonatal intensive care unit admission independently of albuminuria.

As renal function appears to be a risk factor for poorer outcomes, it will be important to further investigate the modifiable risks in order to allow implementation of protective measures aimed at preserving renal function in these young women. This is important, not only for improving pregnancy care, but for long-term complication prevention especially when renal function is so closely related to cardiovascular mortality. [427] Overall, as eGFR is commonly employed in routine clinical settings, it can potentially serve as a readily accessible marker in identifying women with diabetes at a higher risk of developing maternal and fetal outcomes in pregnancies. In addition, understanding how albuminuria can reflect higher risk of adverse pregnancy outcomes can aid clinicians to provide additional pregnancy counselling and more intensive follow up. The independent associations demonstrated between eGFR and albuminuria with selected pregnancy outcomes in this study is encouraging for future research. Studies including a larger cohort of participants being followed up in a longitudinal prospective fashion with stricter biochemical testing protocol may provide further insight into other possible pregnancy related outcomes.

Comparison to previous studies

Chronic kidney disease was linked to poorer maternal and neonatal outcomes in a relatively large demographically matched study, [425] where 13% and 25% of women had diabetes and chronic hypertension. The risk of preterm birth and neonatal intensive care unit admission was increased in our study population, supporting our findings of the association between lower eGFR and adverse pregnancy outcomes. Our study has also provided additional evidence for the association of adverse maternal and fetal outcomes in pregnant women with pre-existing diabetes according to albuminuria status as well as eGFR level. Despite recognition of the limitations of eGFR using the CKD-EPI formula, which underestimates measured GFR [428] and subsequently physiologic pregnancy hyperfiltration; lower eGFR values were associated with increased risk of adverse outcomes.

In individuals with CKD and without diabetes an eGFR > 90 mL/min/1.73m² at baseline in early pregnancy is associated with adverse pregnancy outcomes including preterm delivery, small for gestational age and admission to neonatal intensive care unit. [119] These authors, [120] however, did not detect a difference in outcomes between women who were hyperfiltering in the first trimester compared to those with who were not hyperfiltering, with an eGFR of < 120 mL/min/1.73m². Our findings of the four selected adverse outcomes in what is generally presumed as acceptable renal function (eGFR 90-120 mL/min/1.73m²) was not observed within our healthy group of women but was present within our diabetes cohort with an eGFR 90-120 mL/min/1.73m². This suggests that independent of renal function, other processes including glycemic management and blood pressure, can potentially contribute to preeclampsia. This was specifically demonstrated in recent studies of women with type 1 diabetes. [116, 310] These studies have provided evidence that preeclampsia and likely the resultant preterm birth, were significantly higher in women with diabetic kidney disease.

At this stage, it is not clear if women with diabetes have a reduced ability to achieve hyperfiltration in pregnancy. However, in this present study, we found no difference in peak eGFR during pregnancy in women with and without diabetes. We have demonstrated that stage 1 and stage 2 DKD are associated with higher adverse

outcomes, compared to the group with hyperfiltration ($\text{eGFR} > 120 \text{ ml/min/1.73m}^2$) in diabetes. Park et al has recently demonstrated that adverse pregnancy outcomes in a non-diabetes specific cohort are higher in groups at the extreme eGFR spectrum, that is in individuals with both an $\text{eGFR } 90\text{-}120 \text{ ml/min/1.73m}^2$ as well as those with an $\text{eGFR} > 150 \text{ ml/min/1.73m}^2$, compared to those with an $\text{eGFR } 121\text{-}150 \text{ ml/min/1.73m}^2$. [121] Overall, the protective effect of a higher eGFR in pregnancy in diabetes has yet to be studied and remain to be established. As renal hyperfiltration may have an association with less adverse pregnancy outcomes, averaging the eGFR in the whole pregnancy may have skewed our results, particularly in those with an attenuated ability to achieve peak pregnancy hyperfiltration. Nevertheless, by incorporating all available eGFR results throughout the pregnancy, we believe this allows for reflection of the overall effect of eGFR, rather than trimester specific changes.

The adverse obstetric outcomes in diabetic kidney disease in relation to potential comorbidities may also be related to inflammatory, vascular and oxidative pathways. Serial anti-angiogenic factors such as soluble fms-like tyrosine kinase has been demonstrated to be associated with preeclampsia in women with type 1 diabetes compared to healthy controls. [346] However, overall, there is a paucity of clinical data establishing the potential link of biomarkers in diabetic kidney disease with preeclampsia, and other adverse obstetric outcomes. Perhaps, the future in this area may involve the application of metabolomics or proteomics, where rather than a single biomarker, testing of a combination of diagnostic markers may be required to detect high risk cohorts earlier to enable careful clinical monitoring. A literature review on the potential utility of metabolite profile assisting in identifying pre-eclampsia (in non-diabetes specific cohort) was recently published, with metabolite markers including lipids, amino acids and their derivatives being promising when combined with maternal characteristics [429].

Strengths and Limitations

We recognize the limitations of this study. This was an observational, retrospective study, with potential missing data. However, to enable more complete data collection, we manually extracted and crosschecked information on paper and electronic hospital records, in addition to obtaining information from local practitioners for presumed missing data.

Finally, we had smaller numbers compared to larger multi-center, population-based studies; limiting our study's statistical power. However, the single center approach of data collection minimizes different practices being incorporated into the audit. For example, clinical practice of blood pressure measurement and glucose control are more consistent, and the measurements of biochemistry were performed using a single method or platform. Furthermore, we had detailed data including careful manual review of blood pressure recordings providing clear definition of preeclampsia as a pregnancy outcome. The relatively well-controlled blood pressure and reasonable glycemic management potentially reflect practices within a metropolitan obstetric hospital and may not be applicable extensively. However, our findings are likely to have a potential impact in facilitating diabetes and pregnancy care in the near future widely, as there is increased awareness and focus on improving perinatal outcomes.

It is important to note that for the purpose of this study, we have adapted the KDIGO guidelines for classification of stages of diabetic kidney disease. Of note, we defined stages of DKD purely based on eGFR, and did not incorporate albuminuria status for classification. Additionally, we did not measure serial kidney function or determine structural changes to fulfill the criterion of CKD that is defined as an abnormality of kidney structure or function present for more than three months.

As women without diabetes in our cohort had an eGFR > 90 mL/min/1.73m² and no recorded albuminuria status, we were not able to perform comparison outcome analysis between pregnancies affected by diabetes and those without. The lack of a comparison group of women without pre-existing diabetes limits our understanding on how our findings differ in relation to other pregnancies. Yet, it is clear from our findings that there is a difference between women affected by diabetic kidney disease and those who

do not, and we have taken steps to incorporate albuminuria data to improve our understanding of the impact of renal changes in this study. It is important to note that we were not able to incorporate other variables in the multivariable regression model, such as blood pressure, age, or BMI. These factors resulted in a high co-linearity and affected the performance of the statistical model. We can therefore only postulate the link of adverse outcomes are present with an eGFR $< 120 \text{ mL/min/1.73m}^2$ and ACR $> 31 \text{ mg/g}$. Nevertheless, we postulate that the high co-linearity is likely in part that factors including more advanced age, higher blood pressure and a higher BMI comes hand in hand as part of the phenotype of a high-risk individual with diabetes and a low eGFR.

It is important to consider that eGFR based on the CKD-EPI formula may not reflect true measured renal function in pregnancy, due to physiological renal hyperfiltration. However, we were limited by lack of measured GFR in this study, as this was a clinical audit, and therefore used CKD-EPI eGFR as a surrogate marker of renal function. Establishing the effect of diabetic kidney disease on pregnancies affected by diabetes, particularly type 2 diabetes is essential for safe and effective patient care due to the rising prevalence of women conceiving with pre-existing type 2 diabetes.

10.5 Conclusion

To conclude, we observed CKD-EPI eGFR as a potential renal marker of identifying high-risk pregnancies in diabetes independent from albuminuria status. This is the largest observational study in Australia in recent years with a focus on diabetic kidney disease, and findings will likely aid clinicians to anticipate the risks involved and counsel women with diabetes regarding pregnancy outcomes.

Table 10.3 Major obstetric outcomes according to indicators of diabetic kidney disease in women with type 1 and type 2 diabetes (combined)

	Preeclampsia				Preterm birth < 37 weeks			Preterm birth < 32 weeks			Neonatal intensive care		
	N	OR	95% CI	<i>P</i> value	OR	95% CI	<i>P</i> value	OR	95% CI	<i>P</i> value	OR	95% CI	<i>P</i> value
Urinary albumin⁺													
Normoalbuminuria ⁺ (reference)	112	1.0			1.0			1.0			1.0		
Microalbuminuria	23	5.7	91.8, 17.8	0.003	3.9	1.5, 9.9	0.004	4.1	0.8, 19.5	0.1	2.4	0.9, 6.8	0.1
Macroalbuminuria	9	5.5	2.5, 11.8	<0.001	3.5	1.6, 7.7	0.002	4.2	1.9, 9.5	<0.001	2.4	1.2, 4.6	0.01
DKD stages⁺⁺													
Hyperfiltration ⁺⁺ (reference)	81	1.0			1.0			1.0			1.0		
Stage 1 DKD	97	4.5	1.6, 12.3	0.004	2.3	1.2, 4.6	0.01	1.3	0.4, 4.2	0.6	2.0	0.9, 4.8	0.1
Stage 2+ DKD	8	3.9	1.7, 8.9	0.001	4.9	1.7, 14.5	0.004	5.0	1.7, 14.5	<0.001	4.9	2.0, 11.6	<0.001

⁺Albumin-to-creatinine ratio (mg/g): normoalbuminuria < 31; microalbuminuria 31 to 310; macroalbuminuria > 310

⁺⁺Diabetic kidney disease (DKD) stages defined by glomerular filtration rate estimated using Chronic Kidney Disease Epidemiology Cohort formula (mL/min/1.73m²):

Hyperfiltration > 120; Stage 1 > 90 to 120; Stage 2+ < 90

Univariate regression with results expressed as odds ratios (OR) and 95% confidence interval (CI), in relation to either normoalbuminuria⁺ or hyperfiltration⁺⁺

Odds ratio = OR; confidence interval = CI

Study Six

Chapter Eleven

Functional MRI in predicting diabetic kidney disease

11.1 Introduction

One of the current challenges in clinical practice is the difficulty in identifying individuals with diabetes who are at risk of development for the development and progression of, DKD. Once DKD develops, the optimal way to monitor progression still remains to be defined. The potential of a non-invasive imaging technique to assist with detection and monitoring is therefore attractive. Functional magnetic resonance imaging (MRI), a method that can go beyond anatomical assessment, and can delve into the renal physiology and structure at a microscopic level, is increasingly being investigated for assessment of chronic kidney diseases. Pertinent to DKD, MRI can potentially be employed to quantitatively measure intrarenal oxygenation through blood oxygen level dependent (BOLD) imaging; as well as allowing assessment of renal structural changes at the nephron level through water motion via diffusion tensor imaging (DTI). The parameters that will be investigated in this chapter are [264, 265, 285] :

- i) *Blood Oxygen Dependent (BOLD) imaging* based on deoxy-haemoglobin acting as an endogenous contrast agent to indirectly measure tissue oxygenation through quantitative resonance transverse relaxation rate ($R2^*$) values. Poorer tissue oxygenation (higher $R2^*$, indicating more deoxygenated haemoglobin) reflects greater oxygen consumption and metabolic activity within tissues
- ii) *Diffusion tensor imaging (DTI)* based on the principle of utilizing diffusion of water molecules to characterize the structural changes within tissues. The two measurements derived from DTI are apparent diffusion co-efficient (ADC) and fractional anisotropy (FA)
 - a. ADC measures magnitude of water motion, reflecting capillary perfusion and diffusion (higher ADC scores reflect more motion); and
 - b. FA measures directionality of water motion, with higher FA scores indicating more water molecules moving in the same direction.

The current data on MRI BOLD and DTI in DKD have mainly been reported in type 2 diabetes, and although promising, results to date are inconsistent. Overall, renal hypoxia has been demonstrated in some, but not all pre-clinical and clinical studies

comparing those with and without diabetes [274-277, 279, 280, 430, 431]. More pronounced DTI changes have been reported in those with later stages of kidney disease in diabetes [285, 287, 294, 295]. To date, there have been no studies combining BOLD and DTI for functional renal MRI in type 1 diabetes compared to healthy controls. There are also no known studies investigating renal hyperfiltration in diabetes with functional MRI, which may provide some insight into the mechanism of this process.

This was a prospective exploratory study with a primary aim to evaluate if multi-parametric MRI can detect changes in renal hypoxia utilizing BOLD technique, and renal structural changes utilizing DTI in participants with type 1 diabetes, with varying stages of DKD compared to healthy age and gender matched controls.

An important aspect for interpreting data of this study would be intra- and inter-reliability of the MRI technique. Detailed statistical methods, results and discussion on the inter-reliability, intra-reliability and scan repeatability of the MRI technique in our cohort is described in *Appendix One*. A summary of these aspects will be outlined in *Discussion* of this chapter.

Outcome Measures:

The primary outcome measures were renal functional and structural changes using multi-parametric MRI metrics of BOLD-derived $R2^*$ and DTI-derived apparent diffusion coefficient (ADC) and fractional anisotropy (FA) in type 1 diabetes with varying stages of DKD compared to healthy controls.

Secondary outcome measures were a comparison of other clinical and biochemical markers of DKD severity with functional and structural changes in MRI.

Specific Testable Hypotheses:

1. Compared to normal healthy subjects, participants with type 1 diabetes and DKD will have:
 - (i) functional changes of decreased renal oxygenation, as reflected by an increase in tissue $R2^*$

(ii) structural changes, as reflected by lower FA and ADC

2. These renal functional changes will correlate with:

(i) glomerular filtration rate (GFR) as reflected by direct measurement of GFR and estimates of GFR

(ii) inflammatory and oxidative stress biomarkers (serum and urinary)

11.2 Methods

34 participants with type 1 diabetes and varying stages of diabetic kidney disease and 10 age- and gender- matched controls with normal renal function were recruited in a prospective cross-sectional study. Participants with type 1 diabetes and healthy controls were age and sex matched. Participants with type 1 diabetes were recruited from the Austin Health Diabetes Clinic.

Exclusion criteria were:

- contraindications to MRI,

-presence of anaemia defined by haemoglobin of < 100 g/L to minimize effects of anaemia on BOLD imaging

The Austin Health Human Research Ethics Committee approved ethics for this study. Informed consent was obtained from each participant. Following an overnight fast, a single clinician investigator obtained detailed medical history including medications, and obtained blood pressure, weight and height. Testing included blood tests for biomarkers, 24-hour urine collection and functional MRI with controlled water intake (of a maximum 100mls with a total fasting duration between 8-10 hours).

MRI acquisition and analysis:

The study protocol for MRI acquisition and analysis has been adapted from existing publications on BOLD MRI and DTI [279, 281, 291, 294, 432].

MRI was performed on a 3.0-Tesla magnet (Skyra, Siemens, Germany) at the Melbourne Brain Centre-Austin Campus. The protocol was performed in under 45 minutes and included both anatomical and functional imaging sequences. For anatomical imaging, T2-weighted HASTE images were obtained. 2 runs of BOLD MRI were performed in the axial plane with a single breath-hold acquisition (13 seconds) to mitigate the effects of respiratory motion. A single run of respiratory navigated DTI MRI was acquired. To determine renal artery stenosis, non-contrast magnetic resonance angiography (MRA) using an inversion recovery balanced steady state free precession technique (Native TrueFISP, Siemens, Erlangen, Germany) was performed.

Total MRI acquisition was under 45 minutes. Images were acquired with the following parameters:

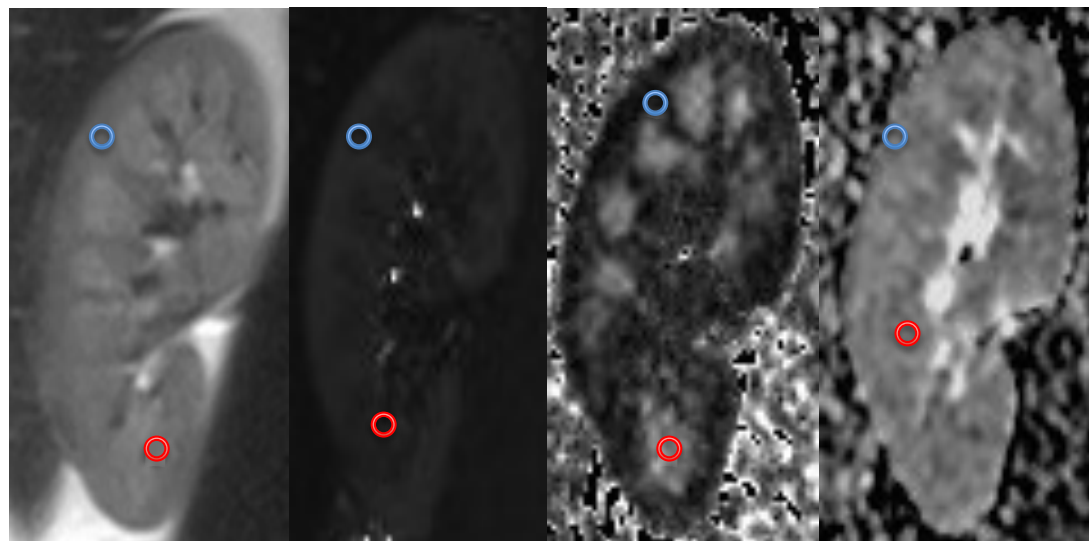
	BOLD MRI (multi-echo gradient-echo)	DTI
Repetition Time and Echo Time (TR/TE)	85/ (4.3-56.7) milliseconds	1800/81 milliseconds
Number of Echoes (TEs)	12	1
Inter-echo spacing	4.8 milliseconds	0.6 milliseconds
Flip Angle	30°	90°
Field of View (mm)	420x420	400x400
Matrix	320x320	340x340
Number of slices	1	7
Slice Thickness	7mm	3mm
B Value	Not applicable	0 and 500 s/mm ²
Directions	Not applicable	12

Images were de-identified and analysed on a dedicated workstation (Multimodality workspace, Siemens, Erlangen, Germany). T2* maps, reflecting the effective transverse relaxation time of the interrogated tissues, were created from BOLD images

for reader analysis. FA and ADC maps from DTI were automatically calculated by the scanner.

Regions of Interest (ROI) of a minimum of 10 pixels for each MRI parameter (T2*, FA and ADC) were defined in the cortex and medulla for both left and right kidneys, with reference to T2-weighted anatomical images (HASTE, Figure 11.1). For each MRI parameter, up to six ROIs were defined per each kidney, with the mean of these computed, resulting in one cortical and medullary value per participant per kidney. Cortical and medullary ROIs were drawn using a freehand tool, avoiding artifacts (motion, vessels, bowel loops, or mass lesions if present) and excluding areas with significant voxel changes or differences.

Figure 11.1 Examples of MRI BOLD and DTI maps with region of Interest



T2 HASTE

T2* map

FA map

ADC map

BOLD(T2*) and DTI maps of right kidney. The blue circles represent region of interest (ROI) defined on the cortex, while the red circles represent ROI on the medulla.

In our initial analysis, to reduce significant bias from measurement variability, each participant's MRI imaging was analysed independently by three inexperienced readers (2 radiologists and 1 endocrinologist). All readers were blinded to clinical information and underwent similar study protocol MRI training by an experienced radiologist with subspecialty training in abdominal MRI. MRI parameters from all 3 readers were obtained and analysed, and expressed as follows:

	Right		Left	
	Cortex	Medulla	Cortex	Medulla
BOLD	RCR2*	RMR2*	LCR2*	LMR2*
DTI (FA)	RCFA	RMFA	LCFA	LMFA
DTI (ADC)	RADC	RMADC	LCADC	LMADC
Right = R, Left = L				
BOLD: CR2* = cortical R2*; MR2* = medullary R2*				
DTI: CFA= cortical fractional anisotropy; MFA= medullary fractional anisotropy				
CADC=cortical apparent diffusion co-efficient; MADC= medullary apparent diffusion co-efficient				

Subsequent Amendment in MRI analysis:

Post-hoc modifications to MRI sequences were implemented with reduced number of echoes for BOLD parameters were employed to minimize effects of susceptibility artefact from bowel gas.

Images were re-analysed by the least experienced (endocrinologist- reader B), and most experienced reader (initial instructor-reader A), who repeated DTI measurements as well. In order to assess intra-rater agreement, ten participants' images were randomly selected by computer software and analysed twice by reader B.

Renal function measurement:

To determine measured Glomerular Filtration Rate (mGFR), each patient with type 1 diabetes underwent ^{99m}Tc -diethylene-triamine-penta-acetic acid (^{99m}Tc -DTPA) plasma clearance with Brochner-Mortensen correction within 12-months of the MRI. Measured GFR is considered clinically relevant and are undertaken in patients with type 1 diabetes at the Austin Health Diabetes Clinic every 5 years to monitor long-term renal function. On the other hand, healthy participants were excluded from undergoing ^{99m}Tc -DTPA plasma clearance; as apart from having an invasive component requiring insertion of an intravenous catheter, the process also involves a small dose of radiation.

Estimated Glomerular Filtration Rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formulation as below [357]. To convert from absolute values to values normalized to 1.73 m^2 body surface area (BSA), results were multiplied by $1.73/\text{BSA}$ [358].

Females:

If serum creatinine $\leq 62\text{ mol/L}$: $144 \times (\text{serum creatinine} \times 0.0113 / 0.7)^{0.329} \times 0.993^{\text{age}}$

If serum creatinine $> 62\text{ mol/L}$: $144 \times (\text{serum creatinine} \times 0.0113 / 0.7)^{1.209} \times 0.993^{\text{age}}$

Males:

If serum creatinine ≤ 80 mol/L: $144 \times (\text{serum creatinine} \times 0.0113 / 0.9)^{0.411} \times 0.993^{\text{age}}$

If serum creatinine > 80 mol/L: $141 \times (\text{serum creatinine} \times 0.0113 / 0.9)^{1.209} \times 0.993^{\text{age}}$

Creatinine clearance was measured using plasma and 24-hour urine collection for both participants with and without diabetes. Detailed calculation of estimated GFR based on the CKD-EPI formula and 24-hour creatinine calculation is outlined in the methods section of Chapter 6: Evaluating renal function in diabetes and pregnancy with inulin plasma clearance.

Hyperfiltration (HF) was arbitrarily defined as a mGFR $> 120 \text{ ml/min/1.73m}^2$

Biomarker Assays:

All collected serum samples were centrifuged at 3500xg for 10min at 4°C. Serum and plasma were divided into aliquots of at least 1ml and frozen in 2ml cryostorage vials at -20°C. For urine, a 10ml aliquot from a 24hr urine collection had 50uL 5M NaOH added to better preserve albumin for long term storage, before dividing into 2x 5ml aliquots & freezing at -20°.

The following markers were assessed in serum: C-reactive protein (CRP), Neutrophil to Lymphocyte ratio (NLR), soluble receptor for advanced glycation end products (sRAGE), soluble tumour necrosis factor receptor type 1 / 2 (sTNFR1/2), monocyte chemoattractant protein 1(MCP1/ CCL2), total 8-Isoprostane, neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule (KIM-1) and soluble Interleukin-2 Receptor (sIL2R).

The following corresponding urinary biomarkers were measured: sTNFR1 and 2, MCP1/CCL2, total 8-Isoprostane, NGAL, KIM1 and, sIL2R. Due to the shortage of assay kits, five serum NGAL samples; along with twenty-six urinary 8-Isoprostane samples were not assayed. These were all type 1 diabetes samples.

Further details on analyte assays are outline in the methods section of Chapter 8: Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy.

Statistical Analysis and Power:

This was a pilot study. An estimate of required sample size was obtained from Yin et al. [275]. A unit difference of approximately 0.36 (19%) in Medullary-Cortical R2* ratio (MCR) between controls and participants with type 2 diabetes, with a mean eGFR $\geq 60 \text{ ml/min/1.73m}^2$ was calculated from the published data. Using this precedent data, a minimum sample of 18 cases and 6 controls would be required to demonstrate such a difference, assuming a 3:1 case-to-control sample ratio, with 80% power at the 5% significance level. Therefore, a sample size of 30 participants with diabetes and 10 controls was proposed.

Results are presented in median and interquartile range unless otherwise stated. P-value significance is <0.05 for data based on comparison between two groups (e.g. Diabetes vs. Controls, or HF vs. Non-HF). P-value significance for multiple testing dataset (e.g. Biomarkers) are based on adjustment of Bonferroni corrections for multiplicity. All P-values are stated in the footnote for each result table.

The following analyses were conducted for MRI parameters (R2*, FA and ADC):

1. Univariate analysis with Mann-Whitney tests, followed by quantile regression to detect differences between
 - a. Univariate analysis of controls and type 1 diabetes, and
 - b. Type 1 diabetes with and without hyperfiltration.Hyperfiltration was defined as a mGFR $>120 \text{ ml/min/1.73m}^2$
2. Spearman correlation (as variables were not normally distributed) for the relationship of MRI metrics with
 - a. renal function: mGFR, eGFR and CrCl
 - b. serum renal biomarkers: NLR, CRP, sRAGE, sTNFR1, sTNFR2, MCP1/CCL2, total 8-Isoprostane, NGAL, KIM 1 and sIL2R

- c. urinary renal biomarkers: sTNFR1, sTNFR2, MCP1/CCL2, total 8-Isoprostane, NGAL, KIM 1 and sIL2R

Urinary biomarker concentrations were corrected for urinary excretion rate using urinary volume over a 24-hour timed collection. Serum and urinary biomarkers were log-transformed for statistical analysis.

11.3 Results

44 subjects were recruited and subsequently completed the study protocol. One participant with type 1 diabetes did not complete the MRI protocol due to claustrophobia. Due to significant respiratory motion artifact, images of another participant with type 1 diabetes (ID35) were non-diagnostic. Subsequent analysis included the data from 32 participants with type 1 diabetes and 10 healthy controls.

Baseline characteristics (Table 11.1, Table 11.2 and Figure 11.2)

Characteristics of participants with and without diabetes are documented in Table 11.1. There were no differences in smoking status between those with and without diabetes. The median duration of diabetes was 18.5 (9, 29) years, with an HbA1c of 7.5 (7.0, 8.5) % [59 (53, 69) mmol/mol]. As expected, participants with type 1 diabetes had higher fasting blood glucose. Participants with type 1 diabetes 26(24,31) kg/m² had higher BMI compared to healthy controls 23(21,26) kg/m², p=0.01. There were no healthy controls with pre-existing hypertension, which was present in 38% of participants with type 1 diabetes, with 8.6% undergoing treatment with renin-angiotensin-aldosterone blockers and 15.6% with diuretics. Systolic blood pressure was similar in both groups (p=0.5), whilst diastolic blood pressure was slightly higher in healthy controls at 76(73,80) mmHg compared to 70 (65, 75) mmHg (p=0.03).

Median mGFR for participants with type 1 diabetes was 105 (77,120) ml/min/1.73m², with 31% meeting the arbitrarily defined criteria for hyperfiltration with an mGFR ≥ 120 ml/min/1.73m². There were no significant differences for estimated GFR (eGFR) (p=0.4) or creatinine clearance (p=0.1) between participants with type 1 diabetes and healthy controls. Within the type 1 diabetes cohort, mGFR correlated with both eGFR and creatinine clearance (Figure 11.2) Despite being within the normo-albuminuria range, albumin creatinine ratio (ACR) was higher in participants with type 1 diabetes 0.8 (0.5, 1.4) mg/mmol in contrast to healthy controls 0.0 (0,0.6) mg/mmol, p=0.004.

Baseline characteristics of participants with type 1 diabetes who were hyperfiltering compared to those who were normofiltering are documented in (Table 11.2) Participants with hyperfiltration were younger (31.5 years vs. 49.5 years, p=0.006). There were no other significant differences between the two groups including gender, duration of diabetes, body mass index or HbA1c.

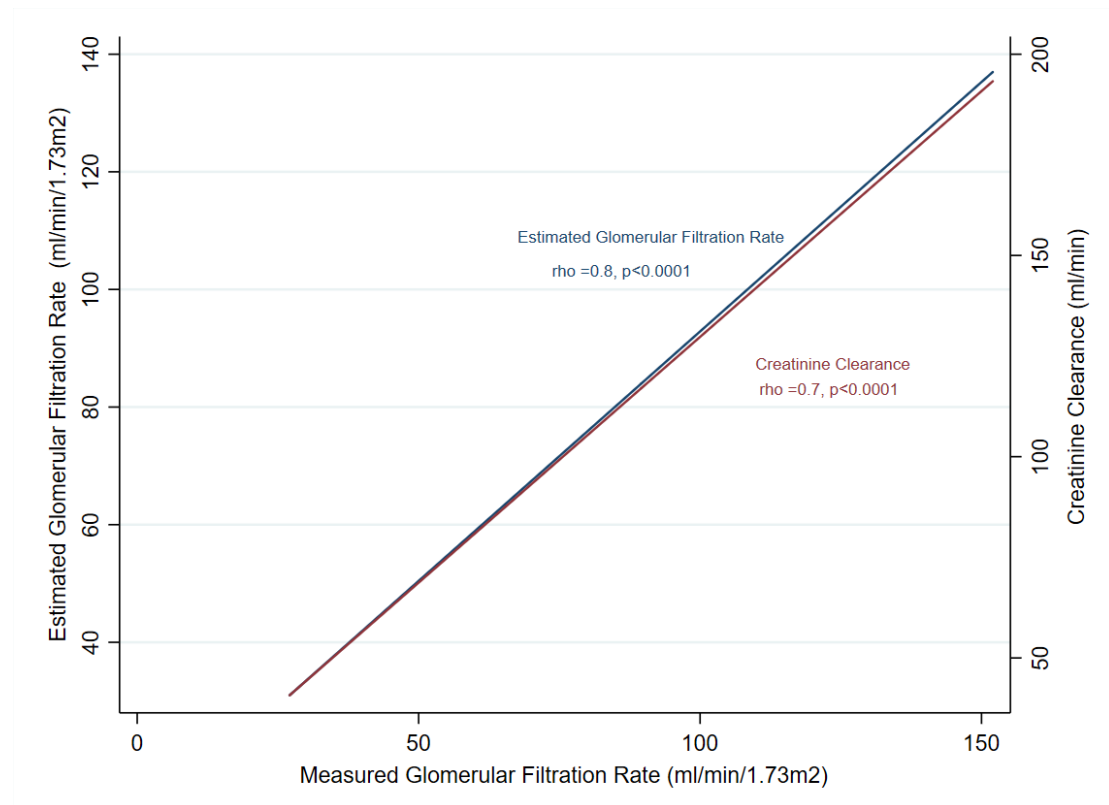
Table 11.1 Baseline characteristics of participants with type 1 diabetes and healthy controls

	Type 1 Diabetes	Healthy Controls	p-value
N	32	10	
Age (years)	45 (32,55)	45 (31,55)	0.8
Sex (M:F)	20:12	5:5	0.5
BMI (kg/m ²)	26 (24,31)	23 (21,26)	0.01
Duration of diabetes (years)	18.5 (9,29)	NA	
Smoker	4 (12.5%)	0	0.2
HbA1c (%)	7.5 (7.0, 8.5)	NA	
(mmol/mol)	59 (52,69)		
Fasting plasma glucose(mmol/L)	8.9 (7.1, 12.1)	4.9 (4.7, 5.8)	0.0001
Systolic blood pressure (mmHg)	128 (120,135)	130 (122, 132)	0.5
Diastolic blood pressure (mmHg)	70 (65, 75)	76 (73,80)	0.03
Serum creatinine (mmol/l)	77(66,88)	82 (75, 87)	0.5
Measured GFR (ml/min/1.73m ²)	105 (77,120)	NA	
≥ 120	10 (31%)		
90-119	12 (38%)		
60-90	7 (22%)		
< 60	3 (8%)		
CKD-EPI eGFR (ml/min/1.73m ²)	100(76,110)	94 (80,99)	0.4
≥120	4 (12.5%)	0	
90-119	16 (50%)	6 (60%)	
60-90	9 (28.1%)	4 (40%)	
< 60	3 (9.4%)	0	
Creatinine Clearance (ml/min)	124 (91,156)	123 (102,154)	1.0
≥120	17 (57)	3 (30)	
<120	13 (43)	7 (70)	
Albumin Creatinine Ratio (mg/mmol)	0.8 (0.5,1.4)	0 (0, 0.6)	0.004
Albumin Excretion Rate (mcg/24hours)	8.5(4.8, 18)	5.6(4.2,6.2)	0.07
Pre-existing hypertension (N/%)	16 (38.1)	0	0.05
RAAS blocker (N/%)	8 (8.6)	0	0.02
Diuretics (N/%)	5(15.6)	0	0.18
Other antihypertensive agents (N/%)	7(22.5)	0	0.1
Diabetic retinopathy (N/%)	6 (18.8)	NA	
Results expressed as Median (25 th , 75 th percentile) or Frequency (%)			
Stages of Diabetic Kidney Disease based on Kidney Disease: Improving Global Outcome Guidelines:			
Hyperfiltration ≥ 120ml/min/1.73m ²			
Stage 1 ≥90-119 ml/min/1.73m ²			
Stage 2 ≥60-90 ml/min/1.73m ²			
Stage 3/4 < 60 ml/min/1.73m ²			

Table 11.2 Baseline characteristics in participants with and without hyperfiltration in type 1 diabetes

	Type 1 Diabetes mGFR >120 ml/min/1.73m ²	Type 1 Diabetes mGFR ≤ 120 ml/min/1.73m ²	p-value
N	10	22	
Age (years)	31.5(29,45)	49.5(37,59)	0.006
Sex (M:F)	8:2	12:10	0.2
BMI (kg/m ²)	27.2(24.1,32.5)	26.1(24.6,29.3)	0.8
Duration of diabetes (years)	12 (2,20)	20(11,32)	0.1
Smoker (N/%)	1(10%)	3(13.6%)	0.8
HbA1c (%)	7.5(7.1,9.0)	7.7(7.1,9.0)	0.6
(mmol/mol)	59(54,75)	61 (54, 75)	
Fasting plasma glucose(mmol/L)	9.4(7.8,13.0)	7.9(9.0,11.4)	0.2
Systolic blood pressure (mmHg)	123(120,135)	130(120,135)	0.5
Diastolic blood pressure (mmHg)	70(68,75)	70(65,80)	0.9
Serum creatinine (mmol/l)	69(62,81)	80(73,95)	0.03
Measured GFR (ml/min/1.73m ²)	124(121,129)	94(70,105)	<0.001
Stage 1 CKD (N=12)		105(98,115)	
Stage 2 CKD (N= 7)		76(65,78)	
Stage 3/4 CKD (N= 3)		35(27,58)	
Creatinine Clearance (ml/min)	163 (125,196)	108(82,130)	0.003
CKD-EPI eGFR (ml/min/1.73m ²)	114(103,125)	87(68,104)	<0.001
Stage 1CKD (N=12)		103(96,107)	
Stage 2 CKD (N=7)		75(65.86)	
Stage 3/4 CKD (N=3)		25(13,56)	
Albumin Excretion Rate (mcg/24hours)	6.5(4.8,18.0)	9.2(4.6,28.9)	0.4
Albumin Creatinine Ratio (mg/mmol)	0.8(0.4,1.4)	0.8(0.6,3.5)	0.4
Pre-existing hypertension (N/%)	3(30)	13(59)	0.1
RAAS blocker (N/%)	3(30)	10(45.5)	0.4
Diuretics	0	5(22.7)	0.1
Other antihypertensive agents (N/%)	0	7(33.3)	0.04
Results expressed as Median (25 th , 75 th percentile) or Frequency (%)			
Stages of CKD (Chronic Kidney Disease) based on Kidney Disease: Improving Global Outcome Guidelines			
Hyperfiltration ≥ 120ml/min/1.73m ²			
Stage 1 ≥90-119 ml/min/1.73m ²			
Stage 2 ≥60-90 ml/min/1.73m ²			
Stage 3/4 < 60 ml/min/1.73m ²			

Figure 11.2 Relationship between measured GFR, estimated GFR and creatinine clearance



MRI parameters: Cortical and Medullary Differences in Type 1 Diabetes and Healthy Controls (Table 11.3)

We conducted univariate analyses to compare cortical to medullary MRI parameters (Table 11.3). In type 1 diabetes, R2* (14.7 (13.7, 15.8) vs. 24.8(21.8,28.2); $p<0.001$) and FA (0.12(0.10,0.14) vs. 0.43(0.37,0.48), $p<0.001$) were lower; while ADC was higher (2.47(2.33,2.61) vs. 2.36(2.13,2.53), $p <0.001$) in the cortex compared to the medulla. Similar significant results were found in healthy controls, with both R2* and FA lower, in contrast to ADC which was higher in the cortex compared to medulla.

Table 11.3 Univariate analysis comparing cortical and medullary MRI parameters for both type 1 diabetes and healthy controls

	Cortical	Medullary	
	Median (IQR)	Median (IQR)	<i>P</i>
Diabetes			
R2* (1/s)	14.7 (13.7, 15.8)	24.8(21.8,28.2)	<0.001
FA	0.12(0.10,0.14)	0.43(0.37,0.48)	<0.001
ADC (10³mm²/s)	2.47(2.33,2.61)	2.36(2.13,2.53)	<0.001
Controls			
R2* (1/s)	15.7(15.1,16.6)	29.3(24.3,32.4)	<0.001
FA	0.11(0.10,0.12)	0.46(0.43,0.49)	<0.001
ADC (10³mm²/s)	2.46(2.37,2.54)	2.26(2.16,2.41)	<0.001
R2*= resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient			
Results expressed as Median (25 th , 75 th percentile).			
Comparisons made between groups with Mann-Whitney test. Significant P-value = 0.05			

MRI parameters: Type 1 Diabetes and Healthy Controls (Table 11.4 and Table 11.5)

Overall results with univariate analysis (Table 11.4) demonstrated that R2* was significantly lower both in the cortex and medulla in participants with diabetes compared to healthy controls. Cortical FA was higher in diabetes; while medullary FA was higher in controls. On the other hand, there were no significant differences in both cortical and medullary ADC in participants with and without diabetes.

Table 11.4 Univariate analysis comparing MRI parameters between type 1 diabetes and healthy controls

	Type 1 Diabetes	Controls	
	Median (IQR)	Median (IQR)	<i>P</i>
R2* CORTEX (1/s)	14.7 (13.7, 15.8)	15.7(15.1,16.6)	<0.001
R2* MEDULLA (1/s)	24.8 (21.8,28.2)	29.3(24.3,32.4)	<0.001
FA CORTEX	0.12 (0.10,0.14)	0.11(0.10,0.12)	0.03
FA MEDULLA	0.43 (0.37,0.48)	0.46(0.43,0.49)	0.004
ADC CORTEX (10³mm²/s)	2.47 (2.33,2.61)	2.46(2.37,2.54)	0.4
ADC MEDULLA (10³mm²/s)	2.36 (2.13,2.53)	2.26(2.16,2.41)	0.6

R2*= resonance transverse relaxation rate; FA=fractional anisotropy; ADC= apparent diffusion coefficient

Results expressed as Median (25th, 75th percentile).

Comparisons made between groups with Mann-Whitney test. Significant P-value = 0.05

We subsequently performed clustered quantile regression analysis taking different variables (readers, anatomical location of left and right, and eGFR) into consideration (Table 11.5). For R2*, the difference in both cortical and medullary R2* persisted, and was 4.7(2.1, 7.2) (1/s) (p<0.001) and 0.9(0.2,1.6) (1/s) (p=0.01) lower in diabetes compared to healthy controls respectively. Similar to results of univariate analysis, there were no significant differences between participants with and without diabetes for cortical and medullary ADC. There were also no significant differences in medullary FA between both groups. On the other hand, rather than a higher cortical FA in diabetes, as with univariate analysis; when confounding factors were taken into account, cortical FA readings were instead 0.04 (0.01,0.07) (p=0.02) lower in diabetes compared to controls.

Table 11.5 Quantile regression of MRI parameters

	Type 1 Diabetes compared to Controls adjusted for eGFR	Type 1 Diabetes Hyperfiltration compared to Non-Hyperfiltration
R2* CORTEX (1/s)	-4.7 (-7.2, -2.1) p<0.001	-0.1 (-1.6,1.7) p=0.9
R2* MEDULLA (1/s)	-0.9 (-1.6, -0.2) p=0.01	-2.7(-5.9, 0.4) p=0.1
FA CORTEX	-0.04 (-0.07, -0.01) p=0.02	-0.01 (-0.02,0.03) p=0.6
FA MEDULLA	0.01(-0.003,0.03) p=0.2	0.17 (-0.06, 0.40) p= 0.2
ADC CORTEX (10³mm²/s)	0.01 (-0.09, -0.23) p=0.4	0.21 (0.09, 0.33) p=0.001
ADC MEDULLA (10³mm²/s)	0.01 (-0.07, 0.09) p=0.9	0.02 (-0.04, 0.07) p=0.5

R2*= resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient

Comparisons made between groups with median quantile regression adjusted for readers, anatomical location of left/ right side. Further adjustment with 2 runs of analysis for R2*

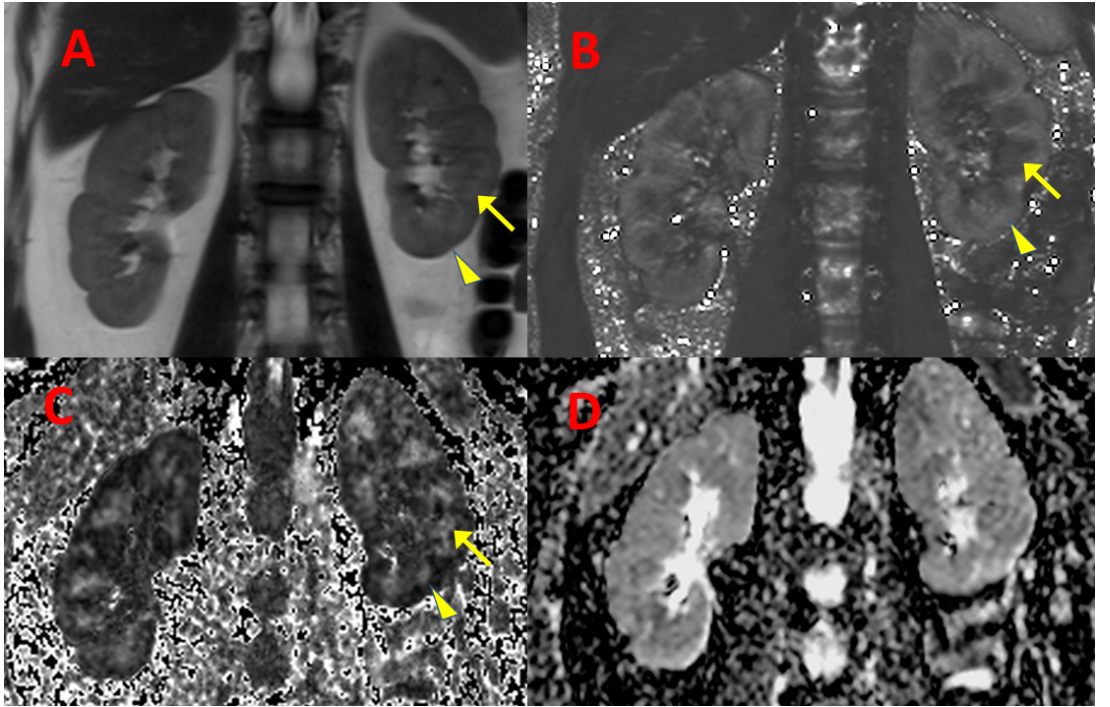
Results expressed as Co-efficient, 95% Confidence Interval

Interpretation example:

For R2*, a co-efficient value of -4.7 indicates that R2* was 4.7 (1/s) lower in type 1 diabetes compared to controls

The images below in Figure 11.3, 11.4 11.5 and 11.6 depict examples of anatomical T2-weighted maps, FA and ADC in those with and without type 1 diabetes with different stages of diabetic kidney disease.

Figure 11.3 Example of MRI images and metrics of a healthy control

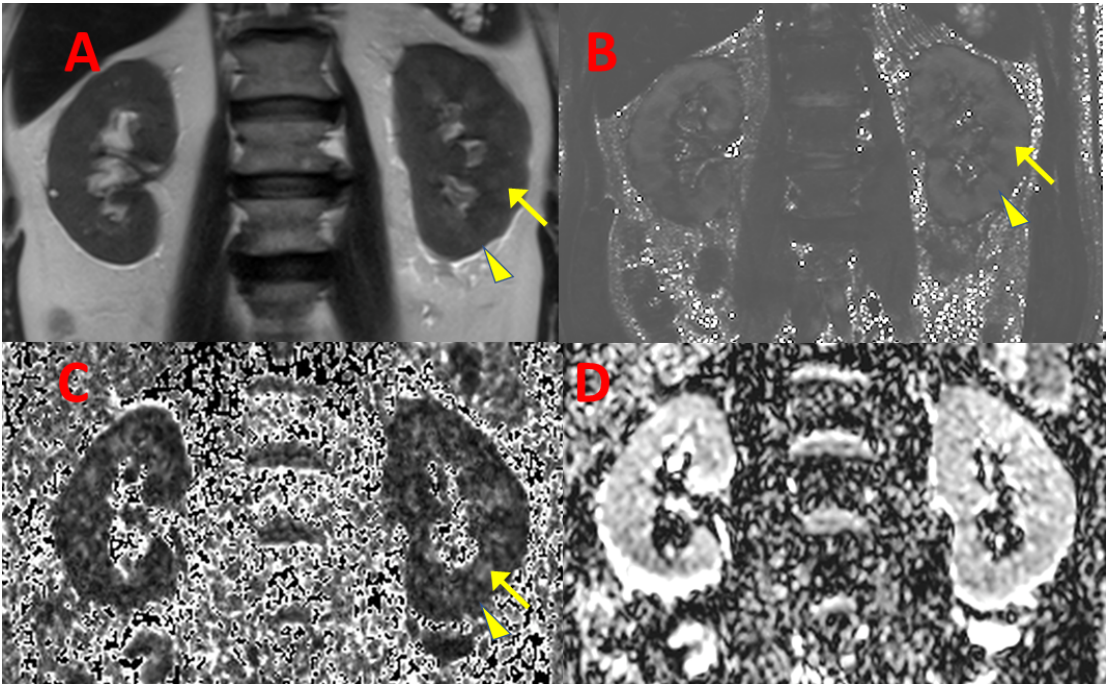


50-year old female healthy control with an eGFR of 83ml/min/1.73m². A) T2-weighted anatomic image; B) T2* map from BOLD imaging from which R2* values are derived; C) FA map; D) ADC map. Cortex (arrow) and medulla (arrowhead) can be readily differentiated with multiple techniques. On A, subtly higher signal of the medulla reflects intrinsic differences in T2 relaxation time of cortex versus medulla. On B, lower medullary signal indicated greater hypoxia (lower T2* and higher R2*). On C, higher medullary signal compared to cortex reflects higher fractional anisotropy, with more orderly structure of the medulla related to tubular microarchitecture. On D, it is more difficult to differentiate between cortex and medulla, with relatively similar ADC values.

MRI metrics of this participant based on Reader A’s readings:

	Right Cortex	Right Medulla	Left Cortex	Left Medulla
R2* (1/s)	15.5	27.6	14.8	30.7
FA	0.11	0.45	0.12	0.31
ADC (10³mm²/s)	2.27	2.14	2.69	2.62
R2*= resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient				

Figure 11.4 Example of MRI images and metrics of a participant with stage one diabetic kidney disease

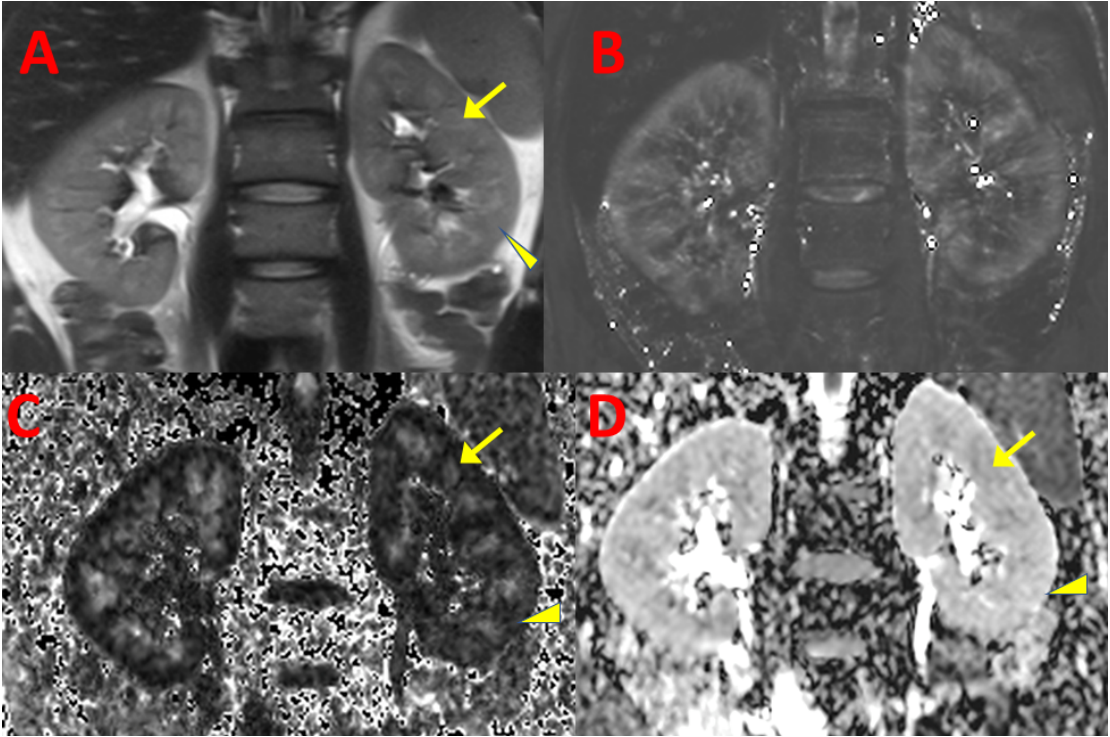


58-year old male with type 1 diabetes with an eGFR of 91 ml/min/1.73m² and mGFR of 98 ml/min/1.73m². A) T2-weighted anatomic image; B) T2* map from while BOLD imaging; C) FA map; D) ADC map. Similar to the healthy control, higher signal on T2-weighted imaging, lower T2*, higher FA is observed in the medulla (arrow) compared to the cortex (arrowhead) for both BOLD (B) and FA map (C) derived from DTI imaging.

MRI metrics of this participant based on Reader A readings:

	Right Cortex	Right Medulla	Left Cortex	Left Medulla
R2* (1/s)	15.6	23.1	16.8	26.8
FA	0.21	0.43	0.13	0.49
ADC (10³mm²/s)	2.13	2.06	2.69	1.93
R2*= resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient				

Figure 11.5 Example of MRI images and metrics of a participant with type 1 diabetes and hyperfiltration



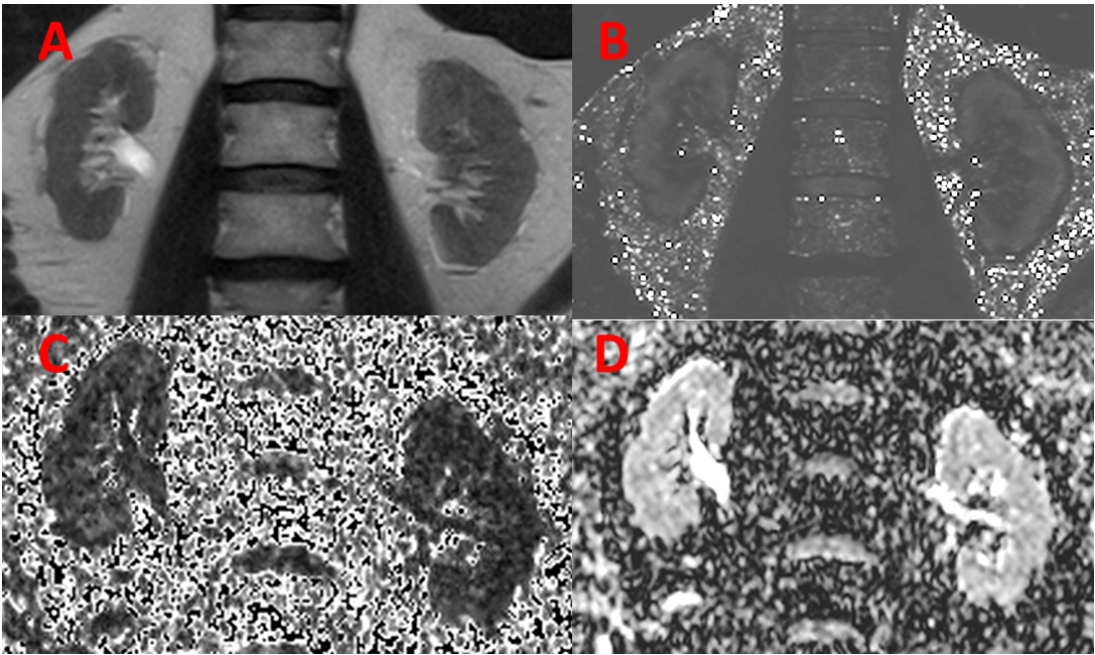
28-year old male with type 1 diabetes and an eGFR of 125 ml/min/1.73m² and mGFR of 129 ml/min/1.73m². A) T2-weighted anatomic image; B) T2* map from BOLD imaging; C) FA map; D) ADC map. Similar to the control subject, there is clear cortico-medullary differentiation on (A), with differences also visible on the ADC map (D), where a lower medullary ADC is apparent (medulla, arrow; cortex, arrowhead).

MRI metrics of this participant based on Reader A readings:

	Right Cortex	Right Medulla	Left Cortex	Left Medulla
R2* (1/s)	16.9	27.3	12.6	32.6
FA	0.91	0.44	0.10	0.51
ADC (10³mm²/s)	2.59	2.45	2.45	2.35

R2*= resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient

Figure 11.6 Example of MRI images and metrics of a participant with stage 4 diabetic kidney disease



70-year old male with type 1 diabetes and an eGFR of 13ml/min/1.73m^2 , and mGFR of 27ml/min/1.73m^2 . A) T2-weighted anatomic image; B) T2* map from BOLD imaging; C) FA map; D) ADC map. There is loss of cortico-medullary differentiation. The kidneys are also small, a relatively late manifestation of CKD.

MRI metrics of this participant based on Reader A readings:

	Right Cortex	Right Medulla	Left Cortex	Left Medulla
R2* (1/s)	14.9	24.6	14.4	19.8
FA	0.19	0.38	0.16	0.39
ADC ($10^3\text{mm}^2/\text{s}$)	2.51	2.54	2.17	1.88

R2*= resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient

***MRI parameters: Type 1 Diabetes hyperfiltration versus non-hyperfiltering group
(Table 11.5 and Table 11.6)***

Univariate comparison (Table 11.6) of MRI parameters between normofiltration (measured GFR<120mls/min/1.73m²) and hyperfiltration (measured GFR≥120mls/min/1.73m²) showed no significant difference for cortical R2*, cortical FA and medullary FA. There was a marginally higher medullary R2* in the hyperfiltration 25.8 (23.3,28.7) (1/s) compared to normofiltration 24.4 (21.4,27.9) (1/s) (p=0.01) group. ADC in the cortex 2.61 (2.53, 2.74) (10³mm²/s) vs. 2.40 (2.29, 2.53) (10³mm²/s) (p<0.001) and medulla 2.46 (2.34, 2.58) vs. 2.27 (2.08, 2.48) (p=0.02) were also observed to be higher in the hyperfiltration group.

Following clustered quantile regression (Table 11.5) the only significant difference between both groups were cortical ADC. Higher cortical ADC 0.21 (0.09, 0.33) (10³mm²/s) p=0.01 persisted in participants with diabetes that were hyperfiltering compared to those who were not. The variation in medullary R2* and ADC attenuated.

Table 11.6 Univariate analysis comparing participants with and without hyperfiltration in type 1 diabetes

Type 1 Diabetes	mGFR ⁺ < 120mls/min	mGFR ⁺ ≥120mls/min	P
R2* CORTEX (1/s)	14.7 (13.8,15.8)	14.7(13.7,16.0)	0.6
R2* MEDULLA (1/s)	24.4 (21.4,27.9)	25.8(23.3,28.7)	0.01
FA CORTEX	0.12 (0.10,0.13)	0.13(0.10,0.14)	0.4
FA MEDULLA	0.43 (0.36,0.48)	0.44(0.39,0.49)	0.2
ADC CORTEX (10³mm²/s)	2.40 (2.29,2.53)	2.61(2.53,2.74)	<0.001
ADC MEDULLA (10³mm²/s)	2.27 (2.08,2.48)	2.46(2.34,2.58)	0.002

⁺Measured Glomerular Filtration Rate (mGFR) based on Tc-99m-DTPA nuclear medicine GFR with Brochner-Mortensen correction. R2*=resonance transverse relaxation rate, FA=fractional anisotropy, ADC= apparent diffusion coefficient. Results expressed as Median (25th, 75th percentile). Comparisons made between groups with Mann-Whitney test. Significant P< 0.05

MRI parameters: Relationship with Renal Function and Biomarkers (Table 11.7, and Table 11.8)

Weak correlations were established between cortical and medullary R2*, medullary FA and cortical ADC with renal function (both estimated and measured GFR).

Some fair associations were observed between all MRI metrics with selected serum and urinary renal biomarkers. Most of these associations were detected with medullary R2*. Urinary sIL2R was undetectable in all samples.

Table 11.7 Correlation between MRI parameters with renal function

	CR2	MR2	CFA	MFA	CADC	MADC
Estimated Glomerular Filtration Rate						
rho	0.2	0.2	0.03	0.2	0.4	0.1
P	0.7	<0.001	0.6	0.002	<0.001	0.4
Measured Glomerular Filtration Rate						
rho	0.1	0.3	0.1	0.3	0.6	0.3
P	0.4	<0.001	0.6	<0.001	<0.001	0.03

Analysis based on Spearman rank correlation coefficient. Results expressed as rho.
C=Cortical, M=Medullary, R2*=resonance transverse relaxation rate, FA=fractional anisotropy,
ADC= apparent diffusion coefficient. Significant P-value = 0.008, adjusted with Bonferroni
corrections

Table 11.8 Correlation between MRI parameters with selected renal biomarkers

	CR2*	MR2*	CFA	MFA	CADC	MADC
NLR	0.01	-0.1	0.1	-0.2	0.02	-0.1
CRP	-0.13	-0.3*	0.2	-0.03	0.1	0.1
sRAGE	-0.2	-0.1	-0.1	-0.2	-0.1	0.1
sTNFR1	-0.2	-0.4*	0.1	-0.2	-0.4*	-0.3*
uTNFR1	-0.2	-0.3*	0.3	-0.1	-0.02	-0.2
sTNFR2	-0.1	-0.4*	0.1	-0.4*	-0.4*	-0.2
uTNFR2	-0.2	-0.3*	0.2	-0.2	0.002	-0.01
sMCP1	-0.2	-0.4*	0.1	-0.2	-0.3*	-0.4*
uMCP1	-0.1	-0.1	0.1	-0.2	0.1	0.04
s8IP	0.04	-0.3*	0.1	-0.3	-0.2	-0.3
u8IP	-0.02	0.3	0.3	0.4	0.3	0.5
sNGAL	-0.5	-0.3*	-0.1	-0.2	-0.3*	-0.03
uNGAL	-0.4*	-0.3*	0.1	-0.3	-0.1	-0.1
sKIM1	-0.2	-0.3*	0.1	-0.4*	-0.3*	-0.2
uKIM1	-0.1	-0.1	0.3	0.002	0.1	-0.01
sIL2R	-0.2*	-0.3*	-0.1	-0.4*	-0.3*	-0.1
uIL2R	-	-	-	-	-	-

Analysis based on Spearman rank correlation coefficient. Results expressed as rho.

* Significant p value < 0.001, adjusted with Bonferroni corrections.

C=Cortical; M=Medullary; R2*= resonance transverse relaxation rate; FA=fractional anisotropy; ADC= apparent diffusion coefficient; NLR: Neutrophil to Lymphocyte Ratio; CRP: C-Reactive Protein; sRAGE: soluble receptor for advanced glycation end products; s/uTNFR1: soluble/urinary tumour necrosis factor receptor type 1; s/uTNFR2: soluble/urinary tumour necrosis factor receptor type 2; s/u MCP: serum/urinary monocyte chemoattractant lymphocyte 1; s/u 8IP: serum/ urinary 8-Isoprostane; s/u NGAL: serum/urinary neutrophil gelatinase-associated lipocalin; s/u KIM1: Kidney Injury Molecule-1; s/uIL2R: serum/urinary Interleukin-2 Receptor

11.4 Discussion

The following discussion focuses on the general considerations, clinical significance, relevance of the study in two separate sections. Firstly, a summary discussion on the inter- and intra-rater reliability of the MRI technique. Detailed discussion on data on reliability is available in Appendix One.

Following this, discussion will focus on findings of MRI parameters, initially, with BOLD imaging and then DTI (FA and ADC).

Summary of intra and inter-reliability results

All three metrics (BOLD, FA and ADC) were assessed by two readers to test inter-rater reliability, along with two runs of BOLD to assess inter-scan concordance. In summary, the results of the *overall* inter- and intra-rater reliability of this MRI technique are acceptable. These results rate “substantial to almost perfect” in accordance to concordance correlation and strength of agreement. Our results of inter and intra rater reliability suggest that interpreting overall readings may provide more accurate results, rather than dichotomizing analysis into different anatomical regions and by kidney, which reduces reliability.

There are established validity and reproducibility studies in the literature. However directly comparing our reliability analysis with the literature is difficult, as there are no prior publications that we know of utilizing the same statistical and analytical method for our target population. Standardisation of BOLD and DTI technique performance and interpretation is lacking in the literature to date, although recent consensus guidelines have been published for both techniques [266, 304]. Hence, it is difficult to translate results from different cohorts in the literature. For example, intra-subject coefficient of variation has been reported to be 3% and 4% for medullary and cortical BOLD MRI readings respectively in healthy volunteers [433] with the study taking 3 repeated measures of each participant, based on only 2 ROI. Another study reported a higher co-efficient of variation of 12.5% and 4.7% for medullary and cortical BOLD MRI readings in ten participants with chronic renal disease, including four with diabetic kidney disease[298].

We performed concordance correlation coefficient and reduced major axis regression for identification of both fixed and proportional biases when comparing data. The primary aim of this study was not to assess reproducibility; however, reliability and agreement analysis contribute to our understanding of how reproducible our technique is and impact the reliability of our primary and secondary aim results.

MRI Derived Parameters

It is acknowledged that the variable results of intra and inter-reliability reflect significant heterogeneity and the potential major issue of reproducibility. Arguably, using solely reader A's analysis may provide more precise results. Nevertheless, to avoid bias, we utilized observations from both readers and both left and right kidney (rather than taking the mean value of both anatomical sides, or selecting only a single side), to provide real world experience, where the experience of readers may vary considerably. This allows us to treat each observation as a “repeated measure” and increases the strength of the analysis. Clustered Quantile Regression was performed taking different variables into consideration. Analyses were adjusted to reader, anatomical side (left and right) as well as eGFR. Notably, there was no significant difference in median eGFR between healthy controls and participants with type 1 diabetes (100 vs. 94 ml/min/1.73m² p =0.4). However, as all healthy controls had an eGFR>90 mls/ min/1.73m², while there were variable stages of diabetic kidney disease in our type 1 diabetes cohort, adjusting to eGFR ensures that the wide degree of renal function did not confound results.

The most pertinent findings of the present study in MRI metrics are summarized in the following format and subsequently discussed in further detail:

BOLD MRI

- a. A higher $R2^*$, reflecting less oxygenation in the medulla compared to the cortex were demonstrated in both the control and diabetes group.
- b. A lower cortical and medullary $R2^*$, reflecting higher medullary oxygenation was detected in type 1 diabetes compared to healthy controls
- c. There were no differences identified between the cohort of type 1 diabetes that were hyperfiltering and non-hyperfiltering
- d. There was a positive correlation between medullary $R2^*$ with measured GFR.
- e. There was a weak relationship between $R2^*$ and selected inflammatory renal biomarkers

DTI MRI

- a. Higher FA and lower ADC, reflecting more complex structure and restricted water motion were found in the medulla compared to the cortex in both the control and diabetes group.
- b. A lower cortical FA, potentially reflecting loss of cortical microstructure or collagen deposition was observed in type 1 diabetes compared to healthy controls.
- c. A lower cortical ADC was detected in those who were not hyperfiltering to those who were hyperfiltering in type 1 diabetes, potentially reflecting water motion restriction in those without hyperfiltration
- d. There was a weak positive correlation between measured GFR and medullary $R2^*$, medullary FA and cortical ADC.
- e. There was a weak relationship between FA and ADC with selected inflammatory renal biomarkers

BOLD: R2* differences between type 1 diabetes and Controls, type 1 diabetes hyperfilterers and type 1 diabetes non-hyperfilterers and the relationship between R2* and renal function

- a. A higher R2*, reflecting less oxygenation in the medulla compared to the cortex were demonstrated in both the control and diabetes group.*
- b. A lower cortical and medullary R2*, reflecting greater oxygenation was detected in type 1 diabetes compared to healthy controls*
- d. There was a positive correlation between medullary R2* with mGFR*

For all three MRI metrics, our study detected clear and significant ($P < 0.001$) regional differences in the cortex and medulla consistent with the literature [298, 434]. A higher R2*, higher FA and lower ADC were found in the medulla compared to the cortex in both the control and diabetes group.

In terms of the participants' characteristics between healthy controls and type 1 diabetes, apart from a lower BMI and the lack of hypertension and accompanying absent RAAS blockade in healthy controls, there were no significant differences between both groups. One of the limitations was the lack of wash out period of medications for the study. There is evidence that RAAS blockade enhances renal oxygenation [435], but given that only 8% of the type 1 diabetes cohort was on a RAAS blocker, the effect was likely too small to have contributed to our results. Of interest, diuretic use that can lead to a transient improvement in oxygenation [281]. Only a small proportion of participants with type 1 diabetes (15%), and none of the healthy participants were on diuretics. The use of RAAS and diuretics were similar between the hyperfiltering and non-hyperfiltering group in type 1 diabetes. Although the hyperfiltering group was significantly younger (31.5 vs. 49.5 years $p = 0.006$), it has been previously observed that there is no correlation between R2* and age ($N = 342$) [267].

A higher R2*, which implies reduced tissue oxygenation likely reflects the higher physiological demands and lower perfusion of the renal medulla. When studying participants with varying degrees of CKD, it was previously found that differences

between cortical and medullary $R2^*$ attenuated at the latter stages of CKD[436]. The authors concluded that this supports the concept that compensatory mechanisms early in the course of renal impairment may not result in significant changes in oxygenation until more advanced injury occurs. As we had a small sample size, we were unable to conduct granular subgroup analysis between different stages of DKD to allow comparison. However, in contrast to the above mentioned study, and several other previous studies in CKD [267, 269, 295] and type 2 diabetes [275, 279, 280] which reported either no difference, or in fact, a higher $R2^*$ in diseased states (including diabetes), we observed lower cortical (14.7 vs. 15.7 1/s, $p < 0.001$) and medullary (24.8 vs. 29.3 1/s, $p < 0.001$) $R2^*$ in type 1 diabetes compared to healthy controls, reflecting greater oxygenation. The changes in $R2^*$ was independent of potential reader, anatomical (right or left of kidney) and eGFR bias (Table 11.5).

Our findings of a lower $R2^*$ in type 1 diabetes, suggesting higher renal oxygenation in diabetes compared to healthy controls was somewhat unexpected but not unprecedented. Wang et al [276] also reported lower medullary (but not cortical) $R2^*$ in moderate to severe DKD compared to controls. Wang's study demonstrated that medullary $R2^*$ was lower in moderate-severe DKD compared to mild DKD; which in turn was lower than healthy controls. Notably, it was not clear if the participants were type 1 or type 2 diabetes, or a combination of both diagnosis. The authors postulated that paradoxical improvement in hypoxia can occur in more advanced renal injury (lower GFR) with lower oxygen consumption, based on experimental models of lower hypoxia inducible factor expression in more advanced renal injury. Interestingly, despite the contrasting findings of higher $R2^*$ in type 2 diabetes (compared to our study and Wang et al), Yin et al [275] also demonstrated that alleviation of medullary hypoxia does occur with more advanced stages of renal failure. Feng et al also demonstrated a higher medullary $R2^*$ in those with type 2 diabetes with normoalbuminuria compared to healthy controls, but a lower medullary $R2^*$ in those with microalbuminuria[305]. In this present study, we also found that the lower the renal function, the lower the $R2^*$ (less hypoxia); with a slight positive relationship between medullary $R2^*$ with mGFR ($\rho = 0.3$, $p < 0.001$). However, we found no relationship between cortical $R2^*$ and mGFR in our study.

The underlying aetiology for why medullary $R2^*$ was lower in diabetes compared to controls remains to be investigated, but we postulate that less hypoxia detected in diabetes may be either due to reduced oxygen utilization or alternatively increased oxygen delivery in diabetes due to higher metabolic demand. To determine if perhaps greater oxygen delivery was present due to increased blood flow is potentially possible by measuring renal blood flow, but this type of investigation was beyond the scope of this current study.

In keeping with our findings of a lower medullary, but unchanged cortical oxygenation with decline in renal function were demonstrated by Li et al in 29 participants with CKD of non-specified causes[436]. Of note, the participants in the study did not have type 1 diabetes. Authors of that postulated that architectural changes in more advanced renal impairment could result in the apparent improvement in medullary oxygenation, perhaps as a reflection of less medullary oxygenation delivery activity. Interstitial fibrosis and peri-tubular capillary changes in advanced renal injury may impair oxygen delivery leading to reduced oxygen extraction affecting $R2^*$ value. The decrease in filtration fraction and reduction in Na^+ pump may also alleviate hypoxia. However, we did not observe any strong evidence of DTI changes between type 1 diabetes and controls apart from a lower cortical FA (to be discussed in more detail below), which would support microstructural variations that would have indirectly affected $R2^*$ values.

In addition, renal oxygen consumption is largely due to the concentrating mechanism of sodium resorption within the medullary thick ascending limb. A pathological state that impairs the urine concentrating ability (reduced sodium resorption leading to salt wasting) can likely lead to increased oxygen bioavailability, by reducing glomerular filtration, increasing blood flow and relieving medullary oxygen insufficiency when the workload capacity is exceeded [437, 438]. It has been observed in young, hypertensive men that medullary oxygenation correlates positively with 24-hour sodium excretion [301]. In our study however, urinary sodium excretion did not significantly differ between type 1 diabetes and controls (157mmol/24 hours vs.138mmol/24 hours $p=0.2$). This lack of sodium excretion does not support the hypothesis of reduced oxygen consumption, or a higher oxygen delivery in type 1 diabetes as a secondary effect to alteration in urinary sodium handling. Nonetheless, sodium resorption is tightly

regulated by many factors, for which we did not adjust, including dietary salt modification, blood pressure and anti-hypertensive agents.

As oxygenation appears to be associated with lower renal function (weak positive correlation between medullary $R2^*$ and measured GFR (ρ 0.3, $p < 0.001$)), it is not clear if incorporating participants with more advanced renal failure resulted in improved renal oxygenation in diabetes compared to healthy controls. However, the majority (20 out of 32) of participants with type 1 diabetes had an $eGFR \geq 90 \text{ ml/min/1.73m}^2$, in keeping with preserved renal function. Therefore, we were unable to clarify if discrepancies in $R2^*$ between healthy controls and participants with DKD from various studies may also be partially explained by the different stages of DKD assessed. Despite including participants with different stages of DKD similar to Yin et al., our study findings of $R2^*$ were dissimilar. Wang's study with predominantly more advanced DKD participants (exact renal function not published but reported to have 14 participants having an $eGFR < 60 \text{ ml/min/1.73m}^2$ and 6 with an $eGFR \geq 60 \text{ ml/min/1.73m}^2$) is in contrast with our cohort with relatively well-preserved renal function with a median $eGFR$ of $100 \text{ ml/min/1.73m}^2$. Type 2 diabetes studies by Economides [279] and Epstein [280], which did not find any $R2^*$ difference with controls had also relatively well preserved renal function. In fact, it is possible that the above two studies (by Economides and Epstein) with participant characteristics with a median duration of diabetes of 5 to 6.5 years, may have incorporated individuals of hyperfiltration, which occurs early on in diabetes.

c. There were no differences identified between the cohort of type 1 diabetes that were hyperfiltering and non-hyperfiltering

Supporting the notion of increased oxygen consumption with tubular sodium reabsorption in hyperfiltration, are findings by Ries et al's experimental model which demonstrated increased renal $R2^*$ in diabetic rats compared to controls [272]. In the above study, MRI was performed on the rats at day five post induction of diabetes, coinciding with the development of renal hyperfiltration.

Nevertheless, our study findings did not prove the hypothesis that higher oxygen consumption or hypoxia occurs in hyperfiltration when taking confounding factors into

account. We failed to demonstrate any significant statistical difference in $R2^*$ between non-hyperfiltering and the hyperfiltration group in type 1 diabetes, despite the trend of a higher medullary $R2^*$ in the hyperfiltering group (25.8 1/s vs. 24.2 1/s, $p = 0.01$) in univariate analysis. The lack of any detectable $R2^*$ differences may indicate that mechanisms apart from hypoxia are behind the process of hyperfiltration in diabetes. It is not clear if the inclusion of participants with predominantly mild renal impairment (50% with stage 2 DKD) within the non-hyperfiltering group may have impacted the $R2^*$ results. Another consideration is that our small sample may also be not statistically powered to observe any difference.

In addition, healthy controls and the type 1 diabetes-hyperfiltering group demonstrated numerically higher $R2^*$ levels in the control group (cortex: 15.7 vs 14.7 1/s, $p < 0.001$; medulla: 29.3 vs 25.8 1/s), again suggesting that pronounced hypoxia may not be observed in type 1 diabetes renal hyperfiltration. Due to the small sample size, sub-analysis comparing those with type 1 diabetes and hyperfiltration only, with healthy controls were not conducted to avoid multiple testing. In addition, eGFR was incorporated into regression analysis for comparison between those with and without diabetes.

e. There was a weak relationship between $R2^*$ and selected renal biomarkers

Of all the MRI metrics, medullary $R2^*$ had the most associations with laboratory biomarkers. Nevertheless, even then, relationship between the candidate biomarkers with medullary $R2^*$ were weak. It is noted however, that association between medullary $R2^*$ with all the biomarkers were consistently negatively correlated, suggesting that better oxygenated medullas are associated with higher concentration of inflammatory biomarkers. Although the exact mechanism remains elusive, this observation of greater oxygenation associated with inflammation, taken together with our findings that a lower medullary $R2^*$ (better oxygenated medullas) was detected in type 1 diabetes compared to healthy controls, might support the theory that the enhanced oxygen level detected with BOLD MRI is a result from compensatory physiological changes in diabetes. This may either be from decreased oxygen consumption or increased oxygen delivery as part of the disease process and pathological state.

The lack of any strong associations is in contrast with a study of chronic allograft dysfunction, which detected significant correlations between cortical and medullary $R2^*$ and oxidative stress markers[439]. Of relevance was the strong positive association between medullary-cortical $R2^*$ (MCR2*) and urinary 8-Isoprostaglandin F2-alpha (correlation coefficient =0.88, $p<0.0001$). However, when the authors assessed the individual relationship between urinary 8-Isoprostaglandin F2-alphaCR2* and MR2*, there was a lack of association. Instead of a positive relationship, urinary nitric oxide negatively correlated with CR2* and MR2*. Inconsistent correlation between endothelial markers with cortical and medullary oxygenation has also been found in a cohort of type 2 diabetes participants [279]. Of interest, CRP correlated positively with cortical oxygenation with the authors suggesting this is due to the increase in blood flow from inflammation.

DTI: ADC /FA differences between type 1 diabetes and Controls, type 1 diabetes hyperfilterers and type 1 diabetes non-hyperfilterers and the relationship between ADC/ FA and renal function

- a. Higher FA and lower ADC. reflecting more complex structure and restricted water motion were found in the medulla compared to the cortex in both the control and diabetes group.*

Consistent with the literature, we demonstrated clear regional differences with reduced ADC and a higher FA in the medulla compared to the cortex. This highlights the structural difference between cortex and medulla with reduced flow and decrease in global diffusivity in the medulla in relation to the cortex.

- b. A lower cortical FA, potentially reflecting loss of cortical microstructure or collagen deposition was observed in type 1 diabetes compared to healthy controls.*

There were no detectable differences between healthy controls and type 1 diabetes for both cortical and medullary ADC. The trend of a lower medullary FA was attenuated when adjusted, while difference in cortical FA persisted between type 1 diabetes and healthy controls. However, while cortical FA readings were higher in diabetes with univariate analysis 0.12 (0.10,0.14) vs. 0.11(0.10,0.12) $p=0.03$; when adjusted and independent from potential confounding factors including eGFR, cortical FA was lower by 0.04(0.01,0.07) in our diabetes cohort compared to controls. The proximity of the median results of 0.12 and 0.11 with overlapping interquartile range may explain the variation.

Our lack of difference in ADC results are similar to a recent published study by Feng et al[305], who evaluated 30 type 2 diabetes participants with and without albuminuria compared to healthy controls. The only DTI changes found in the study was a lower medullary (but not cortical) FA in those with albuminuria and type 2 diabetes. In the study, no associations were found with any of the MRI metrics with either albumin creatinine ratio or eGFR. The lower FA in diabetes compared to controls is relatively consistent with the literature. Overall, despite the inconsistent findings in existing type

2 diabetes and DTI studies, FA and ADC levels are inclined towards being reduced in cohorts with more advanced DKD compared to controls [285, 287, 293, 294].

The exact changes DTI parameters reflect in diabetic kidney disease remain unknown. In renal biopsies of 37 Japanese participants with non-diabetic chronic kidney disease, increased ADC was found to correlate with renal fibrosis[295]. We hypothesize along with other authors that reduced perfusion, tubular flow rate, tubular damage and vascular abnormalities contribute to DTI findings. However, these are postulations that require further validation, with a lack of pre-clinical and histopathology correlation. In an experimental rat model, it was demonstrated that reduction in FA identifies interstitial fibrosis and tubular damage in diabetic kidney disease[440]. In another non-diabetic experimental model based on ureteral obstruction, a reduction in ADC was noted to be related to reduced perfusion associated with renal fibrosis on histopathology[441]. In a non-diabetic chronic kidney disease clinical study of 17 individuals, it was demonstrated that FA correlate negatively with glomerular lesions and tubulointerstitial injury[442]. It was postulated by the authors that glomerular cellular debris and macromolecular proteins can be filtered into the tubules and impact directionality and in turn medullary FA (which changes was not observed in our study). Additionally, FA changes has also been considered to be potentially caused by not only inflammatory cell infiltration but collagen deposition as demonstrated in experimental liver studies[443].

c. There was a weak positive correlation between measured GFR and medullary R2, medullary FA and cortical ADC.*

In this present study, there were inconsistent associations between DTI values and renal function. The associations between MRI parameters with both eGFR and mGFR were similar, with observed associations stronger with mGFR than eGFR (Table 11.7) perhaps reflecting greater reliability of measured as opposed to estimated GFR results. A positive relationship between renal function was observed with cortical ADC and medullary FA; but not with medullary ADC or cortical FA. The lower the mGFR (more renal impairment), the lower the cortical ADC and medullary FA. Fibrosis in more advanced diabetic kidney disease would result in less structured renal histology, and we would expect an overall lower ADC reflecting reduced tissue water diffusion/ water

content; and lower FA, representing a lack of directionality in tissue water motion. The lack of consistency in the association between renal function and architectural changes observed in our study may potentially be explained by regional differences. Previous studies have observed that with fibrosis, reduced ADC appears to preferentially occur within the renal cortex[434]. Our study findings of a reduced cortical FA suggest reduced water motion directionality in type 1 diabetes, and potentially indicate micro-structural renal lesions. The aetiology of the absence of any accompanying corresponding medullary FA changes can only be postulated. Possibly, the changes that occur within the medulla may be present but minute, and the degree may not be sufficient to be detected, particularly as the medulla is more homogenous and structured. This may also be related to the degree of diabetic kidney disease, with the majority of our patients with mild or no renal impairment as reflected by GFR.

The absence of any significant DTI differences apart from cortical FA between healthy controls and type 1 diabetes in our study may be related to the small sample size. However, our cohort of N=45 is comparable to other previous type 2 diabetes studies with a sample size ranging between 15-78 participants. The lack of consistent methods, for example, some studies utilize mean combined cortical and medullary values rather than separate regional readings could also result in discordant results. In addition, despite the variable relationship between DTI parameters with renal function, it may be that the comparable eGFR between healthy controls and type 1 diabetes would attenuate any potential differences between both groups. The study technique may also explain the lack of detectable differences. One of the causes may be that it is not sufficiently sensitive to detect any potential renal architectural changes in type 1 diabetes, particularly with a cohort with relatively well-preserved renal function. Alternatively, difficulty with placement of ROI with loss of cortico-medullary differentiation in the diseased state could have contributed. In most cases, cortico-medullary differentiation is straightforward, however differentiation can be challenging in patients with higher stages of chronic kidney disease (Figure 11.6). However, this is less likely as there were clear regional differences between cortical and medullary MRI derived parameters in both controls and type 1 diabetes.

- d. A lower cortical ADC was detected in those who were not hyperfiltering to those who were hyperfiltering in type 1 diabetes, potentially reflecting water motion restriction in those without hyperfiltration.*

The only difference observed when comparing those with hyperfiltration to those without hyperfiltration in the type 1 diabetes cohort was the lower cortical ADC in those who were not hyperfiltering. This observation may reflect more water content as a consequence of renal hypertrophy, a structural change that can accompany hyperfiltration at the nephron level [444]. However, as renal hypertrophy can involve the tubular lumens, the lack of difference in medullary ADC levels do not support this. Of note, a type 2 diabetes study which defined hyperfiltration as an $\text{eGFR} > 100 \text{ ml/min/1.73m}^2$ in 16 participants, did not observe any difference in ADC parameters compared to healthy controls[294].

On the other hand, rather than changes within the hyperfiltering group, the lower cortical ADC may instead suggest architectural and structural changes resulting in restricted water motion in those who were not hyperfiltering. This contrasts with our hypothesis that there are conceivably DTI changes in hyperfiltration that initiate the process of renal damage in diabetes. It was also observed that a lower cortical ADC was associated with a lower mGFR. Hence, potentially including participants with severe DKD in the non-hyperfiltering group could have resulted in the reduced cortical ADC in the non-hyperfiltering group. Overall, structural cortical changes in later stages of DKD including glomerular sclerosis can possibly result in restricted diffusion and reduced solute and water transfer as suggested by the lower cortical ADC readings.

Importantly, FA changes which could suggest tubular damage with fibrosis was not observed in the hyperfiltration group when compared to those without hyperfiltration. These findings do not support the tubular hypothesis of renal hyperfiltration in diabetes. One explanation for the lack of DTI changes in hyperfiltration may be that at this stage, it is too early in the disease process to allow sufficient cellular damage that may alter directionality and impair diffusion that could be detected by current DTI techniques.

- e. There is a weak relationship between FA and ADC with selected inflammatory renal biomarkers*

In the literature, a weak correlation ($r=0.38$, $p=0.014$) has been found between cortical FA and urinary N-acetyl- β -D-glucosaminidase (NAG)[293], suggesting some involvement of proximal tubular cell injury. In this study, we did not detect any associations between cortical FA with any of the biomarkers to link our findings of a lower cortical FA in type 1 diabetes compared to controls. It was consistently a negative correlation between renal biomarkers and other MRI metrics that were statistically significant ($p<0.001$). However, we did not establish any clear strong associations between selected candidate renal biomarkers with either BOLD or DTI MRI imaging, with ρ all at < 0.5 . One of the possibilities is that the serum and urinary biomarker levels do not reflect true local cortical and medullary concentration.

In addition, there was no single biomarker, or combination of biomarkers that reliably demonstrate associations with all six MRI metrics. Overall, our results do not suggest that MRI metrics can be reliably predicted neither by a single nor combination of the selected candidate renal inflammatory and oxidative stress biomarkers.

Limitations

We acknowledge there are limitations in this study. Firstly, this was a pilot study with a small sample size, which may not be statistically powered to observe true differences, also precluding further subgroup analyses. For example, we were unable to assess MRI changes between different stages of DKD. Moreover, adding to the complexity in interpreting results in the context of the literature to date are the wide and varied study methods of functional MRI studies. Our power calculation was based on previous BOLD data which calculated $R2^*$ based on a medullary-cortical ratio, rather than separate cortical and medullary $R2^*$ as we did. We believe presenting the $R2^*$ data with our approach is clearer, as it displays the oxygenation level in the cortex and medulla separately. This is also more consistent with other more current published data, allowing for comparison with the literature.

Furthermore, the limitations of the MRI technique itself need to be considered. Not all images were diagnostic due to respiratory or bulk motion artifacts and susceptibility artefact caused by adjacent air-filled hollow viscera. We excluded observations that

were non-interpretable (ID35). In addition, as manual selection of region of interest can also introduce observer bias, we selected up to ten circular regions of interest (ROIs) for each image to improve accuracy. However, use of circular ROIs might increase the likelihood of partial volume averaging, sampling structures outside of the cortex, due to the relatively thin curvilinear shape of the renal cortex. Performing ROI measurements that are crescentic that better conform to the cortical shape could have improved the reliability of our results. We used multiple ROIs to sample renal cortex and medulla, to obtain representative average values, as has been advocated in a recent consensus statement for diffusion weighted techniques including DTI [304]. Most current studies have in fact sampled a lower number of regions of interest compared to our study. In addition, having two independent readers and combining two runs of BOLD imaging as we did may have reduced bias in our final analysis.

Another limitation for our BOLD measurement is the lack of a physiologic challenge, for example with water loading, along with the absent of any wash-out period for medications including diuretics, beta blockers and angiotensin receptor inhibitors which could affect oxygen consumption. We did however attempt to minimize any potential external influences by implementing standardized hydration status and excluding participants with anaemia. In addition, assessment of renal blood flow and perfusion that could affect oxygen delivery would be complementary for comprehensive oxygenation status. However, due to technical limitations, we were not able to measure flow velocities accurately. As not all non-contrast MRA images were diagnostic, renal artery stenosis could not be excluded accurately. Undetected renal artery stenosis could potentially result in asymmetric differences in oxygenation. To improve upon this, future considerations are other techniques to measure hemodynamic parameters, for example ultrasound imaging based on photo-acoustic properties. Intravoxel incoherent motion is also another diffusion weighted imaging subtype that can provide more information on perfusion and tubular flow. This technique however is also still in its investigative stage and is not widely applied.

The presence of missing data was also a limitation of our study. In terms of biomarker assays, unfortunately due to kit shortage, there were samples (five serum NGAL and twenty-six urinary 8-Isoprostane) that were not assessed. There were no associations detected between urinary 8-Isoprostane with any of the MRI parameters in those

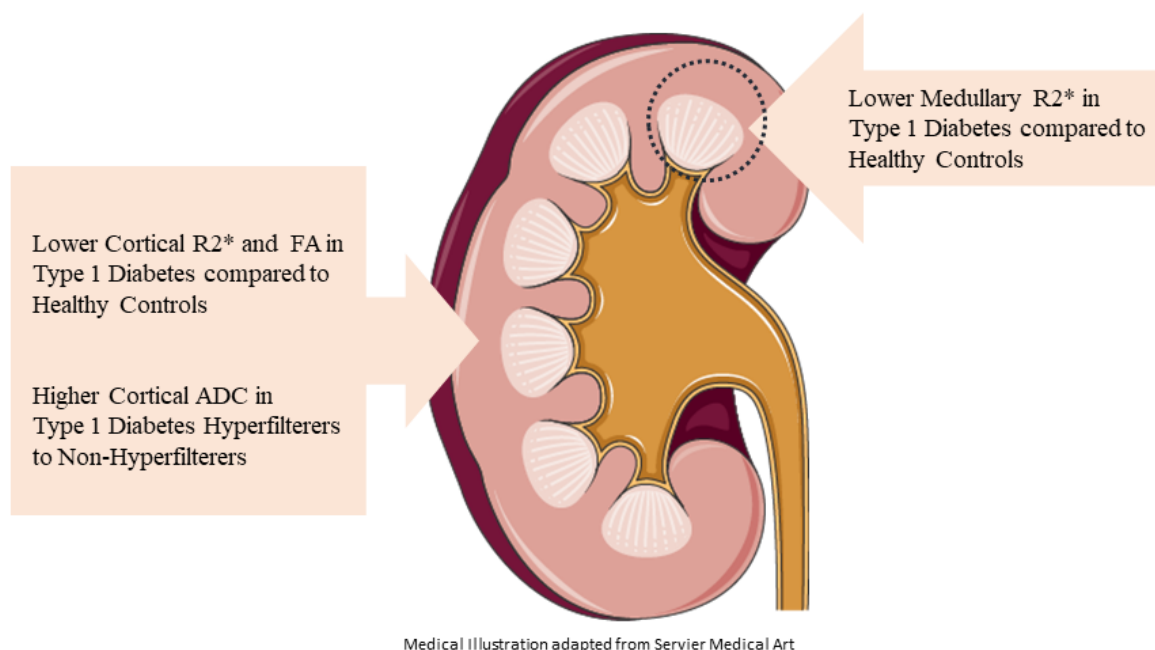
subjects where it was measured. However, based on the trend between the relationship of other renal biomarkers and the MRI metrics, it is unlikely that there would be a strong association detected even if all samples were analysed. Although measured GFR was not acquired in healthy controls, we conducted our analysis based on measured GFR in our diabetes cohort. We emphasize that within our diabetes cohort, measured GFR, urinary creatinine-clearance and CKD-EPI based estimated GFR strongly correlated with each other (Figure 11.2)

Summary

In conclusion, in this present study, we have demonstrated a number of findings (Figure 11.7)

- a) Reduced cortical and medullary $R2^*$ (reflecting higher tissue oxygenation), and reduced cortical FA (indicative of decreased structural integrity) in type 1 diabetes compared to healthy participants.
- b) Reduced cortical ADC (reflecting reduced water diffusion) in those who were non-hyperfiltering type 1 diabetes patients compared to hyperfiltration in type 1 diabetes.
- c) MRI parameters of $R2^*$, ADC and FA values do not reflect the level of renal function in type 1 diabetes consistently.
- d) There are fair associations of $MR2^*$, MFA and CADC with eGFR.
- e) There are no strong associations between selected inflammatory renal biomarkers and multi-parametric MRI.

Figure 11.7 Summary of BOLD-DTI MRI in the cortex and medulla in type 1 diabetes and controls



Based on our results, we were not able to support the notion that hypoxia has a dominant pathogenic effect either in the initiation of DKD with hyperfiltration, or the subsequent progression of DKD in type 1 diabetes. There is no evidence that microstructural changes occur early on in type 1 diabetes, when renal hyperfiltration occurs. In fact, some of these structural changes were detected in those who were type 1 diabetes and not hyperfiltering. In addition, predictive value of MRI for renal function requires further evaluation.

11.5 Conclusion

We are not aware of any other study of multiparametric functional renal MRI in type 1 diabetes compared to healthy controls, as well as examining the relationship of MRI metrics with renal biomarkers. The combination of BOLD MRI and DTI provides more detailed information and insight into the relationship between renal oxygenation and architectural changes. This contributes to our understanding of associations of between multi-parametric MRI with renal function and the pathogenic role of renal inflammatory biomarkers in diabetic kidney disease.

We were able to ascertain that there is a difference in oxygenation as established with medullary $R2^*$ in type 1 diabetes compared to healthy controls. The finding of some alteration in structural properties in type 1 diabetes compared to controls, and in those who were not hyperfiltering suggest that structural changes may be more pronounced, and more readily detected with DTI in advanced DKD. Although not consistent, the presence of associations between certain MRI parameters with renal function in type 1 diabetes does point towards some value of further investigating renal hypoxia and architectural alterations in DKD. Similarly, this applies to the variable associations with inflammatory biomarkers as well. Notably, differences in MRI parameters between those who were hyperfiltering to those who were not were attenuated with quantile regression. Potentially, a larger scale study may be able to elicit persistent differences between the two groups, as this pilot study may not be sufficiently powered to do so.

The mechanisms of hypoxia, architectural changes and inflammation in diabetic kidney disease remains largely undetermined. Nevertheless, we have demonstrated that

functional MRI technology is a promising tool to improve our current understanding of DKD through microstructural changes with DTI and information on oxygenation with BOLD imaging. This is significant, as the functional MRI techniques described are non-invasive, and do not require exogenous contrast, potentially useful for detection and evaluation of progression in DKD. This technology merits attention and further studies will be required to explore the utility of this experimental approach in the clinical setting. Future studies to assess the predictive value of this technology would ideally incorporate a larger sample size followed up in a longitudinal fashion with renal endpoints including correlations with albuminuria, GFR and renal histology.

Study Seven

Chapter Twelve

Renal Biomarkers in Type 1 Diabetes and Renal Hyperfiltration

12.1 Introduction

Activation of inflammatory pathways has been implicated as a driver for the development and progression of diabetic kidney disease (DKD) [445]. Hyperfiltration has also been proposed as an early phase of DKD, but the relationship between hyperfiltration and changes in inflammatory biomarkers remains to be fully defined [446]. Serum soluble Tumour Necrosis Factor alpha-receptors 1 and 2 (TNFR1/2) are one of the most promising inflammatory markers in DKD [143, 144]. A recent study examining the relationship between TNFR and mGFR in T2DM found lower levels of TNFR in patients with hyperfiltration [148]. However, there were no studies examining the levels of TNFR 1/2 in the presence of hyperfiltration in patients with type 1 diabetes. At the time of presenting our findings in the 2016 Australian Diabetes Society annual scientific meeting, it was also not known if serum TNFR1/2 parallels their urinary excretion in diabetes.

In this small, exploratory study, we aimed to assess if inflammation is linked to the process of hyperfiltration in type 1 diabetes.

Outcome Measures:

The primary outcome measures were renal biomarkers including C-reactive protein (CRP), Neutrophil to Lymphocyte ratio (NLR), soluble receptor for advanced glycation end products (sRAGE), paired serum and urinary soluble tumour necrosis factor receptor type 1 / 2 (sTNFR1/2), paired serum and urinary monocyte chemoattractant protein 1(MCP1), paired serum and urinary total 8-Isoprostane, paired serum and urinary neutrophil gelatinase-associated lipocalin (NGAL), paired serum and urinary kidney injury molecule (KIM-1) and paired serum and urinary soluble Interleukin-2 Receptor (sIL2R).

Specific Testable Hypotheses:

We hypothesized that inflammatory and oxidative stress renal biomarkers are elevated in hyperfiltration compared with non-hyperfiltering patients in type 1 diabetes.

12.2 Methods

We recruited 30 participants with type 1 diabetes in a prospective cross-sectional study between 2014-2015 from a single tertiary hospital diabetes outpatient clinic. The Austin Health Human Research Ethics Committee approved ethics for this study. Informed consent was obtained from each participant for clinical assessment, including measurement of mGFR, access to patient medical records, analysis and storage of blood and urine samples.

Clinical characteristics including blood pressure, weight, height, detailed medical history and medication use were collected. Participants were provided with verbal and written instructions to ensure accurate completion of 24h urine collections. After a minimum of an eight-hour fast, fasting blood tests and a timed 24-hour urine collection were collected for routine biochemistry and stored at -20 degrees for subsequent inflammatory marker analysis. To determine mGFR, each participant underwent ^{99m}Tc -DTPA plasma clearance with Brochner-Mortensen correction as previously described [379]. As a comparator group, 10 healthy controls were recruited, and underwent the same protocol except for undergoing ^{99m}Tc -DTPA plasma clearance.

The following markers were assessed in serum:

C-reactive protein (CRP), Neutrophil to Lymphocyte ratio (NLR), soluble receptor for advanced glycation end products (sRAGE), soluble tumour necrosis factor receptor type 1 / 2 (sTNFR1/2), monocyte chemoattractant protein 1(MCP1/ CCL2), total 8-Isoprostane, neutrophil gelatinase-associated lipocalin (NGAL), and kidney injury molecule (KIM-1) and soluble Interleukin-2 Receptor (sIL2R).

The following corresponding markers were measured in urine:

sTNFR1 and 2, MCP1/CCL2, total 8-Isoprostane, NGAL, KIM1 and, sIL2R.

Due to the shortage of assay kits, five serum NGAL samples; along with twenty-six urinary 8-Isoprostane samples were not assayed. Further details on analyte assays are outlined in the methods section of Chapter 8: Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy.

Statistical analysis

The continuous relationship between inflammatory markers and mGFR were analysed with Spearman rank-order correlation. Analysis using Wilcoxon-ranksum was conducted between the following two categories: $\text{mGFR} \geq 120\text{mls/min/1.73m}^2$ and $\text{mGFR} < 120\text{mls/min/1.73m}^2$. Hyperfiltration was defined as an $\text{mGFR} \geq 120\text{mls/min/1.73m}^2$ for the purpose of this study.

Urinary marker concentrations were corrected for urinary excretion rate using urinary volume over a 24-hour timed collection. All markers (apart from NLR) were log-transformed for statistical analysis. Urinary 8-Isoprostane were not analysed due to insufficient samples.

For analysis of serum and urinary biomarkers, a p-value of < 0.003 was considered significant based on Bonferroni corrections.

This study was not powered to adjust for confounding factors. We considered the following factors that may confound concentration of inflammatory markers: age, sex, BMI, duration of diabetes, HbA1c, systolic and diastolic blood pressure, eGFR and smoking status. The mean variance inflation factor (VIF) was 2.0, with individual VIF ranging between 1.1-3.1, in combination with a condition number of 67.5 suggest high co-linearity.

12.3 Results

Renal Biomarkers: Comparing Participants with and without Diabetes

Basic characteristics of participants with and without type 1 diabetes are outlined in Table 12.1. Those with type 1 diabetes had a higher BMI, higher fasting plasma glucose, and more RAAS blockade and anti-lipid medication usage.

Renal biomarkers were not significantly different between participants with type 1 diabetes and healthy controls (Table 12.2).

Table 12.1 Baseline characteristics in participants with and without type 1 diabetes

	Type 1 Diabetes N=30	Controls N=10	p-value
Sex (M:F)	18:12	5:5	0.5
Age (years)	43 (30, 54)	45 (31,55)	0.8
Diabetes Duration (years)	18 (9, 28)	-	-
Smoker (N/ %)	4 (13)	0	0.2
Body Mass Index (kg/m ²)	27 (24,31)	23 (21,26)	0.01
Blood Pressure (mmHg)			
Systolic	130 (120,135)	130 (122, 132)	0.5
Diastolic	70 (65, 75)	76 (73,80)	0.03
Fasting Cholesterol (mmol/L)	4.4 (3.9,4.8)		
HbA1c % (mmol/mol)	7.6 (7.6, 8.5)	-	
Fasting plasma glucose(mmol/L)	8.9 (7.1, 11.5)	4.9 (4.7, 5.8)	<0.001
Estimated GFR* (ml/min/1.73m ²)	100 (77, 112)	94 (80,99)	0.4
RAAS blocker (N/%)	8 (26.7)	0	0.02
Diuretics (N/%)	5(16.7)	0	0.2
Other antihypertensive agents (N/%)	7(23.3)	0	0.1
Anti-lipid medications (N/%)	13 (43)	0	0.01
Aspirin (N/%)	0	0	-
Data expressed as median (interquartile range) or N (%).			
* Estimated GFR based on Chronic Kidney Disease Epidemiology Formula			

Table 12.2 Renal biomarkers in participants with and without type 1 diabetes

	Type 1 Diabetes N = 30	Controls N = 10	p-value
Circulating NLR	1.7 (1.3,2.4)	1.7(1.6,1.9)	1.0
Serum sRAGE (ng/ml)	0.2 (0.1, 0.4)	0.2 (0.1, 0.3)	0.7
Serum IL2R (ng/ml)	0.9 (0.6,1.1)	0.7(0.4, 0.9)	0.1
Serum sTNFR1 (ng/ml)	1.5 (1.2, 2.0)	1.4 (1.2,1.6)	0.5
Serum sTNFR2 (ng/ml)	8.3 (6.7, 9.7)	6.7 (5.7, 8.0)	0.1
Serum MCP1 (pg/ml)	39 (30,50)	36 (27,40)	0.2
Serum 8 Isoprostane (ng/ml)	123 (114,142)	118(104, 132)	0.2
Serum NGAL (ng/ml)	35 (23, 49)	33 (25,37)	0.5
Serum KIM1 (pg/ml)	43 (32,92)	23(18,35)	0.03
Urinary sTNFR1 excretion rate (ng/24 hours)	37070 (2129, 7362)	1675 (1358, 3708)	0.04
Urinary sTNFR2 excretion rate (ng/24 hours)	4471 (2440, 7974)	2819 (2779, 4912)	0.1
Urinary MCP1 (ng/24 hours)	334(222,432)	242(179,319)	0.2
Urinary NGAL (ng/24hours)	9426(3658,1689 1)	3082(1995,11070)	0.1
Urinary KIM1 (ng/24hours)	1.2(0.6,1.7)	0.7(0.6,1.1)	0.7
Comparisons made between groups with Mann-Whitney test. Results expressed as Median (25 th , 75 th percentile). Significant P value ≤ 0.003, adjusted with Bonferroni Correction.			

NLR: Neutrophil to Lymphocyte Ratio, sRAGE: soluble receptor for advanced glycation end products; sTNFR1: soluble tumour necrosis factor receptor type 1; sTNFR2: soluble tumour necrosis factor receptor type 2 IL2R: Interleukin-2 Receptor, MCP: monocyte chemoattractant lymphocyte 1; NGAL: neutrophil gelatinase-associated lipocalin; KIM1: Kidney Injury Molecule-1; IL2R: Interleukin-2 Receptor

Renal Biomarkers in Type 1 Diabetes: Comparing mGFR ≥ 120 ml/min/1.73m² to mGFR < 120 ml/min/1.73m².

Basic characteristics of participants who were hyperfiltering (N=10) compared to those who were not hyperfiltering (N=20) are tabulated in Table 12.3. The median mGFR were 124 (121, 129) ml/min/1.73m² and 91 (65, 110) ml/min/1.73m² in the hyperfiltering and non hyperfiltering group (p<0.001). Hyperfiltering participants were younger compared to non-hyperfiltering participants, however, there were no other differences in the clinical and biochemical characteristics between both groups.

Higher mGFR was associated with lower serum TNFR2 (r=-0.8, p<0.001) and serum TNFR1 (r=-0.7 (p<0.001). There was no association observed between mGFR and all of the other markers: CRP (r=-0.03, p=0.9), NLR (r=-0.3, p=0.3), sRAGE (r -0.3,p=0.1) IL2R (r=-0.5, p =0.004), sMCP1 (r=-0.3, p=0.1), sNGAL (r=-0.4, p=0.1), sKIM1 (r=-0.5, p=0.004), s8IP (r=-0.4,p=0.04), urinary TNFR1 (r=-0.2, p=0.2), urinary TNFR2 (r=-0.3, p=0.2), urinary MCP1 (r=-0.03, p=0.9), urinary NGAL (r=-0.2, p=0.3) and urinary KIM1 (r=0.2, p=0.4).

As shown in Table 12.4, sIL2, sTNFR1/2 were lower in the hyperfiltering group. There were no other significant differences in the serum levels and excretion rate between both groups. Although serum levels for sTNFR1/2 were numerically lower in hyperfiltration compared with non hyperfiltering patients (Figure 12.1), the corresponding urinary excretion rates for both these biomarkers were not significantly different between the two groups of patients. Importantly, there was no relationship between serum levels and urinary excretion for such that as the serum level of TNFR1/2 increased, urinary excretion rates for TNFR1/2 did not alter (Figure 12.2). In contrast, serum creatinine levels were significantly lower and the urinary excretion rates of creatinine were significantly higher in hyperfiltration compared with non-hyperfiltering patients, respective (Table 12.3). There was a lack of a relationship observed for all paired serum and urinary markers: TNFR1 (r=0.3, p=0.1), TNFR2 (r=0.4, p=0.03), KIM 1 (rho=0.1, p=0.5), NGAL (r=0.3, p=0.2) and MCP1 (r=0.2, p=0.4).

Renal Biomarkers in Type 1 Diabetes: Comparing $mGFR \geq 120 \text{ ml/min/1.73m}^2$ to $mGFR$ 90-119 ml/min/1.73m^2 .

We conducted subset analysis *between the hyperfiltering and normo-filtering cohort ($mGFR$ 90-119 ml/min/1.73m^2)*. In the normo-filtering group, there were eleven participants with a $mGFR$ of 105(98,116) ml min/1.73m^2 ($p < 0.0001$), compared to ten participants in the hyperfiltering group with a $mGFR$ of 124 (121, 129) ml/min/1.73m^2 .

Serum sTNFR2 was observed to be lower in hyperfiltering group 6.1 (4.9,6.7) vs. 8.5 (7.3, 8.9) ng/ml , $p < 0.01$. There were no other significant differences in all other biomarkers between both groups.

Table 12.3 Baseline characteristics in participants with and without hyperfiltration in type 1 diabetes

	Measured GFR ≥ 120ml/min/1.73m ² (N=10)	Measured GFR < 120ml/min/1.73m ² (N=20)	P-value
Sex (M:F)	8:2	10:9	0.2
Age (years)	32(29,45)	51 (42,60)	0.01
Diabetes Duration (years)	12 (2,20)	19 (9,35)	0.1
Smoker (N/ %)	1 (10)	3 (16)	0.7
Body Mass Index (kg/m ²)	27 (24,30)	26 (24, 32)	0.7
Blood Pressure (mmHg)	123 (120,135)	130 (120,140)	0.4
Systolic	70 (68,75)	70 (65,80)	0.9
Diastolic			
Fasting Cholesterol (mmol/L)	4.7 (4.2, 5.2)	4.3 (3.9, 4.8)	0.1
HbA1c %	7.5 (6.6, 8.5)	7.0 (7.1,9,0)	0.7
(mmol/mol)	58.5 (49.0, 69.0)	53.0 (54.0, 75.0)	
Fasting plasma glucose(mmol/L)	13.0 (7.8,10.8)	11.3(7.0,9.0)	0.2
Measured GFR (ml/min/1.73m ²)	124 (121, 129)	91 (65, 110)	<0.001
Estimated GFR* (ml/min/1.73m ²)	114 (103, 125)	86 (65,101)	<0.001
Serum Creatinine (umol/L)	68.5 (62.0,81.0)	78.0 (72.0,96.5)	0.05
Urinary Creatinine Excretion (mmol/24hours)	18.8(12.0, 19.3)	14.3(10.3,16.0)	0.03
Albumin Creatinine Ratio (mg/g)	0.8 (0.4,1.4)	0.8(0.6,5.6)	0.4
RAAS blocker (N/%)	3 (30)	9 (45)	0.4
Diuretics (N/%)	0	4 (20)	0.1
Other antihypertensive agents (N/%)	0	6 (32)	0.05
Anti-lipid medications (N/%)	2 (20)	11 (55)	0.07
Aspirin (N/%)	0	0	-

Data expressed as median (interquartile range) or N (%).

* Estimated GFR based on Chronic Kidney Disease Epidemiology Formula

Table 12.4 Renal biomarkers in participants with and without hyperfiltration in type 1 diabetes

	Measured GFR $\geq$ 120 ml/min/1.73m ² (N=10)	Measured GFR < 120 ml/min/1.73m ² (N=20)	p-value
Circulating NLR	1.5 (1.3,2.3)	2.0 (1.3,3.0)	0.3
CRP	1.5 (0.6,3.8)	1.5 (0.5,2.9)	0.8
Serum sRAGE (ng/ml)	0.2 (0.1,0.2)	0.3(0.2,0.4)	0.4
Serum IL2R (ng/ml)	0.6 (0.5,1.0)	1.0 (0.8,1.3)	0.01
Serum sTNFR1 (ng/ml)	1.2 (0.9,1.4)	1.6(1.3,2.7)	0.006
Serum sTNFR2 (ng/ml)	6.1 (4.9, 6.7)	8.7 (8.0,10.8)	<0.001
Serum MCP1 (pg/ml)	37.7(32.7,42.0)	43.6(28.8,54.0)	0.1
Serum 8 Isoprostane (ng/ml)	111.7(108.4,133.2)	127.7(110.1,143.1)	0.1
Serum NGAL (ng/ml)	23.1(22.7,42.1)	36.9(30.7,55.9)	0.1
Serum KIM1 (pg/ml)	44.7(33.9,179.8)	40.5(20.0,49.5)	0.1
Urinary sTNFR1 excretion rate (ng/24 hours)	2699 (1946,5821)	4165 (3174,8740)	0.2
Urinary sTNFR2 excretion rate (ng/24 hours)	3229 (2192, 7251)	4874 (3740, 9037)	0.2
Urinary MCP1 (ng/24 hours)	321.1(221.6,454.1)	323.1(226.5,374.7)	0.6
Urinary NGAL (ng/24hours)	7183(2836,13123)	10401(3949,17417)	0.6
Urinary KIM1 (ng/24hours)	1.5 (0.7,1.9)	1.2(0.6,1.6)	0.4
Comparisons made between groups with Mann-Whitney test. Results expressed as Median (25 th , 75 th percentile). Significant P value $\leq$ 0.003, adjusted with Bonferroni Correction.			

NLR: Neutrophil to Lymphocyte Ratio, sRAGE: soluble receptor for advanced glycation end products; sTNFR1: soluble tumour necrosis factor receptor type 1; sTNFR2: soluble tumour necrosis factor receptor type 2 IL2R: Interleukin-2 Receptor, MCP: monocyte chemoattractant lymphocyte 1; NGAL: neutrophil gelatinase-associated lipocalin; KIM1: Kidney Injury Molecule-1; IL2R: Interleukin-2 Receptor

Figure 12.1 Serum sTNFR1 (top) and sTNFR2 (bottom) in hyperfiltration and non-hyperfiltration

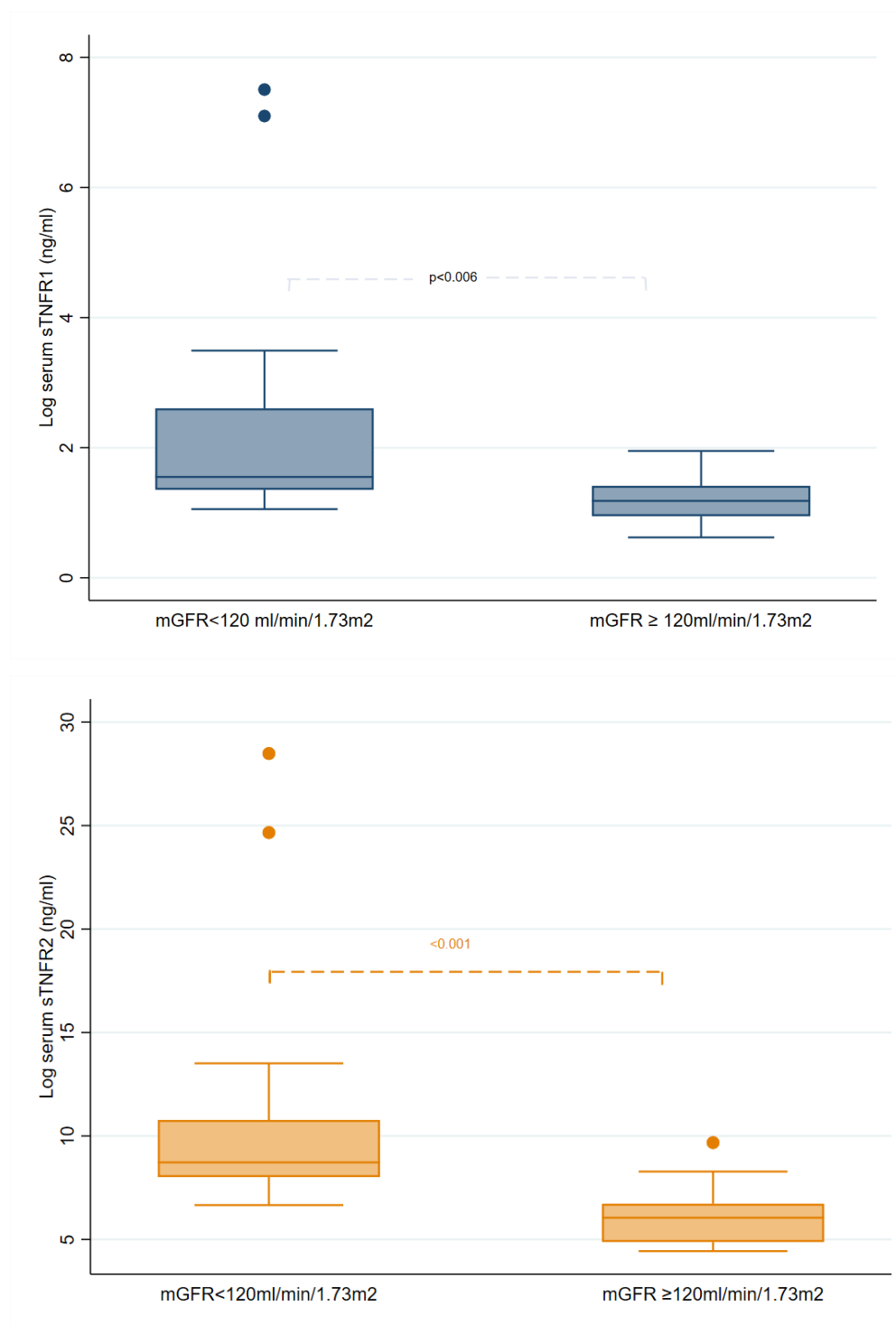
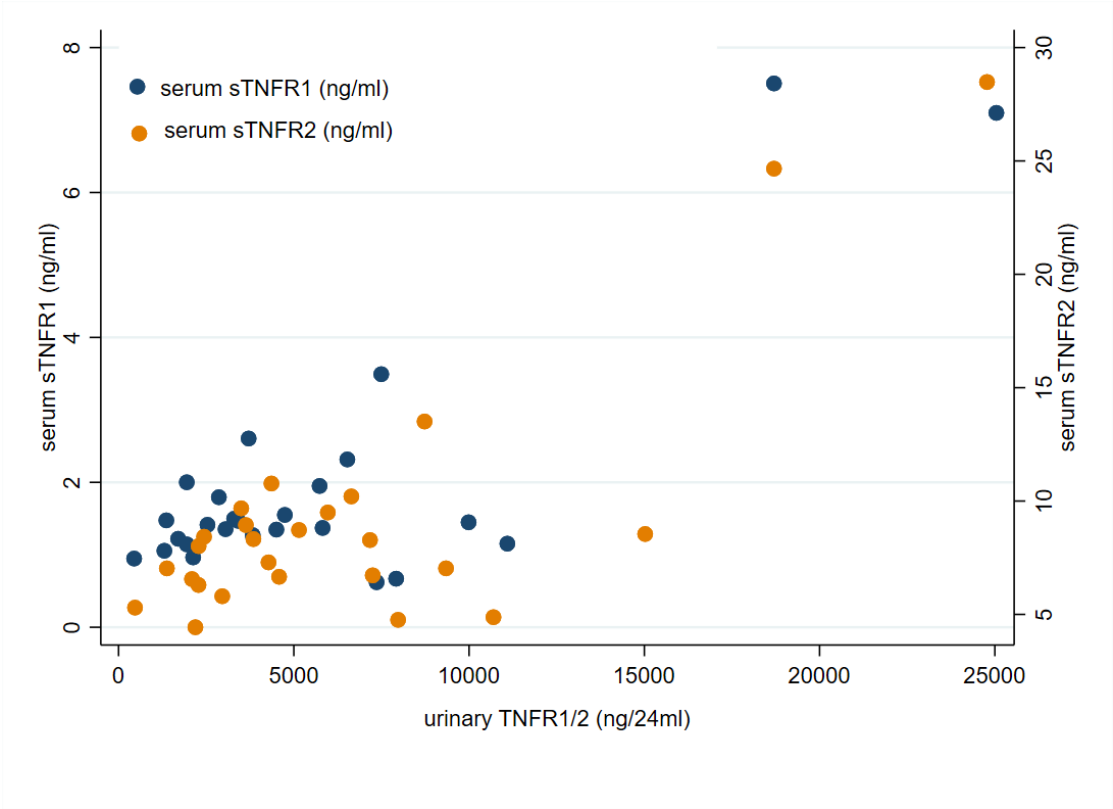


Figure 12.2 Relationship between serum and urinary TNFR1 and TNFR2



12.4 Discussion

Hyperfiltration has been proposed as an early phase of DKD, and the activation of inflammatory pathways are believed to be important in promoting renal damage. The results of this exploratory study suggest that circulating markers of inflammation are not elevated in hyperfiltration compared with non-hyperfiltering patients with type 1 diabetes.

Serum soluble Tumour Necrosis Factor alpha-receptors 1 and 2 have been shown to have a powerful predictive value in detecting the development of ESKD in diabetes [143, 144]. Apart from TNFR, the other candidate inflammatory markers in our study included CRP, NLR, sRAGE, sNGAL and KIM 1 which have all been associated with either decline in GFR or albuminuria in DKD [131, 219, 220, 447, 448]. However, there are limited studies assessing the relationship between these renal biomarkers and hyperfiltration in diabetes. A recent study examining the relationship between TNFR and mGFR in type 2 diabetes found lower levels of sTNFR in patients with hyperfiltration [148]. We know of no studies directly measuring both paired serum and urinary levels of these candidate markers to assess renal handling of these candidate biomarkers in diabetes related hyperfiltration. Therefore, in this study we explored the relationship between the selected candidate inflammatory biomarkers and diabetic hyperfiltration. At the time of presenting our study findings in the 2016 Australian Diabetes Society Scientific Meeting, it was also not known if serum and urinary TNFR1/2 concentration parallel each other in DKD. There has since however, a publication from the Joslin group in 2019 which assessed both serum and urinary TNFR1/2 through the SOMA-scan platform[156].

For this study in type 1 diabetes, there were no correlations between either the two readily assessable markers of inflammation (CPR and the NLR), or novel inflammatory biomarkers that we measured (IL2R, sRAGE, s8IP, sMCP1, uMCP1, sNGAL, uNGAL, sKIM1, and uKIM1) with mGFR.

Concentration of biomarkers were not elevated in the hyperfiltration group compared to those without hyperfiltration. In fact, there were three markers (sIL2R, sTNFR1/2) with significantly lower levels in the hyperfiltration group. Firstly, sIL2R level was

lower in the type 1 diabetes cohort with hyperfiltration. We did not measure sIL2, but Har et al. has previously demonstrated higher sIL2 in adolescents with type 1 diabetes and hyperfiltration compared to those who were normo-filtering[226, 227]. Similarly, the group did not observe any difference in both serum and urinary MCP1, or any of the cytokines measured in those who were hyperfiltering at the threshold of 135ml/min/1.73m² based on measured GFR (inulin). At present, there are very limited studies on both sIL2 and sIL2R in DKD. An experimental mouse model of type 2 diabetes has demonstrated depletion of regulatory T-cells[171], in which sIL2 and its' receptor exert its' effect. Interestingly, these mice exhibited incidental findings of glomerular hyperfiltration and albuminuria. As the main aim of studying these *db/db* mouse model was to characterize regulatory T-cells in insulin resistance, the expression and concentration of sIL2 and its' receptor were not studied. Nevertheless, our findings of a lower sIL2R fits the theory of hyperfiltration associated reduction in sIL2R expression or activation resulting in regulatory T-cells depletion, which suggest a potential pathological link to hyperfiltration. Nevertheless, this is based on a single experimental model and inference should be made with caution. Despite sIL2R levels being significantly lower in our group that was hyperfiltering, a decreasing mGFR does not correlate with elevated sIL2R levels. Possibly the small sample size of our study accounts for the above differences in sIL2R levels with mGFR when assessed on a continuous rather than as a categorical variable.

In contrast to sIL2R, lower levels of two other novel inflammatory biomarkers, sTNFR type 1 and type 2, correlated with higher mGFR and the serum levels of these two biomarkers were significantly lower in the hyperfiltering compared with non-hyperfiltering patients. As an increase in the urinary clearance may influence serum levels of inflammatory biomarkers in hyperfiltration, we also assessed urinary levels of sTNFR type 1 and type 2. We found no significant correlation for either of the biomarker with increasing mGFR. There was also no difference in the excretion rate of both these biomarkers between hyperfiltering and non-hyperfiltering patients. In contrast, serum creatinine levels were lower and urinary creatinine excretion rates were higher in hyperfiltration compared with non-hyperfiltering participants. The discordance between the findings a positive correlation between sTNFR levels in serum but the lack of a significant difference in urinary excretion rates for sTNF receptor in both the hyperfiltering and non-hyperfiltering patients remains to be fully explained.

Urinary excretion rates for both types of sTNF receptors were lower (both by around 35%) in hyperfiltration compared with non-hyperfiltration group, but this difference failed to be statistically significant in our small study. However, our results suggest that factors apart from GFR play an important role in determining serum levels of sTNFR1/2 and that the lower levels of sTNFR1/2 in hyperfiltrating patients are very unlikely to be fully explained by an increased renal clearance of these soluble receptors. This is further supported by findings of higher serum TNFR1/2 in the physiological hyperfiltration state in pregnancy, compared to the non-pregnancy state in healthy controls in our exploratory study in *Chapter 8: Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy*. Of note, the urinary excretion rates of sTNF receptors have recently been shown to be increased in non-hyperfiltering patients with diabetes at risk of progressive DKD[381]. In addition, the study by the Joslin group as mentioned above, also support our findings that serum sTNFR1/2 elevation is not a direct consequence of impaired renal handling[156], where it was demonstrated that both serum and urinary TNFR superfamily in selected type 1 and type 2 diabetes were elevated in individuals at risk of developing ESKD.

As a comparator, renal biomarkers were also assessed in healthy controls. Participants in both groups were sex and age matched, with no significant differences in eGFR. We found that despite the higher plasma glucose, elevated body mass index and higher use of anti-lipid medications, there were no significant differences in biomarker serum concentration and urinary excretion between healthy controls and type 1 diabetes. Similar results were observed in the cohort of women with and without diabetes in *Chapter 8: Presumed pathological hyperfiltration in diabetes compared to physiological hyperfiltration in pregnancy*, both during, and post-pregnancy. Notably however, in the pregnancy cohort, post-partum women with diabetes had serum KIM1 concentration that doubled that of healthy controls ($p=0.002$). KIM1 concentration was also significantly elevated in pregnancy compared to post-partum samples. In this current study, although the concentration of KIM1 was similarly doubled in those with diabetes compared to healthy controls, this did not reach statistical significance. We postulate that taken together, KIM1 that reflects renal tubular injury, may be associated with diabetic kidney disease, and perhaps pregnancy may contribute to further tubular injury.

Study Limitations

Ideally, multivariate analysis should be conducted, but as this pilot study had a relatively small sample size, the high variance inflation factor and condition number did not allow for adjustment for potential confounding factors between the hyperfiltration and non-hyperfiltering group which may influence inflammation. However, duration of diabetes, smoking status, glucose levels, blood pressure, lipid lowering medications and body mass index, which were all not statistically different between the two groups. Participants with hyperfiltration were younger than the non-hyperfiltering group (32 years vs. 51 years, $p=0.01$), however, the exact role of inflammation in aging remains unclear in the literature, specifically with regards to sTNFR [449, 450]. Likewise, this study was also not powered to further stratify the non-hyperfiltering group which included patients with more severe DKD.

We acknowledge that including participants with more severe DKD, which is known to be a pro-inflammatory state can affect results. However, subset analysis comparing the hyperfiltering cohort ($\text{mGFR} > 120 \text{ ml/min/1.73m}^2$) to those who were normo-filtering ($\text{mGFR} 90\text{--}119 \text{ ml/min/1.73m}^2$, $N=11$) did not detect significantly elevated biomarkers within the hyperfiltering group. In fact, concentration and excretion rate of the candidate markers were overall numerically higher in the normo-filtering group. In addition, our threshold of $120 \text{ ml/min/1.73m}^2$ as hyperfiltration could also potentially contribute to the lack of differences observed. However, it was not possible to conduct analysis at a higher threshold, as there were only two participants with a $\text{mGFR} \geq 130 \text{ ml/min/1.73m}^2$. This also highlights the difficulty in recruiting participants with hyperfiltration, as the active recruitment phase was ongoing for 2 years, with all patients attending the diabetes clinic in our centre undergoing $^{99\text{m}}\text{Tc-DTPA}$ plasma clearance as part of routine clinical care to monitor renal function.

12.5 Conclusion

In summary, this study suggests that the state of hyperfiltration in type 1 diabetes is not associated with elevated levels of circulating markers of inflammation. Furthermore, the lack of increase in inflammatory markers in the hyperfiltration state does not appear to be explained by an increase in the urinary clearance of these markers. These results

suggest that the state of hyperfiltration in type 1 diabetes may not necessarily reflect a maladaptive stage of DKD [89]. Indeed, recent studies have questioned the prognostic significance of hyperfiltration as a marker for progressive DKD, with lower levels of both TNFR1/2 found in hyperfiltration [148].

Our findings need to be interpreted with caution and require further validation in a larger cohort of patients adjusted for the effects of relevant covariates and using more stringent criteria for defining hyperfiltration.

Chapter Thirteen

Conclusion, Summary and Final Considerations

Diabetic kidney disease is characterised by a clinically slow and silent, but potentially reversible loss of renal function. The management of diabetic kidney disease incorporates intensifying glycaemic and blood pressure management, especially by targeting the renin angiotensin aldosterone renin system. More recent therapies including sodium glucose co-transporter 2 inhibitors and glucagon-like peptide-1 analogues have demonstrated renoprotective effects likely beyond glucose and blood pressure management, with underlying mechanism which have not as yet to be fully elucidated. Despite continuing therapeutic advances, diabetic kidney disease remains the most common cause of end stage renal failure requiring dialysis and renal transplantation.

The burden of diabetic kidney disease does not only apply to those with advanced diabetes and older individuals with type 1 and type 2 diabetes, but also affects younger populations. Notably in women of child-bearing age, there are potential adverse maternal and pregnancy outcomes in women affected by both type 1 and type 2 diabetes, particularly those with existing diabetic kidney disease. Therefore, understanding the contributors, as well as recognizing ways to detect and target the development and progression of diabetic kidney disease is imperative.

Renal hyperfiltration has been considered as a possible contributing factor to the development of diabetic kidney disease. As it occurs at an early phase of diabetes, elucidating the pathological implication and mechanism is important. If early targeted therapy can be instituted, there is a possibility to prevent the initiation and progression of diabetic kidney disease. However, current evidence on its' significance remain controversial mainly due to the lack of uniformity in studies. This lack of uniformity includes the definition of renal hyperfiltration, methods of assessing renal function, use of variable hard renal endpoints, as well as the duration of follow up of the various studies.

The overall aim for this thesis was to investigate the pathological significance of renal hyperfiltration in diabetes. Initially, I compared hyperfiltration in diabetes to the presumed physiological renal hyperfiltration in pregnancy which has not been previously studied. Additionally, in the process of assessing renal hyperfiltration in pregnancies affected by diabetes, I investigated outcomes in pregnancies affected by type 1 and type 2 diabetes. The focus was on maternal renal function, as it is currently

not known if there is a difference in the degree of hyperfiltration in women with and without diabetes, which may be associated with adverse outcomes. Secondly, I assessed selected candidate inflammatory and oxidative stress renal biomarkers which has shown some associations to the development of diabetic kidney disease. Although there has been a trend in examining these markers in diabetic kidney disease, there is limited research exploring renal biomarkers in hyperfiltration and diabetes. Thirdly, I investigated renal hypoxia and microstructural changes in hyperfiltration and type 1 diabetes using renal functional magnetic resonance imaging. This technique has not been studied in type 1 diabetes, nor has it been employed to investigate hyperfiltration in type 2 diabetes. The most pertinent findings of each of the studies are summarized below.

Study one is a summary of plasma inulin clearance in our study. We found that there was an overall positive correlation between measured GFR and estimated GFR in the second trimester of pregnancy in women with type 1 and type 2 diabetes. There was a lack of correlation in these measurements in healthy controls without diabetes during the second trimester of pregnancy. This study was unfortunately terminated early due to adverse reaction to inulin.

The main findings for *study two* were that pregnancy does not appear to adversely affect maternal renal function in women with type 1 and type 2 diabetes. Overall, physiological peak eGFR in pregnancy, and the rate of recovery in eGFR back to baseline did not differ between women with and without diabetes. However, compared to healthy controls, women with type 1 diabetes and a lower pre-existing eGFR achieved a significantly lower peak eGFR in pregnancy.

The main findings for *study three*, was that women with diabetes had a reduced capacity to hyperfiltrate in pregnancy compared to healthy controls. In addition, we found that the state of diabetic hyperfiltration was not associated with elevated levels of circulating markers of inflammation, suggesting that hyperfiltration may be physiological rather than pathological in diabetes.

Study four highlights that despite current medical advances in an Australian tertiary obstetric hospital, poorer outcomes in pregnancy in type 1 and type 2 diabetes remains higher compared to healthy controls. These outcomes include pre-eclampsia,

emergency caesarean delivery, large for gestational age, small for gestational age, Apgar score less than 7, neonatal intensive care admission, pre-term birth at less than 32 and 37 weeks. Maternal blood pressure, body mass index and renal function were identified as important risk factors of poor outcomes.

Following the above study, we further evaluated renal function as a predictor of selected pregnancy outcomes. The main findings for *study five* were that with every 1 mL/min/1.73m² decline in eGFR, there was 4% higher odds of developing preterm birth < 32 weeks. Furthermore, independent of eGFR, increased albuminuria was associated with preeclampsia. On the other hand, independent of albuminuria, a lower eGFR was associated with preterm birth < 32 weeks and neonatal intensive care unit admission.

In *study six*, using renal functional magnetic resonance imaging in type 1 diabetes, I demonstrated less hypoxia, but reduced cortical structural integrity in type 1 diabetes compared to healthy controls. There was no significant difference in parameters of renal hypoxia and microstructural changes identified in those who were hyperfiltering in type 1 diabetes compared to those who were not hyperfiltering. Magnetic resonance imaging parameters do not consistently reflect the level of renal function in type 1 diabetes, and there were also no strong associations between selected inflammatory renal biomarkers and imaging metrics.

The final study, *study seven* established that circulating markers of inflammation are not elevated in hyperfiltration compared with non-hyperfiltering patients with type 1 diabetes. A summary of results of candidate biomarkers complimenting the current literature are outlined below in Table 13.1

First and foremost, I would like to emphasize that our definition and interpretation of renal hyperfiltration is of the elevation of glomerular filtration rate, based on an eGFR of 120ml/min/1.73m² and above. Although this cut-off is at the lower end of the generally accepted definition of hyperfiltration of *measured* GFR, there is no definite or fixed definition of hyperfiltration for *estimated* GFR. As CKD-EPI based eGFR tends to under-estimate true measured GFR, we deem that this cut-off was not unreasonable. This brings us to the one of the major limitations in this thesis, which is the implementation of CKD-EPI formula based eGFR to assess renal function. However, in instances where it is possible, we have attempted to measure GFR, not

only with ^{99m}Tc -DTPA, but also inulin clearance, which had to be unfortunately withdrawn due to the possibility of triggering allergic reactions. We would also like to highlight that although CKD-EPI based eGFR may not necessarily reflect an unbiased and precise assessment of measured GFR in our study cohorts, it remains one of the most clinically relevant and widely available methods of renal function assessment. Therefore, we are hopeful that results can be extrapolated to real-world data. Overall, through this thesis that examines diabetic kidney disease, we have reached two main conclusions.

Firstly, in summary, in line with current literature I demonstrated that eGFR and albuminuria are independent risk factors for poorer pregnancy outcomes which are still significantly higher in women with type 1 and type 2 diabetes. Although pregnancy do not appear to have long term consequences on maternal renal function in those affected by diabetes, some women with diabetes may have reduced capacity to hyperfiltrate in pregnancy compared to healthy controls, particularly those with a lower pre-pregnancy eGFR. Further validation of these findings with larger sample size, and measured GFR would be important to explore the mechanism and contributing factors that could have resulted in the lack of eGFR rise in these women. In conclusion, there is still substantial progress that needs to be achieved in reducing adverse maternal and fetal outcomes in diabetes. Notably, renal function in diabetes is an important aspect that clinicians need to consider when managing pregnancies affected by type 1 and type 2 diabetes.

Furthermore, in this thesis, although functional MRI demonstrates differences in oxygenation and alteration in structural properties in type 1 diabetes compared to healthy controls and may aid in assisting to delineate the role of hypoxia in DKD; we failed to establish any multi-paramagnetic imaging changes or elevation in inflammatory markers in those who were hyperfiltering in diabetes. We therefore conclude that the state of renal hyperfiltration in diabetes may not necessarily reflect a maladaptive stage of diabetic kidney disease. We infer this in light of one of the recent high quality longitudinal study published last year on the Diabetes Control and Complications Trial/ Epidemiology of Diabetes Interventions and Complications Study (DCCT/ EDIC) cohort which found no association between a higher baseline measured GFR and the long term risk of decreased estimated GFR[89].

Table 13.1 Summary findings of candidate biomarkers in diabetes related hyperfiltration, and healthy pregnancies complimenting the current literature

	<i>Diabetes related Renal Hyperfiltration</i>		<i>Healthy Pregnancies</i>	
	Literature	PhD Findings	Literature	PhD Findings
sRAGE	Not known	Unchanged	↓*[239]	Unchanged
Serum TNFR1/2	↓	↓	↑*[240, 241]	↑
Urinary TNFR1/2	Not known	Unchanged	↑*[240, 241]	Unchanged
Serum sIL2R	Not known	↓	↓*[242] [243]	Unchanged
Urinary sIL2R	Not known	Unchanged	Not known	Unchanged
Serum MCP1	Not known.	Unchanged	↑*[244, 245]	↓
Urinary MCP1	↑	Unchanged	Not known	↑
Serum NGAL	↓	Unchanged	↑*[246, 247]	Unchanged
Urinary NGAL	↑	Unchanged	Not known	Unchanged
Serum KIM1	Not known	Unchanged	Not known	↑
Urinary KIM1	↑	Unchanged	↑*[248]	Unchanged
Serum 8-Isoprostane	Not known	Unchanged	↑[249]	Unchanged
Urinary 8-Isoprostane	Not known	Not known	↑*[250, 251]	Unchanged
Relaxin	Not known	Not known	↑	↑
CRP	Not known	Unchanged	↑*[252]	Unchanged
NLR	Not known	Unchanged	↑*[253]	↑

*Levels increase in pregnancies with complications

soluble advanced glycation end products (sRAGE), soluble Tumour Necrosis Factor alpha-receptors 1 and 2 (TNFR1/2), soluble Interleukin-2 Receptor (sIL2R), monocyte chemoattractant protein 1(MCP1/ CCL2), neutrophil gelatinase-associated lipocalin (NGAL), kidney injury molecule (KIM-1), 8-Isoprostane, relaxin, C-reactive protein (CRP) and Neutrophil to lymphocyte ratio (NLR)

Appendices

Appendix A: Chapter Nine Supplementary Material

Seah JM, Kam NM, Wong L Tanner C, Jerums G, Permezel M, Shub A, Houlihan C, Ekinci E. Risk factors for pregnancy outcomes in type 1 and type 2 diabetes. Intern Med J. 2020;10.1111/imj.14840. doi:10.1111/imj.14840

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Title:

Risk factors for pregnancy outcomes in type 1 and type 2 diabetes

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Abstract:

Objective: Our aim was to explore differences in pregnancy outcomes between women with type 1 and type 2 diabetes, and healthy controls, and to examine the relationships between potential adverse risk factors and pregnancy outcomes in this cohort of women.

Study Design and Methods: This is a 10 year retrospective study of women with type 1 diabetes ($n = 92$), type 2 diabetes ($n = 106$) and healthy women without diabetes (controls) ($n = 119$) from a tertiary obstetric centre. Clinical and biochemical characteristics of women with type 1 and type 2 diabetes were determined and related to major obstetric outcomes using univariate analysis.

Results: Women with pre-existing diabetes had higher adverse pregnancy outcomes (preeclampsia, emergency caesarean section, preterm birth < 32 and 37 weeks, large for gestational age, neonatal jaundice, Apgar score < 7 at 5 minutes, neonatal intensive care admission and neonatal hypoglycaemia) compared to controls. A higher birth weight gestational centile (97.4% vs. 72.4%, $P = 0.001$) and large for gestational age rate (63.4% vs. 35.8%, $P = 0.001$) were observed in type 1 diabetes compared to type 2 diabetes. There were no differences in other outcomes between women with type 1 and 2 diabetes.

Conclusion:

In this exploratory study, risk factors for maternal adverse outcomes differ between type 1 and type 2 diabetes. Maternal and fetal adverse outcomes were higher in

pregnancies affected by diabetes compared to healthy women but occurred with similar frequency in women with type 1 and type 2 diabetes.

BACKGROUND:

Adverse maternal and neonatal outcomes have been reported up to nine times higher in women affected by pre-gestational diabetes compared to those without.^{1,2} Pregnancy outcomes in women with type 1 diabetes have generally been a focus in the literature. However, the incidence of pregnancies affected by type 2 diabetes has surpassed that of type 1 diabetes in Australia.³⁻⁵ This predominance of type 2 diabetes, to almost four times that of type 1 diabetes has also been shown in a large diabetes-pregnancy epidemiology study.⁶ Suboptimal glycaemic management, particularly in the third trimester is associated with preeclampsia in women with type 1 diabetes.⁷ Such a relationship has not been established in pregnancies of women with type 2 diabetes and the presence of pre-existing hypertension in this cohort complicates the picture. Furthermore, a major challenge in interpreting current literature in this field is the lack of homogeneity in outcome measures and determination of risk factors throughout pregnancy.

The aim of this study was to investigate the association of maternal and neonatal outcomes, with established risk factors throughout the pregnancy. We hypothesized that in women attending a tertiary university hospital in metropolitan Melbourne, Australia, those affected by type 1 and type 2 diabetes will have greater risk of adverse outcomes compared to women without diabetes.

METHODS:

Women with type 1 and type 2 diabetes, who delivered in a tertiary obstetric medical centre, over a 10-year period from July 2004 to July 2014 were identified via hospital database. Diagnosis of type 1 and type 2 diabetes was confirmed on clinician entry.

Patients with unclear diabetes diagnosis, gestational diabetes, and inconsistent or lack of baseline measures of glycaemia were excluded. One hundred and nineteen prospectively recruited healthy women as an extension from a separate study were included in the study as the control group.^{8,9} The prospective cohort were healthy pregnant women presenting at or prior to 13 weeks of gestation at the same tertiary obstetric hospital between 2006-2011. Exclusion criteria included women with known type 1 and type 2 diabetes, past history of thyroid disease and positive thyroid peroxidase antibodies, thyroid hormone replacement therapy, past history of intravenous drug abuse or the presence of major systemic illness

We excluded women with multiple-pregnancies, spontaneous or elective abortion within the first 20 weeks of pregnancy. Study investigators reviewed all medical record and pathology entries from the diagnosis of pregnancy until discharge following delivery. Mercy Health Human Research Ethics Committee approved this study.

Anthropometric and Pathology Data

We collected anthropometric data throughout pregnancy, starting from the first antenatal visit. The initial visit entries were considered as baseline data. Data were obtained by manual reviews of the patient medical record. Presence of pre-existing hypertension was based on clinician entry and use of anti-hypertensive agents. Women who were on anti-hypertensive agents solely for the purpose of reno-protection without hypertension were not classified as having pre-existing hypertension.

Blood pressure and all biochemical parameters from each trimester during routine, stable clinical assessments were averaged for each individual. Parameters from every

trimester were included as means for each covariable, as the cumulative exposures throughout pregnancy were considered important for pregnancy outcomes. Biochemical and clinical data obtained during acute and intra-partum admissions were excluded. HbA_{1c} was used to represent glycaemic management. Renal function was measured by serum creatinine. Due to the lack of a direct measurement of glomerular filtration rate, estimated glomerular filtration rate (eGFR) was calculated with the Chronic Kidney Disease Epidemiology Cohort (CKD-EPI) formula.¹⁰

The following risk factors were included in analyses: maternal age, body mass index (BMI), type of diabetes, mean HbA_{1c}, mean eGFR, and mean systolic and diastolic blood pressure. Rather than trimester specific values, the mean HbA_{1c}, blood pressure and eGFR of the whole pregnancy were utilised in the analytical model to take into consideration the overall levels of these risk factors throughout pregnancy. The major obstetric outcome measures were: preeclampsia, preterm birth < 37 weeks and < 32 weeks, large for gestational age (LGA), small for gestational age (SGA) and neonatal intensive care (NICU) admission.

Outcome Measures

For the purpose of this study, maternal and perinatal outcomes were defined using The Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) recommended guidelines¹¹ and clinician diagnosis. Pregnancy induced hypertension was based on the absence of a pre-existing diagnosis of hypertension, as well as systolic blood pressure (sBP) > 140 mmHg with or without diastolic blood pressure (dBP) > 90 mmHg, on three or more measurements. Preeclampsia was defined as a development of sBP > 140 mmHg with or without dBP > 90 mmHg, and proteinuria

(> 0.3 g/24h or ≥ 30 mg/mmol) after 20 weeks gestation. Primary postpartum haemorrhage was defined as a blood loss of either > 500 ml post vaginal delivery or > 1000 ml post caesarean delivery within 24 hours of delivery.

Preterm birth was categorised as delivery at 32-37 weeks and early preterm birth as prior to 32 weeks (with no separation between spontaneous and iatrogenic). Infant birthweight was measured at delivery using standard weighing scales. Birthweight percentiles and z-scores corrected for gestational age were calculated based on a reference population at the same hospital.¹² The z-score was calculated as: (observed birthweight – mean birthweight for gestational age)/ standard deviation of the mean birthweight. LGA and SGA, were defined as birthweights > 90th centile and < 10th centile, respectively. Neonatal jaundice was defined as hyperbilirubinemia requiring phototherapy. Major congenital malformations were those responsible for death, those causing a significant future disability, or those requiring major surgery requiring correction. Neonatal hypoglycaemia was defined as blood glucose of < 2.6 mmol/l in the early neonatal period. Fetal death in utero was defined as fetal death later than 20 weeks gestation.

Statistical Analysis

Univariate analysis was performed with Wilcoxon rank sum test for continuous variables, and Chi-square tests or Fisher's exact tests for categorical variables. Comparisons were made between type 1 and type 2 diabetes pregnancies (Table 1 and 3), and healthy controls with the combined diabetes group (Table 2 and 4).

We undertook univariate logistic analyses to assess associations with established risk factors (including age, blood pressure, BMI, renal function and HbA_{1c}) and four major outcomes of preeclampsia, preterm birth < 32 and 37 weeks, and neonatal intensive care admission in women with and without pregestational diabetes (combined type 1 and type 2 diabetes). Sub-group analysis was also performed comparing outcomes in women with type 1 diabetes to type 2 diabetes. The co-linearity of the model prohibited appropriate inclusion of these risk factors into a multivariable analysis. Due to the multiple outcome measures, we corrected for multiplicity testing using the Bonferroni correction with a threshold of significance at $P = 0.03$.

RESULTS:

Maternal Characteristics

Over the ten-year period, 307 singleton pregnancies were included in the analysis: 119 healthy women without diabetes; 92 women with type 1 diabetes; and 106 women with type 2 diabetes (Table 1 & Figure 1).

Women with type 2 diabetes had more advanced age and higher BMI. Women with type 1 diabetes had statistically significant longer duration of diabetes, marginally higher HbA_{1c}, and higher prevalence of microvascular complications. Lower renal function as reflected by lower eGFR, and higher serum creatinine was evident in the type 1 diabetes cohort (Table 1). Longitudinal data across all three trimesters (Figure 1), demonstrated that women with type 2 diabetes had a significantly lower HbA_{1c} (Figure 1a) but a higher blood pressure (Figure 1b and 1c) compared to women with type 1 diabetes. Women without pre-gestational diabetes were younger and had a lower BMI compared to women with type 1 and type 2 diabetes (Table 2).

Maternal and Neonatal Outcomes

Pregnancy and neonatal outcomes are demonstrated comparing type 1 and type 2 diabetes (Table 3) individually. Furthermore, to illustrate the differences between healthy and pregnancies affected by diabetes, we combined type 1 and type 2 diabetes; comparing the collective diabetes cohort to controls (Table 4). Birth weight gestational centile (median 97.4% vs. 72.4%; $P < 0.001$) and LGA rates (63.4% vs. 35.8%; $P < 0.001$) were higher in women with type 1 diabetes compared to type 2 diabetes. Women with type 2 diabetes had an approximate doubling of SGA compared to type 1 diabetes (15.1% vs. 5.4%; $P = 0.02$). There was no statistically significant difference in other major perinatal and obstetrics outcomes between women with type 1 and type 2 diabetes (Table 3). Sixteen percent of women with diabetes (type 1 and type 2 combined) developed preeclampsia compared to four percent in the control group ($P = 0.003$).

Compared to healthy pregnancies (Table 4), the odds of preeclampsia (odds ratio [OR], 4.4; 95% confidence interval [CI], 1.7-11.6; $P = 0.003$), emergency caesarean delivery (OR, 2.7; 95% CI, 1.5-4.9; $P = 0.001$), LGA (OR, 2.8; 95% CI, 1.7-4.7; $P < 0.001$), Apgar score less than 7 (OR, 5.8; 95% CI, 1.3-25.7 $P = 0.02$), neonatal intensive care admission (OR, 5.2; 95% CI, 2.0-13.7; $P = 0.001$), preterm birth at less than 32 (OR, 12.5; 95% CI, 1.7-94.8; $p = 0.01$) and 37 weeks (OR, 6.3; 95% CI, 3.0-13.1; $P < 0.001$) were all significantly higher in women with diabetes. Only SGA and neonatal congenital malformation was not significantly different between the two groups.

Predictors of adverse pregnancy outcomes in women with pre-gestational diabetes

HbA_{1c} was not associated with major outcomes. A one mmHg increase in systolic and diastolic blood pressure was associated with higher odds of developing preeclampsia (OR of 1.1 [95% CI, 1.0-1.1; $P = 0.001$] and 1.1 [95% CI, 1.1-1.2; $P < 0.001$], respectively) and preterm birth at 37 weeks (OR of 1.1 [95% CI, 1.0-1.1; $P < 0.001$] and 1.1 [95% CI, 1.0-1.1; $P = 0.005$], respectively)

Higher BMI was associated with higher odds of developing pregnancy-induced hypertension (OR, 1.1; 95% CI, 1.0-1.1; $P = 0.01$) and preeclampsia (OR, 1.0; 95% CI, 1.0-1.1; $P = 0.05$). Increasing age (per year) was also associated with higher odds of developing preterm birth < 32 weeks (OR, 1.1; 95% CI, 1.0-1.2; $P = 0.01$). Each ml/min/1.73m² decrease in eGFR ($P < 0.001$) was associated with higher odds of developing preeclampsia (OR, 1.1; 95% CI, 1.0-1.1), preterm birth 32 (OR, 1.1; 95% CI, 1.0-1.1) and 37 weeks (OR, 1.0; 95% CI, 1.0-1.1) and neonatal intensive care admission (OR, 1.1; 95% CI, 1.0-1.1).

Smoking was not associated with any major outcomes apart from the higher odds of developing pre-eclampsia (OR, 1.9; 95% CI, 1.1, 3.1).

As limited by the co-linearity of the model, multivariable analyses for the above risk factors were not conducted.

DISCUSSION:

The most important findings in this study were that the rates of poor outcomes were comparable between type 1 and type 2 diabetes. The measures of poorer outcomes in

pre-existing maternal diabetes were present across most (thirteen out of sixteen) of outcome measures, and consistent with current literature.¹³ In addition, we identified maternal blood pressure, BMI, and renal function to be important predictors of risk for poor outcomes.

Comparison to Previous Studies

We found similar pregnancy outcomes in type 1 and type 2 diabetes. This is in contrast with some studies¹³⁻¹⁶ suggesting more adverse consequences in pregnancies affected by type 2 diabetes. A study by Hillman and colleagues¹⁷ attempting to address this concluded that pregnancy outcomes were better in type 2 diabetes when intensified glycaemic management, as gauged by HbA_{1c}, were as rigorous as in women with type 1 diabetes. In our study, we did not find better outcomes in women with type 2 diabetes despite lower HbA_{1c}. The glycaemic pattern throughout pregnancy from the study by Clausen et al. was not dissimilar to our study, along with others which failed to find any differences in outcomes between type 1 and type 2 diabetes.¹⁴ Of note however, timely intensive glucose therapy was shown to reduce spontaneous abortion and congenital malformation in the diabetes in Complication and Control Trial (DCCT) in women with type 1 diabetes.¹⁸

Findings of LGA rates being significantly higher in pre-gestational diabetes compared to healthy women, supports glucose as a major mediator of growth.¹⁹ Exaggeration of the physiological insulin resistance in pregnancy that occurs in maternal diabetes can promote hyperglycaemia. Independent association of HbA_{1c} with LGA, has been found in previous type 1 diabetes studies.^{20, 21} However a mean HbA_{1c} of 6.5% (48 mmol/mol), which was found to be associated with other poor outcomes in previous

studies including preterm birth before 37 weeks, composite neonatal outcomes and preeclampsia,²⁰ was not found in our cohort. A systemic review of 13 studies in relation to HbA_{1c} and outcomes identified overall poorer, but unpredictable and diverse outcomes from study to study.²² Of note, in the presence of an acceptable HbA_{1c}, higher median glucose readings especially in the second trimester are closely related to LGA in type 1 diabetes.²¹ These inconsistencies linking HbA_{1c} to adverse outcomes illustrate the limitation of using HbA_{1c} as an indicator of fetal glucose exposure during pregnancy. Targeting near normal glycaemia throughout the entire pregnancy is desirable^{23,24} and may be more enabled by further advances in technology of insulin delivery and intensive real time glucose monitoring. In fact, a recent multicentre randomized controlled trial²⁵ demonstrated improved neonatal outcomes for mothers with type 1 diabetes using continuous glucose monitoring during pregnancy, with lower incidence of large for gestational age, fewer neonatal intensive care admissions and fewer instances of neonatal hypoglycaemia.

Obesity has been linked with intra-uterine growth restriction,²⁶ which may partially explain the lower birthweight centile in infants born to type 2 diabetes women in our cohort, with a median BMI within the obese range. The effect of obesity and risk of SGA may be related to the inadequate weight gain of women²⁷ with the introduction of gestational weight gain guidelines which recommends a lower weight gain pregnancy threshold for women with elevated BMIs.²⁸ In contrast, a recent study has shown a different pattern, whereby there appears to be an association with maternal obesity and accelerated fetal growth, especially when combined with gestational diabetes.²⁹ In addition, a recent Australian study established a higher BMI in women with both type 1 and type 2 diabetes with infants more likely to be LGA compared to

healthy controls.^{30, 31} Whether BMI explains the significantly lower birth weight and smaller gestational age in our type 2 diabetes cohort will need to be further investigated, especially with the lack of documented gestational weight changes in our cohort of women, as well as being confounded by the increasing co-morbidities that accompany this, including hypertension.³²

Hypertension has a combined additive effect with diabetes on SGA,³³ which may partly explain the higher rates of SGA in our type 2 diabetes cohort. Intensive blood pressure management in diabetic kidney disease has been shown to benefit outcomes in this group of high-risk pregnancy.^{34, 35}

Supplementing current Australian Pre-gestational Outcome Data

The similar proportion of both type 1 and type 2 women with diabetes in our study may still reflect the under-recognition of the need to direct specialist care for these high-risk women. This is further highlighted with 25% of all type 2 diabetes and 3% of type 1 diabetes patients registered with the National Diabetes Service Scheme were from childbearing age of 21-39 years.^{36, 37} From a study published in 2005, involving ten teaching hospitals in Australia⁵ with comparable numbers (n = 180), the mean age of conception in our study was higher, particularly in type 2 diabetes reflecting current societal trends of deferred pregnancies. The incidence (4.6% vs. 8.1%) of major congenital malformations was also less, however, our study excluded women with spontaneous abortion of pregnancy before 20 weeks and elective terminations. Taking into consideration, findings of a New Zealand study,¹⁶ which demonstrated higher perinatal mortality in type 2 diabetes and gestational diabetes compared to our study, exclusion of gestational diabetes in our study may potentially exclude very small

number of undiagnosed late type 2 diabetes cases that may contribute to neonatal deaths.

In contrast to our findings, a 2010 report based on the National Hospital Morbidity Database ³ found that mothers with type 1 diabetes had higher rate of hypertension. We believe that manually obtaining blood pressure readings, as performed in our study, provided better overall representation of the presence of hypertension than that of a single documentation of self- or clinician reported diagnosis. The findings of higher preterm birth and caesarean-section in type 1 diabetes compare to type 2 diabetes were not replicated by findings of our study. This contrast may be a reflection of different population groups, as our study was conducted at a tertiary centre, and due to improvements in clinical practice over time.

The most recent Australian study reporting pregnancy outcomes in type 1 ³¹ and type 2 diabetes ³⁰ compared to the same cohort of healthy controls highlighted the adverse outcomes in this high risk group, underscoring the importance of HbA_{1c} and glycaemic management. Our study complements the literature by examining the association of outcomes with additional variables of blood pressure, eGFR and other clinical and biochemical data. Risk factors for patients with diabetes were also pooled in our study when compared to healthy controls, which may explain some of the discrepancies in our respective study findings.

Strengths and Limitations

This was an observational, retrospective study and we recognize that there may be incomplete data collection. For example, we excluded women with inconsistent and

absent baseline glycaemia measurement. This may result in missing some cases of pre-gestational diabetes, but on the other hand allow for a low false positive rate on the diagnosis of type 2 diabetes. The cause of preterm birth was also not determined, which would have allowed insight into future management of these cases.

The applicability of findings outside of our institution may also be limited. This includes the relatively well-controlled blood pressure and reasonable glycaemic management which may reflect practices within a tertiary obstetric hospital and affect the overall outcomes. However, as the focus on the importance of obstetric blood pressure and glycaemic management is increasing, our findings may be applicable more broadly with time.

Finally, as this is one of the larger observational studies in Australia in recent years, our findings may be helpful in counselling women with diabetes regarding pregnancy outcomes in contemporary Australian practice.

CONCLUSION:

Both type 1 and type 2 diabetes carry a similar high risk for poorer maternal and fetal outcomes compared to healthy pregnancies. Although estimates of associations between the maternal risk factors and adverse outcomes should be regarded as exploratory in a cohort of this sample size, age, higher BMI, higher systolic and diastolic blood pressure, and lower eGFR predicted poorer outcomes.

CONTRIBUTION TO AUTHORSHIP:

JS, CH, EIE and AS designed the study and conceptualized the research question. JS, NK, LW was involved in data collection. JS, NK and LC analyzed the data. JS and NK drafted the article. All authors critically reviewed and approve the final version of the article.

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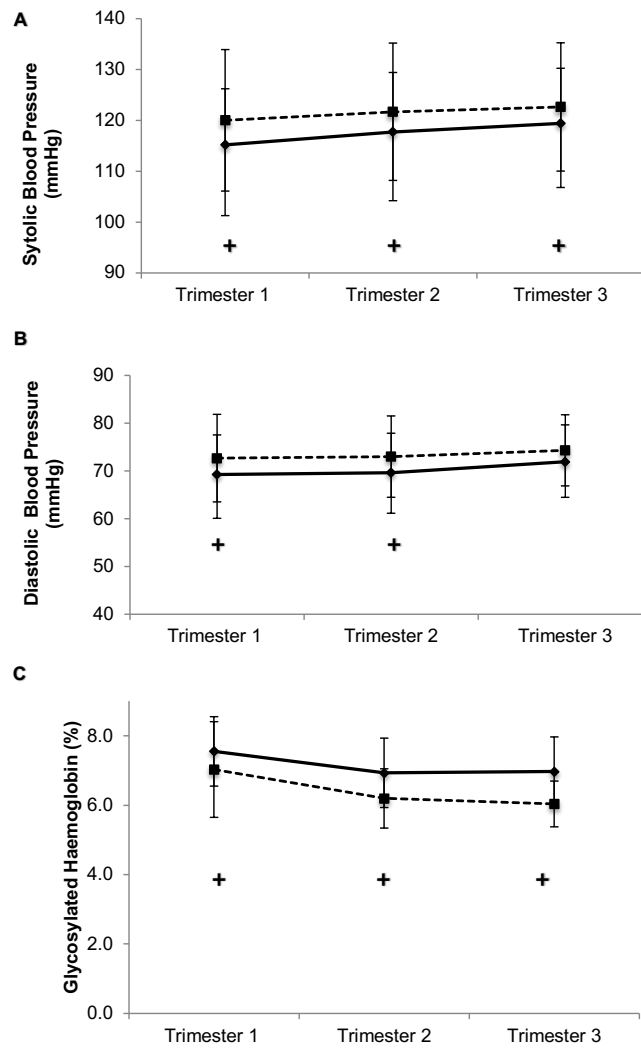
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FIGURES

Figure 1. Comparison of (A) systolic blood pressure and (B) diastolic blood pressure and (C) glycosylated haemoglobin (HbA1c) in women with type 1 diabetes (-◆-) and type 2 diabetes (-■-) during pregnancy.



Data expressed as mean standard error of the mean.
Mann-Whitney U-tests were performed. * $p < 0.05$

TABLES

Table 1. Maternal characteristics in women at baseline (type 1 diabetes vs. type 2 diabetes)

	Type 1 Diabetes	Type 2 Diabetes	<i>P</i> value
<i>n</i>	92	106	
Age (years)	31.0 (27.5, 34.3)	34.5 (31.0, 37.4)	< 0.001
BMI (kg/m ²)	27.2 (23.0, 31.0)	33.0 (29.0, 38.0)	< 0.001
Parity	1.0 (0.0,1.0)	1.0 (0.0,1.0)	0.1
Smoking status at conception			0.9
Current smoker	7 (9.8)	6 (5.7)	
Ex-smoker	9 (7.6)	11 (10.4)	
Non-smoker	76 (82.6)	89 (83.9)	
Duration of diabetes (years)	12.0 (5.0, 19.0)	3.0 (2.0, 5.0)	< 0.001
HbA _{1c} (%)	7.0 (6.5, 8.2)	6.9 (6.0, 7.8)	0.03
HbA _{1c} (mmol/mol)	53.0 (47.5, 66.1)	51.9 (42.1, 61.7)	0.03
Systolic blood pressure (mmHg)	115.0 (110.0, 120.0)	120.0 (110.0, 125.0)	0.02
Diastolic blood pressure (mmHg)	70.0 (65.0, 70.0)	70.0 (70.0, 80.0)	0.02
Serum creatinine (μmol/l)	54.0 (47.0, 59.0)	46.0 (40.5, 51.5)	< 0.001
CKD-EPI eGFR (ml/min/1.73m ²)	120.8 (116.7, 128.5)	124.8 (121.6, 130.6)	0.02
Pre-existing hypertension	10 (10.9)	23 (21.7)	0.04
Renin angiotensin aldosterone System Inhibition at conception	8 (8.7)	8 (7.6)	0.8
Statins therapy at conception	4 (4.4)	9 (8.5)	0.2
Metformin therapy at conception	0 (0)	55 (51.9)	< 0.01
Microvascular complications	21 (22.6)	8 (7.5)	< 0.01

Macrovascular complications	2 (2.2)	0 (0)	0.1

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

Body mass index = BMI; glycosylated hemoglobin = HbA_{1c}; Chronic Kidney Disease

Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR

Table 2. Selected maternal characteristics in women at baseline (diabetes vs. healthy control)

	Diabetes (Type 1 & Type 2)	Healthy Controls	<i>P</i> -value
N	198	119	
Age (years)	33.0 (28.9, 36.2)	31.0 (28.0, 35.0)	0.04
BMI (kg/m ²)	30.0 (26.0, 35.5)	25.0 (23.0, 32.0)	< 0.001
Serum creatinine (μmol/l)	49.5 (43.0, 57.0)	49.0 (44.0, 54.0)	0.8
CKD-EPI eGFR (ml/min/1.73m ²)	123.6 (118.2, 129.4)	125.3 (119.6, 130.6)	0.2
Albumin-to-creatinine ratio (mg/g)	7.1 (4.4, 21.2)	-	

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

Body mass index = BMI; Chronic Kidney Disease Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR

Table 3. Comparison of obstetric and perinatal outcomes in women with type 1 and type 2 diabetes

	Type 1 diabetes	Type 2 diabetes	Odds ratio (95% CI)	P-value
<i>n</i>	92	106		
Obstetric outcomes				
Preeclampsia	12 (12.9)	20 (18.9)	0.6 (0.3, 1.4)	0.3
Pregnancy-induced hypertension	4 (4.3)	11 (10.4)	0.4 (0.1, 1.3)	0.1
Emergency caesarean delivery	32 (34.4)	33 (31.1)	1.2 (0.7, 2.3)	0.5
Postpartum haemorrhage	13 (14)	10 (9.4)	1.6 (0.7, 3.8)	0.3
Perinatal outcomes				
Gestational age (weeks)	37.4 (36.0, 38.1)	37.3 (38.3, 40.4)	-0.1 (-1.0, 0.8)	0.8
Preterm birth < 37 weeks gestation	31 (33.3)	36 (34)	1.0 (0.5, 1.8)	1.0
Preterm birth < 32 weeks gestation	10 (10.8)	9 (8.5)	1.3 (0.5, 3.4)	0.6
Birthweight gestational centile (%)	97.4 (79.2, 99.8)	72.4 (32.4, 94.4)	20.2 (11.7, 28.8)	< 0.001
Large for gestational age	59 (63.4)	38 (35.8)	3.2 (1.8, 5.7)	< 0.001
Small for gestational age	5 (5.4)	16 (15.1)	0.3 (0.1, 0.9)	0.03
Major congenital malformation	3 (3.2)	3 (2.8)	1.2 (0.2, 5.9)	0.9
Fetal death in utero/ in labour	2 (2.2)	3 (2.8)	0.8 (0.1, 4.7)	0.8
Neonatal jaundice	9 (9.7)	17 (16.0)	0.6 (0.2, 1.3)	0.2
Apgar score < 7 at 5 minutes	8 (8.6)	10 (9.4)	0.9 (0.3, 2.4)	0.9
NICU Admission	18 (19.4)	19 (17.9)	1.1 (0.5, 2.3)	0.8
Neonatal hypoglycaemia	25 (26.9)	24 (22.6)	1.3 (0.7, 2.4)	0.5

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using logistic regression.

Data expressed as Odds ratio for categorical variables and Co-efficient variation for continuous variable.

Neonatal intensive care unit = NICU

Table 4. Comparison of obstetric and perinatal outcomes in women with and without diabetes

	Diabetes (Type 1 & Type 2)	Healthy controls	Odds ratio (95% CI)	P-value
<i>n</i>	198	119		
Obstetric outcomes				
Preeclampsia	32 (16.2)	5 (4.2)	4.4 (1.6, 11.6)	0.003
Pregnancy-induced hypertension	15 (7.6)	3 (2.5)	3.2 (0.9, 11.2)	0.07
Emergency caesarean delivery	65 (32.8)	18 (15.1)	2.7 (1.5, 4.9)	0.001
Postpartum haemorrhage	23 (11.6)	11 (9.2)	1.3 (0.6, 2.8)	0.5
Perinatal outcomes				
Gestational age (weeks)	37.3 (36, 38.1)	39.4 (38.3, 40.4)	-2.8 (-3.4, -2.1)	< 0.001
Preterm birth < 37 weeks gestation	67 (33.8)	9 (7.6)	6.3 (3.0, 13.1)	< 0.001
Preterm birth < 32 weeks gestation	19 (9.6)	1 (0.8)	12.5 (1.7, 94.8)	0.01
Birthweight gestational centile (%)	89.5 (61.7, 98.7)	69.7 (41.0, 48.9)	10.8 (3.7, 18.0)	0.003
Large for gestational age	97 (49.5)	30 (25.2)	2.8 (1.7, 4.7)	< 0.001
Small for gestational age	21 (10.6)	9 (7.6)	1.5 (0.6, 3.3)	0.4
Major congenital malformation	6 (3)	4 (3.4)	0.9 (0.2, 3.3)	0.9
Fetal death in utero/ in labour	5 (2.5)	0 (0)	NA	NA
Neonatal jaundice	26 (13.1)	4 (3.4)	4.3 (1.5, 12.6)	0.008
Apgar score < 7 at 5 minutes	18 (9.1)	2 (1.7)	5.9 (1.3, 25.7)	0.02
NICU Admission	37 (18.7)	5 (4.2)	5.2 (2.0, 13.7)	0.001
Neonatal hypoglycaemia	49 (24.7)	4 (3.4)	9.5 (3.3, 27.0)	< 0.001

Data expressed median (interquartile range) or n (%)

Group comparisons were performed using logistic regression.

Data expressed as Odds ratio for categorical variables and Co-efficient variation for continuous variable.

Neonatal intensive care unit = NICU

Appendix B: Chapter Ten Supplementary Material

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TITLE:

The association between maternal renal function and pregnancy outcomes in type 1 and type 2 diabetes

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ABSTRACT:

AIMS: To investigate the prognostic value of estimated glomerular filtration rate (eGFR) and albuminuria in determining pregnancy outcomes in women with type 1 and type 2 diabetes.

METHODS: An observational study of pregnant women with type 1 (n = 92) and type 2 diabetes (n = 106) who delivered between 2004 and 2014 at a single tertiary obstetric centre. Clinical and biochemical characteristics were determined and related to major obstetric outcomes: preeclampsia, preterm birth <32 and <37 weeks, and neonatal intensive care admission. We used univariate analyses and multivariable logistic regression models with eGFR using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation and albuminuria as covariates.

RESULTS: In the pooled diabetes cohort, multivariable logistic regression with eGFR and albuminuria status demonstrated that the presence of albuminuria (albumin-to-creatinine ratio ≥ 3.5 mg/mmol) (OR, 2.7; 95% CI, 1.42-4.99; $P = 0.002$) was associated with preeclampsia, whilst an eGFR of <120 mL/min/1.73m² was associated with preterm birth <32 weeks (OR, 1.04; 95% CI, 1.00-1.09; $P = 0.02$).

CONCLUSIONS: Despite its recognized limitations in pregnancy, lower eGFR values were associated with increased risk of adverse outcomes. Our exploratory data suggest eGFR, along with albuminuria, can aid in identifying women at high risk of developing adverse obstetric outcomes.

KEYWORDS:

diabetes mellitus; neonatal; obstetric complications; diabetic kidney disease; diabetic nephropathy; glomerular filtration rate; albuminuria

INTRODUCTION:

Adverse maternal and neonatal outcomes have been reported to be up to nine times higher than that of pregnancies not affected by type 1 and type 2 diabetes. (1, 2) In addition, in women with type 1 (3, 4) and type 2 diabetes mellitus, (5) pregnancies affected by diabetic kidney disease have a significantly higher obstetric and perinatal risk compared to those without diabetic kidney disease. Specifically, women with type 1 diabetes who have either micro- or macroalbuminuria are at a five to eight times increased risk of developing preeclampsia compared with women with type 1 diabetes mellitus and normal urinary albumin excretion. (4)

Pre-existing chronic kidney disease (CKD), independent of diabetes, has been linked to high obstetric risk. (6) Previous research has demonstrated that the risk of adverse pregnancy outcomes, including preterm birth < 37 weeks, neonatal intensive care unit admission and small for gestational age, is up to threefold higher in pregnancies affected by CKD. Furthermore, this risk was observed even in women with what is generally accepted as “normal” renal function, with an estimated Glomerular Filtration Rate (eGFR) > 90 mL/min/1.73m², categorized as stage 1 CKD using the Kidney Disease Improving Global Outcomes (KDIGO) guidelines. (7)

To the best of our knowledge, there is limited literature studying the utility of eGFR, using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation in predicting adverse obstetric outcomes in type 1 and type 2 diabetes. The aim of this study was to investigate the prognostic value of eGFR and albumin-to-creatinine ratio (ACR) in determining four major obstetric outcomes: preeclampsia; preterm birth at < 32 weeks; preterm birth at < 37 weeks; and neonatal intensive care unit admission. We hypothesize that in women with pre-existing diabetes, lower eGFR and higher albuminuria are independent predictors of poorer maternal and foetal outcomes.

SUBJECTS, MATERIALS AND METHODS:

Women with type 1 and type 2 diabetes, who delivered at the Mercy Hospital for Women (MHW), a tertiary obstetric medical centre in Melbourne, Australia, over a 10-year period from July 2004 to July 2014 were identified via MHW Birthing Outcome Systems database. Diagnosis of type 1 and type 2 diabetes was based on clinical entry by obstetricians or endocrinologists. Study investigators reviewed all medical records and pathology entries from the diagnosis of pregnancy until discharge following delivery. Women with multiple-pregnancies, spontaneous miscarriage or termination of pregnancy within the first 20 weeks of pregnancy, unclear diabetes diagnosis, gestational diabetes, and inconsistent or lack of baseline measures of glycaemia were excluded. One hundred and nineteen healthy women who were prospectively recruited in a separate study were included in the current study as the control group. (8, 9) This study was approved by Mercy Health Human Ethics Committee and these individuals provided written informed consent.

Study participants were reviewed on a regular basis, throughout pregnancy and managed by a multidisciplinary team of obstetricians, endocrinologists, dietitians and diabetes nurse educators. Glycaemia and blood pressure targets were based on the Australasian Diabetes in Pregnancy Society consensus guidelines. (10) Insulin was used as first line therapy for management of type 2 diabetes in pregnancy. Glycosylated haemoglobin (HbA_{1c}) was measured at baseline and approximately every 6 weeks throughout gestation using a turbidimetric inhibition immunoassay on Cobas Integra 800 (Roche Diagnostics, Mannheim, Germany), standardized to the International Federation of Clinical Chemistry reference method. Other biochemistry including serum creatinine, ACR or albumin excretion rate were performed at baseline, and as clinically indicated. Women were generally delivered at approximately 38 weeks gestation, or earlier if clinically indicated.

Anthropometric and biochemistry data

We collected anthropometric data throughout pregnancy, starting from the first antenatal visit. The initial visit entries, generally in the first trimester, were considered as the baseline data. Clinical data were obtained from the patient's medical record. Presence of pre-existing hypertension was based on clinician entry and use of anti-hypertensive agents. Women with normal blood pressure who were on anti-hypertensive agents solely for the purpose of reno-protection were not classified as having pre-existing hypertension. Macrovascular complications included ischaemic heart disease, cerebrovascular disease and peripheral vascular disease. Microvascular complications included retinopathy and diabetic kidney disease.

Blood pressure and all biochemical parameters from each trimester during routine clinical assessments were averaged throughout the pregnancy for each individual. Clinical and biochemical parameters from every trimester were included, as the cumulative exposures throughout pregnancy were considered important for pregnancy outcomes. Biochemistry data that were included in this study comprised of all available results on either the hospital pathology database, or clinical records during the pregnancy. Acute and intra-partum admission biochemical and clinical data were excluded. Glycosylated haemoglobin was used to represent glycaemic management. Renal function was measured by serum creatinine. Due to the lack of an alternative, simple, routine method of measuring glomerular filtration rate; eGFR was calculated based on the CKD-EPI formula. (11) We defined stages of diabetic kidney disease according to the Kidney Disease Improving Global Outcome (KDIGO) guidelines (12) and arbitrarily defined hyperfiltration as an eGFR > 120 mL/min/1.73m². Microalbuminuria was defined as urinary albumin excretion of 30-300 mg/day or urinary ACR of 3.5 to 35.0 mg/mmol. Macroalbuminuria was defined as urinary albumin excretion > 300 mg/day or urinary ACR > 35.0 mg/mmol.

Rather than trimester specific values, the mean HbA_{1c}, blood pressure, eGFR and ACR across the entire pregnancy were utilised in the analytical model to take into consideration the overall levels of these risk factors throughout pregnancy.

Outcome measures

Maternal and perinatal outcomes were defined using The Royal Australian and New Zealand College of Obstetricians and Gynaecologists (RANZCOG) recommended guidelines (13) and clinician diagnosis and entry. For the purpose of this study, we identified four major outcomes based on the literature to avoid multiplicity of testing. The outcome measures included were preeclampsia, preterm birth < 32 weeks and < 37 weeks, and neonatal intensive care unit admission.

Pregnancy induced hypertension was based on the absence of a pre-existing diagnosis of hypertension, and systolic blood pressure (sBP) > 140 mmHg ± diastolic blood pressure (dBP) > 90 mmHg based on three or more measurements. Preeclampsia was defined as a development of sBP > 140 mmHg ± dBP > 90 mmHg, and proteinuria > 300 mg/24h after 20 weeks gestation, as well as clinical diagnosis. 'Preterm birth < 37 weeks' was categorised as delivery from 32 completed weeks to prior to 37 completed weeks, and 'very preterm birth < 32 weeks' as delivery prior to 32 completed weeks (with no separation made between spontaneous and iatrogenic).

Statistical analysis

Univariate analysis was performed with Wilcoxon Rank Sum test for continuous variables, and Chi-square tests or Fisher's exact tests for categorical variables. Comparisons were made between type 1 and type 2 diabetes pregnancies (Table 1 and 3), and healthy controls with the combined diabetes group (Table 2).

We undertook univariate and multivariable logistic analyses to assess the risk of four major outcomes of preeclampsia, preterm birth < 32 and 37 weeks and neonatal intensive care unit admission in women with type 1 and type 2 diabetes. Presence of albuminuria and CKD-EPI eGFR < 120 mL/min/1.73m² were included in the multivariable regression models as categorical and continuous variables. The co-linearity of the model prohibited appropriate inclusion of other established risk factors including age, blood pressure, body mass index (BMI), and glycaemic management in the multivariable analysis. The high co-linearity found in the statistical analysis is likely due to the above-mentioned risk factors all being established diabetes related co-morbidities that is associated with lower eGFR and albuminuria.

RESULTS:

Maternal characteristics

Over the 10-year period, 307 singleton pregnancies were included in the analysis: 119 healthy women without diabetes, 93 with type 1 diabetes, and 106 women with type 2 diabetes. When the maternal characteristics of the diabetes cohorts were compared (Table 1), those with maternal type 2 diabetes had more advanced age, higher BMI, and a higher rate of pre-existing hypertension. Women with type 1 diabetes had longer duration of diabetes, marginally higher HbA_{1c} and higher prevalence of microvascular complications. Rates of albuminuria were not significantly different between type 1 and type 2 diabetes. However, renal function, as measured by CKD-EPI eGFR and serum creatinine values, was lower in the type 1 diabetes cohort (Table 1). Longitudinal data across all three trimesters

demonstrated that women with type 2 diabetes had a significantly lower HbA_{1c} and higher systolic and diastolic blood pressure compared to women with type 1 diabetes. Compared to healthy controls, women with diabetes (combined type 1 & 2 diabetes groups) had higher BMI, but similar eGFR readings (Table 2 & Figure 1).

Albumin-to-creatinine ratio and CKD-EPI eGFR as risk factors

All women in the control group had an eGFR > 90 mL/min/1.73m². Albuminuria status for healthy controls was not available. According to indices of renal status for the diabetes cohort (Table 2), the median ($\pm$ interquartile range) eGFR and ACR were 124 (118, 129) mL/min/1.73m² and 0.8 (0.5, 2.4) mg/mmol, respectively.

The following results on multivariable logistic analysis were based on data in women with type 1 and type 2 diabetes. Analysis on albuminuria and eGFR were based on results that were available for the 144 and 186 women with type 1 and type 2 diabetes

With the selected four predefined outcomes, in women with type 1 and type 2 diabetes, compared to those who were hyperfiltering, women with both stage 1 and stage 2+ diabetic kidney disease had higher odds of developing preeclampsia (OR of 4.5 [95% CI, 1.6-12.3; P = 0.04] and 3.9 [95% CI, 1.7-8.9; P = 0.001], respectively) and of having a preterm birth < 37 weeks (OR 2.3 [95% CI, 1.2-4.6; P = 0.01] and 4.9 [95% CI, 1.7-14.5; P = 0.04], respectively). There was also an additional risk of neonatal intensive care admission (OR, 4.9; 95% CI, 2.0-11.6; P < 0.001) and preterm birth < 32 weeks (OR, 5.0; 95% CI, 1.7-14.5; P < 0.001) in the women with stage 2+ diabetic kidney disease (Table 3).

Compared to women with normoalbuminuria, women with microalbuminuria and macroalbuminuria had a higher odds of developing preeclampsia (OR 5.7 [95% CI, 1.8-17.8; P = 0.03] and 5.5 [95% CI, 2.5-11.8; P < 0.001], respectively) and a higher odds of having a preterm birth < 37 weeks (OR 3.9 [95% CI, 1.5-9.9; P = 0.04] and 3.5 [95% CI, 1.6-7.7; P = 0.02], respectively). In addition, women with macroalbuminuria had higher odds of having a very preterm birth (< 32 weeks) (OR, 4.2; 95% CI, 1.9-9.5; P < 0.01) and of their newborn being admitted to a neonatal intensive care unit (OR, 2.4; 95% CI, 1.2-4.6; P = 0.01).

Furthermore, when analysed as a continuous variable, both eGFR and albuminuria levels were associated with higher risk of all four adverse outcomes. Using multivariable analysis, when adjusted for each other, associations between eGFR and albuminuria with adverse outcomes persisted. Both eGFR (OR, 1.03; 95% CI, 1.00-1.06; P = 0.04) and albuminuria status (OR, 1.95; 95% CI, 1.14-3.35; P = 0.02) were associated with preterm birth < 37 weeks. Specifically, with every 1 mL/min/1.73m² decline in eGFR, there was 4% higher odds of developing preterm birth < 32 weeks (OR, 1.04; 95% CI, 1.00-1.09; P = 0.02) and 3% higher odds of requiring admission to neonatal intensive care unit (OR, 1.03; 95% CI, 1.00-1.06; P = 0.02), but not preeclampsia; whilst, increased albuminuria was

associated with a significantly higher odds of developing preeclampsia (OR, 2.7; 95% CI, 1.42-4.99; $P = 0.002$).

We acknowledge the importance of including HbA_{1c}, systolic and diastolic blood pressure, BMI, age and duration of diabetes in analysis. However, due to the co-linearity of the model, multivariate analyses for potential confounding risk factors were not conducted.

DISCUSSION:

Key findings

The most pertinent findings of this study were that with every 1 mL/min/1.73m² decline in eGFR, there was 4% higher odds of developing preterm birth < 32 weeks. Independent of eGFR, increased albuminuria was associated with preeclampsia; whilst independent of albuminuria, lower eGFR was associated with preterm birth < 32 weeks and neonatal intensive care unit admission.

As renal function appears to be a risk factor for poorer outcomes, it will be important to further investigate modifiable risks in order to allow implementation of protective measures aimed at preserving renal function in these young women. This is important, not only for improving pregnancy care, but for long-term complication prevention especially when renal function is so closely related to cardiovascular mortality. (14)

Overall, as eGFR is commonly employed in routine clinical settings, it can potentially serve as a readily accessible marker in identifying women with diabetes at a higher risk of developing maternal and fetal outcomes in pregnancies. In addition, understanding how albuminuria can reflect higher risk of adverse pregnancy outcomes can aid clinicians to provide additional pregnancy counselling and more intensive follow up. The independent associations demonstrated between eGFR and albuminuria with selected pregnancy outcomes in this study is encouraging for future research. Studies including a larger cohort of participants being followed up in a longitudinal prospective fashion with stricter biochemical testing protocol may provide further insight into other possible pregnancy related outcomes.

Comparison to previous studies

Chronic kidney disease was linked to poorer maternal and neonatal outcomes in a relatively large demographically matched study, (6) where 13% and 25% of women had diabetes and chronic hypertension. The risk of preterm birth and neonatal intensive care unit admission was increased in our study population, supporting our findings of the association between lower eGFR and adverse pregnancy outcomes. Our study has also provided additional evidence for the association of adverse maternal and foetal outcomes in pregnant women with pre-existing diabetes according to albuminuria status as well as eGFR level. Despite recognition of the limitations of eGFR using the CKD-EPI formula, which underestimates measured GFR (15) and subsequently physiologic pregnancy hyperfiltration; lower eGFR values were associated with increased risk of adverse outcomes.

It has been observed in individuals with CKD and without diabetes that an eGFR > 90 mL/min/1.73m² at baseline in early pregnancy is associated with adverse pregnancy outcomes including preterm delivery, small for gestational age and admission to neonatal intensive care unit. (7) These authors, (16) however, did not detect a difference in outcomes between women who were hyperfiltering in the first trimester compared to those with normofiltration with an eGFR of < 120 mL/min/1.73m². The finding of adverse outcomes in what is presumed as acceptable renal function (eGFR 90-120 mL/min/1.73m²) was not observed within our healthy group of women but was present within our diabetes cohort with an eGFR 90-120 mL/min/1.73m². This suggests that independent of renal function, other processes including glycaemic management and blood pressure, can potentially contribute to preeclampsia. This was specifically demonstrated in recent studies of women with type 1 diabetes. (3, 4) These studies have provided evidence that preeclampsia and likely the resultant preterm birth, were significantly higher in women with diabetic kidney disease.

At this stage, it is not clear if women with diabetes have a reduced ability to achieve hyperfiltration in pregnancy. However, in this present study, we found no difference in peak eGFR during pregnancy in women with and without diabetes. We have demonstrated that stage 1 and stage 2 DKD are associated with higher adverse outcomes, compared to the group with hyperfiltration (eGFR > 120 mL/min/1.73m²) in diabetes. Park et al has recently demonstrated that adverse pregnancy outcomes in a non-diabetes specific cohort are higher in groups at the extreme eGFR spectrum, that is in individuals with both an eGFR 90-120 mL/min/1.73m² as well as those with an eGFR > 150 mL/min/1.73m², compared to those with an eGFR 121-150 mL/min/1.73m². (17) Overall, the protective effect of a higher eGFR in pregnancy in diabetes has yet to be studied and remain to be established. As renal hyperfiltration may have an association with less adverse pregnancy outcomes, averaging the eGFR in the whole pregnancy may have skewed our results, particularly in those with an attenuated ability to achieve peak pregnancy hyperfiltration. Nevertheless, by incorporating all available eGFR results throughout the pregnancy, we believe this allows for reflection of the overall effect of eGFR, rather than trimester specific changes.

The adverse obstetric outcomes in diabetic kidney disease in relation to potential co-morbidities may also be related to inflammatory, vascular and oxidative pathways. Serial anti-angiogenic factors have been demonstrated to be associated with preeclampsia in women with type 1 diabetes compared to healthy controls. (18) However, there is a paucity of clinical data establishing the potential link of biomarkers in diabetic kidney disease with preeclampsia, and other adverse obstetric outcomes.

Strengths and limitations

We recognize the limitations of this study. This was an observational, retrospective study, with potential missing data. However, to enable more complete data collection, we manually extracted and crosschecked information on paper and electronic hospital records, in addition to obtaining information

from local practitioners for presumed missing data. By excluding women with inconsistent and absent baseline glycaemia measurement, we may have also missed some cases of pre-gestational diabetes.

Another consideration with data collection was that the acquired data was a combination of routine, as well as tests undertaken for specific clinical indications, which are more likely to be abnormal. For example, including tests immediately prior to delivery in those with pre-eclampsia will bias the results to favour an association of eGFR and albuminuria. Nevertheless, to reduce ascertainment bias and to the best of our ability, we reviewed all available medical records, and excluded biochemical data that was obtained during any acute and intra-partum admission. Finally, we had smaller numbers compared to larger multi-centre, population-based studies; limiting our study's statistical power. However, the single centre approach of data collection minimises different practices being incorporated into the audit. For example, clinical practice of blood pressure measurement and glucose control are more consistent, and the measurements of biochemistry were performed using a single method or platform. Furthermore, we had detailed data including careful manual review of blood pressure recordings providing clear definition of preeclampsia as a pregnancy outcome. The relatively well-controlled blood pressure and reasonable glycaemic management potentially reflect practices within a metropolitan obstetric hospital and may not be applicable extensively. However, our findings are likely to have a potential impact in facilitating diabetes and pregnancy care in the near future, as there is increased awareness and focus on improving perinatal outcomes.

In terms of our comparison cohort, we also acknowledge that the healthy controls were younger, at 31(28,35) years old compared to those in the diabetes group which were 33 (29,36) years old. However, although there is a known association with adverse pregnancy outcomes with advanced maternal age, this is applicable mainly to women above 40 years or older(19). In addition, as women without diabetes in our cohort had an eGFR > 90 mL/min/1.73m² and no recorded albuminuria status, we were not able to perform comparison outcome analysis between pregnancies affected by diabetes and those without. Yet, it is clear from our findings that there is a difference between women affected by diabetic kidney disease and those who do not, and we have taken steps to incorporate albuminuria data to improve our understanding of the impact of renal changes in this study. It is important to note that we were not able to incorporate other variables in the multivariable regression model, such as blood pressure, age, or BMI. These factors resulted in a high co-linearity and affected the performance of the statistical model. We can therefore only postulate the link of adverse outcomes are present with an eGFR < 120 mL/min/1.73m² and ACR > 3.5 mg/mmol.

It is important to consider that eGFR based on the CKD-EPI formula may not reflect true measured renal function in pregnancy, due to physiological renal hyperfiltration. However, we were limited by lack of measured GFR in this study, as this was a clinical audit, and therefore used CKE-EPI eGFR as a surrogate marker of renal function. Establishing the effect of diabetic kidney disease on pregnancies affected by diabetes, particularly type 2 diabetes is increasingly essential for safe and effective patient care due to the rising prevalence of women conceiving with pre-existing type 2 diabetes.

CONCLUSIONS:

To conclude, we observed CKD-EPI eGFR as a potential renal marker of identifying high-risk pregnancies in diabetes independent from albuminuria status. This is the largest observational study in Australia in recent years with a focus on diabetic kidney disease, and findings will likely aid clinicians to anticipate the risks involved and counsel women with diabetes regarding pregnancy outcomes.

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Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Author's Contributions:

Each author contributed important intellectual content during manuscript drafting or revision.

Research concept and study design: JS, CH, EIE, AS; data acquisition: JS, NK, LW; statistical analysis: JS, NK, LC, CT; article drafting and revision: JS, NK, CT; supervision or mentorship: AS, CH, EIE.

All authors have reviewed and approved the final article for submission.

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Table 1. Maternal characteristics in women at baseline (type 1 diabetes vs. type 2 diabetes)

	Type 1 Diabetes	Type 2 Diabetes	<i>P</i> value
N	92	106	
Age (years)	31 (27, 34)	35 (31, 37)	< 0.001
BMI (kg/m ²)	27 (23, 31)	33 (29, 38)	< 0.001
Smoking status at conception			0.9
Current smoker	7 (9.8)	6 (5.7)	
Ex-smoker	9 (7.6)	11 (10.4)	
Non-smoker	76 (82.6)	89 (83.9)	
Duration of diabetes (years)	12 (5, 19)	3 (2, 5)	< 0.001
HbA _{1c} (%)	7.0 (6.5, 8.2)	6.9 (6.0, 7.8)	0.03
HbA _{1c} (mmol/mol)	53 (48, 66)	52 (42, 62)	0.03
Systolic blood pressure (mmHg)	115 (110, 120)	120 (110, 125)	0.02
Diastolic blood pressure (mmHg)	70 (65, 70)	70 (70, 80)	0.02
Serum creatinine (μmol/L)	53.9 (45.1, 59.2)	46.0 (40.7, 52.2)	0.002
CKD-EPI eGFR (mL/min/1.73m ²)	121 (117, 128)	125 (122, 131)	0.02
Albumin-to-creatinine ratio (mg/mmol) *	0.8 (0.5, 2.1)	1.3 (0.8, 3.0)	0.2
Normoalbuminuria	63 (76.8)	49 (77.8)	
Microalbuminuria	13 (15.9)	10 (15.9)	
Macroalbuminuria	6 (7.3)	4 (6.4)	
Pre-existing hypertension	10 (10.9)	23 (21.7)	0.04
Renin angiotensin aldosterone system inhibition at conception	8 (8.7)	8 (7.6)	0.8
Statins therapy at conception	4 (4.4)	9 (8.5)	0.2
Metformin therapy at conception	0 (0)	55 (51.9)	< 0.01
Microvascular complications	21 (22.6)	8 (7.5)	< 0.01
Macrovascular complications	2 (2.2)	0 (0)	0.1

Data expressed median (interquartile range) or n (%)

Conversion factors for units: serum creatinine in μmol/L to mg/dL, ÷ 88.42; albumin-to-creatinine ratio in mg/mmol to mg/g, ÷ 0.113

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

*First trimester albumin-to-creatinine ratio were available for 53/92 (58%) women with type 1 and 40/106 (38%) women with type 2 diabetes

Body mass index = BMI; glycosylated haemoglobin = HbA_{1c}; Chronic Kidney Disease Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR

Table 2. Selected maternal characteristics in women at baseline (diabetes vs. healthy control)

	Diabetes (Type 1 and Type 2)	Healthy Controls	<i>P</i> -value
N	198	119	
Age (years)	33 (29, 36)	31 (28, 35)	0.04
BMI (kg/m ²)	30 (26, 36)	25 (23, 32)	< 0.001
Serum creatinine (μmol/L)	52.2 (43.3, 56.6)	48.6 (44.2, 53.9)	0.8
CKD-EPI eGFR (mL/min/1.73m ²)	124 (118, 129)	125 (120, 131)	0.2
Albumin-to-creatinine ratio (mg/mmol)	0.8 (0.5, 2.4)	-	

Data expressed median (interquartile range) or n (%)

Conversion factors for units: serum creatinine in μmol/L to mg/dL, ÷ 88.4; albumin-to-creatinine ratio in mg/mmol to mg/g, ÷ 0.113

Group comparisons were performed using Chi-square tests and Fisher exact tests for categorical variables and Mann-Whitney U tests for continuous variables

Body mass index = BMI; Chronic Kidney Disease Epidemiology Collaboration equation for estimated glomerular filtration rate = CKD-EPI eGFR

Table 3. Major obstetric outcomes according to indicators of diabetic kidney disease in women with type 1 and type 2 diabetes

	Preeclampsia				Preterm birth < 37 weeks				Preterm birth < 32 weeks				Neonatal intensive care			
	N	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value	OR	95% CI	P value
Urinary albumin⁺																
Normoalbuminuria ⁺	112	1.0			1.0			1.0			1.0					
(reference)																
Microalbuminuria	23	5.7	1.8, 17.8	0.003	3.9	1.5, 9.9	0.004	4.1	0.8, 19.5	0.1	2.4	0.9, 6.8	0.1			
Macroalbuminuria	9	5.5	2.5, 11.8	<0.001	3.5	1.6, 7.7	0.002	4.2	1.9, 9.5	<0.001	2.4	1.2, 4.6	0.01			
DKD stages⁺⁺																
Hyperfiltration ⁺⁺	81	1.0			1.0			1.0			1.0					
(reference)																
Stage 1 DKD	97	4.5	1.6, 12.3	0.004	2.3	1.2, 4.6	0.01	1.3	0.4, 4.2	0.6	2.0	0.9, 4.8	0.1			
Stage 2+ DKD	8	3.9	1.7, 8.9	0.001	4.9	1.7, 14.5	0.004	5.0	1.7, 14.5	<0.001	4.9	2.0, 11.6	<0.001			

⁺Albumin-to-creatinine ratio (mg/mmol): normoalbuminuria < 3.5; microalbuminuria 3.5 to 35; macroalbuminuria > 35; Conversion factors for units: albumin-to-creatinine ratio in mg/mmol to mg/g: $\div 0.113$

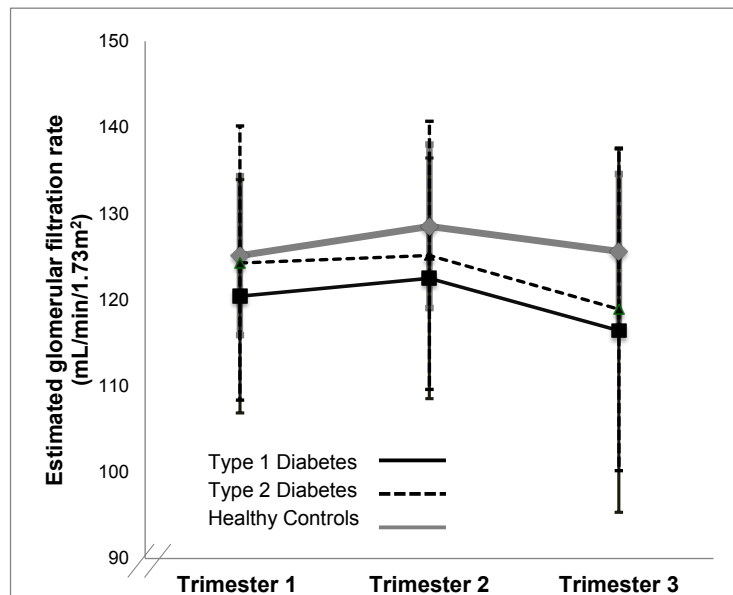
⁺⁺Diabetic kidney disease (DKD) stages defined by glomerular filtration rate estimated using Chronic Kidney Disease Epidemiology Cohort formula (mL/min/1.73m²):

Hyperfiltration > 120; Stage 1 > 90 to 120; Stage 2+ < 90

Univariate regression with results expressed as odds ratios (OR) and 95% confidence interval (CI), in relation to either normoalbuminuria⁺ or hyperfiltration⁺⁺

Odds ratio = OR; confidence interval = CI

Figure 1. Comparison of estimated glomerular filtration rate in women with type 1 diabetes, type 2 diabetes and healthy controls

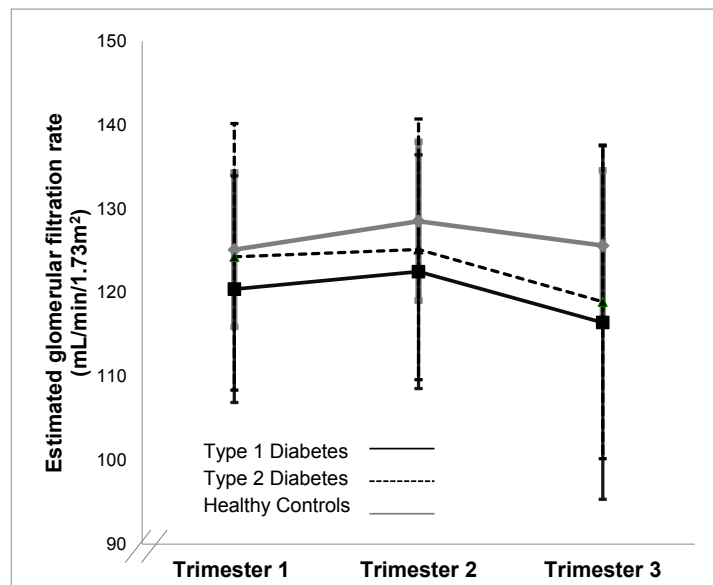


Data performed with Mann-Whitney U-test and expressed as mean + standard error of the mean

Excel spreadsheet used to create figure 1

Figure 1

Figure 1. Comparison of estimated glomerular filtration rate in women with type 1 diabetes, type 2 diabetes and healthy controls



Data performed with Mann-Whitney U-test and expressed as mean + standard error of the mean
Excel spreadsheet used to create figure 1

Appendix C: Chapter Eleven Supplementary Material

This was an extension of Chapter Eleven: Study Six- Functional MRI in predicting Diabetic Kidney Disease. I will outline the statistical methods, results and discussion in relation to inter-rater reliability, intra-rater reliability and scan repeatability of the MRI technique of our cohort.

Statistical Analyses

The following analyses were conducted for MRI parameters ($R2^*$, FA and ADC): Lin's concordance correlation coefficient and reduced major axis regression for Left Cortex, Left Medulla, Right Cortex and Right Medulla individually, and Overall (Combined Left and Right Cortex and Medulla) to assess:

1. Inter-rater reliability - between readers A and B
2. Intra-rater reliability – for BOLD MRI readings performed twice by Reader 2 on ten randomly selected samples
3. Scan repeatability- Both runs of BOLD MRI– for reader A and B individually

The interpretation and suggested concordance correlation and strength of agreement were defined according to Landis and Koch [451]:

0.81-1.00	Almost Perfect
0.61-0.80	Substantial
0.41-0.60	Moderate
0.21-0.40	Fair
0.00-0.20	Slight
<0.00	Poor

In addition, for reduced major axis regression analysis, the slope and intercept assess the presence of proportional or fixed bias in “agreement” of readings, with a slope between 0.9 and 1.1 indicating no meaningful proportional bias, while an ideal intercept will be close to 0.00 (indicating no fixed bias).

Results

Table 11.9 and 11.10 tabulate inter- and intra-rater reliability respectively. Table 11.11 demonstrates concordance agreement between both runs of BOLD MRI.

In terms of inter-rater reliability, overall concordances were highest for FA, followed by BOLD MRI and ADC. Overall readings, taking all the different anatomical regions together, as well as separately (total, left kidney, right kidney, all cortex and all medulla individually) into consideration, had an acceptable inter-rater reliability (concordance > 0.8). However, the reliability declined when evaluating either cortex or medulla by only the left or right kidney. The inter-rater agreement for left cortex was the poorest for BOLD MRI and ADC; whilst right cortex had the poorest inter-rater reliability for FA.

For BOLD MRI, there was high intra-rater reliability based on reader B's ten randomly selected samples on run 1 of BOLD MRI (0.94 for concordance agreement). The left cortex had the poorest concordance agreement at 0.37, and an intercept of 13.8. The average co-efficient of variation for each parameter was similar for both runs: right cortex (8.8% vs. 7.7%), right medulla (16.2% vs. 17.9%), left cortex (7.3% vs. 7.7%) and left medulla (23.6% vs. 22.9%).

For both runs of BOLD MRI, assessing scan repeatability, overall concordance between runs 1 and 2 was almost perfect, with a concordance correlation coefficient for reader A of 0.93 and reader B of 0.83. Reader A had higher reliability compared to reader B for all readings apart from the left cortex, which had the poorest concordance agreement for reader B at 0.46. Reader B's concordance agreement was the poorest for the right medulla at 0.24. Readings for medulla had a better reliability compared to the cortex.

Appendix Table 1 Inter-rater reliability: comparing readings between reader A and B

	LIN's CONCORDANCE CORRELATION (95CI)	SLOPE	INTERCEPT
BOLD MRI			
Overall	0.86 (0.84,0.89)	1.29	-10.5
Left	0.88 (0.85, 0.91)	1.15	-7.4
Right	0.84 (0.80, 0.88)	1.27	-14.8
Medulla	0.97 (0.96, 0.98))	1.01	4.15
Cortex	0.60 (0.51, 0.69)	1.06	-6.0
Left Medulla	0.68 (0.57,0.79)	1.11	-6.8
Right Medulla	0.47 (0.33,0.62)	-6.8	-8.73
Left Cortex	0.36 (0.17,0.52)	0.83	14.3
Right Cortex	0.48 (0.44,0.62)	0.93	8.79
FRACTIONAL ANISTROPY (FA)			
Overall	0.96 (0.95,0.97)	1.04	-0.89
Left	0.96 (0.94,0.97)	1.09	-7.54
Right	0.97 (0.96,0.98)	1.01	4.15
Medulla	0.97 (0.96,0.98)	1.01	4.15
Cortex	0.69 (0.58, 0.80)	0.96	38.6
Left Medulla	0. 68 (0.52,0.83)	0.96	48.6
Right Medulla	0.70 (0.55,0.86)	0.98	17.2
Left Cortex	0.63 (0.45,0.82)	0.86	19.05
Right Cortex	0.46 (0.21,0.70)	0.96	8.76
APPARENT DIFFUSION CO-EFFICIENT (ADC)			
Overall	0.77 (0.70,0.83)	0.99	-2.37
Left	0.74 (0.64,0.84)	1.03	-112.4
Right	0.80 (0.72,0.88)	0.94	114.7
Medulla	0.75 (0.66, 0.85)	0.92	149.9
Cortex	0.71 (0.69,0.81)	1.08	-213.5
Left Medulla	0.75 (0.61,0.89)	0.96	17.3
Right Medulla	0.75 (0.63, 0.86)	0.88	284.7
Left Cortex	0.63 (0.44,0.82)	1.04	-116.6
Right Cortex	0.80 (0.69,0.91)	1.21	-329.9

Appendix Table 2 Intra-rater reliability of BOLD imaging. Assessed from sub analysis of 10 randomly selected patients, Run 1 only

	LIN's CONCORDANCE CORRELATION (95CI)	SLOPE	INTERCEPT
READER 2 (10 PATIENTS, RUN 1 – Read 1 vs Read 2)			
Overall	0.94 (0.91, 0.98)	1.03	-2.19
Left	0.95(0.91,1.00)	1.00	-0.78
Right	0.93 (0.87,0.99)	1.05	-4.05
Medulla	0.89 (0.79, 0.98)	0.99	-0.95
Cortex	0.48 (0.13,0.84)	1.01	-1.13
Left Medulla	0.95 (0.88,1.02)	0.94	1.24
Right Medulla	0.80(0.56,1.04)	1.01	-4.35
Left Cortex	0.37(-0.20,0.95)	0.79	13.8
Right Cortex	0.55 (0.08,1.01)	1.26	-18.2

Appendix Table 3 Scan repeatability: comparing runs 1 and 2 of BOLD readings of reader A and B

	LIN's CONCORDANCE CORRELATION (95CI)	SLOPE	INTERCEPT
READER 1			
Overall	0.90 (0.91,0.95)	0.95	2.10
Left	0.93 (0.90, 0.96)	0.92	4.10
Right	0.93 (0.91, 0.96)	1.00	-0.32
Medulla	0.80 (0.72,0.88)	1.03	-0.71
Cortex	0.53 (0.38, 0.68)	0.88	7.29
Left Medulla	0.79 (0.68,0.91)	1.23	-6.72
Right Medulla	0.79 (0.68,0.90)	0.94	1.65
Left Cortex	0.46 (0.24,0.69)	0.75	15.45
Right Cortex	0.58 (0.38,0.79)	0.98	0.75
READER 2			
Overall	0.83 (0.79,0.88)	1.02	-1.15
Left	0.88 (0.83, 0.93)	1.01	0.06
Right	0.78 (0.69, 0.86)	1.05	-3.00
Medulla	0.47 (0.30,0.64)	1.01	-1.62
Cortex	0.48 (0.33,0.64)	0.75	17.8
Left Medulla	0.24(-0.04, 0.52)	0.96	1.13
Right Medulla	0.59 (0.39,0.79)	1.13	-7.6
Left Cortex	0.52 (0.31, 0.74)	0.79	15.0
Right Cortex	0.43 (0.2, 0.66)	0.70	21.2

Discussion

Overall Inter and Intra-rater reliability of MRI Technique is acceptable

In this study, the results of the *overall* inter- and intra-rater reliability of this MRI technique are acceptable. All three metrics (BOLD, FA and ADC) were assessed by two readers to test inter-rater reliability, along with two runs of BOLD MRI to assess inter-scan concordance. Among the three metrics, FA appears to have the highest concordance.

Based on the variable concordance agreement with inter-rater reliability, results may reflect the different level of radiology experience between both readers. This suggest that experience impacts interpretation, and that further validation of this relatively new technique may be warranted. This is particularly reflected when comparison is made between runs 1 and 2 of BOLD MRI analysis, where reader A had superior inter-scan concordance agreement compared to reader B. Nevertheless, there is an excellent overall concordance agreement of >0.8 for both readers. This provides support to the reproducibility of the BOLD technique.

This is further supplemented by intra-rater reliability evaluation as performed upon repeat reads by reader B. We selected reader B who has less radiology experience, and the overall intra-rater reliability is excellent with a concordance agreement of 0.94 and an acceptable slope and intercept, with other parameters having a concordance agreement >0.8 , apart from the cortex. Moreover, the coefficient variations for each of the parameters were not dissimilar.

Cortical intra and inter-reliability is reduced compared to medullary readings

Understanding the intra and inter-reliability of the study MRI technique in detail are important to aid interpretation. The reduced intra and inter reliability of the cortex compared to the medulla for all three MRI parameters is somewhat unexpected, as renal medulla is more heterogeneous and has a lower signal intensity than the cortex. The anatomical proximity of the cortex to the capsule also allows it to be more readily distinguished. These factors were expected to result in difficulty in observing and

placing ROI in the medulla compared to the cortex. However, kidneys, which are close to the diaphragm, move cranio-caudally and breathing can result in indistinct cortico-medullary differentiation or indistinct cortical margins that might impact cortical readings. We aimed to mitigate effects of respiratory motion with breath-hold BOLD imaging and a respiratory navigated DTI sequence. Visualization is also variable depending on motion artifact and intra-abdominal gas. Another potential explanation for the reduced reliability of cortical readings would be that whilst the medullary pyramids provide a reasonable area to draw ROIs, it may be harder to draw ROIs that do not stray out of the cortex which is thinner than the medulla.

Nevertheless, the cortex and medulla have distinct physiological properties, and it is important to note that the inter-rater reliability was substantial (concordance agreement >0.61) for both cortex and medulla over both kidneys combined. It is unclear why analyzing our data based on left or right reduces our inter- and intra-reliability significantly. It should be noted that as previously published, that there are no significant differences between left or right readings on dedicated reproducibility studies on healthy volunteers [288]. Magnetic field inhomogeneity may cause differences in signal intensity across the image and account for differences in quantitative left and right kidney values, depending upon local causes of inhomogeneity, e.g. gas within the ascending colon (right kidney) or stomach and descending colon (left kidney). Our heterogenous results based on anatomical side are unlikely to be due to diabetic kidney disease, as it is a global process and disease trajectory should not affect one kidney more so than the other. As our sample size was small, we were unable to conduct analysis on controls and participants with diabetes separately to confirm this.

Interpreting overall readings may be more reliable

Our results of inter and intra rater reliability may also suggest that interpreting overall readings may provide more accurate results, rather than dichotomizing analysis into different anatomical regions and by kidney, which reduces reliability. Lower concordance for single region and signal kidney may relate to fewer data points, with greater impact of individual outliers. However, when analyzing MRI metrics, it is important to note that the different physiological properties of the cortex and medulla

result in known differences in BOLD MRI and DTI readings. Hence, it is pertinent to present the data as we have, separating cortex and medulla findings individually.

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