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Intra-Renal Inflammation in Obesity and Type 2 Diabetes Mellitus

A thesis submitted to the National University of Ireland in fulfilment of the Requirements for the degree of

Doctor of Philosophy

By

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September 2013
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Abstract

Introduction: Unhealthy diet and sedentary lifestyle, lead to the constellation of obesity, hyperinsulinaemia and hyperlipidaemia that are risk factors for both chronic kidney disease and type 2 Diabetes mellitus. Renal cellular damage in diabetes is associated with expression of pro-inflammatory cytokines and chemokines, which can lead to activation of multiple immunological cell types that may exacerbate glomerular and tubulointerstitial injury. Methods: In a rat model of pre-diabetic obesity, the presence of cellular inflammation and activation of the IL-6/STAT3 inflammatory pathway within the kidney was investigated. In urine cell samples from patients with type 2 diabetes mellitus and non-diabetic patients with severe obesity, the expression of mRNA transcripts for IL-6, IL-6R, IL-17, STAT3 and SOCS3 was quantified and correlated to clinical and laboratory data. In a cohort of type 2 diabetic outpatients, clinical indices from two time points were statistically analysed to investigate the relationships between obesity and parameters of renal function.

Results: Cellular inflammation, specifically increased macrophages, occurred in the glomeruli of young obese rats and correlated with the expression of SOCS3 - a counter-regulatory protein of STAT3 signalling. Macrophages and SOCS3+ cells were increased in glomeruli with higher expression of the damage marker desmin. In contrast to the glomeruli, the interstitial compartment of obese rat kidney had no evidence of cellular inflammation but did have high expression of SOCS3. In human urine cell samples, contrary to expectations, mRNA levels for IL-6 and other pro-inflammatory mediators were similar to healthy adults. However, the ratio between STAT3 and SOCS3 mRNA levels was found to be decreased in diabetic and obese subjects and correlated with body mass index. In a large cohort of type 2 diabetic patients undergoing active medical management, body mass index was found to correlate positively with kidney function and glomerular hyperfiltration was independently associated with obesity. However, further weight gain during follow up was associated with greater risk of declining kidney function.

Conclusions: Obesity is a pro-inflammatory state and has specific harmful effects on the kidney – primarily the glomerulus. Contrary to some current literature, glomerular inflammation of obesity is not immediately accompanied by tubulointerstitial inflammation – possibly as a result of high-level expression of counter-regulatory proteins such as SOCS3. In treated type 2 diabetics, obesity is linked to hyperfiltration but is not a potent driver of renal injury during the first decade. However, with time, continued weight gain adversely affects the risk of chronic kidney disease.
Acknowledgements

I would like to thank Professor Matthew Griffin for his advice, support, direction and mentorship throughout my time doing this research. I have had a wonderful start to a career as a scientist and a nephrologist. I aspire to use this training to gap the bridge between science and medicine, even if only in a small way.

I would like to acknowledge the support of the staff and researchers at REMEDI who facilitated my fellowship, including Dr. Daniel O’Toole, Mr. Mark Canney, Professor Rhodri Ceredig, Dr. Thomas Ritter, Dr. Aideen Ryan, Dr. Shirley Hanley, Dr. Jana Pindjakova, Ms Malgorzata Zurawska, Dr. Siobhan Gaughan, Dr. Michelle Duffy, Mr Nahidul Islam, Ms Tara Sugrue, Ms. Claire Masterson, Ms Lisa Flynn, Dr. Sonia Prado-Lopez, Ms Michelle Aherne. Their technical advice and assistance was invaluable. Additionally, I would thank Professor Martin O’Donnell, Dr. Francis Finucane, Ms Lena Griffin and the nursing staff at the Diabetes day centre and Clinical research facility for this assistance in enrolling patients for the study. I would also like to thank Professor Jaochim Anders for facilitating my stay at the Ludwig-Maximilian Universität in Munich and Mr Dan Dragonovici for his excellent training in histological techniques.

I would like to acknowledge the support from the funding sources of this project including the Clinical research facility at Galway University Hospitals, Health Service Executive and Health Research Board of Ireland and the Department of Nephrology at Galway University Hospital, Dr David Lappin, Dr Donal Reddan and Dr Louise Giblin.

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A most special thanks to Dr. Andrew Smyth and Ms Andrea Marques –Ribiero whose support crosses oceans and time-zones and never wanes!
Dedications

Tony & Patrick

There is nothing I cannot master with the help of the One who gives me strength

Phillipians 4:13
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<td>3-OMG</td>
<td>3-Omethyl glucose</td>
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<td>ABW</td>
<td>actual body weight</td>
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<tr>
<td>ACEi</td>
<td>Angiotensin converting enzyme inhibitor</td>
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<tr>
<td>ACR</td>
<td>Albumin creatinine ratio</td>
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<td>AGE</td>
<td>Advanced glycosylation end products</td>
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<td>AII</td>
<td>Angiotensin II</td>
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<td>AKI</td>
<td>Acute kidney injury</td>
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<td>AMPK</td>
<td>AMP-activated protein kinase</td>
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<td>ARB</td>
<td>Angiotensin receptor blocker</td>
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<td>AST</td>
<td>Aspartate aminotransferase</td>
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<td>BMI</td>
<td>Body mass Index</td>
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<td>Chronic kidney disease</td>
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<td>C-Reactive protein</td>
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<td>CX3CL-1</td>
<td>C-X3-C motif ligand 1</td>
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<td>DAB</td>
<td>Diaminobenzadine</td>
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<td>Diacylglycerol</td>
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<td>Dendritic cells</td>
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<td>Diabetic kidney disease</td>
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<td>DN</td>
<td>Diabetic nephropathy</td>
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<td>DTPA</td>
<td>diethylene triamine pentaacetic acid</td>
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<td>ECM</td>
<td>Extracellular matrix</td>
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<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
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<td>eNOS</td>
<td>Endothelial nitric oxide synthetase</td>
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<td>F-C gamma chain</td>
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<td>Free fatty acids</td>
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<td>Fat free mass</td>
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<td>FM</td>
<td>Fat mass</td>
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<td>Focal segmental glomerulosclerosis</td>
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<td>GBM</td>
<td>Glomerular basement membrane</td>
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<td>GFR</td>
<td>Glomerular filtration rate</td>
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<td>Glucagon like peptide-1</td>
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<td>Glucose transporter</td>
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<td>GM-CSF</td>
<td>Granulocyte and macrophage colony stimulating factor</td>
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<td>HbA1c</td>
<td>Glycated hemoglobin</td>
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<td>ICAM</td>
<td>Intracellular cellular adhesion molecular</td>
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<td>IFN-g</td>
<td>Interferon gamma</td>
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<td>Interleukin-1</td>
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<td>II-1RA</td>
<td>Interleukin-1 receptor antagonist</td>
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<td>II-6</td>
<td>Interleukin-6</td>
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<td>IL-6ST</td>
<td>IL-6 signal transducer</td>
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<td>IRS-1</td>
<td>Insulin receptor substrate-1</td>
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<td>JAK</td>
<td>Janus kinase</td>
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<td>JNK</td>
<td>J-NH-2 terminal kinase</td>
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<td>KIM-1</td>
<td>Kidney injury molecule -1</td>
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<td>LBW</td>
<td>lean body weight</td>
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<td>LFA-1</td>
<td>Lymphocyte function associated antigen-1</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<tr>
<td>LZR</td>
<td>Lean Zucker rat</td>
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<td>MAPK</td>
<td>Mitogen associated protein kinase</td>
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<td>MCP-1</td>
<td>Monocyte chemoattractant protein-1</td>
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<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
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<td>MDRD</td>
<td>Modification of diet in renal disease</td>
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<td>MIP-1</td>
<td>Monocyte inhibitory protein-1</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate-oxidase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>Nuclear factor-kappaB</td>
</tr>
<tr>
<td>NGAL</td>
<td>Neutrophil gelatinase associated lipocalin</td>
</tr>
<tr>
<td>NTN</td>
<td>Nephrotic serum nephritis</td>
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<tr>
<td>ORG</td>
<td>Obesity related glomerulopathy</td>
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<tr>
<td>OZR</td>
<td>Obese Zucker rat</td>
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<td>PAH</td>
<td>Para-aminohippuric acid</td>
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<td>PAI-1</td>
<td>Plasminogen activator inhibitor -1</td>
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<td>PAMP</td>
<td>Pathogen associated molecular pattern</td>
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<tr>
<td>PBMC</td>
<td>Peripheral blood mononuclear cells</td>
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<td>PDGF</td>
<td>Platelet derived growth factor</td>
</tr>
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<td>PI3K</td>
<td>Phosphatidylinositol-3-kinase</td>
</tr>
<tr>
<td>PIAS</td>
<td>Protein inhibitor associated signalling</td>
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<tr>
<td>pSTAT3</td>
<td>Phosphorylated STAT3</td>
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<tr>
<td>RAAS</td>
<td>Renin-angiotensin aldosterone system</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>RPF</td>
<td>Renal plasma flow</td>
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<td>RT-PCR</td>
<td>Reverse transcriptase polymerase chain reaction</td>
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<td>SAA</td>
<td>Serum amyloid A</td>
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<td>siRNA</td>
<td>Silent Ribonucleic acid</td>
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<td>SNS</td>
<td>Sympathetic nervous system</td>
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<td>SOCS</td>
<td>Suppressor of cytokine signalling</td>
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<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
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<tr>
<td>T1DM</td>
<td>Type 1 diabetes mellitus</td>
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<tr>
<td>T2DM</td>
<td>Type 2 diabetes Mellitus</td>
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<tr>
<td>TGF-beta</td>
<td>Transforming growth factor beta</td>
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<tr>
<td>TNF-alpha</td>
<td>Tumour necrosis alpha</td>
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<td>Abbreviation</td>
<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>tPA</td>
<td>Tissue plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase plasminogen activator</td>
</tr>
<tr>
<td>UUO</td>
<td>Unilateral ureteral obstruction</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cellular adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>WT-1</td>
<td>Wilms-tumour-1</td>
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<td>ZO-1</td>
<td>Zona-occludens-1</td>
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Chapter 1: Obesity, Type 2 Diabetes Mellitus (T2DM) and their Associations with Systemic and Intra-Renal Inflammation
Obesity - Epidemiology
The prevalence of obesity has increased in the last 30 years, with nearly one third of United States (US) adults being obese in a study of the NHANES dataset from 1999-2008 compared to 22% in the time period from 1988-1994 [1]. Nearly 12.5 million children and adolescents are now obese in the US [2]. Although the prevalence of obesity appears to be levelling off in the US, in developing nations the rate of obesity in adults and children continues to increase [3, 4]. In the United Kingdom (UK), rates of obesity trebled from the early 1980’s to 2009, with obesity in men and women increasing from 6% to 23% and 8% to 25% respectively. The largest increase in the prevalence of obesity occurred in younger age groups. The proportion of extreme obesity (BMI>40kg/m²) increased from 0.2% of men and 1.4% of women in 1993 to 0.8% of men and 2.8% of women in 2002. In 2009 in the UK, over 23% of 4–5-year-olds and 33% of 10–11 year-olds were found to be either overweight or obese [5]. In Ireland, 39% of adults are overweight and the trend towards obesity is increasing. For example, between 1990 and 2006, the prevalence of obesity increased by 67%. In children, 10% of 5-12 year olds and 20% of teenagers are obese or overweight [6-9]. It is estimated that there will be 65 million obese adults in the USA and 11 million more obese adults in the UK by 2030. With the associated increase in diabetes, heart disease and stroke, the combined medical costs associated with treatment of these conditions will increase by $48-66 billion/year in the USA and by £1.9-2 billion/year in the UK by 2030[10].

Obesity is associated with a myriad of complications. An obese women is thirteen times more likely to develop type 2 diabetes (T2DM), four times more likely to develop hypertension and three times more likely to have a heart attack than a lean women. For men, the risk of T2DM is five times higher, risk of hypertension doubles and colon cancer risk trebles in obese compared to lean individuals. In addition, the risks for angina, gall bladder disease, liver disease, ovarian cancer, osteoarthritis and stroke are increased [11].

Obesity increases the risk of coronary heart disease, atherosclerosis and certain cancers [12] and is associated with an increased risk of death compared with that of non-obese individuals. A Danish study found that young obese men had a third higher risk for T2DM, cardiovascular morbidity or premature death compared to normal weighted individuals over a 30 year follow-up period [13]. Obesity is associated also with increased diabetes- and kidney disease-related mortality (33,643 excess deaths; 95% CI, 20,640-46,645) [12].
Pathophysiology of Obesity Related Complications

A common pathophysiological factor for complications of obesity is dysregulation of immune and inflammatory responses. Adipose tissue, in addition to being a repository of energy for the body in the form of triacylglycerol, also has endocrine and secretory functions. Fatty acids are the main substances secreted by adipose tissue but additionally, prostanoids, cholesterol, retinol and steroid hormones are synthesised and stored in white adipose tissue. Adipokines are bioactive proteins secreted predominantly by adipose tissue with hormonal and cytokine-like activities. These include classic cytokines, growth factors and proteins involved in regulation of blood pressure, vasculature, lipids and glucose metabolism [14, 15]. Many of these have additional roles in immune system regulation and function and, for this reason, it has been postulated that obesity results in dysregulated activation of the immune system stemming from an increase in production of adipokines [15].

Systemic pro-inflammatory markers associated with obesity including blood neutrophil count [16] and increased concentrations of interleukin-6 (IL-6), C-reactive protein (CRP) [17], tumour necrosis factor alpha (TNF-alpha) [18], transforming growth factor beta (TGF-beta) [19], plasminogen activator inhibitor-1 (PAI-1) [20] and angiotensinogen [21]. Serum concentrations of CRP, IL-6 and TNF-alpha as well as total leukocyte and neutrophil counts have been found to correlate with markers of adipocyte mass [22] though not with presence of metabolic syndrome [16]. Additionally, many of these factors have been found to decrease after bariatric surgery [23].

How and why a strong inflammatory response occurs as fat mass expands in obesity is not clear. White adipose tissue may be providing inflammatory mediators for sites elsewhere in the body as a response to a systemic inflammatory event. Another more likely hypothesis is that white adipose tissue is itself in a state of inflammation due to local events within the tissue and, as adipose tissue mass increases, there is systemic spillover of inflammation affecting other organs[14].

As adipose tissue outgrows its blood supply and collagenase support, hypoxia ensues and contributes to adipose tissue dysfunction and cell death [15, 24-26]. In keeping with this, even as the mass of adipose tissue increases in obese individuals, the blood supply from cardiac output remains the same explaining, at least in part, the development of hypoxia [27]. Feedback pathways from cytokines released by adipose tissue lead to further expression of
additional chemokines and cytokines via pro-inflammatory signalling pathways [28] mediated by toll-like receptors (TLR), advanced glycosylation end-products (AGE) and free fatty acids (FFA) [29, 30]. Among the signalling pathways activated in obesity-associated inflammation are the cJun-NH₂-terminal kinase (JNK) pathway within adipocytes and macrophages [31] and the nuclear factor kappa B (NF-κB) pathway in liver and adipose tissue [31, 32].

Cell death in adipose tissue is higher in high fat fed mice than in lean controls and the necrotic adipocytes are surrounded by macrophages to create ‘crown like-structures’ [33]. Macrophages have been implicated in the inflammatory response to obesity [31]. Tissue macrophages are known to display functional heterogeneity and/or plasticity and current paradigms for macrophage function recognise classically-(M1) and alternatively-activated (M2) phenotypes [31]. Tissue infiltration by macrophages polarized to the inflammatory M1 [34] state is increased in adipose tissue in the setting of obesity and is thought to contribute to local inflammation and insulin resistance [35]. Macrophages located in the stromal vascular fraction of adipose tissue are a source of locally-produced pro-inflammatory cytokines, with, for instance secretion of IL-6 and TNF-alpha being higher from stromal vascular fraction cells than from adipocytes [36]. Deletion of stress-responsive signalling pathway JNK in macrophages using a genetic knock-out mouse model in high fat diet fed mice results in insulin sensitivity, reduced accumulation of M1-polarised macrophages and associated genes in adipose and liver tissue [31]. In keeping with obesity as a driving force behind macrophage polarization, bariatric surgery for weight management leads to the pro-inflammatory profile of macrophages changing to an anti-inflammatory phenotype with increased interleukin-10 (IL-10) and reduced monocyte chemoattractant protein-1 (MCP-1) expression [23]. In contrast, moderate weight loss through dieting did not affect inflammatory profile [16]. Adipose tissue dysfunction leads to an infiltration of pro-inflammatory macrophages in response to chemoattractants [15, 37]. Chemokines like MCP-1 are important for recruitment of macrophages into tissue [38]. MCP-1 has been found to be overexpressed in obese mice compared to their lean counterparts with white adipose tissue being the major source of this chemokine [39]. Although many of the adipokines have obvious immune system function, some have additional complex roles in energy metabolism. For example, MCP-1 affects insulin responsiveness in adipocytes by decreasing insulin-stimulated glucose uptake and the expression of several adipogenic genes [39].
TNF-alpha is a pro-inflammatory cytokine that is secreted by macrophages and adipocytes [18]. It can cause insulin resistance by inhibiting insulin signalling and up-regulating protein phosphatase 2C to suppress AMP-activated protein kinase (AMPK). This results in reduction of acetyl coA carboxylase (ACC) phosphorylation, suppression of fatty acid oxidation and increases of intramuscular diacylglycerol (DAG) and glucose transport [18, 40]. IL-6, regarded as another pro-inflammatory cytokine, is elevated in obese subjects and causes insulin resistance by activating suppressor of cytokine signalling 3 (SOCS3) which inhibits insulin receptor substrate 1 (IRS-1) signalling in the liver and adipose tissue. Chronic infusion of IL-6 into the portal vein of mice to levels similar to that found in obesity led to insulin resistance. In this study, liver tissue was found to have reduced hepatic IRS-1 auto-phosphorylation and tyrosine phosphorylation of IRS-1 and insulin receptor substrate -2 (IRS-2) [41]. It appears, however, that the overall influence of IL-6 on metabolism is complex. Muscle-derived IL-6 improves glycaemia following exercise by influencing glucose uptake and fat oxidation in muscle [42, 43]. IL-6 from both contracting skeletal muscle and white adipose tissue stimulates glucagon like peptide 1(GLP-1) secretion from ileal cells and the pancreas. GLP-1 is an incretin hormone that induces insulin secretion and is secreted from L-cells in the ileum and alpha cells in the pancreas to affect insulin secretion from beta-cells of the pancreas. IL-6 was required for increased GLP-1 following exercise and GLP-1 was required for the glucose sensitizing effects of IL-6. This is thought to be important in preparing the body for food intake following exercise [44]. In both genetic and high fat diet mouse models of obesity, administration of exogenous IL-6 improved glucose tolerance and insulin secretion. Blocking of IL-6 with an IL-6 antibody in db/db mice led to impaired glucose tolerance, lower glucagon and undetectable GLP-1 levels [44] (Figure 1.1). To date, the reported effects of IL-6 on glucose metabolism and insulin activities include some apparently contradictory findings, possibly reflecting differences in administration and timing of IL-6 [45]. Figures 1.1 and 1.2 are summaries of the role of IL-6 in metabolism as well as their role on immune system and inflammation in health and disease.

As noted, epidemiological and experimental data from both human and animal studies indicate that pro-inflammatory activity orchestrated by the immune system leads to end-organ damage associated with obesity [1]. However, obesity is not always associated with detrimental outcomes – a fact that is increasingly recognised in population-based studies [46]. Chronic disease is more often associated with weight loss, with loss of fat stores and breakdown of muscle to provide nutritional support to an active immune system. Therefore, it
is conceivable that pre-existing obesity confers a survival benefit to those with chronic disease, although this protection may come with caveats [47-50]. For example, in a study on causes of mortality in obesity, mortality from infection was found not to be associated with obesity but, on subgroup analysis, grade 1 obesity (BMI 30-35kg/m²) was associated with reduced risk of death from infection, while grade 2 (BMI 35-40kg/m²) and grade 3 obesity (BMI >40kg/m²) were associated with increased risk of mortality due to infection [12]. Similarly while increased mortality risk was not found in obese dialysis patients over the age of 65, there was a two-fold increased risk of death among obese dialysis patients under the age of 65 [47].

Thus, as summarised in this section, the pathophysiology of adipose tissue-related inflammation involves complex cross-talk between inflammatory and metabolic systems, the health implications of which remain to be fully elucidated. Further research is needed to address important unresolved issues in this area such as the role of the immune system in mediating the threshold beyond which an the expanding mass of adipose tissue become harmful to the host and the relative roles of immune/inflammatory mediators produced in adipose tissue and target organs.
Figure 1.1. The Pleiotropic Metabolic Effects of the Cytokine IL-6: Interleukin 6 is released from contracting skeletal muscle and adipose tissue to trigger GLP-1 release from the gut and the pancreas to improve glucose tolerance. Adapted from Allen, T.L., M. Whitham, and M.A. Febbraio, IL-6 muscles in on the gut and pancreas to enhance insulin secretion. *Cell Metab*, 2012. 15(1): p. 8-9. [51] GLP-1 Glucagon like peptide -1.
IL-6 is associated with pro- and anti-inflammatory effects depending on the disease state. In healthy exercising individuals, levels of IL-6 are usually low and increase with physical exercise. IL-6 plays an important role in the adaptive immune function as well as resolving inflammation. Additionally, its role in metabolism especially around exercise is being elucidated but in the acute phase at least, it increases fat oxidation and glucose uptake. Conversely, IL-6 levels are moderately elevated in inflammatory conditions and correlate with disease activity. Adapted from Pederson and Febbraio, Muscle as an Endocrine Organ: Focus on Muscle-Derived Interleukin-6. *Physiol Rev.* 2008 Oct;88(4):1379-406. [42]
Associations between Obesity and Chronic Kidney Disease (CKD)
Inflammatory dysfunction in adipose tissue, initially in the setting of obesity, and additionally with the development of T2DM has important implications for kidney function.

Obesity-Related Glomerulopathy
Obesity is associated with multiple kidney disease risks including glomerulomegaly with or without focal or segmental glomerulosclerosis (FSGS/FGS), diabetic nephropathy, renal carcinoma, nephrolithiasis and increased risk of acute kidney injury (AKI) [52, 53]. Obesity is specifically associated with a syndrome known as obesity-related glomerulopathy (ORG) [54]. This syndrome consists of morbid obesity, marked proteinuria without edema and normal serum albumin. Although ORG has been reported to comprise between 0.89% to 2% of renal biopsies, the incidence of this condition has increased – a trend that is likely to continue with the increasing numbers of obese people worldwide [55, 56].

The number of cases of ORG detected in renal biopsy series has increased in the last 20 years, with a US study finding that it made up 0.2% of biopsies between 1986 and 1990 compared to 2% between 1996 and 2000 [55]. Clinically, patients with ORG tended to have a more indolent course compared to those diagnosed with idiopathic focal segmental glomerulosclerosis (FSGS). In this series, proteinuria was the main indication for biopsy. Despite massive proteinuria in some cases, oedema and other features of nephrotic syndrome such as hypoalbuminaemia and hyperlipidaemia were not present. The main findings of this US cohort, as well as those from other cohort studies, are summarised in Table 1.1. [55-60].

From these case series, it is clear that much of the renal injury observed in the setting of morbid obesity occurs in the glomerulus. The pathological abnormalities of ORG may represent structural and/or functional adaptions of glomerular cell populations in response to hyperinsulinaemia, dyslipidaemia, hyperuricaemia and other metabolic processes associated with obesity. In a key study, Wu et al. used whole-genome microarrays to compare gene expression in microdissected glomeruli in six ORG patients that were carefully selected to exclude other causes of FSGS. Glomeruli from kidneys of healthy, non-obese living donors were used as controls. A further fifteen patients were studied for validation of the microarray analysis. The authors found upregulation of genes related to inflammatory cytokines including TNF-alpha and its receptors, IL-6 signal transducer(IL-6st or gp130), granulocyte-macrophage colony stimulating factor 2 (GM-CSF) and Interferon gamma (INF–gamma). Additionally, there was similarity in expression patterns of genes involved in lipid
metabolism and insulin resistance as well as upregulation of genes involved in extracellular matrix modulation. Expression of LDL receptor, TNF-alpha, vascular endothelial growth factor (VEGF), TGF-beta1, glucose transporter -1(GLUT-1) and leptin receptor within the glomeruli were confirmed with real-time PCR from the validation set of ORG patients compared to two healthy controls. [61]. This study suggests that intra-glomerular adaptations in response to obesity in the setting of ORG involve the upregulation of both inflammatory and metabolic signalling pathways.
## Table 1.1 Biopsy Studies of Patients with Obesity Related Glomerulopathy

<table>
<thead>
<tr>
<th>Study</th>
<th>Number of patients</th>
<th>BMI</th>
<th>Type 2 Diabetes</th>
<th>Clinical findings</th>
<th>Biopsy findings</th>
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<tr>
<td>Kambham et al (ref. 55)</td>
<td>103 patients identified from review of 6818 biopsies at, 71 with clinical details</td>
<td>41.7 kg/m² (range of 30.9 to 62.7 BMI&gt;40 in 38 (53%)</td>
<td>9(12.7%)</td>
<td>34 with nephrotic range proteinuria, only 4 with nephrotic syndrome. Compared to idiopathic FSGS ORG were older, Caucasian, no clinical signs of nephrotic syndrome, higher serum albumin, lower creatinine. In follow up of 39 patients over a mean of 27 months, 8(14.3%) had a doubling of serum creatinine and 2(3.6%) progressed onto end stage renal disease (ESRD), all who progressed were treated with ACE, had FSGS lesions on biopsy and greater BMI did not influence progression.</td>
<td>57 with FSGS and glomerulomegaly and 14 with glomerulomegaly. Mild tubular atrophy and interstitial fibrosis and moderate arteriosclerosis. Mean glomerular diameter of ORG was higher than age and sex matched normal controls (226±24.6mm vs 168±12mm). ORG had fewer segmental sclerotic lesions compared to biopsies from idiopathic-FSGS but percentage of globally sclerotic glomeruli was higher.</td>
</tr>
<tr>
<td>Tsuboi et al (ref. 59)</td>
<td>28 cases from 1999-2008</td>
<td>32.4 ± 2.8 kg/m²</td>
<td>2 patients with impaired glucose tolerance</td>
<td>21(75%) hypertensive, 7(25%) progressed over 6.2 years with progression defined as a 50% increase in serum creatinine or development of ESRD. Progresses were older, had lower renal functional reserve, higher urinary protein excretion and greater interstitial fibrosis and tubular atrophy</td>
<td>29% had Focal glomerulosclerosis 23±19% had mild to moderate interstitial fibrosis and tubular atrophy and global glomerulosclerosis,</td>
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<tr>
<td>Praga et al (ref. 76)</td>
<td>15 obese patients with biopsy proven ORG compared to 15 non obese patients with idiopathic FSGS</td>
<td>35±5.2g/m²</td>
<td>6 patients went onto develop hyperglycaemia on follow up</td>
<td>1/3 had renal insufficiency, 50% had hypertension Although 6(40%) had nephrotic range proteinuria, serum albumin normal in all and no clinical signs of nephrotic syndrome Unilateral renal hypoplasia or unilateral renal agenesis in 3. Patients followed for 82±57 months with 5 (33%) requiring dialysis. Baseline creatinine predicted loss of renal function</td>
<td>100% of the glomeruli (mean 19±23%) showed FSG lesions. The percentage of globally sclerotic glomeruli (GGS) ranged from 0 to 60% (mean 18±18%). Mean glomerular diameter was 256±24nm. Percentage of glomeruli with focal segmental glomerulosclerosis correlated with proteinuria and percentage of glomeruli with global glomerulosclerosis correlated with creatinine clearance.</td>
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<tr>
<td>Chen et al (ref 56)</td>
<td>90 biopsies identified from 10,995 from between 2002-2006 (0.89%)</td>
<td>Mean BMI 31.2±3.3, all patients had visceral obesity</td>
<td>10% had nephrotic range proteinuria and 2.2% had nephrotic syndrome. Patients with severe obesity (&gt;35) had greater creatinine clearance than those with moderate (30-35) or mild (28-30) obesity but prevalence of renal insufficiency trended to be greater in those with moderate obesity compared to severe and mild obesity</td>
<td>Glomerulomegaly present in all specimens, 70% with FSGS lesions, and 13.3% with global sclerosis. Tubular atrophy and interstitial fibrosis were minimal. Foot process lesions on electron microscopy were segmental with 35.6% having foot process fusion</td>
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<tr>
<td>Study</td>
<td>Patients</td>
<td>Body Mass Index</td>
<td>Characteristics</td>
<td>Findings</td>
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<td>Kasiske et al</td>
<td>17</td>
<td>53.6 ± 9.2 kg/m²</td>
<td>Compared to normal weight patients presenting with proteinuria, obese patients had normal albumin levels and better long-term renal prognosis</td>
<td>Renal biopsy found FSGS lesions to be present in 50% of obese patients presenting with proteinuria, with a third having occult diabetic nephropathy</td>
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<tr>
<td>Serra et al</td>
<td>95</td>
<td>59.3 ± 8 kg/m²</td>
<td>17 patients</td>
<td>Protocol biopsy in patients undergoing Bariatric surgery</td>
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<td>Compared to normal weight patients presenting with proteinuria, obese patients had normal albumin levels and better long-term renal prognosis</td>
<td>All had normal renal function, absence of microalbuminuria</td>
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<td>13 (14%) fasting serum glucose between 7 and 11 mmol/l, 36 impaired fasting serum glucose</td>
<td>5 (5.3%) showed FSGS (classic or usual FSGS in three cases and perihilar FSGS in two cases</td>
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<td>56 (59%) with hypertension, 39 patients (41%) had microalbuminuria and 4 (4%) albuminuria &gt; 300</td>
<td>Mesangial matrix expansion, podocyte hypertrophy were more common in the obese biopsies than healthy controls</td>
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<td>All had normal renal function</td>
<td>Glomerulomegaly was observed in 38% of EO patients</td>
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<td>6/69 had positive immunofluorescence</td>
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<td>EM available in 23 nonsclerosed glomerular capillaries with the presence of large-sized podocytes with swollen cytoplasm</td>
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<tr>
<td>Goumenos et al</td>
<td>18</td>
<td>59.3 ± 8kg/m²</td>
<td>Protocol biopsy in patients undergoing Bariatric surgery</td>
<td>Findings similar to early diabetic nephropathy were noted.</td>
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<tr>
<td></td>
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<td>All had normal renal function, absence of microalbuminuria</td>
<td>No patients with FSGS lesions, most had increased glomerular cross sectional area, with patchy thickening of GBM, extensive foot process effacement Scattered or occasional paramesangial deposits were also identified in 9 of 13 patients noted on EM study.</td>
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</table>
Obesity and Chronic Kidney Disease

Epidemiological studies have demonstrated associations between obesity and increased risk of chronic kidney disease (CKD) [62]. In a follow-up on the hypertension detection and follow-up programme, which involved 5897 hypertensive adults without CKD at baseline, obesity was found to be an independent risk factor for kidney disease, with an odds ratio of 1.4 (95% CI, 1.2-1.63) after adjustment for covariates including diabetes mellitus [63]. The physicians healthy study found that a 10% increase in body mass index (BMI) increased the risk of CKD (OR, 1.27; 95% CI, 1.06 to 1.53). In the setting of existing CKD, baseline diastolic and mean arterial blood pressure were higher in overweight and obese patients and frequency of CKD progression, as defined as a >1ml/kg/1.73m² fall in estimated glomerular filtration rate (eGFR), was 79.5% in obese compared to 44.7% in normal weight patients [64, 65]. In a study of 300,000 individuals from the Kaiser Permanente database, North California, there was strong increased relative risk of end stage renal disease (ESRD) in obese individuals even after adjustment for baseline blood pressure and diabetes mellitus [66]. In a community-based longitudinal cohort study of 2585 participants free from pre-existing kidney disease followed for eighteen years, baseline BMI was found to be related to the development of kidney disease (OR 1.23 per 1SD; 95% CI, 1.08-1.41) [67]. Younger age of obese individuals is also associated with a higher risk of developing CKD [68].

It should be noted that, in some but not all epidemiological studies, the association between obesity and risk for CKD is weakened after adjustment for cardiovascular disease and T2DM. For example, in the cardiovascular health study which followed 4295 individuals over the age of 65 for seven years, higher baseline BMI and waist circumference were associated with loss of eGFR following adjustment for age, sex and smoking. However, only waist circumference was significant after adjustment for diabetes, CRP level and hypertension (OR 1.15 95% CI 1.06-1.25) [69]. In 3749 subjects, anthropometric measurements of waist circumference and waist to hip ratio were independently related to higher urinary albumin creatinine ratio (ACR) but not eGFR, although subjects with higher values for these had increased odds of CKD [70]. Waist circumference but not BMI was associated with renal function decline in 454 Koreans followed for six years [71]. In a Japanese study of 100,753 subjects, for each quartile increase in BMI there was an increased risk of developing ESRD but this was attenuated after adjustment for T2DM [72]. In a prospective cohort study of 2767 individuals free from CKD at baseline, after a follow-up of 18 years, obese individuals had a 68% increased odds of developing stage 3 CKD which was no longer observed after adjustment for cardiovascular
disease risk factors [73]. In contrast to the studies discussed so far, Tohidi et al., in a study of risk factors for CKD in an Iranian population, observed that abdominal obesity trended to be protective of CKD [74].

Considering the findings of these epidemiological studies, it appears likely that obesity exerts significant influence on the development and progression of CKD both directly and indirectly through its association with the development of T2DM and atherosclerosis. Additionally, there is evidence that obesity worsens the prognosis of established primary renal conditions. For instance, in a study of 162 patients with IgA nephropathy, BMI >25kg/m² was associated with greater severity of pathological renal lesions (global optical score and vascular, tubular and interstitial indices) and chronic renal failure-free survival was significantly less in patients with higher BMI [75]. Similarly, in a study of patients with unilateral nephrectomy and normal renal function at baseline, 14 out of 73 were noted to be obese. Thirteen (92%) of these obese subjects subsequently developed proteinuria and/or renal insufficiency compared to 7/59 (12%) of those who were not obese at time of surgery [76]. Higher BMI has also been reported to be independently associated with the risk of acute kidney injury (AKI) following cardiac surgery, with a 26.5% increase for every 5kg/m² increase in BMI. Interestingly, this association was lost following adjustment for circulating markers of oxidative stress. This would suggest that oxidative stress mediates the association between post-surgical AKI and obesity [52]. In mechanically ventilated patients, the presence of obesity has also been reported to be associated with increased incidence of AKI [77]. From these studies, it is clear that there is an association between obesity and renal dysfunction. An important unanswered question is the degree to which pathophysiological factors driven directly by obesity contribute to the development and progression of CKD.

**Pathophysiology of Obesity Related Kidney Disease**

The fact that structural and functional changes occur within the kidney in response to obesity has been noted in both human and animal studies. Obesity is associated with increased renal plasma flow (RPF) and glomerular filtration rate (GFR) and these alterations are considered to be instrumental in the development of obesity-related kidney disease. In humans with extreme obesity (BMI >40kg/m²), GFR and RPF measured using inulin and para-aminohippuric acid (PAH) clearance as well as filtration fraction (FF) and mean arterial blood pressure were all higher than in non-obese controls. Of interest, bariatric surgery was
associated with a decrease in GFR and RPF as well as a reduction in albumin excretion rate [78].

Other factors directly induced by increased adipose tissue mass are also thought to be deleterious to the kidney. These include increased production of adipokines and enhanced activation of the renin angiotensin aldosterone system (RAAS) and of the sympathetic nervous system (SNS). Adipose tissue is an important source of angiotensinogen and this, as well as other adipokines and cytokines secreted abnormally in the setting of obesity, contribute to sympathetic overstimulation that also drives hypertension [79]. Hypertension with its associated abnormalities in the RAAS predate the development of diabetes and contribute to the progression of renal disease [21, 80]. The RAAS and SNS are associated with increased renal tubular sodium reabsorption, leading to volume expansion and hypertension. In keeping with this, salt sensitive hypertension is associated with obesity, hyperinsulinaemia and the metabolic syndrome. Adiponectin, an adipokine that decreases with adiposity, has been reported to have a role in podocyte function and adiponectin deficiency may contribute to the initial development of albuminuria in obese individuals. Additionally, adiponectin is considered to be anti-inflammatory and cardioprotective through effects including increased secretion of interleukin-10 (IL-10), interleukin-1 receptor antagonist (IL-1RA), enhancement of nitric oxide bioavailability and reduction of endothelial cell-leukocyte activation[28]. In podocytes from adiponectin-deficient mice, increased permeability to albumin was noted in association with decreased translocation of zona-occludens-1 to gap junctions. This was associated with decreased expression of the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase component NOX4 via the AMPK pathway. Addition of adiponectin increased expression of nephrin, endothelin, PAI-1 and endothelial nitric oxide synthetase (eNOS) in the renal cortex. Furthermore, CRP levels negatively correlated with adiponectin concentration [81]. Adiponectin inhibited TNF-alpha-induced intracellular adhesion molecule 1 (ICAM) and vascular cellular adhesion molecule (VCAM) expression leading to decreased macrophage and monocyte adhesion to endothelial cells [82].

Animal studies of either genetic models of obesity or diet-induced obesity have been used to establish the changes that occur in the kidney in response to obesity. The Obese Zucker rat (OZR) is one such rodent model. The OZR has systemic resistance to leptin as a result of a non-functioning mutation in the leptin receptor. Leptin is an adipokine secreted from adipose
tissue to act both centrally and peripherally to control energy homeostasis [83]. Leptin is additionally thought to be important in linking obesity and chronic kidney disease. The OZR displays hyperphagia, hyperinsulinaemia and severe peripheral insulin resistance associated with elevated triglycerides, cholesterol and hyperinsulinaemia with variable hyperglycaemia [84-86]. These animals develop a spontaneous renal disease characterized by glomerular hypertrophy by 9 weeks of age, albuminuria and evidence of podocyte injury by 12 weeks, mesangial matrix expansion by 14 weeks, glomerulosclerosis by 20 weeks and, frequently, death due to renal failure by 60 weeks [87, 88]. Haemodynamic and metabolic factors are thought to be important in the pathogenesis of the renal disease in the obese rat [85].

Aging Fischer rats, another animal model of obesity, develop spontaneous glomerulosclerosis and proteinuria as they get older. In a study comparing histological changes within the kidneys of lean and obese aging Fischer rats, a progressive structural change in podocytes was noted. These changes included an initial podocyte hypertrophy followed by adaptation of the podocytes manifesting with increased desmin expression. As the glomerulus continues to enlarge, podocyte hypertrophy response leads to compromised podocyte function with reduced production of key proteins necessary for normal podocyte function (e.g., Wilms' tumour 1 protein [WT1], transcription factor pod1, nephrin, glomerular epithelial protein 1, podocalyxin, VEGF, and alpha-5 type IV collagen) associated with widened foot processes and decreased filter efficiency (proteinuria). Finally, podocyte number decreases through cell death and in association with the development of FSGS [89].

In high fat diet-induced obesity of dogs, obesity was shown to be associated with increased kidney weight, mean arterial pressure (MAP) and RPF. Glomerular Bowman’s space was increased and there was increased mesangial matrix and thickening of the glomerular and tubular basement membranes as well as increased proliferation of cells within the glomeruli. These animals displayed hyperinsulinaemia and increased renin activity compared to lean dogs [90].

Rhesus monkeys have a high prevalence of developing obesity and T2DM. Compared to young, metabolically normal aged and hyperinsulinaemic aged monkeys, diabetic monkeys were both found to have glomerulomegaly, glomerulosclerosis and glomerular basement
membrane (GBM) thickening with the development of glomerular hypertrophy occurring in the pre-diabetic hyperinsulinaemic phase [91].

In summary, obesity as measured either by BMI, waist circumference or visceral adiposity [92] is associated with kidney dysfunction. This is supported by epidemiological, in vitro and animal studies. Studies from the OZR suggest that other factors in addition to haemodynamic abnormalities within the kidney in response to increasing mass are important in the development of glomerular injury. The full range of mechanisms leading to ORG and the distinction of ORG-mediated effects from those of diabetes remains to be fully elucidated but are likely to have important implications for the renal health of populations worldwide as the age of onset of obesity continues to fall. These issues represent major themes of the research carried out for this thesis. Mechanistically, some of the key questions that I considered to be of particular importance related to the influence of increased adipose tissue on intra-renal immune cell activity, the signalling pathways that are activated within the kidney in response to obesity and the protective mechanisms which may be in place within the kidney during obesity that serve to prevent rapid progression to CKD in the absence of a superimposed acute injury [52, 77].
Type 2 Diabetes Mellitus-Epidemiology

T2DM is the most common metabolic disease, affecting over 250 million people worldwide [93]. In 2005, it was reported that 129,052 adults in the Republic of Ireland were known to have T2DM. This figure represented 4.3% of the adult population; 3.5% of all men and 5.1% of women. Similarly, it has been reported that 62,287 persons aged 20 years and over in Northern Ireland have T2DM (5.1% of the population) [94]. Most type 2 diabetic patients are overweight or obese [95-97]. Type 2 diabetics who are obese also tend to be younger, with poorer glycaemic control, poorer blood pressure control and more severe dyslipidaemia compared to those with a BMI <30kg/m² [96]. Typically, obesity pre-dates the development of T2DM, often by years, and, as emphasised in the previous section, obesity is being encountered in children and young adults to extents not present in previous generations[2].

While the future risks of developing diabetes, cardiovascular disease, hypertension and dyslipidaemia for obese children that achieve normal weight as adults have been determined to be no greater than those of individuals who were never obese [98], obesity in adolescents tends to persist into adulthood and increased weight in young adults confers an increased risk of excess mortality in later life [99, 100].

Pathophysiology of T2DM

T2DM is a heterogeneous disorder characterized by variable degrees of insulin resistance, impaired insulin secretion and increased glucose production. It is a manifestation of the body’s adaptations to increased and unnecessary nutrition over a long term period of time [101, 102]. Abnormalities in either insulin or glucagon metabolism are involved in the pathogenesis of T2DM with insulin resistance leading to decreased glucose utilization, increased lipolysis and proteolysis and excessive glucagon secretion. This results in increased hepatic glycogenolysis, hepatic gluconeogenesis, as well as decreased glycogen synthesis [103]. In the early stages of T2DM, insulin resistance occurs but glucose tolerance remains normal due to a compensatory increase in insulin secretion by pancreatic beta-cells. However, with prolonged insulin resistance and compensatory hyperinsulinaemia, the pancreatic islets become unable to sustain the hyperinsulinaemic state and impaired glucose tolerance occurs [104]. Hyperglycaemia is thought to be an important primary aetiological factor leading to diverse complications of T2DM. Hyperglycaemia is associated with the formation of AGE resulting in altered protein function. Increased intra-cellular sorbitol and DAG also occur in response to hyperglycaemia and result in activation of signalling pathways which drive transcription of genes encoding cytokines, growth factors, pro-angiogenic factors and
mediators of fibrosis. Additionally, hyperglycaemia places mechanical pressure on cells via alterations in osmolality which leads to activation of cell stress responses [28].

The chronic complications of T2DM affect most organ systems of the body and are responsible for most of the morbidity and mortality associated with the condition. Chronic diabetic complications are divided into microvascular (retinopathy, neuropathy and nephropathy) and macrovascular (cardiovascular disease, peripheral vascular disease and cerebrovascular disease). Although the risk of developing complications increases as a function of the duration of hyperglycaemia, this cannot be the only pathogenic factor as the onset and nature of end-organ complications do not occur uniformly among all diabetic individuals [105]. For patients with T2DM, other specific metabolic abnormalities such as hyperinsulinaemia, hyperlipidaemia, microalbuminuria and abnormalities in cytokines, chemokines, growth and fibrotic factors predate the development of hyperglycaemia and variability in the response to such factors likely influences heterogeneity with regard to the development and severity of complications. Environmental factors that influence the development of complications include age, duration of diabetes, smoking, gender and other underlying medical conditions [106]. Furthermore, there are strong genetic components to the susceptibility and development of complications in response to T2DM although strong, single-gene associations have not, thus far been identified [105].

Among the other advances the have occurred in the understanding of the pathophysiology of T2DM and its chronic complications is the role played by inflammatory responses [28]. Studies of human cohorts have described elevated circulating levels of inflammatory markers such as CRP and a number of cytokines and chemokine in individuals with obesity, pre-diabetes and diabetes. Elevated levels of CRP have additionally been reported to be associated with cardiovascular risk factors as well as risk for developing diabetes [107-109]. Enhanced inflammatory response appears to present at an early stage of the pathogenesis of T2DM as circulating inflammatory markers in obese individuals with pre-diabetes are similar to those with diabetes [110].

Factors suggested to be important in inducing inflammatory response include hyperglycaemia itself [111] and signalling associated with toxic lipids [112], oxidative stress [113] and endoplasmic reticulum stress [114]. Among the adverse consequences of enhanced inflammatory response in obesity, pre-diabetes and T2DM are insulin resistance, cellular
apoptosis, inflammatory cell infiltration, endothelial dysfunction and, from this, microvascular dysfunction. Further implicating inflammation in the pathogenesis of pre-diabetes is the finding that the predominant source of inflammatory cytokines from adipose tissue of obese subjects are inflammatory polarized macrophages [34] residing in the stromal vascular fraction. The number of pro-inflammatory macrophages within visceral adipose tissue has been shown to correlate with degree of obesity [115, 116].

Based on current evidence, it is reasonable to conclude that inflammatory cells and individual molecular mediators of immune response and inflammation molecules are strongly associated with the development of T2DM and its complications. Certain inflammatory cytokines have been found to be elevated in the setting of pre-diabetes in addition to playing important roles in regulating glucose metabolism. These cytokines, which include IL-6 [44], IL-1 [117] and TNF-alpha [18, 44, 118] may represent potential diagnostic and therapeutic targets for future prevention of end-organ complications. However, as the roles for these and other inflammatory mediators in metabolism are further elucidated, it will be important to determine whether they represent markers rather than active participants in the disease process.

**Diabetic Nephropathy—Epidemiology**

Diabetes mellitus, primarily T2DM, is the most common cause of ESRD, accounting for 44% of all new cases requiring renal replacement therapy in 2007 [119]. Since 1996, the incidence of diabetic patients in the US requiring renal replacement therapy has decreased by a third, from 304.5 to 199.1 per 100,000 person with diabetes [119]. This likely reflects increased awareness of CKD and improved management of diabetes and diabetic nephropathy during the time-frame studied.

The natural history of diabetic nephropathy has been better elucidated in type 1 diabetes mellitus (T1DM) than in T2DM, primarily because it is easier to define when the disease started in the former. In contrast to T1DM, microalbuminuria or even overt nephropathy may be present at the time of diagnosis of T2DM and hypertension commonly accompanies T2DM. In T1DM, the cumulative incidence of diabetic nephropathy is 30%. The incidence peaks after 10-15 years and decreases rapidly after this [105]. Glomerular hyperperfusion and renal hypertrophy occur in the first years after the onset of T1DM associated with histological abnormalities such as thickening of glomerular basement membrane (GBM), glomerular hypertrophy and mesangial volume expansion. After 5-10 years, 40% of type 1 diabetics
develop microalbuminuria defined as 30-300 µg/mg creatinine in a spot urine collection. Histological changes that occur in the kidney in response to diabetes have been classified into the glomerular and interstitial/vascular abnormalities. Glomerular abnormalities in increasing grades include: Class I: mild light microscopic changes with glomerular basement membrane (GBM) thickening by electron microscopy, Class IIa: mild mesangial expansion, Class IIb: severe mesangial expansion but without nodular glomerulosclerosis and <50% global glomerulosclerosis, Class III: nodular diabetic glomerulosclerosis without >50% global glomerulosclerosis and Class IV: >50% global glomerulosclerosis. Interstitial and vascular abnormalities include grades of interstitial fibrosis and tubular atrophy (scored 0-3), interstitial inflammation (scored 0-2 based), arteriolar hyalinosis (scored 0-2) and the presence of large vessel arteriosclerosis (scored 0-2) [120].

Histological changes are not uniform in the nephropathy of T2DM. In one study of 53 type 2 diabetics with microalbuminuria, 26% had typical diabetic glomerulopathy, 42% had normal appearing biopsies and a third had mild glomerular changes with disproportionately severe tubulointerstitial lesions, arteriolar hyalinosis or global glomerulosclerosis [121]. Lesions in the kidney were milder that in biopsies of type 1 diabetic patients with considerable heterogeneity among groups [122].

In diabetic nephropathy, progression is not inevitable as regression was reported to occur in 18% of type 2 diabetics in one study [123] and as many as 50% in another. In the latter study from Japan, those regressing had a 50% reduction in death and hospitalisation compared to the 28% that exhibited had progression of nephropathy [124, 125]. There have been many studies showing additive reduction of albumin creatinine ratio (ACR) with dual angiotensin converting enzyme inhibitor (ACEi)/angiotensin receptor blocker (ARB) therapy [123, 124, 126-129], however, a large randomised controlled trial comparing ACE alone with ACE and ARB in patients at high risk for cardiovascular events which included 36% with diabetes found that there was increased risk of renal dysfunction in the combined treatment group [130]. Thus, diabetic nephropathy in T2DM is heterogeneous in its presentation and progression. Furthermore, there is currently no reliable clinical feature or biomarker which identifies those at highest risk of developing progressive nephropathy and this, along with the need for improved understanding of pathogenesis, represent important knowledge gaps in the field.
Pathophysiology of Diabetic Nephropathy

Albuminuria and hypertension are the earliest clinically detectable manifestations of kidney disease in diabetes mellitus, with 25% of type 1 diabetics having microalbuminuria after 10 years followed by an annual rate of progression to overt nephropathy of 3% [131]. Microalbuminuria can occur in the kidney as a primary process due to damaged podocytes or GBM or insufficient uptake by proximal tubular cells [132]. Additionally, microalbuminuria is a manifestation of endothelial dysfunction, in this situation reflecting a more systemic microvascular disorder. Although the development of microalbuminuria is influenced by hyperglycaemia [133], it can occur independently of diabetes and, when present, is associated with increased cardiovascular morbidity and mortality in non-diabetics [134]. Blood pressure strongly influences the development of microalbuminuria and lowering blood pressure reduces the incidence of micro- and macro-albuminuria with associated reduction in subsequent renal events [135]. Additionally, it appears that the effects of lowering blood pressure and improving glycaemic control are independent of each other and are additive. Combination treatment involving fixed doses of an ACEi and a diuretic for blood pressure control and targeting glycaemic control to a HbA1c ≤6.5% in 11,140 type 2 diabetics followed for 4.3 years reduced the risk of new or worsening nephropathy by 33% (95% CI 12-50%, P = 0.005). In this study, new onset of macroalbuminuria was reduced by 54% (95% CI 35-68%, P < 0.0001) and new onset of microalbuminuria by 26% (95% CI 17-34%). Combination treatment was associated with an 18% reduction in the risk of all-cause death (95% CI 1-32%, P = 0.04) [136]. This was also associated with 10% relative reduction in the combined outcome of major macrovascular and microvascular events. This latter effect occurred primarily as a consequence of a 21% relative reduction in nephropathy and was not related to improvement in glycaemic control [137].

In T2DM, the RAAS is instrumental in the development of hypertension, renal injury, atherosclerosis and cardiac hypertrophy by promoting inflammatory, proliferative and fibrotic responses in the kidney [138, 139]. Angiotensin II (AII), through increased tone of the efferent arterioles, causes glomerular hypertension. Associated with glomerular hypertension are glomerular basement membrane thickening, albumin leakage, mesangial matrix expansion, podocyte injury and mechanical strain. As a result of cellular stress in response to this, localised release of cytokines, chemokines and growth factors occurs within the glomerulus and perpetuates injury. It is clear that the RAAS has an important specific role the
pathogenesis of diabetic nephropathy given evidence from multiple clinical trials in which blockade of the system was shown to result in preservation of renal function [140].

**Role of Systemic Inflammation in the Pathophysiology of Diabetic Nephropathy**

In addition to the factors summarised above, there is some evidence that inflammatory responses also play an important role in the development of diabetic nephropathy. Potential sources of inflammatory mediators with the potential to contribute to renal injury in the setting of T2DM include immune and non-immune cells located at distant sites such as adipose tissue, liver, spleen and intestine as well as resident or infiltrating cell populations within the kidney itself [141, 142]. In this regard, it should be noted again that the predominant source of inflammatory cytokines in obese subjects is inflammatory macrophages residing in the stromal vascular fraction of white adipose tissue [34, 115, 116]. Serum levels of several cytokines have been found to be increased in the setting of diabetic nephropathy compared to diabetes with normal renal function. The concentrations of these cytokines in the circulation have been reported to be associated with abnormal urinary albumin excretion and impaired renal function [143, 144]. Animal studies have also shown increased circulating concentrations of TNF-alpha and IL-6 in the setting of diabetes, which correlate with kidney weight and urinary albumin excretion and are reduced by administration of pentoxyfylline or enalapril [141].

Nephropathy is strongly associated with atherosclerotic vascular complications in T2DM and its development could be driven by intra-renal responses to systemic factors generated as part of the pathophysiology of atherosclerosis. For example, in a post hoc analysis of patients treated with pravastatin following myocardial infarction, renal functional decline was greater in those in the highest quartile of CRP and TNF receptor II (TNFRII). This quartile also demonstrated a beneficial effect of pravastatin with regard to rate of renal functional decline [143]. Type 2 diabetes mellitus and obesity are also associated with higher exposure to circulating bacterial endotoxin (lipopolysaccharide, LPS). This may represent effects of unhealthy “Western” diet such as augmentation of LPS translocation across the gut wall and alteration of the intestinal microbiota. Thus, enhanced systemic exposure to microbial LPS and other pathogen-associated molecular patterns (PAMPS) may represent another pathway by which increased inflammation occurs in T2DM and could contribute to the onset and progression of end-organ complications including nephropathy [145, 146].
The release of specific adipokines from inflamed adipose tissue may have deleterious effects on kidney function. For instance, adiponectin, systemic levels of which decrease with increasing obesity, has been shown to be important in maintaining optimal podocyte function [81].

Abnormal coagulation is a feature of diabetes and can also lead to deleterious effects on the kidney. Plasminogen activator inhibitor type 1 (PAI-1), coagulation factor VII and fibrinogen were noted to be elevated in diabetes and with increasing stages of nephropathy [147]. PAI-1 inhibits tissue plasminogen activator (tPA) and urokinase plasminogen activator (uPA), which convert plasminogen to plasmin, a strong proteolytic enzyme. Plasmin activity has been found to be reduced in obesity and diabetic nephropathy due to increased PAI-1 activity and this may be associated with enhanced accumulation of extracellular matrix proteins [20]. In summary, the diabetic state has been shown to be associated with maladaptive activation of immune system in multiple tissues with systemic release of a range of inflammatory mediators. Systemic inflammation, may, therefore, be instrumental in initiating and perpetuating the cycle of damage that contributes to the pathogenesis of diabetic nephropathy. Although associative studies provide evidence for this link, the significance of inflammation as an independent pathophysiological factor and therapeutic target in the early stages of diabetic nephropathy has not been definitively established.

**Intra-Renal Inflammation in Diabetic Nephropathy**

In diabetes, haemodynamic abnormalities (intra-renal/systemic hypertension and SNS activation), metabolic derangements (hyperglycaemia/hyperlipidaemia) and activation of the RAAS are inter-related factors that contribute to renal dysfunction. These processes are sensed by each cell type within the kidney (endothelial, mesangial, tubular and podocyte) to activate inflammatory signalling pathways.

The renal parenchyma comprises approximately 10% glomeruli and 90% tubulointerstitium. It has a complex architecture composed of multiple cell types necessary to carry out the primary functions of the kidney. The glomerulus consists of vascular, epithelial and mesangial components. The tubulointerstitium is mainly composed of tubular epithelial cells, surrounding capillaries and fibroblasts and other supporting cells of the interstitium. Additionally, mononuclear phagocytes, either dendritic cells or macrophages, are resident in the interstitium, forming an intricate immune surveillance system [148]. Although the
glomerulus is often the site of initial damage in kidney disease, tubulointerstitial processes are also important in the progression to functional impairment. Indeed, a kidney biopsy study of human diabetic nephropathy found that renal functional survival was highest for patients in whom the tubules and cortical interstitium appeared normal on biopsy [149]. Cross-talk between glomeruli and tubulointerstitial cells is an important element of the intra-renal pathophysiology of diabetic nephropathy. For example, impaired glomerular permselectivity may allow the escape of tubulotoxic substances into the urinary space. Additionally, altered glomerular haemodynamics may lead to altered post-glomerular blood flow resulting in tubular hypertrophy or ischemia. Inflammatory mediators and activated immune cells leaking from damaged glomeruli represents another mechanism by which glomerular damage may mediate secondary injury in the tubulointerstitial compartment [150].

Interstitial fibrosis, the final common pathway for many renal diseases associated with ESRD, represents the combined effects of the interactions between glomerular, tubular, vascular, and interstitial cells within the kidney. This involves complex molecular pathways activated in response to deviations from normal homeostatic mechanisms, initially sensed by renal parenchymal tissue and communicated to the intra-renal immune system by cytokines, chemokines and growth factors [150, 151].

**Role of Cellular and Innate Immune System in Diabetic Nephropathy**

**Cell types (Macrophages, T-cells, B-cells, Mast cells, renal parenchymal cells)**

In the diseased kidney, activation of the resident mononuclear phagocytic system by a range of stimuli, triggers the production of cytokines, chemokines and adhesion molecules attracting further infiltration of bone marrow-derived immune cells and facilitating their transmigration to areas of the kidney parenchyma under stress [152]. Dendritic cells (DCs) and macrophages make up the renal mononuclear phagocytic system. These have overlapping phenotypes and functions in the kidney to mediate tissue injury surveillance, tolerance and regeneration as well as modulating other inflammatory cell functions [150]. Classification of the populations of intra-renal immune cells both in healthy and diseased kidney is important to understand inflammatory response and resolution in the setting of kidney injury [152]. Intra-renal immune cells are a localised source of cytokines and chemokines and also have additional diverse effector functions in tissue injury and repair. These include phagocytosis of tissue debris and pathogens, remodelling of extracellular matrix (ECM), support of angiogenesis and epithelial regeneration and local polarization and
modulation of both innate and adaptive immune responses to mediate either further pro-inflammatory or anti-inflammatory functions [152]. In the healthy kidney, macrophages are found in the adventitia of large blood vessels while DCs are present in the peri-tubular space and the inner stripe of the outer medulla. These mononuclear phagocytic cells have been shown to be important mediators of renal injury in some experimental models of immune-mediated kidney disease and, in human glomerulonephritis, their numbers correlate with severity of renal impairment [153].

End-organ complications of T2DM are not generally considered to be immunological processes, however, an increase in mononuclear cells in the interstitium is widely recognised in diabetic nephropathy and is known to comprise T-lymphocytes, macrophages, fibroblasts and fibrocytes [120, 149]. In a broader sense, increased numbers of tissue macrophages is recognised as a common feature of T2DM complications, including atherosclerosis, diabetic nephropathy and diabetic retinopathy [154].

Monocytes and T cells are found in the glomeruli and interstitium from the early stages in human diabetic nephropathy [155, 156]. Furthermore, in the db/db mouse model of T2DM, deficiency of ICAM-1, an important binding partner for the integrin lymphocyte function associated antigen-1 (LFA-1) on monocytes and other leukocytes, resulted in reduced numbers of glomerular and interstitial macrophages in association with decreased in albuminuria and glomerular and tubulointerstitial injury [157].

Macrophages from T2DM patients have been found to be activated by high glucose, AGE and oxidised low density lipoprotein (ox-LDL) following which they secrete high levels of cytokines that promote further inflammation and may directly contribute to tissue injury through pro-apoptotic effects [154]. Among the potentially damaging products of activated macrophages are TNF-alpha, IL-1, IL-6, ROS, PAI-1, matrix metalloproteinases (MMP), TGF-beta, platelet derived growth factor (PDGF), A-II and endothelin [158, 159]. In db/db mice, progressive diabetic nephropathy was associated with macrophage accumulation and activation and this correlated with duration of diabetes, hyperglycaemia, albuminuria, glomerular and tubular damage, fibrosis and expression of MCP-1, osteopontin, macrophage inflammatory protein-1 (MIP-1) and macrophage-colony stimulating factor (M-CSF). In this study, macrophages were noted in the glomerular tufts and around dilated tubules [160]. In human T2DM kidney biopsy samples, macrophage accumulation was associated with
glomerulosclerosis [156, 161]. Macrophages and their precursors can also interact with renal parenchymal cells to generate a pro-inflammatory microenvironment that amplifies tissue injury. For example, monocytes have been shown to stimulate expression of cytokines by proximal tubular epithelial cells. Loss of heparan sulphate on proximal tubular epithelial cells allowed binding of monocytes leading to increased TGF-β1 synthesis [30].

Activated T cells are thought to be important effector cells in hypertension and proteinuria, both of which are features of diabetic nephropathy. Their development has been shown to be dependent on angiotensin 1 receptor and NADPH oxidase. T-cell deficient diabetic mice do not have attenuation of histological injury, fibrosis or reduced creatinine clearance compared to wild-type controls but do have reduced albuminuria with preservation of podocytes [162]. The chemokine CCL5 (RANTES), which is a chemoattractant for monocytes and T cells and is expressed by fibroblasts, mesangial cells and renal tubular epithelial cells, was found to be upregulated in tubular cells from human renal biopsies of diabetic nephropathy and its expression correlated with proteinuria and interstitial cellular infiltration [163]. Infiltration of T cells around the juxtaglomerular apparatus has been observed in the biopsies of adolescents with T1DM and was associated with accelerated loss of kidney function [164]. Finally, depletion of regulatory T cells (Tregs), which are associated with resolution of inflammation, in \(db/db\) mice resulted in worsening albuminuria and glomerular hyperfiltration that was reversed by adoptive transfer of Tregs [165].

Neutrophil accumulation is the hallmark of the acute phase response. Abnormal activation of neutrophils has been reported in diabetic patients with kidney disease. Specifically, neutrophils of type 1 diabetics with nephropathy were shown to remain in an activated state with faster exocytosis of primary granules and a persistence of the adhesion molecule CD11b following stimulation when compared to those of type 1 diabetics without nephropathy and normal controls [166].

Additional chemokines such as MCP-1 and CX3CL1 are important for cell adhesion and inflammatory cell recruitment in diabetic nephropathy. Deficiency of MCP-1 in \(db/db\) mice results in reduced renal macrophage accumulation and limited progression of renal injury [167, 168]. Upregulation of MCP-1 is also found to occur in tubulointerstitial lesions of patients with diabetic nephropathy and treatment with ACEi or ARB has been found to
reduce urinary MCP-1 excretion [169]. The chemokine CX3CL1 is a chemoattractant for monocytes, T cells and natural killer cells and was shown to be upregulated in the kidneys of diabetic animals and in glomerular and peritubular capillaries [163, 170].

In summary, there is evidence from multiple studies that diabetic nephropathy is associated with activation of the resident mononuclear phagocytic system of the kidney as well as renal infiltration by bone marrow-derived macrophages, T cells and neutrophils in response to the damage signals emanating from the stressed or apoptotic glomerular or tubulointerstitial cells. In animal models of diabetic kidney disease, human renal biopsies and studies of circulating peripheral blood mononuclear cells (PBMCs), it is evident that activation of immunological cells occurs in response to the diabetic milieu. Within the kidney, the presence of increased numbers of immunological cells correlates with levels of pro-inflammatory and oxidative stress markers, with progressive parenchymal damage and with proteinuria and loss of renal function (Figure 1.3).

Despite the evidence summarised here, it remains to be determined whether intra-renal inflammation and immune cell activation/infiltration is truly an early feature of diabetic kidney disease which, if prevented, could modify the risk for progressive renal functional decline. Thus, an important goal of the research conducted for this thesis was to learn more about the nature and severity of intra-renal immune activities in pre-diabetic obese animals and in human type 2 diabetic subjects with and without clinical evidence of nephropathy.
Overview of Inflammatory Molecules and Signalling Pathways in Diabetic Nephropathy

Figure 1.3 Adapted from Navarro et al Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy [142]
**Key Intracellular Signalling Pathways and their Regulation in Diabetic Nephropathy**

Pro-inflammatory signalling pathways such as NF-κB, JNK, Janus kinase-signal transducer and activator of transcription (JAK-STAT) are known to be activated in obesity and insulin resistance and understanding their roles in metabolism and immune function has furthered our understanding of the pathophysiology of diabetes [171]. Additionally, these pathways are activated in non-insulin-responsive tissues and are important in causing the micro- and macro-vascular damage associated with diabetes. These signalling pathways are important regulators of the expression of a broad range of cytokines and chemokines including IL-1, IL-6, IL-18, TNF-alpha, MCP-1, ICAM-1, VCAM-1, adiponectin, leptin and resistin. As discussed in previous sections, these soluble mediators have a variety of influences on the pathogenic processes underlying diabetic nephropathy including facilitating leukocyte endothelial adhesion and infiltration, promoting ROS production, directly causing cell injury and increased endothelial permeability and stimulating glomerular hypertrophy and vascular smooth muscle proliferation. In addition, many of these factors induce production of other cytokines, thus perpetuating the cycle.

In experimental settings, administration of immunosuppressive agents has shed light on the role of inflammation in nephropathy associated with T2DM. For example, treatment with mycophenolate mofetil, an inosine monophosphate dehydrogenase inhibitor, in obese Zucker diabetic/spontaneous hypertensive heart failure rats demonstrated reduced systemic and renal inflammation, reduced renal size and reduced hyperfiltration and fibrosis despite the lack of effect on glycaemia and proteinuria [172].

Stress-activated protein kinases include protein kinase R, JNK, and p38 mitogen activated protein kinase (MAPK). These represent another class of intracellular signalling pathways that are activated through various extracellular stress stimuli. Activation of the p38 MAPK pathway is associated with development of diabetic nephropathy. Biopsies from patients with T2DM and nephropathy demonstrate evidence of enhanced glomerular and tubulointerstitial p38 MAPK signalling [173]. High glucose concentrations induce activation of p38 MAPK in mesangial cells, podocytes and proximal tubular cells and results in increased rates of apoptosis in the presence of other stimuli such as TGF-beta or AGE [174, 175]. Reduced p38 MAPK activity through genetic deletion of one of its upstream factors in db/db mice resulted in reduced glomerulosclerosis, albuminuria, macrophage infiltration and MCP-1 expression with preservation of podocytes compared to wild-type controls [176].
Components of the JNK signalling pathway are expressed in immune cells and in parenchymal cells of the kidney. When activated, JNK is phosphorylated, translocates to the nucleus and activates transcription factors that increase the expression of proteins involved in inflammation and apoptosis. The JNK pathway is activated by hyperglycaemia, angiotensin II, ROS and pro-inflammatory cytokines [177]. In diabetic nephropathy biopsy samples, JNK activation correlated with interstitial macrophage accumulation, interstitial fibrosis and loss of renal function [178, 179]. Inhibition of JNK in \( db/db \) mice was associated with reduced macrophage accumulation and MCP-1 expression. However, in this study, there was also accelerated loss of nephrin with increased albuminuria. Of interest, macrophage-specific deletion of JNK in \( db/db \) mice resulted in improved insulin sensitivity and reduced systemic inflammation [180].

**The JAK-STAT Signalling Pathways**

The JAK-STAT pathway is an essential intra-cellular mechanism for regulating the expression of genes encoding cytokines and other proteins involved in stimulus-driven cellular activation, proliferation and differentiation. Proteins of the JAK/STAT pathways have been studied for their roles in inflammation, cancer, atherosclerosis and hypertension as well as the vascular and end-organ complications of diabetes. Pharmacological agents that specifically target components of JAK-STAT signalling them have been developed [181-184].

In total, four JAK and seven STAT family members have been described. Each JAK protein contains a conserved kinase domain and a related but catalytically inactive pseudo-kinase domain at the carboxyl-terminus [185]. Proteins of the STAT family are SH2 domain-containing transcription factors located in the cytoplasm of quiescent cells. Receptor engagement activates the associated JAK which phosphorylates the receptor cytoplasmic domain to allow recruitment and tyrosine phosphorylation of STAT proteins. Cytokine-stimulated tyrosine phosphorylation of STAT proteins induces homo- or hetero-dimerization, followed by translocation into the nucleus to activate specific gene expression patterns [186]. In the nucleus, STAT proteins activate defined gene sets that are cell type-specific on the basis of genomic accessibility and co-factors present in the cells. The JAK/STAT pathways are regulated by receptor internalization, protein phosphatases, the protein inhibitor of activated STAT (PIAS) and suppressor of cytokine signalling (SOCS) proteins [187, 188]. The SOCS proteins, particularly SOCS-1 and SOCS-3, regulate the magnitude and duration of JAK/STAT signalling through direct JAK inhibition. Binding of SOCS3 targets the
JAK/STAT protein complex for ubiquitination [187, 189]. The SOCS proteins are recruited to phosphotyrosine residues of the intracellular domain of the cytokine receptor [190]. It is worth noting that the cytokine receptor system is restrictive in that up to forty different cytokine receptors preferentially use one or more JAK/STAT combinations. As a result, the number of receptors that JAK proteins can bind to is large but the number of JAK proteins that transduce the signal is smaller [191]. In the absence of one STAT protein, another may become dominant. For example IL-6R signalling converts to a dominant STAT1 profile in the setting of STAT3 knockdown leading to an IFN-gamma-dominant gene expression profile. In the case of SOCS3 knockdown, both STAT3 and STAT1 signalling pathways are enhanced, but an IFN-gamma expression profile occurs [191].

The Roles of JAK/STAT Signalling in Models of Kidney Injury

Given the importance of JAK/STAT signalling for transduction of inflammatory signals, it is not surprising that this pathway is known to be activated in several forms of kidney disease. In the mouse unilateral ureteral obstruction (UUO) model of renal fibrosis, phosphorylated STAT3 (pSTAT3) was found to be increased in the renal tubular epithelial and interstitial cells of the kidney. Inhibition of STAT3 with a specific inhibitor (S3I-201) resulted in reduced expression of alpha-smooth muscle actin and fibronectin in cultured renal interstitial fibroblasts and was associated with reduced fibroblast proliferation in-vivo. Inhibition of STAT3 signalling was also associated with reduced renal infiltration by neutrophils and reduced intra-renal expression of TNF-alpha, IL-1-beta, ICAM-1 and MCP-1 [192]. Phosphorylated STAT3 was found to be minimally present in healthy kidney with infrequent expression in tubular epithelial cells of collecting ducts [193]. Following UUO, however, expression of pSTAT3 is significantly increased in interstitial cells [194]. In a rat model of experimental mesangial proliferative glomerulonephritis (anti-thy1.1 glomerulonephritis), pSTAT3 was found to co-localise with proliferating mesangial cells. Administration of a PDGF-BB inhibitor attenuated this proliferation in vitro and in vivo with a reduction in pSTAT3. Another compound that has been shown to reduce induced expression of pSTAT3 is the Bcr-Abl kinase inhibitor, STI 571. This compound has also been found to reduce mesangial proliferation in anti-thy1.1 glomerulonephritis [193].

Other forms of kidney disease in which the JAK-STAT pathways have been shown experimentally to play a critical role include HIV-associated nephropathy, in which phosphorylation of the HIV protein nef and activation of STAT3 were linked with podocyte
proliferation. As STAT3-deficient mice show embryonic lethality, a transgenic mouse with either 25% or 75% of normal STAT3 activity was used to assess its role in this disease. In HIV protein transgenic mice with 25% of normal STAT3 activity, reduced proteinuria, glomerulosclerosis, and tubulointerstitial injury was observed [195]. Increased activation of STAT1 and STAT3 has also been noted in ischemia-reperfusion injury of the kidney [196], adriamycin nephropathy [197] and mercuric chloride-induced kidney injury [198]. In these diverse models, administration of the JAK2 inhibitor AG490 was associated with attenuated renal damage and reduced inflammatory cell infiltration. In the setting of acute kidney injury, trans-signalling by IL-6/sIL-6R has been shown to play a key role in the activation of JAK-STAT signalling within renal tubular epithelial cells which do not inherently express the IL-6R [198].

The JAK/STAT pathways in Diabetic Nephropathy
There is evidence from a range of experimental studies that JAK/STAT signalling pathways play important pathogenic roles in multiple aspects of diabetic kidney disease. Exposure of mesangial cells to high glucose concentration for forty eight hours has been shown to enhance the activation of STAT1, STAT3 and JAK2. Furthermore, reduction of JAK2 levels in mesangial cells via small inhibitory RNA (siRNA) prevented A-II-induced proliferation and secretion of collagen IV [199].

Two weeks following streptozotocin-induced diabetes in rats, glomeruli were found to have increased JAK, STAT1 and STAT3 expression with phosphorylation of these proteins present indicating active transcription. This was reduced by administration of the ACEi captopril and by the JAK2 Inhibitor AG-490 [200].

In transgenic mice with either 75% or 25% of normal STAT3 activity, streptozotocin-induced diabetes was associated with reduced diabetic renal injury compared to normal mice, including reductions in proteinuria, mesangial expansion, glomerular cell proliferation and macrophage infiltration. Expression of pro-inflammatory cytokines and markers of fibrosis were also reduced, indicating that STAT3-mediated signalling is directly or indirectly responsible for key intra-renal inflammatory and pro-fibrotic events in this model [201].

Levels of JAK2 have been examined in the C57BL/6 db/db and the DBA/2J STZ-induced mouse models of diabetic nephropathy. These models as associated primarily with glomerular injury and do not develop the significant interstitial fibrosis and decline in renal function that characterizes human diabetic nephropathy. Of interest, therefore, intra-renal expression and activation of JAK2 was found to be no different to that of non-diabetic control animals [202].
Intra-renal JAK/STAT signalling has also been studied in human subjects with diabetic nephropathy. In one such study, gene expression profiling was carried out on seventy four kidney biopsies with diabetic nephropathy of varying severity. Pathway mapping from whole-genome microarrays identified JAK/STAT signalling components to be highly regulated in the glomerular and tubulointerstitial compartment of biopsies showing both early and progressive diabetic nephropathy compared to control patients. Interestingly, several JAK/STAT members (JAK-1, -2,-3, and STAT-1,-3,-4 and -5b) were down-regulated in early diabetic nephropathy but were expressed at higher levels in progressive diabetic nephropathy. Renal function of the diabetic patients in this study, as assessed by eGFR, inversely correlated with tubulointerstitial JAK2, STAT1 and STAT3.

In summary, the JAK-STAT signalling pathways are known to have important pathogenic roles in regulating the responses and fates of multiple renal parenchymal and infiltrating immune cells in response to stressors associated with obesity, insulin resistance and diabetes mellitus. The accumulating evidence favours a conclusion that JAK/STAT signalling, particularly STAT3-mediated signalling is a central mediator of the development of glomerulosclerosis, tubular atrophy and interstitial fibrosis in diabetic patients and is potentially an important target for novel therapies aimed at preventing or improving the outcomes of diabetic kidney disease.

**SOCS proteins, Negative Regulators of Cytokine Signalling**

The SOCS family of proteins regulate expression of cytokines and other gene products that are induced by JAK/STAT signalling. The transcription of genes encoding SOCS proteins is triggered by pSTAT translocation to the nucleus and they then act as negative regulators of JAK/STAT signalling pathways. The SOCS proteins act intra-cellularly and cause inhibition by binding the active receptor-STAT complex and targeting the complex for ubiquitin-mediated degradation. There are eight members in the family, cytokine-inducible: Src homology 2-containing protein (CIS) and SOCS1 to SOCS7 [203]. Increased expression of SOCS proteins may be induced by diverse stimuli including angiotensin II, chemokines, Toll-like receptor ligands, insulin, immunoglobulins and lipoproteins [45, 190, 204-207].

**SOCS3 and its Role in Glucose Metabolism**

The SOCS proteins are known to have a direct role in insulin signalling and are also well positioned to link inflammatory and metabolic events. Insulin responsive tissues including
fat, liver and skeletal muscle express the SOCS family member SOCS3. In the setting of obesity, SOCS3 expression, as well as its effects on cytokine signalling, is altered in these tissues. For example, in adipocytes, SOCS3 expression is induced by both TNF-alpha and by insulin [208]. The expression of SOCS3 in white adipose tissue is apparently sustained by TNF-alpha as SOCS3 expression was reduced in the fat of diabetic, ob/ob mice lacking TNF receptor compared to control ob/ob mice [208].

SOCS3 decreases insulin-induced IRS-1 tyrosine phosphorylation and its association with p85, a regulatory subunit of phosphatidylinositol 3-kinase (PI3K) [208]. In the db/db mouse model of obesity and T2DM, SOCS1 and SOCS3 expression are increased compared to lean mice. Reduction of hepatic expression of SOCS1 and SOCS3 by siRNA modestly improved insulin sensitivity [209]. Additionally, in this mouse model, SOCS3 expression was increased in macrophages and SOCS3 co-immunoprecipitated with IL-4 receptor complexes to a greater extent than in lean mice. These macrophages were found to have reduced IL-1RA production as a result of reduced IRS-2 mediated PI3K activity [210].

Expression of cytokine and cytokine signalling proteins have been found to be increased in the skeletal muscle of the OZR model of obesity, metabolic syndrome and T2DM. Compared to their lean counterparts, OZR were shown to have increased levels of TNF-alpha, pJNK1, JNK 2/3 and SOCS3 in skeletal muscle. Along with decreased rates of insulin stimulated skeletal muscle 3-O-methyl-d-glucose (3-OMG) transport, PI-3K activity and IRS-1 phosphorylation. SOCS3 co-immunoprecipitation with insulin signalling components IRS-beta and IRS-1 was also increased [211].

Similarly, in cardiac tissue of C57BL/6 mice fed a high fat diet for 6 weeks, glucose metabolism was altered with reduced GLUT1, GLUT4 and AMPK expression. Associated with this was an increase in macrophage infiltration and expression of TLR4 and related signalling molecule MyD88, SOCS3, IL-6 and TNF-alpha. This effect was attenuated in IL-6 knockout mice and acute systemic rise in IL-6 to physiological levels lead to insulin resistance through SOCS3-mediated inhibition of IRS-1 [45].

Finally, in obese human subjects, SOCS3 was found to be increased in circulating mononuclear cells compared to healthy non-obese controls and was inversely related to the phosphorylation of the insulin receptor beta-subunit [212]. In a study comparing consumption...
of a high fat, high calorie (HFHC) diet to a healthy high fibre, high fruit iso-caloric diet in non-obese individuals, the HFHC diet resulted in acute increase in ROS, NF-κB DNA binding, MMP-9, SOCS3, TLR2 and TLR-4 expression. In contrast to the healthy diet, plasma LPS concentrations increased during the HFHC diet. In this study, SOCS3 expression in the mononuclear cells was significantly related to TLR4 expression [213].

From these studies it appears that there is an important role played by SOCS3 in insulin and glucose metabolism. Additionally, expression of SOCS3 and the proteins that it has been shown to be associated with becomes altered in various tissues during the development of T2DM in parallel with alterations to insulin- and cytokine-related signalling proteins. From this, it can be proposed that SOCS3 is in a pivotal position to link two features important in the pathogenesis of insulin resistance and T2DM, namely altered insulin/glucagon metabolism and abnormal inflammatory response.

**Inflammation and SOCS3**

Atherosclerosis is accelerated in CKD, T2DM and obesity. The activity of JAK/STAT signalling and the SOCS family of proteins in atherosclerotic lesions provides insight into how these pathways may respond in the kidney. In atherosclerotic lesions, the JAK/STAT pathway is activated by oxidised lipids leading to the induction of pro-inflammatory cytokines. The SOCS family member SOCS3, a negative regulator of STAT signalling, is also expressed in atherosclerotic lesions [214, 215]. The effect of SOCS3 on worsening or resolving atherosclerosis has not been fully resolved. One study reported that loss of SOCS3 expression in T cells lead to increased IL-10 and IL-17 contributing to an anti-inflammatory macrophage phenotype and reduced size of atherosclerotic lesions. Conversely, SOCS3 over-expression resulted in acceleration of atherosclerosis in association with reduced IL-17 expression. The authors also found that, in biopsies of human atherosclerotic plaques, pSTAT3 and IL-17 expression were associated with a more stable fibrous plaque phenotype [216].

Ortiz-Munoz examined SOCS3 and SOCS1 expression in human carotid artery atherosclerotic plaques and observed strong expression in macrophages and vascular smooth muscle cells (VSMC) within the unstable, inflammatory regions of the plaques. *In vitro*, VSMCs over-expressing SOCS3 exhibited reduced STAT3 expression leading to a reduction in the mitogenic effects of IL-6 and LDL and reduced expression of ICAM-1 and MCP-1. In
high fat-fed Apo-E knockout mice, knockdown of SOCS3 did not affect the size or extension of atherosclerotic plaques in the aorta but was associated with increased proliferation of VSMC, infiltration of macrophages and CD4+ cells as well as increased expression of TNF-alpha, IFN-gamma, IL-10, TGF-beta and RANTES [217].

Polarization of macrophages into either classically- or alternatively-activated phenotypes occurs in response to tissue signals in the setting of injury and repair. Both SOCS1 and SOCS3 are rapidly induced in macrophages [218] and are involved in the control of macrophage polarisation and thereby regulating inflammatory responses [219]. SOCS1 is upregulated in M2 macrophages and is important for its repair function while SOCS3 is predominant in the early inflammatory stages of infection in association with M1 polarised macrophages. Knock-down of SOCS1 in macrophages stimulated with IL-4 leads to reduction of M2 polarisation markers (arginase, iNOS) and increased SOCS3 expression [220].

The SOCS family proteins SOCS2 and SOCS3 are required for regulation of macrophage polarisation, with absence of either leading to a fixed polarisation state despite signals that would otherwise alter macrophage phenotype [219]. Macrophage-specific deletion of SOCS3 leads to constitutive STAT6 phosphorylation, decreased STAT1 binding to the promoters of Nos2 and Tnfa and increased STAT6 binding to Arg1 and CCL1 promoters. The results of this include LPS unresponsiveness with increased Treg recruitment during systemic inflammation that was rescued by neutralization of IL-10 and, to a lesser extent, IL-4 [219]. Similar findings were also noted in a study of SOCS3 in nephrotoxic nephritis (NTN). Liu et al observed that SOCS3+ macrophages were rare in healthy rat kidneys but were increased following induction of NTN. In this study there was also a less prominent increase in macrophages expressing SOCS1 and co-expressing SOCS3 and SOCS1. In another study, knockdown of SOCS3 with siRNA in macrophages followed by stimulation with LPS and IFN-γ resulted in enhanced STAT3 signalling, decreased synthesis of nitric oxide, IL-6 and CD86 as well as induction of markers usually associated with alternatively-activated macrophages [221]. Finally, treatment of rats with macrophages over-expressing the alternative activator IL-4 in NTN resulted in reduced number of SOCS3+ macrophages in the kidney at day 1 and day 7 with a reduction in glomerular injury implicating a role for SOCS3 in facilitating macrophage-mediated inflammatory damage within the kidney [221].
However the results of studies examining the effect of SOCS3 deletion in macrophages are not all consistent. For example, in a study by Qin et al, macrophage-specific deletion of SOCS3 had no effect on LPS-induced NF-κB p65 and ERK1/2 activation, but enhanced LPS-induced STAT1 and STAT3 activation. Deletion of SOCS3 in bone marrow-derived macrophages (BMDM) displayed an increased sensitivity to classical M1 polarising stimuli and enhanced the expression of M1- associated cytokines while inhibiting IL-10 expression. This was associated with increased macrophage phagocytic activity as well as increased promotion of Th1 and Th17-type T cell differentiation. In this study, a sublethal dose of LPS was associated with increased mortality in LysMCre-SOCS3 (fl/fl) mice [222]. Furthermore, other studies have shown overexpression of SOCS3 to be protective in endotoxic shock [223] and arthritis [223]. Thus, while it is clear from existing studies that SOCS3 exerts important effects on disease-associated macrophage polarisation, including inflammatory kidney disease, the specific influence of SOCS3 presence and expression level on the M1- and M2-type phenotypes appears to be complex and, possibly, context-dependent.

**SOCS3 and Diabetic Kidney Disease**

Incubation of human glomerular mesangial cells and renal proximal tubular cells under hyperglycaemic conditions has been shown to induce gene and protein expression of SOCS3 in time-dependent fashion when compared with control conditions. In these experiments, SOCS3 expression was maximal following four to eight hours of exposure to hyperglycaemic culture conditions and then decreased to baseline level. In contrast, SOCS1 was expressed later and remained elevated for a longer duration (48 to 56 hours).

Hyperglycaemia is associated with tyrosine phosphorylation of JAK2 and STAT1. Overexpression of SOCS1 and SOCS3 proteins in human mesangial cells resulted in inhibition of high glucose-induced JAK2 and STAT1 tyrosine phosphorylation. This resulted in inhibition of the induction of MCP-1, ICAM-1, IL-6, TGF-beta, collagen IV and fibronectin in both human mesangial cells and in the HK2 proximal tubular epithelial cell line.

In the *in vivo* setting, adenovirus-mediated overexpression of SOCS3 in diabetic rat kidney resulted in reduced phosphorylation of STAT1 and STAT3 in association with normalization of albuminuria, weight and creatinine clearance as well as improvement in glomerular hypertrophy, mesangial expansion, tubular atrophy/dilatation and interstitial fibrosis. In this
study, SOCS3 delivery also resulted in a reduction in the number of glomerular and interstitial macrophages in the diabetic kidneys. In keeping with reduced STAT signalling, intra-renal expression of MCP-1, RANTES, TGF-beta and collagen IV was reduced [224].

In a study of the kidneys of rats with streptozotocin-induced diabetes, SOCS1 and SOCS3 proteins were found to be expressed by glomerular mesangial cells (MCs), podocytes, and proximal tubular cells while the kidneys of non-diabetic rats has minimal SOCS3 expression. In keeping with this, renal biopsy samples from human subjects with diabetic nephropathy have been shown by immunohistochemistry to display SOCS1 and SOCS3 expression in podocytes and mesangial cells of the glomeruli as well as in proximal and distal tubular cells and in inflammatory cells within the interstitium [224].

The AT1 receptor contributes to hypertension, renal injury, atherosclerosis and cardiac hypertrophy by promoting inflammatory, proliferative and fibrotic responses in the kidney [138, 139]. Angiotensin II transduces its signal via G-coupled proteins to activate multiple intra-cellular signalling pathways in sequential fashion. Thus protein kinase C (PKC) signalling is triggered within seconds, MAPK/PI3K within minutes and JAK/STAT signalling within hours of AT1 engagement [225, 226]. Angiotensin II infusion into Wistar rats leads to leukocyte infiltration, mesangial matrix expansion and tubular damage [227]. It is also associated with increased intra-renal expression of SOCS3 and SOCS1 with increased expression being mainly induced in glomerular mesangial cells and tubular cells. Of interest, overexpression of SOCS3 prevented STAT3 activation in response to AII while knock down of SOCS3 by anti-sense RNA lead to increased AII-induced activation of STAT3 activation and c-Fos/C-Jun pathways in renal cells. In-vivo, knockdown of SOCS3 exacerbated AII-induced renal damage with increased fibrosis, tubular atrophy and cellular infiltration [205].

Immunoglobulins (antibodies) have an underappreciated role in renal diseases including diabetic nephropathy. In diabetes, proteins become antigenic as a result of alterations in their structure in response to hyperglycaemia or oxidative stress and the production of immunoglobulins in response to these altered proteins may be associated with immune complex formation and with abnormal proteinuria. In vitro, such immune complexes can stimulate production of MCP-1 and colony stimulating factor -1(CSF-1) and promote glomerular fibrosis [228]. Immunoglobulins complexed with oxidised LDL, can activate complement and induce pro-inflammatory cytokine through p38 MAPK, JNK and PKC.
pathways via ligation of Fcγ receptors (FcRγ) on mesangial cells and macrophages [229, 230]. Signalling through the FcRγ is important in mediating damage in immune and no-immune glomerulonephritis [231]. *In vitro*, mesangial cells treated with immune complexes have rapid induction of SOCS3 that does not occur in the absence of FcRγ. In an immune complex-mediated model of glomerulonephritis, expression of SOCS3 increased in parallel with proteinuria and development of renal lesions. For this model, mice deficient in FcRγ and rats treated with FcRγ blockade had reduced intra-renal SOCS3 and SOCS1 expression. In diabetic ApoE knockout mice, deficiency of the FcRγ chain is associated with less albuminuria, renal hypertrophy, glomerular immune deposits, mesangial matrix expansion and inflammatory cell infiltration along with reduced expression of genes related to leukocyte infiltration, fibrosis and oxidative stress [206].

In summary, expression of SOCS family proteins, particularly SOCS3, by multiple intra-renal cell types appears to play an important role in regulating macrophage infiltration and functional phenotype as well as other important aspects of the localised inflammatory response associated with diabetic kidney disease. Macrophage infiltration is well recognised in diabetic nephropathy and the regulation of macrophage function by SOCS proteins, dampening the further expression of pro-inflammatory cytokines, chemokines and growth factors in response to cellular stress, may be a key protective mechanism employed by the kidney to curtail damage. However, as demonstrated by studies of atherosclerosis, the role of SOCS proteins is complex and possibly alters as the disease progresses. Thus, while intra-renal expression of SOCS3 by non-immune cells may be protective by inhibiting pro-inflammatory signalling pathways, its expression by macrophages may be associated with a predominant pro-inflammatory (M1) effector with potentially negative consequences for disease progression. Better understanding of the expression of SOCS3 in diabetic kidney disease as well as in obesity may help to identify improved strategies for manipulating pro-inflammatory signalling pathways to prevent or slow the progression of kidney disease in these settings.

**Evidence for Increased Inflammation in Human Diabetic Kidney Disease**

In many human studies inflammatory cytokines and chemokines have been reported to be elevated within the kidney, plasma, urine and PBMCs. In some of these studies, cytokine/chemokine levels have been shown to correlate with duration of diabetes and with
urinary albumin excretion. For example, in a study of 296 type 1 diabetics with normoalbuminuria and 296 with microalbuminuria, those with microalbuminuria had higher urine concentrations of IL-6, CXCL-10, kidney injury molecule-1 (KIM-1), neutrophil gelatinase associated lipocalin (NGAL) and CCL2. However, in this study, there was no difference for any of these factors with regard to regression or progression of microalbuminuria [128].

In renal biopsies from patients with diabetic nephropathy, mRNA for IL-6 was found by in situ hybridization to be present in glomerular cells (mesangial cells and podocytes) and to correlate with mesangial expansion as measured by light microscopy. Expression of IL-6 mRNA was highest in biopsies showing moderate mesangial expansion. In the interstitium, infiltrating and atrophic tubules were also positive for IL-6 mRNA and this correlated with level of interstitial injury [232]. In a biopsy study of type 2 diabetics, levels of CRP, fibrinogen, serum amyloid A (SAA) and IL-6 were highest in biopsies of diabetics with overt proteinuria. In this study, there was a positive correlation between GBM width, albumin excretion rate and IL-6 levels [233].

In a study of 94 patients with T2DM (28 without diabetic nephropathy and 66 with diabetic nephropathy), the plasma concentrations of IL-6, TNF-α, IL-18 and CCL2 were higher compared to healthy non-diabetic controls in those with diabetic nephropathy but not in those without diabetic nephropathy [108]. In another study of type 1 diabetic children, significantly higher serum TNF-alpha and urinary IL-6 levels were found in those with microalbuminuria compared to those without microalbuminuria [234]. Furthermore, the concentrations of multiple serum markers of inflammation (including IL-6, CRP, sICAM-1, sPLA2) combined into a score were found to be associated with mortality and diabetic nephropathy in 199 type 1 diabetics with diabetic nephropathy when compared to diabetics with persistent normoalbuminuria [109]. Similarly, in 329 type 1 diabetics with normoalbuminuria, 84 with microalbuminuria and 128 with macroalbuminuria, plasma concentrations of CRP, IL-6 and TNF-α combined into an inflammatory score correlated with albuminuria, retinopathy and cardiovascular disease. Also, each unit increase in inflammatory score was associated with a decrease in GFR [235].
In a cohort of 296 type 2 diabetics of which 18 had progressive worsening of renal function, treatment with irbesartan was associated with a decline in plasma IL-6 levels which correlated with reduction in albumin excretion ratio [236, 237]. In the Finnish diabetic nephropathy study, 194 subjects with T1DM for 10-30 years were studied, of which 67 had normoalbuminuria and no cardiovascular disease, 64 had microalbuminuria and 63 had macroalbuminuria. In these subjects, plasma IL-6 concentration was found to increase with increasing grades of albuminuria and to correlate with ACR, HDL and duration of diabetes [238]. Two other studies have also documented correlations between circulating levels of inflammatory mediators and concomitantly measured GFR as well as subsequent loss of renal function [239, 240].

From these studies, it is evident that markers of inflammation increase as microvascular complications of diabetes occur. Frequently measured markers of inflammation such as IL-6 and TNF-alpha have been shown to correlate with urinary albumin excretion and GBM thickness which represent accepted surrogate markers for the subsequent development of overt diabetic nephropathy. However, the extent to which the measured concentrations of these inflammatory mediators in serum and urine reflect activity within the kidney is unknown. Thus, the question of whether specific inflammatory mediators and the cells responsible for their production represent important primary pathogenic factors in the initiation and progression of diabetic kidney disease remains to be resolved.
Key Knowledge Gaps, Hypotheses and Aims of the Project

Knowledge Gaps: At the time of planning and initiating the research project for this thesis, there was considerable published evidence from epidemiological, clinical, pre-clinical animal model and cellular biology studies that abnormal inflammatory activity, either systemic or intra-renal, occurs in the setting of diabetic kidney disease. While diabetic nephropathy is not typically regarded as an immune-mediated condition, studies which correlated inflammatory markers in the serum and urine as well as genetic and biopsy-based studies suggested a role for inflammation in the progression of kidney disease in type 1 and type 2 diabetics. However, the exact roles of the immune system in the early and later stages of the pathogenesis of diabetic nephropathy and the initiating signals responsible for its activation were not conclusively determined by published research. Indeed, with much of the focus of human clinical research being on patients with established nephropathy, it was not clear whether the detected abnormal inflammation/immune response represented a bystander effect of non-immune to damage incited by other pathogenic factors or a primary driver of renal parenchymal injury in its own right. Furthermore, although the onset of T2DM is typically preceded by years or decades of obesity, which is itself known to be associated with abnormal inflammation and specific forms of kidney injury, the degree to obesity-related intra-renal immune activation contributes to the pathogenesis of diabetic nephropathy was identified as a key unknown during the planning of this research project. The key questions that I set out to address in undertaking the research described the thesis were as follows:

1. When and how does the intra-renal immune-inflammatory system first begin to respond to obesity, hyperinsulinaemia, hypertension and hyperlipidaemia?

2. Is pre-diabetic obesity associated with increased numbers and/or activation state of immune cells within the different compartments of the kidney and, if so, what are the dominant immunological cell types present?

3. Are pre-diabetic obesity and early diabetic nephropathy associated with increased intra-renal IL-6-producing cells and with abnormal activation of the STAT3 intracellular signalling pathway?

4. Can RNA-based assays of urine cells be effectively adapted for non-invasive investigation of pathological intra-renal inflammation, specifically abnormal activation of the STAT3 signalling pathway in humans with obesity and T2DM?

5. Given the links between obesity and systemic inflammation, is there evidence among Irish patients receiving current standard of care for T2DM that obesity is an adverse modifier of the risk for developing diabetic nephropathy and progressive loss of renal function?
Overarching Hypothesis:
Obesity-triggered intra-renal inflammation is an important component of the early pathogenesis of kidney disease in pre-diabetes and T2DM.

Specific Aims and Hypotheses:
Specific Aim 1: A. Quantify and characterise immune cells infiltrating the kidney in the Zucker rat model of obesity and pre-diabetes prior to the onset of established renal parenchymal injury. B. Determine the status of intra-renal IL-6 production and STAT3 signalling pathway activation in the obese Zucker rat model of obesity and pre-diabetes prior to the onset of established renal parenchymal injury.

Hypotheses: A. Glomerular and tubulointerstitial infiltration by activated myeloid and lymphoid cells is an early pathogenic feature of the nephropathy in obese Zucker rats. B. Early tubulointerstitial infiltration of the kidney in the obese Zucker rat is associated with increased production of IL-6 by immune cells and increased STAT3 pathway activation in tubular epithelial cells.

Specific Aim 2: A. Optimise RNA extraction and quantitative RT-PCR from urine cell samples of human subjects with T2DM and preserved renal function. B. Apply qRT-PCR of urine cells to assess intra-renal inflammation in early diabetic nephropathy and obesity with an emphasis on IL-6 and the STAT3 signalling pathway.

Hypothesis: Early diabetic nephropathy in T2DM is associated with increased urine cell mRNA for IL-6, IL-6 receptor and STAT3-induced inflammatory mediators.

Specific Aim 3: A. Determine the relationship between obesity (as reflected in body mass index (BMI)) and renal disease (as reflected in estimated glomerular filtration rate (eGFR) and albumin creatinine ratio (ACR)) among a cohort of patients undergoing active outpatient management of T2DM at an Irish tertiary referral centre. B. Determine the influence of further weight gain on the risk for chronic kidney disease (CKD) among actively managed patients with T2DM.

Hypotheses: A. Higher BMI is independently associated with increased risk for diabetic nephropathy in T2DM. B. Changes in BMI during outpatient follow-up of type 2 diabetic patients modify the risk for progressive chronic kidney disease due to diabetic nephropathy.
Chapter 2: Intra-renal inflammatory Signalling and Cellular infiltration in the Development of Obesity Related Glomerulopathy in the Obese Zucker Rat
Introduction
High abundance, high calorie food coupled with a low energy expenditure lifestyle has led to a dramatic increase in the numbers of adults and children who are classified as overweight and obese in Western nations [2, 241]. Long-term medical complications of obesity include increased risk for development of hypertension, diabetes, cardiovascular disease, reproductive dysfunction and musculoskeletal disease [12]. Morbid obesity is associated with a reduced life expectancy of eight years [242]. In a prospective study of 900,000 adults, it was found that, for each 5 kg/m² increase in BMI, there was a 30% increase in mortality and 60-120% increase in the risk of mortality due to diabetes, kidney or liver disease [242].

Adipose tissue has important roles in insulation, mechanical support of organs, storage of energy substrates, whole body fatty acid homeostasis and hormone production [243]. It secretes biologically-active substances known as adipokines that have important local and systemic effects. The range of substances secreted from adipose tissue also includes cytokines, chemokines, complement components, growth factors and hormones [15]. These are important mediators of the various functions of adipose tissue in addition to facilitating physiological cross-talk between adipose tissue and other organs of the body. However, the effects of adipokines and other factors secreted by adipose tissue may become maladaptive as weight gain progresses and these likely represent key drivers of the detrimental end-organ damage associated with obesity.

Obesity is known to be associated with systemic increases in oxidative stress, inflammation and endothelial dysfunction [52]. Visceral adipose tissue is thought to be most metabolically active and important in secreting factors that mediate end-organ complications of obesity [244]. Visceral adipose tissue from obese individuals shows increased macrophage infiltration localised to areas of inflammation and hypoxia. Clusters of inflammatory macrophages within visceral adipose tissue of obese individuals are directly involved in the excessive secretion of adipokines, cytokines and hormonal mediators [33]. How this inflammation in adipose tissue affects other organs is of high interest as it may explain the increased risk of vascular, metabolic, renal, hepatic and oncologic disorders that occurs with increasing body mass index [242].

As discussed in detail in Chapter 1, an improved understanding of the changes that occur in the kidney in response to obesity is currently of high importance considering the worldwide
increase in prevalence of obesity, the evidence for specific obesity-related kidney disease and the strong links between obesity and the development of T2DM. As is well known, T2DM is a strong risk factor for the development of chronic kidney disease (CKD) and, although the incidence of ESRD due to diabetic nephropathy appears to be decreasing, the number of patients with T2DM continues to increase [119]. Development of diabetic nephropathy is not uniform. While it occurs more often in those with worse glycaemic control, the full range of factors which influence the risk for nephropathy among type 2 diabetics is not fully known [125, 135]. Obesity itself may represent an important amplifier of the risk for damage to the kidney among diabetic individuals, as susceptibility to either AKI or chronic kidney injury is increased in the setting of obesity [52, 75, 76]. Thus, understanding the changes that occur in the kidney in response to obesity may help to identify patients that are likely to develop diabetic nephropathy with the advent of T2DM.

By itself, obesity has been found to be a risk factor for CKD [245]. In similar fashion to diabetes, the development of renal impairment in response to obesity is not uniform [105]. For example, ORG, one of the best-described renal complications of obesity [54], is typically only manifest in a subset of people with extreme obesity [58]. Although the incidence of ORG is increasing, it remains rare despite the dramatic rise in morbid obesity in many countries [60]. More common among obese individuals is increased albuminuria and/or CKD of unspecified aetiology [69]. Although not completely understood, the underlying pathophysiology of these associations is likely to be related, directly or indirectly, to abnormal metabolism and adaptive changes within the kidney to accommodate the demands associated with the increase in body mass [246]. Additionally, differentiating the effects of early diabetes and cardiovascular risk factors from the direct effects of obesity on the kidney remains difficult given the frequent co-association of many of the same risk factors [69, 73].

Alterations to blood flow in the kidney in response to increased metabolic demand due to increased body size represents one mechanism by which obesity may lead to kidney dysfunction. Obese individuals have been found to have higher renal plasma flow (RPF), filtration fraction (FF) and GFR, with the latter occurring as a result of increased transcapillary hydraulic pressure due to afferent arteriolar dilatation [247]. Hyperfiltration is seen in both T1DM and T2DM, occurring early in the course of both conditions. Abnormalities in renal vasodilatation associated with diabetes and obesity also include increased proximal tubular reabsorption of sodium and glucose with suppression of tubulo-
glomerular feedback due to decreased sodium delivery to the macula densa [246].

Hyperfiltration in obesity may also occur as a consequence of glomerulomegaly with expansion of glomerular capillary wall diameter leading to increased tension on the glomerular capillary wall. Additionally, compression of the kidneys due to obesity-associated increased intra-abdominal pressure has the potential to restrict renal tubular flow. Finally, it is not entirely clear if increased glomerular filtration actually occurs within the kidney. While the absolute GFR is higher in obese individuals compared to individuals with ideal BMI, this apparent increase is not present when GFR is indexed for body surface area [248].

The obese Zucker rat (OZR) is a well-established small animal model of obesity-related kidney disease. This rat strain lacks a functional leptin receptor which leads it to be hyperphagic with resultant obesity, hypertriglyceridaemia and hyperinsulinaemia from an early age. Because these rats develop severe hyperinsulinaemia but frequently remain normoglycaemic for prolonged periods, they have proved useful for the assessment of changes in the kidney in response to obesity prior to the onset of T2DM. From 6 weeks of age, OZR display obesity and hyperinsulinaemia. By 14 weeks they typically develop impaired glucose tolerance and, by 24 weeks, are hypertriglyceridaemic. In parallel with these metabolic abnormalities, renal abnormalities develop including glomerular hypertrophy, widening of Bowman’s space, loss of podocytes and podocyte remodelling, all of which are detectable by electron microscopy by 9 weeks [249]. At this stage, within the proximal tubule, there is also loss of basolateral polarity, loss of mitochondrial elongated cristae and decreased basilar invaginating canalicular infolding [250]. At 6 weeks of age, increased albuminuria develops and this increases as the rats age and become more obese [251]. Nephrotic-range albuminuria is usually established in OZR by 12-14 weeks of age and glomerulosclerosis and mesangial matrix expansion are detectable by 24 weeks. Associated with this, glomerular gene expression of collagens and desmin, a marker of damage response in podocytes, are increased [87]. Increased GFR, reflecting hyperfiltration, develops along with proteinuria and glomerulosclerosis. Alterations in angiogenesis also occur in the OZR, with capillary density shown to be increased at 8 weeks but decreased at 22 weeks with associated decreased expression of the angiogenic markers fetal liver kinase (FLK1) and VEGF [252]. Of interest, OZR typically remain normotensive but eventually develop renal failure by 60 weeks of age from which the majority succumb [87].

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Although haemodynamic abnormalities occur within the glomeruli of OZR with development of progressive glomerulosclerosis, these do not appear to pre-date glomerular ultrastructural changes. In a study of early renal structural and functional changes in OZR, the obese animals were found to have normal inulin clearance compared to the lean controls [88]. In micropuncture studies carried out prior to the establishment of focal segmental glomerulosclerosis in OZR and lean controls, the obese rats had non-significantly increased superficial nephron GFR and plasma flow with no difference in intra-glomerular hydraulic pressures. Despite the lack of haemodynamic differences, there was evidence of increased glomerular area and mesangial matrix in both superficial and deep glomeruli in the obese animals [85]. This would indicate that glomerular structural damage in obese rats is driven by factors which are distinct from and occur earlier than haemodynamic abnormalities. At later time points (22-26 weeks age), elevated glomerular capillary pressure is detectable in OZR and the transcapillary hydraulic pressure difference in enalapril-treated obese rats was intermediate between that of lean and obese rats. Enalapril administration in this study was associated with reduced blood pressure, cholesterol, albuminuria kidney weight and incidence of FGS [253]. The authors suggested that non-haemodynamic effects of enalapril were responsible for the observed reduction in transcapillary hydraulic pressure. One candidate mediator of early glomerular functional and structural abnormalities and albuminuria in the OZR and, by extrapolation, in human subjects with obesity, is abnormal immune/inflammatory activity within one or more compartments of the kidney. Although the cellular and molecular components of the intra-renal immune response have not been studied in detail in the OZR model to date, particularly in younger animals in the early stages of kidney involvement, glomerular infiltration with mononuclear cells from 12 weeks of age has been documented [254]. A lack of macrophage infiltration in the interstitial compartment of kidneys of younger OZR has also been reported interstitum [86] although other immune/inflammatory cells and pathways were not evaluated in this study.

For this component of the thesis, the primary motivation for further studying the status of intra-renal immune and inflammatory responses in younger OZR derived from the potential for obesity-associated alterations within the kidney to serve as susceptibility factors for the development of nephropathy with progression of obesity and/or following the onset of T2DM. Based on the literature to date, I posited that the role of specific immunological cell types, cytokine mediators and the key intra-cellular signalling pathway, STAT3, had not been closely examined in this well-established model of “pre-diabetic” obesity-associated renal
disease. Using a combination of techniques which had been successfully applied in my mentors’ laboratories to study intra-renal immune response in a range of rodent kidney disease models [255-257], I set out to more definitively establish whether there is abnormal immunological activity within glomerular and tubulointerstitial compartments of the kidneys of OZR in comparison to lean Zucker rats (LZR) at two relatively early ages (14 and 18 weeks). Stimulated by the reported results from several studies of plasma, urine and kidney samples of diabetic human subjects with and without early evidence of diabetic nephropathy [122, 141, 232, 239], I specifically hypothesised that increased intra-renal expression of the cytokine IL-6 and increased activity of the STAT3 signalling pathway occurs in OZR kidney prior to the onset of overt renal structural damage. Detection of early immunological activity within the kidney of obese individuals would be useful both as a means for risk prediction and early diagnosis of CKD and as a potential therapeutic pathway to prevent progression of kidney disease associated with obesity and T2DM.
Material and Methods

Animals

Six-week-old male obese Zucker rats and control, lean Zucker rats (Charles River Laboratories, UK) were purchased and housed in a controlled environment with a photoperiod of 12 hours light-12 hours dark (lights on from 0700 to 1900) and a temperature of 20 ± 2°C until humane euthanasia at either 14 or 18 weeks of age using carbon dioxide asphyxiation. For blood draws at 12 and 17 weeks, animals were briefly anaesthetised with halothane. Housing, handling, and experimental procedures were approved by the Animal Care Research Ethics Committee of NUI Galway and performed under license by the Department of Health and Children of the Republic of Ireland. All animals were provided with standard rat chow ad libitum and tap water and were cared for by trained technicians under the supervision of a veterinary surgeon.

Measurements of Blood Glucose, Albuminuria

Blood samples were drawn from the left ventricle immediately after euthanasia using a 25 gauge needle and 2 ml syringe. Plasma was obtained by transferring collected blood into 1.5 ml Eppendorf tubes containing 0.05% EDTA followed by centrifugation at 8000 x g for 6 minutes and transfer of supernatants to fresh tubes. Urine samples were obtained by housing rats in a metabolic cage (Techniplast, London, UK) with access to water but not dry food for 24 hours. Urine was separately collected into a funnel with 0.1% sodium azide to prevent bacterial contamination of the sample. Plasma and urine samples were stored at -80°C prior to being thawed for individual analyses. Plasma glucose concentration was measured with a handheld glucometer (Accucheck®, Mannheim, Germany) according to the manufacturer’s instructions using blood obtained by tail vein puncture under halothane induced anaesthesia. Urine albumin concentration was measured using a commercial competitive ELISA kit (Nephrat II®, Exocel, Philadelphia USA). An eight-point, two-fold serial dilution of rat serum albumin was prepared with a starting concentration of 10 mg/dl and was added to wells of the ELISA plate in duplicate. Aliquots of 100 μl/well of the bovine serum albumin (BSA) diluent were added to remaining wells of the plate and 100 μl aliquots of each biological sample were then added to individual wells of the first row of the plate in duplicate. The samples were then further diluted two-fold through the remaining rows. Following this, 100 μl of Nephrat® conjugate were added to each well and the plate was incubated at room temperature for 30 minutes. The plate was emptied then washed in tap water six times with excess fluid blotted off. Next, 100 μl of TBM® colour-developer was
added to each well and the plate was incubated at room temperature for five minutes after which 100 µl of stop solution was added. Colour intensity (optical density) for each well was read at an absorbance of 450 nm on a Wallac 1420 Victor3™ multilabel Counter plate reader (Perkin Elmer, Waltham, MA, USA). Mean values for duplicate wells for standard and sample wells were obtained. A regression equation from the log concentration against linear absorbance was obtained and absorbance for samples substituted into the regression equation to obtain concentrations of albumin.

**Tissue Collection, Processing and Histological Analysis**

At 14 and 18 weeks, animals were euthanized using CO₂ asphyxiation. Following euthanasia, the abdomen of each animal was opened using a scissors and forceps and the left and right kidneys were dissected, decapsulated and cut longitudinally with a scalpel. A portion of kidney and spleen tissue was collected into Dulbecco’s modified eagles medium (DMEM) at 4°C for flow cytometry. Kidney pieces were fixed in 10% neutral buffered formaldehyde for at least 24 hours prior to being processed for histological analysis using a Leica® ASP 300 tissue processor (Wetzlar, Germany). Tissues were wax-embedded in a Leica® EG1150H wax embedder and 2 µM sections were cut using a Leica® RM2235 microtome. The sections were transferred to Superfrost Plus® microscope slides (Fisher Scientific, Dublin, Ireland) and were incubated overnight at 55°C to dry.

**Haematoxylin and Eosin Staining**

Tissue sections were de-waxed in xylene (three changes of 10 minutes each). Xylene was then removed by immersion in absolute ethanol for 5 minutes. The sections were brought to tap water through 95% and 70% ethanol solutions for 5 minutes each then washed in running tap water for 2 minutes. Sections were stained in Mayer’s Hematoxylin (Sigma-Aldrich, St Louis, USA) for 40 seconds and washed in running tap water for 5 minutes followed by staining in Eosin for 5 minutes. The sections were then rinsed in tap water quickly and dehydrated through graded ethanol solutions (70%, 95% and 100% for 3 minutes each). Finally, sections were cleared in xylene (two changes of 10 minutes each) then covered with DPX® mounting medium (Sigma-Aldrich) and cover slips were applied.

**Periodic Acid Schiff staining**

Sections were de-waxed in xylene (two changes of 5 minutes each). Xylene was removed in three changes of absolute alcohol for 2 minutes each. The sections were brought through two washes in 95% alcohol and one wash in 70% alcohol for 2 minutes each. The sections were then washed in phosphate buffered saline (PBS), pH 7.4-7.6 for 7 minutes twice followed by oxidation in 2% periodic acid solution (Sigma-Aldrich) for 5 minutes in the dark. Next, the
sections were washed in distilled water for 5 minutes followed by incubation in Schiff reagent for 20 minutes. Sections were washed in warm water for 7 minutes and counterstained in Mayer’s Hematoxylin for 2 minutes. Sections were washed in tap water for 5 minutes followed by dehydration in 70%, 95% and absolute alcohols for 1 minute each. Finally, the sections were cleared in xylene (two changes of 10 minutes each) and mounted in DPX® medium.

**Gomori’s Trichrome**
Sections were de-waxed in xylene (two changes of 10 minutes each). Xylene was removed in two changes of absolute alcohol for 2 minutes each. The sections were brought to tap water through 95%, 70% and 50% alcohols for 1 minute each. The alcohol was removed in running tap water for 2 minutes. The sections were oxidized in equal parts 0.5% potassium permanganate/0.5% sulfuric acid for 2 minutes then rinsed in tap water and bleached in 2% sodium metabisulphite for 2 minutes. Next, the sections were washed in water for 30 seconds followed by 70% alcohol for 1 minute and then stained in Gomori’s Aldehyde Fuchsin (Sigma-Aldrich) for 1 minute. This was followed by rinsing in water, 95% alcohol and water again for 10 seconds each. The sections were then stained in Celestine blue for 4 minutes, rinsed in water for 30 seconds followed by staining in Mayer’s Hematoxylin for 4 minutes. The sections were washed in water for 30 seconds and differentiated in acid alcohol (247.5ml of 70% absolute alcohol + 2.5ml of HCL) for 20 seconds. The sections were washed in running tap water for 4 minutes, stained in Masson’s cytoplasmic stain for 1 minute, rinsed in water and then differentiated in 1% dodeca-molybdophosphoric acid for 2 minutes. The sections were rinsed in water, counterstained in Fastgreen for 1 minute, differentiated in 1% acetic acid for another minute and then dehydrated through 50%, 70%, 95% and absolute alcohols for 1 minute each. Finally, the sections were cleared in xylene (two changes of 10 minutes each) and mounted in DPX® medium.

**Sirius Red staining**
Sections were de-waxed in two washes of xylene of 5 minutes each followed sequentially by 100% ethanol twice for 5 minutes, 90% ethanol twice for 3 minutes, 70% ethanol once for 3 minutes, distilled water for 5 minutes, Sirius Red solution for 60 minutes, 0.5% acetic acid twice for 30 seconds, distilled water twice for 30 seconds, 90% ethanol for five dips twice and 100% ethanol 5 dips twice. After this, the sections were cleared in Xylene twice for 3 minutes followed by mounting using DPX® mounting medium as described for the other stains.
**Light microscopic analyses**

Evaluation of all slides was performed by an observer who was blinded to the sample identities. Mesangial matrix expansion was defined as increased PAS positive material in mesangial regions while glomerulosclerosis was defined as adhesions and capillary obliteration in one or more segments of individual glomeruli. Using light microscopy (Leica DM4000, Germany) at a magnification of 40X, the degrees of mesangial matrix expansion and glomerulosclerosis were estimated according to the following semiquantitative scoring system: Grade 0: normal; Grade 1: <25% involvement of glomerular tuft; Grade 2: 25-50% involvement of glomerular tuft; Grade 3: 51-75% and Grade 4: >75% of glomerular tuft. A minimum of 30 glomeruli was scored from each tissue section with the average of the scores recorded as the final result for each sample.

Interstitial fibrosis was assessed on Gomori’s Trichrome-stained sections based on bright turquoise-stained areas within the interstitial compartment. At 20X magnification, twenty non-overlapping fields were randomly selected and were scored according to the following semiquantitative scoring system: Grade 0: <2% of the tubulointerstitial area; Grade 1: 2-4%; Grade 2: 5-10%; Grade 3: 11-25%; and Grade 4: >25%. The mean of the twenty scores for each section was calculated and recorded as the interstitial fibrosis grade for that section. In addition, to estimate the extent of collagen fibre accumulation in the interstitium, red-coloured areas of Sirius Red-stained sections were analysed by computer-assisted image analysis (Adobe Photoshop® CS5.5). For this analysis, twenty non-overlapping fields in each section were randomly selected and the total surface area of red-stained material based on a pre-determined colour threshold was calculated. The surface area for red-stained material (collagen) was then expressed as a proportion of the total surface area of the visualised tissue with glomeruli and larger blood vessels excluded. For each section, the average of all twenty readings analysed was recorded as the % Sirius Red positive (% interstitial fibrosis) for that sample.

The extent of interstitial cellular infiltration was determined on H&E-stained paraffin sections at a magnification of 20X in twenty non-overlapping fields from each section. The following semi-quantitative scoring system was used: Grade 0: no interstitial cellular infiltrates observed; Grade 1: cellular infiltrate involving 5% of the total interstitial area visualised; Grade 2: 5-10%; Grade 3:11-25%; and Grade 4: >25%. The average of the twenty
individual scores for each section was calculated and recorded as the final interstitial cellular infiltrate score for that sample.

**Immunohistochemistry**

Two micron sections were de-waxed in xylene (2 changes of 5 minutes each). Xylene was removed in 3 changes of absolute alcohol for 2 minutes each. Slides were brought through 2 washes in 95% alcohol and 1 wash in 70% alcohol for 2 minutes each prior rehydration in two washes of PBS for 7 minutes. Endogenous peroxidase was blocked by incubation of sections in 3% hydrogen peroxide:methanol for 20 minutes in the dark followed by a 7 minute PBS wash. Heat mediated retrieval in a citrate solution was used for antigen unmasking. Slides were placed in boiling 1% antigen unmasking solution (Vector Laboratories, California, USA) in double distilled water for 5 minutes at 121°C. Once cooled, slides were washed in PBS for 7 minutes. Slides were marked with liquid repellent slide marker pen and 1 drop of Avidin blocking solution was applied for 15 minutes to block endogenous biotin. Slides were washed in PBS for 7 minutes followed by application of 1 drop of biotin solution for 15 minutes. Slides were washed in PBS for 7 minutes followed by incubation in 100 μl of diluted primary antibody for 1 hour at room temperature according to Table 2.1. Primary and secondary antibodies were made up in 10% milk in PBS if polyclonal antibody or in PBS if monoclonal antibody. Slides were washed in PBS for 7 minutes followed by addition of 100 μl of diluted secondary antibody for 30 minutes at room temperature. All secondary antibodies were diluted in the same solution as the primary antibody at 1:300 concentrations. Slides were washed in PBS for 7 minutes followed by addition of 100 μl of avidin-biotin complex (ABC) reagent for 30 minutes. Slides were washed in PBS for 5 minutes and TRIS-HCl, pH 7.6 for 5 minutes. Slides were incubated in 3,3-diaminobenzidine (DAB) substrate solution for 2-10 minutes (see Table 2.1 for length of time per antibody) followed by counterstaining in methylgreen for 2 minutes. Slides were incubated in 95% alcohol twice for 10 seconds followed by 3 x 10 second incubations in absolute alcohol then 2 x 10 second incubations in xylene. Slides were mounted in DPX medium. Non-overlapping fields throughout the superficial and deep cortex at 20Xand/or 10X magnification or 40X magnification images of Glomeruli were photographed and subjected to image analysis using Adobe Photoshop® CS5.5.
Analysis of Immunohistochemistry
Evaluation of all slides was performed by a blinded observer who was unaware of the origin of the slides. Images were grey scaled prior to analysis. The number of CD3$^+$ positive T cells and cells positive for phosphorylated STAT3 was counted per high-power field at 20X magnification due to the discrete nature of the staining patterns. For CD68$^+$ cells and SOCS3 expression in glomeruli, more than 30 consecutive cross-sections of glomeruli at 40X magnification were photographed and subjected to image analysis using Adobe Photoshop® CS5.5. Mean values per glomerulus per section or number of glomeruli positive per section were calculated. To analyse planar glomerular areas in renal sections immunostained for desmin, IL-6 and synaptopodin, the outer edges of at least 40 glomerular tufts from each kidney section were traced manually on a video screen and the density of staining in the encircled areas was determined by computerized morphometry (Adobe Photoshop® CS5.5).

To obtain total counts CD68$^+$, expression of STAT3, SOCS3 and albumin, images were converted to greyscale and 20 non-overlapping fields throughout the superficial and deep cortex were photographed at 20X magnification and subjected to image analysis and aerial measurements using Adobe Photoshop® CS5.5. The area of positive signal was measured by an observer blinded to experimental groups and expressed as fraction of the area of the high-power field. Mean area/high power field was calculated.

Table 2.1: Immunohistochemical staining details

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Antigen retrieval</th>
<th>Primary antibody</th>
<th>Milk or PBS</th>
<th>Secondary antibody</th>
<th>DAB time</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD68</td>
<td>Autoclave</td>
<td>1:50</td>
<td>PBS</td>
<td>Mouse IgG1</td>
<td>10 min</td>
<td>Abcam</td>
</tr>
<tr>
<td>CD3</td>
<td>Autoclave</td>
<td>1:200</td>
<td>Milk</td>
<td>Anti-rabbit</td>
<td>2 min</td>
<td>Abcam</td>
</tr>
<tr>
<td>STAT-3</td>
<td>Autoclave</td>
<td>1:600</td>
<td>PBS</td>
<td>Anti-mouse IgG2a</td>
<td>2 min</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>P-STAT3</td>
<td>Autoclave</td>
<td>1:200</td>
<td>Milk</td>
<td>Anti-rabbit</td>
<td>10 min</td>
<td>Cell Signaling</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Autoclave</td>
<td>1:800</td>
<td>Milk</td>
<td>Anti-rabbit</td>
<td>2 min</td>
<td>Abcam</td>
</tr>
<tr>
<td>Desmin</td>
<td>Autoclave</td>
<td>1:800</td>
<td>Milk</td>
<td>Anti-rabbit</td>
<td>5 min</td>
<td>Santa-Cruz</td>
</tr>
<tr>
<td>Synaptopodin</td>
<td>Autoclave</td>
<td>1:600</td>
<td>Milk</td>
<td>Anti-rabbit</td>
<td>5 min</td>
<td>Santa-Cruz</td>
</tr>
</tbody>
</table>

List of antibody concentration, antigen retrieval method, diluting substance, secondary antibody type (all at 1:300 concentration) and length of time of incubation in diaminobenzadine (DAB) and manufacturer.

Collagenase Digestion and Multicolour Flow Cytometry of Spleen and Kidney Tissue

Single Cell Suspension Preparation from Kidney
Cell suspensions of rat kidney tissue were prepared by collagenase and DNase digestion. Freshly dissected, decapsulated kidneys were weighed and diced with a scalpel into pieces of...
approximately 1 mm³. The kidney pieces were incubated at 37°C in sterile 15 ml tubes for 30 minutes in 5 ml of a solution of collagenase 20X Type 1 (Sigma-Aldrich) (0.4 mg/ml) and 40X DNase I (Sigma-Aldrich) with vigorous mixing on a vortex every 10 minutes. Following this, any remaining tissue clumps were crushed with a sterile pestle and the suspension was pipetted up and down repeatedly. The enzymes were then neutralized by addition of 10 ml of culture medium and cells were pelleted by centrifugation at 1500 rpm for 5 minutes. The supernatant was discarded and 4 ml of ACK lysis buffer [(ammonium chloride lysis buffer (10x): ammonium chloride (1.5 M) sodium bicarbonate (100 mM) disodium EDTA (10 mM) adjusted to pH 7.4] were added followed by incubation at room temperature for 15 minutes and neutralisation with 10 ml of FACS buffer (PBS, 2%FBS, 0.05% sodium azide). The cells were re-pelleted then re-suspended in 5 ml of FACS buffer. This final cell suspension was filtered through a 50 μM nylon mesh and viable cells were counted using a standard haematocytometer.

**Single Cell Suspension Preparation from Spleen**
Portions of freshly dissected spleen were weighed and placed into 1.5 ml Eppendorf tubes then cut into pieces with a scissors and re-suspended in 1 ml of FACS buffer. The cell suspensions were then filtered into a 15 ml tubes through a 30 μM mesh. Following this, 6 ml of FACS buffer waere added and the cells were pelleted by centrifugation on a table-top centrifuge at 1500 rpm for 5 minutes. The supernatant was discarded and 1 ml of ACK lysis buffer was added followed by incubation at room temperature for 15 minutes and neutralisation with 10 ml of FACS buffer. The cells were re-pelleted then re-suspended in 5 ml of FACS buffer.

**Staining and Flow Cytometric Analysis of Kidney and Spleen Cells**
For staining of cells prior to flow cytometric analysis, 100 μl aliquots a 2x10⁶ cells/ml single cell suspensions in FACS buffer were added to 3 ml FACS tube (Fisher Scientific) and rat serum (Sigma-Aldrich) was added to 10% followed by incubation for 10 minutes at 4°C. Next, fluorochrome-labelled antibodies were added at previously optimised dilutions followed by incubation in the dark for 20 minutes at 4°C in the dark (See Table 2.2 for details of all antibodies used). At the end of the incubation period, 1 ml of FACS buffer was added to each tube and the cells were pelleted by centrifugation at 1500 rpm for 5 minutes. The cells were washed in FACS buffer then re-suspended in 100 μl of FACS buffer to which 50 μl of Count bright™ (Invitrogen) bead suspension were added immediately before
analysis. Count bright™ absolute counting beads are a calibrated suspension of microspheres that are brightly fluorescent across a wide range of excitation and emission wavelengths and contain a known concentration of microspheres allowing for calculation of absolute numbers of individual cell types.

Flow cytometry was performed using a FACSCanto® flow cytometer (Becton-Diskinson, San Diego, CA, USA) and the resulting data files were analysed using the FlowJo® software package (TreeStar, Olten, Switzerland)
Table 2.2: List of Antibody and Isotype Controls used for Multicolour Flow Cytometry of Single Cell suspension of Rat Spleen and Kidney Tissue

<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Isotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>PE-Cy7 Mouse anti-rat CD45 Clone OX-1 BD Pharamingen</td>
<td>Mouse IgG1 κ</td>
</tr>
<tr>
<td>APC Anti-rat CD45 Clone OX-1 eBioscience</td>
<td>Mouse IgG1 κ</td>
</tr>
<tr>
<td>Rat T Lymphocyte cocktail</td>
<td></td>
</tr>
<tr>
<td>APC anti-rat CD3 Clone 1F4</td>
<td></td>
</tr>
<tr>
<td>PE anti-rat CD4 Clone OX-25</td>
<td></td>
</tr>
<tr>
<td>FITC anti-rat CD6a clone OX-8 BD Pharamingen</td>
<td></td>
</tr>
<tr>
<td>FITC Anti-rat CD45RA Clone OX-33 BD Pharamingen</td>
<td>Mouse IgG1 κ</td>
</tr>
<tr>
<td>PE Anti-rat MHC class II Clone OX-6 Abd sterelec</td>
<td>Mouse IgG1 κ</td>
</tr>
<tr>
<td>APC Anti-rat CD11bc Clone OX-42, Biologend</td>
<td>Mouse IgG2a κ</td>
</tr>
<tr>
<td>RTC Anti rat MHC class II Biologend</td>
<td>Mouse IgG1 κ</td>
</tr>
<tr>
<td>RTC anti rat CD95RA Clone OX-33 Biologend</td>
<td>Mouse IgG1 κ</td>
</tr>
</tbody>
</table>

Quantitative Reverse Transcription and Polymerase Chain Reaction (qRT-PCR)

Tissue samples were stored in RNaLater® solution (Ambion, Texas, USA) in a -80°C freezer prior to processing for RNA extraction and qRT-PCR. Initially, tissue samples were allowed to defrost then were placed into new RNase-free 1.5 ml Eppendorf tubes. Next, the samples were homogenized for 1 minute using a pestle cleaned with RNaseZap® solution (Ambion) then were re-suspended in 1 ml of Trizol® solution (Invitrogen, Paisley UK) and pipetted up and down to dissolve the tissue. The solution was left to stand for 5 minutes before chloroform (Sigma-Aldrich) (200μl) was added and the tubes were shaken vigorously for 30 seconds. Samples were left to stand for a further 2 minutes followed by centrifugation at 12,000 x g for 15 minutes at 4°C. The upper aqueous layer (RNA) was removed carefully and was placed into clean RNase-free Eppendorf tubes following which 500 μl of isopropanol were added. The tubes were gently inverted 4 times, left at room temperature for 10 minutes then centrifuged at 12,000 x g for 10 minutes at 4°C and placed on ice. The isopropanol was removed and 1 ml of 75% ethanol was added. The tubes were inverted 4 times and centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol was removed and the RNA pellets
were left to air-dry for 10 minutes before being re-suspended in 50 µl of RNase-free water and dissolved in a heat block at 55°C for 10 minutes. The quantity and quality of RNA was measured using a Nanodrop ND-1000® spectrophotometer. Equal quantities of RNA from each sample were DNase-treated by adding 10x TURBO® DNase buffer (Ambion) and 1 µl of TURBO® DNase followed by gentle mixing. Samples were incubated at 37°C for 20 minutes. DNase inactivation reagent was added (10% of final volume) and mixed thoroughly. Samples were incubated for 2 minutes at room temperature. Centrifugation was performed at 10,000 x g for 90 seconds and the RNA was transferred to fresh tubes prior to re-measurement of RNA content. Next, 1 µg of RNA from each sample was reverse transcribed using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor® on a GeneAmp 9700 A® Thermal Cycler (Applied Biosystems, Carlsbad, California, USA). The resulting cDNA samples were subjected to Real Time qPCR using inventoried TaqMan® gene expression assays and reagents and a StepOne Plus® Real Time PCR System (Applied Biosystems) according to the manufacturer’s protocols. For qPCR, 10 µl reactions mixtures were prepared in duplicate consisting of 1 µl of cDNA, 5 µl of TaqMan® Universal Master Mix II, no UNG, 0.5 µl of primers and probes, and 3.5 µl of RNase free (all reagents from Applied Biosystems). The comparative CT method was employed to determine relative quantification and controls consisting of ddH2O were included in all runs [258]. Target genes were normalized to β-actin and expressed as fold-change relative to an appropriate reference sample.

**Statistical Analysis**

The Student’s t-test was used for two-group analyses and ANOVA test for three or more groups using GraphPad Prism® v5.0 software package for Windows (San Diego, California USA). Correlations were determined using Pearson’s correlation test. For all experiments, results are expressed as mean ± SEM and p values <0.05 were considered statistically significant.
Results:
Metabolic Characteristics of Experimental Animals
The obese Zucker rats displayed increased body weight at both 14 and 18 weeks compared to their lean counterparts but there was no difference in kidney weight between the groups at either time point (Table 2.3). At 14 weeks age, obese rats had higher blood glucose concentrations compared to lean controls although these were within the normal range. At 18 weeks age, however, average blood glucose concentrations remained within normal range and were not significantly in obese compared to lean rats. At 18 weeks, obese rats had markedly higher urine albumin excretion compared to lean rats (Table 2.3). In keeping with increased albuminuria detected in the obese rats, albumin staining by immunohistochemistry was increased in the tubules of obese rats (Figure 2.2B).

Table 2.3: Metabolic Parameters of Obese and Lean Zucker Rats at 14 and 18 weeks.

<table>
<thead>
<tr>
<th>Weeks</th>
<th>Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (grams)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>292±20</td>
<td>440±16**</td>
</tr>
<tr>
<td>18</td>
<td>386±14.9</td>
<td>528±15.83***</td>
</tr>
<tr>
<td>Kidney weight (mg)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>1538±15.7</td>
<td>1532±35.9</td>
</tr>
<tr>
<td>18</td>
<td>1553±146</td>
<td>1462±87</td>
</tr>
<tr>
<td>Blood glucose (mmol/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>4.33±0.11</td>
<td>5.15±0.19***</td>
</tr>
<tr>
<td>18</td>
<td>4.58±0.31</td>
<td>4.91±0.32</td>
</tr>
<tr>
<td>Urine albumin (mg/mmol)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>27.62±9.8</td>
<td>391±89.55**</td>
</tr>
<tr>
<td>Plasma IL-6 (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>116.6±19.37</td>
<td>122.4±16.12</td>
</tr>
<tr>
<td>Urine IL-6 (pg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>106.6±8.70</td>
<td>88.5±13.43</td>
</tr>
</tbody>
</table>

Body and kidney weights were measured at 14 and 18 weeks. Rats were housed at week 17 in metabolic cages for 24 hours and urine was collected for measurement of urine albumin and urine IL-6 concentration using ELISA technique. Blood glucose concentration was measured from tail vein puncture at 14 and 18 weeks using a glucometer. Plasma IL-6 concentration was measured in serum prepared from blood collected from left ventricle at sacrifice. Data are represented as mean ± SEM for 6 animals per group for each time point. (**p=<0.001, ***p=0.0001 compared with lean Zucker rats, Student’s t-test).
Glomerular Abnormalities-Cellular Infiltration

Despite increased albuminuria at 18 weeks, the kidneys of obese rats showed no overt glomerular abnormalities by light microscopy compared to those of lean rats. Specifically, blinded scoring of histological staining of sections of kidney for mesangial matrix expansion, an early pathological abnormality in diabetic glomerulopathy [120], indicated no significant difference between obese and lean rats at both 14 and 18 weeks (Figure 2.1). Similarly, scoring for glomerulosclerosis, another recognised feature of diabetic nephropathy [120] as well as obesity related glomerulopathy [259], was no different between the two groups (Figure 2.1).

Desmin, an intermediate filament normally restricted to the mesangium, is expressed by podocytes in response to injury and is associated with glomerular hypertrophy [260]. Despite the lack of overt glomerular abnormalities on histologically stained kidney sections of obese rats, immunohistochemical staining and computer-assisted image analysis clearly demonstrated increased glomerular expression of desmin in these animals at 18 weeks (Figure 2.2B). Additionally, as measured by immunohistochemical staining for CD68+, there was evidence of intra-glomerular cellular inflammation in the form of higher numbers of macrophages at 18 weeks but not at 14 weeks (Figure 2.2A). Glomerular infiltration by CD3+ T-cells was a rare event in both groups and was not further enumerated.
Figure 2.1 Scoring for Mesangial Matrix Expansion and Glomerulosclerosis in Obese and Lean Zucker Rats at 14 and 18 weeks: (A) Representative images of 2 µm Periodic acid Schiff stained glomeruli at 14 and 18 weeks at 40X magnification. (B) Using light microscopy at a magnification of 40X, glomeruli were scored for mesangial matrix expansion and glomerulosclerosis in 30 glomeruli per section and the average score per section was calculated. Mesangial matrix expansion was defined as increased PAS-positive material in mesangial regions greater than width of capillaries while glomerulosclerosis was defined as adhesions and capillary obliteration in the same segment of glomerulus. The degree of mesangial matrix expansion and sclerosis was scored adapting the semiquantitative scoring system, consisting of grade 0, normal, grade 1 <25% involvement of glomerular tuft, grade 2, 25-50% involvement of glomerular tuft grade 3, 50-75% and grade 4 occupying >75% of glomerular tuft. Data are represented as mean ± SEM and 6 animals were used per group for each time point. (Obese compared with lean Zucker rats using Student’s t-test)
Figure 2.2 Glomerular CD68\(^{+}\) cells and Expression of Desmin and Albumin Kidneys of Obese and Lean Zucker Rats: (A)  
Upper: Representative images of CD68\(^{+}\) immunostaining in glomeruli of kidney sections from obese and lean Zucker rats at 14 and 18 weeks at 40X magnification. Images were converted to greyscale prior to analysis. Differences in levels of background staining are due to different methylgreen counterstain intensities between 14 and 18 week samples. Lower: Mean counts of CD68\(^{+}\) cells in 40 glomeruli in each section were generated by a blinded observer at both time points. (B) Upper: Representative images for desmin and albumin immunostaining at 18 weeks for lean and obese rats at 40X magnification. Lower: For quantitation of glomerular desmin, images were converted to greyscale, the outer perimeters of the glomeruli were outlined manually on a video screen and the percentage density of staining in the encircled areas was used to quantify desmin. Albumin staining was graded as 0 no staining, 1 minimal staining, 2 moderate staining, 3 heavy staining and 4 very heavy staining on 40 glomeruli in each section by a blinded observer. Average scores were calculated for each tissue section. Data are represented as mean ± SEM of 6 animals per group for each time point. (**p=<0.001 compared with lean Zucker rats for each time point, Student’s t-test).
Analysis of Glomerular Expression of STAT3 and SOCS3

Using immunohistochemical staining, I next examined whether expression of components of the JAK-STAT pathway, a key intracellular signalling pathway for expression of inflammatory cytokines, interferons and the haemodynamic mediator angiotensin, were altered in the glomeruli of obese compared to lean rats [261]. In fact, there was minimal glomerular staining for STAT3 and no detectable expression of phosphorylated STAT3 (pSTAT3) within the glomeruli of either obese or lean Zucker rats at 14 and 18 weeks. As shown later for the tubulointerstitial compartments of these kidneys, this negative result was not due to lack of sensitivity of the staining protocols used. However, the number of glomeruli with positively staining cells for SOCS3 was significantly increased in the glomeruli of obese rat at both time-points (Figure 2.3A). Sequential sections from obese rats at 18 weeks were stained for SOCS3, CD68+ and desmin to assess whether expression levels of these three markers were co-associated in individual glomeruli (Figure 2.3B). It was found that the number of SOCS3+ foci and the number of CD68+ cells within individual glomeruli were correlated with each other. In addition, desmin staining was higher in glomeruli with SOCS3+ foci compared to glomeruli without SOCS3+ foci (Figure 2.3C). Thus, glomeruli in which there was more damage and inflammation as indicated by increased inflammatory cells and desmin staining contained increased expression of SOCS3.

Obesity associated glomerulopathy is associated with an increase in glomerular size with a corresponding decrease in podocyte density and number [262]. Therefore, we assessed the expression of synaptopodin, a podocyte-specific protein. However, there was no difference between obese and lean rat kidneys for glomerular synaptopodin expression at 18 weeks indicating that podocyte number and density was unlikely to be significantly altered despite the presence of increased glomerular macrophages and increased albuminuria (Figure 2.4).
(A) 14 weeks 18 weeks

Obese

Lean

Glomerular SOCS3

% glomeruli with SOCS3

* ***

(B) SOCS3 CD68+ Desmin

Obese

Glomerular SOCS3 and CD68+ in Obese Zucker rats

R² = 0.3129

Desmin expression in glomeruli with and without SOCS3 staining

% area per glomerulus

Negative Positive

***
Figure 2.3: Expression of SOCS3 in Glomeruli of Obese and Lean Zucker rats and Co-expression of SOCS3, CD68+ and Desmin in Glomeruli of Obese Zucker rats

(A) Representative images for SOCS3 immunostaining in sections of kidney tissue from obese and lean Zucker rats at 14 and 18 weeks. Images were converted to greyscale prior to analysis. For each section, 40 glomeruli were selected randomly and the number of glomeruli containing SOCS3 positive foci in each section was calculated by a blinded observer and expressed as a percentage of total glomeruli.

(B) 2 µm sequential sections of obese rat kidneys at 18 weeks were stained for SOCS3, CD68+ and desmin. Sequential glomeruli were identified and scored for the number SOCS3 positive foci, for the number of CD68+ cells and for expression of desmin. Pearson’s correlation test was used to determine correlation between glomerular SOCS3 and CD68+ cells. The same glomeruli with SOCS3 and desmin staining were identified and desmin images were converted to greyscale and the outer perimeters of individual glomeruli were outlined and densitometry was used to quantify desmin staining in at least 40 glomeruli/section. Expression of desmin was quantified separately in glomeruli positive or negative for SOCS3. Data are represented as mean ± SEM for 6 animals per group. (**p=<0.001 compared with lean Zucker rats, ***p=< 0.0001 compared with SOCS3 negative at 18 weeks, Student’s t-test)

Figure 2.4 Expression of Glomerular Synaptopodin and Albumin in Obese and Lean Zucker Rats at 18 Weeks: (Left) Representative images for synaptopodin immunostaining in kidney sections of obese and lean Zucker rats at 18 weeks. Images were converted to greyscale prior to analysis. The perimeters of glomeruli were outlined manually on a video screen and the percentage of the total glomerular area with positive staining was calculated for a total of 40 glomeruli in each section by a blinded observer using computerized morphometry (Adobe Photoshop® CS5.5). (Right) The quantitative data is represented graphically as mean ± SEM for a total of 6 animals per group.
Tubulointerstitial abnormalities- Cellular infiltration

**Multicolour flow cytometry-Kidney**

Cellular infiltration of the tubulointerstitial compartment by bone marrow-derived mononuclear cells is a feature of kidney biopsies from patients with advanced diabetic nephropathy [120]. However, histological analysis is a relatively insensitive method for detecting increased numbers of immunological cells in renal tissue and it is not currently known whether early stages of diabetic nephropathy or obesity-related kidney disease are associated with abnormal interstitial cellular infiltration. To address this, collagenase digestion and multicolour flow cytometry of rat tissue was applied to accurately compare the proportions and absolute numbers of myeloid cells and T cells within the kidneys of obese and lean rats at 14 and 18 weeks of age. On the following pages, Figures 2.5 and 2.6 provide examples of multi-colour flow cytometric analyses of kidney cell suspensions for individual immune cell subtypes along with graphical representations of the absolute numbers of each cell type for the two groups of animals at both time-points. Initially, total bone marrow-derived cells were identified by staining for the common leukocyte marker CD45+ (**Figure 2.5A**). Although there was a trend towards increased numbers of CD45+ cells in the obese rat kidneys at 14 weeks this was not present at 18 weeks (**Figure 2.5A**). Next, the myeloid cell subsets (including monocytes, macrophages and dendritic cells) were analysed by combing staining for CD45+ with staining using antibody with specificity for the myeloid-cell-specific integrin chains CD11b+ and CD11c+. As shown in **Figure 2.5B**, CD45+CD11b/c+ myeloid cells were readily identified and were equally abundant in the kidneys of both lean and obese rats at both 14 and 18 weeks. The total myeloid cells were further subdivided, using a third fluorochrome-labelled antibody, into populations with and without high expression of major histocompatibility complex class II (MHC II) proteins. The CD45+CD11b/c'MHC II' subset represents mature dendritic cells or macrophages while the MHC II' subset is likely to consist primarily of monocytes and other immature myeloid cells. For this analysis also, there were no significant differences between the kidneys of obese and lean rats at either time point (**Figure 2.5C**). Finally, intra-renal T cells were analysed by combining CD45+ staining with staining for CD3+ (Total T cells), CD3+CD4+ (Helper T cells) and CD3+CD8+ (Cytotoxic T cells). As for the myeloid cell analyses, no significant differences were found between the kidneys of obese and lean rats at either 14 or 18 weeks of age (**Figure 2.6A**).

As it was possible that relative differences among the individual immune cell types could be present in the kidneys of obese rats in the absence of differences in absolute cell numbers, the
data generated by multi-colour flow cytometry was also analysed in terms of proportionate representations. This included analysis of the proportions of all immune cells (CD45+) within the total kidney-derived cells as well as proportions of the individual immune cell subtypes within the CD45+ population. **Table 2.4** provides a summary of this data which indicates that there were also no striking differences in the relative abundance of myeloid cell and T cell subtypes in obese compared to lean rat kidneys. This data is consistent with immunohistochemical analysis of kidney tissue (presented subsequently) in which only subtle differences in the absolute and proportional amounts of myeloid and T cells subsets within the tubulointerstitial regions of OZR kidneys. Thus, although it is well recognised that an inflammatory infiltrate becomes prominent as kidney disease progresses in both humans and animals with diabetes [149, 254], it is not evident, at least in the OZR model of pre-diabetic obesity, that early glomerular inflammation and injury is accompanied by increased interstitial infiltration/expansion of immune cells
Figure 2.5 Myeloid Cell Subsets Detected in Kidney Cell Suspensions from Obese and Lean Zucker Rats at 14 and 18 Weeks using Multi-Colour Flow Cytometry. Kidney tissue was digested with collagenase and DNase and the resulting single cell suspensions were stained for myeloid subsets with fluorochrome-labelled antibodies. Using CountBright™ beads, the numbers of cells per mg of tissue were calculated for each subset. (A) Dot plot-based gating examples and numerical data for total bone marrow-derive (CD45+) immune cells in rat kidney cell suspensions at 14 and 18 weeks. (B) Dot plot-based gating examples and numerical data for total myeloid cells (CD45+CD11b/c+). The dot plots shown are gated on CD45+ cells. (C) Dot plot-based gating examples and numerical data for MHC II+ and MHC II- myeloid cells. The dot plots shown are gated on CD45+CD11b/c+ cells. (For all analyses, Student’s t-test used to compare lean and obese rats)
Figure 2.6 T cell subsets detected in kidney cell suspensions from obese and lean Zucker rats at 14 and 18 weeks using multi-colour flow cytometry. Kidney tissue was digested with collagenase and DNase and the resulting single cell suspensions were stained for T cell subsets with fluorochrome-labelled antibodies. Using CountBright™ beads, the numbers of cells per mg of tissue were calculated for each subset. (A) Dot plot examples and numerical data for total T cells (CD3⁺) (upper) and for CD4⁺ and CD8⁺ T cell subsets (lower) at 14 weeks. The dot plots shown are gated on CD45⁺ cells (for total T cells) and on CD45⁺CD3⁺ cells (for CD4⁺ and CD8⁺ T cells). (B) Equivalent data is shown for 18 week samples. (Student’s t-test used to compare lean and obese rats)
Table 2.4: Myeloid and T cell subsets Detected in Kidney from Obese and Lean Zucker Rats at 14 and 18 weeks using Multi-Colour Flow Cytometry.

<table>
<thead>
<tr>
<th></th>
<th>Weeks</th>
<th>14</th>
<th>18</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Lean</td>
<td>Obese</td>
</tr>
<tr>
<td>CD45+ (As a proportion of total cell suspension)</td>
<td></td>
<td>4.29±1.1</td>
<td>2.84±0.26</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.67±0.63</td>
<td>2.99±0.19*</td>
</tr>
<tr>
<td>CD45CD11bc+ (As a proportion of CD45+ cells)</td>
<td></td>
<td>52.82±2.85</td>
<td>56.67±0.84</td>
</tr>
<tr>
<td></td>
<td></td>
<td>53.38±4.8</td>
<td>56.38±0.78</td>
</tr>
<tr>
<td>CD45+CD11bc+MHC class II (As a proportion of CD34+CD11bc+ cells)</td>
<td></td>
<td>47.05±10.5</td>
<td>56.37±1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59.24±2.68</td>
<td>53.45±2.0</td>
</tr>
<tr>
<td>CD3+ (As a proportion of CD45+ cells)</td>
<td></td>
<td>0.47±0.16</td>
<td>0.37±0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.59±0.16</td>
<td>0.50±0.02</td>
</tr>
<tr>
<td>CD3+CD4+ (As a proportion of CD45+CD3+ cells)</td>
<td></td>
<td>37.34±2.54</td>
<td>44.87±2.368</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.82±10.53</td>
<td>48.12±4.16</td>
</tr>
<tr>
<td>CD3+CD8+ (As a proportion of CD45+ CD3+cells)</td>
<td></td>
<td>31.73±11.73</td>
<td>42.02±4.604</td>
</tr>
<tr>
<td></td>
<td></td>
<td>36.02±8.725</td>
<td>41.72±4.894</td>
</tr>
</tbody>
</table>

Proportions of each population of kidney cells detected by flow cytometry at 14 and 18 weeks. Data are presented as mean ± SEM for 6 animals group at 18 weeks and 5 animals per group at 14 weeks *p=<0.05 compared with lean Zucker rats. (Student’s t-test used to compare lean and obese rats).
Multicolour Flow Cytometry-Spleen

Abnormalities in the immune system of obese Zucker rats are known to occur reflecting the role of leptin in regulating some immune activities [263]. Therefore I assessed differences in proportions of myeloid cells and T cells in the spleens of obese and lean Zucker rats at 14 and 18 weeks. There was a small but statistically significant reduction in the proportion of CD45+ cells among the total spleens cells of obese compared to lean rats at 18 weeks but not at 14 weeks (Figure 2.7A). Within the CD45+ cells, the proportions of myeloid cells (CD11b/c+) and, among these, the proportions that were MHC II+ and MHCII- did not differ at either time-point (Figure 2.7B and C). As regards T cells, the proportion of CD3+ cells (total T cells) among the CD45+ cell population was similar for obese and lean rats at both 14 and 18 weeks (Figure 2.8A and B). Among the total T cells, the proportions that were CD4+ (helper) and CD8+ (cytotoxic) were similar at both time points (Figure 2.8B and C).

Overall, the results of immune-phenotyping of spleen cells at 14 and 18 weeks were consistent with minor differences between obese and lean rats at 18 weeks with no evidence of a major abnormality of the splenic compartment. The differences that were observed may reflect a primary effect of leptin receptor dysfunction or a secondary effect of metabolic abnormalities.

This flow cytometry data suggests that there were only subtle changes in the systemic cellular inflammation between the obese and lean Zucker rats at both time points. The Zucker rat model is known to have deficiencies in T cell mediated function but from this data at 14 and 18 weeks it does not appear to be the cause of the lack of difference in intra-renal cellular inflammation at these two time points [264].
Figure 2. Proportional Representation of Total Immune Cells and Myeloid Cell Subsets in Spleens of Obese and Lean Zucker rats at 14 and 18 weeks Analysed by Multi-Colour Flow Cytometry. Single cell suspensions prepared from spleens were stained with antibodies against the common leukocyte antigen CD45 and the myeloid-specific marker CD11b/c. (A) Proportions of CD45+ cells among total viable spleen cells at 14 and 18 weeks (B) Proportions of CD11bc+ cells among the CD45+ cell populations at 14 and 18 weeks. (C) Proportions of mature (MHC II+) and immature (MHC II−) cells among the CD11bc+ cells at 14 and 18 weeks. Data are represented as mean ± SEM for 6 animals per group at 18 weeks and 5 animals per group at 14 weeks. (Student’s t-test used to compare lean and obese rats)
Figure 2.8 Proportional Representation Total T Cells and T Cell Subsets in Spleens of Obese and Lean Zucker Rats at 14 and 18 Weeks analysed by Multi-Colour Flow Cytometry. Single cell suspensions prepared from spleens were stained for total T cells (CD3) and for the helper (CD4) and cytotoxic (CD8) T cell subsets. (A) Proportions of CD3^+ cells among the total CD45^+ cell populations (upper) and proportions of CD4^+ and CD8^+ cells among the total CD3^+ cells (lower) of obese and lean rats at 14 weeks. (B) Equivalent data for obese and lean rats at 18 weeks. Data are represented as mean ± SEM for 6 animals per group at 18 weeks and 5 animals per group at 14 weeks. (Student’s t-test used to compare lean and obese)
Tubulointerstitial abnormalities-Immunohistochemistry for Cellular Infiltration
In addition to macrophages, other cells of the immune system have been implicated in the
development of diabetic complications, with mast cells [265] and T cells found to comprise
elements of the inflammatory infiltrates of adipose tissue in obese subjects and with T cell
number increasing in parallel with the number of macrophages [266]. However, as shown in
the preceding sections, I did not detect an increase in the numbers or proportions of total bone
marrow-derived immune cells or of the major immune cell subtypes in the kidneys of obese
Zucker rats at 14 and 18 weeks using multicolour flow cytometry. Indeed, my findings by
flow cytometric analysis were consistent with previous reports using other techniques for
evaluating interstitial immune cell infiltrates in this model [86, 87]. To further investigate
interstitial macrophage and T cell populations in the obese and lean rats,
immunohistochemistry and quantitative image analysis was carried out on kidney tissue
sections from 14 week and 18 week old rats. As shown in Figure 2.9A, there was a trend
toward increased interstitial CD68+ cells at 18 weeks although this did not reach statistical
significance (obese vs. lean, 0.19 ± 0.03 vs. 0.09 ± 0.03 % area/HPF, p=0.06). A similar
analysis of interstitial T cells by anti-CD3 immunohistochemistry revealed no difference at
either time point (Figure 2.9C).
Figure 2.9: Immunohistochemical Staining and Quantitative Analysis of CD68\(^+\) and CD3\(^+\) cells in Tubulointerstitium of Obese and Lean Zucker Rat Kidneys at 14 and 18 weeks. (A) Representative images for CD68\(^+\) immunostaining at 10X magnification in obese and lean Zucker rat kidneys at 14 and 18 weeks. Differences in background staining are due to batch variation of the methylgreen counterstain. Images were converted to greyscale prior to analysis. For each section, 10 non-overlapping fields of view were randomly selected. Image analysis for density of staining was carried out by a blinded observer using Adobe Photoshop Cs5.5®. (B) Representative images for CD3\(^+\) immunostaining at 20X magnification in obese and lean Zucker rat kidneys at 14 and 18 weeks. Images were converted to greyscale prior to analysis. For each section, 20 non-overlapping fields of view were randomly selected. Counting of CD3\(^+\) cells/high power field was used to calculate expression due to the discrete nature of T cell staining. This was performed by a blinded observer using Adobe Photoshop® Cs5.5. Data are represented as mean ± SEM for 6 animals per group at each time point. (Student’s t-test used to compare lean and obese sections)
Tubulointerstitial Histology and STAT3-SOCS3 Pathway Analysis

Light microscopy

Light microscopy was used to evaluate tissue sections from obese and lean Zucker rat kidneys for tubulointerstitial abnormalities including interstitial fibrosis, increased collagen deposition and interstitial cellularity. Interstitial fibrosis was assessed by blinded semi-quantitative scoring of Gomori’s trichrome stained-sections at both 14 and 18 weeks. This analysis revealed no difference between obese and lean groups at either time-point (Figure 2.10A and B). Interstitial collagen accumulation was calculated for week 18 only from Sirius Red-stained sections. For both groups, Sirius Red staining was predominantly localised to peri-glomerular and peri-arterial regions with only limited peri-tubular staining. Using a colour-based computer-assisted image analysis approach, there was no difference detected in interstitial collagen between obese and lean rat kidneys at 18 weeks (Figure 2.10A and B).
Figure 2.10 Interstitial Fibrosis, Collagen Deposition and Cellular Infiltrates in the Kidneys of Obese and Lean Zucker Rats at 14 and 18 Weeks. (A) Representative images of 2 µm Gomori’s Trichrome and Sirius Red stained section at 20X magnification. (B) 20 non-overlapping fields were scored for interstitial fibrosis by a blinded observer as follows: grade 0 indicating absence of fibrosis, grade 1, fibrosis involving 5% of the total interstitial area, grade 2, fibrosis involving 5-10%, grade 3, fibrosis involving 10-25% and grade 4, fibrosis involving >25%. Average score per high power field was calculated. Sirius Red stain score was calculated in 20 non-overlapping fields at 20X magnification using Image analysis for density of staining. This was carried out by a blinded observer using Adobe Photoshop® Cs5.5. (C) Representative images of 2 µm H&E stained sections from obese and lean rats at 14 and 18 weeks. Interstitial cellular infiltrates were determined from blinded scoring of 20 non-overlapping fields at 20X magnification from each section. The following scoring system was used grade 0, absence of infiltrate, grade 1, infiltrate involving 5% of the total interstitial area, grade 2, infiltrate involving 5-10%, grade 3 infiltrate involving 10-25% and grade 4 infiltrate involving >25%. Data are represented as mean ± SEM for 6 animals per group for each time point. (Obese compared with lean Zucker rats using Student’s t-test)
Immunohistochemistry for STAT3 and SOCS3

Immunohistochemical staining of kidney sections for STAT3 revealed increased distal tubular staining in obese compared to lean Zucker rats (Figure 2.11A) at 18 weeks. Immunostaining for phosphorylated (p)STAT3 demonstrated clusters of pSTAT3+ cells in both distal tubular epithelium and interstitium (Figure 2.11B). This staining was significantly increased in kidneys of obese compared to lean rats at 14 weeks and a similar trend was present at 18 weeks although this did not reach statistical significance (Figure 2.11B). When tubular epithelial and interstitial cell staining for pSTAT3 were separately analysed in the 18 week kidney sections, no significant differences between obese and lean were observed (Figure 2.11C). Immunostaining for SOCS3 demonstrated widespread expression in proximal as well as distal tubular epithelium of both obese and lean Zucker rats. Of interest, the staining pattern was apical in proximal tubules and basal in distal tubular cells. There was a trend toward increased tubulointerstitial staining for SOCS3 in the kidneys of 18 week old obese rat although this did not reach statistical significance (Figure 3.10D).
Figure 2.11 Immunohistochemical Analysis of STAT3, pSTAT3 and SOCS3 Expression in Tubulointerstitium of Obese and Lean Zucker Rats. (A) Representative images of STAT3 immunostaining in kidney sections of 14 and 18 weeks old obese and lean Zucker rats. 20 non-overlapping images at 20X magnification were analysed. Images were converted to greyscale prior to analyses. Image analysis for density of staining was carried out by a blinded observer using Adobe Photoshop® Cs5.5. (B) Representative images of phosphorylated STAT3 immunostaining in kidney sections of 14 and 18 week old in obese and lean Zucker rats. 20 non-overlapping images at 20X magnification were analysed. Images were converted to greyscale prior to analyses. Image analysis for number of cells positive for pSTAT3 staining was carried out by a blinded observer using Adobe Photoshop® Cs5.5. This was possible due to the discrete nature of phosphorylated STAT3 staining in the sections. Average count per high power field was used calculated for each rat at each time point. (C) Representative images of pSTAT3 counterstained with PAS. Sections for pSTAT3 were counterstained with PAS to allow visualisation of basement membrane. 20 non-overlapping fields at 20X magnification were analysed and counts of interstitial and tubular pSTAT3 in obese and lean Zucker rat kidneys at 18 weeks was carried out by a blinded observer. (D). Representative image of SOCS3 immunostaining of kidney sections at 18 weeks in obese and lean Zucker rats. 20 non-overlapping images at 20X magnification were randomly selected for analysis. Images were converted to greyscale prior to analyses. Image analysis for density of staining was carried out by a blinded observer using Adobe Photoshop® Cs5.5. Data are represented as mean ± SEM for 6 animals per group for each time point. (* p=<0.01, i p=0.05, obese compared with lean Zucker rats using Student’s t-test)
Expression of STAT3, SOCS3 and Related Inflammatory Transcripts in Zucker Rat Kidneys

Expression levels of mRNA transcripts for STAT3, STAT1, SOCS3, IL-6 receptor components and related cytokines and chemokines were analysed by quantitative RT-PCR of cortical and medullary kidney tissue from obese and lean Zucker rats at 14 and 18 weeks (results summarised in Table 2.5). Overall, there were no striking differences between obese and lean rat kidney at either time-point although there were trends toward increased STAT3 and CCL2 expression in the renal cortex of obese rats at 18 weeks. Comparisons of mRNA expression levels between the two time-points also revealed no striking changes for either group. However, renal cortical STAT3 and medullary SOCS3 mRNA levels were increased at 18 compared to 14 weeks in obese but not lean rats.
Table 2.5: Summary of Quantitative RT-PCR Analysis of Cortex and Medulla from Obese and Lean Zucker Rat kidneys at 14 and 18 Weeks.

<table>
<thead>
<tr>
<th>Target</th>
<th>Weeks</th>
<th>14 Lean</th>
<th>Obese</th>
<th>18 Lean</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Cortex</td>
<td>1.07±0.06</td>
<td>0.81±0.05*</td>
<td>1.04±0.06</td>
<td>0.84±0.14</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>1.14±0.11</td>
<td>0.95±0.06</td>
<td>1.07±0.06</td>
<td>0.94±0.07</td>
</tr>
<tr>
<td>STAT3</td>
<td>Cortex</td>
<td>1.05±0.05</td>
<td>1.00±0.07</td>
<td>1.01±0.09</td>
<td>1.49±0.22*</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>1.03±0.02</td>
<td>1.12±0.04</td>
<td>1.76±0.42</td>
<td>1.33±0.18</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Cortex</td>
<td>1.02±0.07</td>
<td>1.04±0.34a</td>
<td>0.81±0.26</td>
<td>0.67±0.12a</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>1.37±0.19</td>
<td>1.39±0.19a</td>
<td>3.01±1.27</td>
<td>2.47±0.33a</td>
</tr>
<tr>
<td>CCL2</td>
<td>Cortex</td>
<td>2.23±0.57</td>
<td>1.45±0.53</td>
<td>0.85±0.15</td>
<td>1.49±0.27*</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>1.15±0.11</td>
<td>1.53±0.18</td>
<td>2.19±0.37</td>
<td>2.15±0.69</td>
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<tr>
<td>CXCL1</td>
<td>Cortex</td>
<td>1.88±0.83</td>
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<tr>
<td>IL-6</td>
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<tr>
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<td>1.04±0.20</td>
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<td>0.81±0.08</td>
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<td>1.57±0.23</td>
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<tr>
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<td>Medulla</td>
<td>0.83±0.05</td>
<td>0.94±0.04</td>
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<td>IL-6R</td>
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<td>0.68±0.04</td>
<td>1.17±0.20</td>
<td>1.02±0.21</td>
</tr>
<tr>
<td></td>
<td>Medulla</td>
<td>1.11±0.26</td>
<td>0.99±0.10</td>
<td>1.94±0.48</td>
<td>1.55±0.10</td>
</tr>
</tbody>
</table>

Pieces of kidney cortex and medulla were placed in RNAlater® prior to RNA extraction. Real Time qPCR was used to analyse expression of IL-6 and its related receptor components (IL-6R, IL-6-ST) as well as intracellular signalling components (STAT3, STAT1 and SOCS3) and STAT3-regulated chemokines (CXCL1, CCL2). Results for qPCR from cortex and medulla were normalised to a single lean rat kidney control. Lean and obese cortex and medullary tissue at 14 and 18 weeks were compared using Student’s t-test and ANOVA was used to analyse differences in expression at 14 and 18 weeks. Data are represented as mean ± SEM for 6 animals per group at each time point. (*p<0.05 between lean and obese samples at the same time point, a p<0.05 for lean vs. obese samples between 14 and 18 weeks)
IL-6 Expression in Urine, Serum and Renal Parenchyma of Obese and Lean Zucker Rats

Interleukin 6 is frequently cited as being an important pro-inflammatory cytokine that exhibits systemic and localised increased expression in diabetes, cardiovascular disease and progressive diabetic nephropathy. Given this, assays were performed to determine whether IL-6 was elevated in the urine, plasma or kidneys of obese rats at an early time point preceding the established overt structural damage that is associated with this model. However, no difference was observed in the concentrations of IL-6 in either the urine or plasma of obese rats compared to their lean counterparts (Table 2.6), nor was there any evidence of increased IL-6 protein within the kidneys of obese rats 14 weeks (Figure 2.12). These results suggested that IL-6 is not likely to be active in initiating the early renal damage associated with the obesity in the Zucker rat.

Table 2.6: Plasma and Urine Concentrations of IL-6 in Obese and Lean Zucker Rats at 18 Weeks

<table>
<thead>
<tr>
<th>IL-6 ELISA</th>
<th>pg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>LZR Plasma</td>
<td>116.6±19.37</td>
</tr>
<tr>
<td>OZR Plasma</td>
<td>122.4±16.12</td>
</tr>
<tr>
<td>LZR Urine</td>
<td>106.6±8.70</td>
</tr>
<tr>
<td>OZR Urine</td>
<td>88.5±13.43</td>
</tr>
</tbody>
</table>

IL-6 concentrations in both serum and urine collected from OZR and LZR at 18 weeks was measured using ELISA. Data are represented as mean ± SD for 6 animals per group.
Figure 2.12 Immunohistochemical Analysis of IL-6 Expression in the Kidneys of Obese and Lean Zucker Rats at 14 weeks. Representative images of IL-6 immunostaining at 14 weeks in obese and lean Zucker rat kidneys. 20 non-overlapping images at 20X magnification were analysed for total IL-6 expression. Images were converted to greyscale prior to analyses. Image analysis for density of staining was carried out by a blinded observer using Adobe Photoshop® Cs5.5. For glomerular expression, the outer edges of at least 30 glomerular tufts from each kidney were traced manually on a video screen and the staining in the encircled areas was determined by computerised morphometry (Adobe Photoshop® CS5.5). Mean percentage of area stained per high powered field or glomerulus was recorded for each specimen. Data are represented as mean ± SEM for 6 animals per group.
**Discussion and Conclusions**
Renal cellular damage in diabetes is associated with increased intra-renal expression of pro-inflammatory cytokines and chemokines. This occurs in the context of activation of multiple immunological cell types which then serve to exacerbate glomerular and tubulointerstitial injury leading to albuminuria, hypertension, progressive loss of kidney function and increased risk for cardiovascular morbidity and mortality [142]. Established diabetic nephropathy is known to be associated with infiltration by immunological cells and exacerbated by the activity of pro-inflammatory signalling pathways. However, the role played by cellular inflammation in the early stages of renal injury associated with obesity, metabolic syndrome and T2DM is not well established.

Using a rat model which lacks a functional leptin receptor, leading the hyperphagia, obesity, hyperinsulinaemia and hyperlipidaemia, this project aimed to investigate the presence of cellular inflammation and activation of IL-6/STAT3 inflammatory signalling pathway within the kidney in the setting of obesity and pre-diabetic metabolic abnormalities. The analyses were carried out at time points preceding the development of light microscopically detectable structural changes known to occur within the kidney in this model [86, 87, 254, 267]. As we confirmed here, the kidneys of OZR demonstrate no obvious light microscopically detectable structural changes of the glomeruli such as mesangial matrix expansion or glomerulosclerosis at 14 or 18 weeks. Despite this, the obese rats had higher urinary albumin excretion and greater tubular albumin staining on immunohistochemistry consistent with disruption of glomerular permselectivity. A further indication of podocyte dysfunction was reflected in the detection of an altered pattern of desmin expression in the glomeruli from obese compared to lean rats although synaptopodin staining was preserved.

Podocyte injury in the OZR appears to be an important cause for the development of focal segmental lesions [267]. Ultrastructural changes that have been reported to occur to the podocyte include engorgement with albumin-enriched endocytic vesicles and detachment of podocytes from the glomerular basement membrane. This causes collapse of the capillary loops with progressive disappearance of capillary endothelial cells and formation of hyaline and lipid deposits. Following this, focal adherence of the glomerular basement membrane to the basement lamina of Bowman’s capsule and the synthesis of new matrix occurs [267]. In this project, my analyses were limited to light microscopy and immunohistochemistry with a focus on cellular inflammation and activation of related signalling pathways. Based on
previous studies, it is likely that electron microscopic analysis would have demonstrated distinct ultrastructural abnormalities of the podocytes of obese rats at the time-points examined [87]. There is variability in the development of lesions within this model and this may reflect the degree of hyperglycaemia that is present with greater injury occurring with animals with overt hyperglycaemia. It is of note that some studies of younger OZR report no differences in blood glucose concentration between lean and obese rats [86, 254, 268] while others report significant hyperglycaemia in the young obese animals [269, 270]. In the obese rats I examined for this project, blood glucose remained within normal limits and did not differ between lean and obese at 18 weeks age. Thus, the results of the study, which included subtle inflammatory changes of the glomeruli without clear evidence of cellular inflammation of the tubulointerstitial compartment, should be interpreted as reflecting intra-renal events in the setting of severe obesity and pre-diabetic metabolic abnormalities as opposed to established T2DM.

My findings are in keeping with those of Lavaud et al., who noted that inflammatory changes occurred in the glomeruli but not the tubulointerstitium of obese Zucker rats at an early time point [86, 254]. Our study used both multicolour flow cytometry and immunohistochemical techniques to assess the presence of cellular inflammation, and glomerular and tubulointerstitial damage in the kidney. Immunohistochemical staining demonstrated the presence of increased glomerular CD68+ macrophages in the glomeruli of obese rats. However, while there was a trend towards increased CD68+ cells in the tubulointerstitium at 18 weeks, the application of flow cytometric analysis of kidney cells suspensions to accurately quantify myeloid and T cell numbers indicated no discernible differences between lean and obese rats at either time point. Additionally, I used sequential sections to assess the relationship between glomerular immune cell infiltration and activity of the STAT3-SOCS3 signalling pathway.

Given that there was evidence of glomerular damage and cellular inflammation in the obese rats at both 14 and 18 weeks, I elected to examine whether activation of a key pro-inflammatory intracellular signalling pathway was increased in the glomeruli or tubulointerstitial compartment of obese rats at the same time-points. Based on reported experimental evidence for a pathogenic role of the STAT3 pathway in diabetic kidney disease [195, 197, 198, 201, 202, 203, 224], I performed a series of immunostaining and other analyses to determine expression levels and activation of STAT3 itself along with expression
of a key regulator of the pathway, SOCS3 and of downstream transcriptional targets involved in inflammation. The STAT3 signalling pathway is activated in response to a range of cytokines, hormones and growth factors and leads to the up-regulation of pro-fibrotic and pro-inflammatory gene expression. In addition to the expression of these factors, expression of the JAK/STAT signalling inhibitor, SOCS3 is also increased and serves to negatively regulate these responses. As shown in the results section, there was no evidence for STAT3 pathway activation in the glomeruli of obese rats at either time point as determined by immunostaining for pSTAT3 in the glomeruli of obese rats. What was noted, however, was increased numbers of glomeruli with foci of positive staining for SOCS3 in the kidneys of obese rats. Furthermore, glomeruli with SOCS3 positivity demonstrated greater expression of desmin indicating that SOCS3+ cells were specifically associated with the presence of an activated glomerular stress response. Additionally, the number of these foci correlated with the number of intra-glomerular CD68+ cells. Glomeruli with SOCS3 positive foci were more common in obese rats at 14 and 18 weeks, whereas increased glomerular infiltration with CD68+ was present only at 18 weeks. This would indicate that SOCS3 is activated early on in the course of the development of nephropathy, prior to the inflammatory cell infiltrate that is a feature of the nephropathy. Although not all SOCS3 positive areas corresponded to CD68+ cells, there were many that did co-stain. SOCS3 positive macrophages are a feature of inflammatory processes within the glomeruli in animal models of pauci-immune GN in which the proportions of macrophages that are positive for either SOCS3 or SOCS1 is noted to change as the inflammatory process evolves [221]. Our finding of SOCS3/CD68+ co staining within glomeruli that were more damaged on the basis of desmin staining suggests that there is an inflammatory process active within the glomeruli and that this may be mediated by infiltrating macrophages. Literature regarding the role of SOCS3 as a pro- or anti-inflammatory factor suggests that it is context-dependent [216, 217, 219, 222]. Further phenotypic characterisation of intra-glomerular SOCS3+ cells and elucidation of their role in the inflammatory process associated with obesity (or diabetes) could be achieved through sieving of glomeruli from OZR kidneys followed by fluorescence-activated sorting of myeloid populations or by in situ techniques such as laser capture microdissection.

Interstitial fibrosis, increased collagen deposition and interstitial cellular infiltrates may be important consequences of glomerular inflammation and damage leading to the development of progressive kidney dysfunction. Interstitial and vascular lesions are scored separately from glomerular lesions in the renal pathological classification of diabetic nephropathy. In a biopsy
A series of 488 human diabetic nephropathy sections, normal appearing tubules and interstitium was associated with the most favourable 5 and 10 year clinical outcomes [149]. Cellular infiltration by myeloid cells is a feature present in advanced diabetic nephropathy and has been reported by others to peak within the glomeruli and tubulointerstitium of obese Zucker rats at 14 weeks age and to decrease after this. The fact that I did not observe increased interstitial macrophages or increased expression of inflammation-related transcripts at 14 or 18 weeks in this study may reflect differences in the degree of hyperglycaemia among the obese rats I analysed compared to those studied by others. For example, in the study of Coimbra et al., mild hyperglycaemia was present from 14 weeks and persisted throughout the study [86, 87].

In the study of Lavaud et al., in which the development of interstitial fibrosis in OZR model was specifically examined, accumulation of excess extracellular matrix was found to occur from an age of 12 weeks with collagen type III and fibronectin being the initially detectable matrix proteins at this time-point. Subsequently, increasing accumulation of these and other collagens, including collagens type IV and type I, was shown [86]. From 24 weeks on, collagen type I was detected in large foci and interstitial fibrosis was accompanied by inflammatory cell infiltration [86]. Of note, the obese rats analysed by these authors were not overtly hyperglycaemic throughout the study. In the current project, I assessed the extent of interstitial fibrosis using Masson’s Trichrome and Sirius red staining using non-polarised light in contrast to Lavaud et al. who employed polarised light for quantification fibrosis. While, polarised light results in identification of larger collagen fibril (such as collagen type III and fibronectin) it may also result in underestimation of collagen content within the kidney [271]. Nonetheless, I did not observe differences between the lean and obese animals as regards renal interstitial fibrosis at either 14 and 18 weeks. Although this may reflect differences in the techniques used in comparison to Levaud et al., it is unlikely that an overt increase in interstitial fibrosis was present in the kidneys of the young obese rats studied for this thesis despite the presence of glomerular inflammation and abnormal albuminuria.

Although neither expression of STAT3 nor activation of STAT3 signalling was detected within glomeruli of obese rats, increased tubulointerstitial expression and clusters of activated STAT3 were found at 14 weeks with a trend toward increased activation at 18 weeks. This was despite the interstitial compartment being free from cellular infiltration and overt injury. Furthermore, as noted previously, there were no striking differences in renal parenchymal...
expression of IL-6 and its related receptor components, or of the pro-inflammatory chemokines CXCl1 and CCL2 (MCP-1) between obese and lean Zucker rats. This is consistent with other studies in which inflammatory transcripts have found not to be elevated in the Obese rat unless diabetes is also present [272].

Overall, my results for this project suggest that, despite the presence of glomerular inflammation and albuminuria as well as evidence of focal tubulointerstitial increases in STAT3 signalling at 14 weeks, there was not a progression towards widespread triggering of STAT3 signalling and overt inflammation and fibrosis between 14 and 18 weeks. There are caveats to the OZR model specifically with regard to the role of leptin in the immune system as leptin has been shown to be important for Th-1-type T cell differentiation and to influence the secretion of acute phase reactants [264]. However, it is unlikely that the lack of renal interstitial inflammation in the obese rats represents an anomalous effect of leptin signalling deficiency as there was evidence of intra-glomerular inflammation present that was comparable to that described for other animal models of obesity and for human ORG. Furthermore, as evidenced by spleen flow cytometric analysis of myeloid and T cell subsets and by IL-6 plasma and urine levels, there was no significant alteration to the immune system of these animals.

However, despite the lack of differences in cellular and systemic immune system activity, the model appeared to have a restrained form of inflammation within the kidney that is not associated with progressive kidney damage. This has been noted in multiple other tissue such as pancreas, cardiac and neuronal tissue in the OZR, where progressive tissue damage does not occur until another form of acute of chronic injury is superimposed [273, 274]. This is also noted in obese humans where outcomes following sepsis or mechanical ventilation are worse than those with normal BMI [52, 77]. This raises the possibility that tissue-specific inflammatory activity in obesity and pre-diabetes is held in check at steady-state by counter-regulatory mechanisms. My study shows high expression of SOCS3 in tubular epithelium of obese albuminuric rats and implicates this protein as a potentially important negative regulator of pro-inflammatory signalling in the kidney.

In summary the results of this project, combined with those previously reported by others, favour the following conclusions regarding the role of inflammation in early renal injury in the OZR model of obesity:
(a) Damage begins in the glomerulus, with an initial intra-glomerular inflammatory response evident by 18 weeks age that is not mediated by haemodynamic factors.

(b) Early glomerular injury is manifest by abnormal albuminuria but is not associated with severe podocyte loss or light microscopically evident structural changes in the glomerulus.

(c) Glomerular inflammation and albuminuria do not immediately initiate interstitial inflammation, immune cell infiltration or interstitial fibrosis although there is transient evidence of localised increase of STAT3 pathway activation in tubular epithelial as well as interstitial cells.

(d) The early intra-renal inflammatory process of the Zucker rat occurs in the context of high tubular epithelial expression of SOCS3 which may serve as a key negative regulator of potentially pro-inflammatory JAK/STAT signalling triggered by albuminuria or other metabolic abnormalities.

With additional time and resources, I believe that further important experimental work could be performed to more precisely determine the role of SOCS3 and other potential counter-regulatory mechanisms in determining the nature and consequences of intra-renal inflammation in obesity and T2DM. Additional support for a reno-protective function of SOCS3 comes from a mouse model in which renal injury is induced by 72-hour angiotensin II infusion. In this model, SOCS3 was found to be upregulated within the kidney of infused animals and its suppression by siRNA was shown to be associated with increased expression of STAT3 and its target genes c-jun/c-fos with a resulting increase in renal damage [205]. Experiments to modulate renal tubular SOCS3 expression in the OZR as well as in other animal models of obesity or to study the intra-renal expression of SOCS3 in the setting of superimposed AKI would be of particular interest in order to better determine the significance of this protein as a regulator of inflammation in the kidney.
Chapter 3 Analysis of Urine Cells for Evidence of Intra-Renal Pro-Inflammatory Signalling in Obesity and Type 2 Diabetes Mellitus
**Introduction**

Although diabetic nephropathy represents the most common primary diagnosis among patients receiving renal replacement therapy for ESRD, there is not a reliable method for predicting which individuals with T2DM will develop nephropathy and progressive renal dysfunction [119]. A biomarker is a substance that is objectively measured and evaluated as an indicator of a normal biological process, pathological process or pharmacological response to a therapeutic intervention[275]. Clearly, there is a need for better biomarkers to distinguish patients who are increased risk of developing diabetic nephropathy, thereby allowing earlier intervention [275].

Examples of biomarkers currently used in kidney disease include serum creatinine (SCr) concentration and urine albumin measurements. Although of undoubted clinical value, there are drawbacks to these current biomarkers in diagnosing or predicting progression of chronic kidney disease. For example, creatinine, a non-protein-bound substance that is secreted by muscle and is freely filtered by the kidney, is the current marker by which kidney function is most commonly measured. However, because proximal tubular secretion accounts for 20% of its excretion as well as metabolism by gut bacteria, it can underestimate GFR in the setting of decreased renal function. Additionally, it is an insensitive and non-specific marker for the cause of kidney disease. Furthermore, increase in SCr is a relatively late marker of kidney dysfunction and may only be detectable when renal damage is already well established.

Increased urine albumin excretion is the other biomarker-based test that is commonly employed in patients with T2DM. When functioning perfectly, the glomerulus forms a barrier which prevents excessive loss of albumin into Bowman’s space and excessive albumin content in the urine reflects damage either to the glomerular filtration barrier or to re-uptake mechanisms in proximal tubule. In diabetic patients, increased albuminuria may develop during clinical follow-up but, once detected at a low level (microalbuminuria) may spontaneously revert to normal in up to 40% of cases [125]. Spontaneous remission of albuminuria in diabetic nephropathy is less likely to occur as albumin excretion rate increases [131]. Furthermore, diabetic patients may also present with loss of renal function in the absence of increased albuminuria, often necessitating a renal biopsy to establish the diagnosis. Indeed the rate of decline of GFR in type 2 diabetic patients has been reported to be the same in normo- and micro- and macroalbuminuric patients, indicating that albuminuria
is not a particularly good marker for progression in this setting [276]. Conversely, a substantial proportion of type 2 diabetics with microalbuminuria were found on biopsy to have normal glomerular and tubulointerstitial appearance [277]. Interestingly, albuminuria may be a more accurate marker of increased cardiovascular risk than of progressive nephropathy in T2DM, as recent studies have demonstrated that any amount of albumin in the urine above a negative albumin-creatinine ratio (ACR) is associated with increased cardiovascular risk[134]. Currently, renal biopsy for diabetics is only indicated where the clinical picture is suspicious for an alternative diagnosis to diabetic nephropathy. Importantly, while often considered to be the diagnostic “gold standard” for renal disease aetiology, renal biopsy is also associated with the potential for misdiagnosis on the basis of sampling error.

Thus, despite the availability of well-established clinical testing approaches for diagnosis of diabetic nephropathy, there remains a clear need to establish more accurate, non-invasive biomarker-based tests that reliably diagnose kidney disease at its earliest stage in patients with T2DM. Furthermore, the development of easily performed diagnostic tests that correlate with future risk for progressive loss of kidney function (as well as other adverse outcomes) and that provide additional information regarding the primary pathophysiological process contributing to renal parenchymal injury would be a distinct advance over current clinical standard of care. It should be noted that there are advantages and disadvantages to the measurement of renal disease biomarkers in the serum and in the urine. Urine testing has the advantage that the sampled material has been in direct contact with the tubular and glomerular structures of the kidney, which is not the case for serum-based tests. However, urine samples require correct handling and the biomarker in question may be unstable necessitating rapid transit from the clinical setting to the laboratory. Additionally, the concentration of urinary biomarkers is affected by hydration status and urinary flow rate of the subject. Normalisation against urine creatinine concentration is often used as a means to adjust for this shortcoming but this may lead to misleading results. Furthermore, filtration of the blood by the kidney leads to the presence of many substances, including small proteins and peptides, in the urine whose presence and abundance is not a reflection of the status of the kidney itself. For example, many cytokines are detectable as soluble proteins within the urine in health and disease, but their urinary concentrations may or may not reflect intra-renal inflammatory activity. Therefore, examination of cells that are likely to have originated from the kidney may give a better idea of the state of health of the various cellular compartments within the kidney.
With this consideration in mind, previous studies have focussed on the analysis of cells derived from urine samples for the purposes of better understanding renal disease pathophysiology and of identifying accurate diagnostic and prognostic indicators. Tubular epithelial cells and leukocytes originating from the renal parenchyma are shed in the urine. The integrity of these cells may depend upon physicochemical properties of the urine itself such as pH, density, temperature and the time elapsed between the collection of the urine specimen and the processing of the sample[278, 279]. Measurements of gene expression in these cells using quantitative reverse transcriptase polymerase chain reaction (RT-Q-PCR) have been studied to identify biomarkers of potential clinical value. This technique is used to measure messenger RNA (mRNA) from a gene of interest relative to mRNA from a gene with relatively stable expression (“housekeeping gene”). Differences in gene expression patterns between patient groups of interest can, thus, be quantified using this method. Achievement of reproducible results for RT-Q-PCR analysis of urine cells has been aided by the treatment of urine cell pellets with a proprietary buffer (RNAlater®, Applied Biosystems/Ambion, Austin TX, USA) that improves RNA yield and quality and facilitates mRNA profiling[280]. An example of the application of this approach is the, profiling of urine cells as a non-invasive means to aid diagnosis of early transplant rejection [280, 281]. There are several recent examples of clinical studies of renal allograft recipients in which carefully processed urine cell pellets have been shown to yield quantitative data that correlated closely with renal histological abnormalities and with the clinical course of renal disease [282, 283]. This analytical approach brings the additional advantage that, in comparison to assays or urine itself, there is no need for correction against urine creatinine concentration.

A small number of published studies have reported results for analysis of urine shed epithelial cells in diabetic kidney disease. These have primarily examined expression of mRNA for podocyte markers such as synaptopodin, podocin and WT-1 as well as ACE1 and ACE2. Results of these studies and their major outcomes are in Table 3.1:
<table>
<thead>
<tr>
<th>Reference</th>
<th>Title</th>
<th>Number of patients</th>
<th>Methods</th>
<th>Findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zheng et al (ref. 288)</td>
<td>A pilot trial assessing urinary gene expression profiling with an mRNA array for diabetic nephropathy</td>
<td>6 with DN, 3 controls</td>
<td>mRNA array with 88 genes ∆/∆ for calculating expression No DNase</td>
<td>29 mRNA’s significantly increased including a-actinin4, CDH2, ACE, FAT1, synaptopodin, OL4a, twist, NOTCH 15 fold increase</td>
</tr>
<tr>
<td>Szeto et al (ref. 294)</td>
<td>Messenger RNA expression of glomerular podocyte markers in the urinary sediment of acquired Proteinuric diseases</td>
<td>19 cases of DN (9 with diabetic glomerulosclerosis, 10 with microalbuminuria studied in total)</td>
<td>DNase used ∆/∆ for calculating expression</td>
<td>Nephrin RNA higher in Diabetic Glomerulosclerosis compared to diabetic microalbuminuria</td>
</tr>
<tr>
<td>Szeto et al (ref. 293)</td>
<td>Micro-RNA expression in the urinary sediment of patients with chronic kidney disease</td>
<td>Diabetic nephrosclerosis 22 cases</td>
<td>∆/∆ for calculating expression</td>
<td>Lower miR-15 in Diabetic Glomerulosclerosis compared to hypertensive Glomerulosclerosis and IgA nephropathy Urinary miR-216a correlated with rate of GFR decline</td>
</tr>
<tr>
<td>Zheng et al (ref. 348)</td>
<td>Urinary mRNA markers of epithelial-mesenchymal transition correlate with progression of diabetic nephropathy</td>
<td>Albuminuric diabetic with GFR &gt;60 n=27, GFR 45-60 n=7, GFR &lt;45 n=8 Controls =12 Pre-PCR product of each gene used as standard and included in all runs to calculate relative gene expression</td>
<td>Podocyanxin, synaptopodin, CD2-AP, A-Actinin 4 and podocin increased with progression of DN</td>
<td></td>
</tr>
<tr>
<td>Zheng et al (ref. 349)</td>
<td>Urinary podocyte associated mRNA profile in various stages of diabetic nephropathy</td>
<td>Healthy control n=13, NA n=17, Microalbuminuria n=15, Macroalbuminuria n=19 Pre-PCR product of each gene used as standard and included in all runs to calculate relative gene expression</td>
<td>Glomerular podocyte number correlated with intra-renal expression of nephrin podocyte number. Urinary podocyte markers did not correlate with podocyte loss.</td>
<td></td>
</tr>
<tr>
<td>Wang et al (ref. 351)</td>
<td>Intra-renal and urinary mRNA expression of podocyte associated molecules for the estimation of podocyte loss</td>
<td>21 patients with biopsy proven diabetic nephropathy</td>
<td>∆/∆ for calculating expression DNase</td>
<td>Glomerular podocyte associated mRNA expression for the estimation of podocyte loss</td>
</tr>
<tr>
<td>Wang et al (ref. 437)</td>
<td>Urinary messenger RNA expression of podocyte associated molecules in patient with diabetic nephropathy treated by angiotensin-converting enzyme inhibitor and angiotensin receptor blocker</td>
<td>Type 2 diabetics 71 patient with stable DN on ACE ARB added in 37 Urine at 0 and 12 weeks</td>
<td>∆/∆ for calculating expression DNase</td>
<td>Baseline GFR correlated with urinary expression of nephrin and synaptopodin Lower expression in ACE/ARB group of synaptopodin at 12 weeks</td>
</tr>
<tr>
<td>Wang et al (ref. 80)</td>
<td>Urinary ACE and ACE2 in human type 2 diabetic nephropathy</td>
<td>Type 2 diabetics 26 patient with stable DN on ACE 24 ACE/ARB 9 controls Urine at 0 and 12 weeks</td>
<td>∆/∆ for calculating expression</td>
<td>Proteinuria correlated with urinary ACE and ACE2, GFR with ACE2</td>
</tr>
<tr>
<td>Kubo et al (ref. 303)</td>
<td>Detection of WT1 mRNA in urine from patients with kidney diseases.</td>
<td></td>
<td>Agarose gel electrophoresis</td>
<td>WT1 positive in 1/52 non proteinuric DN, and 8/20 proteinuric DN</td>
</tr>
</tbody>
</table>

DN diabetic nephropathy, ACE Angiotensin converting enzyme, ARB angiotensin receptor blocker, GFR glomerular filtration rate, PCR polymerase chain reaction WT-1 Wilms tumour., BUN blood urea nitrogen, MMP matrix metalloproteinases, FSP-1 fibroblast specific protein SMA smooth muscle actin
Rationale for choice of mRNA targets for the study

Cellular inflammation is thought to be important in the development of diabetic nephropathy as evidenced by inflammatory infiltrates in the tubulointerstitium of biopsies from advanced diabetic nephropathy. Damage within the kidney likely leads to induced expression of chemokines and cytokines which drive this infiltration. What is not currently clear from the current literature is how early in the development of diabetic kidney disease such pro-inflammatory activity occurs and whether intra-renal inflammation instigates and/or perpetuates progressive renal parenchymal damage or is just a marker of response to underlying damage. As discussed in Chapter 2, IL-6-associated signalling pathways have long been linked experimentally with the pathophysiology of diabetes, cardiovascular disease and obesity. Furthermore, epidemiological studies have found that subjects with the aforementioned conditions have elevated IL-6 in their serum and urine compared to healthy subjects or healthier diabetics [22, 233, 284, 285]. With this background in mind, the experimental work for this portion of my thesis was focussed on assessing levels of IL-6 and its signalling receptor (IL-6R) in urine shed epithelial cells of diabetic subjects with varying clinical features of diabetic nephropathy. Of note, IL-6R is typically expressed on the surface of bone marrow derived inflammatory cells making these a direct target for IL-6-mediated signalling. In addition, however, a soluble version of IL-6R facilitates IL-6 signalling on non-inflammatory cells by presenting IL-6 to those cells through a process referred to as “trans-signalling” [198].

An additional selected mRNA, IL-17, is produced by a subset of recently-characterised T cells known as Th17 cells. Production of IL-17 by Th17 cells depends on multiple cytokine, transcription factors and signalling pathways including TGF-β, IL-6, Interleukin-23 (IL-23) and IL-1[286]. Interleukin-17 signals via a heteromeric IL-17RA-IL-17RC complex leading to activation of multiple signalling pathways including NF-κB, mitogen activated protein kinase (MAPK) and CCAAT/enhancer binding pathway (C/EBP). Interleukin-17 stimulates epithelial cells and fibroblasts to produce pro-inflammatory chemokines including IL-8, C-X-C motif ligand-1 (CXCL1), CXCL2, CXCL3, CCL2, CCL3 and CCL20. This leads to the recruitment of neutrophils, monocytes and Th-1 cells[287]. Interleukin-17 also leads to production of granulocyte-monocyte colony stimulating factor (CM-CSF) and G-CSF which activate infiltrating myeloid cells. Interleukin-6 present in the milieu further enhances their inflammatory properties [287, 288]. The primary defensive primary role of IL-17 is to activate immune cells in the presence of microbes and dysregulation of Th17 cells has been
implicated in several autoimmune conditions including multiple sclerosis, systemic lupus
erythematosus and glomerulonephritides such as anti-glomerular basement membrane
disease[287]. With regard to its role in diabetes, abnormalities in IL-17 signalling have been
linked with islet cell dysfunction in models of T1DM [289]. A role of IL-17 and Th17 cells in
diabetic nephropathy has not been described to date but, given the data showing elevated IL-6
in diabetic nephropathy, it is feasible that intra-renal IL-6 could lead to a Th17 type-immune
response within the inflammatory cell infiltrate in the setting of diabetes and that this could
further exacerbate parenchymal injury. Thus, I hypothesised that IL-17 mRNA in urine cells
is elevated in subjects with diabetic kidney disease compared to those without and levels of
IL-17 mRNA increase with worsening disease.

As we found in our study on Obese Zucker rats that STAT3 and SOCS3 were upregulated in
tubular cells and glomeruli respectively, we also aimed, in this study, to assess their
expression patterns in human subjects in the early stages of diabetic kidney disease. I
hypothesised that subjects with diabetic nephropathy have increased activity of the STAT3
signalling pathway reflective of activation of intra-renal inflammatory pathways.
Furthermore, I hypothesised that SOCS3 is also increased in urine cells as a regulatory
mechanism to prevent over-production of inflammatory products in response to the diabetic
milieu.

In similar fashion to the OZR model, obesity is nearly universally present in people with
T2DM and often precedes the manifestation of overt hyperglycaemia by many years [13, 96].
In addition to hyperglycaemia, other clinically quantifiable processes, such as
hyperlipidaemia and hypertension, are important in the development and progression of
diabetic kidney disease. In addition, less readily quantified factors such as aldosterone,
angiotensin II and systemic cytokines and chemokines derived from adipose tissue may also
contribute to renal dysfunction[73, 92]. Finally, in obese individuals, the kidney must respond
to the extra metabolic demand associated with increased total body mass [246]. The capacity
of the kidney to adapt to these factors may be a distinguishing feature of those who develop
progressive kidney dysfunction and those that retain stable renal function.

Therefore, the study performed for this portion of the thesis aimed to develop methodology
for accurate quantification of mRNA for the key gene products described above with a view
to better understanding the status of intra-renal inflammation at varying stages of diabetic nephropathy within a cohort of predominantly obese type 2 diabetic adults.
Material and Methods

Study Design and Procedures
This was a case-control study involving the acquisition and analysis of urine specimens from diabetic and bariatric surgery outpatients at Galway University Hospitals.

Ethical approval
All studies were approved by the Research Ethics Committee of Galway University Hospitals. Written informed consent was obtained from all subjects for collection of clean-catch urine samples to be used for the research study. Patient consent form and information sheets are included in the appendix.

Patient selection and clinical data
207 type 2 diabetic patients and 25 obese non-diabetic patients attending Galway University Hospital’s Diabetes Day Centre were enrolled according to the following inclusion criteria:

**Group 1**: (a) Clinically documented T2DM. (b) Age ≥18 years (c) Hypoglycaemic therapy with insulin or oral or injectable drugs. (d) Renal function documented by estimated glomerular filtration rate (eGFR, 4-parameter MDRD equation) and urine albumin: creatinine ratio (ACR).

**Group 2**: (a) Outpatient evaluation at a bariatric surgery clinic without a diagnosis of T2DM. (b) Body mass index (BMI) ≥35. (c) Age ≥ 18 years

**Group 3**: (a) Age ≥ 18 years. (b) No history of T2DM or kidney disease. For all groups, specific exclusion criteria were: (a) Type I diabetes or diabetes of indeterminate type. (b) Pregnancy. (c) Known or suspected renal or urologic disease other than diabetic nephropathy. (d) Current urine infection. (e) Current use of corticosteroid or other immunosuppressive medication.

Clinical data collected for Groups 1 and 2 included age, gender, body mass index, systolic blood pressure, ACR, eGFR and glycosylated haemoglobin (HbA1c)

Collection of urine samples
Enrolees were identified by medical record review by in advance of or during a scheduled outpatient clinic appointment. Subjects meeting entry criteria were approached for participation in the study. An information sheet was provided with details of the study and the storage and future use of urine samples. For those who agreed to participate after reading the information sheet, an informed consent form was provided. After signing the consent form, participants were given 120 ml sterile, screw-cap polypropylene container (Sarstedt, Wexford, Ireland) along with instructions for collecting a clean catch urine sample. The
sample was returned to the study investigator or research nurse who performed a urine dipstick test (Becton Dickinson, Oxford, UK) for evidence of urine infection. If the sample was negative for leukocytes and nitrites by dipstick it was transported to the laboratory in the Orbsen building on the NUI Galway campus within 4 hours. If the urine sample was positive for leukocytes and nitrates, it was not included in the study. In this case the study investigator or research nurse informed the responsible clinic physician to arrange for appropriate testing and, where necessary, treatment for urine infection. Upon receipt in the laboratory, samples were transferred to sterile 50 ml Falcon tubes and processed by centrifugation at 2000 x g for 30 minutes at 4°C. Supernatant was stored at -80°C for further use. Cell pellet was resuspended in 1ml of sterile PBS and transferred to an RNase-free 1.5ml Eppendorf tube and centrifuged at 16000 x g for 10 minutes at 4°C using a table top centrifuge. The PBS was discarded and pellet was resuspended in 150μl of RNAlater® (Ambion) and stored at -80°C.

**Total RNA extraction**

RNA was extracted by two methods. (a) Qiagen® RNA minikit according to manufacturer’s instructions with and without a DNase step. (b) Trizol®/isopropanol/chloroform method according to the following protocol: Samples were thawed on ice and pelleted by centrifugation at 10,000 x g for 10 minutes at 4°C after which RNAlater® was discarded. Pellets were re-suspended in 800μl Trizol® reagent (Invitrogen) and pipetted up and down gently for 30 seconds. 1μl of glycogen (Invitrogen) was added and the entire volume was brought twice through 26GA 3/8 IN 1ml needle (BD). 160μl of chloroform (Sigma) was added and the tube was shaken vigorously for 30 seconds, after which the sample was incubated at room temperature for 2-3 minutes followed by centrifugation at 12000 x g for 15 minutes at 4°C. The upper aqueous layer (RNA) was removed carefully without disruption to the bottom two layers (phenol (pink) and DNA/protein). This was placed in a new RNase-free Eppendorf tube. To reduce phenol carry-over, a further phase separation step was carried out. This consisted of adding a 1:1 ratio of chloroform to the removed upper aqueous layer followed by vigorous shaking for 30 seconds, incubation at room temperature for 2-3 minutes and centrifugation at 12,000 x g for 5 minutes at 4°C. The upper aqueous layer was again carefully removed and transferred into a clean RNase-free eppendorf tube. 400μl of isopropanol was added. The tubes were gently inverted 3-4 times, left at room temperature for 10 minutes and centrifuged at 12,000 x g for 10 minutes at 4°C. The tubes were placed on ice, the isopropanol was removed and 800μl of 75% ethanol was added. The tubes were then
inverted 3-4 times and centrifuged at 7,500 x g for 5 minutes at 4°C. Ethanol was removed and samples were left to air dry for 10 minutes. Pellets were re-suspended in 20µl of RNase-free water and dissolved in a heat block at 55°C for 10 minutes. RNA was measured using a Nanodrop® ND-1000 spectrophotometer. No DNase step was carried out. RNA quality was also determined using an Agilent 2100 Bioanalyser® according to the manufacturer’s protocol.

**Reverse transcription**

Only samples with A260/280 ratio greater than 1.7 were used for further analysis. For reverse transcription, 14.2µl of RNA was mixed with 2µl of 10X reverse transcriptase buffer, 1µl of 10X random primers, 0.8µl of 25 X dNTP mix, 1µl of MultiScribe™ reverse transcriptase and 1µl of RNase inhibitor as part of High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor® kit (Applied Biosystems). Reverse transcription was performed on an Applied Biosystems Thermal Cycler using the following cycle: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C.

**Quantitative Polymerase Chain Reaction**

Relative abundance of IL-6, IL-6R, IL-17, STAT3 and SOCS3 were quantified using the StepOne Plus® Real Time PCR System (Applied Biosystems). Human beta-Actin was used as a reference housekeeping gene. Taqman® primers and probes sets for each target were purchased from ABI. Primers and probe sets were selected that did not cross exon-intron junctions to reduce likelihood of DNA amplification. Details of the primers and probe sets used set are summarised in Table 3.2. For real-time PCR, 1µl of cDNA, 5µl of TaqMan® Universal Master Mix II, 0.5µl of primers and probes, and 3.5µl of RNase free water were mixed to make a 10µl reaction. All samples were run in duplicate. PCR reaction conditions were as follows: 10 minutes at 55°C, 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Relative expression of each target gene was quantified by a relative standard curve method [290, 291]. The relative standard curve method was used over the Δ/ΔCT method of analysis of gene expression because quality of RNA across samples was not reliable due to low yields and low copy number of RNA. Additionally, because a DNase step was omitted, the Nanodrop analysis was not as useful in determining whether nucleic acids contributing to a 260/280 ratio >1.7 was all from RNA. Purified plasmids carrying the DNA sequence of each target of gene were used as standards and the standard curves were established with a 4 point,
10-fold serial dilution of the product. The standard curves were included in all PCR runs along with a calibrator sample. Controls consisting of ddH2O were also included in all runs.

Table 3.2 Details of the Primers and Probe Sets

<table>
<thead>
<tr>
<th>Target</th>
<th>Catalogue number</th>
<th>Company</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL6</td>
<td>Hs00985641_m1</td>
<td>ABI</td>
</tr>
<tr>
<td>IL17</td>
<td>Hs00936345_m1</td>
<td>ABI</td>
</tr>
<tr>
<td>IL-6R</td>
<td>Hs01075667_m1</td>
<td>ABI</td>
</tr>
<tr>
<td>STAT3</td>
<td>Hs00374280_m1</td>
<td>ABI</td>
</tr>
<tr>
<td>SOCS3</td>
<td>Hs.PT.53a.4303529</td>
<td>Integrated DNA technology</td>
</tr>
<tr>
<td>Actin Beta</td>
<td>Hs99999903_m1</td>
<td>ABI</td>
</tr>
</tbody>
</table>

Taqman® primer and probe sets were commercially bought and were selected so that they did not cross exon-intron junctions to reduce likelihood of DNA amplification.

Creation of Standard Curve

Plasmid Purification

Plasmids containing an Image® clone for each target were purchased from Source Bioscience. Details and on clone number, vector, vector digest, insert size and antibiotic selectivity are given in Table 3.3. Stab cultures received for the targets were streaked on antibiotic-selective solid agar plates and, after 24 hours at 37°C, a single clone was selected and grown in antibiotic-selective liquid broth before proceeding to plasmid purification using a Qiagen® Midi-kit. A single colony from freshly streaked selective plate was used to inoculate a starter culture of 25mls of liquid broth medium containing the appropriate amount of selective antibiotic. It was incubated for eight hours at 37°C with vigorous shaking at 300 RPM.

Bacteria were harvested by centrifugation at 6000 x g for 15 minutes at 4°C. The bacterial pellet was re-suspended in 4 ml of buffer P1 for lysis. RNaseA was added to buffer P1 prior to use. 4 ml of buffer P2 were then added to the solution, which was mixed thoroughly by vigorous inversion four to six times and was then incubated at room temperature for 5 minutes. 4mls of chilled buffered P3 was then added; the solution was mixed immediately by vigorous inversion 4 to 6 times and incubated on ice for 15 minutes. The solution was centrifuged at 20,000 x g for 30 minutes at 4°C. The supernatant containing the plasmid was removed and was re-centrifuged at 20,000 x g for 15 minutes at 4°C. The supernatant was drained through a Qiagen 100-tip that had been equilibrated with 10 ml of buffer QBT. The tip was then washed with buffer QC after which 0.5 ml of buffer QF was used to elute the
The DNA was precipitated by the addition of 3.5mls of isopropanol following centrifugation at 15,000g for 30 minutes. The supernatant was carefully decanted and the pellet was washed with 70% ethanol and centrifuged at 15,000 x g for 10 minutes. The supernatant was discarded and the pellet was allowed to dry then dissolved in DNA Tris EDTA buffer. The yield was determined using Nanodrop® ND-1000 spectrophotometer.

Table 3.3: Details of Plasmids used for Relative Standard Curve Creation from each Target

<table>
<thead>
<tr>
<th>Target</th>
<th>Image ID</th>
<th>Vector</th>
<th>Digest sites</th>
<th>Insert size</th>
<th>Vector sequence size</th>
<th>Size of product</th>
<th>Antibiotic selectivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>3884652</td>
<td>pCMV-SPORT6</td>
<td>5’ SalI/NotI 3’</td>
<td>924</td>
<td>4396</td>
<td>5320</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>IL-6R</td>
<td>40146706</td>
<td>pCR4-TOPO</td>
<td>5’EcoRI</td>
<td>1718</td>
<td>3956</td>
<td>5674</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>IL-17</td>
<td>6971881</td>
<td>pDNR-Dual</td>
<td>5-loxP-SalI/HindIII-loxP-3</td>
<td>592</td>
<td>4938</td>
<td>5530</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>STAT3</td>
<td>3347434</td>
<td>pOTB7</td>
<td>5’ EcoR1 3’Xhol</td>
<td>2389</td>
<td>1815</td>
<td>4204</td>
<td>Chloramphenicol</td>
</tr>
<tr>
<td>SOCS3</td>
<td>30333577</td>
<td>pBluescriptR</td>
<td>5’ Sal-I Xhol 3’BamHI1</td>
<td>677</td>
<td>2998</td>
<td>3675</td>
<td>Kanamycin</td>
</tr>
<tr>
<td>Actin B</td>
<td>100003590</td>
<td>pENTR221</td>
<td>5’-attL1/attL2-3’</td>
<td>1168</td>
<td>2267</td>
<td>3435</td>
<td>Ampicillin</td>
</tr>
</tbody>
</table>

Plasmids containing Image® clone for each target were purchased as a stab cultures from source bioscience and a single clone was grown in antibiotic selected culture broth for plasmid purification using Qiagen® midi-kit.

Verification of Plasmid Inserts

Each plasmid was digested using appropriate endonucleases (see Table 3.3). Briefly, 0.5 µL of endonuclease buffer, 0.5 µL of appropriate enzyme and 4 µL of plasmid solution were incubated for 30 minutes at 37°C. The digest was separated by electrophoresis on a 2% agarose gel at 80V using 1X TAE buffer and a DNA ladder (Invitrogen cat#15429-109) (Figure 3.1). Using the relevant primers and probes sets, amplifications of each plasmid were performed as described above and the resulting PCR products were analysed by agarose gel electrophoresis to ensure that product size was in keeping with that quoted by the manufacturer (Figure 3.1).
Figure 3.1 Agarose Gel Electrophoresis of Plasmid Preparations, ladder marker point for 2000bp for each run (DNA ladder (Invitrogen cat#15429-109). (D)-digested plasmid preparation (U) undigested plasmid preparation.

Using a calculation based on insert sizes and concentrations of the plasmid stock preparation, the volume of plasmid needed to give 1x10⁹ copies/ml was determined. This volume was then used as the starting point for an initial 15-point standard curve. Quantitative RT-PCR reaction was then carried out to assess the most appropriate starting dilution for the standard curve which would cover the range at which expression of transcripts in urine shed epithelial cell samples was detected. The starting dilutions which were most appropriate for each target was 5.5-6 for the aliquots. Aliquots of these starting dilutions were prepared and stored at -80°C so that identical standard curves would be subsequently used for all amplifications.

Calibrator sample

Human peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation using Ficoll-Paque PLUS® (GE Healthcare). Briefly, 30 ml of whole blood were collected into EDTA vacutainer tubes and then diluted 1:1 with Dulbecco’s modified Eagles medium. 30 ml of Blood/DMEM mixture were layered onto 20 ml of Ficoll-Paque PLUS in a 50 ml Falcon tube. This was then centrifuged at 1800 rpm on a table top centrifuge for 30 minutes with deceleration set to zero. The cloudy layer between plasma and Ficoll layers was collected using a Pasteur pipette and transferred into a new 50 ml tube. This was centrifuged at 1600 rpm for 10 minutes with normal deceleration. The supernatant was discarded and the pellet was re-suspended in 5ml red cell lysis buffer then incubated for 2 minutes at room temperature followed by centrifugation for 10 minutes at 1600 rpm. The pellet was then re-suspended in 5 ml of PBS followed by centrifugation twice for 10 minutes at 1600 rpm. The resulting PBMC were counted and 1 x 10⁶ were transferred into the wells of a 6-well tissue culture plate with serum free medium and were activated by addition of 3µl of
a 500µg/ml stock solution of concanavalin A followed by incubation at 37°C for 24 hours. Following this, the cells were removed from culture and centrifuged at 1500 rpm for 5 minutes. RNA was extracted from the pelleted cells using RNeasy® Mini kit. Briefly, 350µl of Buffer RLT was added to the pelleted PBMCs and vortexed vigorously for 60 seconds. 350µl of 70% ethanol was added to the homogenized lysate and mixed well by pipetting. This was then transferred to an RNase spin column placed in a 2ml collection tube. 350µl of Buffer RW1 was added to the spin column which was then centrifuged for 15 seconds at 8000 x g. The flow through was discarded and this process was repeated once. DNase treatment of RNA was carried out using RNase-free DNase (Qiagen). 10µl of DNaseI stock solution was added to 70µl of buffer RDD and mixed by inversion. The 80µl was then added directly to each spin column membrane and placed on the benchtop at room temperature for 15 minutes. After this 350µl of buffer RW1 was added to the spin column and centrifuged for 15 seconds at 8000 x g. The flow through and collection tubes were discarded and the spin column was placed in a new 2 ml collection tube. 500µl of buffer RPE was added to the spin column and it was centrifuged for 15 seconds at 8000 x g. The flow through was discarded and 250µl of 80% ethanol was added to the spin column followed by centrifugation for 2 minutes at 8000 x g. The flow through and collection tubes were discarded and the spin column was placed in a new 2ml tube which was centrifuged with the lid open at 13000 x rpm for 5 minutes. The flow through and collection tube were discarded and the spin column was placed in a new 1.5ml RNase free eppendorf tube. 15µl of RNase-free water was added directly to the centre of the spin column membrane and centrifuged for 1 minute at 13,000 x g to elute RNA. The sample was immediately placed on ice after centrifugation, RNA concentration was determined using a Nanodrop® ND-1000 spectrophotometer after which 1µg of RNA was reverse transcribed as described above using High Capacity cDNA Reverse Transcription Kit with RNase Inhibitor kit (Applied Biosystems). Reverse transcription was performed using the following cycle: 10 minutes at 25°C, 120 minutes at 37°C, 5 minutes at 85°C. Serial dilutions of the cDNA were carried out and quantitative PCR was performed using the selected primer and probes sets to determine an appropriate cDNA dilution at which amplification of all targets of interest occurred within the range of the previously determined standard curves. Aliquots were of these optimised cDNA dilutions of the calibrator sample (activated human PBMCs) were then prepared and stored at -80°C.Subsequently a single aliquot of this calibration sample was used for each plate.
Data analysis:
RNA quantification was generated from a relative standard curve method. Briefly, a relative quantity for both target and reference gene for sample and calibrator was generated from the relative standard curve. The fold difference of expression of each compared to reference gene was calculated and finally the fold difference between sample and calibrator was calculated by dividing these values which was then log transformed [290] The SPSS® 21.0 software package was used for data analysis. Results were expressed as means ± SD or as median (ranges). Information was obtained from patient medical records at the time of sample collection including age, sex, HbA1c, BP control, duration of diabetes and BMI. Baseline data were compared by a one-way analysis of variance (ANOVA) between groups with Bonferroni test to assess for differences between groups. Statistical differences between groups of data distributed parametrically were tested by Student's t-test. As, the distributions of some quantitative measures such as mRNA levels of IL-6, IL-6R, IL-17, STAT3, SOCS3 and ACR were skewed rightward; the significance of any differences in these parameters were assessed by the Mann–Whitney U test or Wilcoxon signed-rank test. These parameters were log transformed resulting in normal distributions and significance was then assessed by nonparametric analysis. To facilitate analysis of samples for which there was no detectable expression by qPCR, a minimal value (0.001) was assigned to such samples prior to log transformation to allow inclusion in the final set of transformed data. Categorical data was compared by the Fisher test or by χ² test. Correlations were determined by the Spearman rank correlation test for categorical variables and Pearson’s correlation test for continuous variables. For all analyses, significance was assigned to p <0.05
Results-

Type 2 diabetes cohort: Urine samples collected and processed

A total of 207 urine samples were collected from subjects with T2DM during the study of which 47 samples were not fully processed (n=14 optimising RNA extraction, n=10 positive leukocytes, n=19 precipitate present in urine sample preventing RNA extraction, n=4 insufficient volume collected). 160 samples were fully processed of which 61 samples had a 260/280 ratio of \( \geq 1.7 \) and 54 samples had a 260/280 ratio of <1.7 and, as such were deemed unsuitable for qRT-PCR analysis. These details are summarised in diagrammatic form in Figure 3.1. An additional 11 samples were collected from healthy adults without diabetes, obesity or hypertension to serve as controls.

Figure 3.2 Flowchart of Type 2 Diabetes Patients Urine samples from patients with type 2 diabetes mellitus recruited from diabetes clinics at Galway University Hospitals were processed for RNA extraction, reverse transcription and quantitative PCR. Number of samples and reasons for exclusion of urine samples from further analysis are also included on the chart.
Results of RNA extraction

Based on published studies, RNA was initially extracted from test samples of urine shed epithelial cells using guanidine-ethanol spin-column based methods (Qiagen® mini-kits)[292-294]. Test samples were extracted with and without a DNase step in order to ascertain its effect on RNA yield and quality. However, due to low RNA yield and poor quality even in the absence of a DNase step, I decided to evaluate alternative RNA extraction methods. Thus, RNA was next extracted from test samples using Trizol® with inclusion of a second phase separation step to reduce phenol contamination. As summarised in Table 3.4, this method yielded improved quality and yield of RNA based on spectrophotometric analysis and was subsequently employed for RNA extraction from all samples included in the final analyses.

Table 3.4: Yield and Quality of RNA as Estimated Using Nanodrop® spectrophotometer.

<table>
<thead>
<tr>
<th>Method of RNA extraction</th>
<th>Concentration (ng/ml) (mean±SD)</th>
<th>260/280 (mean±SD)</th>
<th>260/230 (mean±SD)</th>
<th>% (n) 260/280&gt;1.7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Qiagen® Mini kit + DNase n=18</td>
<td>36.9±90.9</td>
<td>1.37±1.4</td>
<td>0.38±0.8</td>
<td>22% (4/18)</td>
</tr>
<tr>
<td>Qiagen® Mini kit no DNase n=6</td>
<td>17.4±13.0</td>
<td>1.86±0.1</td>
<td>0.27±0.3</td>
<td>50% (3/6)</td>
</tr>
<tr>
<td>Trizol™ n=160</td>
<td>45.9±127.7</td>
<td>1.75±0.3</td>
<td>0.56±0.4</td>
<td>66% (106/160)</td>
</tr>
</tbody>
</table>

Urine samples from patients with T2DM presenting to Galway University Hospital were collected, transferred to the laboratory and centrifuged following which the pelleted material was stored in RNAlater®. Subsequently, RNA was extracted using either Qiagen® mini-kits with or without DNase treatment or Trizol® reagent. Yield and quantity for samples are shown as mean ± SD.

It is important to note that the overall integrity of the RNA extracted from the urine pellets was poor, likely reflecting the RNAse-rich environment to which urine shed cells are exposed prior to pelleting and extraction[258, 280, 291, 295-297]. Assessment of RNA integrity was performed for 59 samples that had RNA extracted using the Trizol® method. This was carried out using an Agilent® 2100 Bioanalyser. Profiles generated on this instrument yield information on concentration, allow a visual inspection of RNA integrity, and generate ribosomal ratios by providing an image of the size and distribution of RNA species. The instrument’s proprietary software generates a numerical read-out [RNA Integrity Number (RIN)] based on the entire electrophoretic trace. The RIN is calculated on a numerical system from 1 to 10, with 1 being the most degraded profile and 10 being the most intact[258].

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Figure 3.3(A) illustrates representative examples of RNA integrity analysis and calculated RINs for RNA samples extracted from urine shed cells of T2DM patients. Figure 3.3(B) shows an example from the literature of electropherograms of RNA with differing levels of RNA integrity [298].
Figure 3.3 Examples of Agilent Bioanalyser Data for Urine Cells (A) Extracted RNA samples from urine cells were assessed for integrity using RNA picochips and an Agilent 2100 Bioanalyser. An RNA ladder is shown on the left and RNA integrity number (RIN) is indicated below each electropherogram. As shown, typical RIN values for samples were between 1 and 4, with some samples too degraded to allow RIN to be calculated. NC=Not calculable (B) Examples of poor, moderate, good and excellent quality RNA as defined by RIN adapted from Parissenti et al [298].
Of the samples analysed using the Agilent Bioanalyser, 36 had 260/280 ratio ≥ 1.7, of which 23 (64%) did not give a readable RIN. There were also 22 samples analysed with 260/280 ratio < 1.7 of which 8 (36%) had unreadable RIN. Where there was an RIN recorded, the average value was 3.24 ± 1.11 (range 1.0 – 5.6) for samples with 260/280 ratio ≥1.7 and 2.46 ± 0.5 for samples with 260/280 ratio < 1.7 (p=0.01, Student’s t-test). Interestingly, in subsequent qRT-PCR analyses, there was no overall difference in actin threshold cycle (C_T) between the sample groups with and without measurable RIN. Thus, the results obtained for spectrophometry and RIN analysis for these samples did not correlate closely and, presumably, reflected different qualities of the RNA extracts that were obtained. Taken together, the analyses of extracted samples provide insight into the important limitations of urine shed cell RNA-based measurements in outpatients with normal or relatively mild abnormalities of renal functional indices. These can be interpreted as including potential for low RNA amount (presumably reflecting low urine cell numbers), variable RNA purity and generally low RNA integrity (both likely reflecting the unfavourable environment within the urine prior to extraction).

**Optimisation of qRT-PCR analyses**

Quantitative (q)RT-PCR using relative standard curve method was selected in preference to the more convenient and commonly used Δ/Δ–CT method which requires that RNA be of relatively high quality to ensure that there is equal hybridization of target and reference probes to the cDNA. Given that the amount and quality of RNA from urine shed epithelial cells was of borderline quality, I concluded that the relative standard curve method would give more reliable quantitative data [290]. Beta-actin was selected as the reference gene of choice over other candidates [hypoxanthine-guanine phosphoribosyltransferase (HPRT) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] as the optimal HPRT primer/probes set typically had threshold cycles >36 in samples for which the beta-actin C_T values were < 25 and a GADPH primer/probe set was not available in a format that did not cross exon-intron junctions. Additionally, for relative standard curve, only one reference gene is required compared to the Δ/Δ–CT method for which multiple reference genes are typically recommended to improve validity of the result [291].

As described in the Methods section, a relative standard curve of each target gene and the reference gene was included on each plate along with cDNA aliquots from a pre-prepared calibrator sample (activated human PBMCs) with known expression of all targets of interest.
Figure 3.4 provides an example from one of the experimental qPCR plate amplifications performed for this study of the 4-point standard curve for beta actin along with the duplicate CT values for the urine cell and calibrator samples. Examples of each target gene standard curve are included in Appendix 4. Table 3.5 summarises the CT values that were obtained for all targets of interest as well as the reference gene throughout the qPCR runs performed for the study.

![Standard Curve for Actin-beta](image)

**Figure 3.4 Standard Curve of Beta-actin with Interpolated Target and Calibrator Sample CT Values.** The values for the calibrator sample duplicates are circled in green and those for a sample with a threshold cycle greater than 25 that was excluded from analysis are circled in red.
Table 3.5: Mean Threshold Cycles for the Calibrator Sample across all Experiment Plates.

<table>
<thead>
<tr>
<th>Threshold cycle for calibrator sample mean±SD</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>30.2±0.96</td>
</tr>
<tr>
<td>IL-6R</td>
<td>28.3±1.57</td>
</tr>
<tr>
<td>IL-17</td>
<td>29.0±0.9</td>
</tr>
<tr>
<td>STAT3</td>
<td>26.29±1.57</td>
</tr>
<tr>
<td>SOCS3</td>
<td>28.4±0.82</td>
</tr>
<tr>
<td>Actin beta</td>
<td>19.9±1.25</td>
</tr>
</tbody>
</table>

Serial dilutions of calibrator cDNA were carried out and quantitative PCR was performed using the selected primer and probes sets to determine an appropriate cDNA dilution at which amplification of all targets of interest occurred within the range of the previously determined standard curves. Aliquots of these optimised cDNA dilutions of the calibrator sample (activated human PBMCs) were then prepared and stored at -80°C. Subsequently a single aliquot of this calibration sample was used for each plate. This table shows the mean ± SD CT for each target for the calibrator sample that was included on each plate. N=12 for IL-6, IL-6R, IL-17 and N=5 for STAT3 and SOCS3.

Comparison between Patients included in Final Analysis and those Excluded due to Unacceptable Urine Sample or RNA quality

Clinical and laboratory characteristics were compared for T2DM subjects for whom RNA deemed to be of adequate quality for qRT-PCR analysis was obtained and those for whom the urine sample or RNA sample was considered unsuitable or inadequate (Table 3.6). This analysis was performed in order to determine whether the eventual study cohort for qRT-PCR analysis had been skewed by clinical factors associated with urine cell number and/or quality. As shown, there were no significance differences between these two groups with regard to SCr, urine ACR, BMI, HbA1c, age or blood pressure at the time of sample collection. However, there was a significantly lower eGFR in the group with adequate RNA samples (66.4±19.5 vs. 73.1±18.6, p=0.02).
Table 3.6: Clinical Details of T2DM Subjects Providing Urine Samples that were and were not eventually included in Final qRT-PCR Analyses.

<table>
<thead>
<tr>
<th></th>
<th>Included in analysis (n=61)</th>
<th>Not included in analysis (n=146)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine µmol/L</td>
<td>103.8±39.0</td>
<td>97.6±40.4</td>
<td>NS</td>
</tr>
<tr>
<td>eGFR mL/min/1.73 m2</td>
<td>66.4±19.5</td>
<td>73.1±18.6</td>
<td>0.02</td>
</tr>
<tr>
<td>ACR mg/mmol</td>
<td>22.3±53.6</td>
<td>28.1±115.2</td>
<td>NS</td>
</tr>
<tr>
<td>BMI kg/m2</td>
<td>31.2±5.3</td>
<td>31.9±5.4</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c mmol/mol</td>
<td>65.3±16.5</td>
<td>60.8±19.7</td>
<td>NS</td>
</tr>
<tr>
<td>SBP mmHg</td>
<td>131.5±17.6</td>
<td>134.8±17.0</td>
<td>NS</td>
</tr>
<tr>
<td>DBP mmHg</td>
<td>72.4±9.6</td>
<td>72.6±9.1</td>
<td>NS</td>
</tr>
<tr>
<td>Age (Years)</td>
<td>65.8±9.1</td>
<td>63.2±11.7</td>
<td>NS</td>
</tr>
</tbody>
</table>

Clinical data was collected for each case at the time of sample acquisition. Student’s t-test was used to compare the groups for each parameter.

Overall Results of qRT-PCR Analysis of RNA-Quality-Selected Urine Cell Samples from T2DM and Bariatric Surgery Clinics Patients

All samples analysed for SOCS3, STAT3, IL-6 and IL-6R had $C_T < 36$ when the beta-actin $C_T$ was $\leq 24$. However for IL-17, of the 61 samples analysed from the diabetic cohort, 17 had no detectable amplification ($CT \geq 40$) despite adequate starting amount of cDNA as indicated by beta-actin $C_T < 25$. There were no significant differences between the 17 diabetic subjects with no detectable expression of IL-17 in urine cell samples and the 44 with detectable expression of IL-17 for SCr, eGFR, HbA1c, Systolic blood pressure (SBP) or BMI. However, ACR was significantly lower in the group with no detectable IL-17 expression (11.2 $\pm 18.1$mg/mmol vs. 41.2$\pm 85.8$ mg/mmol, $p=0.01$). To facilitate the inclusion of the samples with no detectable IL-17 expression in the final analyses of log-transformed RQ values, these samples were assigned a small, nominal value (0.001). Table 3.7 provides a summary of the overall qRT-PCR results for the T2DM and Bariatric Surgery clinic patients that were eventually included in the quantitative analyses.
Table 3.7: Amplification Characteristics for each of the Targets of Interest in the Final Cohorts of RNA Quality-Selected Samples.

<table>
<thead>
<tr>
<th>Target</th>
<th>Threshold cycle Median(range)</th>
<th>RQ Diabetes median (range)</th>
<th>RQ Bariatric median (range) n=16</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>32.2 (23.2-35.6) n=61</td>
<td>-0.31 (4.24)</td>
<td></td>
</tr>
<tr>
<td>IL-6R</td>
<td>28.5 (22.3-35.2) n=61</td>
<td>0.23 (4.41)</td>
<td></td>
</tr>
<tr>
<td>IL-17</td>
<td>35.1 (26.2-40) n=61</td>
<td>-1.96 (4.28)</td>
<td></td>
</tr>
<tr>
<td>STAT3</td>
<td>25.9 (21.8-31.7) n=30</td>
<td>0.058 (0.84)</td>
<td>0.13 (1.10)</td>
</tr>
<tr>
<td>SOCS3</td>
<td>27.3 (21.2-34.6) n=30</td>
<td>0.11 (1.90)</td>
<td>0.22 (1.76)</td>
</tr>
<tr>
<td>Actin beta</td>
<td>21.45 (14.4-23.9) n=91</td>
<td>NA</td>
<td></td>
</tr>
</tbody>
</table>

Threshold cycles (C_T) and relative quantity (RQ) compared to the calibrator sample for targets of interest and the reference gene are presented as median (range) along with the number (n) of samples analysed for each target.

Summary of Conclusions and Decisions made for subsequent qRT-PCR analyses: From preliminary work with samples processed using Qiagen® mini-kits, it was clear that achievement of consistent expression of both target and reference genes would require detailed assessment of RNA quality to ensure the validity of any quantitative analyses. Therefore, RNA extraction of urine shed cells was optimised to produce the highest quality RNA possible from small cell numbers in an unfavourable sampling environment. It was found that RNA extraction with a modified Trizol® protocol designed to reduce phenol contamination was the most successful method.

Subsequently, based on the results of the spectrophotometry and RIN-based quality analysis of multiple Trizol®-extracted samples described above followed by additional first strand synthesis (with random hexamers) and qPCR test runs for target and references genes, I elected to accept all samples with 260/20 ratios ≥1.7 for experimental studies regardless of RIN as it was not apparent that amplification was dependent on RIN. Next, following literature review and another series of test amplifications, I selected the relative standard curve approach (see Methods section) for accurate quantification of mRNA levels in the samples that passed the quality threshold. Finally, of the samples that were subjected to qPCR following first strand cDNA synthesis, those for which the amplification threshold (C_T) for the reference (“housekeeping”) gene beta-actin fell outside the range of the relative standard curve were excluded from the final analysis on the basis that target gene amounts could not be accurately quantified. This resulted in a beta-actin C_T ≥ 25 being selected as a cut-off for acceptance of target gene amplification for inclusion in the quantitative analyses.
The analyses described in the following sections represent the results for patient samples processed, characterised and subjected to qRT-PCR according to these specific optimised methods and selection steps (summarised in Table 3.7 above). In the Discussion section, I consider the implications and limitations inherent in this methodology.

In summary, extra optimisation and selection steps were required to be added to the process of RNA extraction and qRT-PCR of urine shed epithelial cells. This allowed for reliable data to be produced for a significant number of human subjects that measured relative gene expression of several inflammatory markers and their signalling components. The markers of interest, IL-6, IL-6R and IL-17, were selected at the time of initial project planning based on the background of published literature described in Chapter 1, while the additional targets of interest, STAT3 and SOCS3, were subsequently selected on the basis of results from the study on Obese Zucker rats described in Chapter 2. As indicated in Table 3.7, the numbers of samples included in the analysis of STAT3 and SOCS3 mRNA were less than those for the other genes of interest. This was due to the fact that all available cDNA had been used for some samples before the STAT3 and SOCS3 assays were optimised and applied. The results from Chapter 2 also prompted me to collect samples for quantitative analysis of urine cell STAT3 and SOCS3 mRNA from a small cohort of non-diabetic subjects with severe obesity referred for consultation to a bariatric surgery.
Relationships between BMI and Quantitative RT-PCR of Urine Cells for Target Genes of Interest

Based on the qPCR analyses performed on all the urine shed cell RNA samples deemed to be adequate, the relationship between BMI and urine cell expression of the target genes of interest was assessed. Initially, the T2DM patient cohort was subdivided into subgroups based on BMI categories as summarised in Table 3.8. As shown, there were no differences among the grades of obesity with regard to SCr, eGFR, log urine ACR, age, HbA1c or systolic blood pressure at the time of sample collection.

Table 3.8 Clinical details for different grades of BMI

<table>
<thead>
<tr>
<th>BMI Kg/m²</th>
<th>Creatinine µmol/L (Mean±SD)</th>
<th>eGFR ml/min/1.73m² (Mean±SD)</th>
<th>ACR mg/mmol (median(range))</th>
<th>Age Years (Mean±SD)</th>
<th>HbA1c (Mean±SD)</th>
<th>SBP mmHg (Mean±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal &lt;24.9 n=7</td>
<td>93.8±32</td>
<td>69.6±19.2</td>
<td>7.5(65.3)</td>
<td>72.4±6.0</td>
<td>74.8±19.1</td>
<td>129.2±20.6</td>
</tr>
<tr>
<td>Overweight 25-29.9 n=22</td>
<td>107.1±53.0</td>
<td>67.1±20.6</td>
<td>3.3(342.7)</td>
<td>65.5±11.2</td>
<td>62.4±16.9</td>
<td>128.1±16.1</td>
</tr>
<tr>
<td>Obese class 1 30-34.9 n=17</td>
<td>106.1±45.7</td>
<td>66.4±21.8</td>
<td>3.3(56.1)</td>
<td>64.0±9.1</td>
<td>65.2±18.2</td>
<td>136.6±18.2</td>
</tr>
<tr>
<td>Obese class 2 35-40 n=10</td>
<td>107.5±31.7</td>
<td>60.9±23.3</td>
<td>5.3(129.8)</td>
<td>62.8 10.9</td>
<td>65.2±20.2</td>
<td>130.0±16.1</td>
</tr>
<tr>
<td>Obese class 3 &gt;40 n=5</td>
<td>72.4±9.7</td>
<td>81.8±12.1</td>
<td>1.4(76.4)</td>
<td>60.2±8.4</td>
<td>68.0±13.5</td>
<td>141.4±22.5</td>
</tr>
</tbody>
</table>

Patients were classified into WHO grades of BMI. The number, demographic and laboratory parameters for each of these are demonstrated in the table. Data are represented as mean± SD or median (range). Differences across the subgroups were analysed statistically by ANOVA.
Relative expression levels for the targets of interest were compared among the BMI-based subgroups as well as a group of non-obese, healthy adult controls. These are displayed as box-and-whisker plots in Figure 3.5A. As is clear from these graphs, there were no significant gene expression patterns observed across the BMI subgroups and, in general, RQ values for diabetic subjects of varying BMI categories were not strikingly different to those for the non-obese healthy controls. There was a notable increase in STAT3 RQ between the overweight and class 1 obese subgroups but this trend did not continue for the class 2 and class 3 obese subjects.

The relationships between urine cell target gene RQ values and BMI among the entire T2DM cohort were also analysed by logistic regression (Figure 3.5B). Of interest, a significant positive correlation was present by Spearman rank-order between urine cell mRNA expression of STAT3 and BMI (r=0.366, 0=0.04). However, there were no significant correlations between BMI and the urine cell mRNA expression of IL-6, IL-6R, IL-17 and SOCS3.
Figure 3.5 Relationships between IL-6, IL-6R, IL-17, SOCS3 and STAT3 and BMI (A) Box and whisker plots of BMI and mRNA targets of interest among T2DM and non-obese healthy adult (Control) subjects. Kruskall-Wallis test was used to analyse differences in values with increasing BMI. (B) Correlations between expression of mRNA targets of interest and BMI among T2DM subjects. Correlations were assessed using Spearman’s rank correlation test. For all analyses, p < 0.05 was considered significant. The numbers of subjects analysed in each correlation are indicated in the table.

<table>
<thead>
<tr>
<th>Correlation between BMI and expression of mRNA targets of interest</th>
<th>Log IL-6</th>
<th>Log IL-6R</th>
<th>Log IL-17</th>
<th>Log SOCS3</th>
<th>Log STAT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of XY Pairs</td>
<td>58</td>
<td>58</td>
<td>58</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Spearman r</td>
<td>-0.11</td>
<td>-0.03</td>
<td>-0.08</td>
<td>-0.03</td>
<td>0.38</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>-0.36 to 0.16</td>
<td>-0.29 to 0.24</td>
<td>-0.34 to 0.19</td>
<td>-0.39 to 0.34</td>
<td>0.01 to 0.65</td>
</tr>
<tr>
<td>P value (two-tailed)</td>
<td>0.41</td>
<td>0.63</td>
<td>0.55</td>
<td>0.87</td>
<td>0.04</td>
</tr>
</tbody>
</table>
Relationships between Urine ACR and Quantitative RT-PCR of Urine Cells for Target Genes of Interest

Albuminuria was classified according to the Kidney Disease Improving Global Outcomes (KDIGO) guidelines into normal (0-1 mg/mmol), mildly increased (2-3 mg/mmol), moderately increased (3-30 mg/mmol) and highly increased (30 mg/mmol) [299]. There were 25 subjects with albuminuria <3 (of which 9 had ACR <1.0 which is considered normal), 26 with moderately increased and 10 with severely increased albuminuria. Age, SCr and eGFR were significantly different among the groups but there were no differences in BMI or systolic blood pressure (SBP) values among the groups (Table 3.9).

Table 3.9: Clinical Details for Different Grades of ACR

<table>
<thead>
<tr>
<th>Albuminuria mg/mmol</th>
<th>Creatinine µmol/L</th>
<th>eGFR ml/min/1.73m²</th>
<th>Age Years</th>
<th>BMI Kg/m²</th>
<th>HbA1c mmol/mol</th>
<th>SBP mmHg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-3 (25)</td>
<td>76.2±13.4</td>
<td>81.4±10.5</td>
<td>60.2±10.8</td>
<td>31.9±6.1</td>
<td>68.4±20.4</td>
<td>126.9±16.1</td>
</tr>
<tr>
<td>3-30 (26)</td>
<td>120.7±48.9*</td>
<td>57.1±20.6*</td>
<td>68.4±8.4*</td>
<td>29.9±4.2</td>
<td>64.3±14.9</td>
<td>131.7±17.2</td>
</tr>
<tr>
<td>&gt;30 (10)</td>
<td>121.5±44.5*</td>
<td>58.9±21.1**</td>
<td>67.9±8.1*</td>
<td>33.4±6.7</td>
<td>62.2±14.2</td>
<td>137.6±22.5</td>
</tr>
</tbody>
</table>

Patients were classified according to KDIGO guidelines into subcategories of albuminuria [300]. The number, demographic and laboratory parameters for each of these are demonstrated in the Table. Data are represented as mean ± SD or median (range). Cases were compared using ANOVA to analyse differences in values with increasing ACR. P values of <0.05 were considered significant.*=P<0.05 compared to albuminuria 0-3mg/mmol.

Relative expression levels for the targets of interest were compared among the ACR-based subgroups as well as a group of non-obese, healthy adult controls. These are displayed as box-and-whisker plots in Figure 3.6A. As for BMI categories, there was no significant gene expression differences observed across the ACR subgroups. RQ values for diabetic subjects of varying ACR categories were not different to those of the non-obese healthy controls for IL-6, IL-6R, IL-17 and STAT3. However, expression of SOCS3 mRNA was higher in the samples from diabetic subjects compared to the controls albeit without a trend toward increasing or decreasing expression across the ACR-based subgroups.

The relationships between urine cell target gene RQ values and ACR among the entire T2DM cohort were also analysed by logistic regression (Figure 3.6B). Although there was a trend towards a positive correlation between IL-6R expression and ACR, overall there were no significant correlations observed for these analyses.
Figure 3.6 Relationships between IL-6, IL-6R, IL-17, SOCS3 and STAT3 and Urine ACR: (A) Box and whisker plots of ACR and mRNA targets of interest among T2DM and non-obese healthy adult (Control) subjects. Kruskall-Wallis test was used to analyse differences in values with increasing ACR. (B) Correlations between expression of mRNA targets of interest and ACR among T2DM subjects. Correlations were assessed using Spearman’s rank correlation test. For all analyses, p <0.05 was considered significant. The numbers of subjects analysed in each correlation are indicated in the table.
Relationships between Urine HbA1c and Quantitative RT-PCR of Urine Cells for Target Genes of Interest

The mean HbA1c for the cohort at the time of sample collection was 65.6 ± 17, with 16 having a value < 64mmol/mol denoting acceptable control and 44 having a value > 64mmol/mol denoting poor control.

Relative expression levels for the targets of interest were compared among the diabetic subjects with acceptable and poor glycaemic control based on HbA1c values and the healthy adult controls (Figure 3.7A). As shown, there were no striking gene expression differences between the HbA1c-based subgroups or between the diabetic groups and the healthy controls. The relationships between urine cell target gene RQ values and HbA1c among the entire T2DM cohort were also analysed by logistic regression (Figure 3.7B). Of interest, urine cell relative expression of IL-6 and STAT3 showed significant negative correlations with HbA1c while SOCS3 expression demonstrated a positive correlation, although this did not reach statistical significance.
Figure 3.7 Relationships between IL-6, IL-6R, IL-17, SOCS3 and STAT3 and HbA1c:

(A) Box and whisker plots of HbA1c and mRNA targets of interest among T2DM and non-obese healthy adult (Control) subjects. Kruskall-Wallis test was used to analyse differences in values with increasing ACR. (B) Correlations between expression of mRNA targets of interest and HbA1c among T2DM subjects. Correlations were assessed using Spearman’s rank correlation test. For all analyses, p <0.05 was considered significant. The numbers of subjects analysed in each correlation are indicated in the table.
Relationships between SBP and Quantitative RT-PCR of Urine Cells for Target Genes of Interest

Next, analyses were performed to determine whether urine cell expression levels of the selected genes was influenced by blood pressure as represented by the systolic blood pressure (SBP) at the time of sample collection. The diabetic cohort was initially subdivided into those with SBP less than and greater than 140 and these subgroups were compared with the healthy adult control group (Figure 3.8A). Following this, correlative analyses between RQ values and SBP were performed by linear regression as for the other clinical indices (Figure 3.8B). As shown in these figures, there was a trend towards a positive correlation between SBP and relative expression of IL-17 (r= 0.298, p=0.08) with no other clear relationship observed between urine cell gene expression and SBP.
Figure 3.8 Relationships between IL-6, IL-6R, IL-17, SOCS3 and STAT3 and Systolic Blood Pressure: (A) Box and whisker plots of SBP and mRNA targets of interest among T2DM and non-obese healthy adult (Control) subjects. Kruskall-Wallis test was used to analyse differences in values with increasing ACR. (B) Correlations between expression of mRNA targets of interest and SBP among T2DM subjects. Correlations were assessed using Spearman’s rank correlation test. For all analyses, p < 0.05 was considered significant. The numbers of subjects analysed in each correlation are indicated in the table.
Relationships between eGFR and Quantitative RT-PCR of Urine Cells for Target Genes of Interest

Finally, the diabetic cohort was divided into subgroups with eGFR above or below 60 ml/min at the time of urine sample collection. Relative expression levels for the targets of interest were compared among the two eGFR-based subgroups of diabetic subjects and the healthy adult controls (Figure 3.8A) followed by regression analysis to correlate RQ values for each gene of interest with eGFR (Figure 3.8B). As shown, there were no striking gene expression differences between the HbA1c-based subgroups or between the diabetic groups and the healthy controls.

As shown in these figures, there were no significant differences in relative expression of the genes of interest on the basis of eGFR category and no significant correlations between the RQ values and renal function as represented by eGFR.
Figure 3.9 Relationships between IL-6, IL-6R, IL-17, SOCS3 and STAT3 and eGFR: (A) Box and whisker plots of eGFR and mRNA targets of interest among T2DM and non-obese healthy adult (Control) subjects. Kruskall-Wallis test was used to analyse differences in values with increasing ACR. (B) Correlations between expression of mRNA targets of interest and eGFR among T2DM subjects. Correlations were assessed using Spearman’s rank correlation test. For all analyses, p < 0.05 was considered significant. The numbers of subjects analysed in each correlation are indicated in the table.

<table>
<thead>
<tr>
<th>Correlation between Glomerular filtration rate and expression of</th>
<th>Log IL-6</th>
<th>Log IL-6R</th>
<th>Log IL-17</th>
<th>Log SOCS3</th>
<th>Log STAT3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of XY Pairs</td>
<td>57</td>
<td>57</td>
<td>57</td>
<td>30</td>
<td>30</td>
</tr>
<tr>
<td>Spearman r</td>
<td>0.06</td>
<td>-0.03</td>
<td>0.01</td>
<td>0.01</td>
<td>-0.12</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>-0.21 to 0.33</td>
<td>-0.29 to 0.24</td>
<td>-0.26 to 0.28</td>
<td>-0.38 to 0.38</td>
<td>-0.47 to 0.26</td>
</tr>
<tr>
<td>P value (two-tailed)</td>
<td>0.64</td>
<td>0.85</td>
<td>0.93</td>
<td>0.96</td>
<td>0.53</td>
</tr>
</tbody>
</table>
**Bariatric Surgery Clinic Cohort**

Of 28 non-diabetic patients with grade 3 obesity who consented to participate in the study, 5 were unable to provide a urine sample and 4 provided a sample that was positive for leukocytes by dipstick testing and, for this reason, was not processed further. Of the 19 samples that were subjected to pelleting and RNA extraction by the methods previously described, 1 had a 260/280 ratio <1.7 and 2 had beta-actin CT >25 in subsequent qRT-PCR analyses. Once these 3 samples were excluded from further analysis, a total of 16 urine cell RNA samples were used in the final quantitative analysis.

**Clinical parameters for the bariatric surgery clinic cohort**

Of the 16 included in analysis, there were more female than male subjects (10 female and 6 male). The mean age of this group was younger than that of the diabetic cohort (48.9 ± 9.6 vs. 65.0 ± 10.1, p=0.0001). The mean BMI for this cohort was 50.1 ± 7.5, mean SCr was 69.5 ± 14.8, mean eGFR was 60.7 ± 41.2 median ACR was 6 (6-107) and mean HbA1c was 39.9 ± 6.3.

Urine cell relative mRNA expression of STAT3 and SOCS3 in the bariatric surgery cohort

The RNA samples extracted from urine cells of these 16 subjects were analysed for relative expression of STAT3 and SOCS3. Detectable expression of both target genes was present in all samples. Median expression levels of STAT3 and SOCS3 were 0.1332 (-0.37 to -0.72) and 0.22 (-0.55 to 1.21) respectively. The relationships between RQ values for STAT3 and SOCS3 mRNAs and BMI among these subjects was analysed by Spearman’s correlation and Kruskall-Wallis test (**Figure 3.10**).
In contrast to the T2DM cohort (see Figure 3.5B), a significant negative correlation was observed between STAT3 and BMI ($r = -0.546, p = 0.02$). A negative correlation was also observed between the RQ for SOCS3 and BMI although this did not reach statistical significance ($p = 0.2$).

<table>
<thead>
<tr>
<th></th>
<th>STAT3</th>
<th>SOCS3</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>16</td>
<td>15</td>
</tr>
<tr>
<td>Spearman r</td>
<td>-0.55</td>
<td>-0.34</td>
</tr>
<tr>
<td>95% confidence interval</td>
<td>-0.83 to -0.05</td>
<td>-0.73 to 0.21</td>
</tr>
<tr>
<td>P value (two-tailed)</td>
<td>0.02</td>
<td>0.20</td>
</tr>
</tbody>
</table>

**Figure 3.10 Relationships between STAT3 and SOCS3 and BMI in Non-Diabetic Obese Subjects:** Correlations between RQ values for STAT3 and SOCS3 mRNAs and BMI are shown. The degree of correlation was assessed using Spearman rank correlation test. P values of <0.05 were considered significant. Numbers of subjects in each correlation is indicated in the table.

The results for qRT-PCR of urine cells of diabetic and obese subjects with pre-dominantly preserved renal function and variable levels of albuminuria did not provide evidence of overt intra-renal inflammatory activity in these cohorts. As described in Chapter 1, the reported functions of STAT3 and SOCS3 suggest that they have largely opposing actions as regards tissue-specific inflammatory and fibrotic processes. Human and animal model studies would suggest that excessive activation of STAT3 signalling within the tubulointerstitial compartment is associated with progression of renal parenchymal injury and renal functional decline in the setting of diabetes. Furthermore, my results for the OZR model described in Chapter 2 suggested that SOCS3 is highly expressed in the tubulointerstitial compartment of obese, pre-diabetic animals which exhibit glomerular injury and albuminuria but lack tubulointerstitial inflammation and injury. Thus, it is conceivable that intra-renal SOCS3 expression serves as a counter-regulatory factor that prevents widespread, potentially injurious activation of JAK/STAT signalling in the settings of obesity and T2DM. With this in mind, I compared the ratios between STAT3 and SOCS3 mRNA RQ values among healthy
non-obese, diabetic and non-diabetic obese subjects. Interestingly, STAT3/SOCS3 ratios were sequentially lower for these groups (Figure 3.10A) and there was a close inverse correlation between BMI and STAT3/SOCS3 ratio within the combined study cohort (Figure 3.10B).

Figure 3.11 Relationships between STAT3/SOCS3 mRNA ratio and BMI in Healthy Controls, Type 2 Diabetic and Non-Diabetic Obese Subjects. (A) Distributions of STAT3RQ/SOCS3 RQ ratio among control, T2DM and non-diabetic bariatric surgery clinic cohorts are shown. Differences among groups were calculated using the Kruskall-Wallis test. (B) Correlation between STAT3/SOCS3 RQ ratio and BMI. The degree of correlation was assessed using Spearman rank correlation test. P values of <0.05 were considered significant.
**Discussion and Conclusion**
Use of urine shed cells has the potential to diagnose kidney disease in a safe non-invasive manner. Cellular and subcellular shedding from the kidney into the urine has been the focus of much interest due to the development of more sensitive and sophisticated techniques for its analysis. This has improved our understanding of kidney diseases and there are several recent examples of clinical studies of renal allograft recipients in which carefully processed urine cell pellets have been found to yield quantitative data that correlated closely with renal histological abnormalities and the clinical course of renal disease [282, 283].

In this discussion section I address two primary aspects of the research project. Firstly, I focus on the implications and limitations to the methodology with an emphasis on RNA quantity and quality, the effects this had on final patient selection for the study and how this issues has been addressed in similar studies. Secondly, I discuss the clinical relevance of the findings, the possible interpretations of these findings and the limitations inherent to these interpretations.

I applied the strategy of analysis of gene expression from urine shed cells to the clinical settings of T2DM and severe obesity. For the primary study, a patient cohort was selected from attendees at a diabetes outpatient clinic that consisted of individuals with relatively well preserved renal function with and without abnormal albuminuria as well as some individuals with decreased renal function. In my preliminary experiments and optimisation procedures, I found that many samples obtained in the outpatient setting were not of sufficient quality to process further due to either sediment, leukocytes in the urine or insufficient quantity. Clearly this factor would negatively impact the clinical application of testing based on urine cell RNA analysis in addition to having the potential to skew the results of studies investigating renal disease pathogenesis. Furthermore, of the samples deemed to be adequate at the time of collection, a significant proportion did not yield RNA of adequate quantity and/or quality to proceed with qRT-PCR analyses as reflected in the results of spectrophometry and Agilent bioanalyser electropherograms. My findings that RIN did not correlate closely with 260/280 ratio and that lower RIN did not result in poorer end expression by RT-PCR is in agreement with one study in which RIN was examined for RNA extracted from urine shed cells. Mas et al. studied urine cell samples from 165 kidney transplant recipients with chronic allograft nephropathy with and without abnormal proteinuria. In this study, the mean RNA yield per pellet was 1.28 ± 0.77 µg and the mean 260/280 ratio was 1.70 ± 0.05. The integrity of the RNA samples was assessed using the Agilent Bioanalyser with 14% of samples (23/165)
found to have complete RNA degradation despite acceptable 260/280 ratio. Furthermore, 108/165 samples (64.5%) had partial degradation marked by the absence of 2 ribosomal peaks. Finally, only 34/165 (20.6%) had no evidence of degradation. These authors found there was no difference in C_T obtained for the target gene TGF-beta between the partially degraded and the non-degraded samples as measured by RIN [301]. These results obtained for kidney transplant recipients are comparable to the observations that I have made in the current study of diabetic and obese subjects without acute illness or advanced CKD and suggest that meaningful quantitative results can be obtained by qRT-PCR from urine cells if RNA of adequate purity is obtained and despite the frequency of RNA fragmentation.

The assessment of quality of RNA obtained from human urine samples for qRT-PCR analysis has been reported upon in a small number of other published studies. For example, Medeiros et al. reported that the use of the RNA stabilisation solution RNAlater® resulted in higher yields and superior 260/A280 ratios as well as lower threshold cycles for stably expressed reference genes [280]. Other studies report that the detection of bands for 28S and 18S ribosomal RNA on agarose gel electrophoresis may be used to determine integrity of RNA samples prior to qRT-PCR [292, 302, 303]. Finally, in a recent large longitudinal prospective study of mRNA expression of inflammatory and T cell functional markers in kidney transplant recipients, specific criteria were reported for rejection of samples for analysis. In this study, pre-amplified samples analysed by qRT-PCR were required to have a measured 18S rRNA copy number $\geq 5 \times 10^7$ and a measured TGF-beta 1 copy number $\geq 100$ copies per microgram of total isolated RNA to be deemed adequate. Of 4300 urine samples collected from 416 patients, 714 (16.6%) samples did not reach this threshold [297]. Quality of RNA is integral to the correct interpretation of the data produced by RT-PCR in this setting and has taken a more prominent part in reports using RNA extracted from urine shed cells in recent years [297, 304, 305]. In my study, many samples that were collected were not included in final analysis as the RNA expression was too low to fall within the standard curves created on each plate to facilitate measurement of their expression. Despite this, in comparative analyses, I did not observe striking differences in the clinical characteristics of patients that had adequate quality samples compared to those that did not, with the exception that eGFR was higher in the group with poorer quality urine cell RNA. Although not directly examined in this study, this observation may reflect a higher rate of cell shedding into the urine in diabetic subjects with reduced renal function and, presumably, more advanced diabetic nephropathy. In this regard, many of the published studies in this field have been carried out on urine specimens collected from kidney transplant recipients with or without
immunological complications. In these studies, it is likely that numbers of tubular cells as well as immune cells shed into the urine are higher than would be present in healthy adults or those with early/mild native kidney disease with the result that adequate quantities of RNA were more consistently acquired. Additionally, loss of podocytes into the urine is an event that occurs rarely in healthy kidneys (<0.5 podocyte/100µmol creatinine) but has been documented to increase during active glomerular disease (average of 388 podocytes/100µmol creatinine) [306]. As many of the patients in this cohort had near normal kidney function and low urine ACR, I believe that low cell number in the urine samples acquired was a significant limiting factor for the project.

In this study, I hypothesised that inflammation, specifically IL-6/STAT3-mediated inflammation, would be detectable in urine shed cells of type 2 diabetics with early clinical evidence of nephropathy in the form of increased expression of IL-6, IL-6R and IL-17. As outlined in the introduction to this chapter and in Chapter 1, cellular and molecular activation of inflammation is reported to be a feature of diabetic kidney disease. However, the point in the pathogenesis at which abnormal inflammation begins in response to obesity and/or diabetes is not clear. Furthermore, whether activation of immune/inflammatory pathways is a direct driver of the progression of renal functional impairment and, by extrapolation, a promising therapeutic target remains poorly understood. Although my hypothesis was based on extensive review of the literature prior to initiating this project, the results for urine cell expression of IL-6, IL-6R and IL-17 transcripts did not provide evidence to support the hypothesis. Specifically and strikingly, the RQ values for urine cell IL-6 and IL6R were not significantly different for diabetic subjects compared to a group of non-diabetic, healthy adults and did not demonstrate positive or negative correlations with the major clinical indicators of renal status – eGFR and ACR. As the initial selection process restricted the analysis to samples for which adequate quantity/quality of RNA was obtained and for which the transcripts were detected within the range of a carefully optimised standard curve and related to an appropriate calibrator sample, I am confident that abnormally high IL-6/IL-6R expression would have been detected had it been present in these samples. The results for the STAT3-induced pro-inflammatory cytokine IL-17 were similarly negative for associations with renal functional indices. Thus, the results obtained in fact provide evidence for a lack of overt intra-renal inflammation in clinical stable type 2 diabetics with abnormal albuminuria and/or reduced eGFR managed at a specialty diabetes clinic. Although IL-6 has been found to
be elevated in the serum and urine of patients with diabetes and correlates with histological markers of worsening kidney disease [122, 232, 234], its role as a proinflammatory damaging cytokine is not as clear cut as once thought and the current results cast doubt on its role as an early pathogenic factor in diabetic nephropathy. As discussed in Chapter 1, IL-6 is now known to play an important role in regulating glucose metabolism and it may be that reported elevations of plasma and urine IL-6, reflect a systemic response which is directed toward maintaining metabolic homeostasis in the face of increasing obesity and hyperglycaemia as opposed to a primary inflammatory response [28, 44]. Additionally, in the one study in which intra-renal IL-6 was directly examined by in situ hybridisation in biopsies of human diabetic subjects, increased expression was observed in the setting of established nephropathy of moderate severity and, in fact, was not present in advanced disease [232]. Thus, while there may be a phase at which diabetic nephropathy is associated with high numbers of IL-6-secreting inflammatory cells, it now appears less likely that this is a feature of the early pathogenesis of the disease. An important factor to consider, is that current standard of care for diabetic patients, particularly those with micro- or macroalbuminuria, includes the frequent prescription of secondary preventive medications such as RAAS inhibitors (ACEi, ARB renin antagonists), HMG CoA reductase inhibitors (“statins”) and low dose aspirin with known anti-inflammatory effects. Thus, measures of systemic and localised inflammation that have been previously reported to be elevated among diabetic subjects with abnormal albuminuria and/or CKD may normalised by such commonly prescribed therapies.

Regarding the relationship between obesity and intra-renal inflammation, which is also of primary relevance to this thesis, the lack of correlation between BMI and the RQ values for IL-6, IL-6R and IL-17 among this cohort of diabetic subjects provides further evidence against a potent influence of obesity on intra-renal inflammatory activity. This observation is, furthermore, in keeping with my findings in the OZR model in Chapter 2, albeit that the human subjects studied have established T2DM. Again, the potential anti-inflammatory effects of secondary preventive medications should be borne in mind when interpreting these results. Also of some interest, is the finding that glycaemic control at the time of sampling, as reflected in HbA1c level, demonstrated a negative correlation with the quantity urine cell IL-6 mRNA. Given that hyperglycaemia and AGEs have been reported to induce the production of pro-inflammatory mediators by renal tubular epithelial cells, this observation appears surprisingly and, as discussed below, may indicate the existence of counter-regulatory
mechanisms that are actively enhanced within the kidney in the context of adverse metabolic conditions.

In this regard it is notable that the expression of STAT3 mRNA in urine shed cells exhibited significant correlations with BMI and HbA1c among the type 2 diabetic subjects in the final analysis cohort. In the case of BMI, this was a positive correlation while, for HbA1c the correlation was a negative one. Along with this, urine cell mRNA for the STAT3 negative regulator SOCS3 tended to increase with increasing HbA1c, although this finding did not reach statistical significance. Further elucidation of these results, including analysis of a broader range of gene targets and, if possible, direct analysis of kidney tissue, would be necessary to make strong conclusions regarding the status of STAT3-mediated pro-inflammatory signalling within the kidneys of diabetic patients with varying degrees of obesity and glycaemic control. Nonetheless, they raise the possibility that these important clinical factors exert more complex modulatory effects on intra-renal inflammatory responses than has been appreciated thus far. For example, differential effects on the expression level of SOCS3 within tubular epithelial cells may significantly alter the responsiveness of the STAT3 signalling pathway through down-regulation of STAT3 mRNA levels (as suggested by these results) as well as through inhibition of JAK/STAT3 signalling (as indicate by published studies in animal models)[224].

With these results and the findings form the OZR model in hand, I proceeded to analyse urine cell mRNA levels for STAT3 and SOCS3 in a small cohort of non-diabetic obese subjects attending an outpatient clinic for bariatric surgery evaluation. Surprisingly, in this pilot study cohort, the relationship between BMI and RQ values for STAT3 was the converse of that seen in the diabetic cohort (i.e. STAT3 mRNA demonstrated a negative correlation with BMI). As the non-diabetic obese cohort consisted entirely of individuals with BMI > 40 kg/m² while the BMIs of the diabetic cohort were, with only two exceptions < 40 kg/m², it is possible that obesity exerts a biphasic effect on intra-renal STAT3 expression. Alternatively, the hypoglycaemic and secondary preventive therapies to which the diabetic subjects were exposed may have directly or indirectly modulated the relationship between BMI and STAT3 transcription. While the data generated by this study is not sufficient to distinguish between these and other mechanistic explanations, I wished to address the possibility that co-regulation of STAT3 and SOCS3 within the kidney was influenced by obesity within the entire cohort of diabetic and non-diabetic. Therefore, I examined the ratio of RQ values for
STAT3 and SOCS3 for the three subgroups sampled as well as the relationship between BMI and STAT3/SOCS3 ratio among the combined cohort of diabetic and non-diabetic obese subjects. These analyses demonstrated that the median value for STAT3/SOCS3 ratio dropped from healthy non-obese to diabetic to non-diabetic obese subgroups and that there was a strong negative correlation between BMI and STAT3/SOCS3 ratio. This provided me with a potentially important insight into the effect of obesity on the status of STAT3 pathway regulation within the kidney and the unexpected lack of overt inflammatory activity in the urine cell samples studied – that a relative increase in SOCS3 compared to STAT3 levels may serve to prevent widespread pro-inflammatory signalling. Similarly, the unexpected negative correlations between HbA1c and urine cell STAT3 and IL-6 (but not SOCS3) mRNA levels could indicate a counter-regulatory effect induced by hyperglycaemia. I would emphasise here that these results are best viewed as hypothesis generating and, as discussed further in Chapter 5, that there is considerable potential for more precisely pursuing the role of SOCS3 and other counter-regulatory mechanisms in human subjects and animals using additional techniques.

Clearly, there are important caveats to the performance and interpretation of urine cell qRT-PCR analyses for the purposes of investigating kidney disease pathogenesis and developing non-invasive diagnostic/prognostic assays. In the first place, I did not have a “gold-standard” with which to directly compare the gene expression data obtained for the urine shed cells with true intra-renal expression. Secondly, it is not possible to distinguish between individual cell types within the pellet from which RNA is extracted. It is possible, therefore, that the STAT3 and SOCS3 expression quantified and compared in the analysis discussed in the preceding paragraph was derived from different cell types. Thirdly, quantitative data for specific mRNAs may not accurately reflect the expression of the relevant functional protein in urine cells or in the kidney itself. Indeed, where this has been examined in other studies, the level of expression of a given mRNA in urine cells, even when accurately quantified, has not consistently correlated with quantification of the relevant protein by ELISA or IHC. For example, in a study of patients with lupus nephritis, urine cell mRNA expression of CCL2 (MCP-1) was found to correlate well with protein content measured by ELISA and IHC but a similar correlation was not present for TGFβ [302]. In the prospective study of urine shed epithelial cell gene expression to predict transplant rejection, a panel of gene targets was required for accurate prediction of biopsy features of acute rejection [297].
In conclusion, qRT-PCR is a highly sensitive technique that has recently been applied by multiple investigators to the analysis of urine epithelial cells. Among the published studies, this technique has been most convincingly applied to diagnosing/predicting acute rejection in kidney transplant recipients and to identifying transcripts for which expression levels in urine cells correlate with declining function in the setting of CKD. Barriers to its greater application in clinical medicine include all the technical limitations and caveats discussed in the preceding paragraphs as well as the lack of large prospective studies to adequately assess the sensitivity and specificity of such tests. In addition, standardisation of collection conditions to optimise yield and quality of RNA, as well as the optimal methods to assess these key metrics are yet to be broadly developed and applied. Despite these caveats, I believe that the broad consistency of findings between the direct analysis of OZR kidney and the indirect analysis of the kidneys of human subjects via urine cell qRT-PCR have allowed me to disprove the hypothesis that pre-diabetic obesity and T2DM with early clinical evidence of nephropathy are associated with overt parenchymal inflammation involving excess intra-renal IL-6 production and STAT3 pathway activation.
Chapter 4 Relationships between Obesity and Renal Parameters in an Irish Cohort of Type 2 Diabetics Patients
Introduction
Type 2 diabetes is almost uniformly characterized by increased BMI and this is thought to be central to the pathophysiology of the disease. How obesity impacts on the microvascular and macrovascular pathology independent of hyperglycaemia and hyperlipidaemia remains unclear, although obese type 2 diabetics have increased risk for hypertension, hyperlipidaemia and microalbuminuria compared to those who are not obese. [17, 95]

Additionally, the development of complications in response to diabetes and obesity is not uniform and possibly reflects differences in inflammatory response mounted at a molecular level to increased metabolic demands or functional changes in the body stemming from the expanding fat mass. As discussed in Chapter 1, there have been many population-based studies linking increased BMI with the development of CKD. However, the relationship is not entirely clear and there may be confounding based on the methods by which GFR is reported in subjects with extreme obesity [307].

Physiological and structural changes occur within the kidney in response to increased BMI and diabetes. Glomerular hyperfiltration is an absolute increase in GFR and occurs physiologically in response to protein loading, which, in this capacity, is referred to as the renal functional reserve. Although this functional reserve is lost in CKD, glomerular hyperfiltration in type 1 and type 2 diabetics is the first clinically detectable manifestation of diabetic kidney disease, and precedes the development of proteinuria [246]. In diabetes, hyperfiltration occurs at whole-kidney level, with abnormalities in vascular control leading to with increased renal blood flow. This is accompanied by preferential dilatation of the afferent arteriole leading to increased GFR, intra-glomerular pressure and filtration fraction. Systemic factors including insulin, insulin-like growth factors, AGE and atrial natriuretic peptides have been postulated as being mediators of increased glomerular flow rate [90]. Another mechanism by which glomerular hyperfiltration is thought to occur in T2DM is in response to increased proximal tubular sodium and glucose reabsorption which leads to vasodilatation secondary to suppressed tubuloglomerular feedback. Increased proximal tubular sodium reabsorption is also known to occur in obese individuals in the absence of diabetes [308]. This is thought to be due to increased post-glomerular oncotic pressure leading to enhanced sodium reabsorption. It is not clear to what extent hyperfiltration represents a risk factor for the development of diabetic nephropathy. More recent studies suggest that it confers a modest
risk for CKD due to diabetic nephropathy. Whether this enhanced risk is independent of other factors (e.g. glycaemic control, hypertension) is not well documented [90, 246].

Obesity is also reportedly associated with whole-kidney hyperfiltration due to increases in RPF and/or GFR. However, due to the methods by which renal function has been estimated in such studies of obesity, these findings may not reflect true physiological hyperfiltration [84, 85, 88, 253]. For example, some studies reporting increased RPF and GFR in obese compared to non-obese subjects found that correction of these factors using height rather than body surface area resulted in elimination of the differences [309]. Nonetheless, GFR and RPF are conceptually viewed as being proportional to kidney size which should, itself, be proportional to body weight. As heavier individuals have higher fat-free mass (FFM) in addition to fat mass up to a BMI of 35 kg/m², obese body composition is likely to increase metabolic demands and, therefore, waste clearance demands on the kidney. As the maximum number of nephrons remains constant from birth and cannot be increased in response to excess body weight, each nephron must increase its functional capacity to respond to the metabolic demands of obesity. The resultant glomerular hypertrophy and increased glomerular pressure may lead to the development of ORG and secondary FSGS [248, 310, 311]. In a mouse model of reduced nephron mass, diet-induced obesity did not result in striking physiological changes in arterial pressure or creatinine clearance compared to wild-type mice fed the same diet. However, fibrosis within the kidney was accelerated by this diet in nephron-reduced obese mice compared to obese and lean control mice indicating that obesity in the setting of reduced nephron mass has subtle but important effects on kidney structure that are not readily apparent using current measures of kidney function [312].

Identifying the factors responsible for the increased prevalence of CKD in obese individuals is likely to facilitate a better understanding of the functional and structural changes that occur in the kidney in response to obesity. Additionally, accurately categorising CKD in obese individuals will be essential as it may allow selective targeting of more aggressive approaches to managing diabetes and cardiovascular risk factors in high risk patients. Furthermore, obese individuals, when hospitalised, are at increased risk of AKI and have worse outcomes following mechanical ventilation, pancreatitis and sepsis compared to non-obese patients [52, 77, 273]. Finally, adjustment of drug dosing for level of renal function is hampered in obese patients as commonly applied formulae for estimation of GFR were not designed to incorporate the body mass that is frequently being encountered in the intensive
care unit or emergency department today. Thus, obese individuals are likely to be exposed to over or under-dosing of diverse pharmaceuticals such as antibiotics, anticoagulants and chemotherapeutic agents [313-316].

There is much debate in the literature regarding the most appropriate measure of renal function to accurately identify obese individuals with CKD. The formulae that are most commonly used to estimate renal function such as Cockcroft-Gault and MDRD were calculated based on subjects without severe obesity [310, 317]. Estimation of creatinine clearance with these formulae makes the assumption of a regular rate of production of creatinine in proportion to body mass. In obese individuals with BMI $>35\text{kg/m}^2$, the FFM, which is likely to be an important variable in the rate of creatinine production, does not have a predictable relationship with total body mass [311]. This difficulty in GFR estimation among obese subjects has been approached, in some studies, by the analysis of body composition using bio-impedance [316, 318, 319]. Various adjustments to facilitate more accurate estimation of creatinine clearance based on the Cockcroft-Gault formula have been proposed including using ideal body weight [320], no body weight, total body weight and adjusted actual body weight [321], However, these modified formulae result in over- or under-estimation of GFR when compared to ‘gold-standard’ techniques such as iohexol clearance [322], $^{99}\text{mTcDTPA}$ or $^{51}\text{Cr-EDTA}$, and, in fact, have not been well validated in the extreme obese population [311]. Salazar and Corcoran, in a study of 12 obese and 9 normal weighted subjects, established a formula based on creatinine clearance being proportional to FFM [323] which was subsequently validated in obese transplant patients [324]. However, this formula was found to be inferior to Cockcroft-Gault corrected for lean body weight in other populations [318]. In other studies, the commonly used MDRD and CKD-EPI formulae were also shown to have poor accuracy for estimating GFR compared to 24 hour creatinine clearance collections, $^{51}\text{Cr-EDTA}$ and iohexol GFR in individuals with BMI $>30\text{kg/m}^2$ [311, 322, 325]. It is clear, therefore, that estimating true renal function kidney in the setting of extreme obesity is not straightforward and that both over- and under-identification of CKD may occur.

Increased albuminuria is a manifestation of diabetic nephropathy as well as a feature of ORG. Albuminuria in the presence of T2DM increases the relative risk for all-cause mortality, declining GFR and ESRD. In the ADVANCE study, which involved 11,140 type 2 diabetics over the age of 55 with one risk factor for cardiovascular disease, the risk for cardiovascular
event, cardiovascular death and renal events was associated with higher urinary ACR, even within the normal range for ACR [326]. Importantly, however, in current practice, the majority of type 2 diabetics with impaired renal function have urinary albumin excretion within the normal range [327], likely as a result of the almost ubiquitous application of RAAS blockade, lower blood pressure targets and lipid lowering agents [276]. Therefore, utility of measuring albuminuria as a surrogate marker or predictor of CKD in actively managed diabetic (or non-diabetic obese) patient cohorts is questionable. In studies examining the effects of weight loss in obese individuals, diet and pharmacological methods of losing weight were associated with decreases in albuminuria while bariatric surgery led to significant changes in both albuminuria and creatinine clearance [328]. As discussed above, the dramatic changes in FFM and FM that occur in response to bariatric surgery may have confounded accurate estimation of the changes in renal function that have been reported in these studies and, because of this, the benefits of bariatric surgery for risk of CKD and ESRD require more validation [311].

In Chapters 2 and 3 I hypothesised that obesity and T2DM lead to increased kidney injury via intra-renal inflammatory activity. For this section of my thesis, I hypothesised that higher BMI is associated with increased risk for CKD due to diabetic nephropathy with the effect possibly being mediated through obesity-driven functional or structural changes within the kidney. Given current population trends toward ever increasing prevalence of obesity during childhood and adolescence and the known temporal relationship between duration of obesity and development of T2DM, such an enhancing influence of obesity on CKD risk among type 2 diabetics would have enormous public health implications. Additionally, I hypothesised that continued increase in weight during active medical management for T2DM represents a distinct adverse condition for loss of renal function and development of CKD.

In a cohort of almost 1000 type 2 diabetics undergoing outpatient monitoring and management of T2DM at Galway University Hospital within the past 10 years, I examined the relationship between BMI and commonly reported indicators of renal health including GFR estimated by multiple formulae and ACR. My analyses included two specific components. The first of these was a cross-sectional analysis to determine the relationships between eGFR/ACR and BMI within the cohort at a single index time-point, taking into account the potential influence of other variables likely to influence the risk for kidney disease such as age, duration of diabetes, level of glycaemic control, blood pressure and use
of anti-hypertensive therapies. The second component consisted of an analysis of the changes that occurred in BMI and renal functional indices between the initial time-point and a follow-up evaluation 3 to 5 years later. In this latter analysis, I had the opportunity to specifically examine the potential impact of further weight gain on the development of nephropathy among obese adults with T2DM.
Methods

Study Population

Using a computerised database of adult diabetic patients managed by the Endocrinology services of Galway University Hospitals (Diabetes monitoring database, DIAMOND), 939 study subjects with T2DM were identified. Subjects were included in subsequent analyses if the data retrieved from the database included at least one measurement of SCr, ACR and BMI recorded from a single clinic visit. For subjects with multiple such clinic visits, the data from the earliest visit was initially captured including demographic information (age, gender, medication usage, smoking status, duration of diabetes), details of physical examination (BMI, blood pressure), laboratory test results (SCr, HbA1c, total cholesterol, LDL, HDL and triglyceride levels, AST, urine ACR) and number/class of hypoglycaemic, lipid lowering and anti-hypertensive therapies. Individuals with data missing for ACR, SCr and BMI were excluded from the analysis. Follow-up data on 547 of the 939 subjects who had repeated demographic, physical examination and laboratory parameters available in the DIAMOND database were also captured for analysis. Criteria for inclusion in the “follow-up” cohort included the availability of concurrent recorded values for BMI and SCr at a time-point between 3 and 5 years following the index clinic visit.

The modification of diet in renal disease (MDRD) formula [310] as well as the Cockcroft Gault (CG) formula were calculated from the recorded laboratory and clinical variables at each time-point. The CG formula was calculated using the reported SCr, age and body weight as \([140-\text{age (years)} \times \text{body weight (kg)/serum creatinine (µmol/L)}] \times 0.85\) (If female) [317]. Adjusted body weight CG was calculated as \([(\text{Actual body weight (ABW)(0.4) = (total body weight (TBW) - ideal body weight (IBW)(0.4) + ideal body weight})] [321].

The MDRD formula was calculated as estimated GFR (eGFR) (ml/min/1.73m2) =175 X SCr-\text{1.154 X age - 0.203 X (0.742 if female) X (1.212 if Black)} using reported age and creatinine for either baseline or follow up visit [310]. It was necessary to make the assumption that all subjects were non-black as race/ethnicity was not recorded in the database.

The Salazar and Corcoran equation was calculated as for males as Creatinine clearance (CrCl) = \([137 - \text{age}] \times [(0.285 \text{ X weight}) + [12.1 \text{ X height}^2]/(51 \text{ X SCr})]\) and for females as CrCl =\([146 - \text{age}] \times [(0.287 \text{ X weight}) + [9.74 \text{ X height}^2]/(60 \text{ X SCr})]\) [323].
For the purpose of analysis, patients were stratified according to standard Kidney Disease Outcome Quality Initiative (KDIGO) guidelines such that individuals with eGFR <60ml/min per 1.73m² or CrCl > 60cc/min were deemed to have impaired renal function \[310\]. Albuminuria as a marker of kidney damage was also stratified according to KDIGO guidelines as follows: <1 mg/mmol = normal, >1/<3 mg/mmol= mildly increased, 3-30 mg/mmol = moderately increased, >30mg/mmol = severely increased \[300\]. A greater than 5% change in BMI between baseline and follow-up clinic visits was selected as representing a clinically significant weight change in the follow-up cohort analysis. This was based on the results reported by Gelber et al. in which a 10% change in BMI over 14 years was associated with increased risk of CKD \[64\]. Change in eGFR or CrCl per year was calculated based on index and follow-up visit values and the exact time interval between the two visits. Subjects with calculate decrease of 3 ml/min/year were categorised as having accelerated renal functional loss \[329\].

**Statistical Analyses**
Continuous variables are presented as mean ± SD or median (range), as appropriate.
Continuous variables (including ACR and antihypertensive medication number) that did not follow a normal distribution patterns were log transformed prior to analysis. An analysis of variance (ANOVA) and the χ² test were used for between-group comparisons of the continuous and categorical variables, respectively. Pearson’s correlation coefficients were used to assess the simple correlations between clinical parameters and GFR/CrCl and BMI or other indices. Logistic regression was used to estimate the odds ratio (OR) and 95% confidence intervals (CI) for the outcome of CKD (defined as eGFR/CrCl <60). Unadjusted models and models adjusted for log baselineACR, duration of diabetes and HbA1c and serum cholesterol were performed. For all statistical analyses, p<0.05 (two tailed) was considered to be statistically significant. Analyses were performed using SPSS® version 18 for Windows®.
Results

Baseline Characteristics
Within the DIAMOND database, a total of 939 subjects were identified as meeting the study criteria of having a documented diagnosis of T2DM and concomitantly recorded values for BMI, SCr and ACR at an index (“baseline”) Diabetes Clinic outpatient assessment. For this cross-sectional cohort, the mean age was 56.2 ± 12.5 years, the time since first diagnosis of T2DM was 6.1 ± 7.0 years and the proportion that was male was 64.5%. Primary treatment for T2DM was diet alone in 26(2.7%), insulin alone in 87(9.3%), oral hypoglycaemic alone in 635(68.2%), combined oral hypoglycaemic and insulin in 185(19.8%).

Table 4.1 summarises the numbers and proportions for each WHO grade of BMI-based obesity among this cohort. Consistent with expectations, the majority of subjects were overweight or obese at the time of the baseline evaluation.

Table 4.1: Number and Percentage for each WHO Grade of Obesity

<table>
<thead>
<tr>
<th>Grades of BMI (kg/m²)</th>
<th>N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18-24.9</td>
<td>73 (7.8)</td>
</tr>
<tr>
<td>25-29.9</td>
<td>294 (31.2)</td>
</tr>
<tr>
<td>30-34.9</td>
<td>306 (32.5)</td>
</tr>
<tr>
<td>35-39.9</td>
<td>169 (18.4)</td>
</tr>
<tr>
<td>&gt;40</td>
<td>97 (10.3)</td>
</tr>
</tbody>
</table>

Number and percentage of patients included in the cohort at baseline within each grade of Obesity as defined by the WHO [241].

Table 4.2 summarises baseline urine ACR categories for the cohort. The numbers and proportions within each category of albuminuria that also had eGFR (calculated by MDRD formula) < 60ml/min/1.73m² are also indicated. As shown, the cohort consisted predominantly of diabetic patients with normo- or micro-albuminuria with only a small proportion having macro-albuminuria. Nonetheless, the frequency of reduced renal function, at least as reflected by MDRD eGFR, increased through each grade of albuminuria and, in total, 238 (25%) of the cohort had CKD by this criterion.
Table 4.2: Number and Percentage for each Stage of Albuminuria

<table>
<thead>
<tr>
<th>ACR (mg/mmol)</th>
<th>N (%)</th>
<th>N (%) with eGFR &lt;60ml/min/1.73m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal (0-0.99)</td>
<td>261 (27.7)</td>
<td>40 (15.3)</td>
</tr>
<tr>
<td>Mildly increased (1-2.99)</td>
<td>315 (33.5)</td>
<td>70 (22.2)</td>
</tr>
<tr>
<td>Moderately increased (2-29.9)</td>
<td>286 (30.4)</td>
<td>91 (31.8)</td>
</tr>
<tr>
<td>Severely increased (&gt;30)</td>
<td>79 (8.4)</td>
<td>37 (46.8)</td>
</tr>
</tbody>
</table>

Number and percentage of patients included in the cohort at baseline within each category of albuminuria as defined by KDIGO guidelines [299].

Other baseline characteristics of the cohort are shown in Table 4.3.

Table 4.3 Baseline Diabetic and Renal Characteristics of Cohort

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Mean ± SD (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (Kg/m²)</td>
<td>32.3 ± 6.3 (939)</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.46 ± 1.45 (749)</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.03 ± 1.0 (810)</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.9 ± 1.3 (793)</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>28.7 ± 15.6 (217)</td>
</tr>
<tr>
<td>Creatinine (µmol/L)</td>
<td>92.3 ± 37 (939)</td>
</tr>
<tr>
<td>MDRD eGFR (ml/min/1.73m²)</td>
<td>77.2 ± 23.3 (939)</td>
</tr>
<tr>
<td>ACR (mg/mmol)</td>
<td>16.3 ± 66.6 (939)</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>136.2 ± 19 (929)</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>75.6 ± 9.6 (925)</td>
</tr>
</tbody>
</table>

Baseline values for BMI, HbA1c cholesterol, triglycerides, AST, creatinine, eGFR as estimated by MDRD calculated from creatinine and age, ACR, SBP and DBP are presented as mean±SD.

Overall, the cohort had moderately well-controlled diabetes as indicated by mean HbA1c of 7.46 ± 1.45%. The number of subjects with poor glycaemic control (HbA1c > 8%) at the time of the baseline study outpatient visit was 204/749 (27.2%). As shown in the Table, blood pressure control was, on average, good for this treated cohort of type 2 diabetics. The number of subjects with systolic blood pressure that might be considered above a desirable clinical target of ≤140 mmHg was 359/929 (38.6%), however, only 60/925 (6.5%) had a diastolic blood pressure ≥90mmHg. Blood pressure level of <140/90 was selected as an indicator of adequate control based on the most recent KDIGO guidelines which failed to find evidence
that aiming for a lower blood pressure than 140/90 resulted in improved clinical outcomes[330]. The median number of anti-hypertensive agents used was 2 (0-6). RAAS blockade was prescribed for 747/939 (79%) subjects with 658 (69%) receiving a single agent, 86 (9%) receiving dual blockade and 3 subjects receiving three agents that inhibit the RAAS. As regards lipid profiles, total cholesterol level was above the typical clinical target of 5.0 mmol/L in 139/810 (17.2%) subjects.

In summary, the cohort of subjects included in this study had broadly expected characteristics for a sampling of actively-managed, ambulatory, adult type 2 diabetics from a tertiary referral Endocrinology practice in the current era. This included predominant overweight or obesity, satisfactory glycaemic control achieved primarily by prescription of oral hypoglycaemic agents without or with insulin therapy and generally well controlled dyslipidaemia and hypertension. In regard to renal indices, reduced function (MDRD eGFR < 60 ml/min/1.73m2) was relatively common (25%) and its frequency demonstrated an expected relationship with urine albumin excretion as reflected in ACR values. Importantly, and also as expected in current practice, the current use of RAAS blockade for blood pressure control (and presumably also for management of albuminuria) was very common within the cohort while the proportion of subjects with severely increased albuminuria and/or advanced CKD was low.
Correlations between BMI and Renal Parameters within the Cohort

Obesity of varying degrees was present in the cohort as outlined in Table 4.1. In an initial analysis, I questioned whether there was a demonstrable relationship between BMI and indices of renal function and how this was influenced by other pertinent clinical and laboratory factors such as age, duration of diabetes, level of glycaemic control, fasting lipid levels and blood pressure.

This required some consideration of the method to be employed for estimation of renal function at the time of the baseline outpatient visit for the study. As alluded to in the Introduction, there is no strong consensus as to which method is most accurate for estimation of renal function in obese individuals [311]. I initially assessed multiple reported formulae for estimating GFR or CrCl for which the necessary data were available in order to compare the rates of CKD identified by each.

For this analysis, I considered CKD to be eGFR < 60ml/min/1.73m² using the MDRD equation or calculated CrCl < 60cc/min using Cockcroft Gault equation (based on either lean body weight (LBW), actual body weight or adjusted body weight) or using the Salazar-Corcoran formula. Additionally, as hyperfiltration is hypothesised to occur in response to obesity and metabolic stress in T2DM, I also assessed the frequency of eGFR or calculated CrCl > 130 for each equation.

The results of these calculations are summarised in Table 4.4.
Table 4.4: CKD and Possible Hyperfiltration based on Five Different Formulae

<table>
<thead>
<tr>
<th></th>
<th>CrCl/eGFR&lt;60 N(%)</th>
<th>CrCl/eGFR &gt;130ml/min N(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDRD eGFR</td>
<td>238 (25.3)</td>
<td>25 (2.6)</td>
</tr>
<tr>
<td>Cockcroft-Gault CrCl LBW</td>
<td>267 (28.4)</td>
<td>63 (6.7)</td>
</tr>
<tr>
<td>Cockcroft-Gault CrCl ABW</td>
<td>158 (16.8)</td>
<td>223 (23.7)</td>
</tr>
<tr>
<td>Cockcroft-Gault CrCl ABW Adjusted</td>
<td>188 (20.0)</td>
<td>104 (11.1)</td>
</tr>
<tr>
<td>Salazar-Corcoran equation</td>
<td>186 (19.8)</td>
<td>139 (14.8)</td>
</tr>
</tbody>
</table>

Values for eGFR and CrCl were calculated based on reported weight, height and age from the database using appropriate formula for MDRD, Cockcroft-Gault and Salazar-Corcoran equations as outlined in methods section [310, 311, 317]. Creatinine clearance (CrCl), Modification of diet in renal disease estimated glomerular filtration rate (MDRD eGFR), Lean body weight (LBW), Actual body weight (ABW), actual body weight adjusted (ABW adjusted)

In the absence of a gold standard for true renal functional impairment with which to compare the groups, I also assessed the various calculations based on their ability to identify SCr >150 µmol/L as representing CKD. The rationale for this was that, in a predominantly Caucasian population, this SCr value would be considered clinically significant regardless of body weight. The number of subjects with a baseline SCr >150 µmol/L was 46/939 (4.9%). As shown in Table 4.6, all of these were correctly identified as having reduced renal function by the MDRD eGFR formula and the CG formula calculated using LBW. For CG formulae using actual body weight or adjusted body weight or for the Salazar-Corcoran formula, a small number of individuals with SCr >150 µmol/L were not categorised as having CrCl < 60 cc/min.
Given that the MDRD formula was sensitive in identifying SCr > 150 µmol/L as being associated with reduced eGFR and that it is currently the most commonly used formula for estimating kidney function in clinical practice and research studies [311, 313, 318, 325], this value was used in subsequent analyses examining the relationship between BMI and CKD in the cohort. Furthermore, because the MDRD formula does not incorporate body weight into the calculated clearance value, I reasoned that use of this formula would avoid inherent correlations between BMI and estimated GFR/CrCl.

As shown in Figure 4.1, there was a modest, but statistically significant positive correlation between BMI and MDRD formula-based eGFR within this cohort.

<table>
<thead>
<tr>
<th>Creatinine &gt;150 µmol/L: N(%) detected by each formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>eGFR MDRD</td>
</tr>
<tr>
<td>CG-GFR (LBW)</td>
</tr>
<tr>
<td>CG-GFR (ABW)</td>
</tr>
<tr>
<td>CG-GFR (AdjABW)</td>
</tr>
<tr>
<td>Salazar-Corcoran</td>
</tr>
</tbody>
</table>
Thus, without adjustment for other variables, renal function within the full cohort, as reflected in MDRD eGFR, tended to be greater with increasing BMI. This observation could indicate an association between higher BMI and glomerular hyperfiltration among type 2 diabetics but may also reflect the effect of other co-associated variables such as age, duration of diabetes and glycaemic control or, as previously discussed, obesity-related influences on the relationship between eGFR and true GFR.

In regard to hyperfiltration, there were 25 subjects (2.6%) with eGFR >130 ml/min/1.73m². Compared to all others, these subjects were younger (47.4 ± 12.9 vs. 56.6 ± 12.4 years) and had higher BMI (36.5 ± 11.1 vs. 32.3 ± 6.2, p=0.001).

I next performed analyses to determine whether BMI exerted an influence on the presence of reduced renal function (CKD - defined as MDRD eGFR < 60 ml/min/1.73m²) within the cohort at the time of the baseline evaluation. Initially, when BMI was compared for those with and without CKD, no significant difference was observed (32.4 ± 6.4 kg/m² vs. 32.0 ± 6.0 kg/m², p=NS). However, the groups with and without CKD did differ significantly for several other clinical and laboratory variables as shown in Table 4.6.

![Figure 4.1](image-url): Linear Regression analysis of the relationship between BMI and MDRD-estimated GFR among the cohort of 939 type 2 diabetic subjects.
Table 4.6: Comparison of Clinical Laboratory Variables of Type 2 Diabetics without and with Reduced Renal Function.

<table>
<thead>
<tr>
<th>Variable</th>
<th>eGFR &gt;60ml/min/1.73m²</th>
<th>eGFR &lt;60ml/min/1.73m²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>AST (U/L)</td>
<td>30.3 ±16.7</td>
<td>24.0 ± 9.8</td>
<td>0.009</td>
</tr>
<tr>
<td>LogUACR (mg/mmol)</td>
<td>0.81 ± 1.3</td>
<td>1.56 ± 1.8</td>
<td>0.0001</td>
</tr>
<tr>
<td>LogAntihypertensive</td>
<td>0.26 ± 0.23</td>
<td>0.33 ± 0.22</td>
<td>0.0001</td>
</tr>
<tr>
<td>Age (years)</td>
<td>54.2 ± 12.1</td>
<td>62.3 ± 11.7</td>
<td>0.0001</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76.7 ± 9.3</td>
<td>72.6 ± 9.9</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

Thus, although of similar BMI, the group with reduced eGFR were characterised by having higher urine ACR, a greater number of anti-hypertensive medications and greater age than those without.

As an alternative approach, I also applied logistic regression to assess the association between BMI and CKD at the time of baseline evaluation with and without adjustment for other variables. As summarised in Table 4.7, an unadjusted analysis indicated no statistically significant association between BMI and risk for eGFR < 60 ml/min/m². There were no adjustments pre-specified for analysis (glycaemic control, hyperlipidaemia and urine ACR) that revealed an association between BMI and risk of CKD.

Table 4.7: Odds Ratio for CKD Based on BMI showing Unadjusted and Adjusted Values.

<table>
<thead>
<tr>
<th></th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI</td>
<td>1.01</td>
<td>0.98-1.04</td>
<td>0.359</td>
</tr>
<tr>
<td>+ Duration of diabetes</td>
<td>0.97</td>
<td>0.93-1.07</td>
<td>0.11</td>
</tr>
<tr>
<td>+ Duration of diabetes, HbA1c, Log UACR, Cholesterol, SBP</td>
<td>1.01</td>
<td>0.97-1.03</td>
<td>0.95</td>
</tr>
</tbody>
</table>

In contrast, an unadjusted analysis showed a significant influence of BMI on risk for glomerular hyperfiltration (defined as eGFR > 130 ml/min/m²) (OR 1.07, 95% CI 1.03-1.12, p=0.01). This was present after adjustment for duration of diabetes as well as after adjustment for duration of diabetes, HbA1c, cholesterol level, log urine ACR and SBP (OR 1.07, 95% CI
1.01-1.13). By this analysis, for every unit increase in BMI, the OR for glomerular hyperfiltration increased by 7%.

Overall, the cross-sectional analysis of this cohort indicated an overall positive correlation between obesity (as reflected by BMI) and renal function (as reflected by MDRD eGFR). Furthermore, regression analyses with adjustment for relevant co-variables indicated that higher BMI conferred increased risk for concomitant hyperfiltration (eGFR > 130 ml/min/1.73m²) but not CKD (eGFR < 60 ml/min/1.73m²) within the cohort.

Given the limitations of a single time-point and the relatively short average duration of diabetes at the time of baseline outpatient evaluation for the study (6 years), I elected next to perform an analysis of paired data from the baseline outpatient evaluation and from a subsequent evaluation between 3 and 5 years later, the results of which are summarised in the next section.
Follow-Up Study
Data from a second outpatient evaluation was obtained on 547 patients with a mean interval between evaluations of 3.83 ± 0.86 years. Differences between specific baseline and follow-up values for the cohort are shown in Table 4.8.

Of note, systolic and diastolic blood pressure readings and plasma triglyceride levels were lower on follow up, presumably reflecting the influence of on-going active clinical management. There was no overall difference for BMI, MDRD eGFR and urine ACR between the two time-points. Glycaemic control (HbA1c) and plasma cholesterol levels were also unchanged at the follow-up evaluation.

Table 4.8: Clinical and Laboratory Variables at Baseline and Follow-up Outpatient Evaluations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Baseline</th>
<th>Follow-up</th>
<th>n</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>32.5 ± 6.1</td>
<td>32.3 ± 6.3</td>
<td>547</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.5 ± 1.4</td>
<td>7.4 ± 1.5</td>
<td>410</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>4.02 ± 0.9</td>
<td>3.9 ± 0.9</td>
<td>382</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.94 ± 1.0</td>
<td>1.7 ± 1.0</td>
<td>367</td>
<td>0.002</td>
</tr>
<tr>
<td>AST (U/L)</td>
<td>28.47 ± 3.4</td>
<td>26.75 ± 2.43</td>
<td>36</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>136.4 ± 19.1</td>
<td>132.9 ± 16.8</td>
<td>537</td>
<td>0.0001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>76.09 ± 9.6</td>
<td>72.4 ± 8.78</td>
<td>533</td>
<td>0.0001</td>
</tr>
<tr>
<td>ACR (mg/mmol)</td>
<td>11.71 ± 51.6</td>
<td>16.7 ± 92.8</td>
<td>300</td>
<td>NS</td>
</tr>
<tr>
<td>MDRD eGFR ml/min/1.73m²</td>
<td>77.19 ± 23.7</td>
<td>76.3 ± 27.4</td>
<td>430</td>
<td>NS</td>
</tr>
</tbody>
</table>

Baseline and follow up characteristics for patients in whom clinical and laboratory values were recorded from a follow up for a mean of 3.83±0.86 years. Paired Student’s t-test was used to compare groups and a p-value of <0.05 was considered significant.
Relationships between Changes in BMI and eGFR at Follow Up

Although T2DM and its complications are intricately linked to obesity, many patients are not able to lose weight. In the physician’s health study for which self-reported BMI over a 14 year timespan was available, a 10% increase in BMI was associated with an odds ratio of 1.27 for development of CKD (95% CI, 1.06 to 1.53) [64]. Given the shorter follow-up interval for the current study, I elected to categorise those subjects with BMI increase or decrease of ≥ 5% of baseline as subgroups with “weight gain” and “weight loss” respectively. The remaining subjects were categorised as “stable weight”. The numbers and proportions in these categories are shown in Table 4.9 along with the values for baseline BMI. Of note, those in the weight gain category also had higher BMI at baseline. The mean absolute increase in BMI for those in the “weight gain” subgroup was 3.56 ± 2.27 kg/m² compared to an average weight loss of -1.21 ±2.9 kg/m² for the combined “weight loss” and “stable weight” subgroups.

Table 4.9: Subgroups of Type 2 Diabetics with Weight loss, Stable Weight and Weight Gain between Baseline and Follow-up Outpatient Evaluations.

<table>
<thead>
<tr>
<th>Category</th>
<th>n/N(%)</th>
<th>Baseline BMI kg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight Loss</td>
<td>125/547 (22.9)</td>
<td>30.1 ± 5.7</td>
</tr>
<tr>
<td>Stable Weight</td>
<td>219/547 (58.3)</td>
<td>32.0 ± 5.2</td>
</tr>
<tr>
<td>Weight Gain</td>
<td>103/547 (18.8)</td>
<td>35.4 ± 8.5</td>
</tr>
</tbody>
</table>

The subgroups also demonstrated significant differences for baseline HbA1c (7.4 ± 1.3 vs. 7.4 ± 0.2 vs. 8.02 ± 1.7, p=0.001) and baseline logACR (1.38 ± 0.12 vs. 1.37 ± 0.07 vs.1.6 ± 0.16 p=0.009). Only 430/547 (78.6%) of the follow-up group had Scr measured and recorded at the follow up evaluation. Among these, there was no difference between the subgroups for baseline or follow-up MDRD eGFR (or CrCl estimated by other formulae).
Table 4.10 Baseline and Follow eGFR for Groups with Weight loss, Stable or Weight gain.

<table>
<thead>
<tr>
<th>Category</th>
<th>Baseline eGFR ml/min/1.73m²</th>
<th>Follow up eGFR ml/min/1.73m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight Loss</td>
<td>76.6 ± 24.1</td>
<td>77.7 ± 29.4</td>
</tr>
<tr>
<td>Stable Weight</td>
<td>77.6 ± 21.5</td>
<td>77.1 ± 25.5</td>
</tr>
<tr>
<td>Weight Gain</td>
<td>77.6 ± 23.3</td>
<td>76.2 ± 27.4</td>
</tr>
</tbody>
</table>

The mean change of eGFR between baseline and follow-up for the entire group was -0.08 ± 5.25 ml/min/year. In order to define a subgroup with abnormal decline in renal function, an eGFR change of ≥ 3.0 ml/min/year was selected [329]. By this criterion, 112/430 (26%) had abnormal decline in renal function between the two time-points. In comparing these subjects with those who did not have abnormal renal functional decline, a significantly increased baseline logACR (1.52±1.6 (n=103) vs. 0.76 ± 1.2 (n=444) p=0.002) was also noted.

When the frequency of abnormal renal functional decline among the “weight gain” subgroup was compared with that of the combined “weight loss” and “stable weight” subgroups (Figure 4.2), it was evident that a greater proportion exhibited this characteristic (p = 0.02 by χ² test). This was, of course, despite comparable baseline eGFR values for the groups.
Number of cases that had either a decrease or increase BMI as defined as less than or greater than 5% increase in BMI broken into whether or not there was a >3ml/min/year loss in eGFR as measured by MDRD. Chi-square test was used to compare proportions between groups (p = 0.02).

Of note also, for the “weight gain” group, the average rate of change of eGFR was negative (-1.09 ± 5.22 ml/min/year) while that of the other two subgroups combined was slightly positive (0.14 ± 5.24 ml/min/year). Statistical analysis of this difference in renal function change demonstrated marginal significance (p = 0.05).

These results suggested that weight gain during follow-up represents an adverse risk factor for decline of renal function either through direct influences on the pathophysiological processes underlying diabetic nephropathy/CKD or through co-association with other risk factors. Correlations between change in BMI, change in eGFR and other clinical variables were, therefore, next assessed using linear regression. By these analyses (Table 4.11), significant correlations with rate of change of eGFR were observed for baseline HbA1c, logACR and systolic blood pressure. Positive correlations were also observed between rate of change of eGFR and baseline BMI and follow up BMI, although these did not reach statistical significance (0.06 – 0.09).
Table 4.11: Correlations between Clinical Variables and Rate of Change in eGFR

<table>
<thead>
<tr>
<th>Variable</th>
<th>Pearson’s correlation (with ΔeGFR)</th>
<th>p</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline BMI</td>
<td>0.088</td>
<td>0.06</td>
<td>430</td>
</tr>
<tr>
<td>Follow up BMI</td>
<td>0.08</td>
<td>0.09</td>
<td>430</td>
</tr>
<tr>
<td>Δ BMI</td>
<td>-0.09</td>
<td>0.84</td>
<td>430</td>
</tr>
<tr>
<td>Baseline HbA1c</td>
<td>-0.123</td>
<td>0.01</td>
<td>394</td>
</tr>
<tr>
<td>Baseline SBP</td>
<td>-0.103</td>
<td>0.03</td>
<td>425</td>
</tr>
<tr>
<td>Baseline logACR</td>
<td>-0.220</td>
<td>0.0001</td>
<td>430</td>
</tr>
</tbody>
</table>

In correlative analyses for change in BMI from baseline to follow-up evaluation, a significant correlation with follow-up MDRD eGFR was observed (Figure 4.3).

![Figure 4.3: Linear regression of the relationship between BMI change and follow-up eGFR.](image)

Finally, in a logistic regression model, using > 5% increase in BMI from baseline as a categorical variable, the unadjusted OR for abnormal decline in eGFR was 1.83 (95% CI 1.09 - 3.07, p=0.02), consistent with an adverse effect of weight gain on renal functional stability. After adjusting for gender, age, duration of diabetes, logACR, HbA1c, SBP, DBP and number of antihypertensive medications, the OR remained similar (1.74) although the statistical significance became marginal (95% CI 0.96 - 3.18, p=0.06).
T2DM in Young Adults: Effects of Raised BMI

In a final sub-analysis of this cohort, I elected to focus on the renal and other characteristics of young (age < 40 years), obese (BMI >30 kg/m²) type 2 diabetics as this represents a patient group for whom prolonged exposure to the combined effects of obesity and T2DM is likely to occur with, potentially, high risk for eventual development of advanced CKD and ESRD. Within the cohort, there were 76 such subjects for whom baseline data was available.

As shown in Table 4.12, young, obese subjects had higher baseline MDRD eGFR as well as higher DBP, fasting triglyceride and fasting LDL levels and lower SBP compared to all other subjects. 59 were on RAAS blockade (52 on 1 agent, 9 on 2 agent) and median number of anti-hypertensives was 1.5 (0-5). There was no significant difference between the number of anti-hypertensives used in the younger age group compared to the older group.

Table 4.12: Baseline Characteristics of Younger, Obese Type 2 Diabetics compared to all other Cohort Subjects.

<table>
<thead>
<tr>
<th></th>
<th>Age &lt;40, BMI &gt;30</th>
<th>All Others</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²)</td>
<td>36.8±7.7 (76)</td>
<td>31.9±6.6 (863)</td>
<td>0.001</td>
</tr>
<tr>
<td>Duration of diabetes (years)</td>
<td>5.87 ± 7.26</td>
<td>8.69± 9.76</td>
<td>0.001</td>
</tr>
<tr>
<td>MDRD eGFR (ml/min/1.72m²)</td>
<td>89.8 ± 24.2 (75)</td>
<td>79.2 ± 24.8 (854)</td>
<td>0.001</td>
</tr>
<tr>
<td>Log Urine ACR (mg/mmol)</td>
<td>0.99 ± 1.53</td>
<td>1.00 ± 1.53</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>7.48 ± 1.53</td>
<td>7.45 ± 1.45</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>128.6 ± 14.9 (75)</td>
<td>136.9 ± 19.3 (850)</td>
<td>0.001</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>79.5 ± 9.5 (75)</td>
<td>75.3 ± 9.6 (850)</td>
<td>0.001</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>4.45 ± 1.07</td>
<td>4.00 ± 0.99</td>
<td>0.0001</td>
</tr>
<tr>
<td>LDL (mmol/l)</td>
<td>2.35 ± 0.88</td>
<td>1.95 ± 1.10</td>
<td>0.005</td>
</tr>
<tr>
<td>TG (mmol/L)</td>
<td>2.26 ± 1.86 (67)</td>
<td>1.91 ± 1.19 (726)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Of the 76 younger, obese diabetics from the baseline cohort, 42 also had data from a follow-up evaluation available for analysis. Table 4.13 summarises key baseline and follow-up indices for these 42 subjects.
Table 4.13: Comparison of Baseline and Follow-up Indices for 42 Younger, Obese Type 2 Diabetic Subjects.

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Follow-up</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI (kg/m²) (n=42)</td>
<td>37.9 ± 8.6</td>
<td>36.8 ± 8.1</td>
<td>NS</td>
</tr>
<tr>
<td>MDRD eGFR (ml/min/1.72m²) (n=34)</td>
<td>89.1 ± 22.1</td>
<td>94.65 ± 31.4</td>
<td>0.01</td>
</tr>
<tr>
<td>Log Urine ACR (mg/mmol) (n=21)</td>
<td>0.42 ± 0.90</td>
<td>0.19 ± 0.36</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c (%) (n=34)</td>
<td>7.65 ± 1.33</td>
<td>7.95 ± 1.93</td>
<td>NS</td>
</tr>
<tr>
<td>SBP (mmHg) (n=38)</td>
<td>130.6 ± 14.3</td>
<td>135.4 ± 18.4</td>
<td>NS</td>
</tr>
<tr>
<td>DBP (mmHg) (n=38)</td>
<td>80.8 ± 9.2</td>
<td>78.8 ± 10.0</td>
<td>NS</td>
</tr>
<tr>
<td>Cholesterol (mmol/l) (n=32)</td>
<td>4.3 ± 1.0</td>
<td>4.4 ± 1.00</td>
<td>NS</td>
</tr>
<tr>
<td>LDL (mmol/l) (n=30)</td>
<td>2.18 ± 0.81</td>
<td>2.32 ± 0.74</td>
<td>NS</td>
</tr>
<tr>
<td>TG (mmol/L) (n=31)</td>
<td>2.07 ± 1.38</td>
<td>2.05 ± 1.00</td>
<td>NS</td>
</tr>
<tr>
<td>Rate of change of renal function (ml/min/year): +2.52 ± 6.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number (proportion) with eGFR &lt; 60 ml/min/1.73m² at follow-up: 4(4.8%)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The young obese diabetic group, although making up a smaller proportion of the cohort had distinct features including greater BMI and eGFR. At baseline, compared to the rest of the cohort they had poorer lipid control both for cholesterol and triglycerides. Despite this, their systolic blood pressure was lower and urine ACR was not higher. Although the number with follow up data was small, it is interesting to note that eGFR continued to increase while there were no differences in other parameters, including urine ACR and BMI, between baseline and follow up of 3-5 years. The proportion with CKD at follow up was significantly lower compared to the rest of the cohort (4/34 vs. 123/396, p=0.01 by $\chi^2$ test). The observation of increasing eGFR during a 3-5 year follow-up period in this subgroup of young, obese diabetics without concomitant increase in ACR merits further evaluation to determine whether it represents a direct effect of obesity on glomerular filtration and/or a risk factor for eventual renal functional decline.
Discussion and Conclusion
Both obesity and CKD are increasing in prevalence worldwide [331] and many studies have found that obesity is a risk factor for the development of CKD and ESRD [66, 245, 332]. Obesity and CKD have also both been reported to be associated with abnormal inflammation, oxidative stress and endothelial dysfunction [61, 333] and both occur in the setting of other cardiovascular risk factors such as diabetes, hypertension and hyperlipidaemia [334, 335]. However, despite these associations, the direct links between obesity and CKD are not entirely clear. For example, some studies which have addressed this issue have observed that the association between obesity and CKD is either absent or is no longer significant following adjustment for cardiovascular risk factors [73, 336].

Using a cross sectional cohort of type 2 diabetic patients that attended for management at a single tertiary referral centre, I examined the relationship between obesity (as reflected in BMI) and renal function estimated by MDRD and other formulae. As presented in the Results section, this was a typical type 2 diabetic cohort with a large proportion being overweight and obese, hypertensive and hyperlipidaemic. Chronic kidney disease was present in 25% and 7.6% had severe (“macro-”) albuminuria, indicating high risk for progressive renal dysfunction. Of note, a high proportion of the subjects received RAAS blocking therapy. Over the intervening follow-up period, the overall BMI for the group did not change and glycaemic and lipid control as well as overall renal function also remained constant. Systolic and diastolic blood pressure and fasting triglyceride levels were significantly lower on follow-up.

An important challenge in analysing and interpreting the available data from the cohort related to accurately defining factors that are indicative of renal dysfunction. These included setting definitions/cut-off values for clinically relevant CKD and glomerular hyperfiltration in addition to abnormal albuminuria and hypertension. The former two required appropriate calculation based on the data available for the cohort. Unfortunately, the most accurate and clinically relevant method of estimation of renal function is currently not well established in the literature when obese or severely obese individuals are being studied [311]. As outlined in the Introduction, the presence of severe obesity may lead to the underestimation of CKD when GFR is estimated based on SCr [310]. Use of creatinine to detect CKD is limited due to variability in its rate of generation from muscle which is affected by age, ethnicity and racial group [337]. In obese subjects with BMI is >35kg/m², accounting for the effects of body surface area, fat free mass and other weight and height related parameters when estimating
GFR has been shown to be problematic. The gold standard would be measurement of the clearance rate of inulin which is freely filtered by the glomerulus and is neither secreted nor reabsorbed within the tubules. However, this technique is rarely applied in the clinical setting due to expense and inconvenience. Estimating equations such as MDRD and Cockcroft-Gault have been derived to overcome the limitations of SCr in indicating renal function. MDRD-estimated GFR is currently in widespread use for laboratory reporting of renal function to clinicians and for staging of CKD in clinical practice. In some institutions it is not reported alongside the SCr unless the GFR is <60 ml/min/1.73m² in order to prevent inappropriate diagnosis and evaluation of CKD stages 1 and 2. Thus, the presence of hyperfiltration, which may be an important prelude to the development of CKD in obese and/or diabetic patients, is not always clinically evident. In the original study in which it was reported, the MDRD formula was shown to be accurate within 30% of measured true GFR in 91% of cases of CKD [310]. However, it is known to be less accurate in non-CKD populations. For instance, the MDRD formula was found to underestimate GFR in healthy living kidney donors [338] and in type 1 diabetics, compared to iothalamate clearance [339]. As the data available to me from the DIAMOND database did not contain a gold-standard measurement of renal function, I attempted to assess which method of GFR estimation would offer the most useful categorisation of both CKD and hyperfiltration within a predominantly obese cohort. I felt that the presence of SCr ≥150 µmol/l would be a reasonable cut-off mark by which a clinician would consider the impact of therapy on the kidney with more diligence. In this setting, the MDRD formula and the Cockcroft-Gault formula using lean body weight both assigned a value of < 60 ml/min to all subjects with SCr ≥150 µmol/l, whereas the Salazar-Corcoran formula and well as the Cockcroft-Gault formula calculated using adjusted body weight or actual body weight did not do so in all cases. An additional contributing factor to my decision to use MDRD eGFR for subsequent analyses was that, unlike the others considered, this formula does not incorporate weight and, therefore, does not have an inherent relationship to obesity/BMI. It is important to acknowledge that the degree of error introduced into the study and its conclusions by the use of formula-based GFR estimation as a measure of renal function cannot be fully accounted for with the data at hand. Nonetheless, as MDRD-estimated GFR has been extensively validated for the detection of eGFR < 60 ml/min and has not been shown to be inherently biased by body weight or adiposity, I believe that the use of this formula as a basis to categorise CKD within the cohort is a reasonable one.
which permits direct comparison with the majority of published studies in the area as well as interpretation of the results in the context of current clinical practice.

Hyperfiltration is a known feature of diabetes and obesity which, if anything, may be underestimated through use of the MDRD formula for estimation of GFR [340]. Within the entire cohort at baseline, BMI correlated positively and significantly with eGFR. Using a definition of MDRD eGFR > 130 ml/min/1.73m², I identified 25 “cases” of hyperfiltration at baseline and notably, these individuals were younger and heavier than the rest of the cohort. In a binary logistic regression model, BMI predicted the presence of hyperfiltration even after adjustment for duration of diabetes, logACR, HbA1c, cholesterol level and SBP. Although there were only 10 of the 25 cases of hyperfiltration within the follow up cohort, these had a trend towards more rapid decline of eGFR compared to the rest of the cohort (-3.19±7.3 vs. -0.14±5.18ml/min/1.73m²). This observation is consistent with hyperfiltration as an early manifestation of kidney disease in obesity and diabetes which may subsequently be followed by a more rapid loss of renal function. Additionally, the observation that eGFR of young, type 2 diabetics with BMI > 30 kg/m² significantly increased during the follow-up period despite all other clinically relevant indices remaining stable is in keeping with an effect of obesity to induce glomerular hyperfiltration during the first decade of diabetes. These findings should be considered preliminary, however, as they are based on a single creatinine reading for each time point and the accuracy of the MDRD formula for GFR> 130 ml/min in obese subjects have not been fully validated.

Regarding the association between obesity and CKD, the results of the baseline, cross-sectional analysis did not support the hypothesis that obesity is a strong, independent driver of reduced renal function in T2DM – at least during the first decade after diagnosis of diabetes. In evidence of this conclusion, the correlation observed between BMI and eGFR was a positive one and, by unadjusted and adjusted logistic regression analyses, increasing BMI was not shown to increase the risk for CKD. This observation did not, however, rule out the possibilities that obesity represents a distinct adverse condition for loss of renal function over time in type 2 diabetics or that continued increase in weight among obese diabetics is associated with enhanced risk for CKD. With this in mind, the follow-up analysis I performed was focused on exploring trends for BMI, eGFR and other relevant indices in those subjects for whom comparable data were available at a second time-point.
Although there was no overall significant change in BMI or eGFR for the group between the baseline and follow-up evaluations, a subgroup of 18% had further increase in BMI and 26% exhibited rapid loss of renal function on the basis of > 3ml/min/year decrease in eGFR. Thus, it was clear that substantial proportions of these actively managed diabetic subjects had adverse trends for weight and renal function over a 3-5 year time-span. The baseline BMI value was higher for those that continued to gain weight and their glycaemic was poorer compared to the rest of the cohort. There was no strong, overall relationship between the changes in BMI and eGFR from baseline to follow-up evaluation with the exception that there was a modest, but significant, correlation between the change in BMI and the follow-up eGFR. However, the subgroup with >5% increase in BMI during follow-up contained a significantly greater proportion of subjects with eGFR loss > 3 ml/min/year compared to all others and, this subgroup had an average rate of eGFR loss that was > 1 ml/min/year. Furthermore, the categorical variable of weight gain (BMI increase by >5% from baseline) was associated with significantly increased OR (83% unadjusted, 74% adjusted) for rapid renal functional decline. The continuous variables for BMI (baseline, follow-up, change between baseline and follow-up) were also shown to have positive correlations with rate of change of eGFR (as did other clinically relevant variables), but these did not reach statistical significance – possibly reflecting a limitation of the cohort size. Taken together, I interpret the results of the follow-up analysis as providing evidence for a specific adverse effect of on-going weight gain on the risk for development of CKD among obese type 2 diabetics.

Considering also the associations identified in the cross-sectional analysis, this risk may be superimposed upon or follow from an effect of obesity to promote glomerular hyperfiltration.

One conclusion that could also be made from this study, as well as some of the results of Chapters 2 and 3, is that the pathophysiological relationship between obesity and CKD is relatively subtle and not in keeping with an overt intra-renal inflammatory response driven by the systemic consequences of obesity. However, as discussed above, subsets of the group, such as those that continued to gain weight despite on-going medical management, may have a stronger tendency toward CKD. Additionally, the finding that cases with hyperfiltration were more obese and had a trend toward greater loss of renal function over time suggests that specific changes do occur within in the kidney in response to obesity that may progress from glomerular hypertrophy/hyperfiltration to glomerulosclerosis and chronic tubulointerstitial injury in the long term. Clearly such associations are likely to be influenced by medical therapy and it is interesting to note that, while ACR demonstrated the expected positive
correlations with eGFR at baseline, the average ACR values remained low at both time-points. This likely reflected the fact that most subjects received RAAS blockade and other anti-hypertensive agents. Whether these agents, and other elements of current standard of care for diabetic patients, exert strong protective effects against obesity-associated glomerular and other changes is not clear at present. In addition, the potential for specific dietary, pharmaceutical or surgical weight loss interventions to modify CKD risk among obese diabetics independently from their effects on glycaemic control remains to be seen.

I believed that it would be of additional interest to examine baseline and follow-up characteristics of a subgroup of obese type 2 diabetics of younger age to ask whether such individuals show distinct renal functional profiles. As expected, this subgroup had a diagnosis of diabetes for a shorter length of time compared to older subjects. They also had higher BMI and poorer lipid control in keeping with obesity and (presumably) unhealthy diet being key risk factors for earlier onset of T2DM. Reassuringly, however, these subjects did not exhibit further increase in BMI over time, although the number with follow up data was relatively small. Compared to the rest of the cohort, this subgroup had higher eGFR and, interestingly, had further increase in eGFR of approximately 2.5 ml/min/yr during follow-up – an observation that is unlikely to represent an artefact of GFR estimation or other confounding factor given the stability of BMI and other key indices between baseline and follow-up evaluations. As urine ACR did not increase concomitantly with eGFR in these subjects, it will be of high interest in the future to determine longer-term renal functional trends in young, obese type 2 diabetics receiving optimised medical therapy for glycaemic control, hypertension, albuminuria and hyperlipidaemia.

In conclusion, it is clear from this cohort that the overall effects of obesity on the kidney in T2DM are not universally severe or rapidly progressive and are likely to be modified by treatments currently prescribed to most diabetic patients. Indeed, evidence-based therapeutic strategies are now well documented to have reduced the rates at which end-organ complications of diabetes, including ESRD due to diabetic nephropathy, are occurring in current patient populations [119]. Nonetheless, I also show here that a significant proportion of the outpatient population studied had CKD at baseline and rapid loss over renal function over time that may eventually culminate in the need for dialysis and/or transplantation. In those with increasing BMI, the rate of decline of eGFR was greater than in those with stable or no change in BMI, suggesting that, for obese type 2
diabetics, further weight gain may be an important, modifiable risk factor for CKD/ESRD. These observations could be of particular relevance to the management of young adults with T2DM who are otherwise receiving current standard of care for the associated metabolic and cardiovascular abnormalities.
Chapter 5: Discussion
Obesity and T2DM are major public health issues due to their association with stroke, heart disease and cancer [10]. As better management of hyperglycaemia, hyperlipidaemia and cardiovascular risk factors are instituted, it is heartening to see the incidence of severe complications, notably ESRD reducing[119]. However, due to the sheer number of individuals affected by T2DM and obesity, despite optimum treatments, it is likely that these two conditions will continue to utilise a significant proportion of the health care budget for many countries [10].

The discussions in previous chapters have dealt with specific topics related to my findings. In this chapter, I would like to develop some broader themes linking the three project, the limitations of the studies and their potential clinical relevance for further research.

The themes that I will explore include

1. The immune system is intricately linked with metabolism to prevent unnecessary and damaging immune system activation in the presence of metabolic dysregulation.
2. This restraint on inflammation becomes overwhelmed in the setting of acute kidney injury resulting in parenchymal damage within the kidney.
3. Treatment for diabetes is changing from the previously assumed natural history of the condition, such that predicting those who will progress is more difficult
4. Obesity in young people is increasing and it remains unknown if this will result in worse outcomes for kidney function compared to diabetes occurring in later years.

The natural history of chronic kidney disease in response to obesity and T2DM remains poorly understood. Much of our understanding comes from large population studies or selected research populations that have examined urine, blood or renal biopsies to assess damage within the kidney in response to the abnormal metabolism present in pre-diabetes and overt T2DM[109, 236, 237]. Inflammatory changes within the kidney remain an important therapeutic focus for modulating the outcome of diabetic kidney disease[142]. Many of the current treatments such as aspirin, HMGcoA reductase inhibitors and RAAS blockade are credited to have additional anti-inflammatory effects as part of their function in preventing complications related to T2DM [130, 341, 342]. Widespread therapeutic application of these has resulted in improved outcomes for microvascular complications of diabetes and likely has resulted in the change in nature of presentation and progression of diabetic kidney disease.
Many patients progress to ESRD without albuminuria[276]. Finally, in diabetic kidney disease up to a quarter of cases, additional causes for renal damage will be present on biopsy[343].

Levels of proinflammatory cytokines have been linked with obesity, T2DM and diabetic kidney disease in epidemiological studies. IL-6 is one such cytokine particularly linked with diabetes, obesity and cardiovascular disease. IL-6 was found to be elevated in type 2 diabetics and obese individuals with levels increasing with greater involvement of the kidney [239, 344]. IL-6 was found to correlate with sensitive markers of diabetic kidney disease such as glomerular basement membrane thickness, as well as being expressed in biopsy sections from diabetic nephropathy sections[232, 233]. However, in the preceding years, evidence for an important metabolic role of IL-6 has emerged that may explain the paradoxical high levels that are found both in obese type 2 diabetics and athletes post strenuous aerobic exercise. IL-6’s role in regulating GLP-1 secretion and modulating glucose levels in response to food and exercise has added an interesting paradigm in how ‘inflammatory’ cytokines are viewed [28, 44]. This mirrors other studies in which important metabolic functions in addition to immune system regulatory activity are being discovered for cytokines and chemokines[18, 39]. It remains unclear the exact role played by inflammatory markers such as IL-6 in the development and progression of diabetic kidney disease. In studies outlined in chapter 1 found levels of many inflammatory markers increased as microvascular complications accumulated and correlated with clinical indices such as albumin excretion. What the exact role and pathogenicity of these inflammatory molecules is unclear and may change depending on the degree of glycaemic control or other pathological condition such as AKI.

Contrary to my starting hypothesis, using several different experimental techniques, I did not find evidence for active inflammation in the OZR despite the phenotype of obesity and albuminuria being present. There was evidence for subtle activation of the cellular immune system, particularly within the glomeruli of the OZR compared to its lean counterpart. I did not find that IL-6 expression or protein levels were elevated in the kidney, serum or urine of the OZR at the early time points studied. However, both STAT3 and SOCS3, components of IL-6’s downstream signalling cascade were extensively expressed throughout the kidney. This did not translate into increased inflammation evidenced by no strong pattern in inflammatory cytokine and chemokine expression. Additionally, phosphorylated STAT3 expression could only be detected in discrete foci of tubular and interstitial cells throughout
the kidneys at marginally increased rates in the obese rats. The most novel finding of the study was that the negative regulator of the signalling pathway, SOCS3 was increased in glomeruli of OZR compared to the lean rats. Additionally, its expression was greater in glomeruli with increased desmin expression indicating that it was present where there was greater stress. The number of foci of SOCS3 within glomeruli correlated positively with the number of infiltrating CD68+ cells and there was co-expression of SOCS3 and CD68+ cells on sequential sections some but not for all cells. Given the lack of damage detectable by light microscopy despite the phenotype of albuminuria and obesity, I hypothesised that SOCS3 presence was a restraint on STAT3 mediated inflammatory activity in response to the rats’ abnormal metabolism. Lack of inflammatory changes in the OZR at baseline is described in models of this rat used for assessing effects of obesity in pancreatitis and chronic inflammation. Upon initiation of an insult such as periodontitis or pancreatitis, the inflammatory response is exaggerated or maladaptive compared to the lean controls [273, 274, 345]. Additionally, other animal models of diabetic nephropathy have found overexpression of SOCS3 to protect from progression of renal lesions associated with diabetes [224]. I hypothesised that glomerular SOCS3 expression was induced within inflamed glomeruli as a method to restrain further damage both within the glomeruli and its downstream tubule. However, when additional damage occurs within the kidney such as onset of overt hyperglycaemia or acute kidney injury, SOCS3 restraint on inflammation is overwhelmed. SOCS3 has well described activity in regulating glucose/insulin metabolism [45, 212, 346] and is therefore in a prime position to link inflammatory activity with a diseased metabolic state.

I attempted to study further these findings in the OZR, hypothesising that SOCS3 has an important role in the glomerulus in preventing active inflammatory signalling pathways in response to its abnormal metabolic state. I initially elected to use siRNA to knock-down SOCS3 expression in vivo with the hypothesis that this would accelerate the injury in the rats. This was based on several studies examining knock-down of SOCS3 in different animal models of kidney disease: renal damage induced by angiotensin II infusion was accelerated in the presence of SOCS3 siRNA [205] and diabetic nephropathy related lesions were prevented by adenovirus over-expressing SOCS3 siRNA [224]. However, due to technical limitations related to the sizing of the OZR, I was not able to complete this experiment with the budget assigned to the project. An additional limitation of this project is that differences in SOCS3 expression between obese and lean rat glomeruli were only noted by immunohistochemistry.
However, as the expression of SOCS3 in the tubulointerstitium was strong and even by IHC there was only a trend towards a significant difference, assessing for difference in glomerular SOCS3 expression would only have been possible using sieved glomeruli.

Cumulative damage that recurrent episodes of AKI have on long term renal survival is well recognised [347]. AKI can be induced by many factors including NSAIDs, contrast, hypotension or antibiotics. Diabetics and obese patients are increased risk of AKI in the setting of cardiac surgery, sepsis and mechanical ventilation compared to the non obese or diabetic counterparts [52, 77]. From the findings in the OZR, I feel that SOCS3 is in a prime position to regulate the kidneys’ response to injuries such as these. Based on this, one such method to assess SOCS3 role in the OZR could also have included a uni-nephrectomy or unilateral ureteral ligation model, to assess whether its expression decreases as hyperfiltration/obstructive injury occurs and inflammation becomes more prominent. Unfortunately due to budget and time constraints proceeding with these experiments ase not possible.

Predicting who will develop diabetic kidney disease is currently difficult, and even within the 30% that have manifest either albuminuria or hypertension, it is not clear who will progress [128, 131]. From the literature current at the start of this project, it was suggested the pro-inflammatory activity was key to the development of diabetic kidney disease [141]. This was based on epidemiological studies, animal models and in-vitro studies where inflammatory pathways and molecules were found to be elevated in the serum and urine compared to healthier diabetics [128, 236, 237, 239, 344]. Given that cytokine and chemokine expression in biological fluid such as urine and serum may not reflect activity within the kidney, I investigated the hypothesis that early diabetic nephropathy was associated with increased inflammation using mRNA extracted from urine cells and whether this may be used to predict who would likely go onto develop progressive renal dysfunction. Using a cohort of mostly obese type 2 diabetics with varying rates of albuminuria, I assessed the expression of IL-6 and its related inflammatory mediators in urine cells as reflecting inflammation within the kidney. Based on the technique I developed to assay urine cell gene expression, I am confident that cell expression within urine was reliably assessed and any signal in urine cells would be detectable. We studied a cohort of type 2 diabetics who had diabetes at least for several years before samples were taken, with a wide range of BMI, microalbuminuria and hypertension present. Thus, they were a representative group of patients likely to have
inflammation present in the kidney if this was an important factor in diabetic kidney disease development. I found that there were no strong relationships between urine cell expression of IL-6, IL-6R or IL-17 and markers of glycaemia, blood pressure or obesity within the cohort. As this was a group with stable well controlled type 2 diabetics followed in a tertiary referral centre, these findings are in keeping with the lack of inflammation noted in the OZR study. These findings may suggest that inflammation is difficult to detect in well controlled diabetics. It also possibly reflects the effectiveness of current therapeutic regimens for type 2 diabetics modifying inflammation in addition to the clinical targets of their treatment. Additionally, although IL-6 has been noted to be increasingly elevated in serum and urine of obese individuals, diabetics and diabetic nephropathy patients, this may reflect disturbances in metabolism remote to the kidney. Finally assessing for a signal for likelihood to progress from diabetic nephropathy is difficult given the improvement in treatments, as evidenced from the both groups of patients studied from Galway University Hospital. Although there were many patients with both CKD and macroalbuminuria, at follow up, there were only 2 patients with an eGFR less than 10ml/min/1.73m². To study the relationship between BMI and progression of kidney disease in stable diabetics would require a large cohort over a longer period of time. It is likely that the rate of progression, especially in a well managed cohort will be slow. A potential limitation to the interpretation of the data include that it is not clear which cell type is contributing to the expression of these markers. Urine cells may originate from the bladder, ureters and epithelium of the genital tract in addition to the kidney. Studies on podocyte cell retrieval from urine indicate that their presence is low in urine from healthy patients but increases in the setting of kidney disease [306]. There have been many studies assessing podocyte marker expression in urine cells, with the patient groups studied similar to the cohort I examined [292, 348, 349]. Thus I am confident that the cells we studies were likely to have originated from the kidney.

My findings and that of other groups suggest that within the kidney of both obese and diabetic subjects, there is activation of proinflammatory STAT3 pathway [202] but that its activity is held in check by its negative regulator SOCS3[201, 205, 206, 224]. In addition to examining the role of STAT3/SOCS3 in the OZR, I was able to assess STAT3 and SOCS3 expression both within the diabetic cohort and in a non-diabetic obese patient group who were being assessed for bariatric surgery. This represented a wide spectrum of the metabolic phenotypes associated with bariatric surgery. It is likely that with a mean BMI of 51kg/m², that nearly the entire bariatric cohort was hyperinsulinaemic but none had overt
diabetes and all had preserved renal function. Their lack of overt diabetes likely reflects their younger age compared to the diabetic cohort and it is very likely that they would develop this condition in the future without intervention. The expression of STAT3 and SOCS3 in urine cells was compared across these two groups as well as with healthy non diabetic controls. STAT3’s expression in urine cells of T2DM patients correlated positively with their BMI but was found to correlate negatively with BMI in the non-diabetic obese patients being assessed for bariatric surgery. As STAT3 activity within the cell is down-regulated by SOCS3 (increased SOCS3 leads to ubiquitin mediated degradation of STAT3 signalling complexes [203]), I assessed differences in the ratio of STAT3/SOCS3 expression in urine cells between the three groups. There was an inverse correlation between BMI and STAT3/SOCS3 ratio from both the T2DM and bariatric patients. Median expression of STAT3/SOCS3 ratio was lowest in the obese non diabetic cohort and increased in the diabetic group and healthy controls. This suggests that, similar to the OZR, changes in the balance between STAT3 and SOCS3 expression may occur as the metabolic phenotype alters from pre-diabetes and obesity to overt T2DM. Interpreting these findings without a renal biopsy is difficult, but fits with my hypothesis of increased SOCS3 in the glomeruli of OZR being a restraint to further STAT3 induced inflammation. Whether this reflects a change that will be deleterious to the kidney long-term is not clear from this data. The diabetic cohort overall had greater albuminuria, hypertension and lower GFR compared to the obese bariatric group indicating that they were unhealthier, older and had a longer time at risk for developing complications of diabetes. However, the younger, severely obese non diabetic group clearly had changes in urine cell expression compared to diabetics and healthy controls indicating that factors associated with obesity impact on STAT3/SOCS3 signalling pathway within the kidney.

The effect of having obesity and T2DM from a young age on kidney function is unknown. The demographic of extreme obesity and early onset obesity has not existed to this extent before. Assessing young diabetics in the cohort of T2DM patients at GUH indicated that they were heavier with worse lipid control but had a better renal profile: lower blood pressure and higher eGFR. At follow up, although the numbers were small, there was a lower rate of chronic kidney disease at follow up. As noted, this likely reflects that age is an important factor in development of CKD. How important is the finding of hyperfiltration to the development of progressive kidney disease? In a 35 year old male type 2 diabetic with a BMI of 40kg/m2, does an eGFR of 150ml/min/1.73m² reflect stress within the glomerulus? If so, what effects do out current treatments for diabetes have on this? What will be the most
important factors for predicting his kidney disease progression? Obesity confers a worse prognosis in the setting of sepsis, mechanical ventilation and surgery [52, 77]. I propose that there are defensive mechanisms intrinsic to the kidney to prevent metabolically induced activation of inflammation. I hypothesise that hyperfiltration and hyperglycaemia are detected by parenchymal cells of the kidney and this results in priming of the immune system but is held in check by negative regulators such as SOCS3. In the setting of acute kidney injury, in obese diabetics the defence system is overwhelmed resulting in a temporary change in balance towards a more pro-inflammatory phenotype that starts a cycle of damage within the kidney. Inducing the defence system or preventing its downregulation during acute kidney injury is a potential area that could be amenable to therapeutic intervention to prevent pro-inflammatory activation in the short-term in acutely ill diabetics or obese individuals and merits further study.
Appendix
Appendix 1

Galway University Hospitals

Patient Information Leaflet

Principal Investigator’s Name: Professor Matthew Griffin

Project Title: A Study of Interleukin 6 and Interleukin 6-related Gene Expression by Cells Shed in the Urine of Type II Diabetics With and Without Diabetic Nephropathy.

Telephone No. of Principal Investigator: 091-524222 or 091-751131

You are being invited to take part in a clinical research study carried out at Galway University Hospitals. Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP. Take time to ask questions – do not feel rushed or under any obligation to make a hasty judgement. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you – this process is known as Informed Consent.

You are not obliged to take part in this study and failure to participate will have no effect on your future care.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care you will receive from the medical staff.

Why is this study being done?

This study is being done to find new information about why some people with diabetes have damage to their kidneys. The study will test the levels of proteins involved in inflammation in urine samples provided by people with diabetes. If it is found that inflammation is higher in diabetic people with abnormal amounts of urine protein it may be possible to develop new ways of diagnosing or treating diabetic kidney disease. You are being asked to take part because you have diabetes and have had a test done to find out whether or not you have abnormal amounts of protein in your urine.

Who is organising and funding this study?
The study is organised by Professor Griffin, who is a kidney specialist at Galway University Hospitals, and by other doctors at this hospital who specialise in the care of people with diabetes and kidney disease. The funding for the study comes from the NUI Galway/Galway University Hospitals Clinical Research Facility.

How will it be carried out?

People attending diabetes or kidney outpatient clinics at Galway University Hospitals will be asked by a nurse or doctor to take part in the study if they have diabetes, normal kidney function and a recent test for urine protein. In all, 50 people with diabetes who have abnormal amounts of protein in their urine and another 50 people with diabetes but normal amounts of protein in their urine will take part. Each person who agrees to participate in the study will provide a sample of urine during the outpatient clinic visit. The urine sample will be taken to Professor Griffin’s research laboratory at NUI Galway and cells from the sample will be tested to measure the amounts of 4 different inflammatory proteins in them. The difference in amount of inflammation in these cells will be compared for diabetic people with and without abnormal urine protein.

Procedures/ What will happen to me if I agree to take part?

You will first be asked by a doctor or nurse to sign a form indicating that you agree to take part. After this you will be given some simple instructions for collecting a sample of your urine in a clean container. When you return the sample to the doctor or nurse it will be quickly tested by dipstick for any sign of bladder infection. If this test shows no sign of infection the urine sample will saved for transport to the research laboratory later that day. If there is any sign of infection on the dipstick test the sample will not be used for the study but the doctor or nurse will make sure that you have full testing for bladder infection and treatment if necessary. After giving a urine sample you will be finished with the study. The sample will be stored for testing in Professor Griffin’s research laboratory at NUI Galway.

What alternative treatments are available to me?

The study will have no effect on your treatment.

Risks & Discomforts

There is no risk to taking part in the study but there will be a small inconvenience if you have to give an extra urine sample during your clinic visit.

Benefits of this Study
You will not benefit directly from taking part in this study but the researchers may learn more about the cause of kidney damage from diabetes. This could help to develop new tests or treatments for diabetic kidney disease in the future.

**Compensation**

There is no compensation for participating in this study.

**Will there be any additional costs involved?**

There will be no additional costs.

**Confidentiality**

Some information related to your diabetes will be recorded from your medical chart by one of the doctors or nurses involved in the study. Also your urine sample will be stored in Professor Griffin’s research laboratory at NUI Galway. To avoid people who are not involved in the study learning about your medical condition, your name and personal details will be replaced on all study documents and samples by a numbered code known only to the study doctors and nurses. Written documents, computer files and urine samples will be kept in locked cabinets or password-protected computers for a total of 10 years. If you agree to it now, the samples and medical information may be used for other research projects or shared anonymously with other researchers at NUI Galway or elsewhere. After 10 years the written documents and remaining samples from the study will be destroyed by incineration.

**Project Duration**

The project will continue until a total of 80 people have provided suitable urine samples. This is expected to take about 6 months from the start of the study.

**What if something goes wrong as a result of my participation in this study?**

It is very unlikely that anything will go wrong as a result of your participation in this study but if you have a concern you should contact Professor Griffin or Dr. McNicholas at the numbers provided on this information sheet.

**Your responsibility as a participant**

If you believe you may be pregnant please inform the doctor or nurse who is talking to you about the study as pregnancy would exclude you from taking part.

**Our responsibility to you as investigators**

We are responsible for explaining the study carefully to you, for answering any questions or concerns you have about the study at any time, for protecting the confidentiality of your medical information and for making sure that your medical care is not affected in any way by your participation.
If you require further information

For additional information now or any future time please contact:

**Name:** Dr. Bairbre McNicholas

**Address:** Orbsen building, NUI Galway, Galway

**Phone No:** 0872456240
You are being invited to take part as a healthy volunteer in a clinical research study carried out at Galway University Hospitals. Before you decide whether or not you wish to take part, you should read the information provided below carefully and, if you wish, discuss it with your family, friends or GP. Take time to ask questions – do not feel rushed or under any obligation to make a hasty judgement. You should clearly understand the risks and benefits of participating in this study so that you can make a decision that is right for you – this process is known as Informed Consent.

You are not obliged to take part in this study and failure to participate will have no effect on your future care.

You may change your mind at any time (before the start of the study or even after you have commenced the study) for whatever reason without having to justify your decision and without any negative impact on the care you will receive from the medical staff.

Why is this study being done?

You are being asked to take part in this study as a healthy volunteer who will provide a urine sample that will be compared to urine samples from people who have been diagnosed with a kidney condition called diabetic nephropathy.

This study is being done to find new information about why some people with diabetes have damage to their kidneys. The study will test the levels of proteins involved in inflammation in urine samples provided by people with diabetes. If it is found that inflammation is higher in diabetic people with abnormal amounts of urine protein it may be possible to develop new ways of diagnosing or treating diabetic kidney disease.
Who is organising and funding this study?

The study is organised by Professor Griffin, who is a kidney specialist at Galway University Hospitals, and by other doctors at this hospital who specialise in the care of people with kidney disease. The funding for the study comes from a grant to Professor Griffin from the Health Research Board.

How will it be carried out?

The NUI Galway Clinical Research Facility will identify healthy volunteers who may be willing to provide a urine sample for the study. Volunteers will be contacted by phone and asked to come to the Clinical Research Facility on a convenient morning. Once collected, the urine samples will be labelled with the volunteer’s age and gender only and will be taken to Professor Griffin laboratory at NUI Galway cells where the sample will be tested to measure the amounts of 4 different inflammatory proteins in them. The difference in amount of inflammation in these cells will be compared for diabetic people with and without abnormal urine protein as well as healthy controls. In total, 20 healthy volunteers will provide samples – 10 men and 10 women.

Procedures/ What will happen to me if I agree to take part?

You will first be asked by a qualified research nurse to sign a form indicating that you agree to take part. The nurse will then ask you some basic health questions to confirm that you do not have any kidney condition or other major medical condition. If this is the case, you will be given some simple instructions for collecting a sample of your urine in a clean container. When you return the sample to the nurse it will be tested for protein and white blood cells using a dipstick test to make sure that there is no urine infection or other abnormality present. If the dipstick test is normal as expected then it will be saved for transport to the research laboratory later that day. If there is any urine abnormality on the dipstick test, the nurse will explain this to you and will give you clear instructions on what, if anything, you should do to receive appropriate medical attention. After giving this urine sample you will not have to do anything for the study. Each sample will be stored for testing in Professor Griffin and Prof. Joshi’s research laboratory at NUI Galway.

What alternatives are available to me?

You may choose not to provide a sample after reading the information sheet.

Risks & Discomforts
There is no risk to taking part in the study apart from the small inconvenience of collecting a sample of your urine.

Benefits of this Study

You will not benefit directly from taking part in this study. There is a very small chance that you will learn of an untreated urine infection or other condition as a result of the dipstick testing of your urine sample and will be offered advice about how to receive medical care for this.

Compensation

There is no compensation for participating in this study.

Will there be any additional costs involved?

There will be no additional costs.

Confidentiality

Your age and gender will be recorded on the urine sample that is sent to the research laboratory as this information will be important for the study. No other personal information will be recorded for the study. You will not be contacted at a later date by the study investigators and no personal information will be passed on to a third party as a result of your participation.

Project Duration

The project will continue until a total of 20 healthy volunteers and 200 patients with diabetic nephropathy have provided suitable urine samples. This is expected to take about 3 years from the start of the study.

What if something goes wrong as a result of my participation in this study?

It is very unlikely that anything will go wrong as a result of your participation in this study but if you have a concern you should contact Professor Griffin at the numbers provided on this information sheet.

Your responsibility as a participant

Your responsibility as a participant in the study is to let us know if you do not understand any part of the study procedure.

Our responsibility to you as investigators

We are responsible for explaining the study carefully to you, for answering any questions or concerns you have about the study at any time, for protecting your
confidentiality and for making sure that your medical care is not affected in any way by your participation.

**If you require further information**

For additional information now or any future time please contact:

**Name:** Prof. Matthew Griffin  
**Address:** Consultant Nephrologist, Merlin Park Hospital, Merlin Park, Galway  
**Phone No:** 091-524222 or 091-751131
CONSENT FORM

Full title of Project: A Study of Interleukin 6 and Interleukin 6-related Gene Expression by Cells Shed in the Urine of Type II Diabetics With and Without Diabetic Nephropathy.

Name, position and contact address of Chief/Principal Investigator: Professor Matthew Griffin, Consultant Physician, Unit 1 Merlin Park Hospital Dublin Road, Galway.

Please initial box

1. I am an adult taking part in this study.

2. I confirm that I have read and understand the information sheet for the above study and have been given a copy to keep. The information has been fully explained to me and I have been able to ask questions. I understand why the research is being done and any risks involved.

3. I agree to donate tissue sample (urine) for this research project. I understand that given a sample for this research is voluntary and that I am free to withdraw my approval at any time without my medical treatment being affected.

4. I agree to take part in the above study.

Please tick box
Yes            No

5. I give permission for my samples and information collected about me to be stored for possible future research related to this study (including DNA or genetic studies) but only if the research is approved by a Research Ethics Committee

6. I give permission for research personnel to look at my medical records to obtain information. I have been
assured that information about me will be kept confidential.

Name of Participant    Date    Signature

Name of Researcher/ Person Taking Consent

Date    Signature

Date    Signature
Figure a.1

Relationship between BMI, Systolic blood pressure, diastolic blood pressure, Creatinine Cholesterol, Triglycerides and AST as measured using Pearson’s correlation. BMI Body mass index, BPdiastolic Diastolic blood pressure TG triglycerides, AST aspartate aminotransferase.

Figure a.2

Relationship between estimates of glomerular filtration rate or creatinine clearances and body mass index (BMI) using Pearson’s correlation.
Relationship between MDRD estimated GFR and systolic blood pressure, diastolic blood pressure, HbA1c, Low density Lipoprotein (LDL) and cholesterol. LogACR, Cholesterol and AST Triglycerides as measured using Pearson’s correlation. BMI Body mass index, BPdiastolic Diastolic blood pressure TG triglycerides, AST aspartate aminotransferase.
Examples of Standard Curve for targets used in urine cell study with Interpolated Target Sample C_T. A 4 point standard curve was created using the same starting dilution of a plasmid preparation of the target. Individual points are seen as red points and samples in blue.
List of Presentation of Work

1. Irish Nephrology Society Meeting Dublin, May 2012, Oral presentation
2. Royal academy of medicine of Ireland, Galway, June 2013, Oral presentation
3. American Society of Nephrology meeting, San Diego 2012, Poster presentation
4. World Congress of Nephrology meeting, Hong Kong June 2013, Poster presentation
References


20. van Dielen, F.M., et al., *Macrophage inhibitory factor, plasminogen activator inhibitor-1, other acute phase proteins, and inflammatory mediators normalize as a result of weight loss*


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