Renal Hyperfiltration and the Inflammatory Cytokine/Chemokine Signature in Young Adults and Adolescents with Type 1 Diabetes Mellitus

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

Institute of Medical Science
University of Toronto

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2014

ABSTRACT:

Objectives: Current clinical biomarkers lack the sensitivity to detect diabetic nephropathy (DN) prior to glomerular filtration rate (GFR) decline. Inflammatory cytokines/chemokines may be promising candidates for next-generation biomarkers. In two distinct cohorts, the cytokine/chemokine signatures of young, normotensive and normoalbuminuric patients with type 1 diabetes (T1D) and matched healthy controls (HC) were characterized according to hyperfiltration (HF) status, a known risk factor for development of DN.

Results: First, in adults, GFR was measured using inulin clearance. Second, in adolescents from the “AdDIT” study, GFR was estimated using cystatin C. In both cohorts, urinary cytokine/chemokine excretion was broadly increased in T1D vs HC. Step-wise trends were observed from HC to normofilterers to hyperfilterers. Similar trends were observed in the serum of AdDIT, suggesting the source may be from systemic “spill-over”.

Summary: HF represents a distinct physiological condition where cytokine/chemokine levels are elevated from an exaggerated pro-inflammatory state compared to normofilterers and HC.
Acknowledgments

I would like to express the deepest appreciation to my supervisor, Dr. David Cherney. Without his enormous mentorship, leadership, support and patience over the years, this work would not have been possible. Dr. David Cherney’s dedication to his students, talents in educating and passion for his work will be attributes that I will model in my career.

I am most fortunate to have Dr. James Scholey and Dr. Heather Reich on my committee. It is an honor for me to have the opportunity to work with these masterly clinician-scientists. I thank Dr. James Scholey for his sincere welcome for Tuesday journal clubs and the stimulating intellectual discussions. I thank Dr. Heather Reich for her invaluable guidance that has, on many occasions, and continues to better and direct my work.

I will never forget the help I got from members of the Renal Physiology Laboratory. I owe sincere and earnest thankfulness to Vesta Lai, Alana Lee, Maria Maione and Yuliya Lytvyn for all their help and company.

In addition, I would like to thank Dr. Farid Mahmud and Dr. Etienne Sochett for the tremendous opportunity to work on the “AdDIT” study. I am grateful to Yesminno Elia, Livia Deda, Laura Motran, Ria Dekker, Dr. Rahim Moineddin, Cameron Slorach, Wei Hui and members of the endocrinology research team.

Last but not least, I owe my deepest gratitude to my parents, my sister, my family and my friends for their everlasting support.
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Abbreviated Terms

ACEi – angiotensin converting enzyme inhibitors

ACR – albumin-creatinine-ratio

AdDIT - Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial

ANOVA – analysis of variance

ARB – angiotensin-II-receptor blockers

BMI – body mass index

COX - cyclooxygenase

DM – diabetes mellitus

DN – diabetic nephropathy

ERPF – effective renal plasma flow

GFR – glomerular filtration rate

GM-CSF – granulocyte macrophage colony stimulating factor

Hyperfiltration – glomerular filtration of >135 mL/min per 1.73m²

IFN - interferon

IL – interleukin

LDL – low-density lipoprotein

KDIGO - Kidney Disease Improving Global Outcomes
MCP – monocyte chemoattractant protein

MDC – macrophage derived chemokine

MIP – macrophage inflammatory protein

NO(S) – nitric oxide (synthase)

Normofiltration – glomerular filtration of between 90-134 mL/min per 1.73m²

PAH – para-aminohippurate

PDGF – platelet derived growth factor

PG – prostaglandins

P_{GC} - transcapillary hydrostatic pressure

RAAS – renin-angiotensin-aldosterone-system

RANTES – regulated on activation, normal T cell expressed and secreted

RBF – renal blood flow

RVR – renal vascular resistance

sCD40L- soluble CD40 ligand

SD – standard deviation

SGLT – sodium glucose co-transporter

SNGFR – single nephron glomerular filtration rate

T1D – type 1 diabetes mellitus
T2D- type 2 diabetes mellitus

TGF – tubuloglomerular feedback

TNF – tumor necrosis factor
1 Diabetes and Kidney Disease

1.1 The Burden of Diabetes Mellitus

Diabetes mellitus (DM) is a growing global epidemic and the prevalence of DM is estimated to rise by an additional 1.3% from the current 6.4% by 2030 (1). This increase amounts to nearly 160 million additional individuals being diagnosed with DM over the next 20 years. Moreover, the number of individuals with DM is expected to increase by 20% in developed countries and 70% in developing countries, including some of the world's most populous nations such as China and India, reflecting the unprecedented growth of this important health issue (1, 2).

With the growing prevalence of DM worldwide, the cost of treating diabetes and its related complications are major concerns for healthcare policy. In the US and Canada alone, the direct costs of treating diabetes and related complications are $176 billion and $2.1 billion respectively (3, 4). Approximately 43% of this cost is directed towards hospital care relating to late-stage diabetic complications, such as kidney transplantation and dialysis for end stage renal disease. In comparison, the costs during the “silent” years are relatively inexpensive with clinic visits and the routine fulfillment of glycemic control prescriptions such as insulin (3). Therefore, postponing or preventing the progression of diabetic complications, such as diabetic nephropathy (DN), remains a crucial strategy to reduce the economic and social burden of DM.
1.1.1 Natural History of Diabetic Chronic Kidney Disease

DN is a major complication of long-standing DM and the leading cause of end stage renal disease in developed countries (5). In type 1 diabetes (T1D), 20-40% of patients will develop DN (6-8), and those who develop DN have an 18-fold greater risk of death compared to T1D patients without DN (9).

DN usually progresses slowly, and emerges as albuminuria and renal function decline years after the onset of DM. In the classical paradigm of DN, this condition is classified into 5 stages according to the severity of albuminuria and renal injury. The natural history of DN in T1D has been thoroughly reviewed by others and a general overview is provided below (10).

The early stages of DN are characterized by a period of normoalbuminuria with a clinically silent progression of kidney injury. In stage 1, renal hypertrophy and hyperfiltration, an elevation of glomerular filtration rate (GFR), is commonly found in newly diagnosed T1D patients, but is rapidly ameliorated after the introduction of insulin therapy in some patients (10, 11). Unfortunately, many patients develop persistent hyperfiltration despite improved metabolic control with insulin. In addition to hyperfiltration, patients can exhibit abnormal structural morphology in stage 2, reflecting ongoing renal injury even though these abnormalities are clinically silent. For example, renal biopsies collected from T1D patients at this early stage of the disease can show a variety of changes, including increased glomerular basement membrane width and mesangial matrix expansion (12). At follow-up, patients with the greatest increase in glomerular basement membrane width were more likely to advance to later stages of DN.
It is therefore widely accepted that these early glomerular abnormalities, with time, develop into glomerulosclerosis and renal function decline.

After this long silent period, a proportion of T1D patients develop microalbuminuria, which is defined as an urinary albumin excretion of between 30-300 mg/day (10). Microalbuminuria is generally recognized as the first clinical sign of DN and an indicator of increased glomerular permeability. This disruption of permselectivity indicates an escalating degree of structural injury at the glomerular barrier, comprised by the endothelial cell layer, the glomerular basement membrane, the slit diaphragm and podocytes (14). Accordingly, renal biopsies obtained in T1D patients have shown a clear association between the rate of albumin excretion and the severity of podocyte injury (15). Despite this underlying renal injury, GFR usually remains unchanged (normo- or hyperfiltering) at this stage of the classical paradigm, although it has been recently reported that early renal function decline can occur in a significant proportion of patients, even before the onset of albuminuria (16).

Once albumin excretion exceeds 300 mg/day, DN is classified as overt albuminuria or macroalbuminuria (stage 4). At this stage, GFR commonly declines more rapidly due to increasing severity of glomerulosclerosis (10). Once kidney function declines to <15ml/min/1.73m², patients enter the final phase of DN or end-stage renal disease (stage 5), requiring either dialysis or a kidney transplant. Unfortunately, the median wait time for a kidney transplant in 2010 was 3.5 years in Canada, one of the lowest organ donation rates among developed countries (17). Lastly, current clinical markers of early DN risk, such as albuminuria or renal function loss, tend to occur relatively late in the natural history of DN once significant kidney injury has already taken place. Therefore, the identification of novel
biomarkers of early DN risk is of the utmost importance for clinicians to better target high-risk patients with earlier therapies prior to the onset of albuminuria.
Table 1: Classical Natural History of Diabetic Nephropathy in Type 1 Diabetes Mellitus. Development of diabetic nephropathy occurs soon after the onset of diabetes mellitus through to subclinical (stage 1-2) and clinical phases (3-5). Adapted from (10).

<table>
<thead>
<tr>
<th>Stage</th>
<th>Chronology</th>
<th>Structural Lesions/Changes</th>
<th>GFR</th>
<th>RBF</th>
<th>Albumin Excretion Rate</th>
<th>Blood Pressure</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Early renal hyperfiltration and hypertrophy</td>
<td>Present at diagnosis of diabetes</td>
<td>Increased kidney size and glomerular size</td>
<td>Normal or increased</td>
<td>Normal or slightly increased</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>2</td>
<td>Subclinical renal lesions</td>
<td>Detectable after 2 years of diabetes, progression over several years</td>
<td>On renal biopsy, increased glomerular membrane thickness, mesangial expansion</td>
<td>Increased</td>
<td>Normal or slightly increased</td>
<td>Normal</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Normal</td>
</tr>
<tr>
<td>3</td>
<td>Incipient diabetic nephropathy</td>
<td>After 10-15 years (in 20-40% of patients)</td>
<td>Increased glomerular barrier permselectivity, podocyte effacement</td>
<td>Incipient slow decline</td>
<td>Normal or slightly increased</td>
<td>Microalbuminuria (30-300mg/day)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Incipient increase</td>
</tr>
<tr>
<td>4</td>
<td>Clinical overt diabetic nephropathy</td>
<td>After 15-20 years, increased prevalence</td>
<td>Glomerulosclerosis, kidney fibrosis</td>
<td>Rapid decline</td>
<td>Decline</td>
<td>Worsening of macroalbuminuria (&gt;300mg/day)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Abnormal</td>
</tr>
<tr>
<td>5</td>
<td>End-stage renal failure</td>
<td>Final outcome after 25-30 years</td>
<td>Glomerulosclerosis, end-stage kidney</td>
<td>&lt;15 ml/min/1.73m²</td>
<td>Low</td>
<td>Possible decline due to low glomerular filtration</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>High</td>
</tr>
</tbody>
</table>
1.2 Prevention and Treatment of Diabetic Nephropathy

Two main pharmacological therapies have been proven to protect against DN in numerous clinical trials: 1) intensive glycemic control and 2) renin angiotensin aldosterone system (RAAS) inhibition. In the first strategy, intensive glycemic control aims to normalize blood glucose concentrations toward the normal range as safely as possible, thereby reducing the risk of diabetic complications. In the second strategy, RAAS blockade inhibits the action of angiotensin II, which promotes the development of systemic and intraglomerular hypertension leading to cardiovascular and renal complications, as reviewed elsewhere (18).

1.2.1 Intensive Glycemic Control

Benefits of intense glycemic control were first recognized in the Diabetes Control Complications Trial (DCCT) where a large cohort of T1D participants (n=1441) was assigned to intensive glycemic control or conventional glycemic therapy for a mean duration of 6.5 years (19, 20). At the conclusion of DCCT, intensive glycemic control resulted in a 39% reduction in the incidence of microalbuminuria and a 54% reduction in macroalbuminuria compared to the conventional arm.

After the conclusion of the DCCT and the discontinuation of intensive glycemic control, 90% of the original participants were followed observationally in the ongoing Epidemiology of Diabetes Interventions and Complications (EDIC) study (19). Now at 18 years of follow-up, EDIC most recently reported that intensive glycemic control exerts long-lasting benefits, including a 59% reduction in the incidence of microalbuminuria, an
84% decreased risk of macroalbuminuria and a 50% lower risk of developing impaired renal function (19).

Similar observations have been made in type 2 diabetes (T2D) patients who participated in the United Kingdom Prospective Diabetes Study (UKPDS) (n=5102) who underwent intensive rather than conventional glycemic control strategies. The risk of developing microalbuminuria or macroalbuminuria was reduced by 33% in the intensive group compared to the conventional group (18). More recently, the Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation (ADVANCE) (n=11,140) trial reported that intensive therapy reduces the risk of microalbuminuria and worsening nephropathy (21).

Large randomized trials have therefore shown that intensive glycemic control protects against the development of DN. Unfortunately, the use of intensive glycemic control is limited by adverse effects, such as weight gain and hypoglycemia, and a significant proportion of patients still develop renal and cardiovascular complications, highlighting the need to focus on additional protective therapies such as RAAS inhibition.
1.2.2 Renin-Angiotensin-Aldosterone-System Blockade

Angiotensin converting enzyme inhibitors (ACEi) were first conceived to be renoprotective therapy by Brenner in animal studies in the early 1980s and landmark clinical trials confirmed similar protective effects in humans in 1993. The Collaborative study randomized 409 T1D participants to ACEi or placebo and tracked their progression to renal failure end-points consisting of death, dialysis or transplantation. At the conclusion of the study, the investigators reported that ACEi reduced the risk of end stage renal disease by 50% and slowed the rate of GFR decline (22). This success with ACEi led to the introduction of additional RAAS inhibitors called angiotensin-II receptor blockers (ARB), which exert their effects downstream of ACE by blocking the angiotensin II type 1 receptor (14). Like ACEi, the Reduction of Endpoints in NIDDM with the Angiotensin II Antagonist Losartan (RENAAL) study reported a 20% reduction in the risk of end stage renal disease, which was further supported by a similar reduction in the Irbesartan Type II Diabetic Nephropathy Trial (IDNT) (23, 24). Thus, these large clinical trials proved that RAAS blockade was effective at preventing end stage renal disease, reflected by a decrease in death and the need for dialysis or transplantation.

Following the success of RAAS blockade in the prevention end stage renal disease, secondary prevention studies investigated whether RAAS therapy would also prevent the development of overt albuminuria (14). For example, in type 2 diabetes, the Heart Outcomes Prevention Evaluation (MICRO-HOPE) (n=3577) study found a 24% reduction in the risk of developing overt albuminuria after receiving ACEi therapy for 5 years compared to placebo (25). In addition, the Irbesartan in Patients with Type 2 Diabetes and Microalbuminuria (IRMA) study showed a three-fold reduction in the incidence of
macroalbuminuria after a median treatment of 2 years with 300 mg of the ARB irbesartan (14). Lastly, in type 1 diabetes, a meta-analysis including 12 clinical trials with a total of 698 participants reported a 68% reduction in the progression of microalbuminuria to macroalbuminuria, reflecting similar benefits of RAAS inhibition as a renal protective strategy in T1D (14).

Primary prevention of microalbuminuria with RAAS inhibition has been shown to be effective in T2D. The Bergamo Nephrologic Diabetes Complications Trial (BENEDICT) (n=1204) showed that ACEi protected against the development of microalbuminuria, independent of blood pressure control in T2D. The benefits of RAAS inhibition in the prevention of microalbuminuria in T2D was further demonstrated in the recently completed Randomized Olmesartan and Diabetes Microalbuminuria Prevention (ROADMAP) (n=4449), where olmesartan delayed the onset of microalbuminuria by 23% (26).
### Table 2: Major Clinical Trials of RAAS Blockade in Type 1 & 2 Diabetes. Adapted with permission from (14).

<table>
<thead>
<tr>
<th>Study (year)</th>
<th>Patients with end points/total patients (%)</th>
<th>RAAS inhibitor type</th>
<th>Hazard ratio of end point with use of RAAS inhibitor (95% CI)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Preventing ESRD and death</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Collaborative Study (1993)⁷</td>
<td>25/207 (12%)</td>
<td>ACE inhibitor</td>
<td>0.52 (0.16–0.67)</td>
<td>0.007</td>
</tr>
<tr>
<td>RENAAL (2001)²¹</td>
<td>327/751 (44%)</td>
<td>ARB</td>
<td>0.84 (0.72–0.98)</td>
<td>0.02</td>
</tr>
<tr>
<td>IDNT (2001)²²</td>
<td>189/579 (33%)</td>
<td>ARB</td>
<td>0.81 (0.67–0.99)</td>
<td>0.03</td>
</tr>
<tr>
<td><strong>Preventing overt nephropathy</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MICRO-HOPE (2000)²²</td>
<td>117/1,808 (6%)</td>
<td>ACE inhibitor</td>
<td>0.75 (0.64–0.88)</td>
<td>0.027</td>
</tr>
<tr>
<td>LIFE (2002)⁴⁷</td>
<td>27/586 (8%)</td>
<td>ARB</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IRMA 2 (2001)²³</td>
<td>10/194 (5%)</td>
<td>ARB</td>
<td>0.30 (0.14–0.61)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td><strong>Preventing microalbuminuria</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>EUCLID (1997)³⁸</td>
<td>13/213 (6%)</td>
<td>ACE inhibitor</td>
<td>1.30 (0.64–2.70)</td>
<td>0.5</td>
</tr>
<tr>
<td>DIRECT 1 and 2 (2009)¹¹⁰</td>
<td>ND</td>
<td>ARB</td>
<td>0.95 (0.78–1.16)</td>
<td>0.60</td>
</tr>
<tr>
<td>Mauer et al. (2009)⁶⁰</td>
<td>4/94 (4%)</td>
<td>ACE inhibitor</td>
<td>ND</td>
<td>0.96</td>
</tr>
<tr>
<td>Mauer et al. (2009)⁶⁰</td>
<td>16/94 (17%)</td>
<td>ARB</td>
<td>ND</td>
<td>0.01</td>
</tr>
<tr>
<td>J-MIND (2001)⁴¹</td>
<td>23/105 (22%)</td>
<td>ACE inhibitor</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>BENEDICT (2004)⁴²</td>
<td>18/301 (6%)</td>
<td>ACE inhibitor</td>
<td>0.47 (0.26–0.83)</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin-receptor blocker; ND, no data; RAAS, renin-angiotensin-aldosterone system.
1.2.3 Failure of Microalbuminuria Prevention in Type 1 Diabetes

Unfortunately, RAAS inhibition has yielded less positive results as a primary preventative therapy for the development of microalbuminuria in T1D. In the landmark Renin Angiotensin System Study (RASS), 285 patients with T1D were randomized to an ACEi (enalapril 10 mg daily), an ARB (losartan 50 mg daily) or daily placebo (27). The primary outcomes of interest were development of microalbuminuria and evolution of morphologic features of diabetic nephropathy on serial kidney biopsies. After a 5-year trial period, the investigators reported that RAAS inhibition did not reduce the risk of microalbuminuria. Moreover, comparisons between biopsies taken at baseline and 5 years did not show significant improvement in renal structure. Thus, in contrast with T2D, the RASS study failed to find that RAAS inhibition confers renal protective effects in normotensive, normoalbuminuric patients with T1D and GFR in the normal range. Consistent with the RASS study, two large randomized studies, the Diabetic Retinopathy Candesartan Trials (DIRECT)-Protect-1 and DIRECT-Prevent-1, also failed to show protection against development of microalbuminuria after ARB therapy in 3,326 participants after 4.7 years (14, 28). Even though these T1D primary prevention studies were negative, it may be important to note that the study and follow up periods (i.e. 5 years) of these trials, which used microalbuminuria as the endpoint, were relatively brief. In contrast, the natural course of DN can take decades to manifest. Therefore, the benefits of RAAS inhibition may not be apparent without a much longer follow-up period or a longer duration of treatment.
Figure 1: Kaplan-Meier Estimates of Time to Microalbuminuria from RASS Study in Type 1 Diabetes. ACEi and ARB inhibition did not show renoprotection against the development of microalbuminuria. Reproduced with permission from (27), Copyright Massachusetts Medical Society.
1.2.4 Adverse Outcomes and Limitations

Although RAAS blockade has emerged as the cornerstone of renal protection in patients with DM, many patients still develop progressive DN over time despite the use of these drugs (29). A possible reason for the partial effects of these agents relates to compensatory pathways that are activated with the use of RAAS blockade monotherapy, such as chymostatin-sensitive pathways that lead to persistent angiotensin II production in ACEi use (30). Due to these “escape” pathways, dual RAAS blockade, such as with combined ACEi plus ARB treatment, could theoretically offer additional benefits over monotherapy in the reduction of cardiovascular and end stage renal disease (31). However, this strategy is now known to increase the risk of adverse side effects without offering additional renal protection (32). For example, the Ongoing Telmisartan Alone and in Combination with Ramipril Global Endpoint Trial (ONTARGET) reported a 33% and 37% increased risk of renal impairment and renal failure respectively (33). Moreover, The Aliskiren Trial in Type 2 Diabetes Using Cardiorenal Endpoints (ALTITUDE) study was discontinued prematurely due to increased adverse events and also failed to show beneficial effects on renal outcomes (34). Most recently, VA-NEPHRON-D reported no benefit in the long-term progression of DN, despite the reduction of albuminuria, and the clinical trial was prematurely terminated due to an increased risk of acute kidney injury (35). Therefore, treatment of DN by RAAS blockade is limited to monotherapy and, DM patients who are non-responsive to RAAS therapy have limited alternative options.

In recent years, several studies have reported a decline in the prevalence of end-stage renal disease in the DM population, an observation that has been attributed to
improved glycemic control and the increased use of RAAS blockade in clinical practice (6, 36). However, this apparent success should be met with a degree of caution. First, DN remains the most common cause of end stage renal disease (5). Second, the modest decline in the prevalence of DN will likely be counterbalanced by the aforementioned rapid growth in the overall prevalence of DM and accordingly, the absolute number of DN cases will likely continue to rise. Lastly, rather than proactively treating patients with DN, clinicians are reactively treating patients who have already developed glomerular injury. As stated by Vallon et al, “the current strategy of intervening after the onset of albuminuria (i.e., after the onset of sclerosis) may be akin to closing the barn door after the horses are out.” (37) Therefore, further improvements in the risk of developing DN in patients with T1D may depend on earlier detection strategies and an improved ability to identify patients at high-risk for developing DN prior to the onset of clinical manifestations.
1.3 Is Albuminuria a Good Marker of Diabetic Nephropathy?

Albuminuria is the current gold standard biomarker of early DN and one of the first detectable clinical abnormalities of DN prior to the decline of GFR. However in recent years several studies have questioned the validity of albuminuria as a predictor of DN progression due to several limitations:

First, the rate of progression of albuminuria is non-linear and microalbuminuria is a poor predictor of those who will progress to overt albuminuria. In a landmark study by Perkins et al, a large cohort of T1D patients with persistent microalbuminuria was followed-up for 6 years (38). Contrary to the classical paradigm of linear progression, 58% regressed (i.e. 50% reduction within 2 years) in patients with microalbuminuria. Therefore, a majority of T1D patients with microalbuminuria may never progress to overt albuminuria and can stay with persistent microalbuminuria or regress to the normal range.

Second, long-term follow-up from DCCT/EDIC revealed that 24% of patients with renal insufficiency did not develop albuminuria (39). These results illustrate that predicting DN using albuminuria alone leaves one-quarter of the patients untreated until they develop renal function decline. Furthermore, this subset of decliners can exhibit advanced, biopsy-proven renal lesions, hence illustrating some discordance between underlying renal injury and albuminuria (40).

Third, albuminuria detection techniques have yet to be fully standardized and day-to-day variability is high. Current KDIGO guidelines recommend timed urine collection or a spot urine albumin-creatinine-ratio (ACR) to determine albumin excretion rate (41). When compared in the DCCT, the two methods resulted in substantial differences in the classification of albuminuria state (42). Furthermore, other factors such as the time of the
day when the sample was collected may affect ACR results (43). Moreover, day-to-day ACR can vary up to ±467%, ±170% and ±48% in normoalbuminuric, microalbuminuric and macroalbuminuric patients with stable GFR, respectively (44). The variability of albuminuria poses a severe limitation to the use of albuminuria as a marker of DN for clinicians, since the change in ACR must be large in order to detect a clinically significant worsening of albuminuria, which makes it a suboptimal marker for early glomerular injury when changes occur slowly over time.

Notwithstanding these limitations, albuminuria is still a powerful clinical marker of cardiovascular risk, renal disease and all-cause mortality in diabetic patients (29, 45), but may be better utilized as an indicator rather than a predictor of future renal injury, since the use of albuminuria to predict future renal insufficiency leaves much to be desired.
2 The Early Pathogenesis of Diabetic Nephropathy

As discussed, due to the limited clinical utility of albuminuria as a marker of early DN and limitations of current pharmacotherapies, it is imperative to better understand factors that may identify high-risk patients prior to the onset of albuminuria, such as renal hyperfiltration (39). Hyperfiltration is one of the earliest renal abnormalities implicated in the early, pre-clinical phase of diabetes (10). In the 1980s, highly influential work by Barry Brenner proposed the “hyperfiltration hypothesis” as a critical mechanism that underlies early, preclinical renal injury leading to DN.

2.1 Brenner’s Hypothesis to Diabetic Nephropathy

In Brenner’s pioneering micropuncture studies, hyperglycemic streptozotocin (STZ) treated Munich-Wistar rats displayed an elevation in single-nephron glomerular filtration rate (SNGFR) leading to glomerular hyperfiltration (46). Furthermore, this diabetic-induced hyperfiltration was attributed to intraglomerular hypertension due to increased transcapillary hydrostatic pressure (P_{GC}) and abnormal renal vascular resistance (47). Under light and scanning electron microscopy examination, rats with this altered renal hemodynamic profile exhibited renal injury represented by podocyte effacement, mesangial cell expansion and endothelial cell injury (48). When glomerular hypertension was improved leading to lowered SNGFR/ hyperfiltration by a low-protein diet or ACEi, renal injury was significantly reduced (48, 49). Brenner and colleagues were therefore the first to recognize that ACEi, now the gold standard of renoprotection, effectively treated and reduced renal injury in hyperfiltering animals.
With these observations, Brenner proposed a structure-function relationship in the “hyperfiltration paradigm” to explain progression from hyperfiltration to DN (47). The first principle in this schema is that humans are born with a non-regenerative critical number of nephrons that varies between 300,000 and 1.1 million (50). Although some nephrons are lost throughout the aging process, the number of remaining nephrons must provide sufficient overall GFR to support renal clearance throughout an individual’s lifetime. In the setting of glomerular hyperfiltration induced by uninephrectomy, DM, obesity or other glomerular diseases, remaining nephrons are subjected to increased wall tension and endothelial shear stress resulting in premature attrition through structural injury, such as podocyte effacement and mesangial expansion (51). This process may be accelerated in individuals who are born with reduced numbers of nephrons due to maternal illness, diabetes, or environmental stress (51). Since compensatory hypertrophy and intraglomerular hypertension may occur earlier, and to a more severe degree, in these individuals, they may be predisposed to a greater decline in renal function and to renal insufficiency and end stage renal disease (52).

As stated by Brenner et al, “Hyperfiltration occurs in certain pathophysiologic conditions even when renal mass is intact, as in diabetes mellitus. It was shown that hyperfiltration predicts the subsequent development of nephropathy in patients with type 1 diabetes, independently of the degree of metabolic control.” (47) This schema is supported in a recent study by Ruggenetti, where diabetic patients were treated with ACEi for a median duration of 4.0 years (53). Those with persistent hyperfiltration had a two-fold risk of developing clinical albuminuria compared with those with ameliorated hyperfiltration or
normofiltration. The authors noted that ameliorated hyperfiltration was associated with improved cardiovascular outcomes in addition to a stable and slower declining GFR.
Figure 2: Brenner’s Renal Hyperfiltration Schema for Diabetic Nephropathy. Hyperglycemia activates early pathways leading to an increase in intraglomerular pressure ($P_{gc}$) and hyperfiltration. After a period of hyperfiltration, renal function/GFR rapidly declines due to underlying hyperfiltration-induced injury. In the classical paradigm, albumin excretion rate (AER) increases with severity of diabetic nephropathy. Adapted with permission from (52).
Figure 3: Different Phenotypes of Glomerular Injury due to Hyperfiltration. Normal (a) and diabetic (b) glomeruli. Mesangial cell expansion (c), glomerular barrier permselectivity (d), podocyte effacement (e). Brenner proposed that disrupted structure leads to the functional decline in GFR. Reproduced with permission from (54).
2.2 Glomerular Hyperfiltration in Diabetes

In humans, direct measures of SNGFR cannot be performed, hence whole kidney glomerular filtration rate (GFR) is used as a surrogate marker for SNGFR and glomerular hypertension. Hyperfiltration is typically defined as a GFR above 125 mL/min to 140mL/min per 1.73m² or greater than 95th percentile and may be present in up to 60% of patients with T1D (55, 56).

The most comprehensive review of hyperfiltration in T1D is a meta-analysis by Magee et al, which examined the evidence underlying renal risk due to hyperfiltration in studies published in MEDLINE and EMBASE until March 2008 (57). Their search yielded an in-depth analysis of ten patient cohorts with a total of 780 T1D patients with a median follow-up of 11.2 years. After evaluating the combined evidence for hyperfiltration risk, Magee et al reported that T1D patients with hyperfiltration are 2.7 times more likely to progress to overt albuminuria than those with normofiltration. The authors suggested that patients with the “double-hit” of younger age of diabetes onset and hyperfiltration, may be at the greatest risk of developing DN due to the increased exposure to the injurious environment.

For example, in a pediatric study of 308 T1D patients with a follow-up of 10.9 years, children and adolescents with severe hyperfiltration developed microalbuminuria earlier (58). In contrast, children and adolescents without hyperfiltration had a 98.9% chance of remaining normoalbuminuric. Moreover, these differences were independent of long-term glycemic control. Therefore, Amin et al recommended the use of early intervention strategies to reduce GFR to delay or prevent the onset of microalbuminuria.
Glomerular hyperfiltration also occurs in T2D, albeit with a lower prevalence (40%) (59, 60). This difference may be reflective of the heterogeneity in the T2D population and a diabetic milieu that is complicated by insulin resistance, obesity and arterial hypertension. Nevertheless, new onset T2D patients exhibit abnormal renal vascular resistance, glomerular hyperfiltration and a rate of GFR decline, similar to that of T1D (61-63). Lastly, renal biopsies in T2D patients show similar changes observed in T1D including glomerulosclerosis, glomerular basement thickening, podocyte effacement and increased mesangial matrix volume (64, 65). Thus, some patients with T2D and nephropathy demonstrate a parallel hyperfiltration-structure-injury paradigm related to T1D.

Interestingly, hyperfiltration has also been identified in patients with “pre-diabetic” conditions in two separate studies. In the first, Okada et al examined a large Japanese cohort (n=99,140) segregated on the basis of i) no-diabetes ii) stage 1 impaired fasting glucose iii) stage 2 impaired fasting glucose and iv) diabetes (56). The authors found that with increasing severity of impaired fasting glucose, the prevalence of hyperfiltration increased, despite overall clinical similarity between the groups other than their pre-diabetic status. They concluded that hyperfiltration occurs very early in the natural history of the disease or even concurrently with the onset of diabetes. This observation was further confirmed to be independent of age, sex, BMI, blood pressure, smoking status, and insulin levels in a smaller cohort (n=1560) using a continuous measure of fasting glucose, rather than categorical separation (66).

From the above evidence, hyperfiltration is a risk factor for the development of DN and represents a common pathophysiological state that may initiate or accelerate the progression to renal insufficiency (55). Despite what is known about hyperfiltration in
animal models and humans, the primary mechanism responsible for this condition remains controversial (55). There are two hypothesized mechanisms to explain hyperfiltration. The first mechanism is the “hemodynamic/vascular hypothesis” which suggests that hyperfiltration is related to abnormalities in renal vascular tone due to neurohormonal activation by hyperglycemia. The second is the “tubular hypothesis”, which postulates that increased reabsorption of sodium/glucose in the proximal tubules leads to changes in tubuloglomerular feedback.

### 2.3 Hemodynamic Hypothesis of Hyperfiltration

The hemodynamic hypothesis is an extension of Brenner’s observations of abnormal renal hemodynamic function in DM, which has been attributed to activation of the RAAS (angiotensin II), as well as other mediators including cyclooxygenase-2 (COX-2) derived prostaglandins and nitric oxide (NO) (55). In the diabetic milieu, hyperglycemia can activate the production of these factors leading to renal pre-glomerular vasodilatation and post-glomerular constriction. As a result, this abnormal hemodynamic profile leads to increased renal afferent blood flow and glomerular hydrostatic pressure, both of which mediate intraglomerular hypertension and hyperfiltration (55).

At the afferent arteriole, vasodilatation is partly mediated by increased bioavailability of COX-2 derived prostanoids (55). Both isoforms of the COX enzyme (1 and 2) are found in the kidney but under hyperglycemic conditions, the COX-2 isoform is predominantly upregulated (55). In animal models, COX-2 is found at the afferent arteriole and appears to be localized to the endothelial and smooth muscle cells; Komhoff et al suggested that this pattern of localization indicates that COX-2 is a regulator of renal
perfusion and glomerular hemodynamic function (67). COX-2 activity exerts hemodynamic effects by increasing the production of vasodilatory prostaglandins, such as PGE$_2$ and PGI$_2$ (68). In diabetic mice, COX-2 inhibition using non-steroidal anti-inflammatory drugs reduces the urinary excretion of these prostanoids, leading to a decrease in hyperfiltration (69). Furthermore, our laboratory has previously studied COX-2 inhibition with celecoxib and reported that this agent can partially reduce GFR in hyperfiltering T1D patients (55). However, ever after COX-2 inhibition, GFR did not normalize, suggesting that additional factors may be responsible for the hyperfiltration state.

An alternative mediator that is regularly discussed in the hemodynamic theory is NO bioavailability, which can be derived from different NO synthase isoforms (inducible - iNOS, endothelial - eNOS, neuronal - nNOS). Like prostaglandins, NO is a vasodilatory molecule that preferentially dilates the afferent arteriole leading to intraglomerular hypertension. Both animal and humans studies of DM have reported elevated levels of NO metabolites, which suggests an increase in NO production (55). In several animal studies, L-NG-nitroarginine methyl ester (L-NAME), a specific eNOS inhibitor, was shown to reduce urinary NO excretion, reduce afferent arteriolar diameter and normalize GFR, suggesting a role for NO in the hyperfiltration state (68). Similarly in humans, NG-monomethyl-L-arginine, monoacetate (L-NMMA), a non-specific NO synthase inhibitor, partially reduced GFR in hyperfiltering T1D patients but GFR still remained in the hyperfiltration range (70). Interestingly, L-NMMA had no effect on GFR in normofiltering T1D participants and healthy controls. Therefore, this observation reinforces that NO and COX-2 bioactivity are important mediators in hyperfiltration, but pre-glomerular inhibition
does not fully normalize GFR. Thus, efferent constriction may play a critical role in the maintenance of hyperfiltration (68).

At the efferent arteriole, abnormal vascular tone is attributed to the hormonal activation of the RAAS pathway induced by hyperglycemia. For example, a two-fold increase was found in the intrarenal levels of renin mRNA, renin protein and downstream angiotensin II in diabetic rats (71). Moreover, extracellular hyperglycemia stimulated the production of angiotensinogen mRNA and protein expression in in vitro studies using renal proximal tubular cells (72). Thus, both in vitro and in vivo studies suggest that hyperglycemia is a central regulator of RAAS, which upon activation, constricts the efferent arteriole, thereby resulting in renal hyperfiltration (55).

In T1D humans, hyperglycemia similarly induces efferent arteriole constriction leading to an increase in GFR, but these effects are blunted with RAAS blockade, leading to a reduction in hyperfiltration (73). During a longer duration of RAAS blockade of 21 days in an adolescent cohort, T1D participants who were persistent hyperfilterers responded to RAAS blockade with a large reduction in GFR and thus, improved hyperfiltration. However, GFR remained in the hyperfiltration range (74). In contrast, T1D participants with normofiltration did not experience a reduction in GFR.

In summary, the hemodynamic theory postulates that hyperglycemia activates pre-glomerular and post-glomerular hormonal pathways that lead to abnormal renal vascular tone, and the maintenance of the hyperfiltration state. However, COX-2, NO and RAAS inhibition were unable to fully normalize GFR, suggesting that there may be additional factors that contribute to hyperfiltration.
Figure 4: GFR Response to RAAS Inhibition in Young Type 1 Diabetes with and without Hyperfiltration. RAAS ameliorates hyperfiltration by reducing efferent arteriole resistance and intraglomerular pressure. Hyperfilterers show a greater response to ACEi by a decrease in GFR while normofilterers show a mild to no response. Adapted with permission from (75).
**Figure 5:** Hemodynamic Hypothesis of Renal Hyperfiltration. Afferent vasodilation increases afferent blood flow due to upregulation of COX-2, prostaglandins, nitric oxide. Efferent constriction increases renal vascular resistance due to increased RAAS activity. Afferent vasodilatation and efferent constriction results in an increase in intraglomerular pressure leading to hyperfiltration and mechanical strain on endothelial and mesangial cells. Graphics adapted with permission from (76).
2.4 Tubulo-Centric Hypothesis of Hyperfiltration

The second theory, the “tubular hypothesis”, proposes that renal hyperfiltration is mediated by abnormal handling of sodium and glucose in the proximal tubule. The tubular hypothesis is based on the presence of renal hypertrophy and increased proximal reabsorption of sodium, leading to renal hyperfiltration soon after the onset of DM (37). In animal studies, this increase in renal mass is attributed to the growth of the proximal tubules, a site that is highly involved in the reabsorption of sodium and glucose (37).

In healthy and diabetic individuals, blood glucose is freely filtered at the glomerulus and then reabsorbed at the proximal tubule. Glucose reabsorption in the kidneys is highly efficient and accomplished by the sodium-glucose co-transporters which exists in two isoforms - SGLT1 and SGLT2 (77). SGLT1 and 2 reabsorb glucose by passing glucose molecules through the apical side of the proximal tubular cells against the glucose concentration gradient. As the name suggests, this is accomplished through co-transportation with sodium and secondary active-transport resulting in the removal of sodium from the tubular lumen.

In non-diabetic individuals with normal plasma glucose levels, approximately 180 grams of glucose is filtered by the kidneys per day and is completely reabsorbed at the proximal tubule, thus conserving all the filtered glucose (78). In diabetic individuals, however, acute hyperglycemia increases the plasma glucose concentration thereby increasing the amount of glucose that is filtered. Despite this increased glucose load, SGLT1/2 has the remarkable capacity to double the rate of glucose re-absorption prior to saturation and subsequent glycosuria through increased SGLT1/2 expression and activity (78).
In the first element of the “tubular theory”, chronic hyperglycemia therefore stimulates the growth of proximal tubules to more efficiently handle the excess filtered glucose load (37). In urine samples isolated from diabetic individuals, SGLT2 expression is significantly increased in proximal tubular epithelial cells (79). Due to an increase in number and activity SGLT2 transport, basal reabsorption of glucose and sodium is increased. As a result of SGLT2 action and chronic hyperglycemia, sodium concentration is lowered at the distal tubules.

The second element of the tubular theory involves the tubuloglomerular feedback (TGF) feedback, which occurs downstream at the juxtaglomerular apparatus of the distal tubule. In this uniquely organized structure, the distal renal tubule loops back to contact the region between the afferent and efferent arterioles (37). Specialized tubular cells at this location form the macula densa, which is able to detect changes in renal tubular sodium chloride delivery through altered activity of the Na\(^+-K^+\)-2Cl\(^-\) co-transporter (NKCC2). Higher luminal sodium concentration results in higher NKCC2 activity while lower sodium results in lower activity. The NKCC2 transport mechanism is energy dependent, resulting in breakdown of ATP and consequently the generation of adenosine, which causes afferent arteriolar vasoconstriction (37). The kidneys are thereby able to auto-regulate and control GFR by changing afferent arteriole tone through “sensing” the delivery of sodium chloride to the macula densa, which naturally evolved to preserve renal function during states of environment stress including volume depletion (77).

In diabetes, however, distal delivery of sodium to the macula densa is reduced due to increased activity of SGLT2 at the proximal tubule. As a consequence, despite normal GFR levels, lowered sodium delivery results in less sodium transported to macula densa
cells, less NKCC2 activity, less ATP breakdown and less adenosine generation. The decline in adenosine production causes vasodilatation of the afferent arteriole, thereby increasing intraglomerular pressure and the initiation of hyperfiltration.

In humans, non-specific inhibition of SGLT is unfortunately poorly tolerated and hence phlorizin, a known SGLT inhibitor for decades, could not be studied. Therefore, the influence of TGF on hyperfiltration in humans remained unknown (78). However, an emerging class of selective SGLT2 inhibitors, analogs of phlorizin, have most recently been developed and approved for use in humans which allows for the investigation of the “tubular hypothesis”. Preliminary studies in mouse models show that SGLT2 inhibition reduces hyperfiltration, albuminuria and histological evidence of DN thereby, conferring renal protective properties (80). In humans, our laboratory has recently demonstrated that SGLT2 inhibition also attenuates hyperfiltration in young healthy T1D individuals (77). In light of this evidence, tubular factors appear to play a significant role in mediating hyperfiltration in humans and perhaps by extension, early renal injury.
Figure 6: Tubular Hypothesis of Renal Hyperfiltration and the Mechanism of SGLT2 Inhibition to Reduce GFR. Reproduced with permission from (81).
2.4.1 Combination Hemodynamic and Tubular Blockade

Interestingly, Kojima et al demonstrated that combination therapy with a RAAS blocker and an SGLT2 inhibitor leads to additive renal protective effects in animals, suggesting a possible role for combined therapy with agents that target hemodynamic and tubular factors to completely abolish the hyperfiltration state (82). These observations require confirmation in human studies, which also need to determine the safety of this combination and possible adverse effects.

In summary, hyperfiltration is an important factor that may increase the risk of DN in both animal and human studies. Importantly, hyperfiltration is modifiable through the use of medications that target both vascular and renal tubular pathways, although the optimal treatment modality and long term implications of reducing hyperfiltration remain unknown. Nevertheless, from a mechanistic perspective, hyperfiltration appears to increase DN risk through an increase in glomerular wall tension and shear stress, leading to the activation of pro-inflammatory and pro-fibrotic factors.
Figure 7: GFR Response to SGLT2 Inhibition in Young Type 1 Diabetes with Normofiltration (T1D-N) and Hyperfiltration (T1D-H) During Euglycemia (A) or Hyperglycemia (B). SGLT2 ameliorates hyperfiltration by blocking the reabsorption of glucose/sodium and restoring balance to TGF activity. Reproduced with permission from (81).
2.5 Renal Injury Through Activation of Inflammatory Pathways

Traditionally, hemodynamic and metabolic changes at the glomerulus, such as with hyperfiltration and hyperglycemia, were considered to be the primary mechanisms responsible for glomerulosclerosis in diabetes. More recently, however, these conventional pathways are considered to be only a partial cause of DN (83).

Hyperfiltration also causes distention of the glomeruli, due to an increase in intraglomerular pressure, resulting in increased mechanical strain and stretch of glomerular cells such as the mesangial cells and podocytes. In *in vitro* studies, mechanical stretching of mesangial cells, in an effort to mimic hyperfiltration, results in collagen and extra-cellular matrix accumulation - a pro-fibrotic phenotype akin to early glomerulosclerosis (84). Second, glomerular distention induces the production of inflammatory cytokines resulting in the activation of macrophage infiltration (85). Moreover, acute hyperglycemia increases the severity of these two phenotypes (84, 85). Therefore, hyperfiltration-related mechanical stimuli (ie. stretch) may induce a pro-fibrotic and low-grade inflammation phenotype in kidney cells, which may be further exacerbated by hyperglycemia.

While subclinical inflammation is considered to be an important emerging mediator of DN, it is likely related to other mechanisms of injury described above (83). For example, activation of the RAAS pathway is associated with local and systemic production of pro-inflammatory molecules (ex. cytokines), which as previously discussed, can be activated by hyperglycemia and hyperfiltration (73, 86). Therefore, subclinical inflammation is a mechanism of renal injury that “cross-talks” between hyperglycemia, hyperfiltration and
Figure 8: Glomerular Distention and Inflammation. (A) normal sized glomerulus (B) glomerular size after increased perfusion. (C) Immunostaining of macrophage adhesion receptor (ICAM) expression after mechanical stimuli. Increased mechanical stretch leads to greater expression of a pro-inflammatory phenotype. Adapted with permission from (84, 87).
cellular compartments in the kidney. For example, inflammatory cytokines are secreted protein molecules that are critical for immune system signaling and also mediate a number of local biological effects including wound healing, macrophage infiltration, fibrosis/collagen deposition and apoptosis (83, 88). The effects of cytokines can be local, with autocrine or paracrine signaling, or systemic through endocrine secretion into the circulation. Moreover, these effects can be categorized into subsets such as chemokines, which are cytokines involved in the chemotaxis of macrophages and activation of monocytes (89). Hence, the dysfunction of cytokine signaling, by either ligand or receptor binding, may result in renal injury by activating deleterious potential mechanisms of kidney injury such as i) macrophage extravasation, ii) apoptosis and iii) fibrosis.

Indeed, abnormal cytokine signaling has been associated with macrophage infiltration in diabetic animal models and humans. In human biopsy specimens, immunohistological studies reported an accumulation of macrophages during early glomerulosclerosis and tubulointerstitial fibrosis (90). It is interesting to note that, as suggested by the authors, macrophage infiltration may have started much earlier, prior to morphological changes such as glomerular membrane thickening (90). In addition, the intensity of macrophage infiltration correlated with the subsequent decline in renal function (91). This type of renal injury is associated with increased urinary excretion of monocyte chemoattractant protein-1 (MCP-1) in patients with DN (92). Moreover, genetic knock-out and pharmacological inhibition of MCP-1 in diabetic rodents ameliorated glomerular and interstitial macrophage infiltration and renal fibrosis (93, 94). Lastly, RAAS blockade suppresses MCP-1 expression, suggesting that the renoprotective benefits of RAAS blockade may be partially due to a reduction in macrophage infiltration. It is therefore
possible that other cytokines, which are involved in chemotaxis/adhesion/activation (MCP-3, MIP-1α, MDC, GM-CSF, eotaxin, RANTES), may also contribute to the pathogenesis of diabetic kidney injury by promoting macrophage infiltration (95).

While some cytokines may activate macrophage infiltration, others also mediate renal injury by promoting apoptosis of glomerular and tubular cells. In renal biopsies taken from diabetic patients, apoptotic activity was detected in the glomeruli, endothelial cells and in the tubulointerstitium, suggesting that some renal atrophy occurs in the diabetic milieu (96, 97). For example, in diabetic animals, apoptosis of podocytes is detected after stimulation with hyperglycemia, which may explain the depleted podocyte number commonly reported in humans with DN (97). Ultimately, the loss of these glomerular and tubular cells results in structural injury and nephron loss. As suggested by others, the TNF family of cytokines appears to be key mediators of apoptosis and pro-inflammatory response in renal tissues and thus, dysregulation of these cytokines or others involved (IL-2) in the apoptosis pathway may cause renal atrophy and subsequent renal function decline (98).

Alternatively, pro-fibrotic pathways may also be activated by cytokines and contribute to the pathogenesis in the early progression of DN. As proposed by Kalluri and Neilson, cytokines may stimulate renal epithelial cells to “reprogram” into fibroblasts through epithelial-mesenchymal transition (99). These newly epithelial-mesenchymal derived fibroblasts may then synthesize and deposit extra-cellular matrix components including collagen. Although this proposed theory has yet to gain universal support, there is ample evidence to show that fibrosis is associated with diabetes. For example, in urine samples, excretion of collagen fragments is significantly increased in diabetic patients with
DN compared to healthy non-diabetic controls (100, 101). In renal biopsies, mRNA expression of collagen was also significantly increased, suggesting an upregulation of collagen production (102). From a therapeutic perspective, in diabetic rats, collagen accumulation was decreased by RAAS blockade and by increased matrix metalloproteinase activity (103). Taken together, disruption of extracellular matrix production and degradation, may cause collagen deposition in early DN, resulting in glomerular basement thickening and tubulointerstitial fibrosis (104). Over time, these morphological changes may mediate nephron loss leading to renal insufficiency, as previously discussed. Indeed, abnormal production of cytokines involved in fibrosis (IL-6, PDGF) may also contribute to renal injury and subsequent loss of function (104, 105).

Lastly, cytokines may accelerate the progression of DN by maintaining a pro-inflammatory subclinical environment in the kidneys. Previous work has shown that cytokines can create an autocrine loop, so that after an initial release of cytokines, positive feedback triggers second or third or continuous waves of cytokine release (106, 107). Furthermore, paracrine and endocrine signaling can initiate a cascade of deleterious effects in neighboring or systemic cells, respectively. These effects, such as macrophage infiltration, apoptosis and fibrosis, may be mediated through NF-κB or protein kinase C (PKC) signal transduction, which are upregulated in the diabetic milieu (108, 109).
In summary, cytokines may be activated by hyperglycemia or mechanical stimuli, such as hyperfiltration, and may exacerbate renal injury. Preliminary studies in humans have shown an increased urinary excretion of cytokines in T1D and T2D patients who have developed clinical DN (110, 111). Furthermore, baseline inflammation has most recently been reported to predict the development of albuminuria and early GFR decline, suggesting that urine and serum levels of cytokines may be valuable biomarkers for predicting the progression of DN (16, 112). However, the association between hyperfiltration and cytokines is unclear. Thus, two studies as described below investigated the urinary excretion of cytokines in the context of hyperfiltration in young T1D participants.

Firstly, using gold-standard inulin techniques to measure GFR, urinary levels of cytokines/chemokines were measured in young T1D adults analyzed on the basis of whether or not they exhibited renal hyperfiltration, and a healthy control group. The objective was to determine if, similar to experimental models, renal hyperfiltration – a surrogate for intraglomerular hypertension – would be associated with elevated urinary excretion of cytokines/chemokines, suggesting increased renal exposure to these factors. In a second study involving a separate cohort of T1D adolescents, GFR was estimated by cystatin C, which is a more clinically applicable measure of GFR. In this second cohort, urinary cytokine/chemokine excretion was measured and an identical panel of plasma cytokines/chemokines was assessed. The aims were 1) to determine if hyperfiltering patients, defined by a “clinically applicable” estimate of GFR, would reveal a similar association between hyperfiltration and inflammation compared to GFR measured by inulin; and 2) to determine the source of increased urinary cytokine/chemokine excretion – systemic production with renal overflow vs. an intrarenal source.
Figure 9: Schematic of Early Diabetic Nephropathy Paradigm. Hyperglycemia leads to RAAS/Hyperfiltration/Subclinical Inflammation. Cytokines may mediate downstream pathways leading to renal injury. The significance of cytokines in diabetic nephropathy is unclear.
3 The Effect of Renal Hyperfiltration on Urinary Inflammatory Cytokines/Chemokines in Young Adults with Type 1 Diabetes Mellitus

Previously Published as:

The effect of renal hyperfiltration on urinary inflammatory cytokines/chemokines in patients with uncomplicated type 1 diabetes mellitus

Diabetologia 2013, May;56(5):1166-73
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3.1 Abstract:

**Aims/hypothesis:** High intraglomerular pressure causes renal inflammation in experimental models of diabetes. Our objective was to determine whether renal hyperfiltration, a surrogate for intraglomerular hypertension, is associated with increased excretion of urinary cytokines/chemokines in patients with type 1 diabetes mellitus.

**Methods:** Blood pressure, renal hemodynamic function (inulin and paraaminohippurate clearances for glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), respectively) and urine samples were obtained during clamped euglycemia in individuals with type 1 diabetes with either hyperfiltration (GFR determined using inulin \( \text{GFR}_{\text{INULIN}} \geq 135 \text{ ml min}^{-1}\text{1.73 m}^{2} \), \( n=28 \)) or normofiltration (\( n=21 \)) and healthy control individuals (\( n=18 \)).

**Results:** Baseline clinical characteristics, dietary sodium and protein intake and blood pressure levels were similar in the diabetic and healthy control groups. In addition, HbA1c levels were similar in the two diabetic groups. As expected baseline GFR was higher in hyperfilterers than either normofiltering diabetic patients or healthy control patients (165±9 vs 113±2 and 116±4 ml min−1 1.73 m−2, respectively, \( p<0.01 \)). ERPF and renal blood flow were also comparatively higher and renal vascular resistance was lower in hyperfiltering patients (\( p<0.01 \)). Hyperfiltering diabetic patients had higher excretion rates for eotaxin, IFNα2, macrophage-derived chemokine, platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB and granulocyte-macrophage colony-stimulating factor (p≤0.01). Urinary monocyte chemoattractant protein (MCP)-1 and RANTES (regulated on activation, normal T expressed and secreted) excretion was also higher in hyperfiltering vs
normofiltering diabetic individuals (p<0.01) and fibroblast growth factor-2, MCP-3 and sCD40L excretion was elevated in hyperfiltering diabetic individuals vs healthy controls (p<0.01).

**Conclusions/interpretation:** Renal hyperfiltration is associated with increased urinary excretion of inflammatory cytokines/chemokines in patients with uncomplicated type 1 diabetes.
3.2 Introduction:

Inflammation plays a critical role in the pathogenesis of early diabetic nephropathy. In experimental models, this is in part a result of hyperglycemia-induced neurohormonal activation, which increases intraglomerular pressure and shear stress, leading to renal inflammation (87, 113, 114). In *in vitro* studies of human mesangial cells, hyperglycemia increases the mRNA expression of monocyte chemoattractant protein (MCP-1, also known as CCL2) and vascular endothelial growth factor, which have been linked to cell apoptosis, compensatory hypertrophy and hyperfiltration in animal models of diabetes mellitus (115, 116). In patients with type 1 diabetes, we have focused on the effect of acute hyperglycemia on the urinary excretion of cytokines/chemokines rather than tissue expression of these factors, hypothesising that urinary excretion of inflammatory mediators correlates with tissue expression. Similar to observations made in animal studies, we demonstrated that acute hyperglycemia influences urinary cytokine/chemokine excretion, suggesting that the link between intraglomerular pressure, inflammation and renal injury also exists in humans (87, 113, 114, 117).

From a more clinical perspective, we have demonstrated that increased urinary cytokine/chemokine excretion precedes the onset of early clinical manifestations of nephropathy, including microalbuminuria, in young patients with type 1 diabetes (118). In patients with type 2 diabetes and more advanced disease, urinary cytokine/chemokine excretion correlates with the severity of proteinuria and in patients with type 1 diabetes, these factors correlate with declining renal function (110). The factors that influence urinary cytokine/chemokine excretion in patients at an even earlier stage of diabetes, before the onset of proteinuria or renal function decline, remain unclear.
Renal hyperfiltration is one of the earliest detectable abnormalities in humans and is present in approximately 20–60% of patients with uncomplicated type 1 diabetes (75). Due to the potential role of elevated intraglomerular pressure in the pathogenesis of early renal injury in diabetes (57), the aim of this pilot study was to determine whether renal hyperfiltration, used as a surrogate marker for intraglomerular pressure, is associated with elevated urinary cytokine/chemokine excretion in patients with uncomplicated type 1 diabetes. We hypothesised that urinary cytokines/chemokines, which either increase in response to acute hyperglycemia or precede the onset of microalbuminuria, would be elevated in patients with renal hyperfiltration, defined as a glomerular filtration rate determined using inulin (GFR_{INULIN}) \geq 135 \text{ ml/min'/1.73m}^2 (119, 120). If urinary cytokine/chemokine excretion is elevated in high-risk groups, such as patients with hyperfiltration and in those with high-tertile normoalbuminuria (118), then the use of additional, pre-clinical biomarkers in natural history and therapeutic trials may help to clarify the role of earlier interventions in patients with type 1 diabetes.
3.3 Methods:

3.3.1 Participants:

Patients with type 1 diabetes and hyperfiltration (T1D-H, \(n=28\)) or normofiltration (T1D-N, \(n=21\)) and a matched group of healthy individuals as controls (C, \(n=18\)) were recruited from endocrine clinics using local advertisements. Inclusion criteria were: duration of type 1 diabetes \(\geq 5\) years, age \(\geq 18\) years, blood pressure \(<140/90\) mmHg (systolic/diastolic), no history of renal disease or macrovascular disease and participants could not be taking any regular medications other than insulin and had to be normoalbuminuric on a 24 h urine collection. Of the 49 participants with diabetes, 27 had taken part in other physiological studies within 3 years of the present set of experiments and all of these individuals were normoalbuminuric when their albumin excretion was quantified using a 24 h urine collection. Of the remaining 22 participants with diabetes, spot albumin-to-creatinine ratios were normal at their most recent clinic visits but previous 24 h urine collections were not indicated and therefore not performed as part of routine clinical care.

We aimed to study female participants during the early follicular phase of the menstrual cycle, determined by cycle day and measurement of 17β-estradiol levels. None were using oral contraceptive medications. The local Research Ethics Board at the University Health Network (Toronto, Canada) approved the protocol and all participants gave informed consent.
3.3.2 Experimental design:

To suppress the activity of the endogenous renin–angiotensin system and avoid the effect of high dietary protein on renal function, participants adhered to a high-sodium (>140 mmol/day) and moderate-protein (<1.5 g/kg/day) diet during the 7-day period before renal hemodynamic testing and collection of the urine sample, as described previously (Table 3) (119). Furthermore, this restricted diet helped minimize the potential confounding increase in GFR in diabetic participants due to high-protein and low-sodium intake. In patients with diabetes, clamped euglycemic (4–6 mmol/l) conditions were maintained for approximately 6 h preceding and during all investigations, a period of time previously demonstrated to be sufficient to influence vascular function (121). In all phases of the experiment, blood glucose was maintained by a modified glucose clamp technique, as described previously (121). In brief, a 16-gauge peripheral venous cannula was inserted into the left antecubital vein for infusion of glucose and insulin and a second cannula was inserted for blood sampling more distally. Blood glucose was measured every 5–10 min and the insulin infusion was adjusted to maintain euglycemia. In healthy control individuals, studies were performed during normoglycemic conditions. All experiments were performed in the same warm (25°C), temperature-controlled room and in a dark, quiet environment in the supine position.

3.3.3 Assessment of renal variables:

Following maintenance of the euglycemic clamp, a third intravenous line was inserted into the right arm and was connected to a syringe infusion pump for administration of inulin and para-aminohippurate (PAH). After collecting blood for inulin and blank, a
priming infusion containing 25% inulin (60 mg/kg) and 20% PAH (8 mg/kg) was administered. Thereafter, inulin and PAH were infused continuously at a rate calculated to maintain their respective plasma concentrations constant at 2.0 and 0.15 mg/l. After a 90 min equilibration period, blood was collected for measurement of inulin, PAH and hematocrit. Blood was further collected every 30 min for 60 min for inulin and PAH; GFR\text{INULIN} and effective renal plasma flow (ERPF) were estimated by steady-state infusion of inulin and PAH, respectively (121).

### 3.3.4 Sample collection and analytical methods

Blood samples collected for inulin and PAH determinations were immediately centrifuged at 3,000 rpm for 10 min at 4°C. Plasma was separated, placed on ice and then stored at −70°C before the assay. Inulin and PAH were measured in serum by colorimetric assays using anthrone and N-(1-naphthyl)ethylenediamine, respectively (122-124). The mean of two baseline clearance periods represents GFR\text{INULIN} and ERPF, expressed per 1.73 m². Renal blood flow (RBF) was derived using ERPF/(1−haematocrit) and renal vascular resistance (RVR) was derived by dividing the mean arterial pressure by the RBF. All renal hemodynamic measurements were adjusted for body surface area (122, 124).

Upon arrival at the renal physiology laboratory, participants were asked to void and this final volume completed the 24 h urine collection. Participants then voided again mid-morning at approximately 10:00 hours to clear the bladder of urine that was produced while the euglycemic clamp was being stabilised. The subsequent spot urine sample that was used for the analysis was collected before the first set of renal hemodynamic variables was measured. This 50 ml mid-stream sterile urine specimen was used to measure levels of
cytokines/chemokines using an established Cytokine/Chemokine Panel Luminex Assay (Eve Technologies, Calgary, AB, Canada) corrected for urine creatinine concentration (117, 118). Immediately after collection, urine was centrifuged at 96 g for 15 min to remove cells, then separated into 1 ml portions and frozen at –80°C. One day before use, urine was thawed at 4°C. Due to the protocol for handling urine specimens, this analysis only included spot urine samples and not the timed collections that were obtained before the study visit. Furthermore, we only included analytes that either increase in response to hyperglycemia or that are associated with the highest tertile of normoalbuminuria in our previous work (117, 118). Our analysis therefore included eotaxin, fibroblast growth factor-2 (FGF-2), granulocyte–macrophage colony-stimulating factor (GM-CSF), IFNα2, IL-6, IL-12, monocyte chemoattractant protein-3 (MCP-3), macrophage-derived chemokine (MDC), macrophage inflammatory protein-1α (MIP-1α), TNF-β, sCD40 ligand (sCD40L), regulated on activation normal T cell expressed and secreted (RANTES), platelet-derived growth factor (PDGF)-AA, PDGF-AB/BB and monocyte chemoattractant protein-1 (MCP-1). We limited our analysis to these factors to maintain statistical power, to minimise false-positive results and to further elucidate mechanisms that may link high intraglomerular pressure with factors that may promote initiation of renal disease. The investigator performing data analysis was blinded to all study variables.

The accuracy and precision of the urinary cytokine/chemokine assay is available through the vendor at www.millipore.com/userguides/tech1 proto_mpxhcyto-60k (28 January 2013). The detection limits of our assays have been published previously (118).

Urinary albumin excretion rate was determined from a 24 h urine collection by immunoturbidimetry. HbA1c was measured by high-performance liquid chromatography.
Plasma cystatin C was also measured using previously described standard methods, and \( \text{GFR}_{\text{CYSTATIN C}} \) was then calculated using the MacIssac formula (126, 127).

### 3.3.5 Statistical methods

Between-group differences were determined by repeated measures ANOVA, corrected for multiple comparisons, with \( p=0.01 \) (SPSS v.14, Armonk, NY, USA). Our sample size calculation was based on differences in urinary MCP-1 excretion due to the consistent relationship between this cytokine and renal disease (128). Our previous data have shown that the SD for MCP-1 is 17 units (118). To have an 80% power to detect a significant 25-unit between-group difference in MCP-1 (129, 130), for a two-sided test with \( p=0.01 \) and \( Z_{\alpha} = 2.58 \), the sample size should be \( \geq 16 \) per group. Participants with diabetes were analysed on the basis of filtration status determined using inulin clearance as described above. Filtration status was determined at the end of the study once inulin assays were complete for the entire cohort. Between-group differences using \( \text{GFR}_{\text{CYSTATIN C}} \) were not assessed because of the expected underestimation of \( \text{GFR}_{\text{INULIN}} \) within the hyperfiltration range (126). As a consequence, only 7/28 hyperfilterers by \( \text{GFR}_{\text{INULIN}} \) could still be classified as hyperfilterers using \( \text{GFR}_{\text{CYSTATIN C}} \). Urinary cytokine/chemokine excretion was also correlated with \( \text{GFR}_{\text{INULIN}} \) and \( \text{GFR}_{\text{CYSTATIN C}} \) in the entire 49-member group of participants with diabetes using the Spearman correlation coefficient. The University Health Network (Toronto, Canada) Ethics Board approved the protocol and patients gave informed consent.
3.4 Results

3.4.1 Baseline characteristics

T1D-H and T1D-N participants were young, normotensive, normoalbuminuric men and women with suboptimal glycaemic control levels commonly observed in this age group (131) (Table 3). Participants with diabetes were otherwise similar to controls in terms of age, BMI, sex distribution, oestrogen levels and sodium and protein intake.

For baseline hemodynamic variables, as expected, baseline GFR was higher in T1D-H than in T1D-N and healthy control participants (p<0.01). ERPF and RBF were also higher and RVR was lower in T1D-H patients (p<0.01). Renal hemodynamic variables were similar in the control and T1D-N groups. Differences in blood pressure between the three groups were not significant.

3.4.2 Filtration status and urinary excretion of cytokines/chemokines

The urinary excretion of each cytokine/chemokine in each group is represented in Fig. 10, a–k. Compared with the T1D-N and C groups, the T1D-H group exhibited higher urinary eotaxin, IFNα2, MDC, PDGF-AA, PDGF-AB/BB and GM-CSF (Fig. 10, p<0.01). Urinary MCP-1 and RANTES levels were higher in the T1D-H group than in the T1D-N group (p<0.01); between-group interactions for T1D-H vs C were not significant. For FGF-2 and MCP-3, urinary excretion was elevated in the T1D-H group compared with C (Fig. 10, p<0.01), and sCD40L excretion was higher in both diabetic groups compared with C (Fig. 10). Between-group differences in IL-6 excretion for control (0.18±0.05 pmol/l) vs T1D-N (0.28±0.05) vs T1D-H (0.38±0.05) showed the same trend as the other factors but did not reach significance (p=0.09). Urinary IL-2, IL-12 and MIP1α excretion was not
detectable in >50% of samples, and these factors were therefore excluded from the analysis.

In the group of 49 participants with diabetes, GFR_{INULIN} correlated with urinary excretion levels of eotaxin (r=0.467, p=0.001), GM-CSF (r=0.355, p=0.013), MCP-1 (r=0.327, p=0.023), MCP-3 (r=0.298, p=0.04), MDC (r=0.345, p=0.016), PDGF-AB/BB (r=0.296, p=0.046), PDGF-AA (r=0.369, p=0.010), RANTES (r=0.381, p=0.008) and sCD40L (r=0.372, p=0.009). When urinary excretion levels of cytokines/chemokines were correlated with GFR_{CYSYATIN C} in the diabetic cohort, similar interactions were observed between GFR_{CYSYATIN C} and eotaxin, GM-CSF, MCP-1, MCP-3, MDC, PDGF-AA and sCD40L. In addition, GFR_{CYSYATIN C} correlated with FGF2 and TNF-β. In contrast with GFR_{INULIN}, the interactions between GFR_{CYSYATIN C} and RANTES and PDGF-AB/BB were not significant and IL-6 did not correlate with GFR_{INULIN} or GFR_{CYSYATIN C}. 
Table 3: Baseline Characteristics and Biochemistry in Type 1 adults. Reproduced with permission from (132).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Healthy controls ($n=18$)</th>
<th>Diabetes</th>
<th></th>
<th></th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Normofiltration group ($n=21$)</td>
<td>Hyperfiltration group ($n=28$)</td>
<td></td>
</tr>
<tr>
<td>Baseline demographic characteristic</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>23.2±1.2</td>
<td>23.5±1.1</td>
<td>24.6±2.5</td>
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</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>N/A</td>
<td>17.0±7.1</td>
<td>16.0±6.4</td>
<td></td>
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<tr>
<td>BMI (kg/m$^2$)</td>
<td>23.7±3.2</td>
<td>23.8±3.2</td>
<td>23.0±3.0</td>
<td></td>
</tr>
<tr>
<td>Women (%)</td>
<td>56%</td>
<td>52%</td>
<td>54%</td>
<td></td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>N/A</td>
<td>8.8±0.4</td>
<td>9.0±0.2</td>
<td></td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>N/A</td>
<td>72.7±4.9</td>
<td>74.9±4.1</td>
<td></td>
</tr>
<tr>
<td>Oestrogen (pmol/l) in women</td>
<td>314±109</td>
<td>180±34</td>
<td>200±37</td>
<td></td>
</tr>
<tr>
<td>Sodium excretion (mmol/24 h)</td>
<td>191±12</td>
<td>198±18</td>
<td>188±9</td>
<td></td>
</tr>
<tr>
<td>Protein intake (g kg$^{-1}$ day$^{-1}$)</td>
<td>0.93±0.12</td>
<td>1.01±0.15</td>
<td>0.94±0.13</td>
<td></td>
</tr>
<tr>
<td>Albumin excretion rate (mg/24 h)</td>
<td>25±1</td>
<td>19±3</td>
<td>24±3</td>
<td></td>
</tr>
<tr>
<td>Albumin-to-creatinine ratio (mg/mmol)</td>
<td>1.03±0.18</td>
<td>1.11±0.11</td>
<td>1.18±0.12</td>
<td></td>
</tr>
<tr>
<td>GFR$_{\text{CYSYATIN C}}$ (ml min$^{-1}$ 1.73 m$^{-2}$)</td>
<td>99±4</td>
<td>111±2</td>
<td>152±5*</td>
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</tr>
<tr>
<td>Baseline haemodynamic function</td>
<td></td>
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<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>109±3</td>
<td>113±2</td>
<td>108±3</td>
<td></td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>63±2</td>
<td>62±1</td>
<td>61±4</td>
<td></td>
</tr>
<tr>
<td>ERPF (ml min$^{-1}$ 1.73 m$^{-2}$)</td>
<td>641±39</td>
<td>625±25</td>
<td>807±30*</td>
<td></td>
</tr>
<tr>
<td>GFR (ml min$^{-1}$ 1.73 m$^{-2}$)</td>
<td>116±4</td>
<td>113±2</td>
<td>165±9*</td>
<td></td>
</tr>
<tr>
<td>Filtration fraction</td>
<td>0.18±0.01</td>
<td>0.18±0.01</td>
<td>0.21±0.02</td>
<td></td>
</tr>
<tr>
<td>RBF (ml min$^{-1}$ 1.73 m$^{-2}$)</td>
<td>989±60</td>
<td>988±47</td>
<td>1,246±56*</td>
<td></td>
</tr>
<tr>
<td>RVR (mmHg 1$^{-1}$ min$^{-1}$)</td>
<td>0.091±0.009</td>
<td>0.085±0.003</td>
<td>0.065±0.002*</td>
<td></td>
</tr>
</tbody>
</table>

Data are means±SD

A 24 h urine collection was used to evaluate dietary adherence through the determination of urinary sodium and urea excretion. Protein intake was calculated from the formula protein = [(urine urea excretion × 0.18)+14]/weight. RBF was derived using ERPF/(1-haematocrit) and RVR was derived by mean arterial pressure/renal blood flow. Only 7/28 participants classified as T1D-H by inulin remained hyperfilterers when GFR was assessed using cystatin C. GFR$_{\text{CYSYATIN C}}$ was therefore calculated in 7 hyperfilterers and 42 normofilterers. *p<0.01, T1D-H vs healthy control group.
**Figure 10:** Urinary Excretion of Cytokines/Chemokines in Adults with Type 1 Diabetes Mellitus. *p values for T1D-H vs healthy controls: eotaxin p=0.003; IFNα2 p=0.006; MDC p<0.0001; FGF2 p=0.007; PDGF-AA p=0.007; PDGF-AB/BB p=0.001; sCD40L p<0.001; GM-CSF p<0.0001; MCP-3 p=0.001. †p values for T1D-H vs T1D-N: eotaxin p=0.003; IFNα2 p=0.001; MDC p=0.0001; PDGF-AA p=0.01; PDGF-AB/BB p=0.003; RANTES p=0.001; GM-CSF p=0.007 MCP-1 p=0.007. Reproduced with permission from (132).
3.5 Discussion:

Experimental data have suggested the association of high intraglomerular pressure with increased shear stress in diabetes, leading to renal injury (87, 113, 114). The pathogenesis of hyperfiltration remains controversial; both hemodynamic (the ‘hemodynamic hypothesis’) and tubuloglomerular feedback mechanisms (the ‘tubular hypothesis’) have been implicated (55). Animal studies of type 1 diabetes and translational physiology experiments in humans have associated hyperfiltration with lower levels of RVR (119-121, 133-135). This is likely to be based on: (1) hyperglycemia-induced increases in nitric oxide and vasodilatory prostanoid bioactivity in the pre-glomerular, afferent renal circulation (55, 119, 121); (2) changes in tubuloglomerular feedback, due to increased sodium–glucose co-transport at the proximal tubule. Increased proximal tubular sodium reabsorption decreases distal delivery to the macula densa, resulting in afferent vasodilatation (136). Since hyperfiltration, used as a surrogate for intraglomerular pressure in humans, also contributes to the initiation of diabetic nephropathy in some but not all studies (57, 137), we hypothesised that T1D-H patients would also exhibit increased urinary excretion of inflammatory mediators that have been linked with kidney injury. We included a panel of analytes that either increase acutely in response to clamped hyperglycemia or that are associated with the highest tertile of normoalbuminuria in young type 1 diabetes patients (117, 118).

In experimental models of diabetes, hyperfiltration is associated with increased renal tissue cytokine/chemokine mRNA expression (IFN, MCP-1) (115, 138, 139). In animal models in which diabetes is induced by streptozotocin, factors that reduce glomerular hypertrophy and hyperfiltration, such as anti-angiogenic factors, also reduce
renal mRNA expression of TNF, IL-6 and MCP-1 (140). Our major finding in this pilot study was that renal hyperfiltration is also associated with increased urinary cytokine/chemokine excretion in humans with uncomplicated type 1 diabetes. To our knowledge, this is the first time that hyperfiltration, measured using gold-standard inulin clearance techniques, has been linked with renal inflammation in humans. Although, we could not perform between-group comparisons based on filtration status using GFR_{CYSTATIN C}, due to the small numbers of hyperfiltering patients defined by cystatin C criteria, GFR_{CYSTATIN C} also correlated with urinary cytokine/chemokine excretion in participants with diabetes. Previous work in patients with type 1 diabetes has demonstrated that urinary inflammatory mediators correlate with GFR loss and with increased urinary albumin excretion, even when the albumin-to-creatinine ratio values are within the normal range (118, 141). Since hyperfiltration is associated with diabetic nephropathy and occurs early in the natural history of diabetes before the onset of microalbuminuria, our results may identify a potentially high-risk group that can benefit from earlier renoprotective therapies.

Our findings are important from a therapeutic perspective because urine cytokines/chemokines can be influenced by renoprotective medications such as ACE-inhibitors in humans (141-146). Urine cytokines/chemokines may therefore act as future therapeutic targets for novel or existing medications and can be used by clinicians to monitor treatment failure or success. The identification of new biomarkers of renal disease initiation and progression is critical due to the widely recognised limitations of albuminuria as a clinical outcome measure (147).

Previous work has linked diabetes with renal inflammation, possibly through effects of chronic hyperglycemia on intraglomerular pressure (87, 113, 114). We studied patients
under clamped euglycemic conditions and using a controlled dietary preparation to isolate
the effect of filtration status without the influence of acute hyperglycemia, renal
dysfunction or albuminuria (117). Under these conditions, urinary factors that are
associated with either chemotaxis or inflammation/fibrosis were elevated in T1D-H (86,
148, 149). Our results suggest that high intraglomerular pressure may provide a mechanistic
link between factors that cause hyperfiltration, such as hyperglycemia-induced renin
angiotensin system activation (150), and renal inflammation, leading to the onset of clinical
disease.

Although we cannot determine from the present study whether urinary excretion of
cytokines/chemokines was the result of renal tubular secretion or spill-over of systemic
proteins, these preliminary observations may give mechanistic insights into disease
pathogenesis in hyperfiltering humans at a pre-clinical stage of the disease. In light of the
emerging role for cystatin C as a clinical indicator of hyperfiltration and the association
between GFR\textsubscript{CYSYATIN} and urinary cytokines/chemokines, our results suggest that these
factors may be used to monitor the biological effects of therapeutic interventions in high-
 risk patients with type 1 diabetes in future clinical trials (127). Unfortunately, cystatin C
and other indirect GFR measurements, such as creatinine-based estimates, are not yet
accurate or precise enough to study hyperfiltration in large cohorts. As described elsewhere,
the large degree of bias associated with indirect GFR measurements, such as cystatin C,
may account in part for inconsistencies in the literature surrounding the possible role of
hyperfiltration in the progression of renal disease (127, 137). Consistent with our previous
observations suggesting that GFR\textsubscript{CYSYATIN} underestimates GFR\textsubscript{INULIN}, only 7/28 T1D-H
by GFR\textsubscript{INULIN} were still classified as hyperfilterers using GFR\textsubscript{CYSYATIN}, making between-
group comparisons unreliable. Nevertheless, the identification of early ‘pre-clinical’ risk biomarkers, including urinary cytokines/chemokines, in a larger cohort is now more vital than ever given the failure of primary renal prevention strategies using conventional agents, such as renin angiotensin system inhibitors, in an important subset of patients with diabetes (27). Furthermore, recent natural history studies of human diabetic nephropathy have demonstrated that older paradigms involving progression from normoalbuminuria to microalbuminuria to macroalbuminuria to impaired renal function and end-stage renal disease are not accurate in many patients (151). For example, some patients have significant initial deterioration of renal function without proteinuria whereas, in others, microalbuminuria regresses spontaneously (152). The elucidation of novel, non-albuminuria biomarkers is therefore important for the early identification and potential risk-stratification of patients with diabetes.

We attempted to minimise the effect of the small sample size in our study by using homogeneous study groups and by using a careful pre-study preparation phase with a focus on known factors that influence neurohormonal activation, including dietary sodium intake (153). Nevertheless, the small sample size may have limited our ability to detect between-group differences in urinary IL-6 excretion. Furthermore, because we did not study participants under conditions of low dietary sodium intake, we were unable to determine the effect of dietary sodium intake on urine cytokines/chemokines. We also scheduled studies in female participants to coincide with the early follicular phase of the menstrual cycle to avoid confounding effects of oestrogen on vascular function. Although plasma oestrogen levels were higher than expected and more consistent with the mid-to-late follicular phase, oestrogen concentrations were similar in the three groups studied. Finally,
while it is presumed that hyperfiltration may have induced the increase in cytokines/chemokines and thus ultimate renal injury, it is also possible that other factors (i.e. neurohormonal activation, tubuloglomerular feedback) that maintain persistent hyperfiltration during euglycemia may also be responsible for the increase urinary cytokine/chemokine excretion. It is therefore important for future studies to clarify the time course and reversibility of urinary biomarkers using existing and investigational agents. Future work should also determine whether urinary cytokine/chemokine excretion in the T1D-H group is systemically derived (i.e. ‘spill-over’) or due to renal production.

In conclusion, similar to previous findings in animal models of diabetes, renal hyperfiltration during clamped euglycemia was associated with increased urinary excretion of cytokines/chemokines in patients with uncomplicated type 1 diabetes.
The urinary cytokine/chemokine signature of renal hyperfiltration in adolescents with type 1 diabetes


Manuscript in Submission
4.1 Abstract:

Objective: Urinary cytokine/chemokine levels are elevated in adults with type 1 diabetes (T1D) exhibiting renal hyperfiltration. Whether this observation extends to adolescents with T1D remains unknown. Our first objective was to determine the relationship between hyperfiltration and urinary cytokines/chemokines in normotensive, normoalbuminuric adolescents with T1D using GFR\textsubscript{cystatin}. Our second aim was to determine the relationship between urine and plasma levels of inflammatory biomarkers, to clarify the origin of these factors.

Methods: Urine and serum cytokines/chemokines (Luminex platform) and GFR\textsubscript{cystatin} were measured in normofiltering (n=111, T1D-N, GFR<135 ml/min/1.73m\textsuperscript{2}) and hyperfiltering (n=31, T1D-H, GFR\geq135 ml/min/1.73m\textsuperscript{2}) adolescents with T1D (ages 10-16), and in age and sex matched healthy control subjects (HC, n=59).

Results: We noted significant step-wise increases in urinary cytokine/chemokine excretion according to filtration status with highest levels in T1D-H, with parallel trends in serum analyte concentrations. After adjusting for serum glucose at the time of sampling, differences in urinary cytokine excretion were not statistically significant. Only serum IL-2 significantly differed between HC and T1D (p=0.0076).

Conclusions: Hyperfiltration is associated with increased urinary cytokine/chemokine excretion in T1D adolescents, and parallel trends in serum cytokine concentration. The GFR-associated trends in cytokine excretion may be driven by the effects of ambient glycemia. The relationship between hyperfiltration, glycemia, and
variations in serum and urine cytokine expression and their impact on future renal and systemic vascular complications requires further study.
4.2 Introduction:

Diabetes mellitus is the most common cause of end-stage renal failure requiring renal placement therapy in the developed world. Unfortunately, despite the use of renin-angiotensin-aldosterone system (RAAS) blockers and intensive glycemic control, a significant proportion of patients continue to develop diabetic nephropathy. Furthermore, in type 1 diabetes (T1D), RAAS inhibition-based primary prevention of diabetic nephropathy, defined by changes on renal biopsy, has been unsuccessful (27). The failure of current therapies may in part be due to a clinical inability to distinguish high-risk patients from those who may never develop complications. It is therefore critical to identify the role of additional factors that contribute to the initiation and progression of diabetic nephropathy to guide more targeted treatment strategies.

Hyperglycemia is necessary for the development of diabetic nephropathy in experimental models and in humans (53, 55, 57). In young adult patients with T1D, acute clamped hyperglycemia increases the urinary excretion of pro-inflammatory and profibrotic factors implicated in the pathogenesis of diabetic nephropathy, including eotaxin, fibroblast growth factor-2, granulocyte-monocyte colony stimulating factor (GM-CSF), interferon-α2, interleukin (IL)-12, IL-2, monocyte chemoattractant protein-3 (MCP-3), MCP-1, macrophage-derived chemokine (MDC), macrophage inflammatory proteins-1α (MIP-1α), platelet derived growth factor-BB (PDGF-AB/BB), tumour necrosis factor-β and sCD40 Ligand (sCD40L) (117). We have further shown that renal hyperfiltration is associated with higher levels of urinary cytokines/chemokines compared with T1D patients with normal GFR values (T1D-N) and healthy controls (HC) (132). Moreover, the increase in urinary cytokine/chemokine excretion induced by hyperglycemia is blunted by RAAS
inhibition, and this effect is exaggerated in patients with T1D and renal hyperfiltration (T1D-H) (154). However, GFR in this previous work was measured directly by inulin clearances under clamped glycemic conditions, and these techniques can only be used in controlled research laboratory setting. Therefore, this study investigated if renal hyperfiltration, defined with clinically applicable cystatin C-based methods, is also associated with increased urinary cytokine/chemokine excretion in an ambulatory setting (127). Although we have previously noted the limitations of cystatin C, exogenous markers (i.e. inulin, iohexol) were unfeasible in this study due to their associated complexity and the cost. Moreover, cystatin C methods are considered to be a potential replacement for estimating GFR in a clinical setting, less subjected to the effects of age, sex and race, and consistently shown to be a better marker than creatinine (155, 156). Furthermore, cystatin C methods used in this study has been validated in the pediatric population (157).

The identification of urinary biomarkers of preclinical kidney disease that could be used clinically to distinguish adolescents with T1D at increased risk of developing renal disease is an important research goal for clinicians who take care of similar patients in the pediatric setting and as these patients transition to adult care (118). Accordingly, our aim was to determine the relationship between GFR and urinary cytokines/chemokines in normotensive, normoalbuminuric adolescents with T1D and normal renal function. We hypothesized that in an adolescent cohort, urinary cytokines/chemokines would be elevated in patients with T1D-H compared to both T1D-N and a similar group of age and sex matched healthy control participants.
4.3 Research Design and Methods:

Patients were recruited from the longitudinal, observational, non-interventional arm of the Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial (AdDIT), from clinical sites in the Greater Toronto Area. In brief, the Non-Randomized Low-Risk arm of AdDIT is a 4-year observational/natural history study, following adolescents at low and middle risk of developing microalbuminuria (EudraCT Number: 2007-001039-72).

High risk adolescents are recruited into the AdDIT Interventional Study (http://www.clinicaltrials.gov/ct2/show/NCT01581476), which was designed to examine the effect of angiotensin converting enzyme inhibitors and statins on renal, retinal and cardiovascular endpoints. Our study did not include participants involved in the intervention trial, however, as an ancillary component of the Non-Randomized Low-Risk arm of AdDIT we also included high risk subjects who chose not to enter the AdDIT Intervention Study. All analyses in this manuscript were performed using biological specimens and data collected from subjects enrolled in the observational and ancillary arm of AdDIT, specifically baseline data from the study that were obtained at Greater Toronto Area sites.

Normotensive, normoalbuminuric participants with T1D were recruited. T1D patients were analyzed based on whether GFR was in the normal range (n=111, GFR 90-134 ml/min/1.73m²) or hyperfiltration range (n=31, GFR≥135 ml/min/1.73m²) according to Larsson method, as we have previously published (126, 127). Fifty-nine healthy controls with normal renal function were also included for comparison.

Inclusion criteria for T1D patients were: age 10-16, duration of type 1 diabetes ≥1 year, no history of hypertension, proteinuria, renal disease or macrovascular disease. Two
sets of 3 early morning urines were obtained and microalbuminuria was defined as an ACR >3.5 mg/mmol in males and > 4.0 mg/mmol in females in 2 out of the 3 consecutive early morning urines, according to published methods in the Oxford Regional Prospective Study (158, 159). In addition to those delineated in the AdDIT observational protocol, other exclusion criteria included chronic inflammatory disease, anti-inflammatory or corticosteroid medicines or medications that interfere with the renin-angiotensin-aldosterone system (RAAS) (160).

T1D patients were recruited from endocrinology clinics at The Hospital for Sick Children, Credit Valley Hospital and Markham-Stouffville in the Greater Toronto Area (Ontario, Canada), while healthy controls were recruited through local advertisements. The local Research Ethics Board at participating hospitals approved the protocol and all subjects gave informed consent.

4.3.1 Sample Collection and Analytical Methods:

Participants were asked to provide a first morning urine sample. This 50 ml mid-stream sterile urine specimen was used to measure levels of cytokines/chemokines using an established Cytokine/Chemokine Panel Luminex Assay (117, 118). Immediately after collection, urine was centrifuged at 1500 g for 15 minutes to remove cells, then separated into 1 ml aliquots and frozen at -80°C. Urine was then thawed at 4°C one day prior to use. Due to the urine specimen handling protocol designed to avoid protein degradation, this analysis included spot urines and not the timed collections. Furthermore, a priori we only included selected analytes that either increase in response to hyperglycemia or renal
hyperfiltration in our previous work in young adults (117, 132). Our analysis therefore included eotaxin, FGF-2, GM-CSF, IFNα2, IL-2, IL-12, MCP-3, MCP-1, MDC, MIP-1α, TNF-β, sCD40L, PDGF-AB/BB. We limited our analysis to these factors to maintain statistical power, to minimize false positive results, and to further elucidate mechanisms that may link high intraglomerular pressure with factors that promote initiation of renal disease. The urine analytes were adjusted to urine creatinine concentration to correct for dilution variability. The accuracy and precision of the urinary cytokine/chemokine assay is available through the vendor at http://www.millipore.com/userguides/tech1/proto_mp_xhcyto-60k. The detection limits of our assays have also been published previously (118). The investigator performing data analysis was blinded to all study parameters.

Serum cystatin C was measured by a single operator using thawed samples by an immunoassay (Dade Behring Diagnostics, Newark, DE, USA) conducted on a BN Prospec System nephelometer. The between-assay coefficient of variation in samples from the lowest and highest quartiles of the cystatin C distribution was 6.2 and 0.9%, respectively. Cystatin C based GFR was derived using the body-surface area corrected Larsson formula, as described previously, which has superior operating characteristics compared with creatinine-based measurements in the hyperfiltration range (126, 157).

Urinary albumin to creatinine ratio was determined from a spot urine collection by immunoturbidimetry. Hemoglobin A1C (HbA1c) was measured by high-performance liquid chromatography (125).
4.3.2 Statistical analysis

All analyses in the manuscript were based on specimens and data collected at the baseline visit. Descriptive statistics were used to describe the sample. The baseline clinical and demographic characteristics were compared using appropriate test statistics. Between-group differences were determined by ANOVA, adjusted for multiple comparisons using Bonferroni’s correction. The statistical package SAS 9.3 (SAS Institute, Cary, NC, USA) was used to analyze the data. Our sample size calculation was based on differences in urinary MCP-1 excretion due to the consistent relationship between this cytokine and renal disease (117, 118, 132). Our previous data have shown that the SD for MCP-1 is 17 units (117, 118, 132). To have an 80% power to detect a significant 25 unit between-group difference in MCP-1, for a two-sided test with \( p = 0.01 \), the sample size should be at minimum \( \geq 16 \) per group. Participants with diabetes were analyzed on the basis of filtration status determined using cystatin C as described previously (126, 127). Filtration status was determined at the end of the study once cystatin C and creatinine assays were complete for the entire cohort. In the first analysis, *between-group* comparisons were adjusted for age, gender, ACR and HbA1c. In the second analysis, blood glucose at the time of the urine sample collection was included rather than HbA1c, since our previous work has demonstrated that acute, ambient glycemia increases urinary cytokine/chemokine excretion (117, 154). Serum levels of cytokines/chemokines were similarly analyzed using both dichotomous and continuous methods, except that ACR was not included.
4.4 Results:

4.4.1 Baseline characteristics:

Healthy control and T1D participants were generally similar in terms of baseline demographic and biochemical parameters (Table 4). For T1D-N and T1D-H, fasting blood glucose, HbA1c and diabetes duration values were similar.

4.4.2 The effect of renal filtration status on urinary cytokine/chemokine excretion:

After adjusting for baseline clinical characteristics including HbA1c and ACR, the first pattern observed was a step-wise increase from HC to T1D-N to T1D-H for urinary IL-12 (Figure 11, panel a, ANOVA p=0.0005). Pair-wise comparisons were also significant except for HC vs. T1D-N (p=0.0518). For IFNα2 (Figure 11, panel b, ANOVA p=0.0019), pair-wise differences between HC vs. T1D-H and T1D-N vs. T1D-H were significant, while differences in HC vs. T1D-N were not. Finally, levels of IL-2 (ANOVA p=0.0002), sCD40L (ANOVA p=0.001), FGF-2 (ANOVA p=0.0038) (Figure 11, panels c-e), generally increased from HC to T1D-N to T1D-H, but only pairwise differences for HC vs. T1D-N and HC vs. T1D-H reached significance. For TNF-β (ANOVA p=0.0097) and MIP-1α (ANOVA p=0.0174) (Figure 11, panel f-g), only HC and T1D-H group differences were significant. Similar trends for MDC, MCP-3 and GM-CSF did not reach significance.

After adjusting for the same baseline clinical characteristics including plasma glucose rather than HbA1c, between group differences for IL-12 and IFNα2 were no longer significant.
4.4.3 Correlation between urinary cytokines/chemokines and renal function, adjusted for age, gender, ACR and hemoglobin A1c

For HC, only PDGF-AB/BB (β=0.0433, p=0.0019) correlated with GFRcystatin C. In the T1D group, GFRcystatin C correlated with MCP-1 (β=0.3189, p=0.0162) and PDGF-AB/BB (β=0.0231, p=0.0331).

4.4.4 Serum levels of cytokines/chemokines in HC and T1D Groups

For serum markers, between-group differences for IL-2 in HC vs. T1D-N and T1D-H reached significance (Figure 12). In the continuous analysis comparing serum analyte levels with GFR in the HC group, serum MIP-1α (β = -0.2752, p=0.0384), MDC (β = -12.702, p=0.0061), IL-12 (β = -2.1089, p=0.0237) correlated with GFRcystatin C. In the T1D cohort, correlations were also observed between serum IL-12 (β = 4.0269, p=0.0029), IFNα2 (β = 15.8123, p=0.0015), FGF-2 (β = 2.1275, p=0.0032), TNF-β (β = 21.7215, p=0.0028), MDC (β = 20.3889, p=0.0018), GM-CSF (β = 1.1626, p=0.0029), PDGF-AB/BB (β = 20.0651, p=0.0232) and GFRcystatin C.

4.4.5 Correlation between urine and serum levels of cytokines/chemokines in HC and T1D participants

In HC, none of the urine cytokines/chemokines correlated with corresponding cytokines/chemokines in serum. In the T1D group, correlations between urinary and plasma levels of eotaxin (r=0.20, p=0.02), sCD40L (r=0.22, p=0.009) and GM-CSF (r=0.23, p=0.0062) reached statistical significance.
Table 4: Clinical Characteristics and Biochemistry in Healthy Controls and in Patients with Type 1 Diabetes Mellitus with Normofiltration or Hyperfiltration According to GFR\textsubscript{cystatin C} (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Healthy Controls (n=59)</th>
<th>Normofiltration diabetic group (n=111)</th>
<th>Hyperfiltration diabetic group (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clinical characteristics:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male / female</td>
<td>26 / 33</td>
<td>63 / 48</td>
<td>9 / 22</td>
</tr>
<tr>
<td>Age (years)</td>
<td>13.9±2.0</td>
<td>14.5±1.6</td>
<td>15.0±1.6</td>
</tr>
<tr>
<td>Diabetes duration (years)</td>
<td>N/A</td>
<td>7.4±3.1</td>
<td>7.3±3.3</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>54.2±13.0</td>
<td>62.2±14.1</td>
<td>60.4±16.13</td>
</tr>
<tr>
<td>Height (cm)</td>
<td>161.5±11.5</td>
<td>165.1±9.6</td>
<td>160.1±9.6</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>111±10</td>
<td>116±11</td>
<td>114±7</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>68±7</td>
<td>67±7</td>
<td>70±7</td>
</tr>
<tr>
<td>Pulse (beats per minute)</td>
<td>49±6.5</td>
<td>52±9</td>
<td>49±8</td>
</tr>
<tr>
<td><strong>Biochemistry:</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood glucose at the time of urine collection (mmol/L)</td>
<td>4.65±0.74</td>
<td>9.28±4.49</td>
<td>11.47±3.35</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>5.4±0.3</td>
<td>8.4±1.2</td>
<td>8.5±1.1</td>
</tr>
<tr>
<td>HbA1c (mmol/mol)</td>
<td>31±0.06</td>
<td>75±0.15</td>
<td>75±0.15</td>
</tr>
<tr>
<td>Urine albumin / creatinine ratio (mg/mmol)</td>
<td>0.60±0.45</td>
<td>1.14±2.46</td>
<td>1.17±1.36</td>
</tr>
<tr>
<td>Serum LDL (mmol/L)</td>
<td>2.38±0.76</td>
<td>2.29±0.76</td>
<td>2.24±0.51</td>
</tr>
<tr>
<td>Serum triglyceride (mmol/L)</td>
<td>0.92±0.40</td>
<td>0.84±0.40</td>
<td>0.85±0.26</td>
</tr>
</tbody>
</table>
Figure 11: Urinary Excretion of Cytokine/Chemokines in Adolescents with Type 1 Diabetes According to Hyperfiltration Status vs Healthy Controls. Step-wise trends were observed for IL-12, IFNa2, IL-2, sCD40L, FGF-2, TNF-β, MIP-1α, MDC, MCP-3, GM-CSF, adjusted for age, gender, ACR and HbA1c. p-values show pair-wise comparisons with Bonferroni correction. After adjusting for plasma glucose at the time of collection, instead of HbA1c, pair-wise comparisons between normofilterers (T1D-N) and hyperfilterers (T1D-H) were no longer significant.
Figure 12: Serum Cytokine/Chemokine Signature in Adolescents with Type 1 Diabetes Based on Hyperfiltration Status and Healthy Controls. Parallel trends to urinary excretion of cytokine/chemokines were observed although only IL-2 showed significance. p-values show pairwise comparisons with Bonferroni correction. Adjusted for age, gender and HbA1c.
4.5 Discussion:

Albuminuria is an early clinical marker of diabetic nephropathy. Unfortunately, albuminuria is limited as a predictive biomarker because many patients exhibit stable levels of albuminuria and never develop impaired renal function, and spontaneous regression of albuminuria is also common (161). Furthermore, progressive early GFR decline without albuminuria is common (162) and may reflect underlying vascular disease and tubulointerstitial inflammation rather than traditional diabetic glomerulosclerosis (141, 152). The identification of sensitive and specific early biomarkers to predict the development of nephropathy prior to the onset of albuminuria, such as urinary cytokines/chemokines, is therefore of the utmost importance.

Urinary cytokine/chemokine excretion is associated with factors that promote diabetic nephropathy, such as hyperglycemia, as well as hyperfiltration, a surrogate marker for high intraglomerular pressure leading to glomerulosclerosis (117, 132). Despite the promising pathophysiological rationale for the use of cytokines/chemokines as markers of early renal disease, the role of these factors as markers of diabetic nephropathy prior to the onset of albuminuria remains unclear, especially in adolescents (110). Our aim was therefore to determine the relationship between GFR and urinary cytokines/chemokines in a cohort of normotensive, normoalbuminuric adolescents with T1D analyzed on the basis of renal filtration status, since hyperfiltration may identify a group of individuals who are at an increased risk of developing clinical nephropathy in T1D and T2D (53, 57).

Our first major finding was that renal hyperfiltration was associated with higher levels of urinary cytokines/chemokines in T1D adolescents prior to the development of microalbuminuria. There were also parallel trends in serum cytokine concentrations. This
panel of biomarkers was selected due to associations between these factors with 1) renal hyperfiltration and acute responses to clamped hyperglycemia and 2) renal injury leading to diabetic nephropathy, including chemotaxis, inflammation and fibrosis (117, 132) and 3) hyperglycemia-related increases in urinary excretion of cytokine/chemokines, including IFN, PDGF, TNF and MCP-1 (92, 163, 164), leading to chronic kidney disease (165-169). In contrast with our previous work in adults using gold standard inulin clearance techniques to measure GFR under clamped euglycemic and hyperglycemic conditions, we used cystatin C-based measurements, since this estimate of GFR can be used clinically and has superior operating characteristics when compared to creatinine-based estimates within the hyperfiltration range(126, 127, 170).

To account for the influence of ambient hyperglycemia at the time of GFR measurement on urinary cytokines/chemokines, we performed analyses with either HbA1c or plasma glucose as covariables (117). Ambient plasma glucose concentration was an important determinant of urinary cytokine/chemokine excretion. Adjustment for serum glucose at the time of sampling mitigated the observed differences in cytokine excretion. This observation supports the hypothesis that ambient hyperglycemia is an independent determinant of urinary inflammatory biomarkers, and should be accounted for in future work. Chronic glycemic control, reflected by HbA1C, did not have the same confounding influence on cytokine excretion. This suggests that perhaps transient glycemia is more important than chronic glycemic status in determining acute variations in cytokine/chemokine production.

Renal hyperfiltration has been attributed to hyperglycemia-mediated activation of the RAAS causing efferent renal arteriolar vasoconstriction, and also to diabetes related
increases in proximal tubular sodium-glucose cotransport, leading to altered tubuloglomerular feedback and afferent arteriolar vasodilatation (81, 150). Regardless of the responsible mechanism, hyperfiltration is associated with the initiation and progression of nephropathy in T1D and T2D, and is present in 20-60% of young patients with T1D (53, 57, 171). Mechanistically, high intraglomerular pressure is associated with hyperfiltration and increased wall tension in experimental diabetes, promoting renal parenchymal inflammation (172, 173). To determine if a similar relationship exists in humans, we previously compared urinary cytokine/chemokine excretion in adult patients with T1D with or without hyperfiltration defined by inulin-based clearances under controlled laboratory conditions (117, 132). We demonstrated that T1D-H exhibit higher urinary cytokines/chemokines levels vs. T1D-N and HC (132). In a separate study we demonstrated that clamped hyperglycemia, a stimulus for hyperfiltration, also increases urinary cytokine/chemokine excretion (117). To determine if hyperfiltration-related increases in urinary cytokines/chemokines are reversible, we examined the effect of RAAS blockade on these factors and demonstrated 1) a decline in many of these mediators and 2) that these effects were exaggerated in hyperfilterers (154). It was, however, unclear if these observations were applicable outside of a controlled laboratory setting, and if our observations would extend to an adolescent cohort. To our knowledge, the present study is the first to examine the interaction between renal hyperfiltration, the earliest known hemodynamic abnormality related to the development of diabetic nephropathy, and urinary markers of inflammation in adolescents in a clinical setting. These results therefore confirm our previous work in adults with T1D, and suggest that inflammation associated with
hyperfiltration begins much earlier in the natural history of the disease, potentially identifying an opportunity for future primary prevention strategies.

To determine whether the increase in urinary cytokines/chemokines in T1D-H patients is due to high systemic levels resulting in renal “overflow” or instead due to more local production, we also measured serum levels of each factor. Our second major finding was that in contrast with many between-group differences for urinary factors, serum levels of IL-2 differed between the three groups, with highest levels in T1D-H. Although by no means definitive, this observation suggests an interaction between systemic and renal levels of IL-2, possibly reflecting systemic production and consequent renal clearance. Interestingly, urinary levels of IL-12, IFNα2, FGF-2 and TNF-β tended to increase from HC to T1D-N to T1D-H, and serum levels of these factors correlated with GFR, again suggesting a relationship between systemic and renal levels of these factors. Even in the cases of MDC and GM-CSF where between-group differences for urinary levels were not significant, serum levels of these factors still tended to follow the same trend, with a positive correlation with GFR. We and others have previously observed systemic hemodynamic abnormalities in patients with type 1 diabetes and hyperfiltration, including endothelial dysfunction and higher blood pressure, suggesting that hyperfiltration reflects a generalized abnormality of the endothelium and vasculature rather than an isolated renal abnormality (119, 174, 175). Therefore, our findings suggest that at least for IL-2, IL-12, IFNα2, FGF-2 and TNF-β higher urinary excretion rates in T1D-H may have been on the basis of increased clearance from the systemic circulation rather than renal production and subsequent urinary excretion.
In addition to increased risk of developing albuminuria and GFR loss in clinical trials (53, 57), T1D patients with hyperfiltration including adolescents exhibit greater hemodynamic responses to ACE inhibition, reflected by declines in GFR toward the normal range (150). We have also shown that T1D-H exhibit similar effects when tubuloglomerular feedback is activated using the sodium glucose cotransport inhibitor empagliflozin (81). Finally, RAAS blockade results in greater urinary cytokine/chemokine suppression in T1D-H versus T1D-N (154). Thus, it is possible that neurohormonal activation, TGF endothelial dysfunction, mechanical oxidative stress may play additional roles in cytokine/chemokine expression. Hyperfiltration therefore represents a distinct physiological state that identifies a subgroup of patients who may be at an increased risk of diabetic nephropathy, and who also exhibit greater hemodynamic and molecular responses to potential renal protective agents. Regardless of the source and the molecular mechanisms that produce cytokines/chemokines in the present study cohort, our results suggest that T1D-H patients generally exhibit higher levels of factors that have been linked with renal and cardiovascular injury. As such, patients with T1D-related hyperfiltration may therefore represent a high-risk group that should be targeted for earlier therapeutic interventions in future studies.

Our study has some important limitations. First, we were not able to study this large cohort under clamped glycemic conditions. We have demonstrated in previous work that strictly controlled physiologic environment that transient hyperglycemia significantly influences cytokine/chemokine levels. We therefore intentionally sought to study this in a more realistic clinical setting, and accounted for glycemia a priori with our analytic approach. A second limitation was the use of GFR based on estimating equations rather
than direct GFR measures such as inulin. Nevertheless, use of GFR$_{\text{cystatin C}}$ to define hyperfiltration has provided important insights into how urinary cytokine/chemokine excretion rates may be translated into the clinical setting in future work (127). Finally, in this analysis, we hypothesized that between-group differences in urinary cytokines/chemokines were primarily on the basis of systemic overflow, and that increased renal production was also possible. We recognize, however, that children with T1D commonly exhibit evidence of proximal tubular dysfunction (176, 177) and we have recently reported that proximal tubular function may be different in T1D-H vs. T1D-N (81). As such, future work should determine if physiological differences in proximal tubule function, rather than activation of inflammatory pathways, contributes to abnormalities in urinary cytokine/chemokine handling, resulting in step-wise changes from HC to T1D-N to T1D-H observed in this study.

In conclusion, hyperfiltration in adolescents with T1D is associated with higher levels of urinary cytokine/chemokine excretion, an effect that is in part dependent on ambient blood glucose levels. Future work is required to determine if high urinary cytokine/chemokine excretion rates are associated with early renal function decline or the onset of proteinuria. Future studies should also determine if suppression of urinary cytokines/chemokines with RAAS inhibition as observed in physiology studies can also be achieved in a clinical setting and if declines in these factors correlate with improved clinical outcomes (132).
5 Conclusions and Future Directions

The underlying pathological mechanisms responsible for DN are complex and involve both hemodynamic and non-hemodynamic pathways, including the activation of pro-inflammatory molecules, which contribute to renal injury. Increased cytokine levels have been shown to be associated with hyperglycemia (118) and severity of albuminuria (110). In the current sets of experiments, renal hyperfiltration, a distinct physiological phenotype and one of the earliest known risk factors for DN, is associated with increased urinary cytokine/chemokine excretion levels, which may be systemically derived. Since hyperfiltration lies on the causal pathway between neurohormonal activation and tissue inflammation, at least in the kidney, these findings support the concept that hyperfiltration is a central pathogenic factor that should be targeted in patients with early DM, perhaps even in those with pre-clinical nephropathy. Thus, long-term follow-up of young adults and adolescents in the current sets of experiments would further elucidate whether this “pro-inflammatory” profile evolves with aging, longer diabetic duration and the advancement of DN.

In the two described studies, only a subset of cytokines was investigated. However, there are more than a hundred of cytokines that are currently known to exist (88). Thus, there exists the possibility of carefully characterizing the “network” of these cytokines using bioinformatics and eventually, the activated signaling pathways responsible for DN. Studies with RAAS blockade, including those from our laboratory, have previously shown that cytokine levels were lowered in response to treatment (86, 178), suggesting that pharmacotherapy can alter the cytokine/chemokine “signature” associated with DM.
Further work is required to discover if other agents that ameliorate hyperfiltration, such as SGLT2 inhibition, can be safely combined with RAAS inhibitors, and to determine if this can further reduce cytokine levels. In other areas of medicine, blockade of cytokine signaling has shown promising results as therapeutic agents, such as the use of TNF inhibitors to treat autoimmune rheumatoid arthritis (88). Thus, similarly in DM, targeted blockade of relevant cytokine signaling pathways may be a novel strategy of DN therapy by reducing subclinical inflammation.

Currently, albuminuria lacks sensitivity and is a one dimensional biomarker of early renal injury. In contrast, cytokines, as previously mentioned, are dynamic and involved in numerous cellular processes. Some of these processes, such as macrophage infiltration, apoptosis and fibrosis, are upregulated in early DN (110, 111). Cytokines may therefore prove to be valuable clinical biomarkers that could be used to further identify those who are at the greatest risk of developing DN (179). In the future, introducing these novel biomarkers, such as cytokines and protein mass-spectrometry, may be analogous to adding an instrument in a toolbox. For example, cytokine assays in conjunction with early clinical markers, such as hyperfiltration, may help clinicians accurately predict those patients who are at the greatest risk of developing renal function decline prior to developing albuminuria and importantly, without the need for invasive techniques such as renal biopsies. Nevertheless, such composite early risk indices will require long-term refinement and validation studies. However, the present mechanistic studies are important because they may ultimately help to describe a more accurate cytokine/chemokine “signature” in DM, which has yet to be fully understood.
One hypothesis on the origin of these cytokines/chemokines may be from injured endothelial cells. Hyperfiltration causes distention of the glomeruli and elevated intraglomerular pressure, which causes increased mechanical stretch and shear-stress on endothelial cells (74). Injured endothelial cells may then express cell-surface receptors that can activate platelets, which are a potent source of inflammatory and mitogenic substances, including cytokines/chemokines such as PDGF, RANTES, CD40L (180-182). These platelet-derived cytokines/chemokines may then further activate downstream pro-fibrotic, pro-inflammatory effects that contribute to renal and vascular injury. Indeed, evidence of platelet dysfunction has been reported in T2D including enhanced platelet aggregation and sensitivity (183, 184). Therefore, platelet activation may possibly explain the association between renal and cardiovascular risk in DM.

Lastly, pancreatic transplantations have been reported to reverse tubulointerstitial lesions, once considered to be irreversible (185). Thus early identification of DN using hyperfiltration or cytokines, resulting in early intervention, may be an exciting opportunity for clinicians to possibly reverse renal damage prior to the “point of no return”. Moreover, earlier intervention may confer even greater “metabolic memory” which has been proven to have long-lasting benefits of renoprotection (19). In conclusion, DN is the leading cause of end-stage renal disease in developed countries, leading to high financial and societal costs. The early identification of patients at the highest risk for DN progression is therefore critical. While the current series of studies may provide insight into future biomarkers that could be used to stratify renal risk in patients with T1D, similar analyses should be performed in T2D. Furthermore, confirmation of our observations in validation cohorts are required and are currently underway.
Figure 13: Hypothetical Pathway of Cytokine/Chemokine Effects in Hyperfiltration. Hyperfiltration-related mechanical forces may result in endothelial cell injury and platelet activation. Platelets are potent sources of inflammatory substances including cytokines/chemokines. Secretion of cytokines/chemokines may then activate downstream effects leading to deleterious effects associated with renal and cardiovascular injury in DM.
Contributions:

R.L.H Har coordinated recruitment strategies, recruited participants, conducted study visits, performed arterial stiffness testing procedures, handled the biological samples, wrote study visit reports and reported adverse events, created the study database used to store the study parameters, validated the data, collected and analyzed demographic/urine/serum data, performed data analysis and statistical testing, contributed and participated in the intellectual discussion of the results and co-authored the manuscripts. D.Z.I. Cherney, H.N Reich, J.W. Scholey, D. Daneman, D.B Dunger, F.H. Mahmud, E.B. Sochett, R.N. Dalton contributed to the conception and design of the work, acquisition of the data and analysis and interpretation of the data, and co-authored the manuscripts. Y. Elia, L. Deda, V. Lai, R. Dekker coordinated recruitment of participants, contributed to the acquisition of the data and analysis and interpretation of the data. R. Moineddin, M.L. Frtzler M. Ostrovsky contributed to discussion of the results and the analysis and interpretation of the data. All listed authored contributed the drafting and revising of the manuscripts and approved of the manuscripts.
Copyright Acknowledgements

I would like to thank the following publishers for their kind permission to reproduce the figures and tables used in my thesis: Circulation, Current Diabetes Reviews, Diabetes, Diabetologia, Kidney International, Nature Reviews Nephrology, Massachusetts Medical Society, Michael Hortsch and the University of Michigan Medical School, Nephrology Dialysis Transplantation, The American Journal of Pathology. With your kind permission, the figures and tables greatly enhanced my thesis.
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