Sodium Glucose Cotransporter-2 Inhibition and Renal Function

by

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Abstract:

Sodium glucose cotransporter-2 inhibitors (SGLT2i) are approved therapies for renal decline in type 2 diabetes (T2D). Yet there is limited evidence on SGLT2i impact outside of T2D, which this thesis addresses. We hypothesized that 1) the SGLT2i empagliflozin would mediate intrarenal function in type 1 diabetes (T1D), 2) the SGLT2i canagliflozin would be tolerated in renal transplant populations and 3) the SGLT2i dapagliflozin would be protective in focal segmental glomerulosclerosis (FSGS), a subtype of non-diabetic renal disease.

In the first study, we found intrarenal mediation upon SGLT2i therapy in T1D. In the second study, SGLT2i were tolerated in kidney transplant recipients. In the third study, SGLT2i imparted insignificant renoprotection in FSGS. Thus, despite the potential of SGLT2i as a renoprotective therapy outside of T2D, its efficacy may vary across disease subtypes, due to renal SGLT2 regulation. Larger clinical trials are needed to further characterize the renal impact of SGLT2i.

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Commonly used abbreviations

ACE	Angiotensin converting enzyme
ACR	Albumin to creatinine ratio
ARB	Angiotensin receptor blocker
CKD	Chronic kidney disease
DBP	Diastolic blood pressure
DM	Diabetes mellitus
ERPF	Effective renal plasma flow
FF	Filtration fraction
GFR	Glomerular filtration rate
HbA1c	Hemoglobin A1C
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
MAP	Mean arterial pressure
Na^+	Sodium ion
PAH	Paraaminohippurate
RAAS	Renin angiotensin aldosterone system
RBF	Renal blood flow
RVR	Renal vascular resistance
SBP	Systolic blood pressure
TGF	Tubuloglomerular feedback

LITERATURE REVIEW

Chapter 1: INTRODUCTION: SGLT2 INHIBITION AND RENAL HEMODYNAMIC FUNCTION

Selective inhibition of sodium glucose cotransporter-2 (SGLT2) promotes glycosuria, thereby lowering glycated hemoglobin (HbA1c) in patients with type 2 diabetes (T2D) following chronic use. SGLT2 inhibitors (SGLT2i) are approved as antihyperglycemic agents around the world for T2D, including within North America, Europe, and in Japan[1]. More recently in the Empagliflozin Reduce Excess Glucose Outcome (EMPA-REG OUTCOME ClinicalTrials.gov number, NCT01131676) trial, in addition to its favorable benefits on the primary cardiovascular outcome, empagliflozin (an SGLT2i) attenuated nephropathy events in >7000 patients with T2D at high CV risk[2]. Empagliflozin reduced the composite renal microvascular endpoint by 39% (defined as: progression to macroalbuminuria, doubling of creatinine, dialysis or renal death), despite only modest HbA1c lowering effects (-0.4%) in addition to standard of care compared to placebo. These observations suggest thatSGLT2i modify important non-glycemic pathways leading to end-organ protection in patients with T2D.

Mechanistically, progressive kidney injury leading to nephron loss is associated with compensatory reductions in arteriolar resistance at the afferent arteriole and increases in efferent arteriolar resistance in remaining nephrons. This culminates in high intraglomerular pressure, hyperfiltration, and worsening renal injury[3]. Thus, medications that dilate the efferent arteriole (such as angiotensin converting enzyme inhibitors [ACEi] or angiotensin receptor blockers [ARBs], representing the current standard of care) reduce intraglomerular pressure and are protective in patients with DKD and non-diabetic CKD[3]. Unfortunately, despite widespread use of these agents, a significant proportion of patients progress to end-stage renal disease (ESRD)

requiring dialysis or transplantation. Hence, the identification of novel therapeutic strategies that modify glomerular hyperfiltration and attenuate the risk for the progression of CKD to ESRD is critical. In this context, the results of the EMPA-REG OUTCOME trial are of major clinical importance, since the renal protective effects in this study are most likely attributed to non-glucoregulatory, natriuresis-related effects on tubuloglomerular feedback at the afferent arteriole. Moreover, these effects were observed in a patient population wherein the majority (~80%) was already receiving renin angiotensin-aldosterone system inhibitors (RAASi)[4], suggesting that SGLT2 inhibition added on to or stimulated additional renoprotective pathways beyond RAAS. While it is not yet known if these renal benefits extend to patients without diabetes, it is anticipated that natriuresis-related effects on glomerular hypertension and proteinuria with SGLT2i are ubiquitous and independent of diabetes[5].

Accordingly, our first aim was to review physiological mechanisms and clinical data, which support the rationale for SGLT2ias renoprotective therapies in patients with DKD. Secondly, our aim was to demonstrate how these findings in patients with diabetes may support the use of SGLT2i as novel therapeutic strategies in patients with non-diabetic CKD.

1.1 SGLT2i: MECHANISM OF ACTION

Approximately 90% of renal glucose reabsorption is mediated by SGLT2, a high capacity, low-affinity transporter of glucose localized to the renal proximal tubule[6]. In addition to regulating renal glucose reabsorption, SGLT2 also regulates renal sodium excretion. During normoglycemic conditions, SGLT2 is responsible for ~5% of sodium reabsorption, whereas in the setting of chronic hyperglycemia, SGLT2-dependent sodium reabsorption can be as high as 14%, based on increased SGLT2 mRNA expression and greater tubular reabsorptive capacity for

sodium[7]. Thus, given the role of this cotransporter as a mediator of both renal glucose and sodium reabsorption, the physiological effects of SGLT2i are a consequence of both glycosuria (HbA1c lowering, weight reduction) and natriuresis (blood pressure reduction, renal hemodynamic functional effects due to tubuloglomerular feedback).

In healthy individuals, glycosuria is highly regulated, as the kidney actively reabsorbs glucose from the glomerular filtrate even as blood glucose and hence renal glucose filtration rises. Yet the kidney is limited in its capacity to reabsorb glucose – known as the renal threshold for glucose - such that any increases in plasma glucose beyond this limit, results in urinary glucose excretion[8]. At this point (~200 - 250 mg of glucose per 100 ml of plasma in healthy adults, or higher in patients with diabetes), the transporters responsible for glucose reabsorption are fully saturated. By blocking SGLT2, the renal threshold for glucose reabsorption is lowered and leads to a persistent glycosuria, even in non-diabetic individuals with normoglycemia, albeit at a lower level compared to patients with diabetes[9].

In addition to glycosuria, SGLT2 inhibition promotes natriuresis, which has been implicated as a mediator of the renal and cardiovascular protective effects observed in EMPA-REG OUTCOME trial in patients with T2D treated with empagliflozin. The increase in urinary sodium excretion that occurs in patients with diabetes, is associated with a reduction in plasma volume, and may contribute to blood pressure lowering[10](Figure 1). Furthermore, SGLT2 inhibition decreases arterial stiffness, which may further contribute to blood pressure lowering[11]. In preclinical diabetic rodent models, 30-day administration of the SGLT-1/SGLT2 competitive inhibitor phlorizin prevented the development of hypertension, decreased hyperglycemia and decreased SGLT2 activity (but not expression) within the renal brush border membrane vesicles in diabetic rats fed a high salt diet (4%) compared to those not receiving phlorizin[12]. In

normotensive patients with T2D, on average SGLT2i agents reduce systolic blood pressure by ~ -4mmHg[13]. The blood pressure lowering effects with SGLT2i, may be accentuated in hypertensive T2D patients[14] (including those receiving concurrent antihypertensive therapies), which is typically ~ -6 mmHg[15]. Even though changes in urinary sodium excretion only last for several days, the effects of SGLT2i on blood pressure and plasma volume contraction are persistent. Mechanistically, while the initial reduction in plasma volume is stimulated by total urinary sodium excretion, which results in a new steady state in terms of systemic sodium balance, the effects of SGLT2i on the relative fractional excretion of sodium at the proximal renal tubular level persist over time.

Importantly, although SGLT2i-mediated effects on glycosuria and HbA1c lowering are attenuated in patients with CKD as a function of GFR impairment, effects on blood pressure persist down to a GFR of 30 ml/min/1.73m^2[16]. Differential effects on HbA1c versus blood pressure in patients with DKD highlight the discordance between glycosuria-related physiological effects (HbA1c lowering, weight reduction) and those that are primarily due to natriuresis (reductions in blood pressure, eGFR and proteinuria). Since patients with non-diabetic CKD would presumably derive most of the benefit through natriuresis rather than glycosuria-related pathways given the lower filtered load of glucose, the observation that sodium-related physiological effects are preserved in the setting of DKD with impaired eGFR even though glycosuria-related effects are attenuated or lost, is supportive of the use of these agents outside of DKD (Figures 1.1).

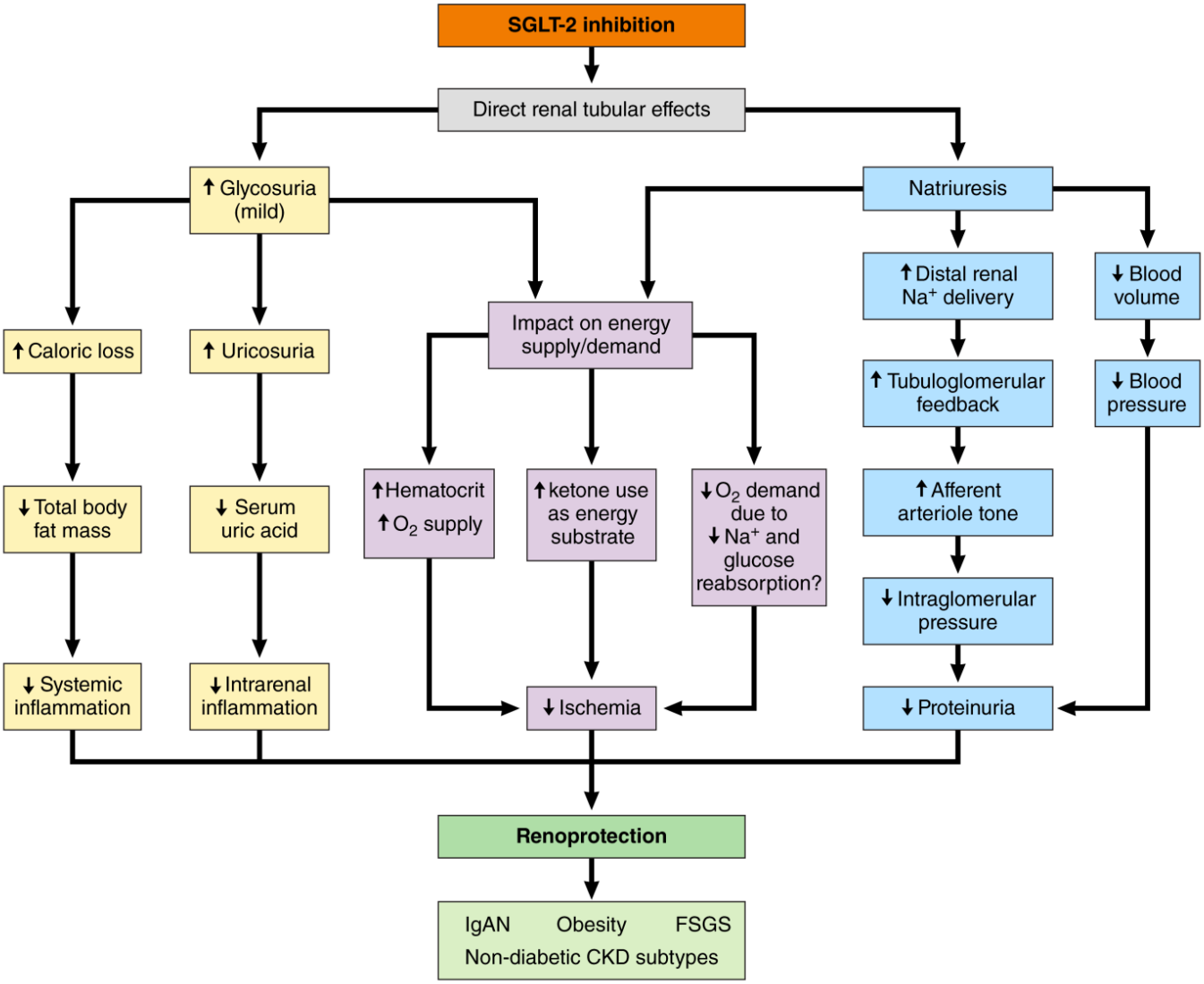

Figure 1.1. Putative renoprotective pathways of SGLT2 inhibition in non-diabetic CKD subtypes

1.2 SGLT2i AND RENAL IMPLICATIONS

Proteinuria is an important prognostic factor for progression of CKD to ESRD[17]. Beyond effects on blood pressure, HbA1c and body weight, SGLT2 inhibition lowers proteinuria. The HbA1c, blood pressure and body weight-independent impact of SGLT2 inhibition with empagliflozin on proteinuria has been reported in patients with micro- or macroalbuminuria and relatively preserved renal function[18]. Similar proteinuria-lowering effects have been demonstrated in patients with impaired renal function with SGLT2i, despite attenuated effects on HbA1c lowering[19]. For example, canagliflozin treatment (100 and 300 mg) reduces albumin-to-creatinine ratios (ACR) in patients with T2D and stage 3 CKD by >20% over 26-weeks[20]. Furthermore, in an analysis of Phase III clinical trials in hypertensive patients with T2D, dapagliflozin (10 mg) reduced albuminuria by approximately 30%; these effects on proteinuria were still statistically significant after adjusting for age, sex, HbA1c, blood pressure, body weight or GFR. Thereby implicating, an independent relationship between SGLT2i and proteinuria that may well be due to intrarenal hemodynamic effects[21].

Mechanistically, the effects of SGLT2 inhibition on proteinuria likely occur predominantly on the basis of a direct proximal renal tubular effect, which stimulates natriuresis. In the setting of diabetes, SGLT2-mediated transport is augmented, leading to increased proximal reabsorption of glucose and sodium, and reduced distal delivery to the macula densa (MD)[22]. Decreased sodium delivery at the MD is sensed by the juxtaglomerular apparatus as a reduction in effective circulating volume, leading to afferent arteriolar vasodilation via tubuloglomerular feedback (TGF), in turn increasing intraglomerular pressure. Thus, by blocking proximal tubular uptake of sodium by SGLT2 inhibition, the resulting increase in distal sodium delivery to the MD, stimulates

TGF-mediated afferent vasoconstriction, and contributes to decreasing intraglomerular hypertension[23]. The precise signaling pathways that link the MD sensing apparatus and the TGF response are not yet known, but may be due to changes in adenosine bioactivity associated to the tubular interstitium, as described elsewhere[24, 25].

1.3 SGLT2i COMPARED TO OTHER EXISTING RENAL THERAPIES

The mechanism(s) of action of SGLT2i are highly distinct as compared to other therapies currently used to treat CKD such as RAASi and diuretics (Figure 1.2). RAASi, such as ACEi and ARBs, are presently first-line agents recommended for non-diabetic CKD management. While they have not yet been studied in non-diabetic patient cohorts, SGLT2i lower blood pressure and proteinuria to a similar magnitude compared to the effects of ACEi observed in patients with diabetes with and without CKD[21, 26, 27]. From a hemodynamic perspective, ACEi and SGLT2i also have similar effects on hyperfiltration. For example, ACEi reduces hyperfiltration in patients with T1D by 35 ml/min/1.73 m^2 which corresponds to a ~19.7% reduction in hyperfiltration[28]. In comparison, SGLT2 inhibition resulted in analogous changes in GFR to those observed following ACEi (33 ml/min/1.73 m^2, corresponding to a ~19.2% reduction in hyperfiltration) in a similar cohort of patients with T1D[29]. It remains challenging however, to decipher the relative attributable impact of SGLT2 inhibition compared to RAASi on albuminuria lowering. Most preceding studies used the combination of both therapies, wherein the addition of SGLT2 inhibition to conventional RAASi therapies further reduces albuminuria in patients with diabetes by ~30-40%[30]. Thus, the protective mechanisms that are responsible for the apparent reduction in intraglomerular pressure appear to be additive. Mechanistically however, these effects are likely distinct between RAASi and SGLT2i[29, 31, 32]. While RAASi modifies mechanisms that are

associated with the "neurohormonal hypothesis" of hyperfiltration and leads to dilation at the efferent renal arteriole, SGLT2i act through "tubular pathways" leading to constriction of the afferent renal arteriole through TGF mechanisms. Consequently, neither RAASi nor SGLT2i alone completely abolish hyperfiltration or albuminuria. Future studies examining the additive or synergistic effects of these two drug classes, specifically within non-diabetic CKD – thereby targeting both neurohormonal (ACEi) and tubular (SGLT2i) mechanisms of hyperfiltration – are therefore of significant interest.

In addition to RAASi, diuretics are frequently used in patients with CKD to lower blood pressure and to enhance anti-proteinuric effects of RAAS blockade[33]. For example, patients with non-diabetic CKD are often prescribed thiazide, loop, or potassium-sparing diuretics for Stages 1-3 CKD, while loop diuretics are the drug of choice for stage 4 CKD[34]. Diuretics potentiate the beneficial effects of RAASi, and these drugs are often prescribed in combination in non-diabetic CKD patients to achieve target blood pressure thresholds and further reduce proteinuria[35].It is therefore clinically important to understand the pharmacodynamic effects of SGLT2i in the setting of diuretic therapy, considering both drug classes lower blood pressure through natriuresis and diuresis, and both drugs will be used concurrently in treating T2D patients, and potentially in non-diabetic CKD subtypes. In pre-clinical studies of 13-week old obese, hypertensive rats with metabolic syndrome, combination of the SGLT2i luseogliflozin with thiazide diuretics (hydrochlorothiazide and furosemide) demonstrated that the natriuretic and blood-pressure lowering effects of SGLT2i are preserved with co-administration of diuretics[36].

Clinical studies in human subjects have observed similar findings. A phase I, open-label, two-period assessment crossover study in normotensive healthy subjects evaluated the pharmacodynamic effects of canagliflozin (SGLT2i) alone and in combination with

hydrochlorothiazide (HCTZ). Mean changes in 24-hour urine volume and urinary sodium excretion, and the fractional excretion of sodium were increased with the SGLT2i + HCTZ combination relative to SGLT2i or HCTZ alone [35]. Interestingly, while the natriuretic effects of SGLT2i + diuretics may be additive relative to either drug alone, pairing of these drugs, seems to, if anything, blunt the blood-pressure lowering effects of SGLT2i. A phase III, randomized, double-blind placebo-controlled study comparing dapagliflozin (SGLT2i) in 449 subjects with uncontrolled T2D (HbA1c ~8%, 7.5yrs duration) and hypertension (SBP ~151 mmHg, ~9.3yrs duration) on ACEi to placebo, examined the effects of dapagliflozin in combination with other antihypertensives on glucose metabolism and seated systolic blood-pressure as co-primary endpoints[37, 38]. At 12 weeks, compared to β-blockers (BB) or calcium-channel blockers (CCB), pairing of thiazide diuretics with SGLT2i had a blunted effect on seated systolic BP change (BB-5.76 mmHg, CCB -5.13 mmHg, diuretics -2.38 mmHg, placebo-subtracted). These apparent differences among the antihypertensive subgroups however, were attenuated in 24-hour ambulatory blood pressure monitoring, a gold-standard method of blood pressure measurement[37, 38].

Taken together, these observations suggest that SGLT2i can be safely combined with other antihypertensive agents, including diuretics, in patients with preserved renal function without expecting dramatic, additive interactive hemodynamic effects. Moreover, these data substantiate that the blood-pressure lowering effects of SGLT2i are maintained irrespective of the class of concomitant antihypertensive[37, 38]. Nevertheless, additional data is required for specific subgroups of patients, such as patients with heart failure and CKD, who may be especially vulnerable to hemodynamic fluid shifts.

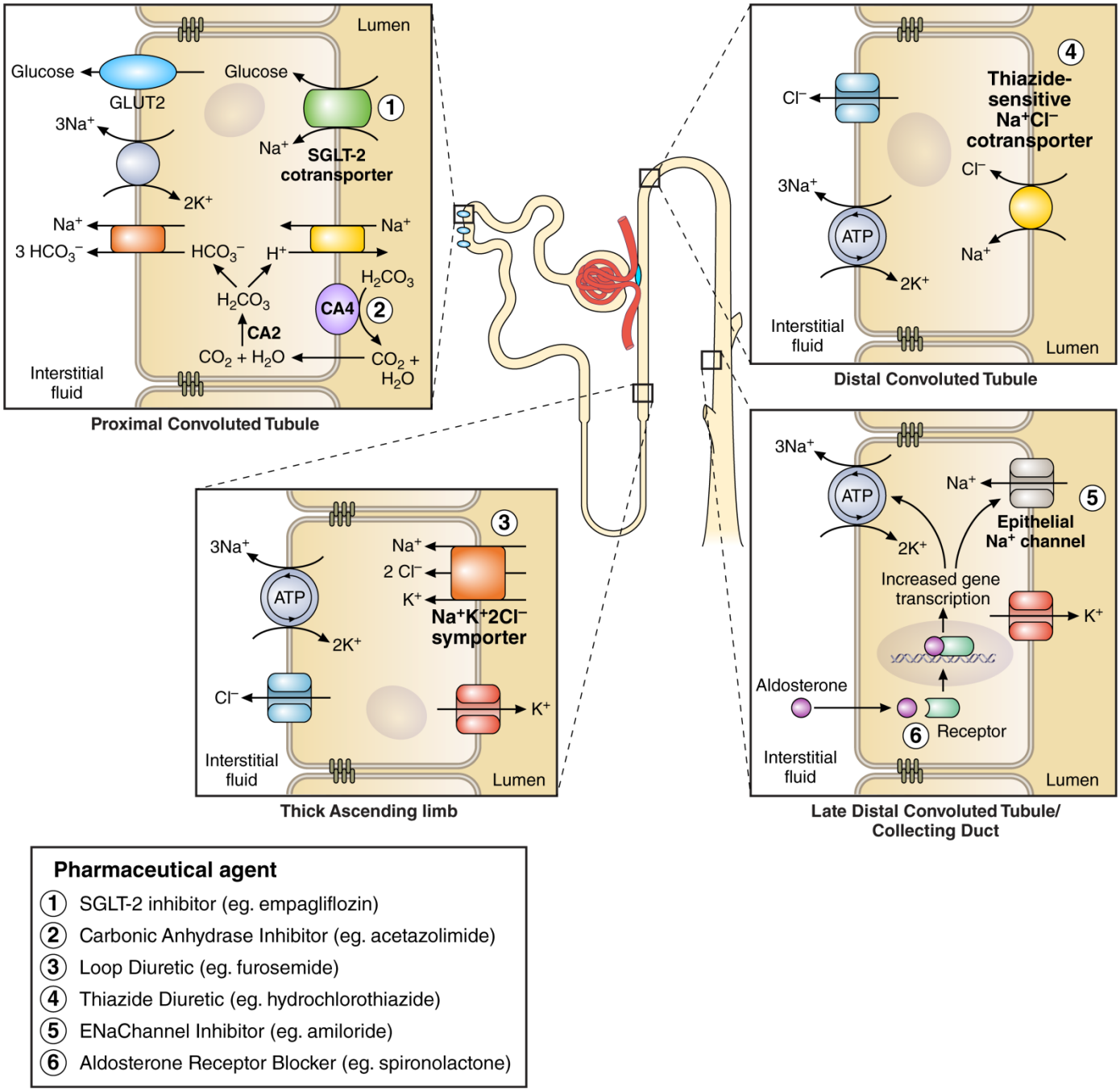

Figure 1.2. Tubular Localization and Mechanisms of SGLT2i and Other Antihypertensives

1.4 RENAL OUTCOME TRIALS WITH SGLT2i

Existing data around the effect of SGLT2i and renal hemodynamic function originated from the “An Open-label 8-week Adjunctive-to-insulin and Renal Mechanistic Pilot Trial of BI 10773 in Type 1 Diabetes Mellitus” (the ATIRMA Trial[31]), which demonstrated that 8-week SGLT2 inhibition with empagliflozin reduces renal hyperfiltration and renal blood flow, and increases renal vascular resistance in patients with T1D. Accordingly, in patients with T2D and either normal or impaired renal function, SGLT2i are associated with a characteristic 4-6 ml/min “dip” in eGFR within ~3-4 weeks, which is likely occurs on the basis of afferent vasoconstrictive effects. In the EMPA-REG OUTCOME trial[2], including 7,020 patients with T2D and established cardiovascular disease, similar eGFR “dip” effects were observed over time. Importantly, after a 34-day washout period, these small changes in eGFR return to baseline levels, demonstrating the reversibility of the hemodynamic effects at the afferent arteriole, even after a treatment period of 192 weeks.

Despite the accruing data supporting the rationale for SGLT2i as renoprotective therapies, primary dedicated renal outcome clinical trials with SGLT2i are not yet complete. For example, the "Evaluation of the Effects of Canagliflozin on Renal and Cardiovascular Outcomes in Participants With Diabetic Nephropathy" (CREDENCE) trial[39] will assess renal and vascular protection in patients with T2D following add-on SGLT2i therapy (canagliflozin) to background RAASi, and is due to report in 2019. Additionally, the "A study of the Effects of Canagliflozin on Renal Endpoints in Adult Participants With Type 2 Diabetes Mellitus" (CANVAS-R) trial[40], investigated the effects of the SGLT2i, canagliflozin, in T2D patients at high cardiovascular and renal risk, and recently reported a significant reduction in the risk of progressive albuminuria and a 40% in the composite renal endpoint (50% decline in eGFR, dialysis or renal death)[41].Primary

cardiovascular safety trials with other SGLT2i (including DECLARE-TIMI[42]for dapagliflozin, CANVAS[40] for canagliflozin, and VERTIS CV[43] for ertugliflozin (an investigational SGLT2i) are ongoing, and will also capture and provide important secondary renal endpoints, as reviewed elsewhere[44].

1.5 SGLT2i AND POTENTIAL USE IN NON-DIABETIC CKD

Beyond DKD, based on prominent non-glycemic effects, potential candidate renal diseases for SGLT2i therapy include those that feature glomerular hyperfiltration, high intraglomerular pressure and progressive proteinuria. Clinical features that are common manifestations in many CKD subtypes, suggesting that SGLT2i therapy may be widely applicable in non-diabetic CKD. We however, have focused on the use of SGLT2i in select CKD subtypes for further discussion that we speculate may be especially favorable including 1) obesity-related kidney disease (glomerulopathy), 2) focal segmental glomerulosclerosis (FSGS), and 3) IgA nephropathy (IgAN) (Figure 1.3).

1) *Obesity-Related Kidney Disease*

Obesity-related hyperfiltration is associated with the initiation and progression of kidney disease, and can present pathologically with or without underlying evidence of focal sclerosis[45]. Clinical studies have demonstrated that maladaptive renal hemodynamic functional changes occur in obese patients, including increases in GFR, effective renal plasma flow, and filtration fraction, through afferent arteriolar vasodilation[46]. Treatment of obesity-related kidney disease often includes the use of RAASi to reduce intraglomerular pressure, along with body weight loss strategies, including bariatric surgery[47]. Hyperfiltration due to obesity is also likely initiated in

part due to increased proximal tubular sodium reabsorption and altered tubuloglomerular feedback, a process that is similar to what occurs in DKD[48], although additional pathways are probably involved including RAAS activation[49].

From the perspective of the afferent arteriole, the proximal tubular diuretic, acetazolamide, significantly reduces GFR in patients with obesity-related hyperfiltration based on modifying afferent arteriole tone[5]. It is not yet known, however, if proximal natriuresis with SGLT2i will exert similar effects on hyperfiltration in obese patients without diabetes. Due to pathophysiological similarities between obesity-related hyperfiltration and diabetes-related hyperfiltration, it seems reasonable to suggest further prospective studies in this clinical area to determine if SGLT2i will modify key factors that promote CKD in patients with obesity, with or without "pre-diabetic" changes in metabolism. Conceptually, the use of SGLT2i in the setting of obesity-related renal disease may be particularly appropriate given ancillary effects on body weight loss and energy substrate utilization.

2) *Focal Segmental Glomerulosclerosis (FSGS)*

Focal segmental glomerulosclerosis (FSGS) is an important cause of glomerulonephritis in North America, and significantly increases the risk for progression towards ESRD[50]. Patients with FSGS are frequently hypertensive and have significant proteinuria[51]. RAAS blockade is a common therapy in FSGS, targeting reduction of blood pressure and proteinuria[51, 52].

Although RAASi have dramatic anti-proteinuric effects in this patient population, there may also be an opportunity for further suppression of remnant proteinuria and improvement in residual renal function with SGLT2i. This may be of particular importance in patients with secondary FSGS due to underlying conditions that promote hyperfiltration, such as sickle cell

disease, obesity and compensatory renal hyperfiltration after loss of nephron mass. Rodent models of type 2 diabetic nephropathy have demonstrated that chronic combined SGLT2i (luseogliflozin) with RAASi (lisinopril) therapy yields greater reductions in blood pressure and proteinuria compared to either treatment alone[53]. In humans, the addition of the SGLT2i dapagliflozin to RAAS blockade similarly leads to additional blood pressure lowering effects in hypertensive patients with uncontrolled type 2 diabetes [38]. Whether such additive effects occur in non-diabetic CKD such as FSGS, remains unknown. To investigate the feasibility of using SGLT2i as an add-on to RAASi in FSGS, small pilot studies such as the "Treating to Reduce Albuminuria and Normalize Hemodynamic Function in Focal ScLerosis with dApagliflozin Trial Effects" (TRANSLATE)[54], are currently underway to investigate the combinatory effects of SGLT2i and RAASi on proteinuria, blood pressure, and renal hemodynamic function. This particular proof-of-concept trial will evaluate the renoprotective influence of dapagliflozin over an 8-week treatment period (10 mg once daily) as an adjunct to RAASi (see Chapter 4).

3) *IgA Nephropathy*

IgA nephropathy is primarily a glomerular-based disorder characterized by the deposition of IgA antibodies in the glomerulus[55]. Patients with IgA nephropathy frequently present clinically with significant proteinuria[56] which correlates with the future risk for renal functional loss[55]. Aside from immunosuppression, current therapy for IgA nephropathy includes blood pressure control and the use of RAASi, which reduce proteinuria and slows the rate of progression of CKD due to a reduction in intraglomerular pressure, even in normotensive patients[57, 58]. Therefore, similar to other proteinuric glomerular-based diseases, the adjunctive use of SGLT2i (as a means of reducing glomerular hypertension) merits further exploration

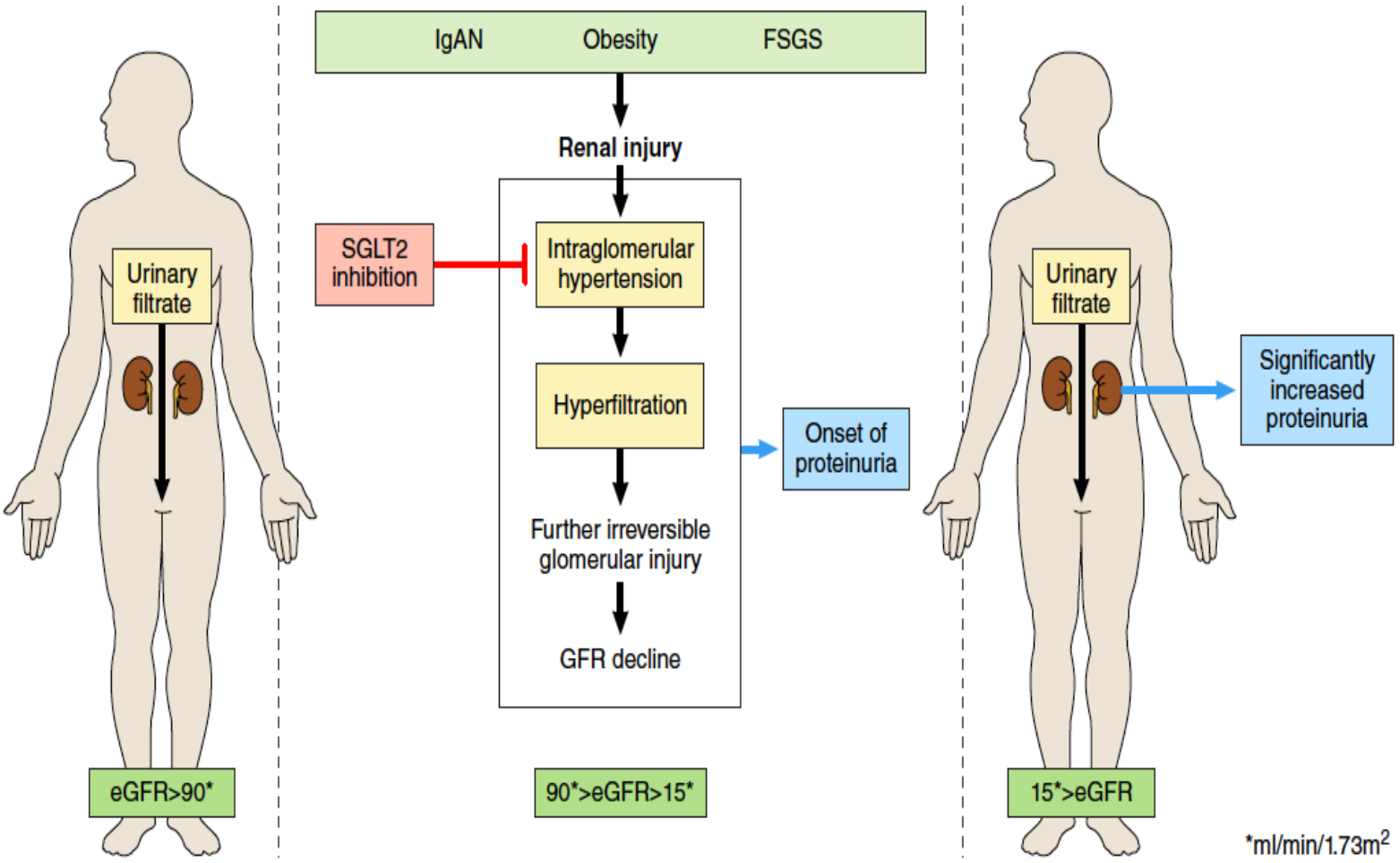

Figure 1.3. Candidate CKD Subtypes for SGLT2i Therapy

based on the mechanism of action as previously described in the two sections above.

1.6 POTENTIAL RISKS OF SGLT2i USE

While SGLT2i may represent a new therapeutic opportunity for several candidate CKD subtypes, exploration of any new drug in new patient populations requires careful clinical vigilance. The use of SGLT2 inhibitors are currently approved only within T2D, primarily for their anti-hyperglycemic effects - there is little data surrounding the sodium handling impact of this drug class on the kidney. Foreseeable investigative obstacles include the potential for hypotension due to additive blood pressure lowering effects with other antihypertensives, lack of efficacy due to optimized natriuresis from other agents, increased cost and polypharmacy. Furthermore, specific patient subgroups not previously recognized may be vulnerable to adverse effects associated with SGLT2i use. For example, recently the US Food and Drug administration identified ~101 cases of acute kidney injury (AKI) associated with SGLT2i use, leading some to speculate that SGLT2i use contributes to medullary hypoxia in the renal parenchyma[59]. These potential associations may be of clinical relevance, particularly in patients with iatrogenic sources of medullary hypoxia including those receiving nonsteroidal anti-inflammatory drugs or radiocontrast dyes. Despite these reports, it is important to keep in mind that SGLT2i-associated AKI is quite rare and occurred less often in EMPA-REG OUTCOME in the SGLT2i-treated group compared to placebo. Importantly, the hazard ratio (HR) in the CANVAS Program also tended to favour canagliflozin (HR 0.68 for serious acute kidney injury, 95% confidence interval 0.45-1.02)[40].

From a broader perspective, SGLT2i have known class-wide adverse effects such as urinary and genital mycotic infections, increased urine frequency and volume depletion (especially

if taken in the context of inter-current illness). Also, drug-specific adverse effects such as bone fractures and disruption of mineral metabolism have been reported with canagliflozin but mechanistically are not yet well understood[60]. The increased risk of lower limb amputation in the CANVAS Program – a risk not seen with empagliflozin or dapagliflozin – is similarly not well understood[40, 41]. While many of these potential adverse effects are rare, they may not represent an acceptable benefit to risk ratio for some patients, especially, such as in elderly or frail individuals with multiple medical co-morbidities commonly present in individuals with CKD. Yet despite these potential shortcomings, SGLT2i show minimal interaction with other drugs and in term of their pharmacokinetics, display rapid oral absorption and long elimination half-life allowing for daily usage in patients[61]. Moreover, SGLT2i are actively catabolized by the liver to inactive metabolites and subsequently cleared. Clearly further evidence is required to discriminate the actual pharmacological risks with SGLT2i across different disease contexts.

1.7 AIMS AND HYPOTHESES

In a general sense, the results of the EMPA-REG OUTCOME trial[2] in patients with T2D at high cardiovascular risk highlight the potential for SGLT2i use beyond diabetic kidney disease. Most lines of evidence indicate that although approved for lowering glycemic index, the major impact of empagliflozin and perhaps other SGLT2ion renal endpoints, likely occur independent of glucose regulation, and instead are related to natriuresis. The renoprotective effects of SGLT2i on renal function may therefore be ubiquitous in individuals with and without T2D, and likely occur because of a reduction in intraglomerular hypertension, which is initiated based on the proximal tubular natriuretic effects associated with SGLT2i. If accurate, SGLT2i may therefore target pathophysiological mechanisms of CKD progression regardless of the underlying cause (similar

in many ways to the impact of RAASi medications). Future data from ongoing clinical trials are needed to help inform clinicians and researchers about the therapeutic potential of SGLT2i use beyond current approved practice patterns in patients with T2D. Moving forward, clinical studies in these novel disease areas should address not only clinical efficacy but substantiate safety, for potential unforeseen risks associated with SGLT2i use beyond T2D.

As such, the specific aim of this present thesis is to better characterize the impact of SGTL2 inhibition on renal hemodynamic function outside of its classical indications in diabetic kidney disease, with particular emphasis on natriuresis-mediated pathways of TGF modulation. In the first study, our aim was to examine the role of intrarenal adenosine, as excreted into the urine, as a putative signalling mediator of TGF in response to SGLT2i administration. We hypothesized that SGLT2i would be associated with increased urinary adenosine levels in patients with T1D, as quantified by a validated method of LC-MS/MS. In this first study, as expected, we saw a significant increase in urine adenosine excretion post-SGLT2 inhibition in our T1D population. In the second study, we hypothesized that SGLT2 inhibition treatment would be well tolerated in renal transplant cohorts, a previously unexplored clinical setting. We discovered mild, albeit non-significant, reductions in GFR, body weight and blood pressure following SGLT2i use across groups of kidney transplant and simultaneous pancreas-kidney transplant recipients. These results convey sufficient tolerability of SGLT2i in this novel context, in addition to modest improvements in renal and metabolic status. In the third study, we examined the possible renoprotective effects of SGLT inhibition in patients with FSGS. We hypothesized that acute SGLT2i therapy would resolve in reductions in renal risk factors of hyperfiltration, proteinuria and blood pressure. However, we largely observed mild attenuations in GFR, urinary protein excretion and blood pressure that did not reach statistical significance. Although upon multiple sensitivity analyses, we

observed significant decreases in GFR and proteinuria among those at lower renal functional risk - which may inform the clinical efficacy of SGLT2i. Moreover, in a *post hoc* examination of kidney biopsy tissue of humans with FSGS, we detected significant downregulation of SGLT2 mRNA as compared to healthy control tissues. This notable finding may in part contribute to the observed neutral impact of SGLT2 inhibition within FSGS. As such, the viability of SGLT2i intervention may vary considerably across clinical contexts due to state-specific modulation of renal SGLT2. Future clinical trials are required to better characterize this phenomenon within subtypes of non-diabetic CKD.

Key Points:

- SGLT2i are approved for the treatment of hyperglycemia in patients with type 2 diabetes
- Due to the mechanism of action of SGLT2i, these agents may reduce hyperfiltration, proteinuria and intraglomerular hypertension, known risk factors that promote CKD progression
- Based on proximal tubular natriuretic effects, SGLT2i have therapeutic potential for use in non-diabetic CKD
- Urinary adenosine excretion is increased post-SGLT2i in patients with type 1 diabetes, supporting its role as a vasoactive mediator of TGF and renal hemodynamic state
- SGLT2i are well tolerated in renal transplant populations, producing mild renoprotection
- SGLT2i has largely neutral impact in FSGS patients which may relate to state-specific modulation of SGLT2 expression and/or alternative neurohormonal compensation
- Clinical investigations are needed to better characterize the use of SGLT2 inhibition outside of diabetic kidney disease

Urinary Adenosine Excretion in Type 1 Diabetes

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2.1 ABSTRACT

In experimental models of diabetes, augmented sodium-glucose cotransport-2 (SGLT2) activity diminishes sodium (Na^+) delivery at the macula densa. As a result, less *vasoconstrictive* adenosine is generated, leading to afferent arteriolar *vasodilatation* and hyperfiltration. The measurement and significance of urinary adenosine in humans has not been extensively examined in states of renal hemodynamic impairment, like that of diabetes. Our aim was to validate a method for urine adenosine quantification in humans and perform an exploratory *post-hoc* analysis to determine whether urinary adenosine levels change dynamically in response to natriuresis in patients with type 1 diabetes (T1D) before and after treatment with the SGLT2 inhibitor (SGLT2i) empagliflozin. We hypothesized that SGLT2i, which reduces renal hyperfiltration through increased Na^+ delivery to the macula densa, would increase urinary adenosine excretion. Urine adenosine corrected for creatinine was measured using our validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method in 40 healthy participants and 40 patients with T1D. In the T1D cohort, measurements were performed during clamped euglycemic and hyperglycemic conditions, prior to and following 8 weeks of SGLT2i therapy. Urinary adenosine was detectable in healthy subjects (0.32± 0.11µmol/mmol Cr) and patients with T1D. In response to SGLT2i, urine adenosine increased during clamped hyperglycemia (0.40±0.11 vs 0.45±0.12µmol/mmol Cr, p=0.005). Similar trends were observed during clamped euglycemia (p=0.08). SGLT2i increases urinary adenosine excretion under clamped hyperglycemic conditions in patients with T1D. The potentially protective role of SGLT2i against glomerular hyperfiltration and its mediation by adenosine in diabetes merits further study.

2.2 INTRODUCTION

Adenosine is an endogenous purine nucleoside, which is associated with paracrine regulation of renal hemodynamic function. Specifically, extracellular adenosine has been identified as a mediator of renal tubuloglomerular feedback (TGF)[62]. These effects are likely accomplished through interactions between adenosine and its cognate A_1 and A_2 adenosine receptors. In response to increased sodium delivery, adenosine is released from sodium sensing macula densa cells at the juxtaglomerular apparatus, activating A_1 receptors at the afferent arteriole. Intracellular calcium influx occurs as a result, leading to vasoconstriction of afferent renal arteriolar smooth muscle cells. This process, known as TGF, maintains stable renal function in response to changes in intravascular volume and distal renal tubular sodium delivery[63]. As a consequence of proximal tubular sodium avidity in the setting of diabetes mellitus, macula densa sodium delivery is reduced, possibly leading to reduced adenosine generation, a process associated with afferent arteriole vasodilatation in the nephron, and hyperfiltration in experimental models of diabetes. In contrast, adenosine-mediated activation of the TGF system reduces hyperfiltration and proteinuria in preclinical studies– however the effect(s) of adenosine activity on renal hemodynamic function in humans has not been extensively examined[62].

Existing urinary adenosine quantification methods vary in efficacy, with some techniques being limited by poor analyte specificity and sensitivity and large sample volume requirements. Such assays include adenosine measurement via adenosine binding to *S*-adenosylhomocysteine (SAH) hydrolase, which is limited by non-specific ligand binding[64]. Other analytical methods, such as high-performance liquid chromatography (HPLC) are also prone to pitfalls, such as low analyte specificity and lengthy sample preparation steps, thereby reducing its utility. Yet, past explorations into liquid chromatography separation coupled to tandem mass

spectrometry (LC-MS/MS) have yielded reliable measurement of urinary nucleosides in humans[65]. An ideal assay for urine adenosine quantification should be robust, precise, free of interference and sensitive. To this end, adapting LC-MS/MS represents a novel clinical application by which urine adenosine may be quantified as related to renal hemodynamic state – and by extension, diabetic kidney disease.

Accordingly, we validated an adenosine-specific LC-MS/MS method for the measurement of adenosine in human urine, with a goal of improving analytical efficacy. We first applied this detection method to measure adenosine excretion in healthy subjects and a cohort of individuals withT1D. In a *post hoc* analysis, we measured urine adenosine excretion in a T1D cohort at baseline and after administration of the sodium glucose co-transport-2 (SGLT2) inhibitor, empagliflozin, under clamped euglycemic and hyperglycemic conditions. We hypothesized that since SGLT2 inhibition increases sodium delivery to the macula densa, that following 8-week empagliflozin administration, extracellular adenosine release would be increased, resulting in increased urinary excretion.

2.3 MATERIALS AND METHODS

Chemicals and Reagents

Adenosine (1 g) was purchased from Sigma-Aldrich (Oakville, ON, Canada). Adenosine-$^{13}C_5$(2.5 mg) internal standard (IS) was from Toronto Research Chemicals (Toronto, ON, Canada). Supelcosil LC-18, 150 x 4.6 mm, 3 µm, and ammonium acetate were also obtained from Sigma-Aldrich. In-house ultrapure water with a high resistivity (>18.2 MΩ.cm) was used. Optima grade acetonitrile was obtained from Fisher Scientific (Ottawa, ON, Canada)

Sample preparation

Prior to the chromatographic analysis, 50 µl of urine was spiked with 25 µl of 4 µM adenosine-$^{13}C_5$ (IS) in water. Then, 175 µl of water was added and the diluted urine was vortexed-mixed for 30 s and centrifuged for 10 min at 14,000 rpm. One hundred and twenty-five µl of sample was transferred to a 96-well plate and analyzed by LC-MS/MS. The remainder was frozen at -20˚C.

LC-MS/MS – Instrumentation and chromatographic conditions

Liquid chromatography separation was performed using an Agilent 1200 series system (Agilent Technologies, US) consisting of a degasser, solvent binary pump, autosampler, and a column oven. The mobile phase consisted of 25 mM ammonium acetate in water (92%) and acetonitrile (8%). Separation was achieved on a reversed phase Supelcosil LC-18, 150 x 4.6 mm, 3 µm column with an isocratic elution. The flow rate was 900 µl/minute and an injection volume of 10 µl was used. Column was maintained at 22 ˚C and the total run time was 6.0 minutes.

The LC system was coupled to an API 5000 triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Concord, ON, Canada) equipped with an electrospray ionization (ESI) probe and was used in positive ion mode by multiple reaction monitoring (MRM). Instrument operating parameters were optimized for adenosine and were as follows: CAD at 4.0 U, CUR 40 U, GS1 50 U, GS2 50 U, IS 5500 V, TEM 550˚C, DP 40 V, EP 8 V, CXP 20 V. The ion-transitions of *m/z* 268.2>136.2 (quantifier) and 268.2>119.0 (qualifier) were monitored to identify and quantitate adenosine, while *m/z*273.1>136.2 was monitored for the internal standard. Dwell time per transition was 200 milliseconds.

The LC system and mass spectrometer were controlled by the Analyst software (version 1.6.2). Data acquisition and analysis were performed with the same software. Adenosine concentrations were corrected based on the internal standard and quantified using the calibration curves that were included in each batch.

Stock solutions were prepared for all analytes. Adenosine and adenosine-$^{13}C_5$ were reconstituted in water at a concentration of 10mM and 9.34µM, respectively. All stock solutions were stored at -80ºC. The calibrators for adenosine at a concentration of 0.01, 0.05, 0.1, 0.5, 1.0, 2.0, 5.0, 10.0, and 20.0 µM were prepared from its stock standard solution in water by serial dilution. Internal standard working solution (4 µM) was prepared in ultrapure water from its stock solution. Quality control samples were three neat urine samples with adenosine concentrations at approximately 0.7, 6.0, and 13.0 µM.

Method validation

Method validation included assessments of: precision, limit of detection (LOD), limit of quantification (LOQ), linearity, analyte recovery, carryover and accuracy. Inter-day precision was determined by analyzing the three-level QCs in 3 replicates for 8days. Intra-day precision was assessed by assaying the same QCs in 10 replicates in a single day. The criterion for acceptability of the precision data was CV < 10% for all 3 QC levels. LOD was determined as the lowest adenosine concentration at which chromatographic peak of the analyte was present in both transitions at expected retention times with a signal-to-noise (S/N) ratio of 3. Subsequently, the LOQ was determined by analyzing four urine samples containing progressively lower concentration of adenosine in 6 replicates, namely 0.79, 0.40, 0.05, 0.002 µM. The criterion for

determining the LOQ was to find the lowest amount of adenosine that can be quantitated with a precision better than 20%, as well as exhibiting S/N ratio of at least 10.

Linearity was assessed by using a spiked urine sample (20 µM) and diluting it 9 times, while each time doubling the dilution and analyzing it in triplicate. Linearity was evaluated with the polynomial regression method. Analyte recovery experiments were performed by spiking adenosine to a patient sample containing 0.91 µM of endogenous adenosine. Specifically, 1, 5, and 10 µM of adenosine was added and samples were assayed in triplicates. Percent recovery was determined by calculating the difference between the observed and expected concentrations of adenosine. Carryover was examined using 3 high samples (urine with spiked adenosine at levels around 70-85 µM) and 3 low samples (adenosine concentration around 0.10 µM). Each pair of high and low samples were run in the following order: High1, High2, High3, Low1, Low2, and Low3 and carryover, k, was calculated using the equation: k= (low1−low3) / (high3−low3). Accuracy of the assay was assessed by analyzing control material obtained from the MCA Laboratory of the Queen Beatrix Hospital (StreekziekenhuisKoningin Beatrix, the Netherlands).

Urine Creatinine Measurements

Creatinine was measured in all urine samples using the modified kinetic Jaffe method on the Abbott Architect C8000 platform. This assay is standardized to the National Institute of Standards and Technology (NIST) standard reference material 967.

Study Participants

Two different cohorts of participants were analyzed in this study for adenosine excretion. The first cohort consisted of 40 healthy volunteers (17 males and 23 females) with an age range of 20-62 years. The second cohort consisted of 40 patients with T1D who participated in a previous

clinical trial. The healthy controls samples were used to develop the assay rather than as a comparison group for the T1D cohort, due to differences between the groups. Urine samples were collected as part of the protocol during clamped euglycemic (4-6 mmol/L) and hyperglycemic (9-11 mmol/L) conditions at baseline and post-SGLT2i for 8 weeks. The local Research Ethics Board at the University Health Network (Toronto, Canada) approved the protocol and all subjects gave informed consent prior to start of study procedures. The study was conducted according to the International Conference on Harmonization on Good Clinical Practice.The specific patient inclusion and exclusion criteria have been reviewed elsewhere[66]. We have also previously reported the effects of empagliflozin on urinary RAAS mediators, systemic and renal hemodynamic function and glycemic control[67].

Statistical Analyses

Data are presented as mean ± standard deviation (SD). *Post-hoc* comparisons of within-patient urine adenosine values from baseline to post empagliflozin treatment were performed using a paired sample t-test statistical analysis. Statistical significance was defined as $p<0.05$. All statistical analyses were performed using SAS v9.1.3 and GraphPad Prism software (version 5.0).

2.4 RESULTS

LC-MS/MS Validation and Development

Figure 1 shows the LC-MS/MS chromatogram obtained for a urine sample containing 0.88 µM of adenosine. Extracted ion chromatograms (XIC) for both the quantifier and qualifier for the analyte as well as the XIC for the IS are shown, demonstrating sharp, symmetrical peaks with high selectivity. Within-day urinary adenosine precision measurement, in which 10 replicates from each of the QC level (QC_{low}, QC_{med}, QC_{high}) were assessed to have percent coefficient of variation (%

CV) values of 7.1, 4.4, and 3.9, respectively. Additionally, for each of the aforementioned QC levels, CV values of 5.0, 3.5 and 3.5%, respectively were obtained for the between-day precision measurements (over 8 days) (Table 1). These results indicate that the developed method has good precision.

Next, 4 patient samples with low adenosine concentrations (0.79, 0.40, 0.05, 0.002 µM) were assayed 6x to determine % CV and to assess the S/N ratio (Table 2) to determine the LOQ of the assay. The LOQ was set to 0.05 µM as it was the lowest amount of adenosine that we were able to quantitatively determine with a precision better than 20%, as well as exhibiting a S/N ratio of at least 10. In addition, the developed assay exhibited excellent linearity, as shown in Figure 2 where the measured adenosine values correlated well across the measuring range to the expected output; correlation coefficient R^2= 0.9992.

The results of method validation also suggest that endogenous urine adenosine, stored at -20ºC is stable for 8-10 freeze-thaw cycles, as demonstrated by the inter-day precision study results. QC material was kept at -20ºC during method validation studies. Prior to analysis, the material was thawed. Following sample preparation, the QC material was placed back into the freezer.

Next, we observed the efficiency with which our analytes were recovered from their native urine matrices (Table 3). Three urine samples, differentially spiked with adenosine, were completely recovered following dilution (average recovery efficiency of 100 %). The carryover values were negligible across all three patients (Table 4).

Finally, accuracy was assessed by assaying MCA quality material, which is lyophilized human urine to which purines and pyrimidines have been added. Calculated consensus value for adenosine in this sample was 68.1 µM, with 95% confidence interval of 66 – 70 µM. Sample was first reconstituted as per instructions in the product certificate and then it was diluted with water

2x, 4x, and 8x. Each diluted sample was prepared in triplicate for LC-MS/MS analysis. 4x and 8x diluted samples had adenosine concentrations that were within the calibration range (0-20 µM), 18.2 µM (2.8 % CV) and 9.2 µM (1.9 % CV), respectively, and we used those values to establish the accuracy of the method based on %BIAS. %BIAS was calculated using the equation: [(Expected c – Average measured c)/Expected c x 100], and it was determined that it was below 10%, namely 7.1 and 8.1% for the two respective dilutions, which met our criterion for the assay accuracy (Table 5). The above results confirm that the developed urine adenosine method by LC-MS/MS is precise (CV <10%), linear (0.05 – 20 µM), accurate and exhibits negligible carryover. The assay is both sensitive (lower reporting limit is 0.05 µM) and specific (free from interference).

Urinary adenosine in healthy subjects

Adenosine:creatinine (ACr) values were measured in urine samples of 40 healthy volunteers (20-62 years of age, 17 males, 23 females). The average ACr value was 0.32 ± 0.11 µmol/mmol Cr, with no significant trends found with regards to age. Overall ACr levels, as well as those in males and females are provided in Tables 6-7.

Urinary adenosine in patients with T1D before and after SGLT2i

Urine adenosine corrected for creatinine was measured in 40 patients with T1D before and after administration of empagliflozin, for 8 weeks. Measurements were performed during clamped euglycemia and hyperglycemia conditions before and after drug. In response to SGLT2i, urine adenosine increased under clamped hyperglycemic conditions (0.40±0.11 vs.0.45±0.12µmol/mmol Cr, p=0.005, Figure 3A). When the participant with the largest change in urinary adenosine excretion (Figure 3B) was excluded from the analysis, the change remained

significant with p=0.01. Similar non-statistically significant differences were observed during euglycemia (0.40±0.12 vs 0.44±0.12μmol/mmol Cr, p=0.08).

2.5 DISCUSSION

In this report, we have described a robust and specified LC-MS/MS method for quantification of urine adenosine both in healthy participants and in those with T1D. The method uses 50 μL of urine and exhibits excellent precision, linearity and accuracy characteristics, along with negligible carryover and a low limit of quantification. This method has the potential for wider use in human physiological experiments designed to assess renal hemodynamic function, especially in response to medications that impact natriuresis and tubuloglomerular feedback. Our major observations were that: 1) urinary adenosine is present and quantifiable in urine samples in healthy controls and individuals with T1D and 2) urine adenosine excretion changes significantly in response to the SGLT2i empagliflozin after treatment duration of 8 weeks under conditions of clamped hyperglycemia within T1D patients.

Adenosine is a naturally occurring metabolite in human urine matrix that has been measured via multiple techniques, but with varied success. In the *in vitro* setting, adenosine can be quantified by enzymatic assays such as SAH hydrolase binding. While this method boasts *within* and *between*-precision measurements of 3.9 and 7.8 % CV respectively, this methodology is also limited due to interference from the adenosine analog, 2'deoxyadenosine, displaying poor specificity[64]. In contrast, our LC-MS/MS method shows a high degree of analyte specificity, by virtue of its tandem mass analysis, in which two separate mass: charge signatures of adenosine, belonging to its parent and product ions, are simultaneously verified. Our method shows no indication of interference from ancillary metabolites.

Simple HPLC methods have also been utilized to detect adenosine both in blood and urine. In whole blood, HPLC shows comparable sensitivity to our method (LOQ of 0.3 µM vs 0.05 µM, respectively), even as it lacks in between-day precision (8.95 vs 4.0 % CV, respectively)[68]. Within urine, although HPLC actually shows high sensitivity and precision, it compares poorly in method run time[69]. In fact, HPLC exhibits total run times of 19 and 18.5 minutes for blood and urine respectively, as contrasted by our shorter LC-MS/MS run time of 6.0 minutes.

Despite less effective methods of adenosine quantification, marked developments in the ability to measure urinary adenosine have emerged following the integration of mass spectrometry techniques. Specifically, LC-MS/MS has been previously used to establish urinary expression profiles of purine and pyrimidine nucleosides in humans. Researchers showed that combining reverse-phase liquid chromatography with the specificity of tandem mass spectrometry could enable accurate detection of more than 30 urinary metabolites, including adenosine. In particular, this LC-MS/MS method featured a run time of 17 minutes, in addition to a LOQ of 0.5 µM for urine adenosine and inter-day precision % CV ranges of 1.6-14.1% (in low creatinine samples) and 1.5-29.1% (in high creatinine samples). Yet, for its shared features with our method, including the polarity of the LC column and type of ion source, this LC-MS/MS method proved subpar to our adenosine-specific LC-MS/MS technique, from precision and sensitivity to sample throughput. A possible reason for this discrepancy might be due to the generalized scope of this particular LC-MS/MS method, in that it targeted 30 unique nucleoside metabolites for quantification. This would have reduced the stringency of test parameters, like that of the ionization efficiency. In fact, as per its protocol, this method's ESI probe cycled its ionization polarity at the beginning of the chromatographic run for an added 2.7 minutes between +5000 V and -4000 V to compensate for the varying physicochemical properties of its multiple target analytes[70]. This variability in solute

volatility may have in turn resulted in ion suppression, ultimately reducing method sensitivity[71]. As such, our validated method represents a novel, and more efficacious, iteration of LC-MS/MS for the purpose of urinary adenosine quantitation.

As mentioned, we used our LC-MS/MS method for measurement of urinary adenosine under clamped euglycemic and hyperglycemic conditions, at baseline and following administration of the SGLT2 inhibitor empagliflozin for 8 weeks in young adults with uncomplicated T1D. We hypothesized that the increase in macula densa sodium delivery would lead to an increase in ATP consumption and adenosine generation. As detected by LC-MS/MS, short-term SGLT2i administration increased adenosine excretion under clamped hyperglycemic conditions in a cohort of patients with T1D– an effect that may contribute to the regulation of renal hemodynamic function.

In contrast, no significant effects were observed during clamped euglycemia. Inhibition of SGLT2 reduces proximal reabsorption of filtering glucose and sodium, thereby increasing distal delivery of sodium to the macula densa. Delivered sodium is subsequently reabsorbed at the macula densa, which is itself associated with extracellular release of adenosine triphosphate (ATP), a precursor of adenosine[72]. ATP is then likely degraded by a series of cell surface ectonucleotidase reactions in the extracellular environment, resulting in adenosine acting at the afferent arteriole as part of the TGF process[73]. While we cannot explain why significant differences in adenosine secretion were found only during clamped hyperglycemia, one possible explanation involves the effect of hyperglycemia on glomerular filtration rate (GFR). It has been previously reported that periods of acute hyperglycemia are associated with notable increases in GFR through inducing increases in intraglomerular pressure[67]. It may be that the resulting increase in GFR during clamped hyperglycemia caused an overall increase in the delivery of

urinary filtrate and sodium to the macula densa, leading to augmented adenosine release and excretion. Alternatively, the expected increase in glycosuria during clamped hyperglycemia in the presence of SGLT2i may have also played a role through an osmotic diuretic effect, also promoting increased delivery of filtrate to the macula densa.

Measurement of urinary adenosine may provide insight into the role of this mediator as a regulator of renal hemodynamic function via effects on renal afferent arteriole tone in response to distal tubular sodium delivery. In addition to validating the current methodology used in this report in patients with T1D, there are other potential applications for the measurement of urinary adenosine. Other disease conditions that feature high levels of intraglomerular pressure, such as patients with chronic kidney disease (CKD), could be especially relevant. In theory, elevated levels of adenosine in the juxtaglomerular apparatus, as detected by its subsequent secretion, may play a protective role in patients with CKD by stimulating renal afferent vasoconstriction, thereby reducing intraglomerular hypertension. Assessing urinary adenosine secretion over time may help clinicians to further characterize CKD progression, while further establishing any differences between diabetic and non-diabetic CKD subtypes. Moreover, the effects of adenosine excretion on renal hemodynamic function could be investigated with regards to other therapies that impact the level of distal natriuresis, thereby altering macula densa-mediated adenosine release, such as incretin-based dipeptidyl peptidase 4 (DPP-4) inhibitors[74].

Finally, in addition to renal considerations, the measurement of urinary adenosine could be important for studies in heart failure, since adenosine is thought to mediate the cardiorenal syndrome. As seen with the adenosine antagonist rolofylline, there was no therapeutic benefit observed after blockade of intrarenal adenosine activity for three days in patients with heart failure[75]. Although adenosine antagonism may lead to a short-term diuretic effects and renal

vasodilatation, it is more likely that greater renoprotection, and subsequent cardiovascular benefit, will be achieved with preservation of renal perfusion after longer-term adenosine antagonism in patients with heart failure. Whether or not our technique captures physiologically relevant data in patients with heart failure is not yet known, but could be important to better assess and risk-stratify patients with heart failure at risk for renal hypoperfusion and the cardiorenal syndrome.

This study also has limitations. A more exhaustive endogenous urine adenosine stability study needs to be conducted, such that it will examine the stability of this analyte across different storage conditions, and after going through freeze-thaws cycles. Preliminary results obtained during method validation suggest that the endogenous analyte is stable across multiple freeze-thaw cycles. In addition, a reference interval should be established for urine adenosine using 120 healthy individuals (assuming no difference in sex or age), as per the minimum statistical standard for reference intervals by the Clinical and Laboratory Standards Institute. Our use of 40 healthy individuals for assay validation, primarily examined distribution of values in a healthy cohort in an exploratory fashion.

Our future studies will also include further investigation of the extract stability, while also investigating the relationship between urinary adenosine and other renal function parameters, such as GFR and effective renal plasma flow. It is also important to recognize that normal variations in GFR levels of tested individuals (i.e. dietary and circadian influences) may confound urine adenosine measures, due to hypothesized effects on macula densa solute delivery. Additionally, as depicted in our healthy control group, there may be a sex-based differences to urine adenosine excretion – with higher ACr expression found in females as compared to males (0.35 ± 0.12versus 0.28 ± 0.08 µmol/mmol Cr respectively). Higher urinary adenosine levels may reflect the impact of sex hormones, which may in turn affect renal perfusion – a possibility that merits further

research. The impact of this and other potential confounders on urinary adenosine excretion has not, to our knowledge, been extensively examined in humans.

In conclusion, this exploratory analysis presents an improved LC-MS/MS method for the reliable quantification of urinary adenosine in healthy humans and in patients with T1D. Furthermore, by utilizing this method we observed that SGLT2i increases adenosine excretion under hyperglycemic conditions –an effect that may contribute to the regulation of renal hemodynamic function in T1D, supporting the role of adenosine as a signalling molecule of TGF within the kidney. This work helps fill a critical knowledge gap regarding the underlying physiological mechanisms by which SGLT2 inhibition impacts kidney function leading to potential renoprotection. To maximize the value of these findings, future studies using this technique may allow for mechanistic insights into how SGLT2i promotes distal tubular sodium delivery to modify renal hemodynamic function outside of the typical diabetic setting. Diabetic kidney disease is but one pathology by which kidneys decline in function. Notably, these other subtypes of non-diabetic chronic kidney disease develop and progress to end-stage renal disease by similar pathophysiological mechanisms. Therefore, it follows that the shared incidence of clinical risk factors like elevated intraglomerular pressure and proteinuria between the setting of a diabetic kidney and that of a non-diabetic diseased kidney may extend to the biochemical expression and activity of intrarenal adenosine.

Patients with renal transplants exhibit an increased risk for weight gain and T2D due to metabolic side effects from immunosuppressive medications, and are at increased cardiovascular risk [75]. Based on weight loss, glycemic control, blood pressure lowering and cardiorenal protective effects with SGLT2i, this class of drugs has advantages over older, traditional antihyperglycemic agents, including insulin, that promote weight gain and hypertension. To better

elucidate the impact of SGLT2i effects in the kidney across different renal and metabolic contexts, the next chapter explores the clinical potential of SGLT2i use in renal transplant patients, which has not been previously described.

Tables and Figures

Table 2.1. Precision Measurements

	Within – Day				**Between - Day**				
Condition	N	Mean Adenosine (µM)	SD	% CV	n (days)	Total Reps.	Mean Adenosine (µM)	SD	% CV
QC $_{low}$	10	0.75	0.05	7.1	8	24	0.7	0.04	5.0
QC $_{med}$	10	6.6	0.29	4.4	8	24	6.3	0.22	3.5
QC $_{high}$	10	12.8	0.50	3.9	8	24	13.0	0.45	3.5

QC $_{low,}$ Low Adenosine Content Quality Group (injected 0.7 µM); QC $_{med}$, Medium Adenosine Content Quality Group (injected 6.0 µM); QC $_{high}$, HighAdenosine Content Quality Group (injected 13.0 µM); N, number of participant measurements; n, range of days; SD, Standard Deviation; %CV, Percent Co-efficient of Variation.

Table 2.2. S/N values of four unique urine samples.

Sample ID*	Adenosine (µM)	Mean (µM)	SD	% CV	S/N
L_1037-3 (1)	0.0044	0.0025	0.0023	89.5	2.3
L_1037-3 (2)	0.0022				1.7
L_1037-3 (3)	0.0048				1.4
L_1037-3 (4)	0.0045				2.3
L_1037-3 (5)	0.0002				2.0
L_1037-3 (6)	0.0010				1.6
L_1002-12 (1)	0.052	0.054	0.003	6.3	13.8
L_1002-12 (2)	0.052				14.5
L_1002-12 (3)	0.055				13.1
L_1002-12 (4)	0.051				12.6
L_1002-12 (5)	0.057				11.7
L_1002-12 (6)	0.060				14.8
L_1003-12 (1)	0.40	0.396	0.015	3.9	129.4
L_1003-12 (2)	0.40				178.8
L_1003-12 (3)	0.39				175.0
L_1003-12 (4)	0.41				160.4
L_1003-12 (5)	0.37				112.6
L_1003-12 (6)	0.39				109.9
L_1022-12 (1)	0.80	0.789	0.025	3.2	195.5
L_1022-12 (2)	0.76				223.3
L_1022-12 (3)	0.80				164.8
L_1022-12 (4)	0.81				243.2
L_1022-12 (5)	0.81				349.6
L_1022-12 (6)	0.80				286.6

Measured values of adenosine signal to noise in four patient urine samples (L_1037, L_1002, L_1003 &L_1022) via LC-MS/MS analysis, one patient pre-SGLT2i and three patients post-SGLT2i (six replicates of each patient).

Table 2.3. Accuracy Measurements via Recovery Exercise

Sample	**Initial (µM)**	**Added (µM)**	**Expected (µM)**	**Total Measured (µM)**	**Recovered (µM)**	**% Recovery**
1-spiked	0.91	1.0	1.9	1.91	1.0	100.0
2-spiked	0.91	5.0	5.9	5.92	5.0	100.2
3-spiked	0.91	10.00	10.9	10.90	10.0	99.9
					Mean	100.0
					SD	0.1
					CV(%)	0.1

Sampled urine from one patient was serially spiked with increasing levels of adenosine (1.0, 5.0 and 10.0 µM), following which their respective recovery efficiencies were determined.

Table 2.4. Carry-over Measurements

Sample ID	Adenosine Content (µM)	Mean Adenosine Content (µM)	SD	%CV	Carryover*, k (%)
A - High (1)	72.9	73.4	0.5	0.6	0.0083
A - High (2)	73.8				
A - High (3)	73.6				
A - Low (1)	0.11	0.1	0	5.4	
A - Low (2)	0.09				
A - Low (3)	0.10				
B - High (1)	81.4	83.1	1.5	1.8	-0.056
B - High (2)	83.5				
B - High (3)	84.3				
B - Low (1)	0.88	0.9	0	2.7	
B - Low (2)	0.89				
B - Low (3)	0.92				
C - High (1)	83.0	85.6	2.3	2.7	0.006
C - High (2)	86.6				
C - High (3)	87.3				
C - Low (1)	0.09	0.1	0	3.8	
C - Low (2)	0.10				
C - Low (3)	0.09				

*Carryover (k) measurements of three unique patient samples (A, B and C), variably spiked with adenosine, in triplicate. Carryover for each sample was calculated as $k=(low1-low3) / (high3-low3)$.

Table 2.5. Accuracy Measurements via Dilution

Sample*	Adenosine (µM)	Mean (µM)	SD	%CV	Expected (µM)	%BIAS
QC - undiluted (1)	63.6	63.7	0.31	0.5	68.1	-6.5
QC - undiluted (2)	63.4					
QC - undiluted (3)	64.0					
QC - 2X dilution (1)	33.0	34.8	1.59	4.6	34.1	2.3
QC - 2X dilution (2)	35.9					
QC - 2X dilution (3)	35.6					
QC - 4X dilution (1)	17.8	18.2	0.51	2.8	17.0	7.1
QC - 4X dilution (2)	18.1					
QC - 4X dilution (3)	18.8					
QC - 8X dilution (1)	9.4	9.2	0.18	1.9	8.5	8.1
QC - 8X dilution (2)	9.1					
QC - 8X dilution (3)	9.1					

Table 2.6. Measurement of Adenosine, Creatinine and Adenosine:Creatinine values.

	Value Range			**Mean**		
Group	Adenosine (µM)	Creatinine (mM)	Adenosine:Creatinine (µmol/mmol Cr)	Adenosine (µM)	Creatinine (mM)	Adenosine:Creatinine (µmol/mmol Cr)
HC	0.6 - 13.4	1.7 - 24.6	0.12 - 0.53	3.0 ± 2.2	9.4 ± 4.9	0.32 ± 0.11
T1D*	0.25 - 8.1	0.57- 29.0	0.19 - 0.66	2.56 ± 1.93	7.13 ± 6.19	0.40 ± 0.12

HC: Healthy Control cohort, T1D: Patients with Type 1 Diabetes * Values are from the pre-SGLT2i, euglycemia condition of patients with T1D.

Table 2.7. Health Control (HC) measured urinary values of Adenosine, Creatinine and Adenosine:Creatinine, stratified by sex. 40 unique participants in total (20-62 years of age, 17 M, 23 F)

HC (Male)	Sample Name	Age	Adenosine c (uM)	Creatinine (uM)	Adenosine (umol/mmol Creatinine)
	RR_1	52	6.1	16564	0.37
	RR_4	36	3.3	13189	0.25
	RR_5	31	1.5	10143	0.15
	RR_11	62	4.1	11415	0.36
	RR_12	55	2.1	6535	0.32
	RR_13	45	4.8	14330	0.34
	RR_18	34	5.8	14566	0.40
	RR_19	60	2.6	11368	0.23
	RR_20	34	0.8	2003	0.40
	RR_21	51	0.7	3114	0.24
	RR_22	49	1.7	5480	0.31
	RR_31	36	1.5	5491	0.28
	RR_33	33	5.2	19177	0.27
	RR_37	49	1.7	11683	0.14
	RR_40	38	0.9	4719	0.19
	RR_41	54	2.5	12097	0.20
	RR_44	37	4.9	15094	0.32
	Mean	**44.5±9.9**	**3.0±1.8**	**10410±4871**	**0.28±0.08**
HC (Female)	**Sample Name**	**Age**	**Adenosine c (uM)**	**Creatinine (uM)**	**Adenosine (umol/mmol Creatinine)**
	RR_2	34	1.5	5438	0.28
	RR_3	39	2.3	8231	0.28
	RR_6	57	1.2	3985	0.30
	RR_7	54	2.4	8584	0.28
	RR_8	45	12.6	24560	0.51
	RR_9	44	2.8	14779	0.19
	RR_10	40	3.9	8399	0.46
	RR_16	59	2.0	3740	0.53
	RR_17	43	3.5	8508	0.41
	RR_23	52	1.0	4073	0.24
	RR_24	43	4.4	11072	0.40
	RR_25	50	2.2	5563	0.39
	RR_26	24	4.7	11792	0.40
	RR_27	20	5.7	11949	0.48
	RR_28	28	4.0	12117	0.33
	RR_29	46	1.4	9315	0.14
	RR_30	48	0.7	1736	0.42
	RR_34	50	1.7	9132	0.19
	RR_35	47	1.2	10437	0.12
	RR_36	50	1.2	4965	0.25
	RR_38	52	1.8	3408	0.53
	RR_39	46	3.7	7861	0.47
	RR_42	28	4.5	10006	0.45
	Mean	**43.4±10.2**	**3.1±2.5**	**8680±4716**	**0.35±0.12**
	Total Mean	**43.9±10.1**	**3.0±2.2**	**9416±4858**	**0.32±0.11**

Figure 2.1. Representative LC-MS/MS chromatograms for adenosine and its labeled analogue measured with our assay in a urine sample containing 0.88 µM of adenosine

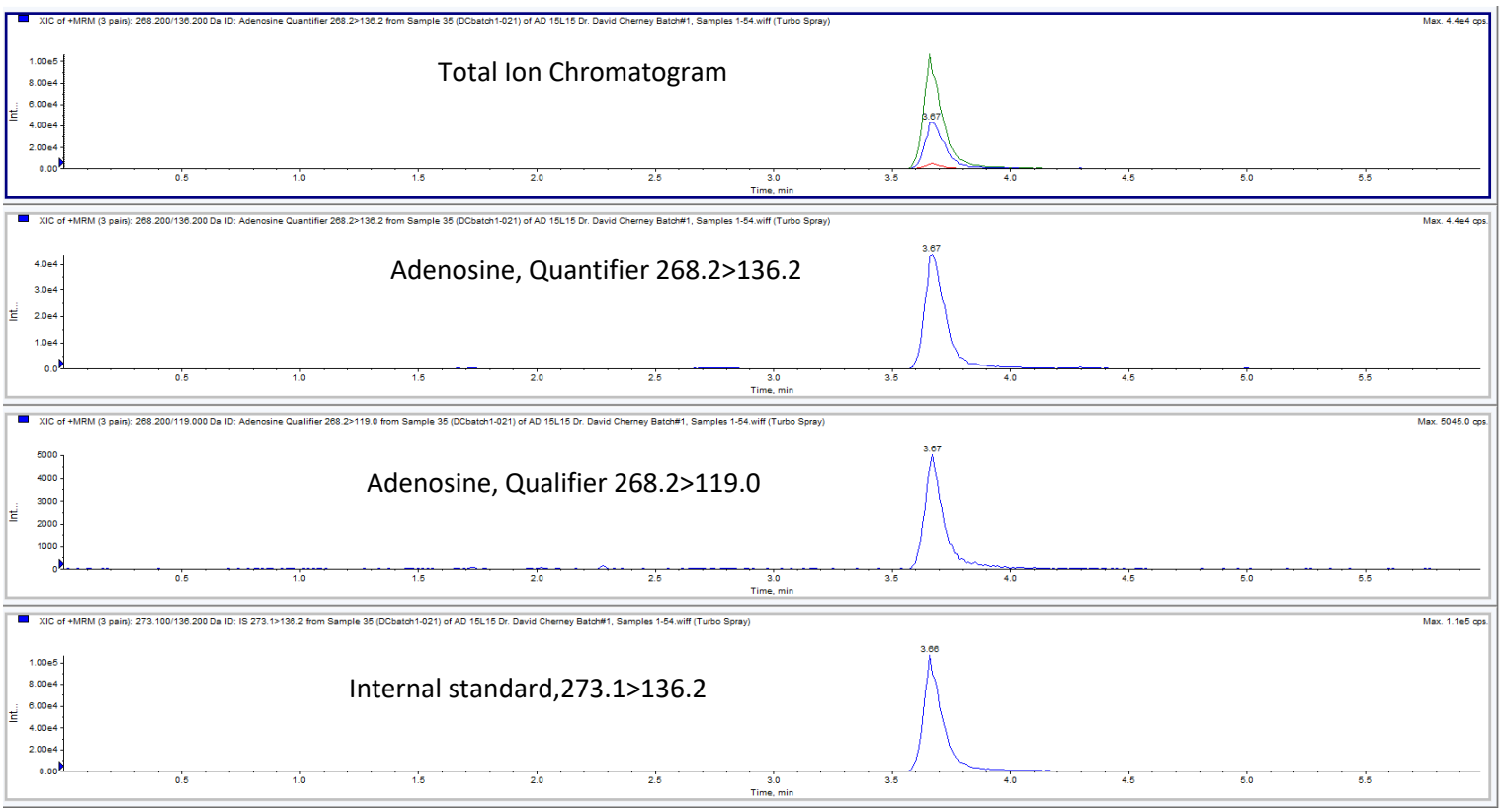


Figure 2.2. Dilution Linearity of Adenosine Measurements; each measured value was an average of three data values.

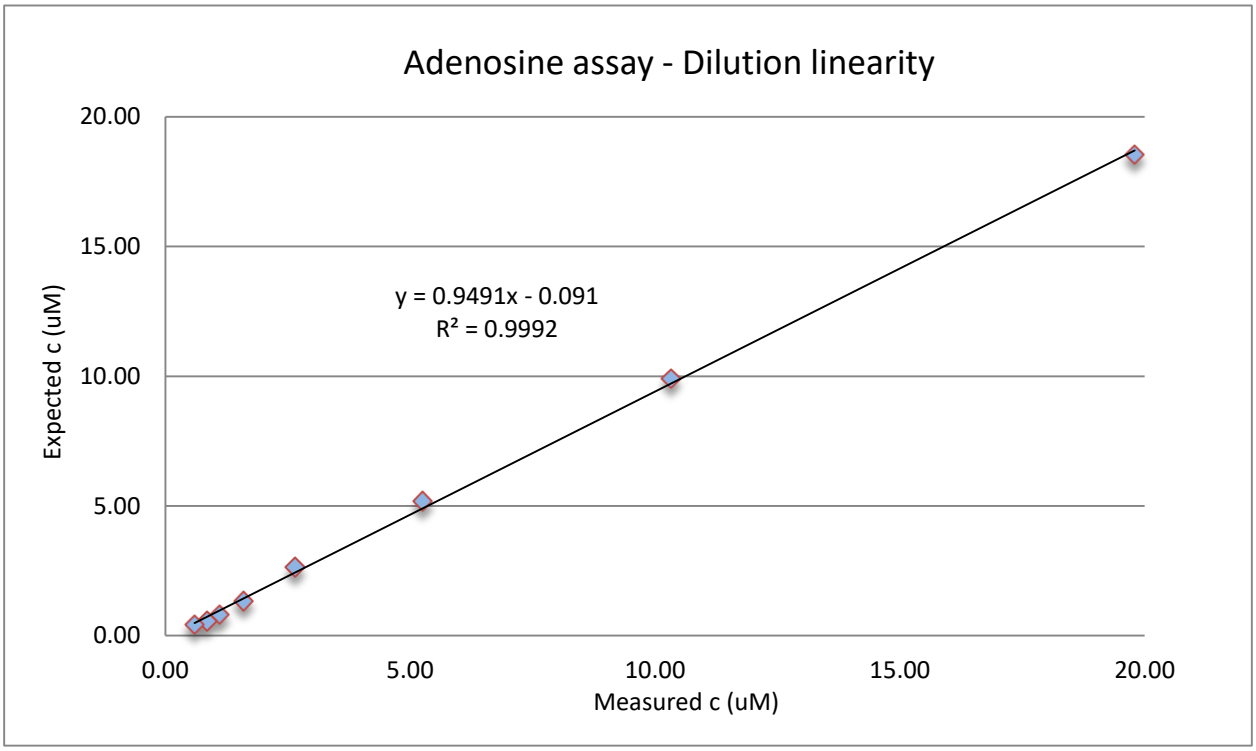
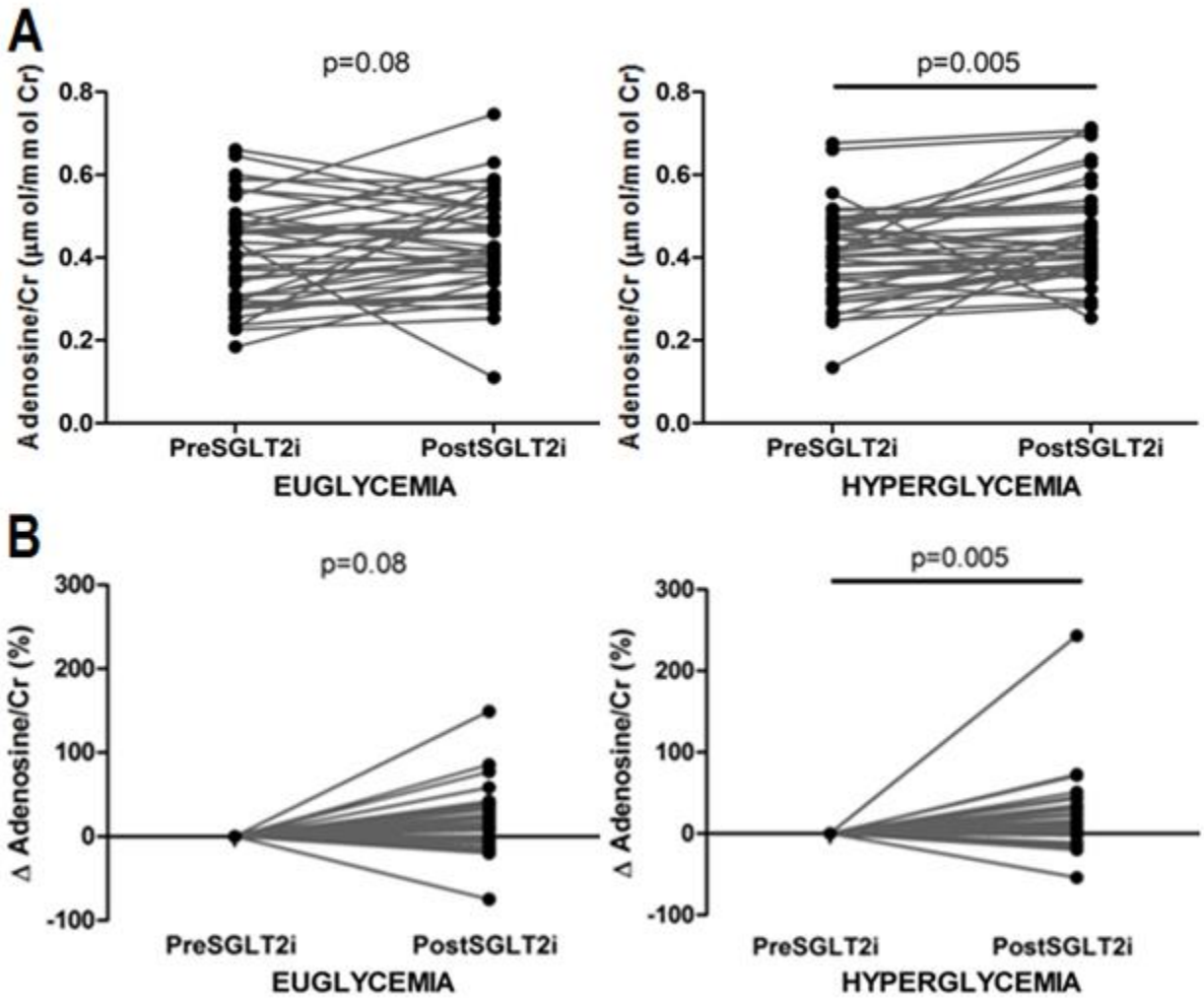


Figure 2.3. Urinary adenosine to creatinine values during clamped euglycemic and hyperglycemic conditions, before and after administration of the SGLT2 inhibitor, empagliflozin in 40 patients with T1D. (A) Absolute adenosine to creatinine ratio values. (B) Percent change in adenosine to creatinine ratio values between pre and post - SGLT2i conditions.



Use of Canagliflozin in Kidney Transplant Recipients for the Treatment of Type 2 Diabetes: A Case Series

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KEY WORDS: Type 2 Diabetes Mellitus, SGLT2 inhibition, Kidney Transplant Recipients

3.1 INTRODUCTION

Diabetes mellitus is highly prevalent in kidney transplant recipients (KTR). Simultaneous pancreas-kidney transplant recipients (SPKTR) are also at risk of developing type 2 diabetes following transplantation, where insulin secretion may be insufficient to maintain normoglycemia. Transplant-specific risk factors associated with the development of type 2 diabetes include the use of diabetogenic immunosuppressive medications, hypomagenesemia and post-transplant weight gain[76].

In non-transplant populations with type 2 diabetes and established cardiovascular (CV) disease, the use of sodium-glucose co-transporter 2 inhibitors (SGLT2i) can improve glycemic control, promote weight loss and reduce the risk of CV events[2]. Given the increased incidence of post-transplant diabetes mellitus and the high CV burden in transplant recipients, the use of SGLT2i in this population is attractive. Of concern, however, is the lack of safety data regarding SGLT2i in transplant recipients. The purpose of this study is to describe our short-term experience of KTR and SPKTR treated with canagliflozin at our institution.

3.2 RESEARCH, DESIGN & METHODS

All adult KTR or SPKTR treated with the SGLT2 inhibitor canagliflozin from 1 January 2015 to 1 November 2016 were included in this study. Baseline demographic and metabolic variables at the time of canagliflozin initiation were collected. Adverse events including hypoglycaemia, acute kidney injury (AKI), hyperkalemia, yeast and urinary tract infections, ketoacidosis, allergic reactions and graft rejection were collected over follow-up.

3.3 RESULTS

Baseline characteristics of study patients and mean changes in metabolic and hemodynamic parameters over 80.5 person-months of follow-up after canagliflozin initiation are summarized in the Tables 3.1 and 3.2 respectively.

3.4 DISCUSSION

Although SGLT2i have been widely used in the non-transplant population, to our knowledge, this is the first report describing the use of these agents in transplant recipients. Given the susceptibility to infectious complications in patients with diabetes and concomitant immunosuppression, clinicians may avoid SGLT2 inhibitors given their side effects. In this small observational cohort, canagliflozin was generally well tolerated.

We did not observe any episodes of AKI. As expected based on data in non-transplant patients, we observed small reductions in eGFR, an effect that has been associated with renal afferent arteriole vasoconstriction due to increased sodium delivery sensing at the macula densa and subsequent modulation of the renal afferent arteriole tone – in a process known as tubuloglomerular feedback[25]. In experimental models, vasoconstriction at the afferent arteriole reduces hyperfiltration – an effect that mitigates renal disease progression in patients with diabetes[66]. While it is not known if such renoprotective effects extend to transplanted kidneys, effects on eGFR in this study suggest the hemodynamic-based eGFR changes occur even though transplanted kidneys are denervated. The characteristic eGFR "dip" is therefore unlikely due to changes in autonomic function and sympathetic activation.

We observed overall improvements in glycemic control, weight and blood pressure, which were similar in magnitude to effects reported in non-transplant cohorts. In non-transplant

populations, SGLT2i reduces diastolic dysfunction, major adverse cardiac events and diabetic nephropathy risk[2, 4, 25]. In transplant recipients, where hypertension and CV disease are commonly depicted, SGLT2i may therefore be an important therapeutic option for its additive clinical benefit.

In conclusion, our data suggest that SGLT2i in KTR and SPKTR is well tolerated, and may have similar therapeutic efficacy compared to non-transplant patients. Our experience highlights the importance of future studies of SGLT2i in a larger cohort of kidney transplant recipients, over an extended period of time. Moreover, our results show that SGLT2i-induced kidney protection can extend to clinical contexts outside of its conventional application in diabetes, possibly via attenuating effects on ubiquitous renal risk factors of renal hemodynamic function.

Our understanding of the cardiorenal effects of SGLT2i has primarily been derived in patients with T2D. However, it seems increasingly clear that the major physiological benefits of SGLT2i in the cardiovascular and renal systems are based on natriuresis (blood pressure and albuminuria lowering and reductions in cardiorenal complications) rather than glucosuria. As a consequence, it is important to better understand whether or not non-hyperglycemic, non-diabetic patients are protected by the natriuretic effects of SGLT2i. To better characterize the impact of SGLT2i on the kidney outside of the context of diabetes, we studied the effects of dapagliflozin in a form of non-diabetic CKD known as focal segmental glomerulosclerosis in afflicted patients.

Tables

Table 3.1. Baseline Characteristics of Study Participants

Parameter	SPK Transplant Recipients (N=4)	Kidney Transplant Recipients (N=6)
Age at time of canagliflozin initiation, years (Mean ± SD)	49.4±8.9	61.6± 12.6
Female Sex, N (%)	2 (50)	1 (17)
PTDM, N (%)	4 (100)	4 (67)
Prior DM Therapy, N (%)	3 (75)	5 (83)
Time from transplant to canagliflozin treatment, years (Mean ± SD)	3.5 ± 3.9	4.4 ± 3.3
Time on treatment, months (Mean ± SD)	5.6 ± 3.4	10.1 ± 4.2
Baseline Hemoglobin A1c, % (Mean ± SD)	7.4 ± 1.1	8.6 ± 1.4
Baseline Hemoglobin A1c, mmol/mol (Mean ± SD)	57 ± 12.0	70 ± 15.3
Baseline eGFR, ml/min/1.73m2, (Mean ± SD)	60 ± 14	78 ± 18.2
Baseline serum Creatinine, μmol/L (Mean ± SD)	108.3 ± 21.6	90.2 ± 22.9
ACEi therapy, N (%)	1 (25)	0 (0)
ARB therapy, N (%)	0 (0)	2 (50)
Diuretic therapy, N (%)	3 (75)	2 (50)
Calcium channel blocker therapy, N (%)	4 (100)	3 (75)
Alpha adrenergic antagonist therapy, N (%)	1 (25)	1 (25)
Beta blocker therapy, N (%)	4 (100)	2 (50)

Table 3.2. Parameter Change following SGLT2i Treatment Across Transplant Patients

Parameter	Mean change over follow-up
Hemoglobin A1c, % (N=9)(Mean ± SD)	-0.84 ±1.2 ; p =0.07
Hemoglobin A1c, mmol/mol (N=9)(Mean ± SD)	-9.2 ±13.1; p =0.07
Weight, Kg (N=8) (Mean ± SD)	-2.14 ±2.8 ; p =0.07
Serum Sodium, mmol/L (N=10) (Mean ± SD)	0.6 ±2.2 ; p =0.4
Serum Potassium,mmol/L (N=10) (Mean ± SD)	0.2 ±0.5 ; p =0.2
Systolic Blood Pressure,mmHg (N=8) (Mean ± SD)	-6.5 ± 10.8; p=0.13
Diastolic Blood Pressure, mmHg (N=8) (Mean ± SD)	-4.8 ±12 ; p =0.3
Hematocrit, % (N=10) (Mean ± SD)	1.6 ±2.5 ; p =0.08
Serum Creatinine,μmol/L (N=10) (Mean ± SD)	9.7 ±14.6 ; p =0.06
eGFR, ml/min/1.73^2(N=10) (Mean ± SD)	-4.3±12.2 ; p =0.3

Dapagliflozin in Focal Segmental Glomerulosclerosis: A Pilot Study

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Abbreviated Title: SGLT2 inhibition in FSGS

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4.1 ABSTRACT:

Background: Focal segmental glomerulosclerosis (FSGS) is an important cause of non-diabetic chronic kidney disease (CKD). Sodium glucose cotransporter-2 inhibition (SGLT2i) therapy attenuates the progression of diabetic nephropathy, but it remains unclear whether SGLT2i provides renoprotection in the context of non-diabetic CKD such as FSGS.

Objective: The primary aim of this pilot study was to determine the effect of SGLT2i with dapagliflozin for 8 weeks on GFR in humans with FSGS. Secondary endpoints were related to changes in renal hemodynamic function, proteinuria and blood pressure.

Methods and Results: GFR (inulin) and renal plasma flow (para-aminohippurate), proteinuria and blood pressure were measured in patients with FSGS (n=10). In response to dapagliflozin, changes in GFR, renal plasma flow and 24-hour urine protein excretion were not statistically significant in our patient cohort. Following drug treatment changes in blood pressure were not statistically significant in study participants (systolic blood pressure: 112.7±8.5 to 109.6±8.9 mmHg, diastolic blood pressure: 71.8±6.5 to 69.1±7.9 mmHg, p=NS), while hematocrit increased to a significant degree (0.40±0.054% to 0.42±0.049%, p=0.03).

Conclusions: Short-term treatment with the SGLT2i dapagliflozin did not modify renal hemodynamic function or attenuate proteinuria our patients with FSGS. Further studies examining the impact of SGLT2i on markers of kidney disease in patients with other causes of non-diabetic CKD are needed.

4.2 INTRODUCTION:

Focal segmental glomerulosclerosis (FSGS) is characterized by proteinuria and renal function decline, leading to chronic kidney disease (CKD) and end-stage renal disease (ESRD)[77]. Approximately 55% of patients with idiopathic FSGS with high grade proteinuria will proceed to ESRD at 10 years following diagnosis[78]. The FSGS lesion can also develop as the end result of a spectrum of sources of glomerular injury, obesity, and reduced nephron mass. Despite current therapies including renin-angiotensin-aldosterone system (RAAS) inhibitors, anti-hypertensives and immunosuppressants, clinical outcomes remain sub-optimal.

Beyond possible immune-mediated pathophysiological mechanisms responsible for development of the primary FSGS, elevated intraglomerular pressure is associated with CKD progression in patients with FSGS[79]. Accordingly, agents that block the RAAS exert renal protection in FSGS, in part via hemodynamic effects to reduce intraglomerular hypertension, single nephron GFR and proteinuria via vasodilatation of the efferent renal arteriole[80]. Unfortunately, RAAS blockade does not abolish glomerular hypertension or proteinuria in patients with CKD[81].

In addition to modulation of neurohormonal pathways, studies in animals and in humans with diabetes mellitus have implicated tubular factors in the pathogenesis of increased glomerular pressure and hyperfiltration leading to CKD progression. According to the tubular hypothesis of hyperfiltration, reabsorption of sodium (Na^+) and glucose by sodium-glucose co-transporter 2 (SGLT2) at the proximal tubule is an important regulator of renal hemodynamic function[82]. Augmented SGLT2 activity in the setting of diabetes reduces Na^+ delivery at the distal macula densa, which is sensed as a reduction in effective circulating volume via a process known as tubuloglomerular feedback (TGF)[22]. As a result, agents that increase sodium delivery to the

macula densa, including proximal tubular diuretics (i.e. acetazolamide) and SGLT2i, lead to afferent vasoconstriction, decreased renal blood flow and reduced intraglomerular hypertension, thereby reducing proteinuria[5].For example, SGLT2 inhibition reduces hyperfiltration and intraglomerular pressure in patients with type 1 diabetes[31]. Notably, monotherapy with either SGLT2i or RAAS blockade fails to abolish hyperfiltration, suggesting that the combined use of agents that target both afferent and efferent factors is required to normalize hyperfiltration and renal abnormalities in patients at risk for CKD. In patients with type 2 diabetes and cardiovascular disease, SGLT2i with empagliflozin reduced the composite nephropathy endpoint[27, 83]. Although it is not known if these benefits extend to patients without diabetes, large clinical trials such as DAPA-CKD (NCT03036150) are underway to determine if SGLT2 inhibition reduces the risk of progressive non-diabetic CKD. These studies are based on the hypothesis that similarly to diabetes, modulation of TGF may reduce kidney injury that may occur in FSGS due to maladaptive renal hyperfiltration, even in the absence of hyperglycemia. Despite interest in this area, especially in patients with diabetes, very little is known about the effects of SGLT2i on proteinuria, renal function or blood pressure in patients with non-diabetic CKD.

Accordingly, the aim of this proof-of-concept pilot study was to examine the effect of dapagliflozin as an adjunct to RAAS blockade for 8 weeks on renal hemodynamic function, proteinuria, blood pressure and body weight in patients with FSGS. As the primary endpoint, we hypothesized that SGLT2i would reduce GFR due to vasoconstrictive effects at the afferent arteriole. For secondary endpoints, we measured the impact of dapagliflozin on glomerular filtration rate (GFR) and effective renal plasma flow (ERPF), proteinuria, blood pressure and metabolic parameters.

4.3 MATERIALS AND METHODS:

Study Participants

The flow chart for participants is shown in Figure 4.1. In this open-label, pilot clinical trial, 10 participants with FSGS were treated with dapagliflozin 10 mg once daily for 8 weeks as an add-on to RAAS blockade therapy to evaluate effects on proteinuria and renal hemodynamic function. Detailed inclusion criteria were as follows: 1) male or female subjects diagnosed with biopsy-proven FSGS ≥ 1 month prior to informed consent; 2) creatinine-based GFR ≥45 ml/min/1.73m^2; 3) age > 18 years; 4) no history of diabetes mellitus; 5) body mass index (BMI) 18.5-45.0 kg/m^2; 6) blood pressure (BP) ≥ 100/60 mmHg at screening; 7) therapy with a RAAS inhibitor (either an ACEi, ARB or direct renin inhibitor) for > 1 month; 8) >30 mg/day and <6 g/day of proteinuria. Exclusion criteria at screening were: 1) leukocyte and/or nitrate positive urinalysis that was untreated; 2) history of organ transplantation; 3) bariatric surgery or gastrointestinal surgeries that induced chronic malabsorption within the past two years; 4) current treatment with systemic corticosteroids, calcineurin inhibitors, or other immunosuppressant therapies; 5) blood dyscrasias or any disorders causing hemolysis or unstable red blood cells; 6) pre-menopausal women who were nursing, pregnant, or of child-bearing potential and not practicing an acceptable method of birth control; 7) participation in another therapeutic trial with an investigational drug within 30 days prior to informed consent; 8) alcohol or drug abuse within three months prior to informed consent that would interfere with trial participation or any ongoing clinical condition that would jeopardize subject safety or study compliance based on investigator judgment; 9) liver disease, defined by serum levels of alanine transaminase, aspartate transaminase, or alkaline phosphatase >3x upper limit of normal as determined during screening; 10) cardiac, lung or peripheral vascular disease or stroke; 11) pancreas, pancreatic islet cell or renal transplant recipient; 12) medical

history of cancer or treatment of cancer in the last 5 years prior to screening; 13) history of allergy or angioedema with RAAS inhibitor exposure; 14) kidney disease due primarily to another identifiable condition aside from FSGS. The local Research Ethics Board at the University Health Network (Toronto, Canada) approved the protocol and all subjects gave informed consent prior to start of study procedures. The study was conducted according to the International Conference on Harmonization on Good Clinical Practice (www.clinicaltrials.gov: NCT02585804).

Clinical Experimental Design

This clinical trial consisted of 5 visits (Figure 4.2): 1) a successful recruitment visit (Visit 1, V1) followed by a 1 week preparation of specified diet: targeting ≥150 mmol/day Na^+ and ≤1.5 g/kg/day protein, to avoid the effects of circulating volume contraction and/or RAAS activation by low Na^+ and hyperfiltration via high protein intake; 2) the first full study visit (Visit 2, V2) starting at 0745 hours, when participants underwent baseline measurements of neurohormonal levels, renal hemodynamic function (GFR, ERPF) and blood pressure; 3) the next day participants started dapagliflozin 10 mg PO daily for 8 weeks, during which they would return to the laboratory at 2 weeks for a safety assessment (Visit 3, V3); 4) the second full study visit (Visit 4, V4) starting at 0745 hours at the end of the 8 week drug treatment period, when participants had all procedures from V2 repeated; 5) a 1-week wash out period, when participants returned for a final appointment (Visit 5, V5) for safety assessments.

During full study visits, V2 and V4, participants fasted for the duration of their stay. On both days, firstly blood samples were collected for inulin and para-aminohippurate blank and RAAS mediators using established techniques[84]. Following these blood and urine tests, blood pressure was measured using an automated sphygmomanometer (Critikon automated

sphygmomanometer, Tampa, Florida, USA) at 30-minute intervals. GFR and ERPF (per 1.73 m^2) were measured in the supine position using inulin and para-aminohippurate clearances[85]. Filtration fraction (GFR/ERPF), renal blood flow (ERPF/[1- hematocrit]) and renal vascular resistance (mean arterial pressure/renal blood flow) were also calculated using standard methods[86, 87]. The Gomez equations were used to calculate efferent R_E and afferent resistance R_A and glomerular pressure, as described elsewhere[86-89].

Data Analysis

Variables were checked for the distributional assumption of normality using normal plots. Variables that were positively skewed were natural log-transformed for the analyses. Paired *t*-tests were used for single measured parameters, and linear mixed models were used for repeated parameters. A two-sided $p<0.05$ was considered statically significant. Analyses were stratified by baseline GFR ($\geq$90ml/min/1.73m^2 or <90ml/min/1.73m^2) and $\geq$ and < median 24-hour proteinuria. Data are presented as mean ± SD for normally distributed variables, and median (range) for positively skewed variables (e.g. renin and aldosterone). Analyses were performed in SAS (version 9.4 for Windows; SAS Institute, Cary, NC). Statistical significance was determined by one-way ANOVA with a Fisher least significance difference test for comparison of multiple groups. Data are presented as mean ± SD.

4.4 RESULTS:

Screening Clinical Characteristics of Study Cohort

The study group comprised of 10 patients with FSGS within an age range of 27–51 years (Table 4.1). All participants had FSGS > 1 month and were taking concomitant RAAS inhibition

for > 1 month (n=3 taking an ACE inhibitor and n=7 taking an angiotensin receptor blocker). One participant failed to proceed to study completion, and stopped drug after 2 weeks of treatment due to non-specific malaise and a feeling of weakness, with no evidence of hypotension, hypoglycemia or genitourinary tract infection. Although no particular severe adverse event was reported, the participant discontinued study participation but did collect and submit a 24-hour urine sample for the primary analysis while on treatment for 2 weeks, which was included in the analysis. Renal hemodynamic parameters were not measured in this patient. All study patients returned to the laboratory approximately 1 week following last drug intake date for follow-up safety analysis across urine and blood examinations.

Effects of Dapagliflozin on Proteinuria, Renal Hemodynamic Function and Blood Pressure in Study Participants

Inulin and PAH-based changes in GFR and ERPF in response to dapagliflozin did not reach statistical significance (Table 4. 1). Similarly, changes in RVR, RBF, FF, R_a, R_e, and P_{GLO} were not statistically significant (Table 4. 1). In a sensitivity analysis, in which study participants were stratified into two subgroups as a function of GFR (GFR<90 or GFR≥90 ml/min/1.73m^{2}at baseline), there was a significant reduction in GFR following dapagliflozin therapy at 8 weeks in those with GFR≥90 ml/min/1.73m^2, and plasma aldosterone increased in this subgroup (Table 4.2).

Dapagliflozin therapy for 8 weeks did not reduce proteinuria in the overall clinical cohort (Table 4.1). In a *post-hoc* sensitivity analysis, patients with 24-hour protein levels below the median (median 24-hour proteinuria - 1.89 grams/day) demonstrated a significant reduction in proteinuria (Table 4.3, Figure 4.2). In response to dapagliflozin, numerical reductions in blood pressure changes did not reach statistical significance (Table 4.1).

Effect of Dapagliflozin on Body Weight, Hematocrit and RAAS Mediators in Study Participants

Body weight did not change significantly following dapagliflozin therapy (Table 4.1). On the other hand, hematocrit values increased significantly from V2 to V4. There were no significant changes in plasma renin or aldosterone levels from V2 to V4 (Table 4.1).

As part of the sensitivity analysis based on GFR, there was a significant increase in plasma aldosterone levels in those with GFR $\geq$ 90 ml/min/1.73m^2 at baseline (Table 4.2). When stratified based on baseline proteinuria, there was a significant increase in plasma aldosterone in those with baseline 24-hour proteinuria above the median (Table 4.3).

4.5 DISCUSSION:

Animal and human models of diabetes mellitus have shown that SGLT2 inhibition-related natriuresis increases macula densa adenosine generation, thereby activating tubuloglomerular feedback and afferent vasoconstriction leading to attenuation of hyperfiltration[18]. Decreased intraglomerular pressure is associated with reduced mechanical stretch and wall tension, suppressing renal inflammation and fibrosis[90]. Despite their routine use as antihyperglycemic agents, intrarenal hemodynamic effects of SGLT2i agents are likely related to natriuresis rather than glucosuria. As a consequence of natriuresis rather than glucosuria, the effects of SGLT2i in the kidney may extend to animals and humans without diabetes or ambient hyperglycemia, due to ubiquitous effects of proximal natriuresis on TGF[5, 91, 92]. In this proof-of-concept clinical study, our aim was to investigate the effect of SGLT2i on renal hemodynamic function in FSGS as a measure of glomerular hypertension, blood pressure and proteinuria.

Our first major observation was that although the intervention was general well tolerated in this relatively unique SGLT2i study cohort, dapagliflozin did not influence renal hemodynamic function in patients with FSGS or in the animal models used in this series of experiments. Despite the lack of statistical significance overall, GFR did decrease numerically in patients with FSGS at 8 weeks in response to SGLT2i to an extent that is similar to that expected in patients with diabetes mellitus[25]. The results of our sensitivity analyses further suggest that renal hemodynamic effects are most prominent in individuals with GFR $\geq$ 90 ml/min/1.73m^2, perhaps due to the greater filtered load of sodium, and in patients with modest proteinuria. Based on this pilot data, patients with FSGS and markers of more mild disease may be more responsive to SGLT2i. Importantly, the impact of changes in proteinuria on CKD progression in secondary FSGS may not be as critically important as they are in primary FSGS. Therefore, significant renal hemodynamic effects in participants with GFR $\geq$90 ml/min/1.73m^2, which may reflect an important physiological reduction in glomerular hypertension, may be physiologically beneficial and preserve renal function over time[93].

Our second major observation was that dapagliflozin did not reduce proteinuria in patients with FSGS. While it is difficult to determine why proteinuria did not decrease in the overall cohort, there are plausible explanations are worth considering. Firstly, the lack of an antiproteinuric effect at 8 weeks may in part reflect the activation of compensatory neurohormonal pathways – such as the RAAS - over time. Secondly, the diminished impact of SGLT2i may be related to substandard bio-availability of drug substrate, like that of the SGLT2 protein itself, specific to this disease context. Significant upregulation of SGLT2 mRNA has been observed in both animals and humans with diabetes, thereby demonstrating possible state-specific modulation of SGLT2[94]. Future work concerning modulatory effects on SGLT2 physiology across various disease contexts

and stimulatory milieus are needed to tailor effective SGLT2i clinical therapy. Moreover, while reduction in proteinuria in glomerular-based disease is a common surrogate marker of renal protection, it is important to note that the mechanisms responsible for renal protection with SGLT2i – including in the setting of diabetes – remain unknown. Therefore, the lack of effect on proteinuria in the setting of FSGS does not necessarily rule out beneficial effects, particularly in light of apparent acute effects on GFR in the preserved renal function range.

In *post hoc* sensitivity analyses, when stratified for GFR at baseline, those individuals who had GFR ≥90 ml/min/1.73m^2 exhibited a significant increase in plasma aldosterone following dapagliflozin therapy. Since RAAS activation can lead to intraglomerular hypertension, increased plasma aldosterone may have attenuated the maximal antiproteinuric effects of dapagliflozin over time[95].

SGLT2 inhibitors typically lead to sustained reductions in SBP by 4-6 mmHg and DBP by 1-2 mmHg in patients with type 2 diabetes and baseline blood pressure values of approximately 130-140 mmHg systolic and 75-85 mmHg diastolic[25]. In patients with type 1 diabetes with baseline blood pressure values (111/64 mmHg) that were similar to those in present cohort, empagliflozin treatment for 8 weeks reduced SBP by ~2.5 mmHg[96]. Therefore, although not statistically significant, the magnitude of the blood pressure reduction observed in patients in the present study was in the expected range. Whether these blood pressure lowering effects would have been accentuated in hypertensive FSGS patients is not known, but should be examined in future work. For the mechanism of blood pressure lowering, dapagliflozin was associated with a significant rise in hematocrit in patients with FSGS. SGLT2 inhibition-associated increases in hematocrit have been reported in studies involving patients with type 1 and 2 diabetes, and likely represent natriuresis-associated hemoconcentration[25, 31], although direct effects on erythropoiesis

have also been hypothesized[97]. In conjunction with studies demonstrating long-term plasma volume contraction using I^{131}-albuminin response to SGLT2 inhibitors, the rise in hematocrit likely reflects hemoconcentration on the basis of natriuresis, leading to blood pressure lowering.

Our study results may have implications for ongoing and planned clinical trials in the setting of non-diabetic CKD. If effects on GFR and glomerular pressure are most prominent in patients with GFR ≥90 ml/min/1.73m^2, then clinical trials focusing on patients with CKD stages 2-4 may miss relevant renal hemodynamic and hence renoprotective effects. On the other hand, patients with lower levels of baseline renal function may exhibit higher baseline BP levels, which may lead to exaggerated anti-hypertensive responses to SGLT2i. Based on our observations, although current trials will recruit patients with diverse etiologies of non-diabetic CKD, FSGS appears to be a setting where SGLT2i-related effects are less likely to be successful. Moreover, previous studies reported similarly neutral effects on renal histology and experimental studies using polycystic and nephrocalcinosis models of non-glomerular, non-diabetic kidney disease, as had a smaller study employing the remnant kidney model[98], suggesting SGLT2i may be ineffective in these settings[99, 100]. A major caveat is that the impact of SGLT2i on proteinuria in FSGS may be limited to those with lower levels of proteinuria, and effects on GFR – reflecting intraglomerular pressure – may only occur in patients with preserved renal function with GFR ≥90 ml/min/1.73m^2. Unfortunately, detecting longer-term benefits in these relatively healthy patients will be difficult in the context of a renal outcome trial due to the prolonged length of time it will likely take to accrue endpoints.

Our experiments do have important limitations worth mentioning. First, the small sample size in the patient study may have reduced our ability to detect significant differences in physiological parameters such as GFR. We tried to minimize the impact of the small sample size

in the patient study by standardizing pre-study conditions, including dietary intake of sodium and protein. Our within-participant study design also helped to minimize physiological variation in parameters such as BMI, allowing individuals to act as their own control over time. Second, we acknowledge that, for ethical and practical reasons, SGLT2 expression was not measured in the kidneys of the participants of the interventional study. Finally, in terms of disease etiology, a further limitation is that our pilot human data included patients with primary FSGS or secondary FSGS. As a result, there is greater pathophysiological heterogeneity among the study participants, obscuring our understanding of the renal impact of SGLT2i. While the clinical distinction of primary versus secondary FSGS can prove challenging, there are established differences. For instance, in addition to exclusion of secondary causes, primary FSGS is associated with nephrotic syndrome (≥3.5 g/day proteinuria, ≤3.5 g/dL serum albumin) and extensive foot process effacement of glomerular podocytes, whereas secondary FSGS diagnoses typically lack significant hypoalbuminemia and podocyte injury[101]. Even still, possible secondary causes for FSGS can vary widely, from diminished nephron mass, to infectious agents and genetic abnormalities. Thus the effect of SGLT2 inhibition, by reduced renal perfusion via attenuated afferent glomerular tone, likely mitigates renal damage inconsistently across the various subtypes of FSGS. As an example, the renoprotection of SGLT2i in oligomeganephronia, a congenital disease of reduced and malformed glomeruli, may yield significant benefit due to its direct hemodynamic effects - compared to non-mechanical stressors of podocyte integrity[102]. Ultimately, the etiology of FSGS is highly varied in its disruption of the glomerular epithelium. Future investigations should accordingly examine the impact of SGLT2i therapy across different subtypes of FSGS.

In conclusion, SGLT2 inhibition did not influence renal hemodynamic function or lower proteinuria in patients with FSGS. The lack of effect on renal endpoints in FSGS is unclear and

merits further investigation. Future mechanistic studies using SGLT2i should consider including patients with hypertension, and those with earlier disease (i.e. higher GFR, less proteinuria), and should also include patients with other etiologies of non-diabetic CKD.

Tables and Figures

Table 4.1: Clinical characteristics, hemodynamic function, biochemistry at baseline and 8 weeks

	Baseline (V2)	Post-Treatment (V4)	P-value
Demographics			
Age, years	37.2±9.2	-	-
Female Sex - n (%)	4 (40)	-	-
Baseline BMI (kg/m^2)	30.0±8.2	-	-
FSGS duration, years	5.6±5.3	-	-
Medications			
Statin, N (%)	3 (30)	-	-
Xanthine Oxidase Inhibition, N (%)	2 (20)	-	-
Calcium Channel Blocker, N (%)	1 (10)	-	-
Proton Pump Inhibitor, N (%)	1 (10)	-	-
β_2 Adrenergic Agonist, N (%)	1 (10)	-	-
Serotonin Receptor Agonist, N (%)	1 (10)	-	-
Histamine 2antagonist	1 (10)	-	-
Diuretics	0	-	-
Renal Hemodynamic Function			
Glomerular filtration rate	93.9 ±18.2	85.9±16.9	0.22
Effective renal plasma flow	513.5 ±161.2	496.6±152.0	0.19
Filtration fraction	0.19±0.035	0.18±0.039	0.85
Renal blood flow	881.7±287.1	853.0±245.6	0.42
Renal vascular resistance	0.11±0.03	0.11±0.03	0.74
Afferent resistance (R_A)	4117.3±1588.0	3832.7±1409.5	0.33
Efferent resistance (R_E)	935.5±255.1	908.7±242.3	0.46
Glomerular pressure (P_{GLO})	44.6±3.8	44.8±4.8	0.78
24 hour urine protein	2.6±1.9	2.4±2.2	0.42
Systemic Hemodynamic, Body Weight			
Systolic blood pressure	112.7±8.5	112.8±11.2	0.99
Diastolic blood pressure	71.8±6.5	69.6±8.4	0.43
Mean arterial pressure	84.3±6.7	84.2±7.8	0.97
Body Weight	88.2±25.1	87.0±25.4	0.11
Plasma Biochemistry			
HbA1c	0.055±0.0051	0.055±0.0056	0.85
Hematocrit	0.40±0.054	0.42±0.049	0.023
Total Protein	64.7±7.2	66.1±6.8	0.16
Plasma Aldosterone*	251.0, 149.0-336.0	350.0, 224.0-376.0	0.17
Plasma Renin*	68.8, 28.0-133.5	121.5, 28.4-174.7	0.077

24-hour urine protein in mg protein/collection period; GFR, ERPF, RBF in $ml/min/1.73m^2$; R_A, R_E in dyn s cm^{-5}; RVR in mmHg/L/min;SBP, DBP, MAP, P_{GLO} in mmHg; Body Weight in kg; Hematocrit in L/L ; Plasma Aldosterone in pM; Plasma Renin in ng/L [*(Median, Q1-Q3)]

Table 4.2. Sensitivity Analysis by GFR_{INULIN} – Change from V2 to V4

	GFR Group	N	Mean difference±SD	p-value
Renal Hemodynamic Function				
△GFR_{INULIN}	<90 ml/min/1.73m^2	4	+4.13±11.3	0.52
	≥90 ml/min/1.73m^2	5	-14.9±11.3	0.042
△ERPF	<90 ml/min/1.73m^2	4	- 20.4±33.2	0.31
	≥90 ml/min/1.73m^2	5	- 31.2±72.7	0.39
Plasma Biochemistry				
△Plasma aldosterone	<90 ml/min/1.73m^2	4	+9.8±334.5	0.92
	≥90 ml/min/1.73m^2	5	+106.0±110.2	0.017

GFR, ERPF inml/min/1.73m^2, plasma aldosterone (pmol/L)

Table 4.3. Sensitivity Analysis by 24 Hour Proteinuria – Change from V2 to V4

	Proteinuria group	N	Mean difference±SD	p-value
Proteinuria				
△24 Hour	24 Hr Protein ≥ Median	5	+ 0.004 ± 0.72	0.99
Proteinuria	24 Hr Protein < Median	5	- 0.27 ± 0.21	0.042
Renal Hemodynamic Function				
△GFR	24 Hr Protein ≥ Median	5	- 11.7 ± 10.5	0.067
	24 Hr Protein < Median	4	+ 0.13 ± 17.7	0.99
△ERPF	24 Hr Protein ≥ Median	5	- 26.8 ± 69.1	0.44
	24 Hr Protein < Median	4	- 25.9 ± 43.2	0.32
Plasma Biochemistry				
△Plasma	24 Hr Protein ≥ Median	5	+ 77.4 ± 19.6	0.019
Aldosterone	24 Hr Protein < Median	4	+ 45.5 ± 30.9	0.88

24 Hour Protein in grams of protein/collection period, GFR, ERPF in ml/min/1.73m^2, Plasma Aldosterone in pmol/L.

Figure 4.1. Flow diagram for study participants.

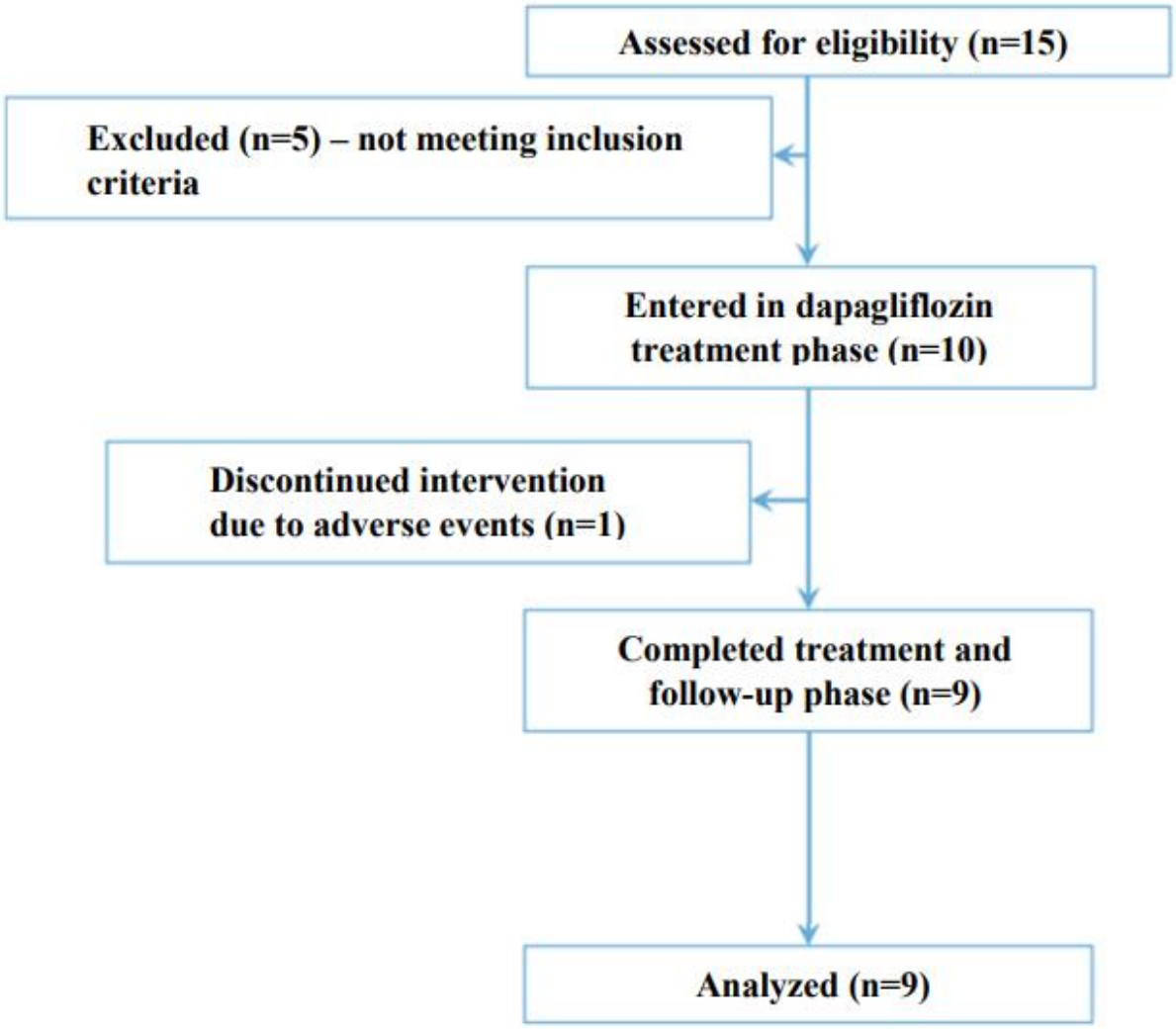
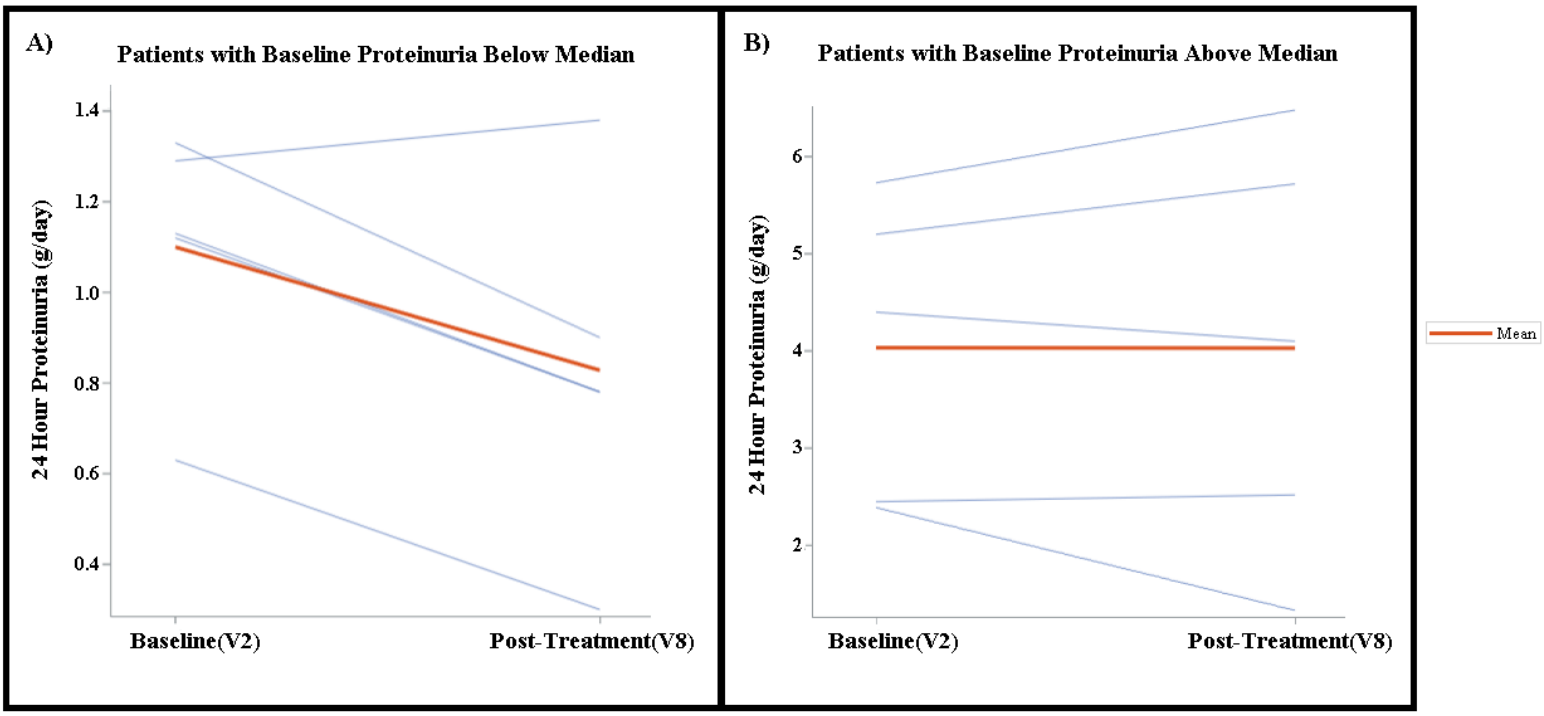


Figure 4.2. Sensitivity Analysis by 24 Hour Proteinuria Below (A) or Above (B) the Median – Individual Participants



Chapter 5: Significance and Future Directions

The aim of the first set of experiments was to investigate the role of intrarenal adenosine as a mediator of tubuloglomerular feedback - by studying the excretion of urinary adenosine following administration of SGLT2i in patients with T1D via a validated LC-MS/MS methodology. We not only found that our novel technique for urine adenosine quantification performed at high accuracy and precision, but further, there was a significant increase in the degree of urinary adenosine excretion post-SGLT2i in patients with T1D. This suggests that adenosine is generated as a paracrine mediator of renal hemodynamic tone through TGF in response to SGLT2i-induced natriuresis in diabetes. Whether this phenomenon extends to other states of renal impairment with similar pathophysiological risk factors outside of diabetic kidney disease, merits future clinical investigation.

The aim of the second set of experiments was to determine the clinical tolerability and applicability of SGLT2 inhibition in renal transplant recipients, which has not, to our knowledge, been reported previously. We observed that SGLT2i was generally well-tolerated - with no episodes of acute kidney injury or alternative detrimental health events recorded following treatment. Moreover, there were mild, yet insignificant, reductions in GFR, body weight and blood pressure despite denervation, indicating possible therapeutic benefit across renal and metabolic risk factors. These results prompt future investigations, both larger and over a longer drug period, to better characterize the clinical value of SGLT2i in transplant recipients.

The aim of the third set of experiments was to investigate the physiological effects of SGLT2 inhibition in patients with FSGS. Contrary to our hypothesis, SGLT2i did not impact renal hemodynamic function, proteinuria or blood pressure in patients with FSGS. Although we did observe modest physiological changes in those with preserved renal function, the trial was neutral overall. Further work is now required to determine if SGLT2 inhibition leads to more significant

hemodynamic, blood pressure and anti-proteinuric effects in other non-diabetic disease states such as IgA nephropathy and obesity-related glomerulopathy, as discussed above.

Importantly, Advani et al have recently examined the impact of dapagliflozin on renal hemodynamic function in subtotallynephrectomized (SNx) rats, which is an experimental model of FSGS. Histologically, kidney injury in this model resembles human FSGS, and develops in part due to raised intraglomerular pressure in remnant kidney tissue[103]. Advani et al also measured levels of SGLT2 mRNA expression in kidney tissue from patients with and without background FSGS to gain additional insight into proteinuric and hemodynamic effects in humans. In this preliminary data, similar to observations in our human cohort, dapagliflozin therapy did not significantly influence renal hemodynamic function or proteinuria, which mirrors our pilot findings in patients with FSGS, and there was no effect on markers of renal histological injury. In contrast, there was a significant decrease in systolic blood pressure in SNx rats. To better understand the direct impact of SGLT2 inhibition on renal physiological parameters, animals were not treated with background RAAS blockade, and were hypertensive. In contrast, patients received standard-of-care agents, and RAAS blockade was not discontinued due to ethical considerations. Whether these blood pressure lowering effects would have been accentuated in hypertensive FSGS patients is not known, but should be examined in future work. In animals, dapagliflozin treatment was also associated with a significant increase in kidney weight in sham-operated rats and a significant increase in kidney weight to body weight ratio in SNx rats. This phenomenon has been described before in rats and may relate to diuresis-induced enlargement of the tubule lumen or to tubule cell hypertrophy as a consequence of increased SGLT1-mediated glucose reabsorption[99]. Whether this effect had any bearing on proteinuria or on histological indices is unclear. Nonetheless, administration of dapagliflozin at the dose employed in the present study

(1mg/kg/day) for the duration of study (8 weeks) in this model of non-diabetic CKD had no significant effect on proteinuria or glomerular or tubulointerstitial injury. We cannot, however rule out the possibility that a higher dose of SGLT2i, longer-term treatment, or the use of less selective SGLT2/SGLT1 inhibitor agents may be associated with greater effects on proteinuria and other markers of renal disease. Overall, despite the small sample size, both human and rodent models of FSGS were consistently neutral for proteinuria outcome following SGLT2i, demonstrating conservation of effects across species as an added value.

In a further set of exploratory analyses in human renal biopsy tissue, Advani et al demonstrated that SGLT2 mRNA expression was lower compared to healthy controls (see Appendix 1 for details). Specifically, a second collaborative *post hoc* study on kidney biopsy tissue from humans with FSGS revealed significant downregulation of SGLT2 mRNA compared to healthy controls. This finding may largely explain the neutral effect of SGLT2i in FSGS. This is, to our knowledge, the first time that SGLT2 mRNA levels have been reported in patients with non-diabetic CKD and may in part explain why dapagliflozin had overall neutral renal hemodynamic and antiproteinuric effects in this non-diabetic condition. The diminution in SGLT2 mRNA levels in the kidneys of patients with FSGS contrasts the upregulation of SGLT2 observed in diabetic animal models[94] and in patients with diabetes[104]. Decreased SGLT2 mRNA expression in kidney tissue from individuals with FSGS may reflect proximal tubular cell injury and/or the absence of a stimulatory hyperglycemic milieu. However, even in two patients with the comorbid FSGS and diabetes, SGLT2 mRNA levels were still diminished. Thus, based on renal functional data and SGLT2 mRNA expression, FSGS may not be the ideal pathophysiological setting in which to examine SGLT2 inhibition effects as a model of non-diabetic renal protection.

This preliminary data from the Advani laboratory is important for two reasons. First, it demonstrates a consistent lack of benefit around renal hemodynamic or anti-proteinuric mechanisms – suggesting that FSGS is not an ideal model for studying SGLT2i in humans in longer-term trials. Second, the reduction in SGLT2 mRNA expression may offer some insight into why dapagliflozin failed to impact parameters of interest in human or animal models: the lack of SGLT2 mRNA may have resulted in less substrate to be blocked pharmacologically. The respective methodologies and results for these pre-clinical studies are presented in detail in Appendix 1.

Future directions for human physiologic studies will include long-term, larger clinical trials of SGLT2 inhibition in patients with other causes of non-diabetic CKD. To this end, in the Effects of Dapagliflozin in Non-diabetic Patients With Proteinuria (DIAMOND) Trial (NCT03190694) will examine the impact of dapagliflozin vs. placebo on GFR and proteinuria is non-diabetic CKD (not limited to FSGS). In addition, "A Study to Evaluate the Effect of Dapagliflozin on Renal Outcomes and Cardiovascular Mortality in Patients with Chronic Kidney Disease" (DAPA-CKD) [NCT03036150] is a long-term trial aimed at evaluating the occurrence of renal outcomes in > 4000 patients with and without T2D in response to dapagliflozin. Moreover, a recent press release announced that the impact of empagliflozin on non-diabetic CKD (n=5000) will also be examined in a separate trial (https://www.boehringer-ingelheim.com/press-release/empagliflozin-be-studied-chronic-kidney-disease). These ongoing and planned trials will provide critical data on the putative renal benefit of SGLT2i in CKD patients, independent of effects of ambient glycemia.

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APPENDIX

A.1 MATERIALS AND METHODS:

1) Effect of Dapagliflozin in Rodents

A) Surgical and Renal Hemodynamic Characteristics

Male Sprague Dawley rats (Charles River, Montreal, Quebec, Canada) aged seven weeks were randomized to undergo sham or subtotal nephrectomy surgery, as previously described [105]. Briefly, rats were anesthetized with 2.5% isoflurane, the right kidney was removed by subcapsular nephrectomy and infarction of approximately two thirds of the left kidney was achieved via ligation of branches of the left renal artery. After surgery, rats were randomly allocated to receive dapagliflozin (1mg/kg/day; AstraZeneca, Mölndal, Sweden) in drinking water or drinking water alone[106]. Animals were followed for 8 weeks with *ad libitum* access to commercial standard rat chow. After 8 weeks of dapagliflozin (or vehicle) treatment, rats were individually housed in metabolic cages for 24 h for determination of urine volume, urine protein (benzethonium chloride method) and urine creatinine (Advia 1650, Siemens Medical Solutions Diagnostics, Tarrytown, NY). Systolic blood pressure (SBP) was determined by tail cuff plethysmography (Powerlab, AD Instruments Colorado Springs, CO) in conscious rats, as previously described[107]. Tail vein venipuncture was used for determination of plasma creatinine (autoanalyzer), hematocrit (XN-9000, Sysmex Canada Inc., Mississauga, Ontario, Canada) and fasting blood glucose (OneTouch UltraMini; LifeScan Canada Ltd.; Burnaby, British Columbia, Canada). The number of animals studied at the end of the 8-week period was as follows: sham + vehicle, n=18; sham + dapagliflozin, n=18; SNx + vehicle, n=17, SNx + dapagliflozin, n=20. GFR was determined by single-shot FITC-inulin clearance and repeated sampling via the tail-vein as previously described[105] in sub-groups of rats (sham + vehicle, n=16; sham + dapagliflozin, n=10; SNx +

vehicle, n=15; SNx + dapagliflozin, n=15).Renal plasma flow was determined in conscious unrestrained rats using an adaptation of previously published methods[108, 109] and in sub-groups of rats (sham + vehicle, n=3; sham + dapagliflozin, n=3; SNx + vehicle, n=4; SNx + dapagliflozin, n=5). Briefly, under 2% isoflurane anesthesia, the right femoral artery and right femoral vein were each cannulated with a heparinized (500IU/ml) PE50 catheter. Animals were recovered from anesthesia and para-aminohippurate (PAH, 11.6mg/ml) (Sigma-Aldrich Canada Co., Oakville, Ontario, Canada) was infused via the right femoral vein with a priming dose of 8mg/kg and a constant maintenance rate of 0.0267ml/min. After an equilibration phase of 105 minutes, three blood samples were obtained from the right femoral artery, one every 15 minutes, for determination of PAH clearance. All experimental procedures adhered to the guidelines of the Canadian Council on Animal Care and were approved by the St. Michael's Hospital Animal Care Committee.

1) Effect of Dapagliflozin in Rodents

B) Histological Characteristics

Rats were anesthetized with an i.p. injection of pentobarbital sodium, 60 mg/kg (Boeringer– Ingelheim, North Ryde, NSW, Australia). The right renal artery was clamped and the kidney removed, decapsulated, sliced transversely, and immersed in 10% neutral buffered formalin (NBF) for 24 h. Blood film examination was performed in at least three rats per group by using Wright–Giemsa staining. Glomerulosclerosis index was determined semi-quantitatively in periodic-acid Schiff stained kidney sections as previously described and in approximately 60 glomerular profiles for each kidney section[107].

Immunohistochemistry was performed as previously described with antibodies in the following concentration: collagen IV 1:500 (EMD Millipore, Darmstadt, Germany), JG-12 1:1000

(Bender Medsytsems GmbH, Vienna, Austria) and ED1 (Bio-Rad, Hercules, CA)[107]. Incubation with phosphate buffered saline in place of the primary antibody served as the negative control. After incubation with the appropriate horseradish peroxidase conjugated secondary antibody, sections were labeled with Liquid Diaminobenzidine and Substrate Chromogen (Dako North America Inc., Carpinteria, CA) before counterstaining in Mayer's hematoxylin. Slides were scanned (Leica Microsystems Inc., Concord, Ontario, Canada) and analyzed using ImageScope 11.1 software (Leica Microsystems Inc.).

The proportional glomerular area positively immunostaining for collagen IV or with the JG-12 antibody was determined in 30 randomly selected glomerular profiles from each kidney section using ImageScope. Cortical tubulointerstitial ED1 immunostaining was determined in 10 non-overlapping cortical fields (excluding glomeruli) using the ImageScope 20X zoom. All histological analyses were performed by an investigator masked to the study groups.

2) Gene Expression in Human Kidney Tissue

For the determination of SGLT2 mRNA levels, kidney biopsy tissue was examined from six individuals with secondary FSGS (biopsy-proven and clinically-correlated obesity-related secondary FSGS) and compared to that of kidney tissue obtained at the time of live kidney transplant from six healthy donors with normal kidney function[110]. The study was approved by the Institutional Research Board of the Health Sciences Centre, University of Manitoba and was conducted in accordance with the Declaration of Helsinki. RNA was isolated using a Paradise Plus Reagent System (Arcturus, Mountain View, CA). Real-time PCR was performed using SYBR green (Wisent Bio Products, St.-Jean-Baptiste, Quebec, Canada) on a ViiA7 PCR system (ThermoFisher Scientific, Rockford, IL) and with the following primer sequences: SGLT2, forward GCTGGAGAGAATGGAGCAA, reverse AGACCACAAGCCAACACCAA; RPL32,

forward CAACATTGGTTATGGAAGCAACA, reverse TGACGTTGTGGACCAGGAACT. Samples were analyzed in duplicate and data analysis was performed using the Applied Biosystems Comparative C_T method.

Statistical Parameters

For rodent and human gene expression studies, statistical analyses were performed using GraphPad Prism 6 for Mac OS X (GraphPad Software Inc. San Diego, CA). Data are presented as mean ± SD except skew distributed rat proteinuria data which are shown as median (range) and which were analyzed using a Kruskal-Wallis test with a Dunn's post-hoc comparison. Gene expression changes in human tissue were compared by Student *t*-test.

A.2 RESULTS:

1) Effect of Dapagliflozin in Rodents

Table A.1. shows the physiological parameters in sham-operated and SNx rats treated with dapagliflozin (or vehicle) for 8 weeks. As expected, GFR was reduced in SNx rats in comparison to sham-operated rats. Dapagliflozin treatment had no effect on GFR in either sham or SNx rats. Similarly, ERPF tended to be lower in SNx rats than sham-operated rats but the difference was not statistically significant, likely because of the relatively small number of rats studied (n=3-5/group) and the multiple groups comparison. Dapagliflozin did not modify ERPF in either sham or SNx rats. As expected, urine protein excretion was increased in SNx rats in comparison to sham-operated rats. Similar to effects in humans, dapagliflozin treatment did not impact 24h urine protein excretion. Twenty-four-hour urine volume was increased in sham-operated rats treated with dapagliflozin and in vehicle-treated SNx rats compared to vehicle-treated sham rats. However, there was no difference in urine volume between dapagliflozin-treated sham and SNx rats, whereas the increase in urine volume in SNx rats treated with dapagliflozin was not

statistically significance (p=0.0504 vs. SNx + vehicle, Table A.1.). Body weight tended to be lower with dapagliflozin treatment and in SNx rats, but only the comparison of body weight between vehicle-treated sham rats and dapagliflozin-treated SNx rats achieved statistical significance. Fasting blood glucose did not differ between the study groups. SBP was significantly higher in SNx rats compared to sham-operated rats. Whereas dapagliflozin treatment had no effect on SBP in sham rats, SBP was significantly lower in dapagliflozin-treated SNx rats than in vehicle-treated SNx rats. Kidney weight was increased both after SNx surgery and with dapagliflozin treatment, with the largest kidney weight seen in dapagliflozin treated SNx rats. Kidney weight:body weight (%) was significantly larger in dapagliflozin treated SNx rats than in vehicle treated SNx rats (p=0.0491).Renal histological parameters in sham and SNx rats treated with vehicle or dapagliflozin were assessed as: i) the degree of glomerulosclerosis on periodic acid-Schiff stained kidney sections (Figure A.1a); ii) the proportional glomerular area positively immunostaining for Type IV collagen (Figure A.1b); iii) glomerular capillary density, determined as the proportional glomerular area immunostained with the monoclonal antibody JG-12, that detects aminopeptidase P expressed on rat renal microvascular endothelial cells (Figure A.1c)[111]; and iv) cortical tubulointerstitial macrophage infiltration, determined at the proportional area of cortical tubulointerstitium immunostained with the monoclonal antibody ED1, that recognizes CD68 expressed on rat macrophages (Figure A.1d). In comparison to sham-operated rats, SNx rats exhibited increased glomerulosclerosis, increased glomerular collagen IV deposition, loss of glomerular capillaries and increased infiltration of the tubulointerstitium by macrophages (Figure A.1). Dapagliflozin treatment did not affect any of these parameters in either sham or SNx rats (Figure A.1).

2) Gene Expression in Human Kidney Tissue

Based on the neutral effect of dapagliflozin on proteinuria in animals and patients with FSGS, we measured SGLT2 mRNA levels in archived biopsy kidney tissue from individuals with FSGS and in healthy controls. The clinical characteristics of the individuals with FSGS have been described previously[110]. All of the patients had obesity-related secondary FSGS and two of the patients had concurrent diabetes mellitus. Normal control kidney biopsy tissue was obtained from transplanted kidneys before implantation. All of the controls had normal kidney function (eGFR>60ml/min/1.73m^2) and none had concurrent diabetes. SGLT2 mRNA levels were markedly diminished in kidney tissue from individuals with secondary FSGS (Figure A.2).

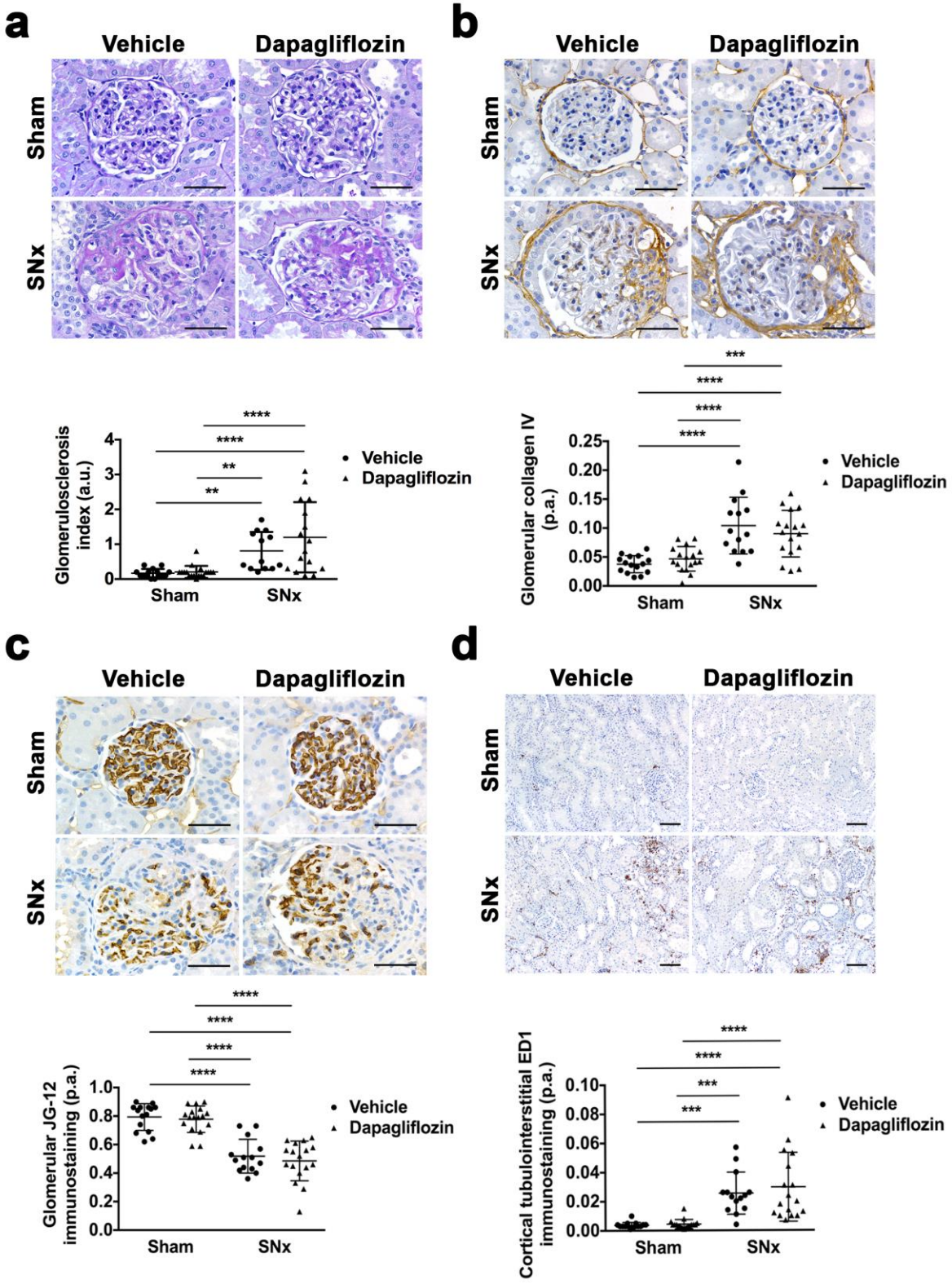
Tables and Figures

Table A.1. Effects of dapagliflozin on renal and metabolic parameters in sham-operated and subtotallynephrectomized (SNx) rats.

Variable	Group	N	Value	Group	N	Value
Body weight (g)	**Sham + vehicle**	18	614±59	**Sham + 'dapa'**	18	570±55
	SNx + vehicle	17	562±65	**SNx+ 'dapa'**	20	542±87[a]
GFR (ml/min/kg)	**Sham + vehicle**	16	5.4±2.2	**Sham + 'dapa'**	10	4.9±1.7
	SNx + vehicle	15	1.8±0.6[bd]	**SNx+ 'dapa'**	15	2.3±1.2[be]
ERPF (ml/min/kg)	**Sham + vehicle**	3	57.3±15.9	**Sham + 'dapa'**	3	58.2±26.8
	SNx + vehicle	4	34.2±3.3	**SNx+ 'dapa'**	5	47.2±43.0
Systolic blood pressure (mmHg)	**Sham + vehicle**	18	122±14	**Sham + 'dapa'**	18	124±13
	SNx + vehicle	17	196±26[bd]	**SNx+ 'dapa'**	20	165±33[bdf]
Fasting blood glucose (mmol/L)	**Sham + vehicle**	18	6.3±0.7	**Sham + 'dapa'**	18	6.1±1.0
	SNx + vehicle	17	6.6±0.7	**SNx+ 'dapa'**	20	6.4±0.6
24h urine volume (ml)	**Sham + vehicle**	18	25±12	**Sham + 'dapa'**	18	48±13[b]
	SNx + vehicle	17	38±12[ac]	**SNx+ 'dapa'**	20	46±12[b]
24h urine protein (mg)	**Sham + vehicle**	18	15.1 (11.2-38.0)	**Sham + 'dapa'**	18	20.1 (10.8-82.2)
	SNx + vehicle	17	118.4 (18.3-658.4)[be]	**SNx+ 'dapa'**	20	88.9 (9.2-230.9)[bc]
Hematocrit (L/L)	**Sham + vehicle**	18	0.434±0.059	**Sham + 'dapa'**	18	0.428±0.037
	SNx + vehicle	16	0.394±0.041[g]	**SNx+ 'dapa'**	18	0.379±0.05[4ah]
Left kidney weight (g)	**Sham + vehicle**	18	1.72±0.17	**Sham + 'dapa'**	18	2.27±0.25[a]
	SNx + vehicle	17	2.51±0.62[b]	**SNx+ 'dapa'**	20	2.72±0.76[bh]
Left kidney weight: body weight (%)	**Sham + vehicle**	18	0.28±0.04	**Sham + 'dapa'**	18	0.40±0.05[i]
	SNx + vehicle	17	0.45±0.10[b]	**SNx+ 'dapa'**	20	0.51±0.14[bef]

Note: 'dapa' is shorthand for dapagliflozin. Values are expressed as mean±SD. [a]$p<0.01$ vs. sham + vehicle, [b]$p<0.0001$ vs. sham + vehicle, [c]$p<0.05$ vs. sham + dapagliflozin, [d]$p<0.0001$ vs. sham + dapagliflozin, [e]$p<0.001$ vs. sham + dapagliflozin, [f]$p<0.05$ vs. SNx + vehicle, [g]$p<0.05$ vs. sham + vehicle, [h]$p<0.01$ vs. sham + dapagliflozin, [i]$p<0.001$ vs. sham + vehicle

Figure A.1. SGLT2 inhibition with dapagliflozin does not affect histological parameters of renal injury in subtotallynephrectomized (SNx) rats.



(a) Representative photomicrographs of periodic acid-Schiff stained kidney sections (original magnification x400) from sham-operated (sham) and SNx rats treated with vehicle or dapagliflozin and quantitation of glomerular injury (glomerulosclerosis index, GSI; sham + vehicle, n=15; sham + dapagliflozin, n=18; SNx + vehicle, n=13; SNx + dapagliflozin, n=16). (b) Representative photomicrographs of kidney sections (original magnification x400) from sham and SNx rats treated with vehicle or dapagliflozin stained for collagen IV and quantitation of glomerular collagen IV (sham + vehicle, n=15; sham + dapagliflozin, n=17; SNx + vehicle, n=14, SNx + dapagliflozin, n=17). (c) Representative photomicrographs of kidney sections (original magnification x400) from sham and SNx rats treated with vehicle or dapagliflozin stained with the JG-12 antibody, which detects rat renal microvascular capillaries, and quantitation of glomerular capillary density (sham + vehicle, n=15; sham + dapagliflozin, n=17; SNx + vehicle, n=14; SNx + dapagliflozin, n=17). (d) Representative photomicrographs of kidneys sections (original magnification x100) from sham and SNx rats treated with vehicle or dapagliflozin stained with the ED1 antibody that detects rat macrophages and quantitation of cortical tubulointerstitial ED1 immunostaining (sham + vehicle, n=15; sham + dapagliflozin, n=17; SNx + vehicle, n=14; SNx + dapagliflozin, n=17). (a-c) Scale bar = 50μm; (d) scale bar = 100μm. a.u. = arbitrary units; p.a. = proportional area. ** P<0.01, *** P < 0.001, **** P < 0.0001.

Figure A.2. SGLT2 mRNA levels are decreased in kidney biopsy tissue from patients with obesity-related secondary focal segmental glomerulosclerosis (FSGS). The triangles indicate mRNA levels in the two patients with FSGS who also had diabetes. a.u. = arbitrary units. *** $P<0.001$.