Advanced glycation end products as predictors of renal function in youth with type 1 diabetes

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To examine if skin autofluorescence (sAF) differed in early adulthood between individuals with type 1 diabetes and age-matched controls and to ascertain if sAF aligned with risk for kidney disease. Young adults with type 1 diabetes (N = 100; 20.0 ± 2.8 years; M:F 54:46; FBG-11.6 ± 4.9 mmol/mol; diabetes duration 10.7 ± 5.2 years; BMI 24.5(5.3) kg/m²) and healthy controls (N = 299; 20.3 ± 1.8 years; M:F-83:116; FBG 5.2 ± 0.8 mmol/L; BMI 22.5(3.3) kg/m²) were recruited. Skin autofluorescence (sAF) and circulating AGEs were measured. In a subset of both groups, kidney function was estimated by GFR\textsuperscript{CKD-EPI} CysC and uACR, and DKD risk defined by uACR tertiles. Youth with type 1 diabetes had higher sAF and BMI, and were taller than controls. For sAF, 13.6% of variance was explained by diabetes duration, height and BMI (P\textsubscript{model} = 1.5 × 10\textsuperscript{–12}). In the sub-set examining kidney function, eGFR and sAF were higher in type 1 diabetes versus controls. eGFR and sAF predicted 24.5% of variance in DKD risk (P\textsubscript{model} = 2.2 × 10\textsuperscript{–9}), which increased with diabetes duration (51%; P\textsubscript{model} < 2.2 × 10\textsuperscript{–16}) and random blood glucose concentrations (56%; P\textsubscript{model} < 2.2 × 10\textsuperscript{–10}). HbA\textsubscript{1c} and circulating fructosamine albumin were higher in individuals with type 1 diabetes at high versus low DKD risk. eGFR was independently associated with DKD risk in all models. Higher eGFR and longer diabetes duration are associated with DKD risk in youth with type 1 diabetes. sAF, circulating AGEs, and urinary AGEs were not independent predictors of DKD risk. Changes in eGFR should be monitored early, in addition to uACR, for determining DKD risk in type 1 diabetes.

The presence of kidney disease (DKD) is the strongest predictor of mortality in individuals with diabetes\textsuperscript{1}. In type 1 diabetes, it is increasingly appreciated that future risk for DKD and cardiovascular disease (CVD) may be evident as early as adolescence\textsuperscript{2}, exacerbated by difficulties in maintaining adequate glycemic control at that time\textsuperscript{1}. Risk for DKD is defined as an increase in urinary albumin excretion during puberty, preceding micro- and macroalbuminuria, as seen in adolescents with type 1 diabetes in the upper third of urinary albumin excretion\textsuperscript{4}. However, best practice regimens for adults, targeting hypertension and dyslipidemia were ineffective at preventing microalbuminuria over a few years, in a Phase III clinical trial in adolescents with type 1 diabetes\textsuperscript{4}. This suggests that other pathological factors may be at play during the early development of DKD.

Despite there being increasing scrutiny of childhood and adolescence, there is a paucity of data available examining risk for DKD in young adults with type 1 diabetes prior to the onset of chronic complications. These young people are often lost to follow up in the transition from pediatric to adult clinical care\textsuperscript{5}, and commonly...
adolescents and young people have mostly shown increases in circulating or urinary AGE concentrations. Studies in cystatin C > 0.8 mg/mL eGFR = 133 × min(Scys/0.8, 1)−1.328 × 0.996Age × 0.932 [if female]. In individuals with age at diagnosis, diabetes duration, mean systolic blood pressure and a morning non-fasted and second void sample of care devices. Quantikine ELISA kits were used for the measurement of serum cystatin C (R&D Systems, Minneapolis, USA) according to the manufacturer’s instructions. Estimated GFR was calculated from serum cystatin C using the Chronic Kidney Disease Epidemiology Collaboration eGFR CKD-EPI-CysC equation, if serum cystatin C ≤ 0.8 mg/mL eGFR = 133 × min(Scys/0.8, 1)−0.499 × 0.996Age × 0.932 [if female], and if serum cystatin C > 0.8 mg/mL eGFR = 133 × min(Scys/0.8, 1)−1.238 × 0.996Age × 0.932 [if female]. In individuals with type 1 diabetes, the second morning urine void was collected for uACR and measured by the Mater Pathology routine laboratory. In addition to this uACR, we utilised the uACR measurements from two previous clinic visits to calculate mean urinary ACR values and define tertiles of ACR for each of the renal study participants based on previous studies. Individual risk for DKD was allocated as: 0 = no diabetes (Control, 49 subjects), 1 = diabetes + lowest uACR tertile (Low Risk uACR ≤ 0.66 mg/mmol; 27 subjects), 2 = diabetes + middle uACR tertile (Medium Risk uACR = 0.67–1.16 mg/mmol; 29 subjects) or 3 = diabetes + upper uACR tertile (High Risk uACR ≥ 1.17 mg/mmol; 33 subjects). Albuminuria was defined according to international guidelines as uACR > 2.5 mg/mmol in males and > 3.5 mg/mmol in females.

Statistical analyses. Data were expressed as mean ± SD or median (interquartile range) unless otherwise stated. Normality testing (Shapiro–Wilks) was performed on all data. Parametric data were analysed by one-way
Results

Recruited individuals (Fig. 1A) were, on average, 20 years of age (Table 1). Baseline characteristics did not differ between the full sAF cohort and the Renal sub-set (Table 1). Those with diabetes were taller, with greater BMI, random blood glucose concentrations and sAF and with median diabetes duration of 10 years (Table 1, Fig. 1B). Holm’s corrected Spearman’s correlations in the full sAF and renal sub-set showed that sAF was positively associated with age, BMI and diabetes duration and negatively correlated with height (Fig. 1C). The duration of diabetes was also related to weight and consequently BMI (Fig. 1C).

General linear modelling for sAF with sequential addition of covariates, is shown in Table 2 for the full cohort. In Model 1 (Full cohort; N = 399 participants), diabetes duration and BMI were significant independent positive predictors, whilst height was a negative independent predictor of sAF (Adjusted $r^2 = 0.14, P = 1.47 \times 10^{-12}$). This model was not appreciably improved by the addition of age and sex (Model 2; Adjusted $r^2 = 0.14, P = 8.77 \times 10^{-12}$).

In those who had biological samples taken (Renal sub-set; Suppl. Table 1; Fig. 1; N = 148), individuals with type 1 diabetes had significantly higher eGFR and lower serum cystatin C and progressive increases in uACR, as DKD risk increased (Fig. 2A), as predicted. In this Renal sub-set, 12.8% of males and 10.3% of females with type 1 diabetes had microalbuminuria. BMI was greater in young individuals with type 1 diabetes (Fig. 2B).

When examining AGE burden in the renal sub-set, sAF remained greater, as per the full cohort, in individuals with type 1 diabetes versus control (Fig. 2C, $P = 0.0004$) and was modestly higher in subjects at greatest risk for DKD. Using general linear modelling with sequential addition of covariates, Model 1 explained 17.3% of variance in sAF (Adjusted $r^2 = 0.17, P = 1.0 \times 10^{-6}$), where BMI, diabetes duration and height were independent variables (Suppl. Table 2). This was strengthened by the addition of random BG in Model 2 (Adjusted $r^2 = 0.20$, $P = 3.6 \times 10^{-7}$) but not further improved by the addition of age and sex in Model 3 (Suppl. Table 2; Adjusted $r^2 = 0.20, P = 1.0 \times 10^{-6}$).

Serum soluble RAGE (sRAGE) concentrations differed between control and low ($P < 0.05$), but not medium or high risk individuals (Fig. 2C; $P = 0.069$). Overall, circulating sRAGE was lower in those youth with type 1 diabetes (973 ± 349 pg/ml) versus controls (1184 ± 379 pg/ml, $P = 0.0245$). The glycosylated proteins HbA1c and fructosamine albumin, each indicative of longer-term glycaemic control, were greatest in the high versus low risk tertile for DKD (Fig. 2C). Urinary excretion of both the protein bound and free AGEs, MG-H1 and CML, fructosamine albumin, each indicative of longer-term glycaemic control, were greatest in the high versus low risk tertile for DKD (Fig. 2C). Urinary excretion of both the protein bound and free AGEs, MG-H1 and CML, did not differ between risk groups (Suppl. Fig. 1A). A Holm’s corrected Spearman’s correlation matrix showed that sAF was positively related to age, BMI and risk for DKD in the renal sub-set. Risk for DKD was positively related to BMI, diabetes duration, eGFR and random BG (Suppl. Fig. 1A). Soluble RAGE concentrations were negatively associated with BMI and diabetes duration (Suppl. Fig. 1A).

Using univariate analysis in just those individuals with type 1 diabetes, sAF was positively associated with age ($r = 0.37, P = 0.0008$), diabetes duration ($r = 0.22, P = 0.047$ and BMI ($r = 0.27, P = 0.017$) but not with any indices of glycaemic control, including HbA1c, or other AGE measurements. uACR was most strongly related to plasma sRAGE ($r = 0.30, P = 0.0068$) and urinary MG-H1 concentrations ($r = 0.33, P = 0.0025$) in individuals with type 1 diabetes. These associations did not persist following Holm’s adjustment.

eGFR remained independent to all patient variables collected in this sub-analysis of individuals with type 1 diabetes. Indeed, even a model containing uACR, diabetes duration, age, sex, BMI, HbA1c, SBP, random BG and total cholesterol explained only ~ 4% of variance in eGFR in youth with type 1 diabetes and was not significant (Adjusted $r = -0.0375, P_{\text{model}} = 0.7293$). Addition of urinary CML, MG-H1, sAF and circulating sRAGE did not appreciably improve the model.

General linear modelling for DKD risk with sequential addition of covariates in the renal sub-study, is shown in Table 2. Firstly, eGFR predicted 23% of the variance in DKD risk (Adjusted $r = 0.25, P = 2.3 \times 10^{-9}$). In Model 1 (Adjusted $r = 0.25, P = 2.2 \times 10^{-9}$), eGFR ($P = 5.5 \times 10^{-9}$) and sAF ($P = 0.04$) were significant independent predictors of DKD risk. With additional adjustment for diabetes duration (Table 2; Model 2, Adjusted $r = 0.51$, $P < 2.2 \times 10^{-16}$), the ability of the model to predict DKD risk variance doubled to greater than 50%. eGFR remained an independent predictor of DKD risk in this model ($P = 1.2 \times 10^{-4}$). With the addition of random blood glucose in Model 3, diabetes duration and eGFR remained independent predictors of DKD risk but the prediction of DKD risk by this model only modestly increased (Table 2, Model 3; Adjusted $r = 0.56, P < 2.2 \times 10^{-16}$). With the addition of height and sex in Model 4, diabetes duration, FBG and eGFR remained independent predictors of DKD risk, but overall the prediction of DKD risk did not appreciably increase (Table 2, Model 4; Adjusted $r = 0.56, P < 2.2 \times 10^{-16}$). The addition of circulating sRAGE and urinary AGEs did not alter the explained variance in DKD risk in any of these models.

**Ethics approval statement.** This research protocol was approved by the Human Research Ethics committees of Mater Misericordiae Limited (Approval: HREC_15_MHS_35T1D) and the University of Queensland, Brisbane, Australia (Approval: 2016-02-066-PRE-3; UQ 2015-000-958). All investigation was conducted according to the principles of the Declaration of Helsinki.

**Patient consent statement.** Written informed consent was obtained from all participants and if under 18 years of age, from their legal guardian in addition to the participant assent, prior to inclusion in the study.

**Statistical analysis.** All variables were tested for normality and adjusted as appropriate. Continuous variables were compared by Student’s t-test, Mann-Whitney test or ANOVA with Tukey’s post hoc for multiple comparisons. Non-parametric data were analysed by Kruskal–Wallis and Dunn’s post hoc. Univariate modelling with Holm’s correction was used to determine interdependence of variables in the two cohorts. General linear models were used to examine the associations between sAF and were sequentially adjusted for covariates identified by univariate analyses in the full sAF cohort. Risk for DKD in the renal sub-study was also studied using general linear modelling.
Figure 1. Overview of study design and baseline summary of the full cohort. (A) Flow chart of participant numbers. (B) Baseline characteristics of participants in the Full cohort for control individuals without diabetes (N=299) and for individuals with type 1 diabetes (N=100; Median IQR). ***P≤0.001, ****P≤0.0001 versus Control (No diabetes). (C) Spearman’s univariate correlation matrix corrected by Holm’s method. Significant r coefficients for positive associations are shown in dark blue—P≤0.001 and light blue P≤0.01 and significant negative relationships in red—P≤0.001. BMI body mass index, sAF skin autofluorescence, Duration, diabetes duration.
Table 1. Baseline clinical and anthropometric characteristics. Data are median (IQR) or N (%). Participants included in the renal sub-set groups were from the full cohort who had biological samples taken (as per Fig. 1A). nd—not determined. Comparisons within full cohorts or sub-sets were by two-tailed Mann–Whitney Testing. Proportions were analysed by Fisher’s Exact test. **P < 0.01 versus control counterpart; ***P < 0.001 versus control counterpart; ****P < 0.0001 versus control counterpart; §N = 74 for random BG. BG—blood glucose.

Table 2. General linear modelling for sAF predictors in the full cohort and DKD risk in the renal sub-study. For full cohort—Control, N = 299; Diabetes, N = 100 individuals. For renal sub-study—Control, N = 49; Diabetes, N = 89 individuals. DKD risk is defined on a scale of 0–3, where 0 = No diabetes; 1 = type 1 diabetes and lowest uACR tertile; 2 = type 1 diabetes and middle uACR tertile and 3 = type 1 diabetes and highest uACR tertile. SE standard error, sAF skin autofluorescence, BG blood glucose, eGFR estimated glomerular filtration rate. *P < 0.05; **P < 0.01; ***P ≤ 0.001.
Discussion

In this present study, biomarkers of DKD related to advanced glycation were investigated in youth. Greater gGFR, random blood glucose concentrations and diabetes duration were independent markers for DKD, accounting for >55% of variation in this young renal cohort. Risk prediction models were not further improved by the addition of skin autofluorescence (sAF), nor other AGE or sRAGE measurements. In our larger population where biological samples were not available, diabetes duration, BMI and height were significant independent predictors of sAF. Height may have been influenced by a modestly greater proportion of males and was not related to sex in univariate analysis. However, the inverse relationship between sAF and height may also be explained by previous studies suggesting that after diabetes diagnosis, those adolescents with the poorest glycaemic control, end up with the greatest deficit in final height\(^24,25\). Certainly those with poorer glycaemic control would not only be more likely to have higher sAF but also greater risk for chronic complications\(^25\). However, together these covariates explained only ~13% of sAF variance in our entire population and ~20% in the renal sub-study with the addition of random blood glucose concentrations. This implies that other major factors are contributing to
sAF in young adults at the age groups we are examining that were not identified in this study. Indeed, while sAF is known to increase in parallel with age and diabetes duration in young people, height has not been previously identified as a predictor of sAF in this group.

sAF was significantly greater in young people with type 1 diabetes compared to controls, which is consistent with previous work in adults and in children and adolescents. However, when considering the renal sub-study who had biological samples taken, sAF was an independent predictor of DKD risk in a model that included eGFR. Certainly in a previous study, sAF did increase according to chronic kidney disease stage and within the DCCT/EDIC study, a slightly older cohort than ours, predicted the development of both micro- and macrovascular disease, including kidney disease. With addition of diabetes duration to our models, where sAF has shown dependency in previous studies, the variability in DKD risk significantly improved to >50%. Indeed, diabetes duration has been consistently shown as an important determinant of complication risk in diabetes as shown in the present study.

Glycaemic control is the most commonly targeted risk factor for DKD, but target control is notoriously difficult to achieve in youth. Certainly, addition of random blood glucose concentrations to diabetes duration, eGFR and sAF explained more of the variance in DKD risk in the renal cohort (up to 56%). Other measures of glycaemic control, namely fructosamine albumin, which are both glycated proteins but early advanced glycation adducts, were elevated with diabetes to a greater degree in high risk individuals. Unfortunately, these measurements were not available in our control subjects. However, adding these variables did not appreciably improve DKD risk prediction in individuals with type 1 diabetes. It is possible that youth in the present study were assessed too early in the course of disease for measures of advanced glycation, such as sAF, to differentiate those at higher risk for DKD. Indeed, we had specifically excluded youth with previously diagnosed kidney disease.

Further, most previous studies demonstrating that sRAGE and AGEs as independent predictors of DKD or macrovascular disease in diabetes, were performed in older people. Interestingly, in the Adolescent Type 1 Diabetes Cardio-Renal Intervention Trial (AdDIT), HbA₁c measurements were remarkably similar among groups and did not align with greater DKD risk. However, in recent follow up of these adolescents from AdDIT at a similar age to our cohort, HbA₁c concentrations were significantly higher in those individuals previously allocated to the upper tertile of uACR, and predicted to be at greatest risk for DKD. This agrees with HbA₁c concentrations in our youth with type 1 diabetes, which were higher in those at greatest risk for DKD. Additionally, in young people with type 1 diabetes, complications including DKD, often initially progress without changes in conventional risk factors, such as HbA₁c. This suggests that the development of DKD in the early stages may not be as reliant on poor glycaemic control as previously thought and abnormalities in uACR may, in fact precede worsening glycaemic control.

Surprisingly, a model which explained eGFR variance (to greater than 5%) in individuals with type 1 diabetes using sequential addition of all of the covariates collected, could not be found in this cohort of 15–25 year olds. This suggests that other factors may be driving eGFR changes at this early time point in the development of diabetic kidney disease. Furthermore, youth with type 1 diabetes had significantly higher eGFR values compared to those with normal glomerular filtration. However, other studies in young people have not identified a link between hyperfiltration and microalbuminuria. In young people and adults with type 1 diabetes, complications including DKD, often initially progress without changes in conventional risk factors, such as HbA₁c. This suggests that the development of DKD in the early stages may not be as reliant on poor glycaemic control as previously thought and abnormalities in uACR may, in fact precede worsening glycaemic control.

In addition to eGFR, BMI was also significantly higher in youth with type 1 diabetes compared to controls. However risk did not vary among DKD risk tertiles, which is in agreement with the recent AdDIT follow up study, where BMI also did not significantly differ among uACR tertiles. In the present study, BMI was positively associated with sAF and was an independent predictor of sAF in a model that included diabetes duration and height. This positive association has been previously described in adults and without diabetes. The increased AGE accumulation in people with higher BMI could be a result of increased dietary intake of AGEs or oxidative stress, but some factors known to affect sAF were not assessed in the present study. Indeed, lower sRAGE concentrations in our youth with type 1 diabetes, could adversely impact AGE clearance. Lower sRAGE also consistently associates with greater BMI and a causal link between obesity and DKD in adults with type 1 diabetes has been demonstrated.
of diabetes per se. Additionally, the limitations of the AGE reader used to measure sAF have been previously described, but it has been validated as a reliable surrogate marker of AGE burden in an adolescent population. Finally, given that this was a cross-sectional observational study, we did not have follow up data for these individuals to determine if/when they developed diabetic kidney disease.

**Conclusion**

Taken together, these studies suggest that greater eGFR and diabetes duration in youth with type 1 diabetes without previously diagnosed complications are markers of DKD risk, which are not improved by measurement of sAF nor other markers of AGE burden. However, there may be some utility for the routine measurement of early glycation adducts such as fructosamine albumin, in addition to more routine HbA1c. Further, early changes in eGFR during diabetes should be monitored alongside uACR to better stratify those young people with type 1 diabetes at greatest risk for DKD and CVD. This is of significant interest, as current methods for prediction of early DKD are poor, which often delays appropriate clinical management until more advanced complications develop. Additional longitudinal studies are required to better establish risk factors for GFR decline, since this could not be ascertained in this study and appeared independent of uACR.

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Author contributions
J.M.F. designed the experiments, analyzed and interpreted data and prepared the manuscript and is responsible for the contents of the article as guarantor. S.L.B. analyzed and interpreted data and prepared the manuscript. K.B., S.R., T.B., J.N., A.M., S.T., N.d.S., H.B., T.O.M.S. assisted type 1 diabetes clinical cohort design and collaboration. K.M.M., L.A.G., L.A.G., J.B., A.K.F. collected the control cohort data and samples. D.A.Mc., A.Z., K.B., L.A.G., S.R., S.L., S.L.B. performed experiments. T.J., J.C., K.D., M.I., D.J. assisted in study conceptualization and design and gaining financial support. P.D. and L.D. completed the urinary A.G.E. analyses. All authors edited and approved the final manuscript.

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Competing interests
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