Evidence That Differences in Fructosamine-3-Kinase Activity May Be Associated With the Glycation Gap in Human Diabetes

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Diabetes 2018;67:131–136 | https://doi.org/10.2337/db17-0441

The phenomenon of a discrepancy between glycated hemoglobin levels and other indicators of average glycemia may be due to many factors but can be measured as the glycation gap (GGap). This GGap is associated with differences in complications in patients with diabetes and may possibly be explained by dissimilarities in deglycation in turn leading to altered production of advanced glycation end products (AGEs). We hypothesized that variations in the level of the deglycating enzyme fructosamine-3-kinase (FN3K) might be associated with the GGap. We measured erythrocyte FN3K concentrations and enzyme activity in a population dichotomized for a large positive or negative GGap. FN3K protein was higher and we found a striking threefold greater activity (323%) at any given FN3K protein level in the erythrocytes of the negative-GGap group compared with the positive-GGap group. This was associated with lower AGE levels in the negative-GGap group (79%), lower proinflammatory adipokines (leptin-to-adiponectin ratio) (73%), and much lower prothrombotic PAI-1 levels (19%). We conclude that FN3K may play a key role in the GGap and thus diabetes complications such that FN3K may be a potential predictor of the risk of diabetes complications. Pharmacological modifications of its activity may provide a novel approach to their prevention.

Nonenzymatic protein glycation is aetiologically important to diabetes complications (1) and for diabetes diagnosis, control, and treatment surveillance using glycated proteins, such as glycated hemoglobin (HbA_{1c}) or fructosamine (2). We, and others, have demonstrated a glycation gap (GGap) (3–6), the discrepancy between average glycaemia determined by HbA_{1c} and fructosamine, and have shown the following: a methodology for its measurement (4), its consistency among individuals (5), its potential for clinical error (4), and associated morbidity and mortality (6), noting similar adverse outcomes in Action to Control Cardiovascular Risk in Diabetes (ACCORD) (7).

Variation in hemoglobin (Hb) glycation is multifactorial (8,9), for example, relating to red blood cell factors. Excluding those, we are considering here the possibility of a systematic variation of the glycation phenotype within the human population that is due to factors independent of blood glucose concentration, for which the GGap estimation is a metric. One such factor might be deglycation. Glycation is a nonenzymatic process dictated by glucose concentration. While the generation of the Schiff base and ketoamine Amadori products is reversible in normal reaction kinetics (10), there is a fructosamine-3-kinase (FN3K)-catalyzed pathway (11,12) removing ketoamines and preventing AGE production, including HbA_{1c}. Accordingly, FN3K gene knockout mice show increased protein glycation (13). In human diabetes, single nucleotide polymorphisms alter FN3K activity affecting HbA_{1c}, the onset of type 2 diabetes, and pathogenic mechanisms related to its complications (14,15). A potential mechanism for the variation in glycation, measured as the GGap, may be FN3K-related glycation/deglycation shift

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Received 13 April 2017 and accepted 17 October 2017.

through deglycation of intracellular proteins, such as Hb, precisely because it is primarily intracellular and thus does not affect fructosamine-assessed circulating protein glycation.

We hypothesized that GGap-positive patients (higher HbA_{1c} levels than average glycemia) would have lower FN3K activity than GGap-negative patients and that FN3K differences would reflect in factors related to diabetes complications. We therefore measured erythrocyte FN3K protein concentration and activity, AGEs, E-selectin, and thrombomodulin (endothelial function), PAI-1 (prothrombotic marker), and leptin and adiponectin (pro- and anti-inflammatory adipokines, respectively) in patients characterized as consistently GGap positive or negative, aiming for a de novo demonstration of biochemical mechanisms explaining the GGap and its link to diabetes complications.

RESEARCH DESIGN AND METHODS

Patient Selection

In our previous study among those with GGap estimations on two or more occasions (6), we identified two GGap groups that were distinctly dichotomized, with a consistently positive or negative GGap (greater than +0.5% or greater than −0.5% HbA_{1c}), in order to explore biochemical mechanisms. Individuals were invited in cohorts starting from the extremes of GGap (GGap consistently greater than ±2), and 150 individuals attended. Two patients were excluded, as fructosamine estimation was not possible because of lipemia, with no other inclusion or exclusion criteria. The 148 individuals comprised 81 and 67 individuals with a negative and positive GGap, respectively. An a priori power calculation estimated the minimum sample size as 67.

GGap Calculation

GGap is the difference between the HbA_{1c} and fructosamine-predicted HbA_{1c} ($FHbA_{1c}$). $FHbA_{1c}$ was calculated from the simultaneously measured fructosamine standardized to the HbA_{1c} distribution as follows: $FHbA_{1c}$ = ([(fructosamine − mean fructosamine)/SD fructosamine] × SD HbA_{1c}) + mean HbA_{1c} (4,5).

Analytical Methods

Blood samples were taken into heparinized tubes and centrifuged immediately. Erythrocytes were stored at −80°C until they were used for assay. Plasma was also stored at −80 C.

FN3K Assay

Erythrocyte FN3K protein expression was measured by immunoassay (Cloud-Clone Corp., Houston, TX), with an intra-assay and inter-assay CV of <10 and 12%, respectively; detection range (1:5 diluted samples) was 1.6–100 ng/mL, equivalent to 0.8–910 ng/g Hb. The same assay used for FN3K protein in undiluted plasma gave a range of detection of 0.31–20 ng/mL. Enzymatic activity was assayed using the high-performance liquid chromatography method of Krause et al. (16), which involves following the conversion, catalyzed by erythrocyte lysate FN3K, of a synthetic substrate N^{α}-hippuryl-N^{ε}-(1-deoxy-D-fructosyl)-lysine to N^{α}-hippuryl-N^{ε}-(3-phosphofructosyl)-lysine by quantifying the product using separation on reversed-phase high-performance liquid chromatography and ultraviolet detection. CV was 12%, and lower and upper limits of quantification were 0.2 mU/g Hb and 15 mU/g Hb, respectively. Measurements were normalized to the Hb content of the erythrocyte lysate.

Other Assays

AGE plasma levels were determined by ELISA (OxiSelect Advanced Glycation End Product Competitive ELISA Kit, category no. STA-817; Cell Biolabs, Inc., San Diego, CA) (the kit uses AGE-BSA as a standard and immunogen and can detect AGEs including N-carboxymethyl-lysine and pentosidine), while soluble E-selectins, thrombomodulin, leptin, adiponectin, and PAI-1 levels were measured using appropriate ELISAs (all from R&D systems, Minneapolis, MN), and soluble E-selectins, thrombomodulin, leptin, adiponectin, and PAI-1 levels were measured using ELISA (all from R&D systems). Plasma FN3K, AGE, PAI-1, and soluble E-selectin were measured in all 148 subjects, whereas, owing to technical limitations, erythrocyte FN3K concentration and activity were measured in a random subset of 98 subjects (55 GGap positive and 43 GGap negative).

Routine Metabolite Assays

Routine metabolite assays including HbA_{1c} and fructosamine were measured according to standard methodologies in our NHS quality-controlled routine laboratory (6).

Statistical Methods

Data were analyzed on SPSS, version 24, using χ^2 test for proportions and Student t test for means. The Mann-Whitney U test was used where log conversion failed to normalize skewed data distribution. Bootstrap methodology tested analysis robustness, and the observed power was calculated. Effect of independent variables on a dependent variable was by univariate analysis with Bonferroni correction. ANCOVA was used to fit regression lines. Results are mean ± SD or otherwise percentages. Statistical significance threshold was $P < 0.05$.

Ethics Approval

Ethics approval was by the National Research Ethics Service Committee (ref. no. 11/WM/0224).

RESULTS

The groups were similar for age, sex, ethnicity (black subjects: GGap negative, 7 of 81 [8%]; GGap positive, 3 of 67 [5%]; not significant [ns]), smoking status, type of diabetes, duration of diabetes, and diabetes treatments (Table 1). The subjects in the positive-GGap group were heavier and had higher levels of urinary albumin-to-creatinine ratio (UACR) but were similar for serum creatinine, retinopathy status, and macrovascular status.

As expected, the groups differed for HbA_{1c} ($P < 0.001$) and GGap ($P < 0.001$) (Table 2). Plasma FN3K concentrations

Table 1—Demographic and clinical characteristics of two groups characterized according to GGap status

	Negative GGap	Positive GGap	*P*
n	81	67	
Age (years)	61.3 ± 10.4	64.4 ± 9.3	ns
Sex (% male)	64	60	ns
Ethnicity (% white)	72	72	ns
Smoking status (% never smoked)	56	49	ns
BMI (kg · m^{-2})	30.2 ± 5.2	35.4 ± 6.7	<0.001
Weight (kg)	87.8 ± 18.3	99.1 ± 21.4	<0.01
Type of diabetes (% type 2)	84	91	ns
Duration of diabetes (years)	15 ± 10	15 ± 9	ns
Metformin use	54	68	ns
Any oral hypoglycemic agent	58	69	ns
Insulin therapy (% yes)	69	81	ns
Retinal status (% with any retinopathy)	72	71	ns
UACR (μg/μmol)	4.4 ± 18.5	9.7 ± 29.7	<0.05*
Creatinine (μmol/L)	86 ± 22	82 ± 21	ns
Cholesterol (mmol/L)	4.3 ± 1.2	4.2 ± 1.3	ns
Vascular status (% with macrovascular disease)	29	31	ns

Data are mean ± SD unless otherwise indicated. *Significant only after analysis of log-converted data.

were not different. The negative-GGap group had significantly higher concentrations of erythrocyte FN3K protein, although Hb adjustment was borderline significant ($P = 0.05$ by t test, but Mann-Whitney U test, $P < 0.01$); higher levels of erythrocyte FN3K enzyme activity and erythrocyte FN3K activity-to-concentration ratio; and lower AGE and PAI-1 levels and leptin-to-adiponectin ratio (LAR), with no difference for E-selectin or thrombomodulin.

In univariate analysis, only GGap grouping attained significance affecting any FN3K measure. Specifically for FN3K activity-to-concentration ratio, the overall model $F = 12.54$, $r^2 = 0.41$, $P < 0.001$; GGap $F = 36.36$, $P < 0.001$; HbA_{1c} $F = 0.07$, ns; UACR $F = 2.24$, ns; BMI $F = 0.01$, ns; and ethnic category $F = 1.52$, ns, with Bonferroni $P < 0.008$, bootstrap $P < 0.01$, and observed power = 1).

In ANCOVA, erythrocyte FN3K activity versus concentration (Fig. 1), using $\log_{10}$ conversion for normalizing distribution, showed significant between-group differences ($r^2 = 0.66$, $F = 90.68$, $P < 0.001$) with slope parameters close to unity ($B = 0.90$, $t = 9.219$, $P < 0.001$). Separate regression lines fitted to the two groups were significantly displaced ($F = 61.79$, $P < 0.001$). The difference between negative-GGap (mean = $\log_{10}$ 0.248) and positive-GGap (mean = $\log_{10}$ −0.262) groups was $\log_{10}$ 0.51 (SEM ±0.07, $t = 7.861$, $P < 0.001$), and (log − log) this is a ratio of increased enzyme activity in the negative-GGap group of 3.23 or 323%, consistent with raw data outcomes FN3K activity unadjusted (2.4 of 0.5 = 4.8), Hb adjusted (4.1 of 0.8 = 5.1), and then FN3K concentration adjusted (0.013 of 0.003 = 4.3) (Table 2).

DISCUSSION

FN3K may represent an intracellular system controlling nonenzymatic protein glycation, AGE production, and, hence, diabetes complications (17). FN3K is highly expressed in erythrocytes (18) with an evidenced role in HbA_{1c} variation (13). We demonstrate, de novo, a significant relationship between erythrocyte FN3K and the GGap. FN3K enzyme activity and protein levels were both significantly higher in the negative-GGap group, with further analysis revealing a threefold difference in FN3K activity-to-concentration ratio. We hypothesize that these differences may be accounted for by variations in the FN3K gene (17,19,20) potentially affecting enzyme activity or producing transcript splice variants encoding products of differing activity. This is the subject of our ongoing research.

This novel finding of enzyme activity associated with AGE level potentially links with GGap-associated mortality and morbidity (6). The FN3K difference was accompanied by fivefold change in PAI-1, which would probably be associated with cardiovascular risk (21). Raising the possibility of FN3K impacting the ratio of adipokines via AGE production, a substantially higher LAR was present in the positive-GGap group, consistent with previous studies showing LAR reduction by restriction of AGE levels (22).

These observations sit coherently with interrelationships of glucose, glycation, AGE production, oxidative stress, and inflammation in the genesis of diabetes complications (23), enhanced by the evidenced link between adipose dysmetabolism, inflammation, and the shift in LAR as a key orchestrator of such dysmetabolism (24). Regarding inflammation, we only measured PAI-1, but others have shown a relationship of CRP with hemoglobin glycation index (HGI) (25) and, further, with carotid artery intimal thickness (26).

We acknowledge the study limitations. It is small-scale, with results requiring confirmation. The groups were matched for ethnicity and diabetes treatment, factors that may influence GGap status (5,7,27,28). However, we emphasize differences in BMI, UACR, and prior glycemic control. Neither BMI nor UACR had statistical impact. We note that selection was by divergent GGap status, and groups differed significantly for HbA_{1c} but also (marginally) fructosamine, so there was no glycemic control measure demonstrating equal prior glucose exposure. This affects any conclusion relating glycation/deglycation to FN3K outcomes, although we demonstrate no statistical effect of HbA_{1c} on observed FN3K concentration or activity outcomes. We accept that further studies are required in groups matched for prior glycemia; these must use fructosamine or glucose but clearly not HbA_{1c}.

Table 2—The further metabolic characteristics of two groups characterized according to GGap status

	n	Negative GGap	Positive GGap	*P*	Bootstrap	Observed power
HbA_{1c}, % (mmol/mol)	81, 67	7.5 ± 1.7 (57 ± 21)	9.7 ± 1.7 (83 ± 19)	<0.001	<0.01	1.00
Fructosamine (μmol/L)	81, 67	331 ± 79	302 ± 60	<0.05	<0.05	0.70
$FHbA_{1c}$ (% HbA_{1c})	81, 67	8.8 ± 1.8	8.2 ± 1.3	<0.05	<0.05	0.70
GGap (% HbA_{1c})	81, 67	−1.3 ± 0.7	1.5 ± 0.6	<0.001	<0.01	1.00
Plasma FN3K concentration (ng/mL)	81, 67	3.3 ± 3.5	2.3 ± 2.5	ns	<0.05	0.47
Erythrocyte FN3K concentration (ng/mL)	55, 43	223 ± 78	176 ± 60	<0.01	<0.01	0.90
Adjusted erythrocyte FN3K concentration (ng/g Hb)	55, 43	351 ± 481	239 ± 295	ns*	ns	0.27
FN3K activity (mU/mL)	55, 43	2.4 ± 2.4	0.5 ± 0.4	<0.001	<0.01	1.00
Adjusted FN3K activity (mU/g Hb)	55, 43	4.1 ± 7.4	0.8 ± 1.2	<0.01	<0.05	0.82
Ratio of FN3K activity to concentration (adjusted)	55, 43	0.013 ± 0.017	0.003 ± 0.002	<0.001	<0.02	0.97
Plasma AGE (ng/mL)	81, 67	63 ± 42	79 ± 43	<0.05	<0.05	0.60
PAI (ng/mL)	81, 67	17.2 ± 17.7	93.1 ± 55.7	<0.001	<0.01	1.00
E-selectin (ng/mL)	81, 67	30.5 ± 15.1	28.7 ± 12.8	ns	ns	0.12
Adiponectin (nmol/L)	77, 65	2.8 ± 0.3	2.7 ± 0.5	ns	ns	0.21
Leptin (nmol/L)	77, 65	1.5 ± 0.8	2.0 ± 1.2	<0.01	<0.01	0.91
LAR (nmol/nmol)	77, 65	0.53 ± 0.27	0.72 ± 0.41	<0.01	<0.01	0.93
Adiponectin/BMI (nmol/L/kg · m^{-2})	77, 65	0.095 ± 0.022	0.079 ± 0.020.	<0.001	<0.01	0.99
Leptin/BMI (nmol/L/kg · m^{-2})	77, 65	0.048 ± 0.022	0.055 ± 0.028	ns	ns	0.36
LAR BMI (nmol/nmol/kg · m^{-2})	77, 65	0.53 ± 0.27	0.72 ± 0.41	<0.01	<0.01	0.91

Data are mean ± SD or percentages unless otherwise indicated; numbers in the *n* column appear as negative GGap, positive GGap, respectively. *Significant only after analysis with nonparametric statistics (see text).

A methodological consideration is the differing algorithms for calculating the deviation of HbA_{1c} from prevailing glycemia. Some are derived from the relationship of HbA_{1c} with fructosamine, referred to as the GGap, while for HGI the relationship is with glucose. In our view, the crucial understanding is whether any value is derived by regression analysis. For the HGI, the correlation coefficient between glucose and HbA_{1c} was $r = 0.71$, $r^2 = 0.50$ (29), meaning that 50% of the variance is unexplained. Use of such analysis will be subject to a degree of mathematical error. Some fructosamine-derived GGap methods also use correlation (3). We have used the standardized normal deviation approach in which fructosamine values remain the actual value (not an estimated value) retaining its rank order in the fructosamine distribution as it is converted to an HbA_{1c} equivalent. These methodologies require triangulation by cotesting, and we note that published comparisons of GGap and HGI show comparable findings (30,31). Both methods yield metrics (GGap or HGI) that allude to a potential underlying mechanism for glycation variation, with the intent to distinguish those exposed to equivalent glycemia who differ in glycation.

Another issue relates to our hypotheses of altered glycation/deglycation balance. While deglycation occurs nonenzymatically (10), we propose that FN3K enzymatic activity shifts deglycation sufficiently to account for the GGap. It is held that FN3K deglycates lysine residues with little effect at the HbA_{1c}-defining N-terminal valine. It might follow that FN3K cannot contribute to GGap genesis. Logically, we cannot definitively conclude that FN3K activity

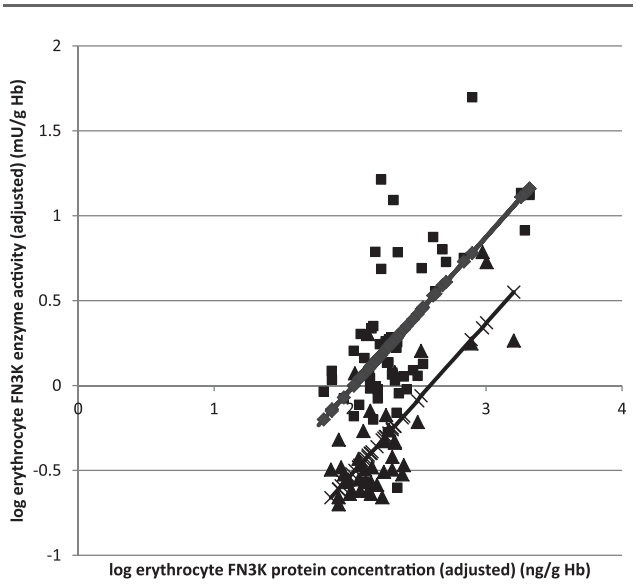

Figure 1—Relationship of erythrocyte FN3K protein and enzyme activity in patients with diabetes with positive and negative GGaps. Patients with a negative GGap (■) ($n = 55$) showed a significantly much higher FN3K enzyme activity in relation to FN3K protein compared with patients with a positive GGap (▲) ($n = 43$) ($P < 0.001$).

variation accounts for the GGap, since we have described association, not causation, albeit for the first time ever. However, the specificity of FN3K comparing N-ε-fructosyl-lysine (FruLys) and "N-terminal" N-α-fructosyl amino acids reportedly ranges from 100 times to 10 times lower affinity (11,32). Lower affinity implies a slower reaction but not a zero-rate reaction, especially at a factoring of 10, which may thus permit some deglycation at the N-terminal valine. Furthermore, published affinity values for FruLys comprise the free amino acid and the protein-bound or histone-bound FruLys, whereas for N-α–bound Amadori products only the free amino acids have been examined (11,33). Thus, the evidence in how FN3K reacts with the N-terminal valine of the hemoglobin protein may not be definitive. Finally, a link between low HbA_{1c} and high FN3K activity in the frequency of a single nucleotide polymorphism associated with high FN3K is reported (14,34). Whilst our data might prompt a reevaluation of FN3K action, we accept it is currently held that FN3K has negligible effect at the Hb β-chain N-terminus and that if this is correct, our hypothesis may be unsustainable.

In conclusion, we suggest that information on an individual's GGap and FN3K status not only may be important to diagnosing and monitoring diabetes but also may assist with assessment of an individual's risk of diabetes complications. Pharmaceutical interventions may become possible using agents that modify FN3K activity.

Funding. This study was partly funded by the Wolverhampton Diabetes Trust.
Duality of Interest. No potential conflicts of interest relevant to this article were reported for any of the authors.
Author Contributions. S.J.D. and B.M.S. designed the study, analyzed data, and wrote the manuscript. A.S.A.-D. and A.U.N. researched data. A.N. assisted with study design and sample collection. A.M.N. provided expert statistical analysis. A.H. provided specialist advice and materials for the FN3K enzyme assay. A.M. researched data. P.K. provided supervisory support for PhD research of A.S.A.-D. J.E.B. assisted with data interpretation and provided supervisory support for PhD research of A.S.A.-D. All authors participated in data interpretation and commented on the draft of the manuscript and approved the final version. S.J.D. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Prior Presentation. Parts of this study were presented in abstract form at the 77th Scientific Sessions of the American Diabetes Association, San Diego, CA, 9–13 June 2017.

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