Evidence that differences in fructosamine-3-kinase activity may be associated with the glycation gap in human diabetes

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Abstract

The phenomenon of a discrepancy between glycated haemoglobin levels and other indicators of average glycaemia 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 (AGE) products. We hypothesised 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 dichotomised for a large positive or negative GGap. FN3K protein was higher and we found a striking 3-fold greater activity (323%) at any given FN3K protein level in the erythrocytes of the negative compared with positive GGap groups. This was associated with lower AGE levels in the negative GGap group (79%), lower pro-inflammatory adipokines (Leptin/Adiponectin ratio) (73%) and much lower pro-thrombotic 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 potential predictor of the risk of diabetes complications. Pharmacological modifications of its activity may provide a novel approach to their prevention.

Non-enzymatic protein glycation is aetiologically important to diabetes complications (1) and for diabetes diagnosis, control and treatment surveillance using glycated proteins, such as glycated haemoglobin (HbA1c) or Fructosamine (2). We, and others, have demonstrated a "glycation gap" (GGap) (3, 4, 5, 6), the discrepancy between average glycaemia determined by HbA1c and Fructosamine, and have shown: methodology for its measurement (4); its consistency amongst individuals (5); its potential for clinical error (4); associated morbidity and mortality (6), noting similar adverse outcomes in ACCORD (7).

Variation in haemoglobin glycation is multi-factorial (8, 9), for example relating to red cell factors. Excluding those, what we are considering here is the possibility of a systematic variation of the glycation phenotype within the human population that is due to factors independent blood glucose concentration, for which the GGap estimation is a metric. One such factor might be deglycation. Glycation is a non-enzymatic process dictated by glucose concentration. Whilst the generation of the Schiff base and ketoamine Amadori products is reversible in normal reaction kinetics (10), there is a Fructosamine-3-kinase (FN3K) (11, 12) catalysed pathway removing ketoamines and preventing AGE production, including HbA1c. Accordingly, FN3K gene knock-out mice show increased protein glycation (13). In human diabetes, single nucleotide polymorphisms (SNPs) alter FN3K activity affecting HbA1c, 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 through deglycation of intracellular proteins such as haemoglobin, precisely because it is primarily intracellular thus not affecting Fructosamine assessed circulating protein glycation.

We hypothesised that GGap positive patients (higher HbA1c levels than average glycaemia) 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, AGE, E-Selectin and Thrombomodulin (endothelial function), PAI-1 (pro-thrombotic marker) and leptin and adiponectin (pro- and anti-inflammatory adipokines respectively) in patients characterised as consistently GGap positive or negative, aiming for a *de novo* demonstration of biochemical mechanisms explaining the GGap and its link to diabetes complication.

Research Design and Methods

Patient selection

In our previous study amongst those with GGap estimations on two or more occasions (6) we identified two GGap groups that were distinctly dichotomised with a consistently positive or negative GGap (> +0.5 % or > -0.5 % glycated HbA1c) in order to explore biochemical mechanisms. Individuals were invited in cohorts starting from the extremes of GGap (GGap consistently > ±2) and 150 individuals attended. Two patients were excluded as Fructosamine estimation was not possible because of lipaemia 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 HbA1c and Fructosamine predicted FHbA1c. FHbA1c was calculated from the simultaneously measured Fructosamine standardized to the HbA1c

distribution as follows: FHbA1c = (((Fructosamine – mean Fructosamine)/SD Fructosamine) x SD HbA1c) + mean HbA1c (4, 5).

Analytical methods

Blood samples were taken into heparinised tubes and centrifuged immediately. Erythrocytes were stored at -80 C until 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, USA); with an intra-assay and inter-assay CV of <10 & 12%; 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 0.31-20ng/mL. Enzymatic activity was assayed using the HPLC method of Krause *et al*., 2006 (16), which involves following the conversion, catalysed 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 RP-HPLC and UV-detection. CV was 12%, lower and upper limits of quantification were 0.2 mU/g Hb and 15 mU/g Hb respectively. Measurements were normalised to the haemoglobin content of the erythrocyte lysate.

Other assays: AGE plasma levels were determined by ELISA (OxiSelect™ AGE-BSA competitive ELISA kit: STA 817 from Cell Biolabs Inc, San Diego, CA, USA; 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, USA) and soluble E-selectins, Thrombomodulin, Leptin, Adiponectin and PAI-1 levels were measured using ELISA (all from R&D systems, Minneapolis, MN, USA). Plasma FN3K, AGE, PAI-1 and soluble E-selectin were measured in all 148 subjects whereas, due to technical limitations, erythrocyte FN3K concentration and activity were measured in a random sub-set of 98 subjects (55 GGap positive and 43 GGap negative).

Routine metabolite assays: including HbA1c and Fructosamine were measured as standard in our NHS quality controlled routine laboratory (6).

Statistical methods

Data were analysed on SPSSv24 using Chi square test for proportions and Student's t-test for means. The Mann Whitney U was used where log conversion failed to normalise 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. Analysis of covariance (ANCOVA) was used to fit regression lines. Results are mean± SD or otherwise percentages. Statistical significance threshold was $p<0.05$.

Ethical approval

Ethics approval was by the National Research Ethics Service Committee (REC reference 11/WM/0224).

Results

The groups were similar for age, gender, ethnicity (black subjects: GGap negative - 7/81(8%); GGap positive - 3/67 (5%); ns), smoking status, type of diabetes, duration of diabetes and diabetes treatments (Table 1). The positive GGap group were heavier, had higher levels of urinary albumin creatinine ratio (UACR) but were similar for serum creatinine, retinopathy status and macrovascular status.

As expected, the groups differed for HbA1c ($p<0.001$) and GGap ($p<0.001$) (Table2). Plasma FN3K concentrations were not different. The negative GGap group had significantly higher concentrations of erythrocyte FN3K protein although haemoglobin adjustment was borderline significant ($p=0.05$ by t test but MWU, $p<0.01$), higher levels of erythrocyte FN3K enzyme activity and erythrocyte FN3K activity /concentration ratio, lower AGE and PAI-1 and Leptin/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/concentration ratio, the overall model was $F=12.54$, $r^2=0.41$, $p<0.001$: GGap, $F= 36.36$, $p<0.001$; HbA1c, $F=0.07$, ns; UACR, $F=2.24$, ns; BMI, $F=0.01$, ns; ethnic category, $F=1.52$, ns; Bonferroni $p<0.008$, Bootstrap $p<0.01$, observed power =1).

In ANCOVA, for erythrocyte FN3K activity versus concentration (Figure 1), using log10 conversion for normalising distribution, this 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 2 groups were significantly displaced ($F=61.79$, $p<0.001$). The difference between negative (mean = $\log^{10}$ 0.248) and positive GGap groups (mean = $\log^{10}$ -0.262) was $\log^{10}$ 0.51 (SEM ±0.07, $t=7.861$, $p<0.001$) and, (log minus log), this is a ratio of increased enzyme activity in the negative GGap group of 3.23 or 323%, consistent with raw data outcomes of FN3K activity unadjusted (2.4 / 0.5 = 4.8), Hb adjusted (4.1 / 0.8 = 5.1) and then FN3K concentration adjusted (0.013 /0.003 = 4.3) (Table 2).

Discussion

FN3K may represent an intra-cellular system controlling non-enzymatic protein glycation, AGE production and hence diabetes complications (17). FN3K is highly expressed in erythrocytes (18) with an evidenced role in HbA1c 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, further analysis revealing a 3-fold difference in FN3K activity / concentration ratio. We hypothesise 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 on-going 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 5 fold 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, there was a substantially higher leptin/adiponectin ratio (LAR) in the positive G-Gap 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 to HGI (25) and further to 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 glycaemic control. Neither BMI nor UACR had statistical impact. Noting selection was by divergent GGap status, groups differed significantly for HbA1c but also (marginally) Fructosamine, so there was no glycaemic control measure demonstrating equal prior glucose exposure. This affects any conclusion relating glycation / deglycation to FN3K outcomes, although we demonstrate no statistical effect of HbA1c on observed FN3K concentration or activity outcomes. Accepting further studies are required in groups matched for prior glycaemia, these must use Fructosamine or glucose but clearly not HbA1c.

A methodological consideration is the differing algorithms for calculating the deviation of HbA1c from prevailing glycaemia. Some are derived from the relationship of HbA1c with Fructosamine referred to as the GGap whilst 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 HbA1c was $r = 0.71$, $r^2 = 0.50$ (29) meaning that 50% of the variance is unexplained. Utilising such analysis will be subject to a

degree of mathematical error. Some Fructosamine-derived GGap methods also utilise correlation (3). We have used the standardised normal deviate approach in which Fructosamine values remains the actual value (not an estimated value) retaining its rank order in the Fructosamine distribution as it is converted to an HbA1c equivalent. These methodologies require triangulation by co-testing, noting 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 glycaemia who differ in glycation.

Another issue relates to our hypotheses of altered glycation/deglycation balance. Whilst deglycation occurs non-enzymatically (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 HbA1c defining N-terminal valine. It might follow that FN3K cannot contribute to GGap genesis. Logically, we cannot definitively conclude FN3K activity 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-epsilon-fructosyllysine (FruLys) and "N-terminal" N-alpha-fructosyl amino acids reportedly ranges from 100 times to 10 times lower affinity (32, 11). 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-alpha-bound Amadori products only the free amino acids have been examined (11,33). Thus, the evidence on how FN3K reacts with the N-terminal valine of the haemoglobin protein may not be definitive. Finally, a link between low HbA1c and high FN3K activity in the frequency of a SNP associated with high FN3K is reported (14, 34). Whilst our data might prompt a re-evaluation

of FN3K action, we accept it is currently held that FN3K has negligible effect at the haemoglobin beta chain N-terminus and that if this is correct our hypothesis is unsustained.

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

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Table 1

The demographic and clinical characteristics of 2 groups characterised according to their Glycation Gap status. The results are presented as mean ± SD or percentages and ns = not significant. * Symbol denotes significance only after analysis of log converted data.

Glycation Gap Category	**Negative**	**Positive**	**P**
n	81	67	
Age (years)	61.3±10.4	64.4±9.3	ns
Gender (% male)	64%	60%	ns
Ethnicity (% white)	72%	72%	ns
Smoking status (% never smoked)	56%	49%	ns
Body mass index (kg/m^{-2})	30.2±5.2	35.4±6.7	p<0.001
Weight (kg)	87.8±18.3	99.1±21.4	p<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 Hypoglycaemic Agent	58%	69%	ns
Insulin therapy (% yes)	69%	81%	ns
Retinal status (% with any retinopathy)	72%	71%	ns
Urinary albumin creatinine ratio (ug / umol)	4.4±18.5	9.7±29.7	p<0.05 *
Creatinine (μmol/l)	86±22	82±21	ns
Cholesterol (mmol/l)	4.3±1.2	4.2±1.3	ns
Vascular status (% macrovascular disease)	29%	31%	ns

Table 2

The further metabolic characteristics of 2 groups characterised according to their Glycation Gap status. The results are presented as mean ± SD or percentages and ns = not significant. * Symbol denotes significance only after analysis with non-parametric statistics (see text). Numbers in each group are shown in column two as negative GGap,positive GGap respectively.

Glycation Gap Category	n	Negative GGap	Positive GGap	P	Bootstrap	Observed power
HbA1c (% glycated) (mmol/mol)	81, 67	7.5 ± 1.7 (57±21)	9.7 ± 1.7 (83±19)	p<0.001	p<0.01	1.00
Fructosamine (µmol/l)	81, 67	331 ± 79	302 ± 60	p<0.05	p<0.05	0.70
Fructosamine derived HbA1c (% glycated HbA1c)	81, 67	8.8 ± 1.8	8.2 ± 1.3	p<0.05	p<0.05	0.70
Glycation gap (% glycated HbA1c)	81, 67	-1.3 ± 0.7	+1.5 ± 0.6	p<0.001	p<0.01	1.00
Plasma FN3K concentration (ng/ml)	81, 67	3.3 ± 3.5	2.3 ± 2.5	ns	p<0.05	0.47
Erythrocyte FN3K concentration (ng/ml)	55,43	223 ± 78	176 ± 60	p<0.01	P<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	p<0.001	p<0.01	1.00
Adjusted FN3K activity (mU/g Hb)	55,43	4.1 ± 7.4	0.8 ± 1.2	p<0.01	p<0.05	0.82
Ratio of FN3K activity / concentration (adjusted)	55,43	0.013 ± 0.017	0.003 ± 0.002	p<0.001	p<0.02	0.97
Plasma AGE (ng/ml)	81, 67	63 ± 42	79 ± 43	p<0.05	p<0.05	0.60
PAI (ng/ml)	81, 67	17.2 ± 17.7	93.1 ± 55.7	p<0.001	p<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	p<0.01	p<0.01	0.91
Leptin/ Adiponectin ratio (nmol/nmol)	77,65	0.53 ± 0.27	0.72 ± 0.41	p<0.01	p<0.01	0.93
Adiponectin / BMI (nmol/l/kg.m-2)	77,65	0.095 ± 0.022	0.079 ± 0.020.	p<0.001	p<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
Leptin/ Adiponectin ratio BMI (nmol/nmol/kg.m-2)	77,65	0.53 ± 0.27	0.72 ± 0.41	p<0.01	p<0.01	0.91

FIGURE LEGEND

Figure 1: Relationship of erythrocyte FN3K protein and enzyme activity in diabetic patients with positive and negative GGaps. Patients with a negative GGap (black squares, n= 55) showed a very significantly higher FN3K enzyme activity in relation to FN3K protein compared with patients with a positive GGap (black triangles, n=43) ($p<0.001$)