Glycated nail proteins: a new approach for detecting diabetes in developing countries

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Abstract

OBJECTIVE   To assess glycation of nail proteins as a tool in the diagnosis of diabetes.

METHODS   Glycation of nail proteins was assessed using a modified photometric nitroblue tetrazolium-based assay, which provides information about average glucose values of the last 6–9 months. Analysis is possible on 10 mg of nail clippings with a within-run coefficient of variation (CV) of 11%. The analyte is extremely stable. The reference range for glycated nail protein (0.55–3.60 μmol/g nail) increases upon ageing.

RESULTS   In diabetics ($n = 112$), values for glycated nail protein are significantly higher (median: 4.07 μmol/g nail, IQR: 2.37–6.89 μmol/g nail, $P < 0.0001$) than in non-diabetics ($n = 116$). ROC analysis shows an AUC of 0.848 (specificity 93.1%; sensitivity 68.9%).

CONCLUSION   This affordable method is a simple alternative for diagnosing diabetes in remote areas as the pre-analytical phase (including all processes from the time a laboratory request is made by a physician until the sample is ready for testing) is extremely robust.

keywords fructosamine, glycation, hair, keratin, nails

Introduction

In developing countries, diabetes prevalence is rising dramatically due to rapid urbanisation, growth and ageing of the population (Mbanya et al. 2010)), as well as by eating behaviours, food supply and fast food facilities in urban areas. In sub-Saharan Africa, type 2 diabetes accounts for >90% of the cases and population prevalence proportions range from 1 to 12%. Besides infectious conditions, timely diagnosis and screening for noncommunicable diseases deserve attention, as both have an important impact on local economies and population morbidity and mortality. Primary prevention and treatment can reduce the incidence of both diabetes and related diseases such as retinopathy, cataract, atherosclerosis, nephropathy and diabetic neuropathy (Hall et al. 2011).

Recently, an International Expert Committee with members of the American Diabetes Association, the European Association for the Study of Diabetes and the International Diabetes Federation revised the diagnostic criteria for diabetes mellitus. Currently, diagnosis of

diabetes is based on plasma glucose concentrations [either a fasting plasma glucose (FPG) concentration ≥126 mg/dl (7.0 mM), a random plasma glucose concentration ≥200 mg/dl (11.1 mM) or a 2-h plasma glucose value ≥ 200 mg/dl in the 75 g oral glucose tolerance test (OGTT)] or a haemoglobin (Hb) A1c ≥48 mmol/mol (The International Expert Committee 2009). Venous blood glucose remains a widely used tool for diagnosing and monitoring of patients with diabetes, but this analysis is subject to pre-analytical variation (pre-analytical conditions for blood prelevations: fasting; pre-analytical conditions of transport: an expected loss of glucose of ±10 mg/dl/h) (Janssen & Delanghe 2010). Although HbA1c is now considered as a good diagnostic tool with high specificity and a low sensitivity (Zemlin et al. 2011), various haemoglobinopathies (e.g. thalassaemias) factors that impact red blood cell survival and red blood cell age, uraemia, hyperbilirubinaemia and iron deficiency may influence the results (Herman & Cohen 2012). The cost of analysis limits the use of HbA1c in certain regions of sub-Saharan Africa. In view of the particular problems of diabetes diagnosis in Africa, there is a call for an

affordable method, which is adapted to regional hospitals in developing countries, requiring only simple equipment and chemicals.

Serum fructosamine assays measure the glycation of all serum proteins and reflect glycaemia over ±2 weeks (Mosca *et al.* 1987; Lindsey *et al.* 2004). This analyte presents several advantages to traditional methods (FPG and OGTT) including a greater pre-analytical stability and a correct interpretation in patients with haemoglobinopathies or anaemia (Schnedl *et al.* 2000; Narbonne *et al.* 2001). Although multiple studies have demonstrated a significant correlation between fructosamine and HbA1c, differences between fructosamine and HbA1c in various studies have also been reported (Islam *et al.* 1993; Braatvedt *et al.* 1997; Cohen *et al.* 2003). This finding can be explained by the fact that both markers reflect a glycaemic status over greatly different time frames as a consequence of very diverse half-lives (Nayak *et al.* 2011).

As the protein content in a keratinised structures (including nails) is approximately 80% of the total mass (Goldsmith 1983), glycation of nail proteins has been observed (Bakan & Bakan 1985; Masuta *et al.* 1989; Seven *et al.* 1993; Márová *et al.* 1995; Kobayashi & Igimi 1996; Yajima *et al.* 2005). Nail clippings can be easily obtained and finger nail fragments rather than blood could be considered as specimens for the analysis. In this pilot study, we explored the possibilities of measuring glycation of nail proteins as a non-invasive method to diagnose and monitor diabetes.

Methods

Subjects

Finger nail clippings from 116 healthy subjects (36 males, 80 females; median age: 36 years, range: 1–91 years) and 112 diabetics (62 men; 50 women, median age: 62 years, range: 21–91 years) were cut into small pieces (size: 2–3 mm) before investigation. The study was approved by the local ethics committee (Ghent University hospital BUN: B670201215602).

Laboratory analysis

After weighing, the nail clippings were transferred into a standard 10-mm pathway cuvette. One ml fructosamine reagent [0.25 mM nitro blue tetrazolium (NBT) (Sigma, St. Louis, USA) in a 0.1 M sodium carbonate/ bicarbonate buffer (pH 10.3) containing 0.1% Triton X-100 (Fluka, St. Louis, USA)] was added to the clippings. After incubation (37 °C, 60 min), photometric readings occurred at 530 nm in a UV-1800 photometer

(Shimadzu, Kyoto, Japan) (Johnson *et al.* 1983). The spontaneous rate of hydrolysis of the NBT dye is very low and below the detection limit within the observation period (60 min). A commercial fructosamine standard (Roche, Mannheim, Germany) was used for standardising the assay. Results were expressed as μmol of fructosamine per g of nail. HbA1c was assayed on EDTA blood specimens using a Menarini 8160 HPLC system (Menarini, Firenze, Italy). Within-run CV, which is defined as the ratio of the standard deviation to the mean and is a measure for test reproducibility, was investigated by aliquoting nail fragments ($n = 10$) in various cuvettes. Stability of the analyte was investigated by storing nail clippings in an incubation oven at 37 °C for 2 weeks.

Retinopathy–nephropathy evaluation

Retinopathy scoring was based on eye fundus examination. Fundus photographs were taken from every patient with diabetes. Diabetic retinopathy was scored by an experienced retina specialist based upon the ETDRS scale (Early Treatment Diabetic Retinopathy Study Research Group 1991). Diabetic nephropathy was assessed according to the KDOQI guidelines (KDOQI 2007).

Statistical analysis

Statistical analyses were performed using MedCalc (MedCalc, Mariakerke, Belgium). Values are expressed as median + interquartile range (IQR). Differences between groups were evaluated using Student's *t*-test. Reference values were determined by defining the 2.5 and 97.5 percentile of a non-diabetic control population. Differences of $P < 0.05$ were considered to be statistically significant. Receiver operating characteristics curve (ROC) analysis was used for calculation of cut-off values. An ROC curve is a graphical plot which illustrates the performance of a binary classifier system as its discrimination threshold is varied. It is created by plotting the fraction of true positives out of the positives (TPR = true-positive rate) *vs.* the fraction of false positives out of the negatives (FPR = false-positive rate), at various threshold settings. TPR is also known as sensitivity, and FPR is one minus the specificity or true negative rate. ROC analysis provides tools to select possibly optimal models independently from (and prior to specifying) the cost context or the class distribution. ROC analysis is related in a direct and natural way to cost/benefit analysis of diagnostic decision-making. When using normalised units, the area under the curve (AUC) is equal to the probability that a classifier will rank a randomly chosen positive instance

higher than a randomly chosen negative one (assuming 'positive' ranks higher than 'negative').

Results

Analytical aspects

In comparison with serum fructosamine, the glycated nail proteins slowly reacted with the nitroblue tetrazolium. Figure 1 shows the kinetics of the photometric reaction in a typical nail preparation. A stable end point of the reaction was reached after ±60 min. Samples as small as 10 mg could be analysed. Within-run CV of the assay was 11%. Storage of nail clippings ($n = 10$) at 37 °C for 2 weeks in an incubation oven did not significantly change fructosamine results.

Effects of clippings size

We carefully analysed the dose dependency of the NBT reaction and the effect of the size of the nail clippings. In five test subjects (four women, one man; age: 24–56 years), three finger nail fragments variable in size were clipped in each individual, ranging from 0.3 to 2.8 mg per fragment. For each individual, the within-run CV value was calculated and compared with the method's CV. The mean CV ($n = 5$) of the series (each series of three sizes of the same subject) with variable size of clippings was 8.97%, which is in the same magnitude as the method's CV. Thus, the contribution of size variance to total variance is negligible. As nails are flat structures (plates), it can be assumed that the diffusion rate of the reagent into the nail is mainly determined by the thick-
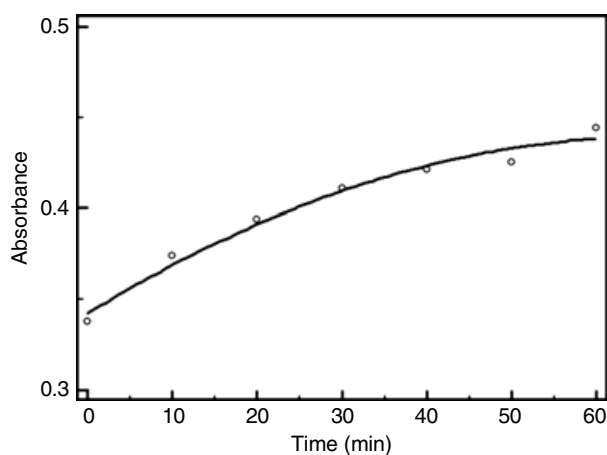

Figure 1 Kinetics of the reaction following the incubation of 8.6 mg of nail fragments in a non-diabetic subject. Photometric readings occurred at 530 nm.

ness of the nail (and not by the size of the clipping). As the variation in nail thickness in man is extremely small, the size of nail clipping is not a critical factor in the analysis. Similarly, we studied in a group of eight volunteers (seven non-diabetics and one diabetic, age: 20–60 years) the effect of 'nail dose' on the test result. The total amount of nail clippings varied between 1 and 10 mg per cuvette. The result was expressed as amount of glycated nail protein/mg nail. A within-run CV of 13% was calculated, which is in the same magnitude as the overall study.

Effects of detergents

An additional experiment was carried out to test the hypothesis that pre-treating nails with detergent (soap) could affect test results. In contrast to personal hygiene procedure, we submerged the nail fragments in Triton X-100-containing solutions for prolonged incubation times. This means that the exposure of the nail proteins in this experiment exceeds by far the conditions encountered related to personal hygiene. Nail clippings originating from 12 subjects were treated with detergent (Triton X-100, Fluka, St. Louis, USA, 0.5% in water) for 1 h to reduce the surface tension and compared with the results of the untreated clippings. Incubation with detergent did not result in a significant change of glycated nail protein concentrations. Untreated nails gave a value of 1.07 ± 0.35 µmol/g nail, and in treated nails, we found a value of 1.02 ± 0.25 µmol/g nail. A paired *t*-test analysis did not demonstrate a statistically significant difference between both groups. To be sure, we tested the nails of professional cleaners with a daily exposure to (acidic/alkaline) cleaning products. In this case, we did not observe abnormal values for glycated nail proteins either.

Reference range

The reference range for glycated nail protein was 0.55–3.60 µmol fructosamine equivalent/g nail (median: 1.75 µmol/g nail, IQR: 1.23–2.28 µmol/g nail). Reference values for males (median: 1.81 µmol/g nail, IQR: 1.18–2.24 µmol/g nail) and females (median: 1.74 µmol/g nail, IQR: 1.25–2.29 µmol/g nail) were not significantly different. Reference ranges for men (0.58–3.80 µmol/g nail) were comparable with those for women (0.55–3.32 µmol/g nail).

Glycated nail proteins in diabetes

In diabetics ($n = 112$), values for glycated nail proteins (median: 4.07 µmol/g nail, IQR: 2.37–6.89 µmol/g nail)

were significantly ($P < 0.0001$) higher than in the reference population (median: 1.75 μmol/g nail, IQR: 1.23–2.28 μmol/g nail). Figure 2 summarises the glycated nail protein concentration in finger nails from healthy subjects and diabetics. There was a marked age dependency of the glycated nail protein values in both non-diabetics, where a 10-year age increase corresponded to a fructosamine increase of 0.07 μmol/g nail ($r = 0.155$) and in diabetics, where a 10-year age increase corresponded to a glycated nail protein increase of 0.06 μmol/g nail ($r = 0.297$). Figure 3 illustrates the age dependency of nail protein in the reference group. In diabetic subjects presenting with diabetic nephropathy ($n = 42$; median: 5.64 μmol/g nail, IQR: 3.28–6.86 μmol/g nail) or retinopathy ($n = 20$, median: 5.65 μmol/g nail, IQR 3.2–7.5 μmol/g nail), glycated nail protein values were higher than in subjects without target organ damage ($n = 50$; median: 3.77 μmol/g nail, IQR: 1.82–5.21 μmol/g nail). Figure 4 shows the correlation of glycated nail protein values with HbA1c: y (glycated nail protein, μmol/g nail) = 0.058 × (HbA1c, mM) + 4.33; $r = 0.08$.

ROC analyses yielded an AUC of 0.848 [95% confidence interval (CI): 0.795–0.891] and a cut-off point of 3.14 μmol/g nail (corresponding with a specificity of 93.1% and a sensitivity of 68.9%). In older subjects, (age >60 years), the AUC further increased to 0.885 (95% CI: 0.785–0.949) (specificity: 91.7%, sensitivity: 78.9%; cut-off value = 3.48 μmol/g nail, Figure 5).

Discussion

Nail proteins are subject to non-enzymatic glycation (Bakan & Bakan 1985; Masuta *et al.* 1989; Seven *et al.* 1993; Márová *et al.* 1995; Kobayashi & Igimi 1996;
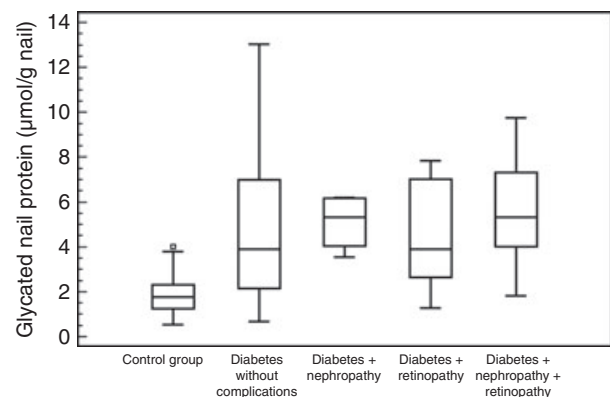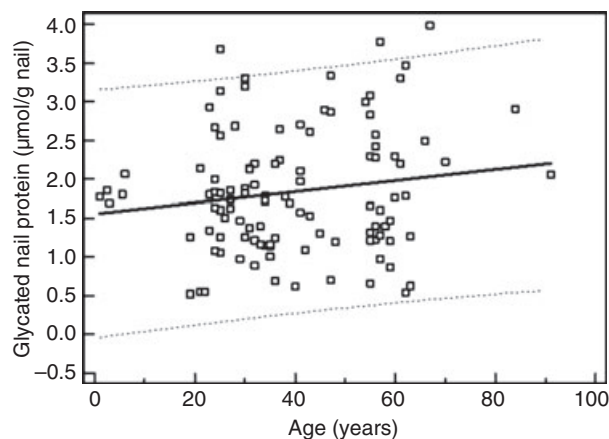
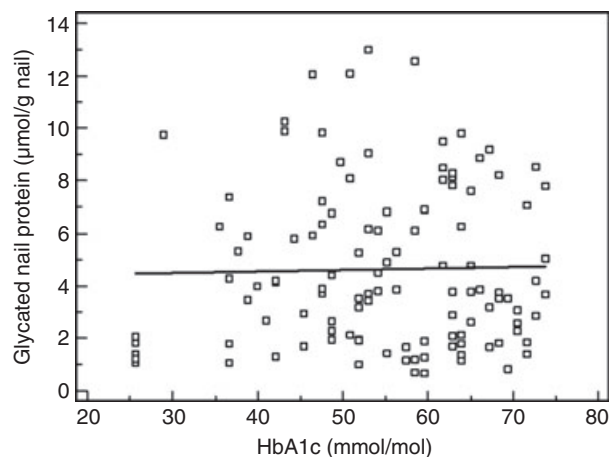**Figure 3** Illustration of the age dependency of nail fructosamine in the reference group.

Figure 4 Correlation of the glycated nail protein values with HbA1c: y (glycated nail protein, μmol/g nail) = 0.058 × (HbA1c, mM) + 4.33, $r = 0.08$.

Yajima *et al.* 2005). The present study shows, for the first time, the successful application of a nitroblue tetrazolium-based test for measuring nail protein glycation in human fingernails. The presented glycated nail protein assay turned out to be reproducible. In contrast to glucose determinations (Chan *et al.* 1989), assaying nail protein glycation does not imply important pre-analytical problems, as even prolonged storage (up to 2 weeks) at 37 °C did not affect test results.

The nail is a specialised keratinous skin appendage that grows approximately 2–3 mm per month, with complete replacement achieved in 6–9 months (Cashman & Sloan 2010). As variation in human nail length is smaller than hair length, this test can be better standardised. The
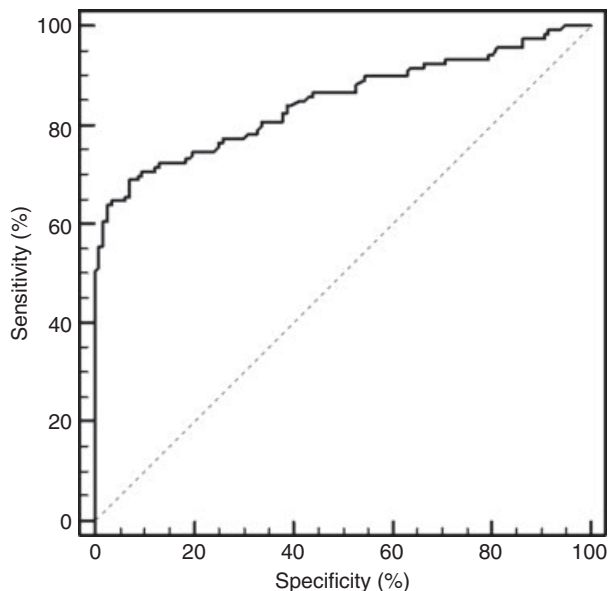
Figure 2 Relative concentration of glycated nail proteins in diabetics ($n = 112$) *vs.* control group ($n = 116$). ($P < 0.01$).

Figure 5 ROC curve of glycated nail proteins. Area under the curve (AUC) = 0.848, cut-off point: 3.14 μmol fructosamine/g nail; sensitivity: 68.9%; specificity: 93.1%).

choice of the finger is not critical, as nail growth velocity is comparable for all fingers (Yaemsiri *et al.* 2010). Use of toe nails is not recommended as diabetic foot injury is associated with trophic disturbances and nail problems (Tantisiriwat & Janchai 2008). Although glycation of nail proteins could also be determined in hair, hair is less suited as a sample because of variation in its length, which creates standardisation problems. Also previous treatment of hair with shampoos might cause analytical interferences.

As the protein content of human nails is about 80% (Goldsmith 1983), the physiological fructosamine/protein ratio is on the order of 2–2.5 μmol/g nail protein, which is a bit lower than the values found by Bakan and Bakan (1985) who used a thiobarbituric acid method, a different standardisation (based on fructose and 5-hydroxymethyl-furfural) and reference values for plasma protein glycation. Typical fructosamine/protein ratios of 3.5 μmol/g protein were found. It is clear that diffusion of molecules in nails (Walters & Fly 1983; Elkeeb *et al.* 2010) is slower than in serum, which explains the lower degree of glycation in nails, despite the longer life span of a finger nail segment.

The test provides information about the period that the nail was exposed to extracellular fluid glucose. The presented method may be regarded as an alternative for assessing long-term regulation of glycaemia. Our data are in agreement with literature data on nail protein glycation obtained in stratum corneum, nails (Bakan & Bakan 1985) and hair (Masuta *et al.* 1989; Seven *et al.* 1993; Kobayashi & Igimi 1996). The increase in reference values upon ageing may be explained by the slower growth rate of nails (Cashman & Sloan 2010), by altered chemical composition of nail proteins (Dittmar *et al.* 2008) and by the increasing reference range for serum glucose upon ageing (Ghasemi *et al.* 2011).

The sensitivity obtained in the present study for glycated nail proteins was better than that of the trivial assessment of urinary glucose (van der Sande *et al.* 1999). Also, the pre-analytical care (often a problem in rural communities in developing countries) is far less critical for glycated nail proteins. Glucosuria is strongly affected by diet and diuresis. In this respect, analyses of glycation in nails definitely offer advantages.

Despite the marked differences observed in glycated nail protein values between diabetics and non-diabetics, glycated nail protein values correlated poorly with HbA1c values. Discordances between HbA1c and other measures of glycaemic control are common in clinical practice as these markers reflect different exposure times, but these differences remain incompletely understood (Cohen *et al.* 2003). In contrast to HbA1c formation, which is a slow intracellular process occurring during the entire life span of an erythrocyte (about 120 days), exposure of nail proteins to extracellular glucose is a longer process (>6 months), as the transfer of the basal zone to the distal part of the nail takes several months. Interestingly, HbA1c is not a substrate for deglycating enzymes as HbA1c values depend on glycation of valine linked glycation. Lysine-linked glycation is a substrate for fructosamine 3 kinase (Delpierrre *et al.* 2004; Delplanque *et al.* 2004). This enzyme was not discovered at the time HbA1c was introduced as a long-term diabetes marker. Currently, we know that fructosamine 3 kinase is polymorphic. Besides biochemical reasons, also glucose diffusion rate from the blood vessel to the nail bed may show variation. These differences may contribute to the observed lack of correlation between glycated nail protein and HbA1c.

The presented method may have an important clinical value in sub-Saharan Africa. In rural Africa, where many people have an aversion to blood sampling, collecting fingernail clippings is an interesting alternative because of its much lower psychological threshold. Moreover, collecting nail clippings does not require medically trained personnel. In comparison with conventional glycaemia determinations, the pre-analytical phase is much less critical as the nail clippings can be stored for a long period, even at 37 °C. As the reagent cost for our glycated nail

protein assay is very small (reagent cost: ±0.1 dollar/ test), the reagents can be easily prepared and the required equipment is simple. Thus, the test is excellently suited for developing countries. Our results suggest that the determination of glycated nail proteins may have the potential to serve as a diagnostic marker of diabetes mellitus and may provide useful information for management of diabetic complications in situations where blood sample analysis is not possible. As this is a pilot study, further studies are needed to investigate the *in vivo* kinetics of nail protein glycation and to assess the clinical value of the assay. It could be interesting to further investigate the correlation between glycated nail protein and other markers of glycaemic control, such as estimated average glucose, fructosamine and glycated albumin.

Acknowledgements

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