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Glycation in human fingernail clippings using ATR-FTIR spectrometry, a new marker for the diagnosis and monitoring of diabetes mellitus

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ABSTRACT

Objectives: Although HbA1c is a good diagnostic tool for diabetes, the precarity of the health system and the costs limit the use of this biomarker in developing countries. Fingernail clippings contain ± 85% of keratins, which are prone to glycation. Nail keratin glycation may reflect the average glycemia over the last months. We explored if attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) can be used as a non-invasive tool for assessing glycation in diabetes.

Design and methods: Using ATR-FTIR spectroscopy, glycation and deglycation experiments with fructosamine 3-kinase allowed to identify the spectrum that corresponds with keratin glycation in fingernail clippings. Clippings of 105 healthy subjects and 127 diabetics were subjected to the standardized ATR-FTIR spectroscopy method.

Results: In vitro glycation resulted in an increased absorption at 1047 cm^{-1}. Following enzymatic deglycation, this peak diminished significantly, proving that the AUC between 970 and 1140 cm^{-1} corresponded with glycated proteins. Within-run CV of the assay was 3%. Storage of nail clippings at 37 °C for 2 weeks did not significantly change results. In diabetics, glycated nail protein concentrations (median: 1.51 μmol/g protein, IQR: 1.37–1.85 μmol/g protein) were significantly higher than in the controls (median: 1.19 μmol/g protein, IQR: 1.09–1.26 μmol/g protein) ($p < 0.0001$). ROC analysis yielded an AUC of 0.92 at a cut-off point of 1.28 μmol/g nail (specificity: 82%; sensitivity: 90%). No correlation was observed between the glycated nail protein concentrations and HbA1c.

Conclusions: Protein glycation analysis in fingernails with ATR-FTIR spectroscopy could be an alternative affordable technique for diagnosing and monitoring diabetes. As the test does not consume reagents, and the preanalytical phase is extremely robust, the test could be particularly useful in developing countries.

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1. Introduction

Based on current estimations, the prevalence of diabetes mellitus will rise dramatically in the coming decades. The largest increase is expected in developing countries. Several determinants of incident prediabetes and diabetes mellitus type 2 in developing nations have been identified: unhealthy diet, waist gain, obesity, hypertension, hypertriglyceridemia, parental history and living in rural area [1]. The number of adults with diabetes mellitus in developing countries will probably rise with 69% between 2010 and 2030. Moreover, mainly young people are affected by the disease in these countries [2,3].

According to the revised criteria of the European Association for the Study of Diabetes, the American Diabetes Association and the International Diabetes Federation, the diagnosis of diabetes mellitus is based on plasma glucose concentrations or on a hemoglobin (Hb) A1c value [4]. At this moment, the analysis of the glucose concentration in venous blood is still widely used for the diagnosis of diabetes mellitus and its monitoring [5].

Glycation markers like fructosamine allow monitoring of average glucose levels over the last weeks [6]. As the protein content in keratinized structures (including nails) is approximately 80% of the total mass [7], glycation of nail proteins has been observed [8–14]. As fingernail clippings can be easily obtained, these specimens have been

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considered as a novel approach instead of the classical blood analysis. So far, chemical assays have been used to investigate keratin glycation [14–16]. However in the present study, we wanted to explore the possibilities of measuring glycation of nail proteins using infrared (IR) spectroscopy as a non-invasive alternative for diagnosis and monitoring of diabetes mellitus.

2. Material and methods

2.1. Subjects

127 healthy subjects (53 males, 74 females; median age: 55 years, IQR: 50–64 years) and 105 diabetics (47 males, 58 females, median age: 59 years, range: 51–69 years) were included in the present study, which was approved by the local ethics committee (Ghent University hospital BUN: B670201215602). Healthy subjects were screened for diabetes mellitus with analysis of HbA1c < **6.5% (48 mmol/mol)**. The group of diabetics consisted of long standing diabetics **under treatment** with a mean HbA1c value of 7.5 ± 1.4% (59 ± 15 mmol/mol).

2.2. Routine laboratory analysis

HbA1c was assayed on ethylenediaminetetraacetic acid (EDTA) blood specimens using a Menarini 8160 high-performance liquid chromatography (HPLC) system (Menarini, Firenze, Italy). The coefficient of variation of the Menarini 8160 HPLC system was 1.5%. The internal QC was based on third party BioRAd QC material. For the external QC assessment, the lab has participated in the joint Belgian/Dutch HbA1c external quality control program (result: pass).

2.3. In vitro glycation study

Fingernail clippings (about 20 mg) of 9 healthy subjects were collected in eppendorf tubes and washed by adding 1 mL of distilled water. The tubes were placed in a sonication bath at 21 °C (60 min). The fingernail clippings were subsequently dried in an incubator (37 °C) during 12 h and pulverized with a dental drill. Afterwards, 3 groups of 3 nail powders were incubated with respectively 1 mL of 0.9% sodium chloride [Mini-Plasco NaCl B. Braun 0.9% (B. Braun Medical NV/SA, Diegem, Belgium] solution, 5% glucose [D(+)-glucose-monohydrate (Merck KGaA, Darmstadt, Germany] solution and 10% glucose solution at 37 °C. After 4 weeks, the samples were centrifuged during 10 min at 10,000 rpm and the supernatant was removed. After addition of 1 mL of distilled water to the powders, samples were again placed in a sonication bath at 21 °C (60 min). The supernatant was removed, which was followed by a drying phase in an incubator at 37 °C (12 h) and a washing step with 1 mL distilled water. After vortexing during 10 s, the supernatant was removed and the samples were finally dried in an incubator (37 °C, 12 h). This was followed by analysis with ATR-FTIR spectroscopy [Spectrum Two IR Spectrometer (PerkinElmer, Inc., Shelton, USA)] in the range from 4500 cm^{-1} to 450 cm^{-1}.

2.4. In vitro deglycation study

After incubation of 5 nail powders (about 20 mg) with a 5% glucose solution at 37 °C (48 h), 40 μL of recombinant fructosamine 3-kinase (0.25 mg/mL, Fitzgerald Industries International, USA) and 40 μL of a 1:1 solution of 5 mM ATP and 2 mM $MgCl_2$ (Sigma-Aldrich, St Louis, Missouri, USA) were added. Incubation at 37 °C during 3 h was followed by a washing step with 1 mL distilled water and transfer in a sonication bath during 60 min. This was followed by a drying step in an incubator at 37 °C (12 h) and analysis by ATR-FTIR spectroscopy.

In addition, the fructosamine concentration was determined before and after the incubation step with fructosamine 3-kinase, as described by Kishabongo et al. [14]. About 20 mg of nail powder was transferred into a standard 10-mm pathway cuvette. One milliliter 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 nail powder. After incubation (37 °C, 60 min), photometric readings occurred at 530 nm in a UV-1800 photometer (Shimadzu, Kyoto, Japan) [17]. 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 standardizing the assay.

2.5. Standardization process of fructosamine determination for the estimation of glycated nail proteins using attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR)

After creating 5 serum pools (one pool consisting of 10 healthy subjects), 1 mL serum was transferred into a tube. 400 μL aceton (Sigma-Aldrich, St. Louis, MO, USA) was added to the serum and afterwards the serum was vortexed for 10 s (Vortex-Genie 2®, Scientific Industries, NY, USA). After centrifugation in a Universal 320 Centrifuge (Hettich Zentrifugen, T912) during 10 min at 3000 *g* and after removing the supernatant, the precipitate was dried in a Jouan EB18 incubator (37 °C) during 12 h. The obtained precipitate was pulverized in a mortar and analysed with ATR-FITR spectroscopy [Spectrum Two IR Spectrometer (PerkinElmer, Inc., Shelton, USA)] in the range from 4500 cm^{-1} to 450 cm^{-1}. The absorbance spectra were normalized on the highest peak in the resulting spectrum (amide I band at 1645 cm^{-1}) and the area under the curve (AUC) was calculated between 970 and 1140 cm^{-1} using the Spectrum 10TM Software. Within-run CV of the assay was investigated.

In addition, the fructosamine concentration was determined in the different serum pools on a Cobas® 8000 analyzer (Roche Diagnostics, Basel, Suisse). A commercial fructosamine standard (Roche, Mannheim, Germany) was used for standardizing the assay. In this way, it was possible to calculate a conversion factor between the AUC data and the fructosamine concentrations.

In a second phase, fingernail clippings from 105 healthy subjects and 127 diabetics were collected in Eppendorf tubes and washed by adding 1 mL of distilled water. The tubes were placed in a sonication bath at 21 °C (60 min). The fingernail clippings were subsequently dried in an incubator (37 °C) during 12 h, pulverized with a dental drill and analysed with ATR-FITR spectroscopy.

2.6. Statistical analysis

Statistical analyses were performed using MedCalc (MedCalc, Mariakerke, Belgium). D'Agostino-Pearson test and Kolmogorov-Smirnov test were performed to test the normal distribution of the data. Values were expressed as median + interquartile range (IQR). Differences between groups were evaluated using the Mann–Whitney *U* test. 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.

3. Results

3.1. Analytical aspects

Fig. 1 shows the IR spectrum of nail powders after incubation with respectively 1 mL of 0.9% sodium chloride solution, 5% glucose solution and 10% glucose solution. The absorbance spectra were normalized on the highest peak in the resulting spectrum (amide I band at 1645 cm^{-1}). A clear difference in the area under the IR peak was observed at wavenumber 1047 cm^{-1}. This band was used for measuring the degree of keratin glycation. In addition to the recombination band (1047 cm^{-1}), overtone bands were observed at 2095 and 3140 cm^{-1}. Using the Spectrum

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