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Analysis of protein glycation in human fingernail clippings with near-infrared (NIR) spectroscopy as an alternative technique for the diagnosis of diabetes mellitus

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Abstract

Background: Glycated keratin allows the monitoring of average tissue glucose exposure over previous weeks. In the present study, we wanted to explore if near-infrared (NIR) spectroscopy could be used as a non-invasive diagnostic tool for assessing glycation in diabetes mellitus.
Methods: A total of 52 patients with diabetes mellitus and 107 healthy subjects were enrolled in this study. A limited number (n = 21) of nails of healthy subjects were glycated *in vitro* with 0.278 mol/L, 0.556 mol/L and 0.833 mol/L glucose solution to study the effect of glucose on the nail spectrum. Consequently, the nail clippings of the patients were analyzed using a Thermo Fisher Antaris II Near-IR Analyzer Spectrometer and near infrared (NIR) chemical imaging. Spectral classification (patients with diabetes mellitus vs. healthy subjects) was performed using partial least square discriminant analysis (PLS-DA).
Results: *In vitro* glycation resulted in peak sharpening between 4300 and 4400 cm^{-1} and spectral variations at 5270 cm^{-1} and between 6600 and 7500 cm^{-1}. Similar regions encountered spectral deviations during analysis of the patients' nails. Optimization of the spectral collection parameters was necessary in order to distinguish a large dataset. Spectra had to be collected at 16 cm^{-1}, 128 scans, region 4000–7500 cm^{-1}. Using standard normal variate, Savitsky-Golay smoothing (7 points) and first derivative preprocessing allowed for the prediction of the test set with 100% correct assignments utilizing a PLS-DA model.
Conclusions: Analysis of protein glycation in human fingernail clippings with NIR spectroscopy could be an alternative affordable technique for the diagnosis of diabetes mellitus.

Keywords: glycation; infrared spectroscopy; keratin; nails; partial least square discriminant analysis.

Introduction

The rising prevalence of diabetes mellitus is creating a global public health problem [1]. According to the current international criteria, diagnosis of diabetes mellitus is based on plasma glucose concentrations (either a fasting plasma glucose [FPG] concentration ≥7.0 mmol/L, a random plasma glucose concentration ≥11.1 mmol/L or a 2-h plasma glucose value ≥11.1 mmol/L in the 75 g oral glucose tolerance test [OGTT]) or a hemoglobin (Hb) A_{1c} level ≥48 mmol/mol [2]. Blood glucose remains widely used for the diagnosing and monitoring of diabetes mellitus, but this analysis is invasive and subject to preanalytical variation [3]. Although HbA_{1c} is now considered as a good diagnostic tool [4], various hemoglobinopathies (e.g. thalassemias), factors that impact erythrocyte survival and age, hyperbilirubinemia, uremia and iron deficiency may influence the results [5].

Glycated keratin allows monitoring average glucose levels over the previous weeks. In diabetes mellitus, an increased glycation of nail keratins has been observed [6–8]. Finger nails, consisting of keratin, are simple to collect and can be easily investigated. Earlier methods to assay nail keratin glycation were based on chemical analysis of nail clippings either by wet chemistry [8] or mid-infrared (MIR) spectroscopy [9]. More specifically,

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Coopman et al. [9] demonstrated that attenuated total reflection-Fourier transform infrared (ATR-FTIR) spectroscopy could successfully be used to measure the concentration of glycated nail proteins in human fingernails. According to *in vitro* glycation of nail clippings, the region between 970 and 1140 cm^{-1} was identified as the region of interest, attributed to the CO stretching band. The previous published approaches were hampered by the time-consuming character of the analysis or by the necessity to powder the nail clippings prior to analysis, which prevents the application of these methods into routine clinical practice. Near-infrared (NIR) spectroscopy is an alternative, non-invasive and fast tool to assess glycation. The possibility to connect fiber optic probes to NIR equipment makes this technique more flexible and allows measurement on complete fingernails [10]. In the present study, we wanted to explore the possibilities of measuring glycation of nail proteins using NIR spectroscopy in combination with partial least square discriminant analysis (PLS-DA) [11] as a non-invasive alternative for the diagnosis of diabetes mellitus.

Materials and methods

Collection and preparation of nails

A total of 107 healthy subjects and 52 patients with diabetes mellitus were included in this study. The demographics of both groups are presented in Table 1. Healthy subjects were screened for diabetes mellitus through analysis of HbA_{1c} (limit set at <6.5% [48 mmol/mol]). The group of patients consisted of individuals with a long-standing diabetes, both diabetes mellitus type 1 and 2, under treatment and were recruited at the Department of Endocrinology of the Ghent University Hospital. The study was approved by the local Ethics Committee. Fingernail clippings (free of nail polish) of healthy subjects and of patients with diabetes mellitus were collected in Eppendorf tubes and washed by adding 1 mL of distilled water. The tubes were placed in a sonication bath (Branson 3510DTH, Sigma-Aldrich, St. Louis, MO, USA) at 21 °C for 60 min. Subsequently, the nail clippings were removed from the Eppendorf tubes and dried by using tissues. When a hyponychium was discovered, this was removed. Finally, the nails were dried in an incubator (Jouan EB18®, Saint-Herblain, France) at 37 °C over 12 h.

Table 1: Demographics of investigated population.

	Healthy subjects (42 males and 65 females)	Patients with diabetes mellitus (38 males and 14 females)	p-Value
Diabetes type 1 (n)		12	
Diabetes type 2 (n)		40	
HbA_{1c}, mmol/mol		55 (50–60)	
Age, years	31 (28–34)	58 (50–63)	$p<0.0001$
Height, m	1.72 ± 0.09	1.71 ± 0.08	N.S.
Weight, kg	69 (64–72)	81 (74–89)	$p<0.0001$
BMI, kg/m^2	23 (22–24)	29 (27–30)	$p<0.0001$

N.S., not significant.

In vitro glycation study

Fingernail clippings of about 20 mg of 21 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 for 60 min. The fingernail clippings were subsequently dried in an incubator at 37 °C over 12 h. The clippings of nine randomly selected subjects were pulverized with a dental drill. Afterwards, three groups of either three nail powders and three nail clippings were incubated with, respectively, 1 mL of 0.9% sodium chloride solution (Mini-Plasco NaCl B. Braun 0.9% [B. Braun Medical NV/SA, Diegem, Belgium]), 0.278 mol/L glucose solution (D(+)-glucose-monohydrate [Merck KGaA, Darmstadt, Germany]) and 0.556 mol/L glucose solution at 37 °C. For the complete nail clippings, an extra solution of 0.833 mol/L glucose was prepared for incubating nail clippings of three subjects. After 4 weeks, the samples were centrifuged for 10 min at 3000 *g*, followed by removing the supernatant. Subsequently, the powders were washed by adding 1 mL of distilled water to the powders. The samples were placed in a sonication bath at 21 °C for 60 min. After removing the supernatant, a drying step was performed in an incubator at 37 °C for 12 h, which was followed by another 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).

NIR spectroscopy

NIR spectra were recorded off-line using a Fourier-transform NIR spectrometer (Thermo Fisher Scientific, Nicolet Antaris II Near-IR analyzer) equipped with an InGaAs detector and a quartz halogen lamp. Fingernails are usually convex, which has implications for reflectance spectroscopy measurements. When radiation strikes a surface it may be reflected, transmitted or absorbed. The relative amounts of reflection and transmission are determined by the refractive index of the nail vs. the air and the angle of incidence. This means that the position of the radiation beam onto the convex sample has to be carefully selected in order to obtain an appropriate number of reflected beams onto the detector to reach a sufficient intensity of the signal. To avoid this difficulty for the preliminary experiments and to exclude spectral variation due to the angle of incidence, the measurements were executed using the integrating sphere. The integrating sphere allows measuring an extended sample area (depending on the sample port size) and provides uniform light collection independent of the sample orientation [12]. The prepared nail clippings were placed upside down, i.e. with the upper-side directed to the integrating sphere module of the instrument. The spectra were computed across the spectral range 10,000–4000 cm^{-1} and run in triplicate for each patient. In order to be able to select the appropriate resolution and number of scans, the measurements were executed at the resolutions 4, 8 and 16 cm^{-1} and the number of scans varied from 32 to 64 to 128 scans. Instrument control was performed with the Result Integration software (Thermo Fisher Scientific, Dublin,

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Ireland). The spectra were recorded at ambient temperature. Besides using the Integrating sphere module, spectra were also measured using the MR non-contact probe (Kaiser Optical Systems, Inc., Ann Arbor, MI, USA).

NIR chemical imaging

Ten fingernail clippings were analyzed using NIR-chemical imaging (NIR-CI) in order to visualize the homogeneity of glycation of the keratin on the surface of the nails. The NIR spectral images of the nails were collected using a Pushbroom line-scanning hyperspectral camera (SWIR, Specim Ltd., Oulu, Finland). The nails were put on a black background. The camera scans a row of 320 spatial pixels at a time (spatial resolution was 32 µm) and disperses the incoming light from each pixel in the spectral range 5880–11,100 cm^{-1} onto one column of the 320 × 256-pixel mercury-cadmium-telluride (MCT)-detector (14-bit readout, cooled to −40 °C with a 4-stage 6 Peltier system to reduce noise). The lens-to-camera distance was 96 mm. The nails were illuminated with one set of halogen lamps (V = 16.0 V [voltage controlled] – I = 0.08 A, Specim Ltd., Oulu, Finland). Hence, the set of lamps shine light onto the sample surface at an angle of 45° and the camera looks directly down towards the sample. From the total NIR-CI image of each nail sample, a square (50 × 50 pixels) was randomly selected. A mean spectrum was calculated from this square. The mean spectrum was SNV corrected and the first derivative was applied.

Spectral analysis

NIR spectral data analysis was performed using SIMCA P+ version 14.1. (Umetrics, Umeå, Sweden). Different preprocessing methods were examined. These include standard normal variate (SNV), Savitzky-Golay smoothing and derivatives. The use of SNV preprocessing eliminates the additive baseline offset variations and multiplicative scaling effects in the spectra, which may be caused by possible differences in sample density and different sample-to-sample measurement variations. Savitzky-Golay smoothing can be performed to reduce the level of noise, while keeping the spectral details. Derivatives accentuate small structural differences between similar spectra and can be used to reduce baseline effects, which will facilitate spectral discrimination. Furthermore, derivatives cause spectral resolution enhancement, increasing the apparent resolution of overlapping spectral bands. The first derivative is a very effective method for removing linear ordinary offsets. The second derivative is a very effective method for removing a sloping baseline from a spectrum [13, 14].

Furthermore, PCA-class and PLS-DA models were developed based on the NIR spectra from the different sample types (diabetes mellitus and control nails). PLS-DA models are able to accomplish a rotation of the projection to give latent variables a focus on class separation, i.e. discrimination. This type of model takes into account the class membership of observations and is developed from a training set of observations with known classes (sample pretreatments) [15]. The aim was to use the PLS-DA models to predict the class membership of future samples. The ability of each created PLS-DA model to classify observations was evaluated using a misclassification table. Such a table shows the proportion of correct classification of the tested new observation set. The calculated misclassification rates are considered as good indicators of model performance [16, 17].

Statistical analysis

Statistical analyses were performed using MedCalc (MedCalc, Mariakerke, Belgium). Data are expressed as mean ± standard deviation or as median (95% confidence interval). Differences between patient groups were evaluated using the t-test or the Mann-Whitney U test. A p-value of <0.05 was considered to be statistically significant.

Results

In vitro glycation of nails

In order to evaluate whether NIR spectroscopy has the potential to measure increasing concentration of glycated nail proteins, *in vitro* glycated pulverized fingernails incubated with 0.278–0.556 mol/L glucose solution were investigated with the NIR-integrating sphere. Most spectral variation could be observed in the region between 4150 and 6150 cm^{-1} (Figure 1). Table 2 gives an overview of the wavenumber of interest and the corresponding band assignment. Especially, peak 4666 cm^{-1}, attributed to the $CONH_2$ stretching band, became broader when the glycation increased and the RCO_2H band shifted from 5253 cm^{-1} at 0.278 mol/L glycation to 5272 cm^{-1} at 0.556 mol/L glycation.

In order to be able to prove that NIR might be a suitable technique to measure complete fingernails instead of pulverized nails, these *in vitro* glycation experiments were repeated with unpulverized complete nail clippings. The analysis of the spectra collected after *in vitro* glycation of unpulverized nails revealed the possibility of measuring increasing levels of glycation in complete fingernail clippings. Here, the most expressed spectral variation could be observed in the region between 4150 and

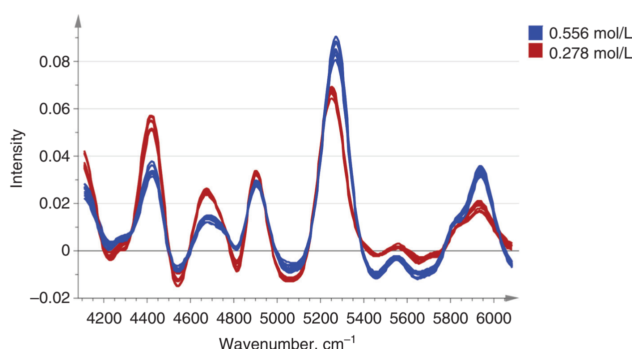

Figure 1: NIR spectra (4150 and 6150 cm^{-1}) of *in vitro* glycated pulverized nails with 0.278 mol/L (red) and 0.556 mol/L (blue) glucose solution.

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Table 2: Spectral band assignments.

Wavenumber, cm^{-1}	Band assignment
970–1140	C-O stretching band
4280	CH stretching band
4321	Combination band of C-H stretch, C-H bending and O-H bending of α-anomer of glucose
4435	Combination band of CH- and OH-related modes of α-anomer of glucose
4666	$CONH_2$ stretching band
5071	Combination of NH stretching and bending
5253	RCO_2H band
6900	Overlapping bands due to the combinations of OH antisymmetric and symmetric stretching
7190	OH stretching overtone
7420	First overtone of C-H combinations

5700 cm^{-1} and between 6600 and 7500 cm^{-1} (Figure 2). Besides the increasing intensity observed for the higher level of glycation, the peak located at 4280 cm^{-1} became sharper (Figure 2A). Peak 5071 cm^{-1} (0.278 mol/L glycation) shifted to 5077 cm^{-1} and 5081 cm^{-1} for 0.556 mol/L and 0.833 mol/L glycation, respectively. This is demonstrated in Figure 2B and D. Furthermore, the RCO_2H band located at approximately 5270 cm^{-1}, which was shifted for the pulverized glycated nails, became broader and two new peaks appeared at 5384 and 5415 cm^{-1}. Also in the region between 6600 and 7500 cm^{-1}, spectral deviations owing to glycation could be detected (Figure 2C). Especially, the peak located at 7190 cm^{-1} became more expressed when the glycation level increased and the peak 7420 cm^{-1} shifted and decreased in intensity. The peak 6900 cm^{-1} (0.278 mol/L glycation) shifted leftwards when the glycation became higher and reached 6880 cm^{-1} at 0.833 mol/L glycation. The difference in spectral variations between the pulverized nails and complete nail clippings can be explained by the fact that the structure (powder vs. nail) of the sample has a large influence on how the laser beams will be reflected. Furthermore, a nail is composed of several layers, which are homogenized in the pulverized nails.

In vivo glycation

Preliminary experiments

Initially, a preliminary experiment was performed to investigate whether NIR spectroscopy could be used as

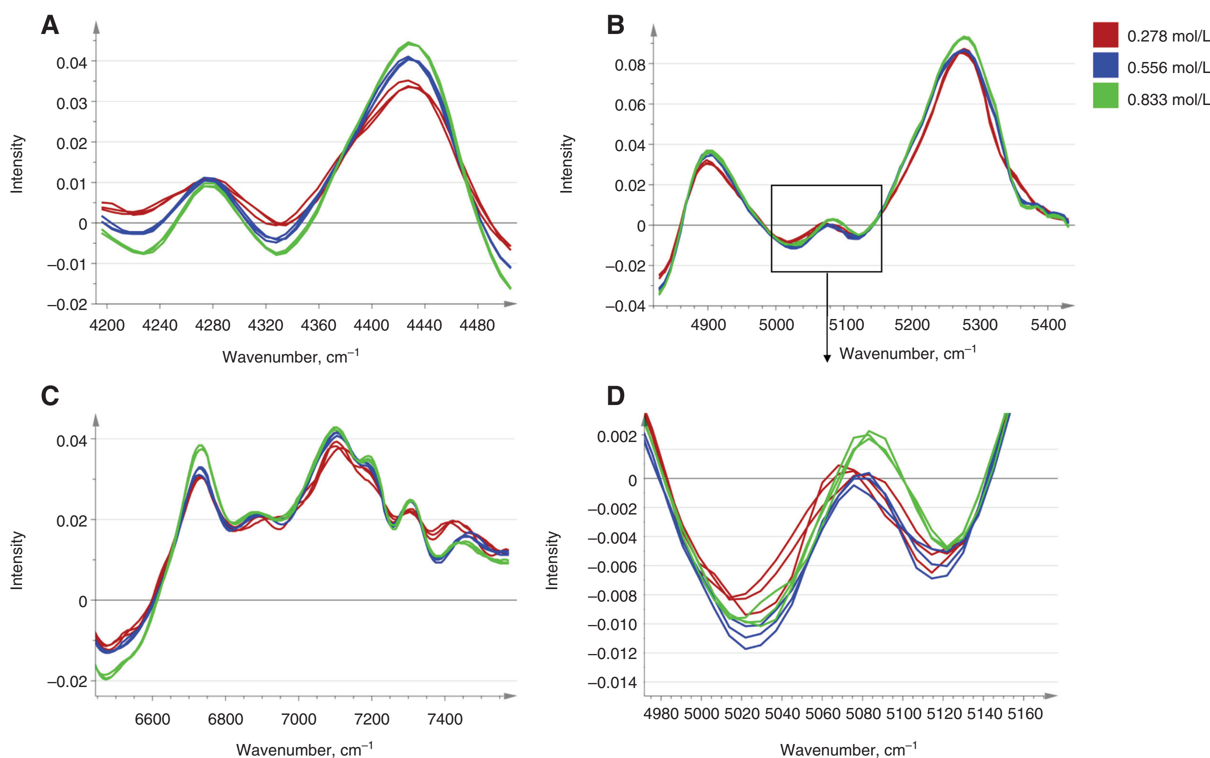

Figure 2: NIR spectra of *in vitro* glycated complete nail clippings with 0.278 mol/L (red), 0.556 mol/L (blue) and 0.833 mol/L (green) glucose solution.
A–C represent partial NIR spectra: 4200–4500 cm^{-1} (A), 4800–5500 cm^{-1} (B), and 6500–7500 cm^{-1} (C); D represents spectral details between 4980 and 5160 cm^{-1}.

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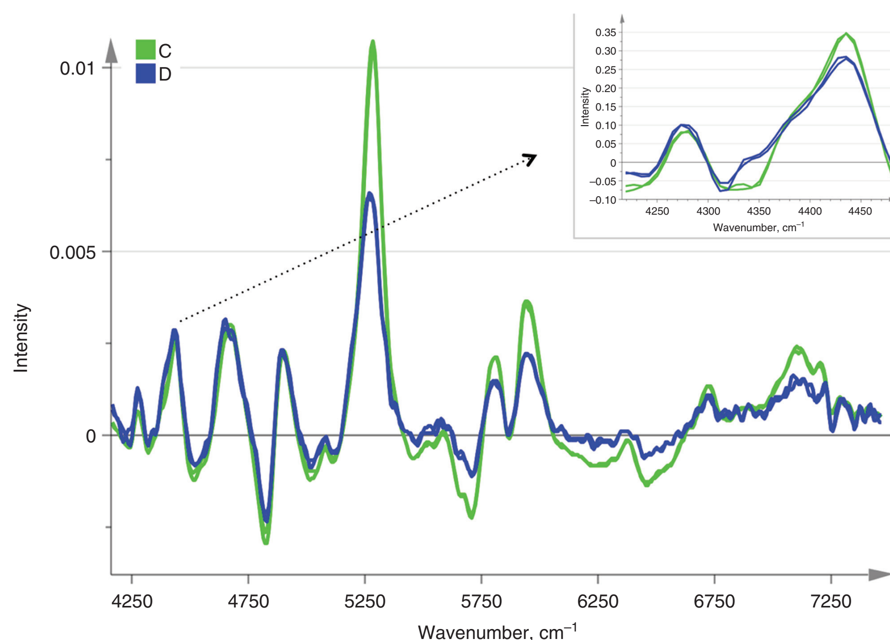

Figure 3: NIR spectra collected from nail clippings of healthy subjects (green) and patients with diabetes mellitus (blue).

a diagnostic tool for diabetes mellitus. The initial experiment was performed on complete nail clippings of 20 patients, including healthy subjects (n = 10) and patients with diabetes mellitus (n = 10). From each patient three nails were collected and measured with the NIR integrating sphere using a resolution of 16 cm^{-1} with a total of 32 scans. The spectra revealed variations in the spectral range between 4200 and 7500 cm^{-1}. Clear deviations could be observed between 6250 and 7300 cm^{-1} due to the NH and OH first overtone stretching. However, the spectra did contain a lot of noise in this area. It was possible to distinguish healthy subjects from patients with diabetes mellitus, based on the spectral region between 4200 and 6000 cm^{-1} using SNV and first derivative as preprocessing techniques (Figure 3). The clearest variation could be observed at the peak 4435 cm^{-1} with the extra broadening at 4335 cm^{-1}. Furthermore, a general trend of peak broadening could be seen for the samples of patients with diabetes mellitus.

Parameter optimization

As the number of samples included in the preliminary experiments was low, the experiments were repeated including more patients. However, the more extended experimental database complicated the distinction between the two groups. Hence, it was necessary to optimize the data collection procedure. The spectra of 10 patients with diabetes mellitus and 10 healthy subjects were collected at resolutions 4, 8 and 16 cm^{-1} at a total of 32, 64 and 128 scans. However, using a resolution of 4 cm^{-1} was useless as this resulted in very noisy spectra. In order to select the most appropriate combination, the prediction ability of every combination (resolution and number of scans) was estimated. The data were divided in a training and test set. The training set served to create the model, which was used to predict the sample class of the unknown spectra of the test set. These experiments were executed for different spectral ranges, being 4000–7500 cm^{-1}, 4150–5350 cm^{-1} and 4150–4650 cm^{-1}. The results demonstrated that the combination of resolution 16 cm^{-1} and 128 scans was the most promising, having a maximum prediction ability of 85%.

Confirmation of distinguishing ability

The extended dataset was performed on 107 healthy subjects and 52 patients with diabetes mellitus (Table 1). From each patient, five spectra (16 cm^{-1} and 128 scans) were collected from different fingernail clippings. Different preprocessing techniques were executed and multiple spectral regions were investigated in order to select the optimal preprocessing techniques and best spectral region. Using SNV, Savitsky-Golay smoothing (7 points) and first derivative preprocessing on the spectral region 4000–7500 cm^{-1} allowed to predict the test set with 100% correct assignments (Figure 4). Therefore, a PLS-DA model was used. This can be seen in the

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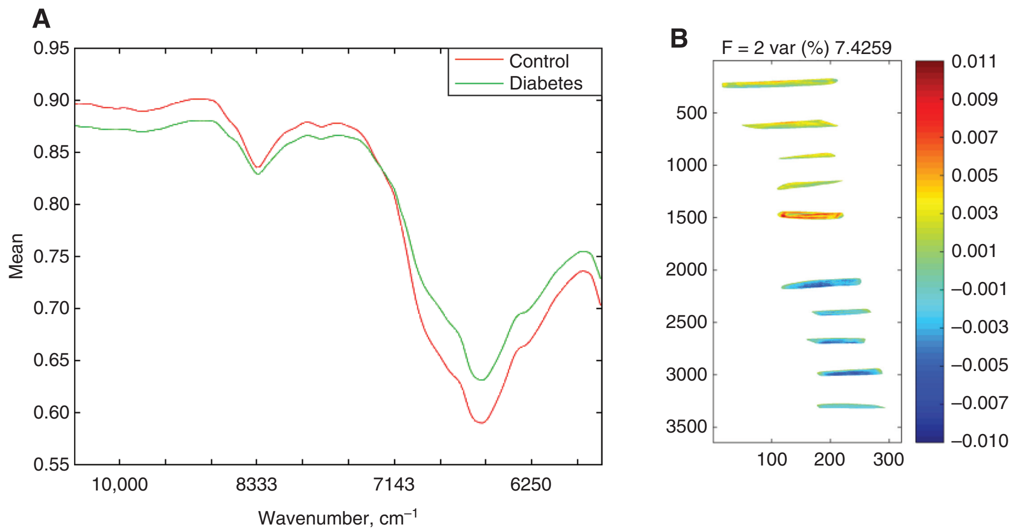

Figure 4: A compares spectra between controls and diabetics, B shows spectral images composed of multiple spectra with 32 µm intervals, showing a clear distinction over the complete nail clipping.
Analysis according NIR-CI with (A) the range of interest (B) visualization of distinction between healthy subjects (green-red) and diabetes patients (blue) which occurred in a homogeneous way.

Table 3: Misclassification table of the extended data set showing an excellent performance of the model with 100% correct classification.

	Members	Correct, %	Healthy subjects	Patients with diabetes	No class
Healthy subjects	107	100	107	0	0
Patients with diabetes	52	100	0	52	0
Total	159	100	107	52	0

misclassification table (Table 3). No confounding could be observed.

Non-contact probe and NIR-CI

In addition, the experiments were also executed using the NIR probe and NIR-CI. The NIR-probe was used in order to investigate whether the experiment could be executed on fingernails still attached to the hand of patients instead of using the integrating sphere to analyse fingernail clippings. As the fiber optic cable connected to the probe starts to transport data from 4500 until 7000 cm^{-1}, the current used fiber optic cable used was not optimal. Although, as only five patients of each class were used, it was possible to distinguish between patients with diabetes mellitus and healthy subjects with 100% prediction ability.

Besides the NIR probe, the experiments were also executed using NIR-CI on complete fingernail clippings from five healthy subjects and five patients with diabetes mellitus. Every 32 µm, a spectrum was taken and this was executed over the whole surface of the nail clipping, resulting in a spectral image of the nail. This allows visualizing the homogeneity of the spectral variations due to glycation on the complete nail surface. The NIR-CI equipment measures in a range between 5880 and 11,100 cm^{-1}. Hence, it was not possible to make a distinction between the two classes based on the band located between 4300 and 4400 cm^{-1}. However, it was possible to distinguish the two classes based on the peak between 7140 and 6250 cm^{-1}, attributed to the NH and OH first overtone stretching bands (Figure 4A). As a result, the NIR image on Figure 4B shows a clear color difference between the fingernail clippings from patients with diabetes mellitus (green-red) and the fingernail clippings from the healthy subjects (blue). This color difference is clear over the complete nail clipping due to the homogeneous pattern of the glycated state. This means that the location of measurement with a NIR probe can randomly be chosen.

Discussion

The present study shows for the first time the successful application of NIR spectroscopy for estimating the degree

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of glycated nail proteins in human fingernails. Using this interesting method, we were able for the first time to distinguish patients with diabetes mellitus from healthy subjects with a 100% certainty in a non-invasive way. Our results suggest that the determination of glycated nail proteins by NIR reflectance spectroscopy may have the potential to serve as a diagnostic marker of diabetes mellitus in situations where blood sample analysis is not possible. Direct NIR illumination of fingernails has the advantage of a very quick analysis time, which allows diabetes mellitus screening in third world countries (e.g. in sub-Saharan Africa), in which the diagnosis of diabetes mellitus is at the lowest rates in the world [18].

The human nail is a specialized keratinous skin appendage that grows approximately 2–3 mm per month, with a complete replacement achieved in 6–9 months [8]. The choice of the finger is not critical, as nail growth velocity is comparable for all fingers [19]. Use of toe nails is not recommended as a diabetic foot injury is associated with trophic disturbances and nail problems [20]. The presented test provides information about the period that the nail was exposed to glucose in the extracellular fluid. In contrast to blood glucose concentration measurements [3], assaying nail protein glycation does not imply important preanalytical problems, as even prolonged storage (up to 2 weeks) at 37 °C did not affect test results [9]. As there is no reagent cost for the described assay, this proposed screening strategy is very economical. In addition, the non-invasive character of the proposed technique results in a much lower psychological threshold without a need of medically trained personnel.

In the *in vitro* part of the study, we demonstrated that the degree of nail glycation in pulverized fingernail clippings could be determined with NIR spectroscopy. In comparison with the ATR-FTIR spectroscopy [9], in which the CO stretching band was the region of interest, the $CONH_2$ and the RCO_2H bands captured our attention using NIR spectroscopy. Subsequently, *in vitro* glycation of unpulverized fingernails identified two regions of interest between 4150 and 5700 cm^{-1} and between 6600 and 7500 cm^{-1}. The peak 4321 cm^{-1} can be assigned to the combination bands of the CH related modes of glucose, whereas the band near 5070 cm^{-1} is due to a combination of NH stretching and bending [21]. The more pronounced peak located at 7190 cm^{-1} corresponds with the OH stretching overtone, whereas the shifted and decreased peak at 7420 cm^{-1} represents the first overtone of CH combinations. The band centered near 6900 cm^{-1} is composed of a number of overlapping bands due to the combinations of OH antisymmetric and symmetric stretching [22]. The spectral variations between 6600 and 7000 cm^{-1} are owing to the NH first overtone stretching.

In the *in vivo* part of the study, an extra broadening at the peak 4435 cm^{-1} was observed, which correlates to the spectral variance detected after *in vitro* glycation of complete fingernail clippings, which showed peak sharpening at 4321 cm^{-1} when glycation increased. According to Awichi et al., the absorption features at 4300 and 4400 cm^{-1} are assigned to the combination band of C-H stretch, C-H bending and O-H bending of glucose. Their results suggested that the 4350 cm^{-1} band may belong to the α-anomer of glucose [23, 24].

Further studies are needed to investigate the *in vivo* kinetics of nail protein glycation and to assess the clinical value of the assay. In addition, it would be interesting to also use this novel method for monitoring the metabolic control over time in patients with diabetes mellitus. Therefore, not only the fingernail clipping, but rather the complete nail should be investigated to see whether therapy reaches success or not. Finally, the pilot study experiments using the NIR probe and NIR-CI should be confirmed in an extended dataset using a fiber optic cable with optimal transport properties for the spectral range of interest.

Conclusions

Analysis of protein glycation in human fingernail clippings with NIR spectroscopy could be an alternative affordable technique for diagnosis of diabetes mellitus. PLS-DA allows an excellent discrimination between patients with diabetes mellitus and healthy subjects. As the test does not consume reagents, the proposed test could be particularly useful in developing countries. However, the present method does not bring quantitative information at this step of development. The obtained results should be confirmed by a study comparing the classical strategies using validated criteria to this novel approach.

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