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Original article

Identification of urine metabolites associated with 5-year changes in biomarkers of glucose homoeostasis

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A B S T R A C T

Aim. – Metabolomics provides information on pathogenetic mechanisms and targets for interventions, and may improve risk stratification. During the last decade, metabolomics studies were used to gain deeper insight into the pathogenesis of diabetes mellitus. However, longitudinal metabolomics studies of possible subclinical states of disturbed glucose metabolism are limited. Therefore, the aim of this study was to analyze the associations between baseline urinary metabolites and 5-year changes in continuous markers of glucose homoeostasis, including fasting glucose, HbA_{1c} and homoeostasis model assessment of insulin resistance (HOMA-IR) index values.

Methods. – Urine metabolites in 3986 participants at both baseline and 5-year follow-up of the population-based Inter99 study were analyzed by ^{1}H-NMR spectroscopy. Linear regression and analyses of covariance models were used to detect associations between urine metabolites and 5-year changes in markers of glucose homoeostasis.

Results. – Higher baseline levels of urinary alanine, betaine, N,N-dimethylglycine (DMG), creatinine and trimethylamine were associated with an increase in HbA_{1c} from baseline to follow-up. In contrast, formic acid and trigonelline levels were associated with a decrease in HbA_{1c} over time. Analyses of 5-year changes in fasting glucose and HOMA-IR index showed similar findings, with high baseline levels of lactic acid, beta-D-glucose, creatinine, alanine and 1-methylnicotinamide associated with increases in both parameters.

Conclusion. – Several urine metabolites were found to be associated with detrimental longitudinal changes in biomarkers of glucose homoeostasis. The identified metabolites point to mechanisms involving betaine and coffee metabolism as well as the possible influence of the gut microbiome.

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Introduction

For the past several decades, type 2 diabetes mellitus (T2DM) has represented one of the main worldwide health problems set to become more and more important due to the steadily increasing prevalence of overweight and obesity. Over the last 10 years, the profiling of small molecules—called 'metabolomics'—has been performed to gain deeper insights into the pathogenesis of diabetes and to identify early biomarkers of T2DM [1–3]. There are several cross-sectional metabolomics studies with respect to diabetes, but prospective studies are still limited. First, Wang et al. [4] used mass spectrometry (MS)-based metabolomics to reveal that branched-chain and aromatic amino acids (AAs), such as leucine, valine and phenylalanine, are associated with incident T2DM in a prospective study of 2422 individuals. Further prospective studies confirmed these findings [5–8] and also detected further AAs, including alanine and glycine [9–11], associated with T2DM in different settings. However, the predictive ability of these markers over the classic clinical markers is still a subject of discussion [5,10].

Beside AAs, other metabolomics-based detected markers of incident diabetes have included trigonelline [9], 2-aminoadipic acid [12], acylcarnitines [10,11] and lyso-glycerophospholipids

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[6,10,13,14], which reflect processes contribute to the development or progression of diabetes involving different metabolic pathways. The majority of these studies [4–7,10–14], however, applied MS to measure blood metabolites, whereas only two previous investigations used nuclear magnetic resonance (NMR) spectroscopy and only one study used urine as a biofluid [9]. As previously reviewed [1,3], both methods have their strengths and weaknesses. However, NMR provides highly reproducible results, and is inexpensive and fast and, therefore, highly suitable as a screening tool, especially combined with urine specimens.

Nevertheless, a serious concern of such studies is the limited number of incident cases (< 200) [4–7,9,12–14] and lack of assessment of the possible subclinical states of perturbed glucose metabolism. Thus, the aim of the present investigation was to analyze the associations between baseline urine metabolites measured by NMR spectroscopy and 5-year longitudinal changes in continuous markers of glucose homoeostasis, including fasting glucose, glycated haemoglobin (HbA_{1c}) and homoeostasis model assessment of insulin resistance (HOMA-IR).

Methods

Study population

The Inter99, a population-based non-pharmacological lifestyle intervention study, was initiated in March 1999 and carried out by the Capital Region of Denmark Research Centre for Prevention and Health. The study design and intervention have been described in detail elsewhere [15], and can also be found on the website www.inter99.dk. Subjects were drawn randomly from the Civil Registration System. The study population (n = 61,301) comprised all individuals in specific age groups (30, 35, 40, 45, 50, 55 and 60 years) from a defined area of Copenhagen. From this study population, 13016 were randomly drawn for the intervention, of whom 6784 (52.5%) were examined at baseline. Of these, 4511 subjects participated in a 5-year follow-up examination. The present project was approved by the Ethics Committee of the Capital Region of Denmark (H-15004167) and Danish Data Protection Agency.

Baseline NMR measurements were available for 4117 subjects who participated in both the baseline and 5-year follow-up examinations. Of these, subjects with missing values for baseline/follow-up HbA_{1c} or baseline confounders were excluded, resulting in a total of 3986 subjects included in the analyses.

Measurements

Information on lifestyle, such as smoking habits and coffee consumption, were assessed by questionnaire. Blood and urine spot samples were collected after an overnight fast. Height and weight were measured without shoes and with light clothing. Body mass index (BMI) was calculated as weight (kg) divided by height squared (m^2). All participants had their blood pressure measured twice with a mercury sphygmomanometer (Mercuro 300; Speidel & Keller GmbH & Co, Jungingen, Germany) and appropriate cuff size after 5 min of rest, in supine position, and the average of the two recorded measurements was used. HbA_{1c} was measured in all participants using ion-exchange high-performance liquid chromatography (VARIANT™ Hemoglobin A_{1c}, Bio-Rad Laboratories, Hercules, CA, USA). Fasting glucose concentrations were analyzed by hexokinase/glucose-6-phosphate dehydrogenase assay (Roche Diagnostics Corporation, Indianapolis, IN, USA). Insulin levels were measured by a fluoroimmunoassay technique (Dako Diagnostics Ltd., Ely, Cambridgeshire, UK), and the HOMA-IR index was calculated as: [fasting glucose (mmol/L) $\times$ fasting insulin (mU/L)]/22.5. Low-density lipoprotein (LDL) cholesterol was measured using enzymatic colorimetric methods (Roche Diagnostics).

^{1}H-NMR spectroscopy analysis of urine specimens

After thawing, urine specimens were centrifuged for 5 min at 3000 g, and the supernatant was used for spectroscopy analysis: 450 μL of urine was mixed with 50 μL of phosphate buffer to stabilize urine pH at 7.0 ($\pm$ 0.35). The buffer was prepared with D2O and contained sodium 3-trimethylsilyl-(2,2,3,3-D4)-1-propionate (TSP) as reference. Spectra were recorded at the University Medicine Greifswald, Germany, on a Bruker DRX-400 NMR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany), operating at a ^{1}H frequency of 400.13 MHz, and equipped with a 4-mm selective inverse flow probe (FISEI; 120 μL of active volume) with z gradients. Specimens were automatically delivered to the spectrometer *via* flow injection. Acquisition temperature was calibrated to 300 $\pm$ 0.1 K, and a standard one-dimensional ^{1}H-NMR pulse sequence with suppression of water peak (NOESYGPPR1D) was used: RD—P(90°)—4 μs—P(90°)—tm—P(90°)—acquisition of free induction decay (FID). For each sample, the non-selective 90° hard pulse [P(90°)] was individually calibrated in full automation, using the Bruker automation program PULSECAL. Relaxation delay (RD), mixing time (tm) and acquisition time were set to 4 s, 10 ms and 3.96 s, respectively, resulting in a total recycle time of $\sim$ 8.0 s. Low-power continuous-wave irradiation on water resonance at a radiofrequency field strength of 25 Hz was applied during the RD and tm for presaturation, and 1-ms z gradients were applied between RD and P(90°) and between tm and P(90°) to further reduce the residual solvent signal. After application of four dummy scans, NS 32 were collected into 65536 (64 K) complex data points, using a spectral width of 20.689 parts per million (ppm) and a receiver gain (RG) setting of 128. FIDs were multiplied with an exponential function corresponding to a line broadening of 0.3 Hz before Fourier transformation. TopSpin Version TS2.1pl6 was generally used for automated data acquisition and data processing. Spectra were automatically phase-corrected and referenced to the internal standard (TSP–0.0 ppm), using Bruker's processing programme APK0.NOE.

Bucketing of ^{1}H-NMR spectra

Processed spectra were segmented into 500 consecutive integrated spectral regions (buckets) of fixed bucket width (0.018 ppm), covering a range of 0.5 ppm to 9.5 ppm (R version 3.0.1, R Foundation for Statistical Computing, Vienna, Austria). The 4.5–5.0 ppm chemical shift region (28 buckets) was left out of the analysis to remove the effects of variations in suppression of water resonance and variations in the urea signal, caused by partial cross-solvent saturation through solvent-exchanging protons. To account for urine dilution based on the remaining 472 buckets, a dilution factor for each sample was obtained by probabilistic quotient normalization (PQN) [16]. This procedure involves calculation of a median reference spectrum within the population (overall Inter99 observations) and, subsequently, estimation of the median quotient of each sample to this reference. Thereafter, the derived dilution factor was used for normalization of the buckets. In addition, the Fourier-transformed and baseline corrected spectra were processed, using the published FOCUS workflow, including a proposed alignment and peak-picking algorithm called RUNAS [17]. Again, for this purpose, spectral regions containing the water peak (4.5–5.0 ppm) were excluded and the algorithm was run with the default parameters, whereas the threshold for such peak occurrences was adapted to the present signal intensities. As a result, 153 distinct peak entities that markedly reduced the undesired shift in ppm signals due to slight differences in pH or molecular interactions were obtained. The FOCUS-aligned peaks were also PQN normalized.

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