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ARTICLE *in* ANALYTICAL CHEMISTRY · SEPTEMBER 2009

Impact Factor: 5.64 · DOI: 10.1021/ac9020598 · Source: PubMed

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Anal. Chem. **2009,** *81,* 9188–9193

Potential of Human Saliva for Nuclear Magnetic Resonance-Based Metabolomics and for Health-Related Biomarker Identification

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In the present study, the ability of ^{1}H nuclear magnetic resonance (NMR) for metabolic profiling of human saliva samples was investigated. High-resolution ^{1}H NMR spectra were obtained, and signals were assigned to various metabolites mainly representing small organic acids and amino acids. In addition, the use of human saliva for metabolomic studies was evaluated, and multivariate data analysis revealed that the 92 morning and night samples from 46 subjects could be discriminated with a predictability of 85%. The diurnal effect on the salivary metabolite profile were ascribed to changes in intensities of several metabolites including trimethylamine oxide (TMAO), choline, propionate, alanine, methanol, and *N*-acetyl groups. No effects of gender and body mass index (BMI) on the salivary metabolite profile were detected. The relationships between the salivary metabolome and glycated hemoglobin, systolic and diastolic blood pressure were investigated; however, no significant correlations could be established.

Metabolomics, the term describing the global analysis of metabolites, has emerged as a powerful approach used in many disciplines to study how a stimulus causes perturbations in the metabolite profile. Nuclear magnetic resonance (NMR) is one of the principal analytical technique for metabolomic purposes,[1,2] and blood and urine are the biofluids most frequently studied in these investigations.[3,4] In contrast, the use of saliva in metabolomic studies are rare;[5,6] however, recently a comprehensive investigation of the salivary metabolome was reported.[7] This is despite the fact that the use of saliva has an important advantage, as it is easily accessible and can be sampled noninvasively without the introduction of stress or pain. A strong correlation between salivary and serum concentrations of caffeine has been demonstrated, indicating the prospective use of saliva for monitoring circulating levels of small molecules.[8] Thus, saliva has been proposed to be useful for diagnosis of various diseases and endocrine disorders.[9,10] Metabolomics often aims at identifying biomarkers, in particular there is an interest in establishing biomarkers related to health, which can be used as diagnostic tools. Therefore, the aim of the present study was (1) to elucidate the information obtained from high-resolution ^{1}H NMR spectra of human saliva samples, (2) to elucidate the use of human saliva for metabolomic studies by evaluating the intra- and interindividual differences and by examining the impact of fundamental parameters such as gender and body mass index (BMI) on the metabolite profile of population-based saliva samples from 46 healthy Danes in the age group between 43 and 68 years, and ultimately (3) to elucidate a potential correlation between the human salivary metabolome and important indicators of cardiovascular diseases (CVD) risk including glycated hemoglobin and diastolic and systolic blood pressure (BP).

EXPERIMENTAL SECTION

Subjects and Sampling. Samples from a total of 46 subjects were included in the study. These subjects were a part of a follow-up study on occupational health and salivary cortisol initiated in 1998. At that time, all participants were in good health.[11,12] The follow-up study, from which saliva samples for the present study were taken, took place between October 2008 and January 2009. The local scientific ethical committee approved the study. All participants gave informed consent before the examination.

Saliva samples were obtained by chewing a polyester tampon (Salivette, Sarstedt, Nürnbrecht, Germany) for about 60 s, which was subsequently stored in the internal vial of the double-chamber tube, capped, and stored at 4 °C until transport to the laboratory.

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10.1021/ac9020598 CCC: $40.75
Published on Web 09/25/2009

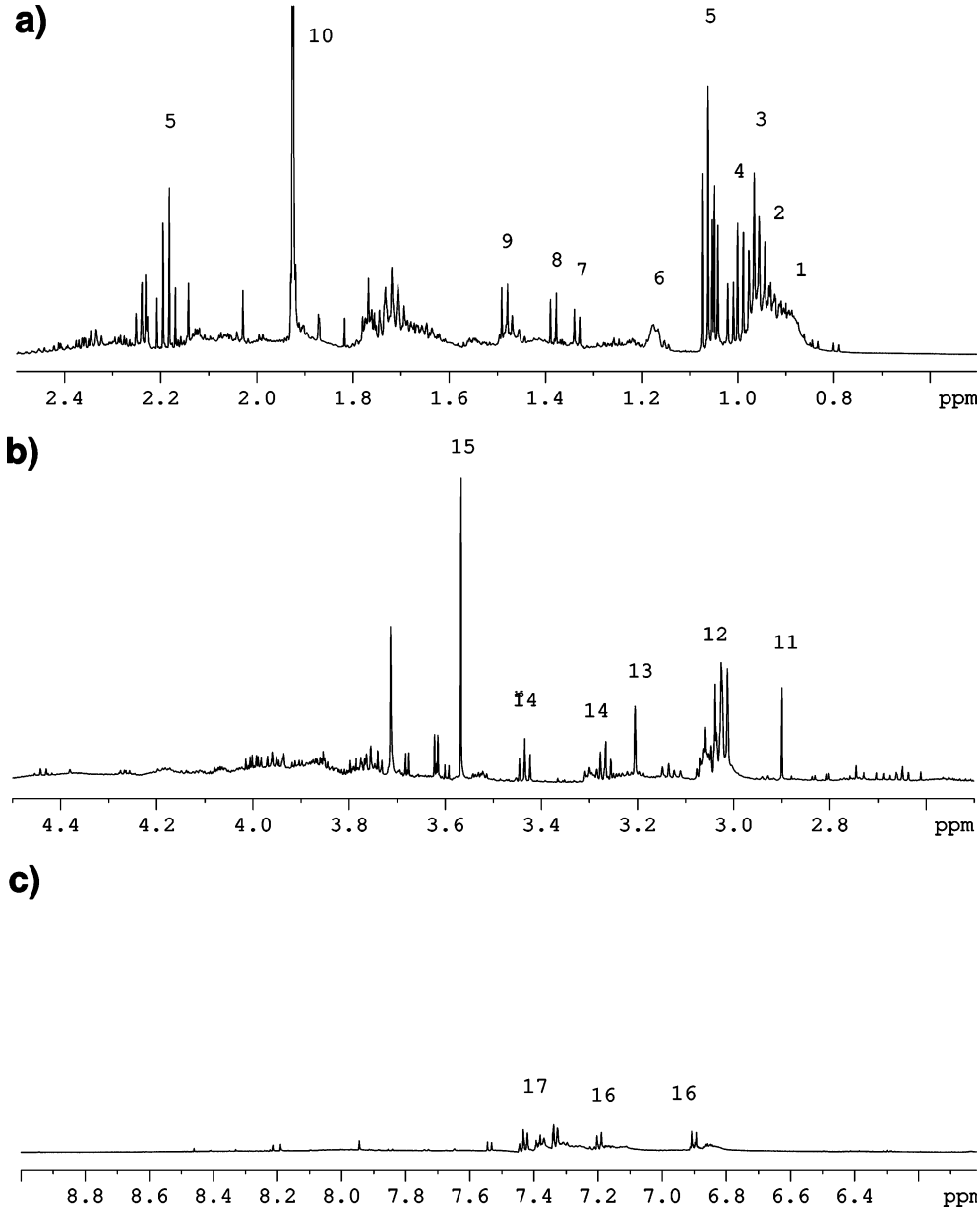

Figure 1. Example of 1D ^{1}H NMR spectrum of a human saliva sample: (a−c) expanded regions of 0.5−2.5, 2.5−4.5, and 6.0−9.0 ppm regions, respectively. Assignments: 1, CH_3 in fatty acids; 2, valine; 3, butyrate; 4, leucine; 5, propionate; 6, CH_2 in fatty acids; 7, lactate; 8, threonine; 9, alanine; 10, acetate; 11, trimethylamine; 12, tyrosine; 13, choline; 14, taurine; 15, glycine; 16, tyrosine; 17, phenylalanine.

The salivettes were centrifuged at 2000*g* for 10 min, and aliquots were immediately stored in vials and placed in a freezer (−20 °C) until analysis. The participants were instructed to take the first sample immediately after awakening, while they were still in bed, and at 18.00 p.m. The participants were instructed to write down the time at which each sample was taken, to ensure that the samples were taken at the correct times. The participants were instructed to fast, to refrain from smoking, and to not brush their teeth before taking the morning samples.

The participants' blood pressure was measured after 10 min of rest on the back by the use of an automatic blood pressures apparatus (Dinamap 845 Vital Signs Monitor, Critikon Inc.).[13] With the use of tourniquet, blood samples were drawn immediately hereafter to be analyzed for HbA1c as the routine method in the laboratory at Hillerød hospital.

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NMR Measurements. The NMR measurements were performed at 310 K on a Bruker Avance III 600 spectrometer, operating at a ^{1}H frequency of 600.13 MHz, and equipped with a 5 mm ^{1}H TXI probe (Bruker BioSpin, Rheinstetten, Germany). Prior to the measurements, saliva samples were thawed, and 500 μL aliquots were mixed with 100 μL of D_2O. Sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate (TSP) was added as an internal chemical shift standard (0.0083% w/w). Standard one-dimensional (1D) ^{1}H NMR spectra were acquired using a single 90° pulse experiment with water presaturation using a recycle delay of 3 s. Each data set was averaged over 64 transients using 32K time domain points. The data were Fourier transformed, and spectra were referenced to the TSP signal at 0 ppm.

To aid spectral assignment, 2D ^{1}H−^{1}H total correlation spectroscopy (TOCSY), 2D ^{1}H−^{1}H nuclear Overhauser effect

Table 1. ^{1}H NMR Assignments for Human Saliva at 600 MHz

compound	δ_H ppm (multiplicity, assignment)/δ_C ppm
acetate	1.93 (s, CH_3)/24.0
butyrate	0.90 (t, CH_3)/16.0; 1.56 (m, CH_2)/ 22.0; 2.16 (t, CH_2)/41.0
choline	3.20 (s, $N(CH_3)_3$)/55.0; 3.52 (m, NCH_2)/70.0
ethanol	1.20 (t, CH_3)/20.0; 3.65 (q, CH_2)/64.0
fatty acids	0.9 (m, CH_3); 1.18 (m, CH_2)
formate	8.45 (s, CH)
glycine	3.55 (s, CH)/43.0
histidine	3.02 (m, CH_2)/30.0; 3.12 (m, CH_2)/ 30.0; 7.03 (s, C4H, ring)/118.0; 7.89 (s, C2H, ring)/137.0
lactate	1.32 (d, CH_3)/21.0; 4.11 (q, CH)/70.0
methanol	3.35 (s, CH_3)/50.0
N-acetyl groups	2.05 (m, CH_3)
propionate	1.05 (t,CH_3)/12.0; 2.18 (q, CH_2)/34.0
pyruvate	3.36 (s, CH_2)/32.0
succinate	3.38 (s, CH_2)/35.0
trimethylamine-*N*-oxide (TMAO)	3.26 (s/ $N(CH_3)_3$)/60.0
tyrosine	3.0 (m, CH)/37.0; 3.83 (d, CH)/57.0; 6.88 (m, C3H, ring)/117.0; 7.18 (m, C2H, ring)/132.0

spectroscopy (NOESY), and 2D ^{1}H−^{13}C heteronuclear single quantum coherence (HSCQ) spectra were recorded on selected saliva samples. The TOCSY spectra were acquired with a spectral width of 6250 Hz in both dimensions, 2K data points, 512 increments with 32 transients per increment, and 60 ms spinlock period. The NOESY spectra were acquired with a size and number of data points similar to that of the TOCSY and a mixing time of 800 ms. The HSCQ spectra were acquired with a spectral width of 6250 Hz in the F2 dimension and 21 128 Hz in the F1 dimension, a data matrix with a size of 2K × 512 data points and 64 transients per increment.

Multivariate Data Analysis. The spectra in the region 10.0−0.5 ppm were subdivided into 0.0074 ppm integral regions and integrated, leaving out the region 5.0−4.6 ppm and 3.73−3.66 ppm, which included the residual water and signals originating from a compound in the tampons, respectively. The acetate signal at ∼1.93 ppm was enclosed in two integral regions, which therefore were combined to a single integral region. The reduced spectra consisting of 1225 independent variables were normalized to the TSP signal, and further analysis was performed using the Unscrambler software version 9.2 (Camo, Oslo, Norway). Principal component analysis (PCA) was applied to the centered data to explore any clustering behavior of the samples, and partial least-squares regression discriminant analysis (PLS-DA) was performed to explore intrinsic biochemical dissimilarities between morning and night samples. During all regressions, Martens' uncertainty test[14] was used to eliminate noisy variables, and all models were validated using full cross-validation.[15]

RESULTS

A 600 MHz 1D ^{1}H NMR spectrum of a typical human saliva sample is shown in Figure 1. The spectrum shows several peaks arising from low molecular weight compounds, and Table 1 lists compounds assigned based on the 1D and 2D spectra.

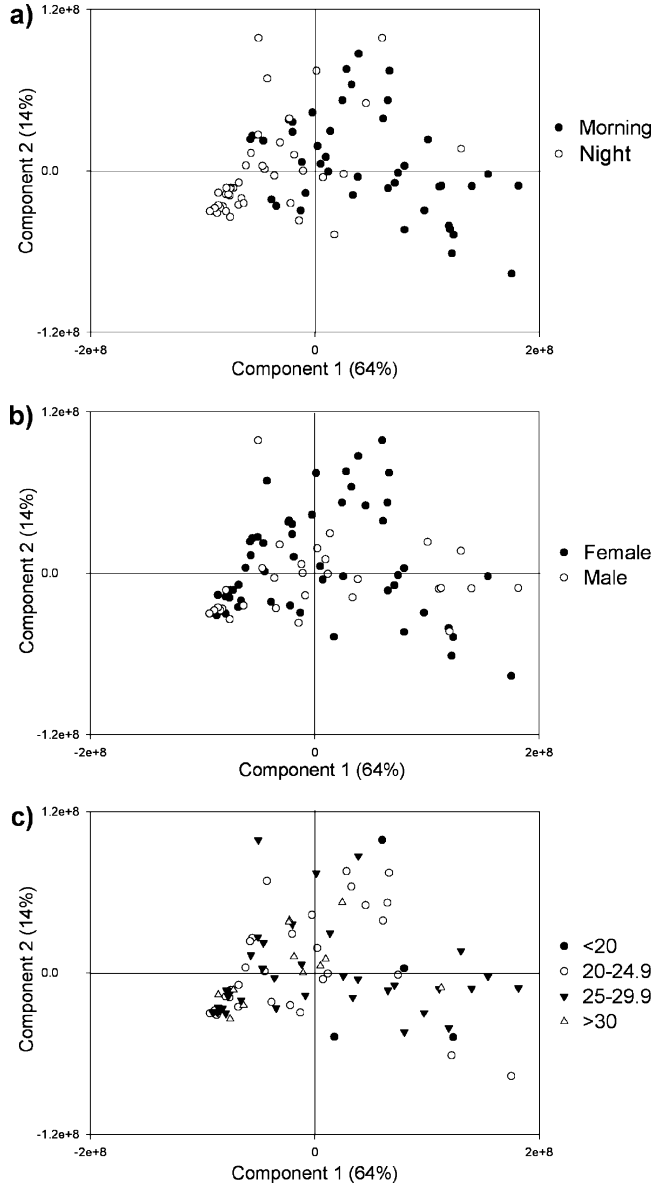

Figure 2. Score plots from PCA carried out on 1D ^{1}H NMR spectra data obtained on human saliva. The samples are labeled according to (a) sampling time, (b) gender, and (c) BMI.

The score plot obtained from PCA on the 1D ^{1}H NMR spectra is shown in Figure 2. The first two principal components in the PCA explain 64% and 14% of the variation in the data, respectively. A tendency for grouping according to sampling time is seen along the first principal component (Figure 2a), while no clear grouping according gender or BMI is observed (Figure 2b,c). Consequently, for a further elucidation of the effect of sampling time on the NMR metabolite profile of saliva, PLS-DA was carried out with the 1D ^{1}H NMR data as *X*-variables and sampling time as the response variable. A good discrimination of the two sampling time is obtained (Figure 3a) (R^2 = 0.54); 14 out of 92 samples was misclassified, which corresponds to a predictive ability of 85%. The discrimination between morning and night samples can be ascribed to several features in the NMR metabolite profile including lower intensities of signals assigned to trimethylamine oxide (TMAO), choline, propionate, and alanine in night

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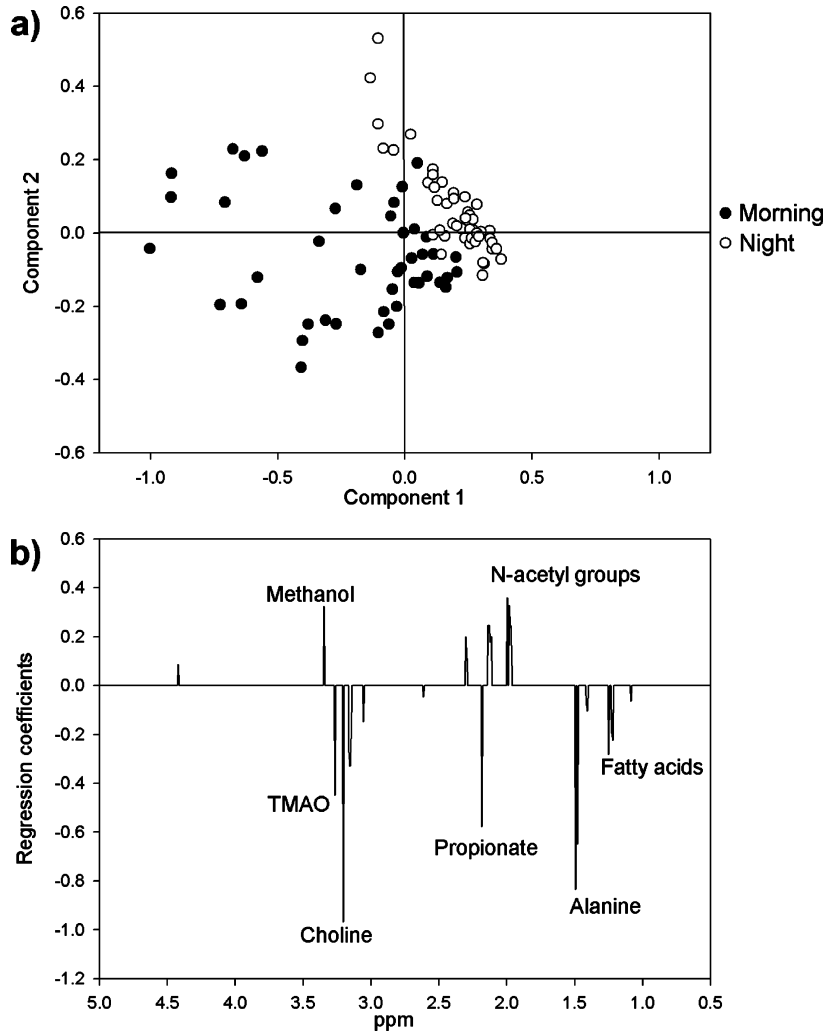

Figure 3. (a) PLS-DA scores plot from analysis of NMR spectra obtained on morning (●) and night saliva samples (○) and (b) the regression coefficients.

samples compared to morning samples (Figure 3b). In contrast, higher intensities of signals assigned to methanol and *N*-acetyl groups are observed in night saliva samples compared with morning samples (Figure 3b).

In order to investigate a potential correlation between the salivary metabolome and indicators of risk of developing cardiovascular diseases, PLS regressions were carried out with the NMR salivary metabolome as *X*-variables and various parameters including systolic and diastolic blood pressure (BP) and glycated hemoglobin as *y*-variables. For none of these three risk indicators included was it possible to establish a correlation (Figure 4).

DISCUSSION

In the present study, the use of human saliva samples for NMR-based metabolomics was evaluated. High-resolution ^{1}H NMR spectra could be obtained, and based on 2D experiments the major signals could be unambiguously assigned consistent with earlier studies.[16,17] In addition, the potential of human saliva for metabolomic studies was elucidated by evaluating the intra- and interindividual differences and by examining the impact of fundamental parameters such as gender and body mass index (BMI) using multivariate data analysis. In a proteomic study on saliva, an unknown peptide was found to correlate with BMI.[18] Nevertheless, in the present study principal component analysis (PCA) revealed grouping of samples according to sampling time (morning vs night), while BMI was not found to have any effect on the grouping of samples. Different results have been reported regarding the effect of BMI on the blood plasma metabolite profile.[19,20]

Gender has been shown to have a pronounced effect on the ^{1}H NMR metabolite profile of blood plasma,[18] and a recent ^{1}H NMR study could to some extent discriminate female and males based on partial least-squares regression discriminant analysis (PLS-DA) of the salivary metabolome.[7] However, in the present study unsupervised PCA indicated no strong gender effect on the salivary metabolome. Thus, no supervised analyses such as PLS-DA were attempted in relation to gender.

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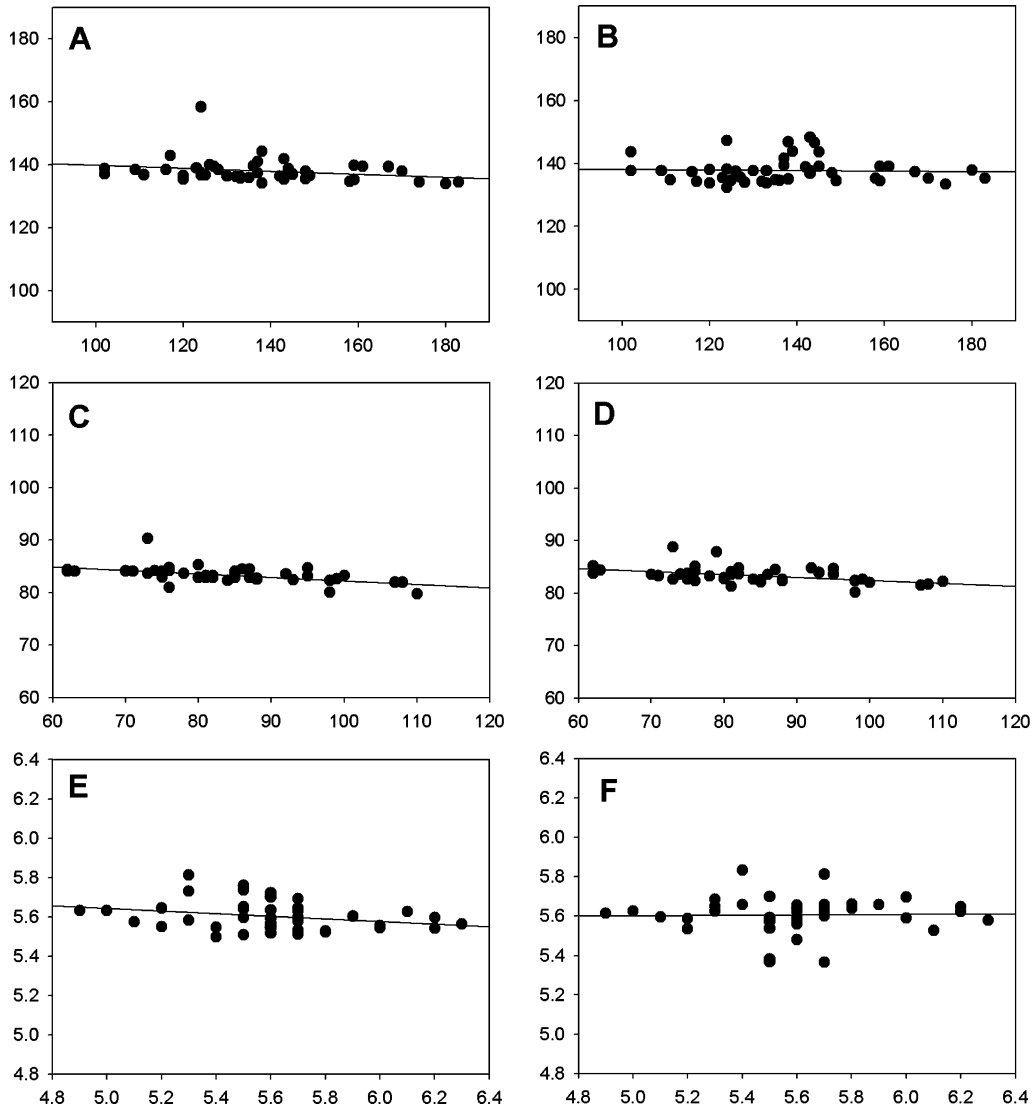

Figure 4. Predicted versus measured values of systolic blood pressure (a, b), diastolic blood pressure (c, d), and glycated hemoglobin (e, f). Values are predicted from the NMR metabolome measured on saliva samples collected either morning (a, c, e) or night (b, d, f). Values of blood pressure are expressed in mm Hg, while values of glycated hemoglobin are expressed in % of total hemoglobin.

In order to elucidate the intraindividual differences and diurnal effects, partial least-squares regression discriminant analysis (PLS-DA) was employed to discriminate between morning and night samples. A model with a predictive ability of 85% was obtained, which is consistent with values reported previously in a study on 30 healthy individuals.[5] The discrimination between morning and night samples could be ascribed to lower intensities of signals assigned to trimethylamine oxide (TMAO), choline, propionate, and alanine in night samples compared to morning samples, and higher intensities of signals assigned to methanol and *N*-acetyl groups in night saliva samples compared with morning samples. Propionate probably reflects the growth, preponderance, and metabolism of micro-organisms present in the oral cavity.[21] TMAO is presumably derived from the trimethylamine oxidase-mediated oxidation of dietary trimethylamine.[16] A study, which investigated the relationship between amino acids in plasma and saliva, found no relationship between alanine concentrations in plasma and saliva,[22] and there is also evidence that saliva alanine does not originate from bacteria or foods.[23] Thus, it is likely that alanine is produced in salivary glands, which are metabolically very active.[24] Methanol is derived from the passive or direct inhalation of cigarette smoke,[17] and since the study included smokers, the increase in methanol at night time can most likely be ascribed to smoking. *N*-Acetylsugars are probably derived from the actions of the bacterial enzymes hyaluronidase and neuraminidase.[17] Further studies are needed to elucidate the significance of the observed metabolic perturbations between morning and night samples.

There is a huge interest in identifying biomarkers related to health, in particular indicators of CVD, and a relationship between urinary metabolites and BP has been demonstrated in a large NMR-based metabolomic investigation.[25] Systolic and diastolic BP and glycated hemoglobin were determined on the subjects included in the present study. Accordingly, it was possible to

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investigate if there were any correlations between these CVD risk indicators and the salivary metabolome. However, no correlation could be established for any of the risk indictors investigated. Thus, the present study indicates that the salivary metabolome is not useful for identification of biomarkers related to CVD risk indictors. However, studies enclosing a larger number of subjects are needed to substantiate the present finding.

CONCLUSIONS

In conclusion, the present study demonstrated that high-resolution ^{1}H NMR spectra could be obtained on human saliva samples. Multivariate data analysis showed that while gender and BMI had no detectable effect on the salivary metabolite profile, morning and night samples could be discriminated, revealing a diurnal effect on the salivary metabolite profile.

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ACKNOWLEDGMENT

The Danish Technology and Production Research Council (FTP) is thanked for financial support through the project "Advances in food quality and nutrition research through implementation of metabolomic technologies" (Project No. 274-09-0107).

Received for review August 18, 2009. Accepted September 16, 2009.

AC9020598