Available online at www.sciencedirect.com
ScienceDirect
Metabolism Clinical and Experimental 58 (2009) 1039–1045
Metabolism
Clinical and Experimental
www.metabolismjournal.com

Nuclear magnetic resonance–based metabonomics reveals strong sex effect on plasma metabolism in 17-year–old Scandinavians and correlation to retrospective infant plasma parameters

Hanne Christine Bertram[a,*], Jens Ø. Duus[b], Bent O. Petersen[b], Camilla Hoppe[c], Anni Larnkjær[c], Lene Schack-Nielsen[c], Christian Mølgaard[c], Kim. F. Michaelsen[c]

[a]*Faculty of Agricultural Sciences, Department of Food Science, Research Center Årslev, University of Aarhus, PO Box 102, DK-5792 Årslev, Denmark*
[b]*Carlsberg Laboratory, DK-2500 Valby, Denmark*
[c]*Faculty of Life Sciences, Department of Human Nutrition, University of Copenhagen, DK-1958 Frederiksberg C, Denmark*

Received 23 January 2009; accepted 31 March 2009

Abstract

Nuclear magnetic resonance (NMR)–based metabonomics was carried out on plasma samples from a total of seventy-five 17-year–old Danes to investigate the impact of key parameters such as sex, height, weight, and body mass index on the plasma metabolite profile in a normal, healthy population. Principal component analysis identified sex to have a large impact on the NMR plasma metabolome, whereas no apparent effects of height, weight, and body mass index were found. Partial least square regression discriminant analysis and quantification of relative metabolite concentrations by integration of NMR signals revealed that the sex effect included differences in plasma lipoproteins (mainly high-density lipoprotein), glucose, choline, and amino acid content. Accordingly, the present study suggests a higher lipid synthesis in young women than young men and a higher protein turnover in young men compared with women. Data on plasma content of triglyceride, lipoprotein fractions, and cholesterol at an age of 9 months were available for selected individuals (n = 40); and partial least square regressions revealed correlations between these infant parameters and the NMR plasma metabolome at an age of 17 years. In conclusion, the present study demonstrates the feasibility of NMR-based metabonomics for obtaining a deeper insight into interindividual differences in metabolism and for exploring relationships between parameters measured early in life and metabolic status at a later stage.

1. Introduction

Lifestyle diseases have become a common cause of death in the Western world. It is well known that interindividual metabolic variations exist between humans as a result of genetic, nutritional, and environmental factors, giving rise to different risks of developing lifestyle-related diseases. Moreover, the presence of diverse groups such as athletes, infants, and pregnant women increases such variations; and lifestyle factors including dietary habits may contribute further to the variation. Accordingly, it follows that a further differentiation of the nutritional recommendations for the general population could be advantageous; and personalized nutrition and personalized medicine are receiving increasing attention. In fact, although research traditionally has aimed at understanding the requirements of an entire population, it has recently turned into focusing more and more on individual needs. However, before personalized nutrition and personalized medicine can be introduced, it is a prerequisite to understand individual differences including effects of physiologic factors such as sex and age.

The global analysis of metabolites, which has been termed *metabonomics*, provides research with a new powerful tool to assess individual metabolic differences in an explorative way, thereby supplying a deeper insight into the relations between individual differences and individual needs. Proton nuclear magnetic resonance (NMR) spectroscopy is a commonly used technique for metabonomic studies. The technique provides concurrent detection of all hydrogen-containing molecules in a sample; and, in combination with multivariate data analysis, it captures utmost data on the metabolic status

Disclosure statement: None of the authors have any potential conflicts of financial interest in the scientific project related to the manuscript.

* Corresponding author. Tel.: +45 89 99 33 44; fax: +45 89 99 34 94.
E-mail address: hannec.bertram@agrsci.dk (H.C. Bertram).

0026-0495/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.metabol.2009.03.011

by measurements on a biofluid, thereby providing an efficient explorative tool. Nuclear magnetic resonance–based metabonomics has been widely applied in the research fields of pharmacology and toxicology [1]. In addition, the technique has exciting opportunities for elucidating how diet elicits changes in metabolite profile [2-8]; and a recent study showed applicability of NMR-based metabonomics for phenotyping humans for dietary preferences [9], demonstrating that it is a superior technique for obtaining a deeper insight into metabolic differences between subgroups within the population.

The call for personalized nutrition and personalized medicine is strongly connected with the fact that humans are extremely diverse. Accordingly, it is necessary to understand the factors that contribute to normal physiologic variation; and it is hypothesized that these factors are manifested in the NMR metabolite profile. Therefore, the aim of the present study was to elucidate the NMR metabolite profiles in population-based plasma samples from a total of 75 healthy 17-year–old Danes to study the impact of key parameters such as sex, height, weight, and body mass index (BMI) as a first step toward understanding the interindividual differences giving rise to individual nutritional needs. In addition, the correlation between the plasma metabolome at 17 years of age and key infant parameters measured earlier in life on the same subjects was investigated.

2. Materials and methods

2.1. Subjects

This was an observational follow-up study of 17-year–old adolescents in the Danish Copenhagen Cohort Study on Infant Nutrition and Growth. This cohort followed infants born from 1987 to 1988 during the first year of life with subsequent follow-up at 10 and 17 years. A detailed description of the selection and characteristics of the study group has been published elsewhere [10]. Briefly, the inclusion criteria were as follows: parents of Danish origin, singleton birth, gestational age between 37 and 42 weeks, birth weight for gestational age between 10th and 90th percentiles, and no neonatal disease or malformation. Of the 251 infants fulfilling the inclusion criteria, 142 infants completed the study.

Participants were invited to participate in the second follow-up studies when they were 17 years old. At the follow-up, 109 subjects agreed to participate; and 75 subjects (29 boys and 46 girls), from whom plasma samples were available, were included in this study. The mean BMI was 21.12 ± 2.24 kg/m^2 (range, 16.05-26.61 kg/m^2). The study was approved by the Ethics Committee for Copenhagen and Frederiksberg (KF 01-226/97).

2.2. Measurements

Venous blood samples were obtained at 9 months, and serum was stored at −20°C until analysis. Data were available for 40 infants (9 boys and 31 girls). Concentrations of cholesterol, high-density lipoprotein (HDL), low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), and triglyceride were measured by routine methods with an autoanalyzer (SMAC; Technicon Instruments, Tarrytown, NY). At the 17-year follow-up examination, fasting blood samples were drawn from vein puncture. Serum was stored at −80°C until analysis. Plasma concentrations of triglyceride were determined by an automated enzymatic colorimetric test with kits from Roche Diagnostics (12016648) on Cobas Mira (Roche Diagnostic System, Basel, Switzerland). Height was measured to the nearest 1 mm by a wall-mounted stadiometer (Chasmore, London, United Kingdom), and weight was measured to the nearest 0.1 kg by an electronic scale (Lindeltronic 8000, Lindells Inc, Kristianstad, Sweden).

2.3. NMR measurements

The NMR measurements were performed at 310 K on a Bruker 800 spectrometer, operating at a ^{1}H frequency of 799.40 MHz and equipped with a 5-mm ^{1}H observe cryoprobe (Bruker BioSpin, Rheinstetten, Germany). Before the measurements, plasma samples were thawed; and 400-μL aliquots were mixed with 200 μL D_2O. Sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate was added as an internal chemical shift standard (0.15% wt/wt). The ^{1}H NMR spectra of plasma samples were obtained using 2 different pulse sequences: (*a*) standard 1-dimensional spectra were acquired using single 90° pulse experiment, and (*b*) 1-dimensional spectra were acquired with a Carr-Purcell-Meiboom-Gill (CPMG) delay added to attenuate broad signals from high–molecular-weight components. The total CPMG delay was 50 milliseconds; and a total of 64 scans were acquired, whereas in the standard spectrum, a total of 32 scans were acquired. In both cases, water suppression was achieved by irradiating the water peak during the relaxation delay of 5 seconds; and 16-K data points spanning a spectral width of 13.03 ppm were collected. An exponential line-broadening function of 0.3 Hz was applied to the free induction decay before Fourier transformation. All spectra were referenced to the sodium trimethylsilyl-[2,2,3,3-$^2H_4$]-1-propionate signal at 0 ppm. The spectra were subdivided into 0.002-ppm integral regions and integrated, reducing each spectrum into 4480 independent variables in the region 0.1 to 4.4 and 5.0 to 10.0 ppm. The reduced spectra were normalized to the whole spectrum to remove any concentration effects, 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 square regression discriminant analysis (PLS-DA) was performed to explore intrinsic biochemical dissimilarities between sexes. In addition, PLS regressions were used for prediction of metabolite concentrations using NMR spectra as x variables and biochemical determinations of metabolite concentrations as y variables.

During all regressions, the Martens and Dardenne [11] uncertainty test was used to eliminate noisy variables; and all models were validated using full cross validation [12]. In addition, relative concentrations of selected metabolites were determined as NMR spectral integrals; and statistics were performed using the SAS system 8.2 (SAS Institute, Cary, NC). The PROC ANOVA procedure was used, and the models included the fixed effect of sex.

3. Results

Fig. 1 displays spectral features of the 2 different types of NMR metabolomic data obtained on the plasma, corresponding to the standard ^{1}H spectrum with broad overlapping resonances and the ^{1}H CPMG spectrum with special emphasis on low–molecular-weight metabolites. The score plot obtained from PCA on the NMR CPMG low–molecular-weight metabolome is shown in Fig. 2. The first 2 principal components in the PCA explain 69% and 15% of the variation in the data, respectively. A clear grouping according to sex is seen along the second principal component (Fig. 2A), whereas labeling according to plasma triglyceride content shows that the first principal component mainly describes variations in this parameter (Fig. 2B). No clear grouping according to BMI (Fig. 2C) was found in any of the principal components, and the same is valid for weight and height (data not shown).

For a further elucidation of the strong sex effect apparent on the NMR plasma metabolome, PLS-DA was carried out with the ^{1}H NMR CPMG data as x variables and sex as response variable. An unambiguous discrimination of the 2 sexes is obtained (Fig. 3A), which can be ascribed to several features in the NMR metabolome (Fig. 3B). Table 1 summarizes the tentative assignment of the major spectral regions included in the PLS-DA loadings. Sex effects on lipids signals corresponding to lower intensities of HDL signals (0.82 and 1.23 ppm), higher intensities of VLDL/LDL signals (0.89 ppm), and lower intensities of unsaturated lipid signals (5.26 ppm) for boys compared with girls are observed. In addition, plasma glucose (~3-4 ppm) is found to be higher in boys compared with girls. Boys are also found to have a higher plasma content of the amino acids valine, leucine, and isoleucine, which are evident in the spectral region 0.95 to 1.04 ppm. The characteristic signal from choline (3.20 ppm) representing lipid membrane constituents is found to be lower in plasma from boys than girls.

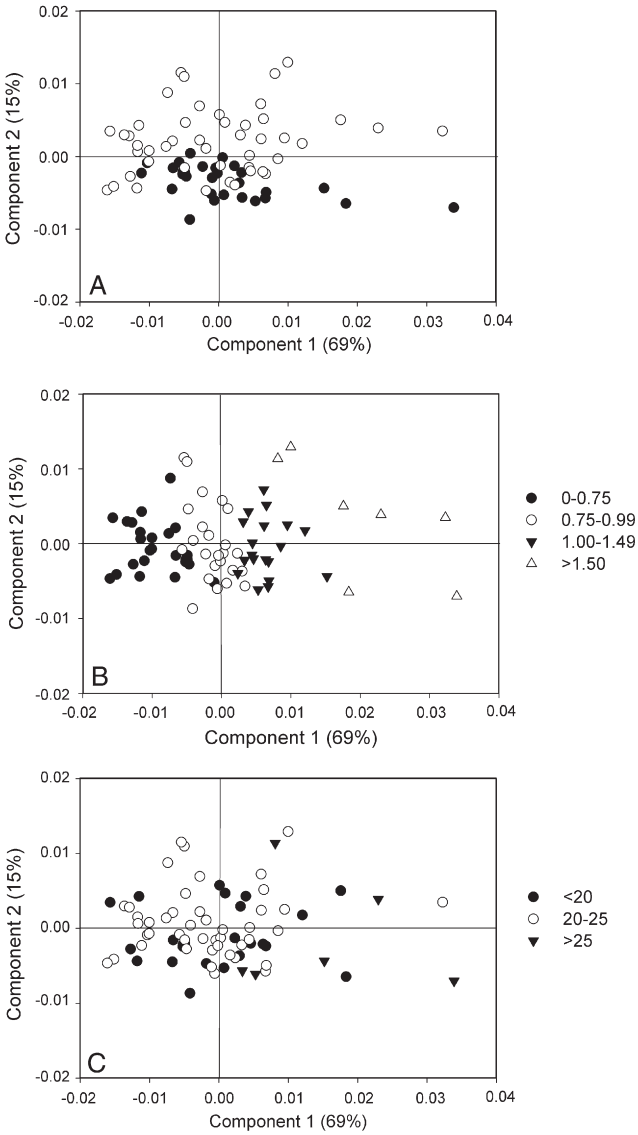

Fig. 2. Score plots from PCA carried out on ^{1}H NMR CPMG (low–molecular-weight) metabolomic data. A, The samples are labeled according to sex; open signals represent female subjects and closed signals represent male subjects. B, The samples are labeled according to plasma triglyceride content. C, The samples are labeled according to BMI.

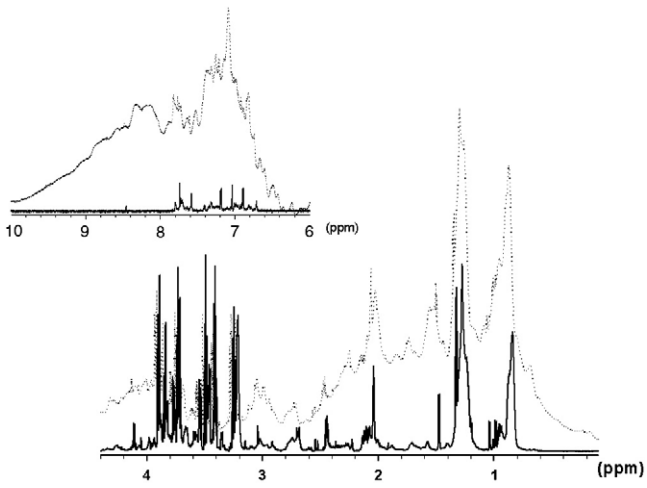

Fig. 1. Characteristic ^{1}H NMR plasma metabolome window. Dotted line represents the standard ^{1}H NMR spectrum; and full line represents the ^{1}H NMR CPMG spectrum, which attenuates the contribution from high–molecular-weight metabolites. The insertion in the upper left corner shows the aromatic region, which is magnified 10 times in intensity.

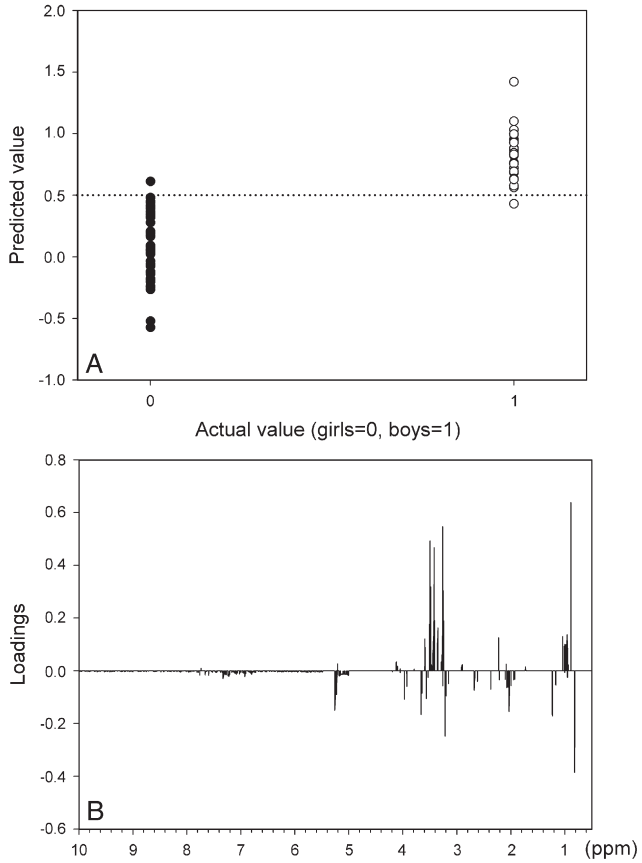

Fig. 3. Results from PLS-DA with ^{1}H NMR low–molecular-weight spectra (CPMG) as *x* variables and sex as response variable. A, Predicted values obtained in a PLS-DA model with female = 0 and male = 1. Values that are on the wrong side of 0.5 would classify to the wrong group. B, The loading plot obtained after selection of significant variables by jackknifing [11].

For a further quantification, relative intensities of NMR regions representing metabolites identified by PLS-DA to vary between sexes were determined (Table 2). Significant

Table 1
Major plasma metabolites displaying sex differences in PLS-DA

δ_H ppm	Effect	Tentative assignment
0.82	F > M	HDL
0.89	F < M	VLDL + LDL
0.95-1.04	F < M	Valine, leucine, and isoleucine
1.17	F > M	*β*-Hydroxybutyrate
1.23	F > M	HDL
1.72	F < M	Lipid CH_2-CHCO
1.93	F > M	Acetate
2.0-2.1	F > M	*N*-Glycoproteins
2.37	F > M	Pyruvate
3.20-3.21	F > M	Choline
3.35	F < M	Proline
~3.4-3.6	F < M	Glucose
3.93	F > M	Creatine
3.97	F > M	Histidine
5.26	F > M	Unsaturated lipid (CH = CH)

F < M corresponds to higher values in male compared with female subjects; and F > M, vice versa.

Table 2
Relative metabolite concentrations of metabolites identified to vary between sex based on PLS-DA

δ_H ppm	Metabolite	Male (n = 29)	Female (n = 46)	*P* value (sex effect)
0.83-0.85	HDL (CH_3 signal)	3.41 (0.12)	4.27 (0.09)	<.0001
0.86-0.88	LDL + VLDL (CH_3 signal)	2.69 (0.09)	2.77 (0.07)	NS
1.22-1.24	HDL (CH_2 signal)	30.93 (1.08)	37.57 (0.85)	<.0001
1.27-1.29	LDL (CH_2 signal)	74.35 (4.96)	70.99 (3.94)	NS
0.93-0.97	Isoleucine + leucine	6.55 (0.11)	6.00 (0.08)	<.0001
0.98-1.00	Valine	4.73 (0.15)	4.14 (0.12)	.0028
1.19-1.20	*β*-Hydroxybutyrate	7.95 (0.62)	8.52 (0.49)	NS
1.92	Acetate	2.97 (0.23)	2.64 (0.18)	NS
2.04	*N*-Glycoproteins	26.33 (0.64)	26.75 (0.51)	NS
2.37	Pyruvate	3.40 (0.12)	3.33 (0.09)	NS
3.21	Choline	34.56 (1.62)	43.79 (1.28)	<.0001
3.35-3.36	Proline	3.86 (0.13)	2.99 (0.10)	<.0001
3.41	Glucose	4.68 (0.13)	4.22 (0.14)	.0082
3.42	Glucose	3.70 (0.10)	3.31 (0.08)	.0032
3.49	Glucose	4.46 (0.13)	4.10 (0.11)	.0338
5.23	Glucose	3.08 (0.08)	2.84 (0.07)	.0286
5.28	Unsaturated lipid	8.48 (0.21)	10.25 (0.17)	<.0001

The relative metabolite concentrations are determined as integrals of the normalized NMR spectral intensities. The LS mean values are given. Standard errors are given in parentheses. NS indicates not significant.

effects of sex on the intensities of HDL, unsaturated lipid, choline, isoleucine, leucine, valine, proline, and 4 characteristic signals arising from glucose signals were found.

Data on plasma content of triglyceride, HDL, LDL, VLDL, and cholesterol at an age of 9 months were available for selected individuals (9 boys and 31 girls); and PLS regressions were carried out with the NMR plasma metabolome measured at an age of 17 years as *x* variables and infant parameters at *y* variables. Correlations could be established between the 17-year NMR plasma metabolome and plasma triglyceride, VLDL, and cholesterol content at an age of 9 months (Fig. 4), whereas no correlations could be established between the 17-year plasma metabolome and HDL as well as LDL at an age of 9 months (data not shown).

4. Discussion

Epidemiologic studies have the following advantages: a relatively large number of subjects can be included, they facilitate an elucidation of the factual variation within a population, and long-term effects and correlations can be studied. In the present study, the plasma metabolome was characterized in seventy-five 17-year–old Scandinavians in a cohort study using proton NMR-based metabonomics. This explorative approach revealed that the plasma triglyceride content is the major factor causing variation in the plasma metabolome of the 17-year–old subjects studied (Fig. 2). In addition, it was found that sex had a strong impact on the plasma metabolome, explaining the second largest part of the variation observed in the plasma metabolome between the 17-year–old subjects studied (Fig. 2). A previous study

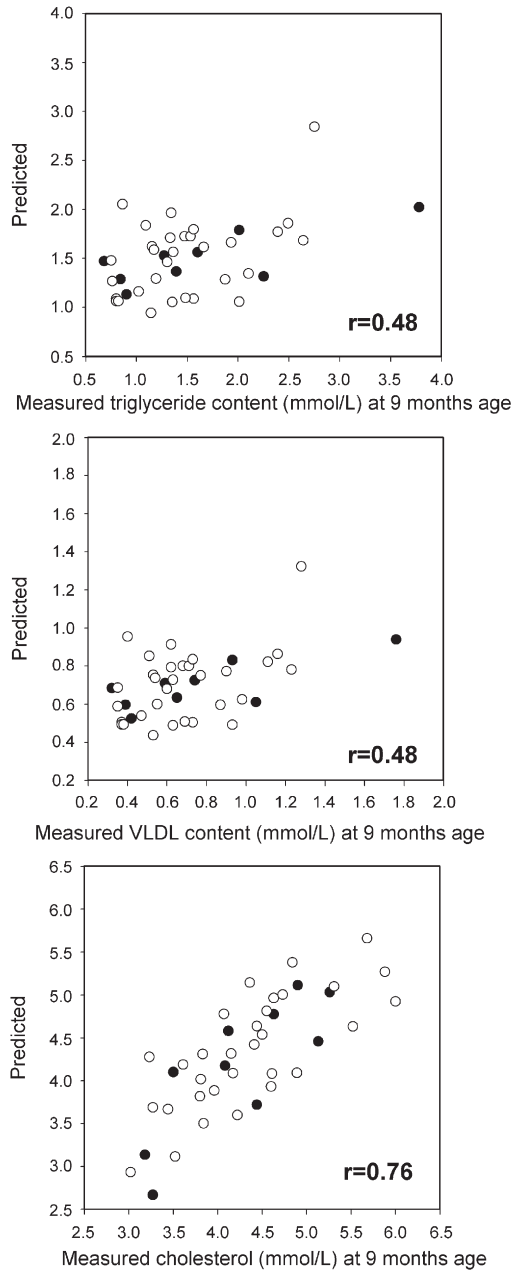

Fig. 4. Scatter graphs showing the correlation between plasma parameters measured at an age of 9 months and values predicted from PLS regressions on the NMR plasma metabolome measured at an age of 17 years. Female and male subjects are shown as open and closed symbols, respectively. The quoted *r* values are correlation coefficients.

demonstrated sex effects on the plasma profile of humans with a wide spread of age from 18 years and up using NMR metabonomics on biofluids [13]. The present study was carefully designed and included subjects all with an age of 17 years to investigate and elucidate the effect of sex and BMI; and to our knowledge, the present study for the first time demonstrates by NMR-based metabonomics that sex has a substantial influence on the plasma metabolome already early in adulthood.

Intriguingly, PLS-DA enabled the prediction of sex from the NMR plasma metabolome with a high success rate; only 1 of the 45 girls and 1 of the 30 boys included in the study were misclassified (Fig. 3). This finding reveals that, in a young population at the same age, NMR-based metabonomics can efficiently discriminate sex, which has strong impact on the metabolite profile. The distinction between the 2 sexes could be ascribed to differences in the content of lipoproteins and glucose; and moreover, boys were also found to have a significantly higher content of the amino acids valine, leucine, isoleucine, and proline than girls. These amino acids give rise to distinct signals in the ^{1}H NMR spectrum, making detection of variations easier than for many other amino acids; and the finding probably reflects a general higher content of amino acids in the plasma from boys compared with girls. Accordingly, the results suggest that the girls synthesized more lipids and had lower protein turnover than the boys. Compliant findings on protein metabolism have also been reported earlier in comparative studies of young men and women [14,15]; for example, a higher amino acid oxidation in plasma has been reported in 25-year–old men than women of the same age, even when compensating for differences in muscle mass [16]. In addition, the significantly lower plasma glucose and the higher HDL content in girls than boys, the latter suggesting a higher lipid synthesis, seems to be in agreement with existing knowledge, as it has been demonstrated that women have lower fasting plasma glucose and higher fasting plasma free fatty acid [17-19]. Moreover, the findings also seem to be consistent with women being reported to oxidize more fat and less carbohydrate than men during exercise [20].

The characteristic NMR signal arising from protons in $N(CH_3)_3$ groups containing the choline moiety, principally phosphatidylcholine [21], was also found to be significantly affected by sex. The finding can be expected to reflect a higher overall plasma membrane turnover in girls and/or be specifically related to lipoprotein metabolism and the higher content of lipoproteins found in the plasma from girls, as the $N(CH_3)_3$ signal also includes phosphatidylcholine groups located at the surface of the lipoprotein particles. Sex-related differences in phosphatidylcholine homeostasis have also been reported in mice [22]. The observed effect on the $N(CH_3)_3$ signal might also be a coupled to the higher content of unsaturated lipid in plasma of girls than boys, reflecting that the phospholipids are also more unsaturated in girls compared with boys. Furthermore, it is worth to notice that oxidation of lipoproteins has been shown to affect the $N(CH_3)_3$ signal [23]; and the sex effect on the $N(CH_3)_3$ signal could possibly also be reflecting an overall difference in lipoprotein physical/chemical structure between the 2 sexes.

Principal component analysis of the obtained NMR plasma metabolite profiles did not identify any apparent effect of weight, height, or BMI. Applying PLS-DA, a previous study was able to discriminate lean and obese individuals with a BMI less than 21 kg/m^2 or greater than 25 kg/m^2, respectively, based on the NMR plasma metabolite

profile [13]. However, no large variation existed in the BMI of the subjects included in the present study. Only 6 of the persons included had an iso-BMI greater than 25 kg/m^2 [24], which probably explains why BMI did not have any apparent influence on the NMR plasma metabolite profiles.

There is a huge interest in understanding how early-life conditions affect our health later in life. The present study included subjects where key plasma parameters had been determined at an age of 9 months. Accordingly, it was possible to investigate if there were any correlations between these early-life parameters and the plasma metabolome at the age of 17 years. Interestingly, although not being very strong, correlations could be established between the 17-year plasma NMR metabolome and total cholesterol, total plasma triglyceride, and VLDL at the age of 9 months. These intriguing findings suggest an early programming of cholesterol metabolism in adulthood, which has also been proposed in other epidemiologic studies [25]. During the first year of life, nutrition is known to influence lipid levels markedly, as breast milk contains a high level of cholesterol, which is why breastfed infants have higher levels of cholesterol. The degree of tracking of triglycerides, LDL cholesterol, and HDL cholesterol into late childhood is probably therefore much stronger from 2 years than 1 year of age [26]. Based on this, lipids measured after infancy may be able to explain a higher degree of variation in the metabolome at 17 years compared with lipids measured at 9 months of age as in our study. No correlations were found between HDL and LDL at an age of 9 months and the plasma metabolome at the age of 17 years. Accordingly, it seems that infant VLDL is most important for status later in life.

In conclusion, the present study demonstrated that variations in plasma triglyceride content were the major cause of individual differences in the NMR plasma metabolome of healthy 17-year–old Danes. However, PCA also identified that, for a young population of the same age, sex has a strongly dominating impact on the NMR plasma metabolome, whereas no apparent effects of height, weight, and BMI were found. The sex differences could be ascribed to significant effects on plasma lipoproteins (mainly HDL), glucose, choline, and amino acid content. Accordingly, the present study suggests a higher lipid synthesis in young women than young men and a higher protein turnover in young men compared with women, and demonstrates the feasibility of NMR-based metabonomics for obtaining a deeper insight into interindividual differences in metabolism.

Acknowledgment

The Danish Technology and Production Research Council is thanked for financial support through the project “NMR-based metabonomics on tissues and biofluids” (project no. 274-05-339). The 800-MHz spectra were obtained using the Bruker 800 spectrometer of the Danish Instrument Center for NMR Spectroscopy of Biological Macromolecules.

References

[1] Lindon J, Holmes E, Nicholson JK. Metabonomics techniques and applications to pharmaceutical research & development. Pharm Res 2006;23:1075-88.
[2] Gibney MJ, Walsh M, Brennan L, Roche HM, German B, van Ommen B. Metabolomics in human nutrition: opportunities and challenges. Am J Clin Nutr 2005;82:497-503.
[3] Solanky KS, Bailey NJC, Holmes E, Lindon JC, Davis AL, Mulder TPJ, et al. NMR-based metabonomic studies on the biochemical effects of epicatechin in the rat. J Agric Food Chem 2003;51:4139-45.
[4] Bertram HC, Bach Knudsen KE, Serena A, Malmendal A, Nielsen NC, Fretté X, et al. NMR-based metabonomic studies reveal changes in the biochemical profile of plasma and urine from pigs fed high-fibre rye bread. Br J Nutr 2006;95:955-62.
[5] Solanky KS, Bailey NJC, Beckwith-Hall BM, Davis A, Bingham S, Holmes E, Cassidy A, et al. Application of biofluid ^{1}H nuclear magnetic resonance–based metabonomic techniques for the analysis of the biochemical effects of dietary isoflavones on human plasma profile. Anal Biochem 2003;323:197-204.
[6] Daykin CA, Van Duynhoven JPM, Groenewegen A, Dachtler M, Van Amelsvoort JMM, Mulder TPJ. Nuclear magnetic resonance spectroscopic based studies of the metabolism of black tea polyphenols in humans. J Agric Food Chem 2005;53:1428-34.
[7] Wang YL, Tang HR, Nicholson JK, Hylands PJ, Sampson J, Holmes E. A metabonomic strategy for the detection of the metabolic effects of chamomile (*Matricaria recutita* L.) ingestion. J Agric Food Chem 2005;53:191-6.
[8] Bertram HC, Hoppe C, Petersen BO, Duus JØ, Mølgaard C, Michaelsen KF. An NMR-based metabonomic investigation on effects of milk and meat protein diets given to 8-year–old boys. Br J Nutr 2007;97:758-63.
[9] Rezzi S, Ramadan Z, Martin FPJ, Fay LB, van Bladeren P, Lindon JC, et al. Human metabolic phenotypes link directly to specific dietary preferences in healthy individuals. J Proteome Res 2007;6:4469-77.
[10] Michaelsen KF. Nutrition and growth during infancy—the Copenhagen cohort study—preface. Acta Paediatr 1997;86:R4.
[11] Martens H, Dardenne P. Validation and verification of regression in small data sets. Chemometr Intell Lab Syst 1998;44:99-121.
[12] Martens H, Martens M. Modified jack-knife estimation of parameter uncertainty in bilinear modelling by partial least squares regression (PLSR). Food Qual Prefer 2000;11:5-16.
[13] Kochhar S, Jacobs DM, Ramadan Z, Berruex F, Fuerholz A, Fay LB. Probing gender-specific metabolism differences in humans by nuclear magnetic resonance–based metabonomics. Anal Biochem 2006;352:274-81.
[14] Galante A, Angelico F, Crocchioni G, Pennetti V. Intersexual differences in the serum-free amino acid pattern of young adult, normal obese aged subjects. Nutr Metab 1978;33:119-26.
[15] Proenza AM, Crespí C, Roca P, Palou A. Gender related differences in the effect of aging on blood amino acid compartmentation. J Nutr Biochem 2001;12:431-40.
[16] Volpi E, Lucidi P, Bolli GB, Santeusanio F, De Feo P. Gender differences in basal protein kinetics in young adults. J Clin Endocrin Metab 1998;83:4363-7.
[17] Haymond MW, Karl IE, Clarke WL, Pagliara AS, Santiago JV. Differences in circulating gluconeogenic substrates during short-term fasting in men, women, and children. Metabolism 1982;31:33-42.
[18] Hojlund K, Wildner-Christensen M, Eshoj O, Skjaerbaek C, Holst JJ, Koldkjaer O, et al. Reference intervals for glucose, β-cell polypeptides, and counterregulatory factors during prolonged fasting. Am J Physiol Endocrinol Metab 2001;280:E50-8.
[19] Soeters MR, Sauerwein HP, Groener JE, Aerts JM, Ackermans MT, Glatz JFC, et al. Gender-related differences in the metabolic response to fasting. J Clin Endocrinol Metab 2007;92:3646-52.
[20] Riddel MC, Partington SL, Stupka D, Armstrong C, Rennie MA. Substrate utilization during exercise performed with and without

glucose ingestion in female and male endurance trained athletes. Int J Sport Nutr Exerc Metab 2003;13:407-21.

[21] Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, et al. Rapid and non-invasive diagnosis of the presence and severity of coronary heart disease using ^{1}H-NMR–based metabonomics. Nat Med 2002;8:1439-45.

[22] Li Z, Agellon LB, Vance DE. A role for high density lipoproteins in hepatic phosphatidylcholine homeostasis. Biochim Biophys Acta 2007;1771:893-900.

[23] Soininen P, Öörni K, Maaheimo H, Laatikainen R, Kovanen PT, Kaski K, et al. ^{1}H NMR at 800 MHz facilitates phospholipid follow-up during atherogenic modifications in low density lipoproteins. Biochem Biophys Res Comm 2007;360:290-4.

[24] Cole TJ, Bellizzi MC, Flegal KM, Dietz WH. Establishing a standard definition for child overweight and obesity worldwide: international survey. BMJ 2000;320:1240.

[25] Singhal A, Cole TJ, Lucas A. Early nutrition in preterm infants and later blood pressure: two cohorts after randomised trials. Lancet 2001; 357:413-9.

[26] Boulton TJC, Magarey AM, Cockington RA. Tracking of serum lipids and dietary energy, fat and calcium intake from 1 to 15 years. Acta Paediatr 1995;84:1050-5.