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SHORT COMMUNICATION

Gut microbiome-derived metabolites characterize a peculiar obese urinary metabotype

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Obesity is a complex multifactorial disease involving genetic and environmental factors and influencing several different metabolic pathways. In this regard, metabonomics, that is the study of complex metabolite profiles in biological samples, may provide a systems approach to understand the global metabolic regulation of the organism in relation to this peculiar pathology. In this pilot study, we have applied a nuclear magnetic resonance (NMR)-based metabolomic approach on urinary samples of morbidly obese subjects. Urine samples of 15 morbidly obese insulin-resistant (body mass index > 40; homeostasis assessment model of insulin resistance > 3) male patients and 10 age-matched controls were collected, frozen and analyzed by high-resolution ^{1}H-NMR spectroscopy combined with partial least squares-discriminant analysis. Furthermore, two obese patients who underwent bariatric surgery (biliopancreatic diversion and gastric bypass, respectively) were monitored during the first 3 months after surgery and their urinary metabolic profiles were characterized. NMR-based metabolomic analysis allowed us to identify an obesity-associated metabolic phenotype (metabotype) that differs from that of lean controls. Gut flora-derived metabolites such as hippuric acid, trigonelline, 2-hydroxyisobutyrate and xanthine contributed most to the classification model and were responsible for the discrimination. These preliminary results confirmed that in humans the gut microflora metabolism is strongly linked to the obesity phenotype. Moreover, the typical obese metabotype is lost after weight loss induced by bariatric surgery.
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Introduction

Obesity is a complex multifactorial disease arising from the interplay between a wide range of variables such as genetic susceptibility factors, nutritional habits and physical activity, set within a social, cultural and environmental landscape.[1]

Recent studies have suggested that the gut microbiota may have a role in obesity through the regulation of energy metabolism by several mechanisms (that is, energy harvest from the diet, regulation of fat storage, lipogenesis and fatty oxidation, modulation of afferent gastrointestinal peptide hormones, induction of metabolic endotoxemia).[2–4]

All these findings lead to the concept of human beings as a 'superorganism' in which the metabolism is the resultant of the integration of the host metabolic processes with the microbiome ones. The symbiotic metabolic complexity of the individual host–microbiome co-metabolism is likely to be reflected in a specific chemical signature of biofluids.[5–8]

In this study, we sought to characterize the metabolic status of morbidly obese patients as a whole, through a nuclear magnetic resonance (NMR)-based metabonomic approach. For this purpose, we analyzed the urine samples of obese patients by high-resolution proton NMR (^{1}H NMR) spectroscopy in combination with multivariate statistics. Furthermore, we investigated the obese metabolic phenotype (metabotype) changes in relation to two different bariatric surgery procedures (biliopancreatic diversion and Roux-en-Y gastric bypass)[9,10] that are detailed in the Supplementary Information.

Patients and methods

Fifteen morbidly obese insulin-resistant (body mass index $49.58 \pm 8.30\,kg\,m^{-2}$; homeostasis assessment model of insulin resistance 11.49 ± 12.92) male patients and 10 age-matched controls (body mass index $24.63 \pm 1.90\,kg\,m^{-2}$; homeostasis assessment model of insulin resistance

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1.24 ± 0.39) were enrolled in this observational study. None of the patients had gastrointestinal symptoms or a history of chronic gastrointestinal or renal problems; none of the patients received any antibiotic, probiotic or prebiotic agents in the 3-month period before the collection of urine samples. The study protocol did not specify a standard diet but placed some restrictions on diet and alcohol consumption. Each patient signed an informed consent to participate in the study, which was approved by the ethics committee of the Catholic University in Rome.

Physical and metabolic characteristics of the participants are listed in Supplementary Table 1.

Two obese patients who underwent bariatric surgery were monitored during the first 3 months after surgery. Urine samples were collected at 30 and 90 days after biliopancreatic diversion and Roux-en-Y gastric bypass surgery, respectively.

Morning urine samples were collected, centrifuged, sterilized by 0.22 μm filtration (Millipore, Billerica, MA, USA) and frozen at −80 °C. Samples for NMR analysis were prepared by taking an aliquot of 700 μl, and adding 70 μl of a solution of 20.2 mM TSP in D_2O (2 mM final concentration). pH was adjusted to 2.5 with HCl 2 N.

^{1}H-NMR spectra of urine samples were obtained on an Avance 400 spectrometer (Bruker Spectrospin, Karlsruhe, Germany) under full relaxation conditions; presaturation was used to suppress the solvent signal. Details are provided in the Supplementary Information. Signal assignments were performed by using two-dimensional ^{1}H-NMR spectra (TOCSY) and by comparison with literature.[11,12]

^{1}H NMR spectra were processed and binned (bin size = 0.0125 p.p.m.) by using the 1H-Manager ver. 11.0 software (Advanced Chemistry Development, Inc., Toronto, Ontario, Canada). Data from urine spectra were divided by the methyl peak integral of creatinine (Crn) at 3.05 p.p.m., because Crn is an indicator of the concentration of urine commonly used in clinical chemistry.[13] Multivariate data analysis was carried out using Unscrambler 9.0 Software (CAMO, Oslo, Norway). Spectral data were mean centered and scaled before analysis.[14] Principal components analysis (PCA) was used to examine inherent clustering and to identify outliers. Partial least squares-discriminant analysis was used to assess the correlation between the observed NMR data and class membership information (that is, an external variable that indicates to which class the subjects belong, of which the values are 0 for lean and 1 for obese subjects) as the response variable.[14] Unpaired Student's *t*-test was applied to the discriminant variables obtained from partial least squares-discriminant analysis; a P value <0.05 was considered significant.

Results and discussion

A clear separation of the obese and age-matched lean subjects was obtained by partial least squares-discriminant analysis of the ^{1}H-NMR spectra of urine samples ($R^2 = 0.976$; cross-validation = 0.848 and four significant components; Figure 1).

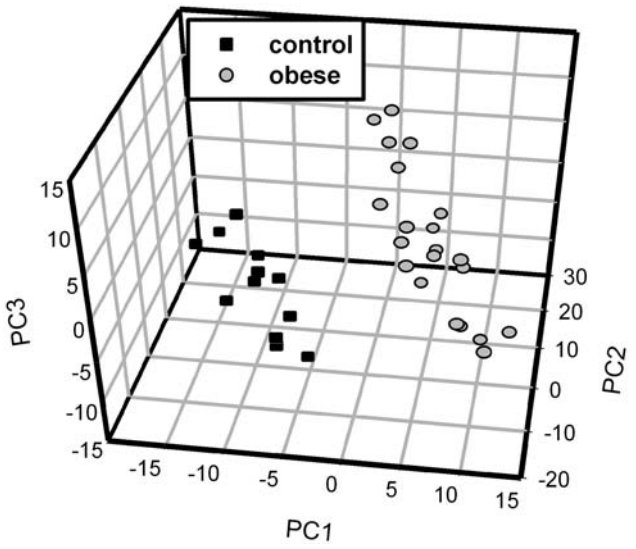

Figure 1 Partial least squares-discriminant analysis score plot of the first three factors distinguishing obese subjects (gray circles) from lean controls (black squares).

The metabolites involved in the discrimination between groups were hippuric acid, trigonelline, 2-hydroxyisobutyrate and xanthine. In particular, the obese urinary metabolic phenotypes (metabotypes) were characterized by a lower concentration of hippuric acid (180.7 ± 69.1 and 361.3 ± 91.7 μmol mmol^{-1} of Crn in obese and control subjects, respectively; $P<0.001$), trigonelline (11.3 ± 7.2 and 26.2 ± 8.9 μmol mmol^{-1}Crn; $P<0.001$) and xanthine (7.9 ± 2.4 and 14.5 ± 4.8 μmol mmol^{-1} Crn; $P<0.001$), and a higher concentration of 2-hydroxyisobutyrate (12.1 ± 3.8 and 9.8 ± 2.1 μmol mmol^{-1} Crn; $P<0.001$), with hippurate being the most markedly different metabolite between the obese and control groups.

Hippurate is a gut microbial mammalian co-metabolite of benzoic acid that can be generated by a range of gut microbes from low-molecular-weight aromatic compounds and polyphenoles in the gut, subsequently conjugated with glycine in the mitochondria, and finally excreted in the urine.[15] Urinary levels of hippurate have been shown to correlate with the obese phenotype in different animal models[16–19] and to associate with the fecal counting of several bacterial species in a rat model of obesity;[16] moreover, lower levels of hippurate were found in the urinary metabolic phenotypes of human type-2 diabetes mellitus patients.[17] Hippurate has also been inversely linked to blood pressure, suggesting a further connection with diet and obesity.[20]

The changes in urinary excretion of trigonelline (*N*-methylnicotinate) indicate a class-specific metabolism of niacin, which is an essential vitamin involved in major

physiological functions such as a coenzyme in tissue respiration, and carbohydrate and lipid metabolism. Niacin requirements are satisfied by both dietary sources and biosynthesis through a tryptophan-mediated metabolism ensured by the liver and the gut microflora.[21] Trigonelline is also a byproduct of the conversion of *S*-adenosylmethionine to *S*-adenosylhomocysteine and its decrease has been related to a depletion of *S*-adenosylmethionine, as it is consumed in the trans-sulfuration pathway in order to regenerate glutathione stores that are depleted by obesity-related metabolic stress.[22] It is noteworthy that lower levels of *N*-methylnicotinate were found in urine samples from rodent models of type-2 diabetes mellitus as well as human sufferers.[17]

Urinary excretion of 2-hydroxyisobutyrate, deriving from the microbial degradation of dietary proteins that escape digestion in the upper gastrointestinal tract, has been associated with the presence of some microbial members (for example, *Faecalibacterium prausnitzii*) in the colon.[6]

Therefore, the observed differences in hippurate, trigonelline and 2-hydroxyisobutyrate excretion demonstrated significant functional differences in the microbiome metabolic activity between obese and lean individuals and confirmed recent findings on the relationships between gut bacterial composition and the obese host phenotype. Indeed, recent works have shown that the human microbiome composition varies among healthy people, as well as between genetically obese and lean rats. At the same time, metagenomic studies on humans have highlighted that obesity is associated with phylum-level changes in the microbiota, reduced bacterial diversity, and altered representation of bacterial genes and metabolic pathways, including those involved in nutrient harvest.[23,24]

Furthermore, in the obese group the lower urinary levels of xanthine were associated with elevated serum levels of uric acid ($>8\,mg\,dl^{-1}$; hyperuricemia is commonly found in obese patients[25]), suggesting a significantly higher activity of xanthine oxidase, the rate-limiting enzyme of the purine degradation pathway.

Bariatric surgery is currently the only available treatment for morbid obesity that consistently achieves and sustains substantial weight loss.[26] A recent study revealed that this surgical procedure alters the intestinal microbial community.[27]

To investigate whether the obesity-related metabolic profile changed after bariatric surgery, we monitored two obese patients during the first 3 months after bariatric surgery (biliopancreatic diversion and Roux-en-Y gastric bypass, respectively) and characterized their urinary metabotypes.

The postoperative course of the two patients was in agreement with the results of a recent meta-analysis on bariatric surgery.[26,28] Surgical treatment resulted in a significant weight loss and body mass index reduction, and in a marked improvement of insulin sensitivity, hypercholesterolemia and hypertriglyceridemia irrespective of the surgical technique used (data not shown).

The urinary metabolic phenotypes showed marked differences after bariatric surgery. In particular, spectra from the urine samples of both groups showed substantial variations in the levels of metabolites responsible for the discrimination between obese and lean metabotypes.

Again, hippuric acid was the metabolite that changed most, showing up to a 30-fold increase in the urine of the patient who underwent biliopancreatic diversion and a threefold increase in the patient who underwent Roux-en-Y gastric bypass during the 3 months after surgery (Figure 2). In addition, trigonelline levels increased markedly while both xanthine and 2-hydroxyisobutyrate concentrations approximated to the lean subject values. Interestingly, the post-surgery increased urinary concentrations of xanthine were associated with a decreased serum concentration of uric acid ($<5\,mg\,dl^{-1}$).

In conclusion, these preliminary results demonstrated that obese individuals are characterized by a urinary metabolic phenotype (metabotype) that differs from that of lean subjects, with gut microflora-associated metabolites being

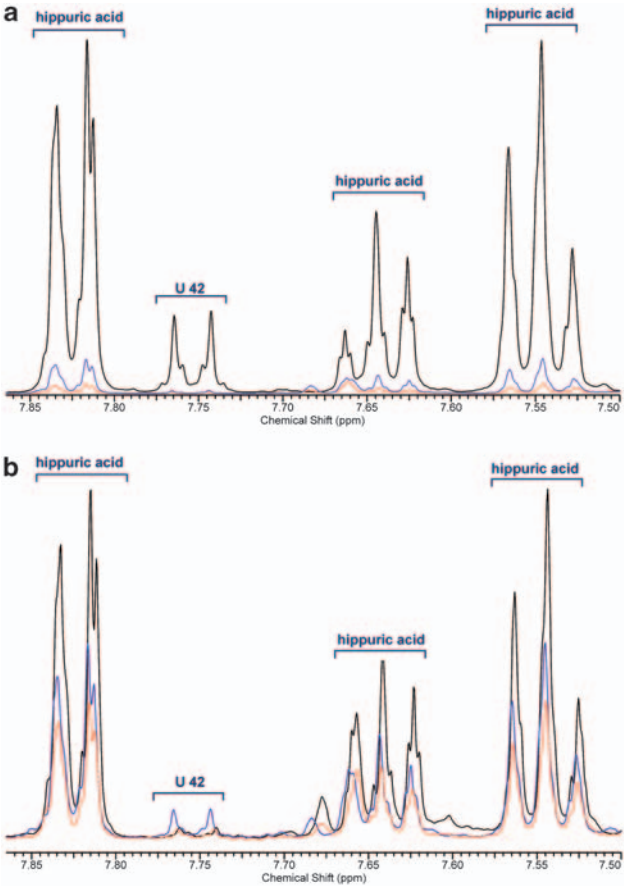

Figure 2 ^{1}H NMR spectral regions relative to hippuric acid signals of urine samples of biliopancreatic diversion (**a**) and Roux-en-Y gastric bypass (**b**) patients (red = pre-operative sample; blue = 30 days post surgery; black = 90 days post surgery).

the prominent contributors to this difference. Moreover, our findings showed that those metabolites responsible for the discrimination between obese and lean metabotypes significantly changed after bariatric surgery in the two subjects analyzed; this may reflect the physiological changes caused by the surgical procedures irrespective of the surgical technique. Obviously, further studies with a greater number of subjects are needed to confirm these findings.

Conflict of interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on International Journal of Obesity website (http://www.nature.com/ijo)