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Can Salivary Phosphate Levels Be an Early Biomarker to Monitor the Evolvement of Obesity?

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Abstract

Phosphate is an essential nutrient required for important biological reactions that maintain the normal homoeostatic control of the cell. The adverse effects of phosphate metabolism in obesity have not been studied in detail, chiefly because such an association is thought to be uncommon. However, in some animal models of obesity, serum phosphate levels were noted to be higher than the nonobese controls. For example, leptin-deficient (ob/ob) mice become severely obese and have high serum phosphate levels. In this study, we analyzed the phosphate content in saliva collected from children (n = 77; 10.5 ± 1.8) to evaluate association with body mass index; there is a significant increase of salivary phosphate content in obese compared to normal-weight children (ANOVA $p < 0.001$). The correlation coefficient (r) between BMI and phosphate was 0.33 ($p = 0.0032$). Our results suggest that the human salivary phosphate level may be an early biomarker of the genesis of obesity in children. The diagnostic importance lies in the fact that the salivary phosphate level could provide a noninvasive predictive marker in the development of obesity. Further studies will be required to understand the underlying mechanism of increased salivary phosphate accumulation in obese and overweight children. Nevertheless, its occurrence without systemic changes could be of diagnostic value, particularly in monitoring evolvement of obesity.

The increasing occurrence of obesity and its related complications, including type 2 diabetes mellitus and cardiovascular anomalies, is alarming and becoming a major public health problem. Despite significant improvement of our understanding of underlying molecular mechanisms of adipose tissue remodeling and eventual development of obesity, potential factors that might serve as a biomarker to monitor evolvement of obesity is not yet clearly identified. Earlier studies have found that the composition of salivary bacteria changes in overweight women [1]. In particular, the percentage of the bacterium *Selenomonas noxia* was capable of identifying 98.4% of studied 313 overweight women from a group of generally healthy individuals, with a remarkably high degree of diagnostic power [1]. Whether change in the composition of salivary content could serve as biological marker of a developing overweight condition is an area that needs further studies. Of even greater

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interest, and the subject of future research, is the possibility of using easily available saliva as a diagnostic and prognostic marker of systemic diseases, including obesity.

A clinical association between phosphate imbalance and metabolic diseases has been reported elsewhere. For instance, serum phosphate levels were positively correlated with insulin sensitivity (but not with insulin secretion) in a study conducted in 881 individuals without diabetes mellitus [2]; such a correlation was independent of age, sex, proportion of body adipose content, serum calcium and serum creatinine levels [2]. In a separate study of 298 children and adolescents aged 6–12 years old, in which 190 individuals with obesity and 108 controls without obesity were compared, reduced phosphate serum levels were significantly associated with the development of insulin resistance in children with obesity [3]. Moreover, in a leptin-deficient animal model of obesity, higher serum phosphate levels were noted, as compared to the nonobese controls [4–6]. Summarizing the above-mentioned evidence, it appears likely there might be an association between phosphate metabolism and adipose tissue turnover. This study was designed to study whether the phosphate content of saliva might be clinically useful in monitoring the development of obesity.

The physiological phosphate balance is mostly accomplished by cross-organ talk among kidney, intestine, and bone, where sodium-dependent phosphate transporters (Na/Pi) play a key role in the absorption and reabsorption of consumed phosphate [7–12]. The Na/Pi-2b is mostly present in the luminal side of the intestinal cells where it helps in intestinal phosphate absorption according to the body's need of the phosphate [13]. Almost 80% of the filtered phosphate in the kidney is reabsorbed in the proximal tubular epithelial cells and is also partly accomplished by sodium-dependent phosphate uptake through Na/Pi-2a and Na/Pi-2c transporters. Of relevance, Na/Pi-2b transporters are also expressed in the salivary gland; studies have identified the expression of Na/Pi-2b mRNA and protein in the salivary duct cells [14, 15]. In fact, the ductal cells mostly demonstrated apical Na/Pi-2b expression in salivary glands, whereas the acinar cells showed mainly basolateral expression, suggesting that there might be phosphate secretion by the acini and phosphate reabsorption by the ducts [15]. Moreover, klotho, a cofactor for circulating fibroblast growth factor 23 (FGF23), a master regulator of phosphate metabolism [11, 16–18], is also present in the salivary gland [19], and is therefore providing the necessary machinery for a local salivary regulation of phosphate metabolism, dependent or independent of systemic regulation. This study provides the data to show that the phosphate content in saliva collected from children has a close association with BMI.

Materials and Methods

Saliva and Blood Collection from Children

Saliva and blood samples were collected from 77 children (59% male), aged 10.5 ± 1.8 years, under fasting conditions. Height, weight, blood pressure, heart rate and fitness were measured and recorded. Fitness was measured by heart rate elevation to a Queens College step test [20]. BMI percentile was used to define the children into body weight categories. This study was reviewed and approved by the Forsyth Institute institutional review board. Informed consent was obtained from parents or guardians and assent was obtained from each child. The study was conducted in Portland, Me., USA.

Measurement of Phosphate Contents

Paired 100-μl samples of saliva and plasma from each child were prepared and maintained frozen at −80 °C until assayed. Phosphate was measured on a nontargeted metabolic profiling platform (Metabolon, Durham, N.C, USA) employing gas chromatography/mass spectrometry (GC/MS) [21, 22]. For each sample, 100 μl of plasma or saliva was used for

analyses. Using an automated liquid handler (Hamilton LabStar, Salt Lake City, Utah, USA), protein was precipitated with methanol that contained four standards to report on extraction efficiency. The resulting supernatant was split into equal aliquots for analysis. One aliquot, dried under nitrogen and vacuum-desiccated, was subsequently derivatized to a final volume of 50 μl for GC/MS analysis using equal parts of bis(trimethylsilyl)trifluoroacetamide and the solvent mixtureacetonitrile:dichloromethane:cyclohexane (5:4:1) with 5% triethylamine at 60°C for 1 h. Mass spectrometric salivary phosphate data were tested and found non-normal using the Shapiro-Wilk statistics and were also both skewed and kurtotic. Saliva phosphate levels were also determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test (Stanbio Laboratory, Boerne, Tex., USA) as detailed earlier [18, 23, 24]. Statistical evaluations of these data were performed by the Mann-Whitney test. BMI percentile was computed from software from the Center for Disease Control [25]. Body weight categories were assigned using their percentile recommendations (normal healthy weight <85%, overweight ≥85–95% and obese ≥95%). Two children were classified as underweight (percentile <5%). These were included in the normal healthy weight group for analysis.

Leptin-Deficient Obese Mice

Heterozygous leptin-deficient obese [C57B1/6J *lep*^{ob} (+/−)] mutants were obtained from Jackson Laboratory, Me., USA and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Mouse breeding was performed according to the protocols approved by the institution's subcommittee on animal care (IACUC). Routine PCR using genomic DNA extracted from tail clips was performed for genotyping of the various groups of mice, as detailed earlier [23, 26, 27]. The total body weight of wild-type and *ob/ob* mice were taken weekly starting at 3 weeks of age until 30 weeks. At least three or more mice from each genotype were sacrificed at 9 weeks and retroperitoneal, mesenteric and epididymal fat tissue weights were recorded. In addition, liver weights of the wild-type and *ob/ob* mice were taken before fixing part of the liver for histological analysis. Fasting blood glucose levels were determined in all four genotypes. In addition, serum isolated from blood obtained by cheek-pouch bleeding of wild-type and *ob/ob* mice was isolated and stored at −80°C. Serum phosphate levels were determined by colorimetric measurements using the Stanbio Phosphorus Liqui-UV Test [26, 28, 29]. Statistically significant differences between groups were evaluated either by the Student's t test or the Mann-Whitney U test for a comparison between two groups. All values are expressed as mean ± SE. $p < 0.05$ was considered to be statistically significant.

Results

Animal Studies

Leptin-deficient obese (ob/ob) mice develop hyperglycemia, glucose intolerance, and obesity. Since the ob/ob mouse could not produce leptin, its food intake is uncontrolled due to the lack of feedback signaling [30]. Mutant ob/ob mice are indistinguishable from their unaffected wild-type littermates at birth, but gain weight rapidly throughout their lives, reaching a weight three times that of unaffected wild-type mice (fig. 1, upper left panel). Compared to the wild-type controls, the increased body size of ob/ob mice is also associated with signifi-candy increased fat accumulation, as determined by analysis of retroperitoneal, mesenteric and epididymal fat tissue weights. Increased accumulation of fat is also obvious in the livers obtained from ob/ob mice (fig. 1, right panel). The ob/ob mice also develop hyperglycemia [31] with increased serum levels of phosphate (fig. 1, lower left panel). Although increased serum levels of phosphate in ob/ob mice could pardybe related to the increased food consumption, it is, however, an intriguing association that needs further exploration.

Human Study

Biometric characteristics of the 77 children admitted into the study are shown in table 1. Differences between normal and obese children (p 1:3) were all statistically significant except for diastolic blood pressure and heart rate. The acceptance criteria (10 ± 1 year) was expanded (10 ± 2 years) for overweight and obese volunteers to obtain a sufficient number willing to provide both saliva and blood samples. This resulted in small but significant differences in age for heavier weight groups. The correlation and statistical significance between salivary phosphate and other parameters are presented in table 2. We have found that different obesity parameters, including BMI, waist circumference, fitness and weight, along with cardiovascular parameter (systolic blood pressure) were significantly associated with salivary phosphate content. Saliva determination by nontargeted mass spectrometric analysis revealed that phosphate was significantly higher in the saliva of obese children. By comparison, however, plasma phosphate was not associated with obesity (fig. 2), suggesting that changes in salivary phosphate levels could occur even before serum changes become apparent, and thereby could be used as a biomarker for monitoring evolvement of obesity. Of relevance, an r^2 of 0.70 and a p of 0.003 for prediction of phosphate from the spectrographic response by the quantitative phosphate levels (mg/dl) = 5.03 MSR + 4.92. Thus, the median mass spectrographic value for saliva of 1.0 would roughly equivalent to 9.95 mg/dl (fig. 3).

Discussion

Child obesity has accelerated over the last decade [32]. As a result, investigation of biomarkers that can serve as early warning signs has become increasingly important. Salivary analysis ranks high in consideration due to ease of collection in a population of children that are understandably reticent to provide blood samples. In this report, we provide evidence that salivary phosphate is elevated in obese children. By contrast, plasma phosphate was not found to be elevated in these children. Taken together, these observations suggest that phosphate metabolism might be associated with fat cell turnover, and that salivary phosphate could be an early marker of metabolic disease associated with development of obesity.

In this study, we presented values for phosphate that were obtained by colorimetric phosphate test from the supernatant of unstimulated saliva (fig. 2). The similar phosphate values in our study are in the similar range compared to other published studies; for instance, studies that were done on healthy children presenting a range of salivary phosphate concentration of 10.5–17 mg/dl [33–35]. No data on BMI were presented in these published studies, except for one study that stated that children with metabolic disorder were excluded [34]. It is, therefore, not possible to estimate the accurate phosphate concentration from these studies, as some children might have been overweight. In a similar line of study that was done on children, adolescents and young adults who have chronic kidney disease showed salivary phosphate values for the healthy subjects of 15.8 mg/dl and patients (on predialytic) of 20.77 mg/dl [36].

Why salivary phosphate levels are high in obese children is not yet clear, and might be related to the altered functions of phosphate-regulating machineries present in the salivary glands. It is important to note that type II sodium-phosphate cotransporters (Na/Pi-2b) are present in the salivary gland [14, 15]. In addition, the type III sodium phosphate cotransporters, PiT1 and PiT2, have been detected in human salivary glands [37]. The possible role of PiT1 and PiT2 in the salivary gland has not been well studied, but a number of studies demonstrated the role for both PiT1 and PiT2 in phosphate reabsorption in the kidney. Depending on the sodium phosphate cotransporter activities in the salivary gland, it is likely that there might be a local regulation of phosphate metabolism that is independent

of systemic phosphate regulation. In fact, such possibilities are raised from our observations of increased salivary phosphate contents in obese children, despite their normal plasma phosphate levels. It is important to note that despite recognition that serum phosphate level is the gold standard to estimate the overall phosphate status of the body, the amount of intracellular phosphate or phosphate storage is not taken into consideration in such traditional methods of phosphate measurement. Both human and experimental studies have shown clearly that certain features of phosphate toxicity might appear even in normophosphatemic conditions [38], thereby exposing the limitation of serum phosphate measurements to detect early events of phosphate toxicity. In that context, our result of increased salivary phosphate content without any change in plasma levels provides a meaningful early marker for eventual obesity development.

Since consumption of soda drinks and fast foods are well connected with childhood obesity [39], it will be interesting to know whether such phosphate-rich foods and drinks, by local regulation, might increase the salivary phosphate content that we found in our obese cohorts. Moreover, obese children exhibit more bone resorption than healthy normal-weight children [40], and thereby could induce altered mineral ion metabolism. Further studies will explain the underlying mechanism of increased salivary phosphate accumulation in obese and overweight children, with its biological and clinical significances. This particular study, although limited in numbers of subjects involved, provides the evidence of potential clinical importance of salivary phosphate in monitoring the evolvement of obesity.

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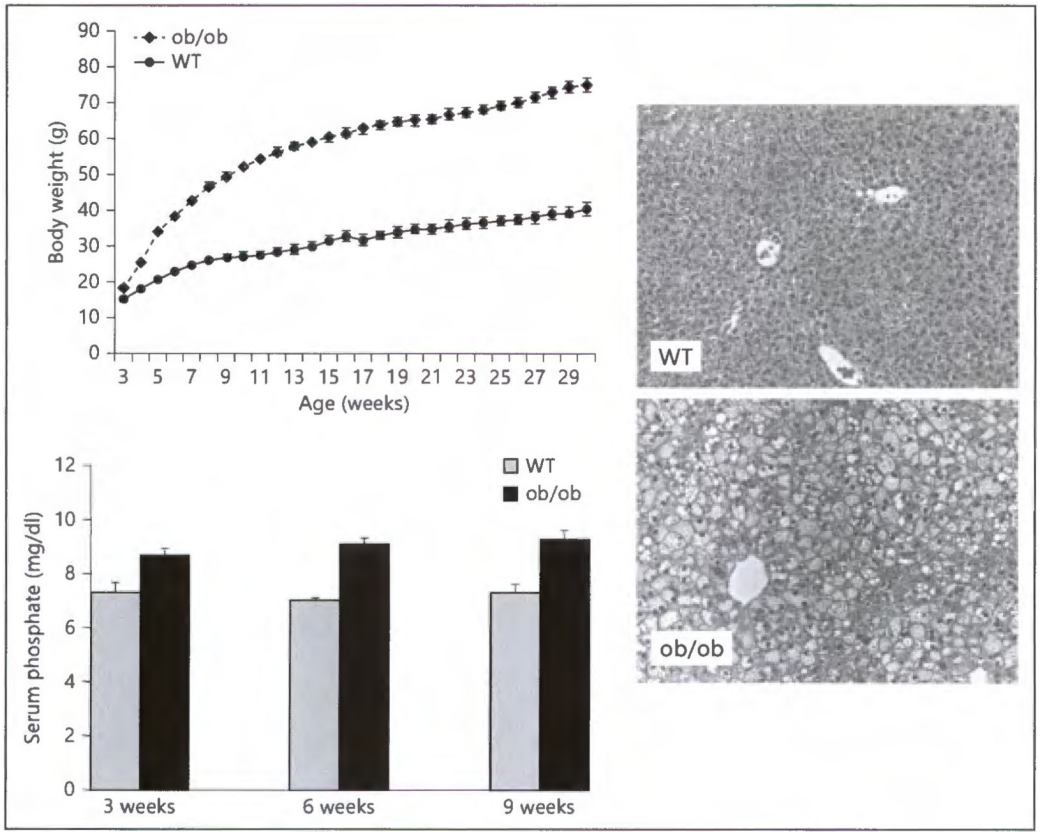

Fig. 1.

Upper left panel: The body weight chart of wild-type and ob/ob animals. Note that compared to the wild-type control animals, the ob/ob mice are significantly heavier due to an excessive accumulation of fat tissues. Right panel: In accord with the relatively increased liver weight in ob/ob mice over wild-type controls, the histological analysis of the liver shows increased fat tissue accumulation in ob/ob mice. Lower left panel: Biochemical analysis of serum phosphate levels. Note that the serum phosphate levels are significantly higher in ob/ob mice compared with the wild-type (WT) mice at 3, 6 and 9 weeks of age. * $p < 0.05$ vs. WT. Serum samples collected from at least 4 or more mice were used for the measurements.

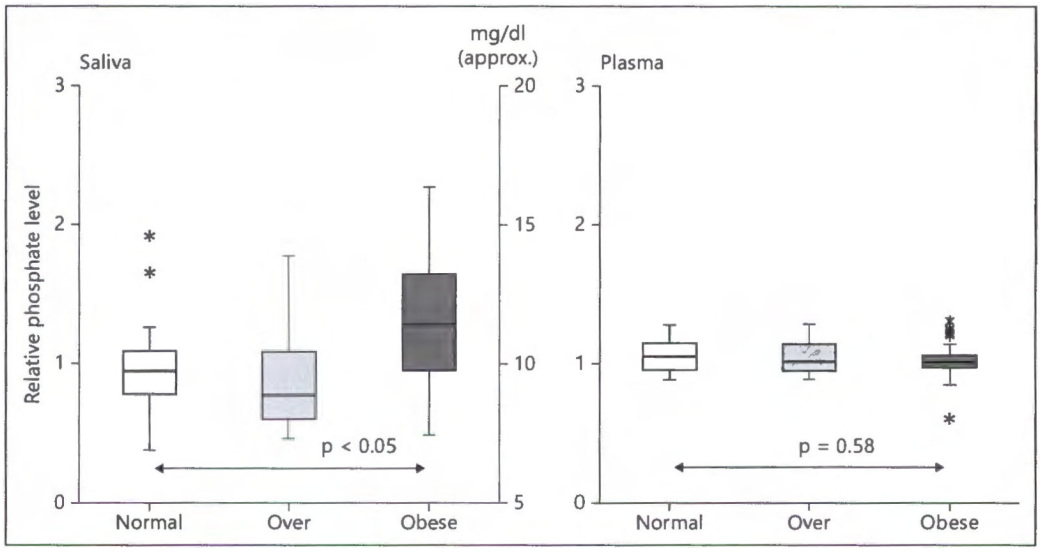

Fig. 2.

Relative phosphate levels in saliva and plasma by mass spectroscopy. Central bars are the median, rectangles are the intraquartile range, whiskers are ×1.5 intraquartile range, asterisks are outside values and p is computed using the Mann-Whitney test. Relative phosphate values were obtained by dividing the spectrograph response by the median of all values. This comparison indicates that salivary phosphate levels in obese children were significantly greater than in normal healthy weight children whereas plasma levels were not. Approximate concentrations (mg/dl) were computed from colorometric determinations of aliquots.

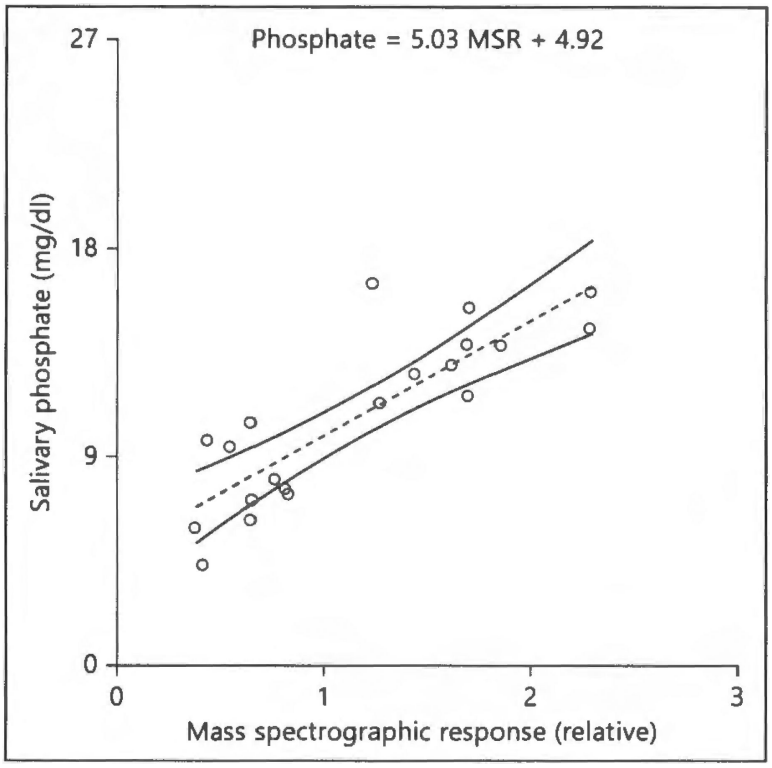

Fig. 3.

Least-squares fit linear function and 95% CIs. The relationship has an r^2 of 0.72 and a $p < 0.0001$ for prediction of phosphate from the spectrographic response by the function phosphate (mg/dl) = 5.03 MSR + 4.92. Thus, the median mass spectrographic value for saliva of 1.0 would be 9.95 mg/dl.

Table 1

Characteristics of children investigated in this study

	Normal weight (n = 36)			Overweight (n = 15)			Obese (n = 26)		
	median	intraquartile range	p = 1:3	median	intraquartile range	p = 1:2	median	intraquartile range	p = 2:3
Weight, lb	74.4	24.4	<0.0001	106.1	22.4	<0.0001	151.5	50.1	0.002
Height, inches	56.5	5.0	0.004	58.5	4.9	0.07	60.1	9.5	0.3
Age, years	9.6	2.4	0.02	10.5	1.9	0.02	10.8	2.5	0.8
BMI	17.2	2.2	<0.0001	21.1	2.4	<0.0001	27.4	7.6	<0.0001
Waist, inches	24.0	3.5	<0.0001	27.5	5.0	<0.0001	34.8	9.0	0.004
Systolic BP, mm Hg	117.5	15.2	0.02	122.0	12.0	0.03	121.2	14.7	0.9
Diastolic BP, mm Hg	67.3	8.5	0.1	69.0	6.7	0.2	71.2	8.7	0.9
Heart rate, BPM	77.3	15.5	0.7	73.3	23.3	0.3	81.8	20.3	0.6
Fitness, BPM	24.7	16.7	0.002	37.2	25.5	0.02	35.1	15.3	0.9
Salivary flow, ml/h	37.3	27.4	0.1	40.3	25.3	0.9	32.7	27.5	0.3

Values listed include median, intraquartile range and the significance p value of differences between body weight categories (1 = normal, 2 = overweight, 3 = obese) by Mann-Whitney analysis.

Table 2

Correlation (r) and statistical significance (p) between salivary phosphate and each measure

Measure	r	p
BMI	0.33	0.003
Waist, inches	0.31	0.007
Fitness, BPM	0.29	0.011
Weight, lb	0.27	0.018
Systolic BP, mm Hg	0.22	0.051
Heart rate, BPM	0.21	0.067
Diastolic BP, mm Hg	0.17	0.139
Height, inches	0.09	0.433
Age, years	0.01	0.913
Salivary flow, ml/h	–0.24	0.035

Results indicate that measures of obesity (BMI, waist circumference, fitness and weight), one cardiovascular parameter (systolic blood pressure) and salivary flow were significantly associated with salivary phosphate.