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ORIGINAL ARTICLE

Urinary markers of nucleic acid oxidation in Danish overweight/obese children and youths

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ABSTRACT

Urinary excretion of the RNA and DNA oxidation markers, 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) in newly diagnosed adult type 2 diabetics are reported to be long-term predictors of mortality independent of conventional risk factors. In the current study, we investigated the relationships between urinary markers of nucleic acid oxidation concentrations and the degree of obesity and glucose metabolism in overweight compared to lean children. Forty-two (24 girls) overweight and 35 lean (19 girls) children and adolescents were recruited from the Registry of the Danish Childhood Obesity Biobank. Anthropometric measurements were collected at baseline and glucose metabolism was assessed by an oral glucose tolerance test. A urine sample was obtained during the test. Linear regression did not demonstrate any associations between the urinary markers and the degree of obesity or glucose metabolism in lean and obese children. However, sub-analyses adjusted for age, sex, and the degree of obesity showed positive associations between the 2 h glucose and the urinary markers, 8-oxoGuo ($p = 0.02$, $r^2 = 0.63$) and 8-oxodG ($p = 0.046$, $r^2 = 0.48$), and between the insulinogenic index and 8-oxoGuo ($p = 0.03$, $r^2 = 0.60$) in the 12 obese children exhibiting impaired glucose tolerance. Excretion of the urinary markers of nucleic acid oxidation and the degree of obesity or the glucose metabolism were not associated in this study. Nevertheless, obese children with impaired glucose tolerance seem to exhibiting an increased oxidative stress level, but due to the small sample size in this study, further investigations are required to elucidate this correlation.

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Child; glucose metabolism; obesity; 8-oxodG; 8-oxoGuo

Introduction

The increasing prevalence of type 2 diabetes in overweight children and adolescent has become an increasing health challenge worldwide during the last decades [1,2]. Obesity-related impaired glucose metabolism and insulin resistance lead to a higher risk of developing type 2 diabetes [3,4]. However, screening tools in the clinical setting to identify obese children with a higher risk of developing type 2 diabetes are still insufficient.

An increasing degree of obesity has been associated to the development of low grade systemic inflammation and cellular oxidative damage to the DNA and RNA [5,6]. The oxidative damage to nucleic acids has been suggested to be a marker depicting the development of diabetes, progression of insulin resistance, β-cell dysfunction, and cardiovascular complications in adults [7–10].

Oxidative stress occurs when increasing concentrations of reactive oxygen species (ROS) overwhelm the cell's protective mechanisms against oxidatively generated damage resulting in cell damage [7]. During the cellular repair/degradation of the nuclear oxidized DNA and RNA, 8-oxo-7,8-dihydroguanosine (8-oxoGuo) and 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) are excreted into the urine, which may be interpreted as a reflection of the average rate of oxidatively generated damage to DNA and RNA in the body [8].

Increased concentrations of urinary 8-oxodG in adult type 2 diabetic patients have been reported to be correlated with glycosylated hemoglobin (HbA1c) and other medical complications including nephropathy and

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retinopathy have also been reported [9,10]. Interestingly, urinary concentrations of the RNA and DNA oxidation markers in newly diagnosed adult type 2 diabetics have shown to be long-term predictors of mortality independent of conventional risk factors [11], and increased levels of urinary 8-oxoGuo during the first 6 years after the diagnosis of type 2 diabetes mellitus are also associated with mortality [8,11,12].

In the pediatric population, excretion of nucleic acid oxidation in urine has been reported to be elevated in children with chronic diseases, i.e. type 1 diabetes and celiac disease, compared to healthy children [13,14]. In contrast, excretion of the urinary marker 8-oxodG has been reported to be significantly lower in overweight and obese children compared to lean controls [15].

Since these intracellular biomarkers can be assessed non-invasively in the urine, it makes them available as potential identification, risk stratification, and treatment markers in individual obese children and youths during treatment in order to monitor the potential progression towards type 2 diabetes and diabetes- or obesity related medical complications.

In this study, we investigated the hypothesis that creatinine adjusted urinary 8-oxoGuo or 8-oxodG concentrations are associated with glucose metabolism and the degree of obesity in Danish children and youths.

Methods and materials

Design and study population

The study was a cross-sectional pilot study, including 77 children and adolescents from the Registry of The Danish Childhood Obesity Biobank. Forty-two overweight and 35 lean children and adolescent aged 7–17 years were recruited. The common inclusion criterion was the ability to complete an oral glucose tolerance test (OGTT). Furthermore, the inclusion criteria for the overweight/obese children were (1) body mass index standard deviation score (BMI SDS) >1.28 and (2) enrolled in childhood obesity treatment. The inclusion criterion for the lean children was a BMI SDS between −1.2 and 1.2. Informed written consent was obtained from their parents and informed assent was obtained from each participant. The study was carried out in accordance with the ethical principles of the Declaration of Helsinki 2013 and approved by the Danish Data Protection Agency (REG-06-2014) and the Ethics Committee of Region Zealand, Denmark (SJ-104), and is registered at ClinicalTrials.gov (NCT00928473).

Sample collection

Anthropometric measurements were collected at baseline. Height was measured by stadiometer to the nearest 1 mm and weight was measured to the nearest 0.1 kg on a Tanita WB-100 medical scale (Tanita Corp., Tokyo, Japan) without shoes and wearing light indoor clothes. The degree of obesity was estimated by BMI SDS, which was calculated from height and weight by the LMS method [16,17].

A standard OGTT (1.75 g of glucose/kg of body weight with a maximum dose 75 g) was performed after a 10-h overnight fast and 3 days of normal food intake and non-excessive exercise. Blood samples were drawn from a peripheral venous catheter at baseline and in 30 min intervals during the test. The blood samples collected at baseline included plasma glucose, serum insulin, and whole blood HbA1c. A sample of fresh voided urine was obtained from the participants during the test day and immediately stored at −80 °C until the analyses.

Analyses

Biochemical

Plasma glucose samples were collected in fluoride containing tubes and stored at room temperature for less than 30 min after sampling before being centrifuged at 4 °C. The biochemical analyses of plasma glucose were performed on a Dimension Vista® 1500 analyzer (Siemens, Munich, Germany). Serum insulin was collected in a tube containing serum separating gel and stored at room temperature for 30–60 min after sampling before being centrifuged at 4 °C. The biochemical analyses of serum insulin were performed on a Cobas® 6000 analyzer (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Whole blood HbA1c was analyzed on a Tosoh high-performance liquid chromatography G8 analyzer (Tosoh Corporation, Tokyo, Japan).

Urinary markers

8-oxodG and 8-oxoGuo concentrations were measured from urine using a validated ultra-performance liquid chromatography and tandem mass spectrometry on the Biomek 3000 deck (Beckman Coulter, Brea, CA) [18]. 8-oxodG and 8-oxoGuo were normalized against the urinary creatinine concentration. The creatinine concentrations were quantified by using the method of Jaffé [19].

Glucose metabolism

In order to describe different aspects of the glucose metabolism, surrogate measures were obtained from

Table 1. Characteristics of the overweight/obese and lean group.

	Overweight/obese	Lean	*p* Value
Sex, Boys/Girls	18/24	16/19	0.81
Age, years	12.9 (7.2–17.6)	11.4 (7.4–17.8)	0.21
BMI SDS	2.86 (1.30–4.09)	0.14 (−0.59 to 1.18)	<0.0001
Waist for height ratio	0.58 (0.47–0.79)	0.45 (0.37–0.52)	<0.0001
HbA1c, mmol/mol/%	34.0/5,3% (28.0–44.0)/(4.7–6.2%)	34.5/5,4% (27.0–39.0)/(4.6–5.7%)	0.40
Plasma glucose $t = 0$, mmol/L	5.2 (4.3–6.7)	5.1 (4.2–5.9)	0.55
Plasma glucose $t = 120$, mmol/L	6.6 (5.3–10.1)	6.0 (3.9–9.7)	<0.0001
WBISI	8.5 (1.9–40.1)	11.5 (6.3–29.9)	0.006
Insulinogenic index	207.7 (87.8–447.5)	144.9 (34.1–433)	0.007
Disposition index	1705 (388–5681)	1591 (1038–12,920)	0.65
8-oxo Guo level (RNA), nmol/mmol creatinine	2.11 (1.04–4.35)	1.99 (0.93-–3.15)	0.90
8-oxodG level (DNA), nmol/mmol creatinine	1.34 (0.54–2.45)	1.42 (0.55–3.04)	0.73

Values are median with (range). BMI SDS, body mass index standard deviation score; WBISI, whole body insulin sensitivity.

the OGTT. Fasting plasma glucose described the steady state, plasma glucose after 2 h demonstrated the glucose metabolism after stress. The peripheral insulin sensitivity was estimated by the whole body insulin sensitivity index (WBISI) calculated by the Matsuda and DeFronzo formulae [20], β-cell function was estimated by the insulinogenic index (IGI) [21], and the β-cell capacity was estimated by the oral disposition index (DI) [22] using the concentrations of plasma glucose and serum insulin during fasting and after 120 min (2 h glucose) of the OGTT. The β-cell capacity was evaluated with the oral DI as the product of the IGI and the WBISI. The oral DI has been validated in adults during OGTT, elaborating the relationship between the β-cell function and the peripheral insulin sensitivity [22]. Impaired glucose tolerance (IGT) was, according to the International Society for Pediatric and Adolescent Diabetes (ISPAD) guidelines, classified as 2 h glucose levels of 7.8–11.0 mmol/L during the OGTT [23].

Data analysis

R version 3.1.2 (https://cran.r-project.org) was used as statistical software. The Wilcoxon rank sum test was used to compare the lean and overweight/obese children. We used linear regression analyses to investigate the associations between the concentrations of the urinary markers and the degree of obesity and the glucose metabolism. Sub-analyses adjusted for age, sex and degree of obesity were used to investigate associations between the urinary marker and the glucose metabolism in children exhibiting IGT. A p value <0.05 was considered statistically significant.

Results

The baseline characteristics of the 42 (24 girls) obese and 35 lean (19 girls) children are shown in Table 1.

Glucose metabolism

No significant differences between the lean controls and the overweight group were observed in baseline concentrations of fasting plasma glucose ($p = 0.55$) or HbA1c ($p = 0.40$) (Table 1).

Oral glucose tolerance test

Compared to the lean controls, the overweight group exhibited a higher IGI ($p = 0.007$) and a lower WBISI ($p = 0.006$) (Table 1). However, no significant difference in DI between the two groups was observed ($p = 0.65$) (Table 1). Twelve (10 females) overweight/obese children and adolescents exhibited impaired glucose tolerance (IGT).

Urinary markers

Across the overweight/obese and lean children and adolescents, linear regression did not show any significant associations between BMI SDS and the urinary 8-oxoGuo ($p = 0.51$) or 8-oxodG ($p = 0.50$); nor in the overweight group ($p = 0.90$ and $p = 0.32$) or the lean group ($p = 0.52$ and $p = 0.43$), respectively.

No significant associations between the 8-oxoGuo and the 2 h glucose ($p = 0.08$), IGI ($p = 0.33$), WBISI ($p = 0.47$), DI ($p = 0.22$) were observed across the overweight/obese and lean children. Likewise, the urinary 8-oxodG did not correlate with neither WBISI ($p = 0.82$), IGI ($p = 0.38$), DI ($p = 0.09$), nor the 2 h glucose ($p = 0.54$).

When analyzing the subgroup of overweight/obese children exhibiting IGT, a multiple linear regression model, adjusted for BMI SDS, age and sex, showed that urinary RNA marker 8-oxoGuo was positively correlated with the 2 h glucose concentration (multiple R-squared (r^2) = 0.63, $p = 0.02$) (Figure 1). The same correlation was demonstrated between The DNA urinary marker 8-oxodG and 2 h glucose ($r^2 = 0.48$, $p = 0.046$) (Figure 2).

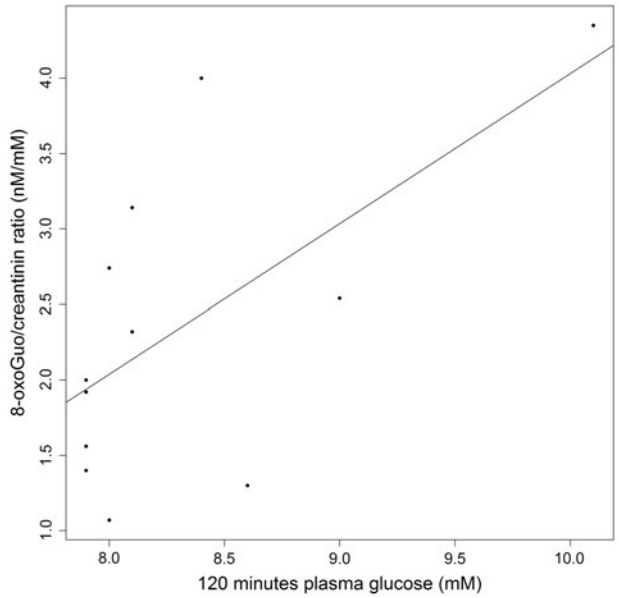

Figure 1. Association between the urinary RNA-marker and 2-h glucose. The correlation between urinary 8-oxo-7,8-dihydroguanosine and 2-h glucose in the 12 obese children and adolescents exhibiting IGT ($p = 0.02$, r^2=0.63).

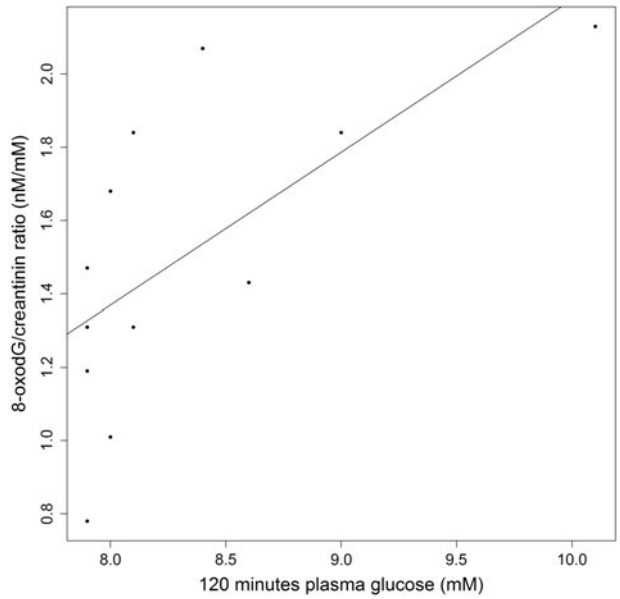

Figure 2. Association between the urinary DNA marker and 2-h glucose. The correlation between urinary 8-oxo-7,8-2′-deoxyguanosine and 2-h glucose in the 12 obese children and adolescents exhibiting IGT ($p = 0.046$, r^2=0.48).

Sub-analyses in this subgroup also demonstrated a positive association between the 8-oxoGuo and the IGI ($r^2 = 0.60$, $p = 0.03$) (Figure 3).

Discussion

Across overweight and lean children, we did not demonstrate any association between the excretion of

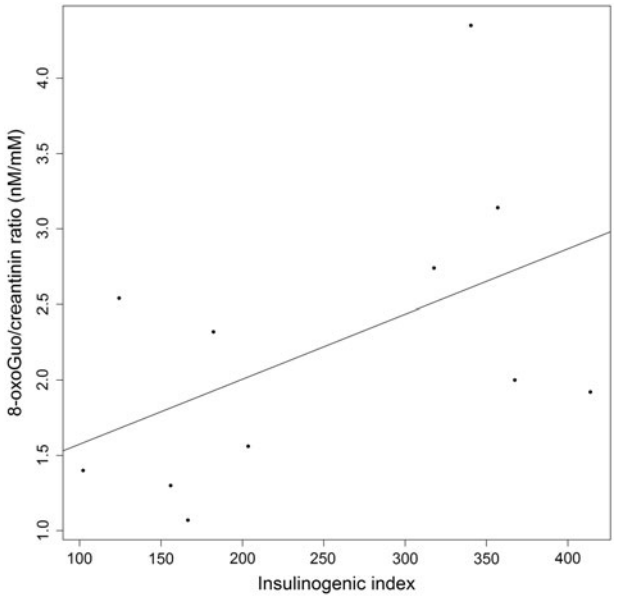

Figure 3. Association between the urinary RNA-marker and IGI. The correlation between urinary 8-oxo-7,8-dihydroguanosine and IGI in the 12 obese children and adolescents exhibiting IGT ($p = 0.03$, r^2=0.60).

urinary marker of RNA or DNA nucleic acid oxidation and the degree of obesity. When separately analyzing children exhibiting IGT, we observed a positive association between the concentrations of the urinary markers with the level of 2 h glucose, independent of age, sex, and the degree of obesity, suggesting that impaired glucose metabolism is linked to an increased oxidative stress level in children. Furthermore, the present study also assessed a relationship between the concentration of the RNA urinary marker and the surrogate measure of β-cell function, expressed by IGI. There were no further associations reported between the concentrations of the urinary markers and the other surrogate measures of the glucose metabolism in the obese and lean children and adolescents.

The correlations between an impaired glucose tolerance and a higher excretion of 8-oxodG in children are in line with other reports in adults diagnosed with type 2 diabetes describing positive associations between the levels of 8-oxodG and hyperglycemia and HbA1c [9,10]. Independent of known conventional risk factors, the urinary marker 8-oxoGuo has also been demonstrated to be a predictor of mortality in 970 adults diagnosed and treated with type 2 diabetes, as well as changes in the concentration of 8-oxoGuo during the first 6 years after diagnosis are associated with mortality [11,12].

In the present study, the degree of childhood obesity *per se* was not associated with an increased oxidative stress by this urine evaluation, albeit the results might indicate that intermittent high glucose level associate with oxidatively generated RNA and DNA damage.

This explanation is supported by other reports who demonstrate how intermittent high glucose levels as well as chronic hyperglycemia can induce generation of ROS by activation of the NAD(P)H oxidase [24]. In adults diagnosed with type 2 diabetes, acute glycemic fluctuation has been reported to have a more triggering effect on oxidative stress than chronic sustained hyperglycemia [25]. The intermittent high glucose level might possibly relate to a higher degree of mitochondrial dysfunction in the children with IGT, hypothesized to be an important culprit in diabetes pathogenesis [8,26]. *In vitro* studies in different cell lines (from patients diagnosed with type 2 diabetes) exposed to high glucose concentrations have shown to increase the level of oxidative damaged DNA [27–29]. As examples, increased levels of 8-oxodG have been detected in islets of pancreatic β-cells in type 2 diabetic humans [29], in the vitreous humour of adults with diabetic retinopathy [28] and in human endothelial cells [27]. In our study, the children with overweight or obesity had HbA1c concentrations in the normal range. HbA1c is used not only as diagnostic criteria, but also as an important treatment marker in adults and children with diabetes, because it reflects the average long-standing glucose level. However, in children with overweight or obesity exhibiting IGT, there is a discrepancy regarding the level of HbA1c. Several studies have demonstrated HbA1c to be normal or near-normal in their daily lives and only when the children are challenged with an OGTT, they become hyperglycemic [23]. Due to the low sensitivity and specificity of the Hba1c in this particular group of individuals, conflicting views on whether this measurement should be used alone in the screening for impaired glucose metabolism in children and adolescents with overweight or obesity have arisen [30–32].

Although we did demonstrate a positive association between the excretion of the urinary oxidative markers and the glucose metabolism, further investigations, in larger study samples of overweight children and youths exhibiting impaired glucose tolerance, are needed to elucidate the associations between the glucose metabolism and the excretion of nucleic acid oxidations markers in urine.

The pathophysiological mechanisms behind the development of diabetes and its medical complications remain unclear. Defects in the oxidative phosphorylation system in the mitochondria leading to oxidatively generated damage to DNA and RNA have been demonstrated in obese adults and patients with type 2 diabetes [33–35]. These mitochondrial dysfunctions have been suggested to play a pivotal role in the biochemical abnormalities leading to insulin resistance, β-cell dysfunction, and vascular complications in diabetes [26].

In addition, genetic studies have shown reduced expressions of oxidative phosphorylation genes in family members of persons with type 2 diabetes and in adults patients with impaired glucose tolerance [33,35].

Associations between the urinary markers and different forms of chronic disease have been reported in the pediatric population. In very low birth-weight infants, 8-oxodG associated with weight, mental development, and the development of bronchopulmonary dysplasia [28,29] and in studies of children, the level of 8-oxodG correlated with type 1 diabetes, celiac disease, and urinary tract infection with renal complications [13,14,36].

The knowledge and use of these urinary markers as potential biomarkers to provide additional information for progression or effect of treatment in different diseases is becoming more evident independent of age and conventional risk factors in adults [8,13,14,36,37].

Among the limitations of the present study, we did not adjust for multiple hypothesis testing. Nevertheless, the numbers of tests for association were planned beforehand according to our a priori hypotheses. Another limitation is that we used spot urinary samples to study the excretion of nucleic acid oxidation instead of a 24-hour collection of urine, which is the gold standard. However, we found the spot urine sample acceptable since the diurnal variation in 8-oxodG excretion is relatively small [38].

In conclusion, excretion of the urinary markers of nucleic acid oxidation and the degree of obesity or the glucose metabolism were not significant associated in this study. However, obese children exhibiting impaired glucose seems to have a correlation between the urinary marker and the glucose tolerance, but further investigations are required to elucidate this correlation.

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Disclosure statement

All authors declare that there are no conflicts of interest. This study is part of the research activities in TARGET (The impact of our genomes on individual treatment response in obese children; http://metabol.ku.dk/research-project-sites/target/) and BIOCHILD (Genetics and systems biology of childhood obesity in India and Denmark; http://biochild.ku.dk/).

In addition, this study is part of The Danish Childhood Obesity Biobank.

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