

Oxidative stress parameters in paediatric non-alcoholic fatty liver disease

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Abstract. We have investigated the presence and the possible clinical implications of oxidative stress in children with non-alcoholic fatty liver disease (NAFLD). The present study was an observational study of oxidative stress parameters in the progression of paediatric NAFLD. We observed the role of oxidative stress in children diagnosed with NAFLD by evaluating: serum protein carbonyls, hepatic expression of 8-hydroxy-2-deoxyguanosine (8-OHG), and circulating antibody against malondialdehyde adducted human serum albumin (MDA-HSA). Forty consecutive children with biopsy-proven NAFLD (27 male; 13 female) referred to Bambino Gesù Children's Hospital, Rome, Italy, from January 2007 to April 2008 were included in the study. Serum variations of protein carbonyls, 8-OHG, and circulating antibody against MDA-HSA were evaluated. Elevated protein carbonyls were evident in 33 subjects (83%) irrespective of obesity and insulin resistance. Moreover, liver biopsies of NAFLD patients positive for circulating protein carbonyls also showed a significant increase in the nuclear staining for 8-OHG ($p=0.006$; 95% CI 3.1-17.7). Anti-MDA-HSA IgG above control threshold was detected in 25 (63%) children. Although protein carbonyl levels were unrelated with disease severity, patients with elevated anti-MDA-HSA IgG had scores for lobular inflammation significantly higher ($p=0.019$) than subjects with antibodies within the control range, while steatosis, hepatocyte ballooning and fibrosis were similar. High anti-MDA-HSA reactivity was also associated with a 13-fold increased risk (OR=12.9; 95% CI

1.5-113.8; $p=0.013$) of a NAFLD activity score (NAS) ≥ 5 . These results demonstrate that oxidative stress has an high prevalence in children with NAFLD and is associated with an increased severity of steatohepatitis.

Introduction

Paediatric non-alcoholic fatty liver disease (NAFLD) has become the most frequent chronic liver disease in children and adolescents of industrialized countries due to the growing prevalence of childhood obesity and overweight (1-3). Presently, NAFLD prevalence among children and adolescents ranges between 2.6-9.8% and is especially high among obese subjects. Such a high prevalence of NAFLD and the likelihood of evolution to cirrhosis warrant increased attention toward this disease (4,5). NAFLD progression toward hepatic fibrosis and cirrhosis depends upon the combination of necro-inflammation and increased fibrogenesis that characterize non-alcoholic steatohepatitis (NASH). In addition, obesity and older age are independently associated with more advanced fibrosis (6). Thus, a key issue in unravelling the pathogenesis of NASH is the characterization of the mechanisms promoting necro-inflammation and healing responses in fatty livers.

Increasing evidence from experimental models of NAFLD/NASH suggests that oxidative stress plays a key role in mechanisms leading to the death of fat-laden hepatocytes and contributes to activating hepatic stellate cells to matrix-producing myofibroblasts. Moreover, redox changes are responsible for increasing Kupffer cell response to pro-inflammatory stimuli (7,8). Accordingly, antioxidant supplementation reduces liver injury in experimental rodent models of NASH (9,10). The relevance of these observations in humans is supported by several studies showing increased liver and serum content of oxidative stress markers, such as lipid peroxidation end-products, 8-hydroxy-2-deoxyguanosine (8-OHG), and nitrotyrosine in adult patients with NAFLD/NASH (11-13). Moreover, microarray analysis of liver biopsies from patients with NASH demonstrated lower mRNA expression of different antioxidant enzymes (14). However, information regarding the involvement of oxidative stress in paediatric NAFLD has been, to date, quite scattered (2). This is

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an important limitation considering that antioxidant supplementation is under evaluation for the therapy of paediatric NAFLD (15,16).

In the present study we investigated the presence and the possible clinical implications of oxidative stress in children with NAFLD/NASH.

Patients and methods

Patients. A total of 40 consecutive patients diagnosed with NAFLD (27 male; 13 female) referred to Bambino Gesù Children's Hospital, Rome, Italy, from January 2007 to April 2008 were included in the study. Inclusion criteria were, persistently elevated serum aminotransferase levels, diffusely echogenic liver or imaging studies suggestive of fatty liver and biopsy consistent with the diagnosis of NAFLD (15-17). Exclusion criteria were hepatic virus infections (Hepatitis A, B, C, D, E and G, cytomegalovirus and Epstein-Barr virus), alcohol consumption, history of parenteral nutrition, and use of drugs known to induce steatosis (e.g. valproate, amiodarone or prednisone) or to affect body weight and carbohydrate metabolism. Autoimmune and metabolic liver disease, Wilson's disease, and α -1-antitrypsin-associated liver disease were ruled out using standard clinical, laboratory and histological criteria. Informed consent was obtained from each patient or responsible guardian. The study was approved by the Ethics Committee of the Bambino Gesù Children's Hospital and Research Institute, Rome, Italy.

The body mass index (BMI) and BMI Z-score were calculated according to established criteria (18,19). Metabolic syndrome was defined by the presence of three or more of the following five criteria: abdominal obesity (defined by waist circumference \geq 90th percentile for age), hypertriglyceridemia (triglycerides $>$ 95th percentile for age, gender and race), low HDL cholesterol (HDL cholesterol $<$ 5th percentile for age and gender), elevated blood pressure (defined as systolic or diastolic blood pressure $>$ 95th percentile for age and gender), and impaired fasting glucose or known type 2 diabetes mellitus.

A 2-h oral glucose tolerance test (OGTT) was performed with the standard 1.75 g of glucose per kg of body weight, or a maximum of 75 g. Glucose tolerance status was determined according to the classification of the American Diabetes Association in which fasting plasma glucose levels up to 99 mg/dl are considered normal; impaired fasting glucose (IFG) is defined by a fasting plasma glucose of 100-125 mg/dl; impaired glucose tolerance (IGT) is defined by a 2-h plasma glucose of 140-199 mg/dl; diabetes mellitus is defined by a fasting plasma glucose \geq 126 mg/dl, or a 2-h plasma glucose \geq 200 mg/dl (20,21). Plasma glucose was measured in triplicate by the glucose oxidase technique on a Beckman glucose analyzer (Beckman, Fullerton, CA) and plasma insulin was measured by a specific radioimmunoassay (Myria Technogenetics, Milan, Italy). The degrees of insulin resistance and sensitivity were determined, respectively, by the homeostatic model assessment insulin resistance (HOMA-IR) using the formula: $IR = (\text{insulin} \times \text{glucose}) / 22.5$; and by the insulin sensitivity index (ISI) derived from OGTT using the formula: $ISI = [10,000 / \text{square root of } (\text{fasting glucose} \times \text{fasting insulin}) \times (\text{mean glucose} \times \text{mean insulin during OGTT})]$ (22,23).

Liver histology. Liver biopsy was performed in all NAFLD children, after an overnight fast, using an automatic core biopsy 18 Gauge needle (Biopince, Amedic, Sweden) under general anaesthesia and ultrasound guidance. A Sonoline Omnia Ultrasound machine (Siemens, Germany) with a 5-MHz probe (5.0 C 50, Siemens) was employed. Two biopsy passes within different liver segments were performed for each subject. The length of the liver specimen (in mm) was recorded. Only samples with a length \geq 15 mm and including at least 10-11 complete portal tracts were considered adequate for the purpose of the study (24). Biopsies were evaluated by a single liver pathologist. Sections of liver tissue, 5 μ m thick, were stained with hematoxylin-eosin, Van Gieson, PAS-D, and Prussian blue staining. Immunohistochemical staining with antibodies against α -1-anti-trypsin was used to exclude α -1-anti-trypsin deficiency-associated liver disease. Liver biopsy features were graded according to the NAFLD activity scoring (NAS) system proposed by Kleiner *et al.* (25). Briefly, grade of steatosis was scored as 0, $<$ 5%; 1, 5-33%; 2, $>$ 33-66%; 3, $>$ 66%; grade of lobular inflammation was scored as 0, no foci; 1, $<$ 2 foci/x200 field; 2, 2-4 foci/x200 field; 3, $>$ 4 foci/x200 field; and grade of ballooning was scored as 0, none; 1, few ballooning cells; 2, many cells/prominent ballooning. The grade of steatosis (0-3), lobular inflammation (0-3), and ballooning (0-2) were then combined to determine the NAFLD activity score (0-8). Fibrosis was scored as 0, none; 1, periportal or perisinusoidal fibrosis; 2, perisinusoidal and portal/periportal fibrosis; 3, bridging fibrosis; and 4, cirrhosis. Fibrosis score for stage 1 was extended to include a distinction between delicate perisinusoidal fibrosis (stage 1A), dense perisinusoidal fibrosis (stage 1B), and portal-only fibrosis without perisinusoidal fibrosis (stage 1C) (25).

Assessment of oxidative stress in NAFLD children. Serum levels of protein carbonyls were measured by reactions with 2,4 dinitrophenylhydrazine using the Protein Carbonyls Assay Kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the manufacturer's instructions. Serum samples from 18 normal-weight children matched for gender and age and without echographic evidence of fatty liver undergoing routine surgery were used as controls. Threshold values were calculated as 95th percentile in the controls. Oxidative injury was confirmed by the immunohistochemical detection of 8-OHG in paraffin-embedded liver biopsies using a monoclonal anti-8-OHG antibody (Japanese Aging Control Institute, Shizuoka, Japan) (final dilution 5 μ g/ml) as previously described (26). The percentage of stained hepatocyte nuclei was evaluated in a blinded manner in ten different microscopical fields.

Measurement of antibody titres against malondialdehyde-derived adducts. MDA adducted with human serum albumin (MDA-HSA) was prepared by reacting for 2 h at 37°C 2 mg/ml HSA with 100 mmol/l MDA as previously reported (27). For the evaluation of immune response induced by oxidative stress polystyrene microwell plates for enzyme-linked immunosorbent assay (ELISA) (Nunc-Immuno Maxi-Sorb, Nunc, S/A, Roskilde, Denmark) were coated for 4 h at 37°C with 0.05 mg/ml of either modified or native HSA solubilised in

Table I. Clinical and biochemical characterization of NAFLD children investigated.

Parameters	Patients	
Patients (male/female)	40.0	(27/13)
Age (years)	11.57	(10.04-12.89)
BMI	24.6	(22.7-28.9)
HOMA-IR (n.v. <3)	2.18	(1.61-3.06)
ISI (n.v. >6)	4.21	(3.13-5.70)
AST (U/l - n.v. 5-40)	60.0	(45.24-78.0)
ALT (U/l - n.v. 5-40)	44.0	(32.0-54.50)
γ -GT (U/l - n.v. 5-45)	18.5	(15.75-34.25)
Fasting glucose (mg/dl - n.v. <100)	83.0	(76.0-91.25)
Cholesterol (mg/dl - n.v. <200)	149.5	(130-176)
Triglycerides (mg/dl - n.v. <160)	80.0	(59.0-99.25)
Steatosis score	2.0	(1-3)
Inflammation score	1.0	(0-3)
Ballooning score	0.0	(0-2)
Fibrosis score	1.0	(0-3)
NAS score	4.0	(1-6)

Values are expressed as median and inter-quartile range (IQR). For histological scores the range of variability is included. BMI, body mass index; AST, alanine aminotransferase; ALT, aspartate aminotransferase; γ -GT, γ -glutamyl transpeptidase; HOMA-IR, homeostatic model assessment-insulin resistance; ISI, insulin sensitivity index; n.v., normal values; NAS, NAFLD activity score.

0.1 M bicarbonate buffer, pH 9.6. After incubation, solutions were removed and replaced by 0.3 ml of coating buffer containing 3% bovine serum albumin (BSA) in phosphate buffered saline (PBS), pH 7.4. Plates were further incubated for 1 h at 37°C to block non-specific binding sites. Coated wells were washed three times with PBS containing 0.25% Triton X-100. Human sera (0.20 ml, dilution 1:50 in the coating buffer) were added in duplicate and incubated for 1 h at 37°C. After washing three times with PBS-0.25% Triton X-100, antibody binding was revealed using peroxidase-linked goat anti-human IgG (dilution 1:6,000) (Dako S.P.A., Milano, Italy) as previously described (27). The results were expressed by subtracting background reactivity from unmodified HSA. Threshold values were calculated as 95th percentile in the control population.

Data analysis and statistical calculations. Statistical analyses were performed by SPSS statistical software (SPSS Inc. Chicago IL, USA) using one-way ANOVA or Kruskal-Wallis test for non-parametric values. Confidence intervals were calculated using the CIA software (by T. Bryant, University of Southampton, UK). Relative risk and Fisher's Exact Tests were used for the comparison of frequency data. Significance was taken at the 5% level. The independent effect of significant variables was assessed using stepwise logistic regression analysis. Normality distribution was preliminary assessed by the Kolmogorov-Smirnov and the Shapiro-Wilk tests and corrections were performed by logarithmic transformation.

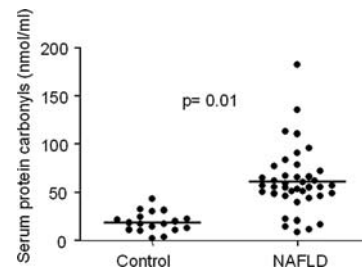


Figure 1. Individual levels of serum protein carbonyls in 40 children with NAFLD and 18 age and gender matched controls. Horizontal bars represent medians.

Results

For this study we enrolled 40 paediatric biopsy-proven NAFLD patients (27 male; 13 female), mean age 11.5 years (range, 5.8-19.1 years). The major clinical and biochemical parameters are reported in Table I. Obesity was present in 25 subjects (62%), while insulin resistance, as estimated by HOMA-IR and ISI indexes, was detected in 30 subjects (75%). Hypercholesterolemia and hypertriglyceridemia were observed in 1 patient (3%), respectively. The presence of NAFLD was confirmed histopathologically in all subjects. Extensive steatosis (>66%) was evident in 16 subjects (40%). Twenty-six patients (65%) showed mild-moderate peri-sinusoidal or periportal fibrosis, 1 (3%) had perisinusoidal and portal/periportal fibrosis and 3 (8%) bridging fibrosis.

Serum protein carbonyls were significantly increased in NAFLD patients (61.0 ± 33.4 vs 20.7 ± 13.8 nmol/ml; $p < 0.0001$ 95% CI 27.7-52.8) as compared to 18 gender- and age-matched healthy controls (Fig. 1). Furthermore, protein carbonyl values above the control threshold were evident in 33 subjects (83%). Protein carbonyl levels were unrelated with age, gender, obesity, insulin resistance, circulating triglyceride and glucose or the biochemical or histopathological severity of the disease (data not shown). Immunohistochemistry 8-OHG, a marker of oxidative DNA damage confirmed the liver specificity of oxidative damage, as 8-OHG nuclear staining was significantly higher ($p = 0.006$; 95% CI 3.1-17.7) in liver biopsies from NAFLD patients positive for circulating protein carbonyls than in those who were negative (Fig. 2).

Recent data from a rodent model of NAFLD indicate that hepatic oxidative stress leads to the development of an antibody response against protein modified by the reaction with end-products of lipid peroxidation, namely malondialdehyde (MDA) (10). Similar antibodies have also been detected in adult NAFLD patients and are an independent predictor for advanced fibrosis (28). These observations prompted us to investigate whether antibodies against MDA-derived antigens were associated with paediatric NAFLD. As shown in Fig. 2, 25 out of 40 NAFLD patients (63%) had circulating IgG against MDA adducts with human serum albumin (MDA-HSA) above the control threshold. The presence of anti-MDA-HSA IgG was not influenced by age, gender, obesity, insulin resistance, aminotransferase or GGT release (data not shown). However, at the histology, patients with elevated anti-MDA-HSA antibodies showed that scores of lobular inflammation were significantly higher ($p = 0.019$) than subjects with antibodies within control range (Fig. 3). Lobular

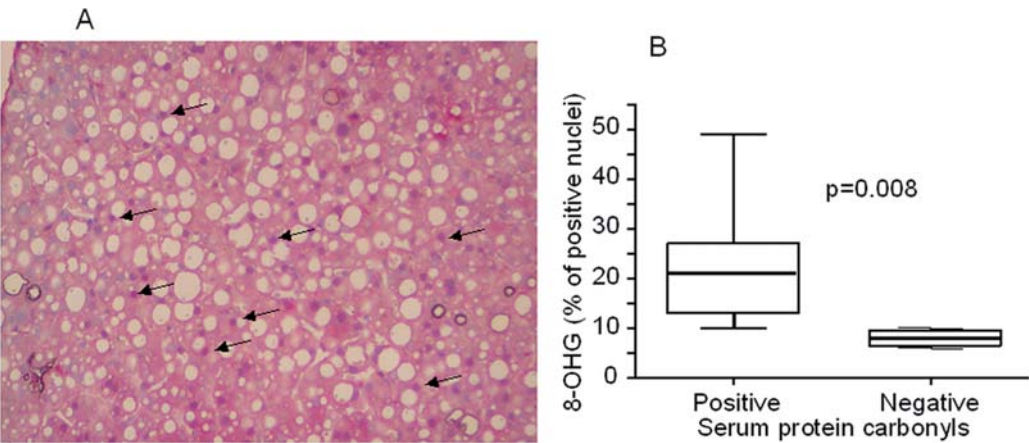


Figure 2. Immunohistochemical detection of 8-hydroxydeoxyguanosine (8-OHG) in liver biopsies form NAFLD children. (A) Predominant localization of 8-OHG in the nuclei of parenchymal cells is indicated by arrows. (B) Difference in the percentage of 8-OHG-positive nuclei in liver biopsies from NAFLD children with or without increased circulating protein carbonyls. Boxes include values within 25th and 75th percentile, while horizontal bars represent medians. Eighty percent of the values are comprised between the extremities of the vertical bars (10th-90th percentile).



Figure 3. Distribution of the individual IgG reactivity against malondialdehyde-modified human serum albumin (MDA-HSA) among 40 children with NAFLD. Results are expressed as optical density (o.d.) at 490 nm, as reported in Patients and methods. Horizontal bars represent threshold values calculated at the 95th percentile in a control population.

Figure 5. Distribution of the individual values of NAFLD activity score (NAS) among 40 children with NAFLD sub-grouped according to the presence (Anti-MDA IgG+) or absence (Anti-MDA IgG-) of elevated IgG reactivity against malondialdehyde-modified human serum albumin. Horizontal bars represent median.

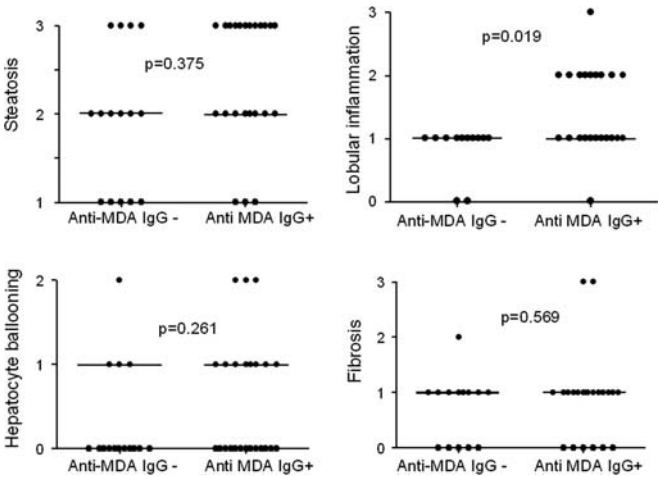


Figure 4. Distribution of the individual histological score for steatosis, lobular inflammation, hepatocyte ballooning and fibrosis among 40 children with NAFLD sub-grouped according to the presence (Anti-MDA IgG+) or absence (Anti-MDA IgG-) of elevated IgG reactivity against malondialdehyde-modified human serum albumin. Horizontal bars represent medians.

inflammation score >1 was also prevalent among NAFLD children with elevated anti-MDA-HSA IgG (0% vs 40%; $p=0.006$; 95% CI 1.2-2.3). No significant differences were observed in the extension of steatosis, frequency of hepatocyte ballooning or severity of fibrosis (Fig. 4). NAFLD activity score (NAS) was higher (median 4; range 1-6) in NAFLD patients with anti-MDA-HSA antibodies as compared to the remaining patients (median 3; range 2-6; $p=0.006$) (Fig. 5). Moreover, a NAS score ≥ 5 , considered indicative of overt NASH, 25 was 13-fold more frequent (OR= 12.9; 95%CI 1.5-113.8; $p=0.013$) among subjects with elevated anti-MDA-HSA IgG.

Discussion

Several studies have implicated oxidative stress in adult NAFLD/NASH (11-13), however, very little is known about the involvement of oxidative damage in paediatric NAFLD (2). Our observations demonstrate that a high proportion (83%) of children with NAFLD show signs of oxidative injury, as evaluated by elevated circulating levels of protein carbonyls. Protein-bound carbonyls arise from free radical-mediated

protein oxidation and are a widely used generic marker of oxidative stress (29). Although protein carbonyls can also originate from protein glycoxidation, elevated serum protein carbonyls are unrelated to glycemia or insulin resistance, indicating that they are expression of oxidative injury (29). Children with elevated serum protein carbonyls also show an increased hepatocyte nuclear staining for 8-OHG, a marker of oxidative DNA damage. Interestingly, 8-OHG presence in parenchymal cells along with cytosolic accumulation of the lipid peroxidation product 4-hydroxynonenal have been documented in adult NASH (11). Thus, circulating protein carbonyls in NAFLD children likely reflect hepatic oxidative injury consequent to fat accumulation. Indeed, mitochondrial alterations caused by hepatocyte free fatty acid overload along with the induction of cytochrome P450 2E1 (CYP2E1) are regarded as the main causes for oxidative injury in NAFLD (8). In agreement with such an interpretation, previous reports have shown that paediatric NAFLD is characterized by a decrease in the plasma antioxidant content and by an abnormal glutathione metabolism (30-32).

In a significant fraction of NAFLD children (63%) hepatic oxidative injury is associated with the development of antibodies towards protein adducted by malondialdehyde (MDA). Similar antibodies have also been observed in ~40% of adult NAFLD patients, as well as in subjects with advanced alcoholic liver disease (ALD) and chronic hepatitis C, two other liver diseases characterized by the involvement of oxidative stress (26,27,33-36). Moreover, ~35% of ALD patients also have circulating CD4⁺ T-lymphocytes recognizing MDA adducts, indicating that oxidative mechanisms can promote both humoral and cellular immune responses (37).

At present the mechanisms responsible for oxidative stress-driven immunity in NAFLD are still poorly understood. The phagocytosis of oxidatively-modified hepatic proteins by dendritic cells in intraportal lymphoid follicles might lead to the priming of naïve B and T cells (38). This process is likely facilitated by the capacity of the scavenger receptors and of some pattern recognition receptors to recognize oxidatively-modified proteins and lipids (39). An additional factor in the development of immune response in NAFLD may involve the impairment of immuno-regulatory mechanisms, such as steatosis and oxidative stress lower hepatic regulatory T cells (Tregs) in mice fed a high fat diet (40). Indeed, Treg lowering is now regarded as an important event in promoting lymphocyte activation against oxidized LDL during the evolution of atherosclerosis (41).

Previous studies have shown that the intra-hepatic detection of lipid peroxidation products is prevalent in patients with NASH and correlates with necro-inflammation (11-13). Several lines of evidence in ALD indicate the involvement of immunological mechanisms in fuelling hepatic inflammation during the progression of the disease (27). In particular, we have observed that heavy drinkers with high circulating levels of oxidative stress-induced antibodies have a 5-fold higher prevalence of elevated plasma TNF- α levels and 11-fold greater risk of advanced ALD than heavy drinkers with these antibodies within the control range (26). In the present study, NAFLD children with immune responses against MDA-derived antigens show more severe lobular inflammation and have a 13-fold higher prevalence of overt NASH (NAS score

≥ 5). Conversely, no association is evident between elevated serum protein carbonyls and liver histology. Altogether these results suggest that the activation of adaptive immunity against defined oxidative stress-derived antigens contributes to hepatic inflammation in pediatric NAFLD. In contrast to adult NAFLD, we did not observe any significant association between the presence of anti-MDA-HSA antibodies and the severity of fibrosis. This might be due to the low prevalence of extensive fibrosis in the population sample investigated (27).

In conclusion, these results demonstrate that oxidative stress is present in a high proportion of NAFLD children and leads to the development of lipid peroxidation-related antibodies that are associated with elevated NAS scores. If confirmed by prospective studies, the evaluation of IgG reactivity towards MDA adducts may become a useful marker for the identification of NAFLD children at risk of disease progression who might benefit from targeted antioxidant therapy.

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