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Oxidative Stress in Subjects Affected by Celiac Disease

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In order to study the role of oxidative stress in celiac disease, protein carbonyl groups, thiobarbituric acid-reactive substance and pentosidine were evaluated in the plasma of nine patients with asymptomatic celiac disease and in a control group ($n = 25$). Plasma alpha-tocopherol, retinol and lipids were determined in the same samples. The levels of markers of oxidative stress derived from both protein (carbonyl groups) and lipids (thiobarbituric acid-reactive substances) were significantly higher in celiac disease patients, whereas lipoproteins and alpha-tocopherol were significantly lower. These data indicate that in celiac disease, even when asymptomatic, a redox imbalance persists; this is probably caused by an absorption deficiency, even if slight. Dietary supplementation with antioxidant molecules may offer some benefit and deserves further investigation.

Keywords: Celiac disease, oxidative stress, antioxidant vitamins, carbonyl group, TBARS, pentosidine

Abbreviations: TBARS, thiobarbituric acid-reactive substance; HPLC, high pressure liquid chromatography

INTRODUCTION

It has been reported that increased oxidative stress is responsible for cell damage in several diseases,[1–3] including diabetes mellitus, uremia, ischemic cardiac disease and many others. The source of reactive oxygen species may be endogenous or exogenous;[4] the increased production of free radicals leads to an unbalanced redox equilibrium; conversely, the reduced uptake of antioxidant molecules and an impaired production of antioxidant enzymes are linked to oxidative injury.[5,6] Indeed, diet provides the micronutrients essential for maintenance of an optimal antioxidant counterbalance.[7]

Recent studies have reported that malabsorption is associated with a low plasma antioxidant status and higher levels of markers of oxidative damage in plasma proteins.[8,9] The aim of the present study was to establish whether a deficiency of defense against oxidative stress is also present in celiac disease and whether markers of this imbalance are existing in plasma.

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SUBJECTS AND METHODS

Venous plasma samples were obtained from nine patients (mean age 23 ± 1 years; range 16–28 years, 4 men and 5 women) affected by celiac disease and from 25 age-matched controls (mean age 24 ± 1 years; range 18–30 years, 10 men and 15 women). Samples were collected in the morning (7.00–9.00 a.m.) in tubes containing EDTA as anticoagulant and stored at −20°C until determination (within 8 weeks). The diagnosis of celiac disease had been made on intestinal biopsy during infancy (age range of diagnosis: 0–3 years). In all subjects further intestinal biopsies were performed at adolescence (11–12 years) and at 15–18 years: in all cases the initial diagnosis was confirmed. In all patients at the time of the study the antigliadin IgG and IgA antibodies titers were less than 1/10 and the IgA antiendomysium antibody level was below the sensitivity of the assay. However increases in serum antigliadin antibody levels have been documented when gluten was included in the diet, and sometimes in patients with low compliance to the free-gluten diet. One patient showed antibody against thyroid peroxidase (TPO), thyroglobulin (TGA) and insular cells (ICA) suggesting a multiple autoimmune endocrinopathy.

None of the subjects studied was diabetic (mean glycated hemoglobin: 4.1 ± 0.2%, range of normality: 2.8–4.7%) or presented acute illness at the time of the present evaluation and all had a body mass within the normal limits, though in the lower range (IBW: 85 ± 2%, range 81–98).

Plasma total cholesterol and plasma triglycerides were determined with enzymatic–colorimetric methods (Cholesterol and GPO-Trinder, Sigma Diagnostics Chem. Co., St. Louis, MO, USA).

Plasma retinol and alpha-tocopherol were measured by high-pressure liquid chromatography (HPLC) according to the procedure of Miller:[10] plasma samples were added to an equal volume of 100% ethanol containing 0.01% of butylated hydroxytoluene and 10 µl of retinyl acetate as internal standard, and to a double volume of hexane; samples were extracted twice. After centrifugation, the hexane layers were evaporated under nitrogen and the residue was resuspended in the 100% mobile phase.

The HPLC system consists of two Wat510 pumps, a U6K injector (Waters, Millipore S.p.A., Milan, Italy) and a computer directed programmable UV wavelength detector (Waters 486); Prodigy (Phenomenex, Labservice, Bologna, Italy) was the reverse-phase analytical column and methanol (100% with 0.01% ammonium acetate) was the eluting solvent (1.2 ml/min). Retinol and retinyl acetate were detected at 320 nm and tocopherol at 290 nm. Samples were handled under dimmed light and, whenever possible, on ice.

Plasma protein content was determined by the bicinchoninic acid method (Pierce Chem. Co., Prodotti Gianni, Milano, Italy). Carbonyl content was evaluated following the 2,4-dinitrophenylhydrazine assay[11] with slight modifications. Briefly, one milligram of plasma proteins was precipitated with 20% trichloroacetic acid (1 : 1 v/v) in Eppendorf tubes and then vortexed (30 s); after centrifugation, the clear supernatant was discarded and the pellet was resuspended in 500 µl of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl and let stand at room temperature for 60 min, vortexing every 10–15 min to facilitate the reaction with proteins. During derivatization, the pellet was submitted to slight sonication (5 bursts of few seconds each) to provide complete resuspension without loss of material.

The proteins were precipitated again with 20% trichloroacetic acid and the pellet washed three times with 1 ml of ethanol : ethyl acetate (1 : 1 v/v). The pellet was dissolved in 0.6 ml 6 M guanidine hydrochloride solution at 37°C.

After centrifugation (6000 × *g*) for 5 min, the samples were read against complementary blank by spectral scanning: the peak was discernible at 365 ± 2 nm.

The samples were processed in triplicate and carbonyl content was calculated in nanomoles

per mg of protein, using an absorption coefficient of 22,000 M^{-1} cm^{-1} at 365 nm.

Protein recovery was checked on the HCl blank pellets using a bovine serum albumin standard curve in guanidine solution read at 280 nm: the recovery was 90 ± 2%.

Thiobarbituric acid-reactive substances (TBARS) were assayed by the method of Young and Trimble.[12] As a standard, pure malondialdehyde (monosodium tri-hydrate) was obtained from 1,1,3,3-tetra-ethoxy-propane by hydrolysis, following the procedure of Nair. [13] The reaction was carried out by mixing 750 µl of phosphoric acid (0.44 M) with 50 µl of sample. Two-hundred-fifty µl of TBA solution (42 mM) was added to the sample and then distilled HPLC-grade water was used to adjust the volume to 1.5 ml. Tubes were capped tightly and placed in hot bath water (100°C) for 60 min; after the incubation, samples were cooled and kept in ice until HPLC analysis was performed.

About ten minutes before injection onto the column, all samples were neutralized with 0.5 ml of methanol–NaOH solution (4.5 ml of NaOH 1 M plus 50 ml HPLC-grade methanol) for protein precipitation; 50 µl of clear supernatant was injected into a 3.9 × 300 mm C18 µBondapak (Waters S.p.A., Milan, Italy); the mobile phase contained 50% methanol and 50% of 25 mM phosphate buffer (pH 6.5); the flow rate was 0.8 ml/min. The detection system (Waters 470 fluorimeter) was set at 532 nm excitation and 553 nm emission. TBARS were calculated on the basis of a MDA standard calibration curve.

The amount of pentosidine contained in plasma was determined in acid hydrolyzed samples by reverse phase HPLC.[14] Briefly, 5 mg of protein was hydrolyzed in 2 ml of HCl 6 N for 16 h at 110°C in borosilicate screw-capped tubes flushed with N_2.

Acid was evaporated in a Modulyo lyophilizer (Edwards Alto Vuoto S.p.A., Milan, Italy) and each sample was reconstituted in water containing 0.01 M heptafluorobutyric acid (Pierce, Prodotti Gianni, Milan, Italy). A volume equivalent to 3 mg of protein was injected into the HPLC apparatus after filtration by 0.45 µm filter (Ultrafree MC, Millipore, Milan, Italy).

Separations were made on 25 cm × 0.46 cm Vydac type 218TP (10 µ) C18 column (Separation Group, Hesperia, CA, USA) with a linear gradient program of 10–17% acetonitrile (Delchimica Scient. Glassware S.r.l., Naples, Italy) from 0 to 22 min, containing water (MilliQ, Millipore, Milan, Italy) and heptafluorobutyric acid as a counterion.

The effluent was scanned by a Waters 470 fluorescent detector; the excitation and emission wavelengths were 335 and 385 nm, respectively. The pentosidine area was collected, dried and resuspended for further separation.

Two mg of the collected sample was injected onto a cation exchange column (SP-5PW Waters) with a mobile phase, characterized by a curvilinear gradient of NaCl from 0 to 0.06 M in sodium acetate 0.02 M (pH 4.7). The fluorimeter was set as previously reported for the reverse-phase column. A pentosidine synthetic standard was injected at the beginning of every run to quantitate the pentosidine level in the sample by peak area comparison.

Data are presented as mean and standard errors (see Figure 1). Comparisons between groups were performed with the Mann–Whitney U test for unpaired groups. Linear regression analysis was performed by non-parametric Spearman test. A two-tailed P value lower than 0.05 was considered to be significant.

RESULTS

In Table I, the results obtained in patients affected by celiac disease, who were asymptomatic at the time of the study, are compared with those of age-matched controls. The group of subjects affected by celiac disease shows higher levels of TBARS (2.03 vs. 1.14 nmol/ml; $U = 52$, $P < 0.05$), carbonyl groups (1.52 vs. 1.02 nmol/mg protein; $U = 4.0$, $P < 0.001$) and pentosidine in plasma proteins

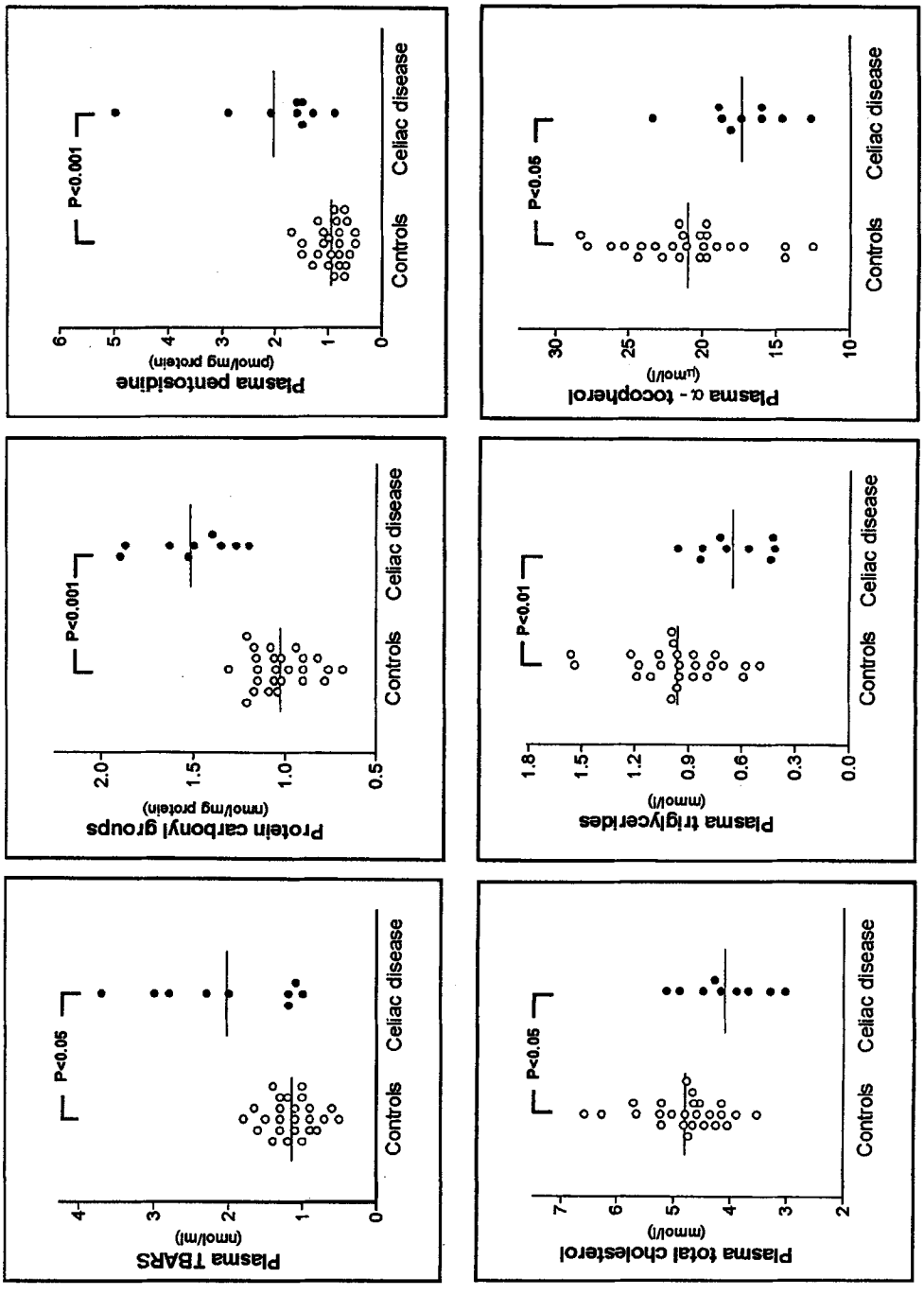

FIGURE 1 Data spread (bar is the mean value) of plasma TBARS, carbonyl groups of protein, plasma pentosidine, plasma total cholesterol, plasma triglycerides and alpha-tocopherol in celiac patients and healthy controls. Statistical significance is reported in the figure.

TABLE I Mean values ± standard error of studied parameters in healthy controls and in patients affected by celiac disease

	Control subjects	Celiac disease
n (m/f)	25 (15/10)	9 (4/5)
Age (years)	24 ± 1	23 ± 1
TBARS (nmol/ml)	1.14 ± 0.06	2.03 ± 0.33*
Carbonyl groups (nmol/mg protein)	1.02 ± 0.03	1.52 ± 0.08‡
Pentosidine (pmol/mg protein)	0.95 ± 0.08	2.04 ± 0.41‡
Total cholesterol (mmol/l)	4.77 ± 0.16	4.07 ± 0.26*
Triglycerides (mmol/l)	0.95 ± 0.6	0.67 ± 0.8†
Plasma protein (mg/ml)	73.6 ± 3.23	70.8 ± 1.50
Retinol (μmol/l)	2.05 ± 0.17	1.68 ± 0.16
Alpha-tocopherol (μmol/l)	21.0 ± 0.8	17.3 ± 1.0*

*$P < 0.05$, †$P < 0.01$, ‡$P < 0.001$.

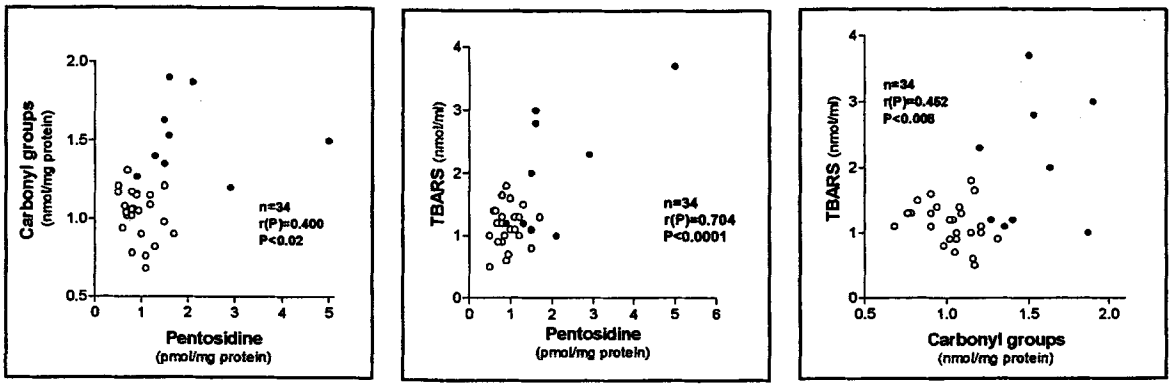

FIGURE 2 Data plot and coefficient of Pearson correlation *r*(*P*) between carbonyl groups of protein, pentosidine and TBARS in celiac disease patients (●) and controls (○) (all $n = 34$).

(2.04 vs. 0.95 pmol/mg protein; $U = 21.5$, $P < 0.001$). Plasma total cholesterol and plasma triglycerides, on the contrary, are lower than in the control group (4.07 vs. 4.77 mmol/l and 0.67 vs. 0.95 mmol/l; $U = 56.5$, $P < 0.05$ and $U = 35.0$, $P < 0.01$ respectively); total plasma proteins, show no substantial difference (71 vs. 74 mg/ml; $U = 86.5$, *P* ns) between the two groups.

Interestingly, retinol (1.68 vs. 2.05 μmol/ml; $U = 85$, *P* ns) and alpha-tocopherol levels are both lower in patients with celiac disease, but only alpha-tocopherol achieves a significant difference (17.3 vs. 21.0 μmol/ml; $U = 47.0$, $P < 0.05$) in comparison with the control group.

Statistically significant relationships (Figure 2) are found between TBARS and pentosidine levels ($R = 0.70$, $P < 0.001$) and between TBARS and carbonyl group content of proteins ($R = 0.45$, $P < 0.01$); moreover, pentosidine correlates with carbonyl group levels (R=0.40, $P < 0.02$).

A significant correlation between lipids and alpha-tocopherol is also present (plasma triglycerides and total cholesterol vs. alpha-tocopherol $R = 0.34$ ($P = 0.05$) and $R = 0.52$ ($P < 0.001$), respectively).

DISCUSSION

Celiac disease is a gluten-sensitive enteropathy characterized by a toxic injury induced by gliadin, a component of gluten, or by similar molecules. A genetic predisposition and an immune mechanism have been suggested, but

intrinsic mechanism seems to be complex and is characterized by a wide spectrum of clinical patterns of intolerance.[15–17]

The involvement of small bowel is diffuse and histological alterations, mainly consisting of a flat mucosal surface with complete absence of normal intestinal villi, have been described;[18–20] a decrease in enzyme production and concentration and in absorptive capacity has been exhaustively described. Malabsorption of nutrients commonly involves fat, lipid-soluble vitamins, iron, calcium and protein.[21,22]

During strict gluten withdrawal, a significant improvement in symptoms and a decline in malabsorption are observed, though some non-responsive subjects have been reported.[23]

In the patients involved in the present study, the diagnosis was made during infancy and the early introduction of a gluten-free diet enabled them to lead a fairly normal life, avoiding gastrointestinal or systemic symptoms. However, the relatively low body mass index was a sign of the imperfect absorption of nutrients.

In this study, the investigation of plasma lipid (TBARS) and protein (carbonyl groups) oxidation markers and of blood antioxidant vitamins demonstrate that a derangement in the redox balance is detectable and that a significant damage to proteins and lipids exists.

Furthermore, the significantly higher levels of pentosidine, a well-known marker of glycoxidation,[24] substantiates this interpretation. Surprisingly, celiac disease is one of the diseases in which oxidative stress is able to produce an increase, albeit small, in plasma pentosidine in the presence of normal renal function and in the absence of a chronic hyperglycemia, main factor in the synthesis of advanced glycation endproducts.[25,26] The association between carbonyl group levels and TBARS strongly suggests the presence of greater oxidative stress in these subjects, both in the hydrophobic and hydrophilic compartments. A weakening of the defenses rather than an increased production is most likely to be the main cause and the lower absorption of lipid-soluble vitamins and other hydrophilic antioxidant molecules, such as ascorbic acid or polyphenols[27] may play a leading role.

Interestingly, pentosidine, a glycoxidation end-product, is well correlated with both parameters, thus suggesting a close interrelationship of all non-enzymatic and spontaneous reactions. Consequently, it is possible that also in non-hyperglycemic patients advanced glycation end-products might result from the activation of the above reactions.

In celiac disease the likely hypothesis for the activation of oxidative stress is that even a slight malabsorption occurs in these patients, possibly maintained by a continued gluten ingestion, either intentional or unintentional. The presumption of absorption deficit is confirmed by the low levels of circulating lipoproteins and lipid-soluble vitamins. It is noteworthy that a deficit in lipid-soluble vitamins has been previously described in asymptomatic adult patients with celiac disease.[28]

Analysis of the retinol/lipid and alpha-tocopherol/lipid ratios, however revealed no significant differences between controls and celiac patients, thus, supporting the speculation of inadequate absorption together with a normal assembly of lipoproteins.

Furthermore, a recent paper reported that celiac disease is a chronic and lifelong disorder; that spontaneous recovery leaves the disease in a latent or silent state, and that during such periods the absence of clinical symptoms does not correlate with the histologic damage.[29]

There is considerable evidences that the incidence of *malignant disease* and especially intestinal T cell lymphoma and esophageal cancer is greater in patients with celiac sprue than in general population.[30–32] Furthermore evidence[33,34] indicates that free radicals are implicated in the biological processes leading to malignant cell transformation.

In patients with celiac disease the malabsorption of lipid-soluble vitamins, even during a

period of remission or improvement, likely persists and leads to a redox imbalance which significantly exacerbates damage to biological molecules.

In our opinion, it is consequently advisable to integrate the diet of these subjects with a surplus of antioxidant molecules and to verify the long-term effect. In this regard dietary integration in these patients is under consideration.

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