



Oxidative stress, DNA stability and evoked inflammatory signaling in young celiac patients consuming a gluten-free diet

Javier Diaz-Castro^{1,2} · Carlota Muriel-Neyra^{1,2} · Rafael Martin-Masot³ · Jorge Moreno-Fernandez^{1,2} · José Maldonado³ · Teresa Nestares^{1,2}

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Abstract

Purpose Celiac disease (CD) is a multifactorial, autoimmune, gluten-sensitive inflammatory disorder of the small intestine. Taking into account the pathogenesis of CD, a strict gluten-free diet (GFD) is the only treatment able to restore epithelium integrity and eliminate complications. The current study was designed to assess whether the use of a GFD is sufficient for maintaining a correct oxidative/antioxidant balance and ameliorating the evoked inflammatory signaling in young patients with CD.

Methods The study covered 80 children, aged between 7 and 18 years, attending the Gastroenterology Service of the Gastroenterology, Hepatology and Child Nutrition Service from the Virgen de las Nieves Hospital in Granada. Children with CD diagnosed were included in the celiac group who followed a strict GFD for 2 years ($n = 40$) and the control group ($n = 40$) included healthy children, with negative serological screening. Soluble superoxide dismutase 1 and 2, total antioxidant status, 8-hydroxy-2'-deoxyguanosine, cortisol, melatonin and inflammatory parameters in plasma, 15-F2t-isoprostanes in urine, and DNA breaks in peripheral blood lymphocytes were analysed.

Results No differences were found in oxidative stress between CD patients and controls; however, IFN- γ , IL-1 α , IP-10 and TNF- β were higher in the CD patients. VEGF was also higher than in the control group.

Conclusion The GFD in the CD patients is enough to reduce the oxidative stress; however, in the case of the inflammatory signaling, the initial exposure to gluten prior to establish the GFD is strong enough to induce an inflammatory state which is maintained (even when consuming the GFD); meanwhile the increase in VEGF recorded in the CD group could be a compensatory mechanism to restore the damaged mucosa and duodenal villous atrophy, due to its role in endothelial activation and generation of new functional and stable vascular networks.

Keywords Celiac disease · Gluten-free diet · Oxidative stress · Inflammatory signaling

Introduction

Celiac disease (CD) is a multifactorial, autoimmune, gluten-sensitive inflammatory disorder of the small intestine, which occurs in people with a genetic predisposition.

It is one of the more common genetic diseases, with a prevalence of 1–3% of the population worldwide [1]. The pathogenesis of CD is complex and still not fully understood. Besides genetic predisposition, immune response plays a vital role in the pathogenesis and disease development. It has been assumed that oxidative stress, because of an increase in the concentration of reactive oxygen species (ROS), a decrease of antioxidant capacity, and the pro-inflammatory state are the main processes possibly involved in gliadin toxicity. The mucosal damage in celiac patients is considered to be induced by the interplay between innate and adaptive immune responses to dietary gluten. Gluten peptides in enterocytes induce certain signal transduction pathways by accumulating in lysosomes and increase the levels of ROS, impairing the oxidation

✉ Teresa Nestares
nestares@ugr.es

¹ Department of Physiology, Faculty of Pharmacy, Campus Universitario de Cartuja, University of Granada, 18071 Granada, Spain

² Institute of Nutrition and Food Technology “José Mataix Verdú”, University of Granada, Granada, Spain

³ Gastroenterology, Hepatology and Child Nutrition Service, University Hospital Virgen de las Nieves, Granada, Spain

redox equilibrium [2]. In this sense, oxidative stress plays an important role in the pathogenesis of many intestinal symptoms of CD not only in adults but also in children, a currently expanding group affected by these diseases [3]. Nevertheless, data concerning ROS status and antioxidant defense in children patients with CD are scarce [4].

Gliadin gene sequence contains regions that play an important role in CD pathogenesis by exerting cytotoxic or immunomodulatory activity. Other regions within the gene are responsible for triggering oxidative stress and inducing the release of proinflammatory cytokines [5, 6]. It has been reported that the interaction of peripheral blood mononuclear cells of CD patients with gliadin produces interleukin 1 β (IL1 β) [7]. Recently, interleukin 15 (IL-15) was also found to be upregulated in the epithelium and lamina propria of CD patients [8]. Th1 response increases interferon gamma (IFN γ), leading to intraepithelial lymphocyte toxicity and the onset of CD [9]. Various epitopes of gliadin can stimulate tumor necrosis factor (TNF), and proinflammatory cytokines (IFN), due to adaptive immune response. Such immunological changes enhance the permeability of the intestine, thereby causing harm to the intestinal mucosa [9]. Many of the pathways that lead to the production of such inflammatory mediators may be initially induced by oxidative stress because these interleukins can overload cells and cause excessive mitochondrial oxidation, resulting in increased production of ROS and oxidative stress [10]. Furthermore, some CD patients may also experience psychological and emotional stress caused by everyday implications of gluten-free diet (GFD) [11], resulting in increased cortisol release and melatonin reduction, facts that could also influence the antioxidant status.

As a consequence, the treatment with a GFD results in the improvement of several clinical biomarkers in CD patients [12]; however even with the high amount of information about this pathology in the scientific literature, to date still it is not well elucidated if the compliance with GFD will be enough to prevent the oxidative stress and inflammatory signaling in patients with CD.

Young CD patients are a very vulnerable group for research due to the implications of the CD manifestations in growing and developing, and due to the lack of relevant research data, it is necessary to carry out additional studies to build on the amount of previously conducted studies. While the diagnostic criteria of CD are well known and well established, it remains difficult to define the correct use of available biomarkers to assess the progression of the disease during follow-up. The current study was designed to assess whether the use of a GFD in young patients with CD is sufficient for maintaining a correct oxidative/antioxidant balance and ameliorating the evoked inflammatory signaling.

Materials and methods

Subjects

The study was carried out according to the principles of the Declaration of Helsinki and its later amendments and approved by the Ethics Committee of the University of Granada (Ref. 20120240000697). The study covered 40 children, between 7 and 18 years, attending the Gastroenterology, Hepatology and Child Nutrition Service from the University Hospital Virgen de las Nieves in Granada. The control group included 40 healthy children, whose serological screening was negative and who had no history of any chronic disease. These children attended to this Service due to minor symptoms related to for chronic functional constipation, according to the Rome IV criteria. After verifying that was due to transitory gastrointestinal symptoms (functional constipation), they were included in the control group. Once the constipation was overcome, the absence of serum IgA and IgG anti-transglutaminase (tTG) antibodies was assessed. Inclusion criteria for the control group were: age between 7 and 18 years, absence of serum IgA and IgG anti-transglutaminase (tTG) antibodies, normal weight for the age, absence of gastrointestinal disorders in the previous year and normal appetite. Children with CD diagnosed in accordance with the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) were included in celiac group ($n=40$). These were children on a strict GFD during at least 2 years, as evidenced by the absence of serum IgA and IgG anti-transglutaminase (tTG) antibodies during the last year (at least) [13]. Exclusion criteria for both groups were: liver or kidney diseases, acute and chronic inflammation, inflammatory bowel disease, diabetes, chronic asthma, consumption of dietary supplements containing substances with antioxidant activity. We also excluded obese patients (according to criteria of the International Task Force) [14], and those who did not sign the informed consent. Written informed consent was obtained from all parents. Anthropometric characteristics (weight, height, mineral bone density and body fat in control and celiac subjects receiving GFD were assessed (Table 1).

Experimental design

Anthropometric measurements

Weight, height, bone mineral density and body fat were collected to assess nutritional and development status of the participants.

Table 1 Anthropometric characteristics in control and celiac subjects receiving GFD

	Control group (<i>n</i> =40)	Celiac group (<i>n</i> =40)
Age (years)	11.42 ± 3.55	10.06 ± 3.12
Sex		
Boys (%)	52	55
Girls (%)	48	45
Weight (kg)	41.86 ± 13.77	34.75 ± 10.53
Height (cm)	147.25 ± 19.16	138.88 ± 15.58
Mineral bone density (g/cm ²)	0.80 ± 0.12	0.77 ± 0.11
Body fat (%)	27.58 ± 8.07	29.47 ± 6.23

Data are shown as the mean values ± SEM

Blood sampling

For measurements, venous blood (5.0 mL) was taken in the morning hours from fasting patients. Blood was collected into anticoagulated tubes with sodium heparin. To obtain plasma, the blood was centrifuged at 2500×*g* at 4 °C for 10 min. Plasma samples were frozen (−80 °C) until further measurements.

Soluble superoxide dismutase (SOD) 1 and 2

Soluble isoforms of SOD1 and SOD2 were determined in plasma using the HND3MAG-39K Milliplex MAP Human Neurological Disorders Magnetic Bead Panel 3 (Millipore Corporation, Missouri, USA), based on immunoassays on the surface of fluorescent-coded beads (microspheres), following the specifications of the manufacturer (50 events per bead, 50 µL sample, gate settings: 8000–15,000, time out 60 s). Plate was read on LABScan 100 analyzer (Luminex Corporation, Texas, USA) with xPONENT software for data acquisition. Average values for each set of duplicate samples or standards were within 15% of the mean. Standard curve: SOD1: 0.04–30 ng/mL, SOD2: 0.03–25 ng/mL. Soluble enzymes concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

15-F2t-isoprostanes

The isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids. Isoprostanes in urine were measured using a commercial kit Enzyme Immunoassay for Urinary Iso-prostane (Oxford Biomedical Research, Oxford, England), a competitive enzyme-linked immunoassay (ELISA) for determining levels of 15-F2t-isoprostane (the best characterized isoprostane) in urine samples. Urine samples are mixed with

an enhanced dilution buffer that essentially eliminates interference due to non-specific binding. The 15-F2t-isoprostane in the samples or standards competes with 15-F2t-isoprostane conjugated to horseradish peroxidase (HRP) for binding to a polyclonal antibody specific for 15-F2t-isoprostane coated on the microplate. The HRP activity results in colour development when substrate is added, with the intensity of the colour proportional to the amount of 15-F2t-isoprostane-HRP bound and inversely proportional to the amount of unconjugated 15-F2t-isoprostane in the samples or standards. Plate was read spectrophotometrically (Bio-tek, Vermont, USA) at 450 nm.

Total antioxidant status (TAS)

To determine plasma TAS levels, peripheral blood was placed in pre-cooled test tubes on the examination day. Plasma was immediately separated in refrigerated centrifuge aliquoted and stored at −20 °C until further use. Freshly thawed batches of plasma were analysed using TAS Randox kit (Randox laboratories, Ltd, Crumlin, UK). Results were expressed in mM of Trolox equivalents. The reference range for human blood plasma is given by the manufacturer as 1.30–1.77 mmol/L. The linearity of calibration extends to 2.5 mmol/L of Trolox. Measurements in duplicate were used to determine intra-assay variability.

Cortisol and melatonin

Cortisol and melatonin were determined in plasma using the HNCSMAG-35K Milliplex MAP Human Circadian/Stress Magnetic Bead Panel (Millipore Corporation, Missouri, USA), following the specifications of the manufacturer. Plate was read on LABScan 100 analyzer (Luminex Corporation, Texas, USA) with xPONENT software for data acquisition. Standard curve: cortisol: 686–500,000 pg/mL; melatonin: 3.4–2500 pg/mL. Analytes concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

8-Hydroxy-2'-deoxyguanosine (8-OHdG)

8-OHdG is an oxidized nucleoside of DNA, widely used as a biomarker for DNA oxidative damage. 8-OHdG in plasma was measured using a commercial kit (8OHdG Check, Japan Institute for the Control of Aging, Shizuoka, Japan) which is a competitive *in vitro* enzyme-linked immunosorbent assay (ELISA) for quantitative detection of the oxidative DNA adduct 8-OHdG. To separate interfering substances, filtration of serum using an ultra filter (cut off molecular weight 10,000) was done. Results were read at 450 nm on a microplate reader (Bio-tek, Vermont, USA).

Alkaline single cell gel electrophoresis (Comet assay)

For comet measurements, a small amount of blood sample (100 µL) was obtained fresh by digital puncture. Among the available genotoxicity tests, comet assay is recognized due to their robustness sensitivity and statistical power to evaluate DNA breaks. DNA instability (strand breaks) was measured in isolated lymphocytes, according to the method previously described [15]. Briefly, isolated lymphocytes were embedded in agarose and lysed to produce nucleoids which were electrophoresed in a 260-mm-wide horizontal electrophoresis tank (Consort, Parklaan, Belgium) at 25 V for 30 min at a temperature of 4 °C. The slides were then washed three times for 5 min each with 0.4 mol/L of Tris–HCl (Sigma Diagnostics), pH 7.5, at 4 °C before staining with 20 µL of 4,6-diamidine-2-phenylindol dihydrochloride (DAPI) (Sigma-Aldrich, St. Louis, MO, USA). DAPI-stained nucleoids were scored using a Leica DMLS fluorescence microscope (Leica Microsystems, Wetzlar, Germany using computerized image analysis (Komet 3.0; Kinetic Imaging Ltd, Liverpool, UK) and the percentage of fluorescence in the comet tail was measured.

Inflammatory parameters

Epidermal growth factor (EGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α2, IFN-γ, interleukin (IL)-10, IL-12P40, IL-12P70, IL-13, IL-15, IL-17A, IL-1RA, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, interferon-inducible protein (IP)-10, monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, tumour necrosis factor (TNF)-α, TNF-β and vascular endothelial growth factor (VEGF) were determined in plasma using the HCYTMAG-60K-PX29 Milliplex MAP Human Cytokine/Chemokine Magnetic Bead Panel (Millipore Corporation, Missouri, USA), following the specifications of the manufacturer. Plate was read on LABScan 100 analyzer (Luminex Corporation, Texas, USA) with xPONENT software for data acquisition. Standard curve: 3.2–10,000 pg/mL. Cytokines

concentrations in plasma samples were determined by comparing the mean of duplicate samples with the standard curve for each assay.

Statistical analysis

Data are reported as mean ± standard error of the mean (SEM). Statistical analyses were performed using the SPSS software (version 24.0, 2016, SPSS Inc., Chicago, IL, USA). Taking into account previous studies, in oxidative stress [3] and inflammation [31] in CD patients, to obtain an asymptotic relative efficacy on the 90% power in the contrast of the null hypothesis $H_0: \mu_1 = \mu_2$, taking into account a level of significance of 5% and assuming the averages and standard deviations previously mentioned, it would be necessary to include 33 subjects per group. If we consider a possible drop out of 20%, the number would be a minimum of 40 individuals per group. The appropriate difference significance tests, such as unpaired Student's *t* test for variables with normal distribution and homogeneity of variance as well as the Mann–Whitney *U* test for variables with non-normal distribution, were used to assess the differences between children with CD and the controls. Differences were considered significant at $P < 0.05$.

Results

The anthropometric characteristics of subjects participating in the study are summarized in Table 1. There were no statistically significant differences in the age, sex, weight, height, mineral bone density or body fat between control and celiac patients.

With regard to the oxidative stress-mediated antioxidants (SOD1, SOD2, melatonin and TAS) and biomarkers related to damage to the prostaglandins (15-F2t-isoprostanes), no statistical differences were observed between control and CD groups, nor in cortisol levels (Table 2).

No differences were found in DNA oxidation (measured through the quantification of to deoxyguanosine sites (8-OHdG) (Fig. 1a) nor in DNA stability in lymphocytes

Table 2 Oxidative/antioxidant biomarkers in control and celiac subjects receiving GFD

	Control group ($n=40$)	Celiac group ($n=40$)
SOD1 (pg/mL)	52.52 ± 1.62	52.77 ± 1.37
SOD2 (pg/mL)	61.03 ± 5.04	57.25 ± 3.05
15-F2t-isoprostanes (pg/mL)	8.99 ± 0.25	9.03 ± 0.26
TAS (mmol/L)	1.69 ± 0.06	1.57 ± 0.05
Melatonin (µg/mL)	639.88 ± 110.44	477.02 ± 97.71
Cortisol (µg/mL)	49.55 ± 6.14	53.33 ± 40.25

Data are shown as the mean values ± SEM

SOD1 superoxide dismutase 1, SOD2 superoxide dismutase 2, TAS total antioxidant status

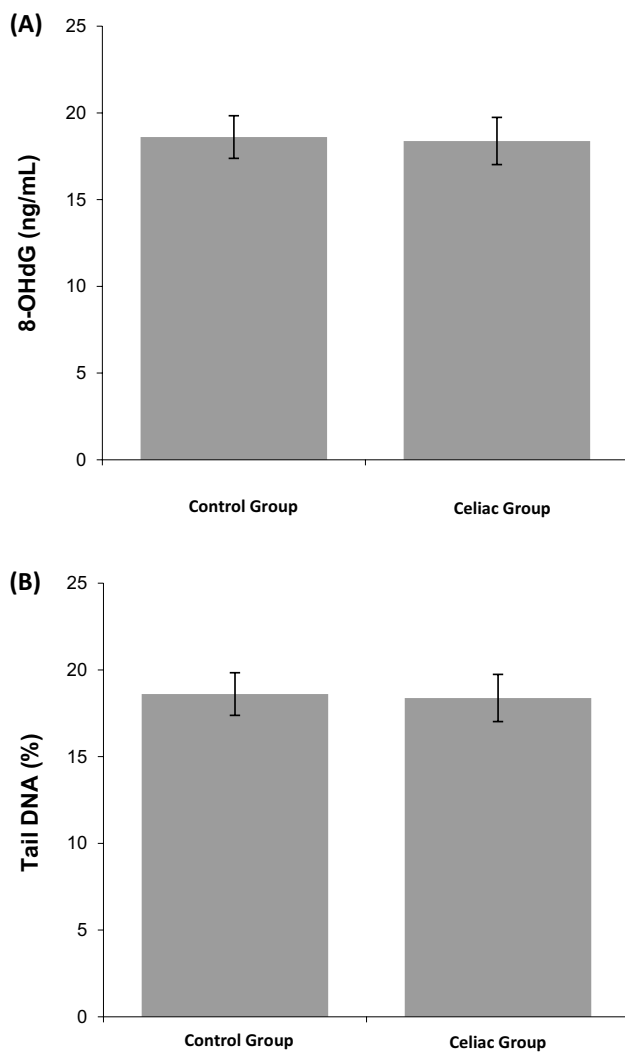


Fig. 1 DNA stability parameters in control and celiac subjects receiving GFD. **a** 8-OHdG 8-hydroxy-2'-deoxyguanosine (8-OHdG, ng/mL); **b** tail DNA (%)

of peripheral blood (Tail DNA, Fig. 1b), revealing that oxidative stress is not present in the celiac group following a GFD.

Pro- and anti-inflammatory cytokines are shown in Table 3. IL-1 α , TNF- α , TNF- β , IFN- γ , IL-12, IL-18 and G-CSF, GM-CSF and IP-10 are well characterized as pro-inflammatory cytokines whereas IL4, IL-10, IL-13 and IFN- α 2 are recognized as anti-inflammatory cytokines. We point out that this classification is far too simplistic and a given cytokine may behave as a pro- as well as an anti-inflammatory cytokine. Indeed, the cytokine amount, the nature of the activating signal and the timing are parameters which greatly influence cytokine properties. No differences

Table 3 Inflammatory signaling in control and celiac subjects receiving GFD

	Control group (n=40)	Celiac group (n=40)
EFG (pg/mL)	18.67 \pm 1.52	20.77 \pm 1.82
Eotaxin (pg/mL)	79.83 \pm 3.91	96.63 \pm 4.66**
G-CSF (pg/mL)	50.35 \pm 5.98	57.47 \pm 5.98
GM-CSF (pg/mL)	12.95 \pm 1.54	15.22 \pm 1.94
IFN- α 2 (pg/mL)	134.19 \pm 9.77	130.94 \pm 7.98
IFN- γ (pg/mL)	34.18 \pm 2.38	51.01 \pm 3.51***
IL-10 (pg/mL)	12.02 \pm 1.43	14.90 \pm 1.72
IL-12P40 (pg/mL)	38.34 \pm 4.45	38.91 \pm 3.83
IL-12P70 (pg/mL)	8.77 \pm 0.94	9.67 \pm 0.82
IL-13 (pg/mL)	45.19 \pm 15.65	50.49 \pm 12.05
IL-15 (pg/mL)	5.71 \pm 0.63	6.17 \pm 0.78
IL-17A (pg/mL)	9.75 \pm 3.45	9.49 \pm 1.55
IL-1ra (pg/mL)	47.86 \pm 4.08	51.71 \pm 3.69
IL-1 α (pg/mL)	31.05 \pm 2.76	42.23 \pm 2.35***
IL-1 β (pg/mL)	4.82 \pm 0.46	4.95 \pm 0.50
IL-2 (pg/mL)	3.29 \pm 0.37	3.67 \pm 0.38
IL-3 (pg/mL)	9.66 \pm 0.95	9.10 \pm 0.84
IL-4 (pg/mL)	25.49 \pm 3.72	23.76 \pm 4.32
IL-5 (pg/mL)	3.17 \pm 0.52	3.98 \pm 0.60
IL-6 (pg/mL)	15.20 \pm 4.64	17.05 \pm 3.67
IL-7 (pg/mL)	21.00 \pm 1.66	19.90 \pm 1.38
IL-8 (pg/mL)	7.65 \pm 1.22	8.06 \pm 0.90
IP-10 (pg/mL)	505.17 \pm 21.19	599.33 \pm 20.99**
MCP-1 (pg/mL)	312.57 \pm 17.02	357.03 \pm 23.18
MIP-1 α (pg/mL)	6.40 \pm 0.62	7.53 \pm 0.95
MIP-1 β (pg/mL)	28.58 \pm 1.59	31.94 \pm 1.51
TNF- α (pg/mL)	23.79 \pm 1.10	26.27 \pm 1.71
TNF- β (pg/mL)	18.29 \pm 2.94	28.42 \pm 2.78**
VEGF (pg/mL)	81.32 \pm 6.70	98.95 \pm 8.37*

Data are shown as the mean values \pm SEM

EFG endothelial growth factor, G-CSF granulocyte/colony stimulating factor, GM-CSF granulocyte/macrophage colony stimulating factor, IFN interferon, IL interleukin, IP interferon-inducible protein, MCP monocyte chemoattractant protein, TNF tumour necrosis factor, VEGF vascular endothelial growth factor

*Significantly different from the control group ($P < 0.05$, Student's t test)

** Significantly different from the control group ($P < 0.01$, Student's t test)

***Significantly different from the control group ($P < 0.001$, Student's t test)

were found in most of the biomarkers studied; however, eotaxin ($P < 0.01$), IFN- γ ($P < 0.001$), IL-1 α ($P < 0.001$), IP-10 ($P < 0.01$), TNF- β ($P < 0.01$) and VEGF ($P < 0.05$)

were higher in the celiac group compared to the control group.

Discussion

Several studies have shown that oxidative stress is one of the major mechanisms involved in the pathology of many diseases, including a number of gastrointestinal ones [16]. Particularly, some gliadin peptides are able to penetrate the cells, accumulate into the lysosomes and lead to the activation of transduction pathways, increasing reactive oxygen species (ROS) levels [17]. Furthermore, the impairment of redox equilibrium induces severe damage in proteins, lipids and DNA. Gluten exposure results in an intracellular oxidative imbalance, characterized by increased levels of lipid peroxidation products [18]. Finally, ROS can induce the formation of oxidative DNA lesion products (8-OHdG), single or double-strand breaks, mutations and chromosome abnormalities [19]. Therefore, it is clear that ROS have a key role in the pathogenesis of CD; in fact, CD patients feature a severe reduction of antioxidant capacity (including antioxidant vitamins) [20]. On the other hand, the results of the current study have demonstrated that the compliance with GFD in the CD patients could help, at least partially, to reduce the levels of gliadin and, therefore, reducing the amount of ROS production in the gut. This phenomenon, together with the TAS and levels of SOD1 and SOD2, which are similar to the control group, explain the protection of the GFD, which reduces the levels of free radicals, leading to cell integrity. This mechanism is linked to the reduction of peroxides and free radicals that damage different cell components, including proteins, lipids and DNA, avoiding the formation of a large number of pyrimidine and purine lesions in the DNA [21]. The expression of 8-OHdG and 15-Ft isoprostanes in the CD group, also similar to their counterpart controls, suggests that oxidative stress is not present due to the compliance to the GFD. Furthermore, some CD patients may also experience psychological and emotional stress caused by everyday implications of GFD due to social, economical and other reasons [11]; however, in the current study, no differences were found in cortisol, nor in melatonin, facts that could explain the absence of difference in the antioxidant status.

It is known that ROS signaling can enhance the synthesis of inflammatory mediators such as TNF- α and IL-1 [22], which, in our study are increased in the CD group. However, in our case, the synthesis of these pro-inflammatory mediators was not mediated by oxidative stress, because as previously mentioned, oxidative stress is not present due to the compliance to the GFD. Data available about apoptosis in patients with CD are conflicting, but increased apoptotic cell death of intestinal epithelial cells has been reported in

untreated CD, as detected by DNA damage in small intestinal biopsies; however apoptosis and DNA damage returned to normal in patients treated with GFD [23], as occurs in the current study, therefore, we can assume that the source of the deleterious pro-inflammatory cytokines was the remaining inflamed mucosa and duodenal villous atrophy.

Patients with CD show an autoimmune reaction to gliadin. The glutamines in gliadin are converted to glutamic acid by tissue transglutaminase. Hereupon, antigen-presenting cells present these gliadin peptides on to gluten-specific CD4+ T cells in the lamina propria [24]. This step induces a strong inflammatory response resulting in small intestinal enteropathy [25]. Interestingly, although no intraepithelial T cells are gluten-specific [26], CD is associated with profound changes in intraepithelial T cells function, resulting in increased cytotoxic effect or function and increased pro-inflammatory state [27]. In addition, histological studies have suggested a change in intraepithelial T cells distribution in patients with CD. In CD, an increase in number and villi from the junction of the crypts was observed (resulting in an increase in number of intraepithelial T cells towards the villous tips) [28, 29] and this occurs even before disease symptoms appear. Taking into account the results of the current study, it seems that these changes in intraepithelial T cells are maintained, at least in part even after adhering to a GFD, because IFN- γ , IL-1 α , IP-10 and TNF- β are still elevated in the CD patients compared with the counterpart controls. This can be explained because even after recovery of normal villous structure, duodenal lymphocytosis can persist for > 10 years [30], therefore, keeping a pro-inflammatory state. After the recovery of normal villous structure, intraepithelial T cells remain elevated [31], which might contribute to re-establishing epithelial homeostasis [32]. Thus, although a clear pathogenic role exists for this pro-inflammatory state, the increase in intraepithelial T cells and the cytokines released might have an important role in epithelial repair. In addition, it has also been reported that patients with CD on GFD, showed the persistence of duodenal damage despite clinical improvement and evident decline in celiac antibodies [33].

One of the key cytokines in the development and pathology of CD is IFN- γ , which has been reported to be elevated in CD and was also found to correlate with tissue damage [34]. A balance between T cell types may be important for maintaining a healthy adaptive immune system. There are reports on an imbalance between the helper T cells Th1 and Th2 in CD [26]. The Th1 response is characterized by a high secretion of IFN- γ , which results in T-cell activation and intestinal tissue damage [34]. Nilsen et al. [35] also studied the gluten specific T cells and found them to produce large amounts of IFN- γ . In the current study, IFN- γ remains elevated even though the CD patients follow a strict GFD, revealing a close connection with immune system

impairment [36] and tissue damage [34], indicating once more, that in spite of the compliance of a strict GFD, the mucosa remains damaged and duodenal villous atrophy is still present.

Interestingly, a gap has emerged between the clinical and mucosal recovery, since when re-biopsing treated CD patients, only half of them had healed mucosa, despite the negativity of celiac antibodies [37, 38], reason that also could explain the persistence of some pro-inflammatory cytokines in the celiac group compared with their counterpart controls in the current study. Following the GFD, the clinical symptoms and mucosal architecture usually improve very quickly in some patients [39], while in a mixed population, the recovery of duodenal mucosa assessed by histology requires longer time to heal [40]. These previous observations are in agreement with the results of our study because some pro-inflammatory cytokines persisted despite the disappearance of specific antibodies due to the compliance of the GFD for 2 years.

Finally, VEGF is the master regulator of vascular growth both in development and disease and, upon expression as a single factor, is capable of initiating the cascade of events leading from endothelial activation to the generation of new functional and stable vascular networks, inducing the growth of new blood vessels from pre-existing ones, is a process that can be targeted to restore blood supply to ischemic tissues [41], therefore, the increase of this soluble factor in the CD group could be a compensatory mechanism to restore the damaged mucosa and duodenal villous atrophy.

Conclusion

The results of the current study demonstrate that the compliance with GFD for 2 years in the CD patients is enough to reduce the amount of ROS production demonstrating that it has a beneficial effect on the molecules and biologic mediators of oxidative stress. However, in the case of the inflammatory signaling, the autoimmune reaction in the mucosa induces a strong inflammatory response which is maintained, at least in part even after consuming GFD, because IFN- γ , IL-1 α , IP-10 and TNF- β are still elevated in the CD patients compared with the counterpart controls. On the other hand, the increase in VEGF recorded in the CD group could be a compensatory mechanism to restore the damaged mucosa and duodenal villous atrophy, due to its role in endothelial activation and generation of new functional and stable vascular networks restoring blood supply to ischemic villi.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest. The funding sponsor had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

- Green PH, Leibold B (2011) Mesalazine for refractory celiac disease: an old medicine for a new disease. *J Clin Gastroenterol* 45(1):1–3
- Zimmer KP, Fischer I, Mothes T, Weissen-Plenz G, Schmitz M, Wieser H, Büning J, Lerch MM, Ciclitira PC, Weber P, Naim HY (2010) Endocytotic segregation of gliadin peptide 31–49 in enterocytes. *Gut* 59:300–310
- Stojiljkovic V, Todorovic A, Radlovic N, Pejic S, Mladenovic M, Kasapovic J, Pajovic SB (2007) Antioxidant enzymes, glutathione and lipid peroxidation in peripheral blood of children affected by celiac disease. *Ann Clin Biochem* 44:537–543
- Pascual V, Dieli-Crimi R, López-Palacios N, Bodas A, Medrano LM, Núñez C (2014) Inflammatory bowel disease and celiac disease: overlaps and differences. *World J Gastroenterol* 20:4846–4856
- Green PHR, Jabri B (2006) Celiac disease. *Ann Rev Med* 57:207–221
- Högberg L, Webb C, Fälth-Magnusson K, Forslund T, Magnusson KE, Danielsson L, Ivarsson A, Sandström O, Sundqvist T (2011) Children with screening-detected coeliac disease show increased levels of nitric oxide products in urine. *Acta Paediatr* 100:1023–1027
- Palova-Jelinkova L, Danova K, Drasarov H, Dvorak M, Funda DP, Fundova P, Kotrbova-Kozak A, Cerna M, Kamanova J, Martin SF, Freudenberg M, Tuckova L (2013) Pepsin digest of wheat gliadin fraction increases production of IL-1 β via TLR4/MyD88/TRIF/MAPK/NF- κ B signaling pathway and an NLRP3 inflammasome activation. *PLoS One* 8:e62426
- Abadie V, Jabri B (2014) IL-15: a central regulator of celiac disease immunopathology. *Immunol Rev* 260:221–234
- Serena G, Camhi S, Sturgeon C, Yan S, Fasano A (2015) The role of gluten in celiac disease and type 1 diabetes. *Nutrients* 7:7143–7162
- Dandekar A, Mendez R, Zhang K (2015) Cross talk between ER stress, oxidative stress, and inflammation in health and disease. *Methods Mol Biol* 1292:205–214
- Makharia GK (2014) Current and emerging therapy for celiac disease. *Front Med (Lausanne)* 1:6
- Ferretti G, Bacchetti T, Masciangelo S, Saturni L (2012) Celiac disease, inflammation and oxidative damage: a nutrigenetic approach. *Nutrients* 4(4):243–257
- Husby S, Koletzko IR, Koletzko S, Korponay-Szabó IR, Mearin ML, Phillips A, Shamir R, Troncone R, Giersiepen K, Branski D, Catassi C, Leigeman M, Mäki M, Ribes-Koninckx C, Ventura A, Zimmer KP, ESPGHAN Working Group on Coeliac Disease Diagnosis, ESPGHAN Gastroenterology Committee, European Society for Pediatric Gastroenterology, Hepatology, and Nutrition (2012) European Society for Pediatric Gastroenterology, Hepatology, and Nutrition guidelines for the diagnosis of coeliac disease. *J Pediatr Gastroenterol Nutr* 54:136–160

14. Cole TJ, Bellizzi MC, Flegal KM, Dietz WH (2000) Establishing a standard definition for child overweight and obesity worldwide: international survey. *BMJ* 320:1240–1243
15. Diaz-Castro J, Alferez MJ, Lopez-Aliaga I, Nestares T, Granados S, Barrionuevo M, Campos MS (2008) Influence of nutritional iron deficiency anemia on DNA stability and lipid peroxidation in rats. *Nutrition* 24(11–12):1167–1173
16. Dugas B, Dugas N, Conti M, Calenda A, Pino P, Thomas Y, Mazier D, Vouldoukis I (2003) Wheat gliadin promotes the interleukin-4-induced IgE production by normal human peripheral mononuclear cells through a redox-dependent mechanism. *Cytokine* 21:270–280
17. Luciani A, Vilella VR, Vasaturo A, Giardino I, Pettoello-Mantovani M, Guido S, Cexus ON, Peake N, Londei M, Quarantino S, Mairuri L (2010) Lysosomal accumulation of gliadin p31–43 peptide induces oxidative stress and tissue transglutaminase-mediated PPARgamma downregulation intestinal epithelial cells and coeliac mucosa. *Gut* 59:311–319
18. Katar M, Ozugurlu AF, Ozyurt H, Benli I (2014) Evaluation of glutathione peroxidase and superoxide dismutase enzyme polymorphisms in celiac disease patients. *Genet Mol Res* 13:1030–1037
19. Marnett LJ (2000) Oxyradicals and DNA damage. *Carcinogenesis* 21:361–370
20. Diosdado B, van Oort E, Wijmenga C (2005) Coelionomics: towards understanding the molecular pathology of coeliac disease. *Clin Chem Lab Med* 43:685–695
21. Olinski R, Gackowski D, Rozalski R, Foksinski M, Bialkowski K (2003) Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development? *Mutat Res* 531:177–190
22. Haddad JJ, Olver RE, Land SC (2000) Antioxidant/pro-oxidant equilibrium regulates HIF-1alpha and NF-kappa B redox sensitivity. Evidence for inhibition by glutathione oxidation in alveolar epithelial cells. *J Biol Chem* 275:21130–21139
23. Moss SF, Attia L, Scholes JV, Walters JR, Holt PR (1996) Increased small intestinal apoptosis in coeliac disease. *Gut* 39:811–817
24. Jabri B, Sollid LM (2017) T Cells in celiac disease. *J Immunol* 198:3005–3014
25. Green PHR, Cellier C (2007) Celiac disease. *N Engl J Med* 357:1731–1743
26. Meresse B, Malamut G, Cerf-Bensussan N (2012) Celiac disease: an immunological jigsaw. *Immunity* 36:907–919
27. Abadie V, Discepolo V, Jabri B (2012) Intraepithelial lymphocytes in celiac disease immunopathology. *Semin Immunopathol* 34:551–556
28. Goldstein NS, Underhill J (2001) Morphologic features suggestive of gluten sensitivity in architecturally normal duodenal biopsy specimens. *Am J Clin Pathol* 116:63–71
29. Steenholt JV, Nielsen C, Baudewijn L, Staal A, Rasmussen KS, Sabir HJ, Barington T, Husby S, Toft-Hansen H (2017) The composition of T cell subtypes in duodenal biopsies are altered in coeliac disease patients. *PLoS One* 12:1–17
30. Tuire I, Marja-Leena L, Teea S, Katri H, Jukka P, Päivi S, Heini H, Markku M, Pekka C, Katri K (2012) Persistent duodenal intraepithelial lymphocytosis despite a long-term strict gluten-free diet in celiac disease. *Am J Gastroenterol* 107:1563–1569
31. Kutlu T, Brousse N, Rambaud C, Le Deist F, Schmitz J, Cerf-Bensussan N (1993) Numbers of T cell receptor (TCR) alpha beta+ but not of TCR gamma delta+ intraepithelial lymphocytes correlate with the grade of villous atrophy in coeliac patients on a long term normal diet. *Gut* 34:208–214
32. Chen Y, Chou K, Fuchs E, Havran WL, Boismenu R (2002) Protection of the intestinal mucosa by intraepithelial gamma delta T cells. *Proc Natl Acad Sci USA* 99:14338–14343
33. Piatak-Guziewicz A, Ptak-Belowska A, Przybylska-Felus M, Pasko P, Zagrodzki P, Brzozowski T, Mach T, Zwolinska-Wcislo M (2017) Intestinal parameters of oxidative imbalance in celiac adults with extraintestinal manifestations. *World J Gastroenterol* 23(44):7849–7862
34. Wapenaar MC, van Belzen MJ, Franssen JH, Sarasqueta AF, Houwen RH, Meijer JW, Mulder CJ, Wijmenga C (2004) The interferon gamma gene in celiac disease: augmented expression correlates with tissue damage but no evidence for genetic susceptibility. *J Autoimmun* 23:183–190
35. Nilsen EM, Lundin KE, Krajci P, Scott H, Sollid LM, Brandtzaeg P (1995) Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Th1 or Th0 profile dominated by interferon gamma. *Gut* 37:766–776
36. Kumar V, Gutierrez-Achury J, Kanduri K, Almeida R, Hrdlickova B, Zhernakova DV, Westra HJ, Karjalainen J, Ricaño-Ponce I, Li Y, Stachurska A, Tigchelaar EF, Abdulahad WH, Lähdesmäki H, Hofker MH, Zhernakova A, Franke L, Lahesmaa R, Wijmenga C, Withoff S (2015) Systematic annotation of celiac disease loci refines pathological pathways and suggests a genetic explanation for increased interferon-gamma levels. *Hum Mol Genet* 24(2):397–409
37. Lanzini A, Lanzarotto F, Villanacci V, Mora A, Bertolazzi S, Turini D, Carella G, Malagoli A, Ferrante G, Cesana BM, Ricci C (2009) Complete recovery of intestinal mucosa occurs very rarely in adult coeliac patients despite adherence to gluten-free diet. *Aliment Pharmacol Ther* 29:1299–1308
38. Leibold B, Granath F, Ekbom A, Smedby KE, Murray JA, Neugut AI, Green PH, Ludvigsson JF (2013) Mucosal healing and risk for lymphoproliferative malignancy in celiac disease: a population based cohort study. *Ann Intern Med* 159:169–175
39. McNicholl B, Egan-Mitchell B, Stevens F, Keane R, Baker S, McCarthy CF, Fottrell PF (1976) Mucosal recovery in treated childhood celiac disease (gluten-sensitive enteropathy). *J Pediatr* 89:418–424
40. Wahab PJ, Meijer JW, Mulder CJ (2002) Histologic follow-up of people with celiac disease on a gluten-free diet: slow and incomplete recovery. *Am J Clin Pathol* 118:459–463
41. Yla-Herttuala S, Rissanen TT, Vajanto I, Hartikainen J (2007) Vascular endothelial growth factors: biology and current status of clinical applications in cardiovascular medicine. *J Am Coll Cardiol* 49(10):1015–1026