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Alimentary Tract

# Gliadin effect on the oxidative balance and DNA damage: An *in-vitro*, *ex-vivo* study

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ABSTRACT

*Background:* Gliadins are involved in gluten-related disorders and are responsible for the alteration of the cellular redox balance. It is not clear if the gliadin-related oxidative stress can induce DNA damage in enterocytes.
*Aim:* To investigate any possible genotoxicity caused by gliadin and to assess its relationship with oxidative stress *in vitro* and *ex vivo*.
*Methods:* Caco-2 cells were exposed for 6–12–24 h to increasing concentrations (250 μg/mL–1000 μg/mL) of digested gliadin. We investigated: cytotoxicity, oxidative balance (reactive oxygen species, ROS), DNA damage (comet assay and γ-H2AX detection), transglutaminase type 2 (TG2) activity and annexin V expression. H2AX and 8-OHG immunohistochemistry has been evaluated on duodenal biopsies of celiac subjects and controls.
*Results:* Gliadin induced a significant increase (+50%) of ROS after 12 h of exposition starting with a 500 μg/mL dose of gliadin. Comet assay and γ-H2AX demonstrated DNA damage, evident at the gliadin concentration of 500 μg/mL after 24 h. TG2 activity increased in chromatin and cytoskeleton cellular compartments at different gliadin doses (250/500/1000 μg/mL). The γ-H2AX and 8-OHG immunohistochemistry was altered in the duodenal biopsies of celiac patients.
*Conclusions:* Gliadin induces cellular oxidative stress, DNA damage and pro-apoptotic stimulation in Caco-2 cells and in the duodenal mucosa of celiac patients.


## 1. Introduction

Gluten is the major structural protein of wheat, composed by two main fractions: gliadins (alcohol-soluble) and glutenins (acid and alkali-soluble). Recently, wheat components, especially gliadins, have been considered as the environmental factors underpinning different diseases and syndromes, the so-called "gluten-related disorders" (GRDs), celiac disease (CD), wheat allergy and non-celiac gluten sensitivity (NCGS) [1–3].

CD is characterized by a chronic duodenal inflammation in which the increased secretion of inflammatory cytokines may in turn derange intestinal permeability and produce large amounts of reactive oxygen species (ROS), altering the redox state at the cellular level thus reducing the cellular antioxidant capability [4–7].

Oxidative stress is defined as the imbalance between the production of ROS and the antioxidant defenses of the cells in favor of the oxidants, leading to potential damage. ROS production occurs during the cellular metabolic processes; if the production of ROS overwhelms a cell's antioxidant (AO) capability, a condition known as oxidative stress occurs [8].

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The association between small-intestinal inflammation and oxidative stress is well known; in fact, the inflammatory response can lead to the recruitment of activated leukocytes, which may lead to an increased $O_2$ uptake, called "respiratory burst," inducing the release of high quantities of ROS [9,10].

Previous studies have showed that oxidative stress is one of the major mechanisms involved in the pathology of many diseases, including a number of gastrointestinal ones [11,12]. Particularly, some gliadin peptides (13-mer) are able to penetrate the cells, accumulate into the lysosomes and lead to the activation of transduction pathways with an increase of free radicals levels (ROS and reactive nitrogen species, RNS) [5]. Increased levels of ROS reduce the transglutaminase type 2 (TG2) degradation by the ubiquitin–proteasome system, thus leading to increased TG2 protein levels. TG2 is implicated in different biological functions, including signal transduction, survival, apoptosis and differentiation: TG2 plays an important role in CD [13–15].

Furthermore, the impairment of redox equilibrium proved to cause severe damage in proteins, lipids and DNA. Several studies showed that gluten exposure results in an intracellular oxidative imbalance, characterized by increased levels of lipid peroxidation products [4-hydroxy-2(E)-nonenal (4-HNE)], increased oxidized (GSSG)/reduced (GSH) glutathione ratio and decreased number of protein-bound sulfhydryl groups [16,17].

Finally, ROS can induce the formation of oxidative DNA lesion products (8-oxodG), which is considered as a mutagenicity marker. Oxidative damage can lead to single or double-strand breaks, point and frameshift mutations and chromosome abnormalities [18]. There is a considerable amount of circumstantial evidence that oxidative DNA damage may play an important role not only in carcinogenesis, being used as a predictive marker of cancer development [19,20], but also in aging [21]. Nowadays, no studies concerning oxidative damage on DNA have developed with regard to CD or GRDs.

In the present study, we have investigated the possible gliadin-induced genotoxic damage and its correlation with oxidative stress *in vitro*; moreover, we have evaluated the DNA damage in the duodenal biopsies of celiac patients.

## 2. Methods

### 2.1. Cell culture

The human colon carcinoma Caco-2 cell line was grown in Dulbecco's modified Eagle's medium (DMEM, Euroclone) supplemented with 0.2 mM L-glutamine, 1% non-essential amino acids (Hy-, Euroclone), 5 U/mL penicillin, 5 μg streptomycin and 10% (v/v) foetal bovine serum (Euroclone), maintained in a humidified incubator with 5% $CO_2$ at 37 °C.

In all the experiments, the cells were exposed to different concentrations of digested gliadin (from 125 μg/mL to 1000 μg/mL) for 24 h.

### 2.2. Enzymatic digestion of gliadin

The enzymatic digestion of gliadin developed as described by Friis et al. [22]. Briefly, 1 g gliadin was dissolved in 10.0 mL 0.1 mol/L HCl, 20 mg pepsin was added and after digestion (2 h, 37 °C) the pH was adjusted to 8 by 5.0 mol/L NaOH. The gliadin was then digested further with 20 mg trypsin (4 h, 37 °C) with constant stirring. The inactivation of trypsin was achieved by heating (90 °C, 3 min). Insoluble material was removed by centrifugation (10,000 × g, 30 min) and the supernatant was sterilized by filtering through a 20-mm pore membrane.

### 2.3. Cytotoxicity assay

Caco-2 cells were seeded in 96-well plates by adding 200 μL/well of a suspension of $3 \times 10^4$ cells/well and they were treated with different concentrations of digested gliadin (250 μg/mL to 1000 μg/mL). The medium was removed and 50 μL MTT (5 mg MTT with 1 mL PBS) was added. After incubating for 4 h the medium was removed and 150 μL DMSO was added to each well. The spectrophotometric absorbance of each well was analyzed by means of a microplate reader at 550 nm (Perkin Elmer Wallac Victor Multi-label Counter 1420).

### 2.4. Reactive oxygen species

Cells were cultured in black 96-well plates; at 80% confluence the medium was removed and the cells were treated by protocol. The medium was removed and the cells were incubated with 100 μL DCFH-DA (2′,7′-dichlorofluorescin diacetate) 20 μM in the loading medium in 5% $CO_2$ at 37 °C for 30 min. The cells were washed with 100 μL PBS into each well; fluorescence was measured at excitation and emission wavelengths of respectively 485 and 530 nm with a Wallac Victor 1420 Multi-label Counter [23]. The ROS formed were normalized to the protein content in each well for statistical analysis. The protein concentration was measured according to the Lowry et al. method [24].

### 2.5. Alkaline comet assay

DNA damage was estimated by alkaline single cell gel electrophoresis (comet assay) according to the method of Collins et al. [25].

Briefly, $2 \times 10^4$ cell/mL were re-suspended in 200 μL 0.5% low-melting agarose (LMA) and the suspension was pipetted onto the precoated 1% of normal melting agarose on microscope slides. Slides were immersed in cold lysis solution (2.5 M NaCl, 100 mM Na EDTA, 10 mM TRIS, 250 mM NaOH, 10% DMSO, 1% Triton X-100 pH 10) for 30 min at 4 °C. To allow DNA denaturation, the slides were kept in alkaline electrophoresis buffer (pH = 13) for approximately 20 min. Then electrophoresis started at 25 V, 300 mA for 20 min. Slides were neutralized and stained with ethidium bromide (20 μg/mL). Observations were made using a fluorescence microscope (Axioplan, Zeiss) attached to a digital camera connected to a personal computer. Images were analysed by TriTek CometScore™ software.

### 2.6. Enzyme-modified comet assay

Three slides per sample were prepared, one enzyme buffer control, one with FPG and one with Endonuclease III. This assay is based on the alkaline comet assay, but after cell lysis, the gels were rinsed three times with an enzyme buffer (40 mM Hepes, 100 mM KCl, 0.5 mM $Na_2$ EDTA, 0.2 mg/mL BSA, pH 8.0), and incubated with the enzymes FPG (100 mU per gel; 25 μL; 30 min; 37 °C), or Endonuclease III (100 mU per gel, 25 μL, 30 min, 37 °C). Endonuclease III detects the oxidized pyrimidines and FPG recognizes the common oxidized purine 8-oxo-7,8-dihydroguanine. After enzyme treatment the slides were placed in a horizontal electrophoresis chamber for DNA unwinding.

This kind of comet assay is able to show the genotoxic damage induced only by oxidative stress, calculated as the Δ tail moment between samples treated with and without enzymes [26].

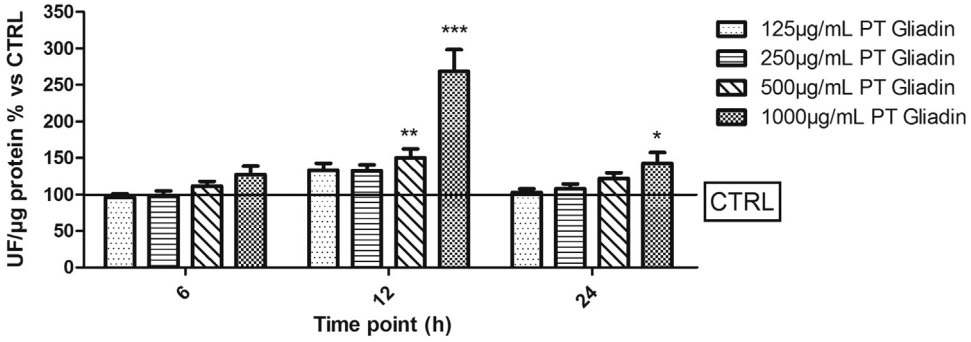



**Fig. 1.** Effect of the digested gliadin (PT gliadin) treatment on ROS production. Cells were incubated with different concentrations and levels of ROS measured after each endpoint (6/12/24 h). The ROS formed were normalized to the protein content in each well. *p < 0.05, **p < 0.01, ***p < 0.001. Results are shown as UF/mg protein% compared to the control.

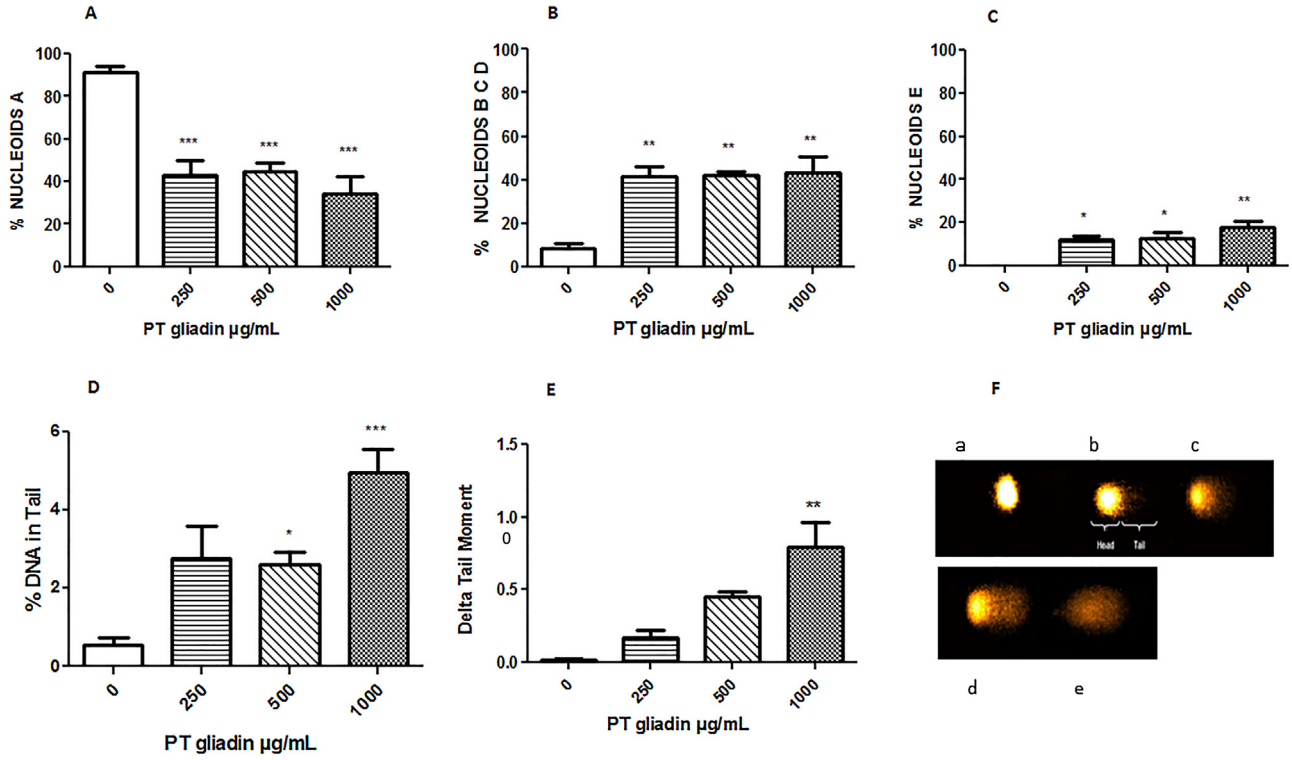



**Fig. 2.** DNA damage detected by means of comet assay. Panels A, B, C show the distribution of Caco-2 cells referring to each class of damage. Panel D reports the induction of DNA damage (relative% DNA in tail, assessed in the *in-vitro* alkaline Comet assay). Panel E shows the induction of DNA strand breaks with ENDO III enzyme as assessed in the enzyme-modified Comet assay. Panel F shows the Nucleoids classification: (a) undamaged, (b) lowly damaged, (c) moderately damaged, (d) damaged, (e) highly damaged (ghost).
Caco-2 cell treated with PT digested gliadin (250–1000 µg/mL) for 24 h. *p < 0.05; **p < 0.01, ***p < 0.001 *vs.* CTRL.

### 2.7. Detection of γ-H2AX

After the 24 h-long treatment the Caco-2 cells grown on glass slides were fixed in cold methanol for 10 min. The cells were rehydrated in PBS (3 × 5 min) and permeabilized in PBS containing 0.5% Triton X-100 for 20 min. After blocking for 30 min with 3% BSA in PBS, the cells were incubated with mouse anti-γ-H2AX (Millipore, 1:150 in 1% BSA) for 2 h at room temperature after PBS washing (3 × 5 min) with a secondary anti-mouse Alexa Fluor 488 antibody (1:500 Life Technology) for 1 h at room temperature. The nuclei were counterstained with DAPI (VECTASHIELD Mounting Medium). Images were acquired (60×) utilizing a Nikon Eclipse E600 equipped with a DXM1200 digital camera (Nikon, Tokyo, Japan). The number of γ-H2AX foci/nucleus was determined using the ImageJ software and the cells were classified in three groups: 0–5, 6–10, >10 foci/nucleus.

### 2.8. Annexin V

After 24 h some supernatant samples were picked up and cells were washed with PBS, detached with trypsin and finally collected with their supernatant. The cells were centrifuged for 5 min at 2000 rpm and re-suspended in 1 mL PBS + 5% FBS, counted by the trypan blue method ($1 \times 10^6$ cells/mL) and centrifuged again. The supernatant was discarded and 100 µL Annexin Binding Buffer (1:10 in 0.1% sodium citrate) was added to each sample (except for the control cells and only PI stained cells). The Caco-2 cells were double-stained with 5 µL Alexa Fluor 488 Annexin V and 1 µL PI (100 µg/mL) and kept for 15 min at room temperature in the dark.

After incubation, 400 μL Annexin Binding Buffer 5× (1:10 in 0.1% sodium citrate) was added to each sample and the samples were analyzed by flow cytometry (BD FACScalibur, Becton Dickinson) at an excitation wavelength of 496 nm and an emission wavelength of 516 nm.

### 2.9. Western blot analysis of transglutaminase type 2 in the total cellular lysate

The cells were washed in PBS, scraped and homogenized. Aliquots of homogenate (each containing 40 μg of protein) were boiled for 5 min and electrophoresed on a 10% SDS polyacrylamide gel. The resolved proteins were then transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked by incubation in 4% non-fat milk in TBS at room temperature for 1 h, and then incubated overnight with primary antibody (transglutaminase 2 antibody, CUB7402 Life Technologies 1:500) at 4 °C, followed by an alkaline phosphatase-conjugated secondary antibody (GE Healthcare) for 1 h at room temperature. Antibody binding was detected using an enhanced chemiluminescence Western blotting detection kit (ECL Star, Euroclone). The blots were analyzed by means of image analysis software: the results were shown as the percentage of the optical densities relative to the expression in untreated Caco-2 cells normalized to 100%.

### 2.10. Transglutaminase type 2 activity in different cellular compartments

Caco-2 cells were treated with digested gliadin (250 μg/mL to 1000 μg/mL) for 24 h. The cells were washed with PBS and they were detached with cold PBS. We used the Sub-cellular Protein Fractionation Kit for cultured cells (Thermo Scientific 78840) following the manufacturer's instruction. Subsequently, TG2 activity was evaluated in the different cellular compartments by means of the transglutaminase Colorimetric Micro-assay kit (opr0001, ABCAM) according to the manufacturer's instructions.

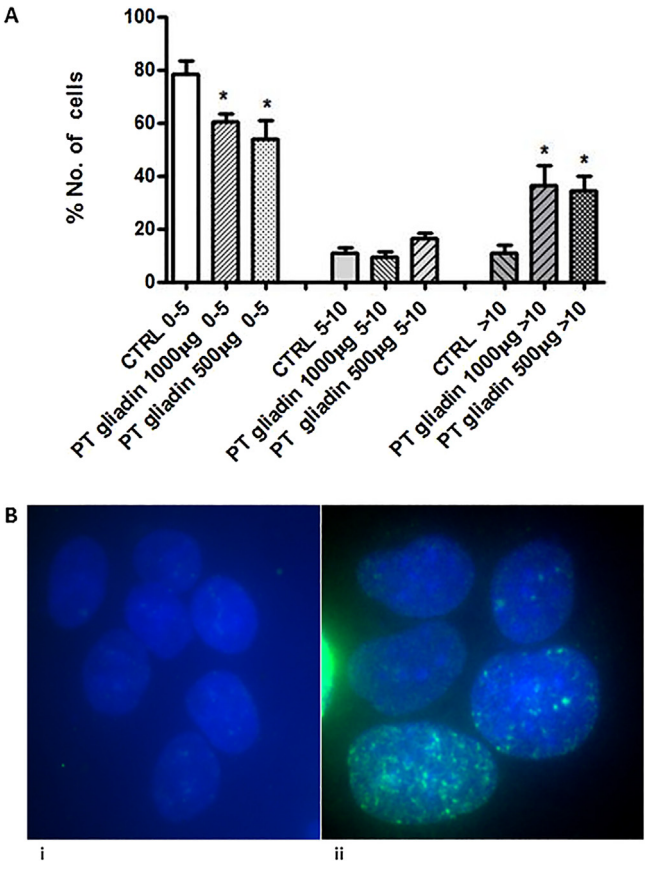
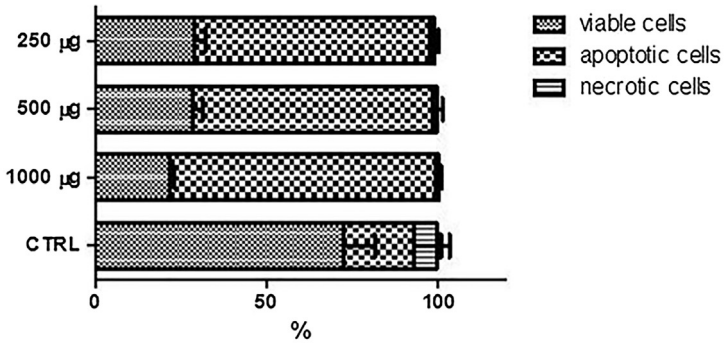


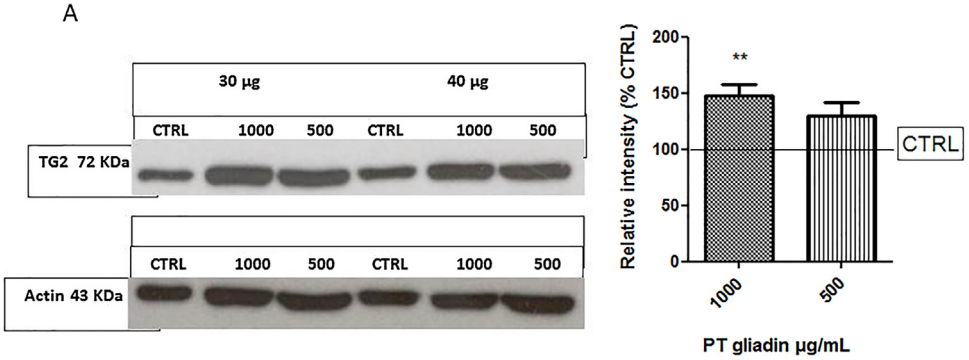

**Fig. 3.** DNA damage detected by means of immunofluorescence. Panel A shows the DNA damage (Double breaks) detected by γ-H2AX immunofluorescence staining. Number of foci: 0–5, 6–10, >10 in control cells and treated with PT gliadin. Panel B shows (i) untreated Caco-2 cells and (ii) PT digested gliadin 1000 μg/mL treated cells. *p < 0.05; **p < 0.01, ***p < 0.001 *vs.* CTRL.




| GROUPS | VIABLE CELLS | APOPTOTIC CELLS |
|---|---|---|
| CTRL VS 1000 PT Gliadin | *** p<0.001 | ***p<0.001 |
| CTRL VS 1000 PT Gliadin | ***p<0.001 | ***p<0.001 |
| CTRL VS 1000 PT Gliadin | ***p<0.001 | **p<0.001 |

**Fig. 4.** Evaluation of apoptosis in Caco-2 cells after different treatments with PT digested gliadin. *p < 0.05; **p < 0.01, ***p < 0.001 *vs.* CTRL.

A




B

| | CTRL | 1000µg/mL | 500µg/mL | 250µg/mL |
|---|---|---|---|---|
| MEMBRANE | 15.75 ± 2.60 | 18.60 ± 6.54 | 17.75 ± 4.92 | 9.00 ± 1.00 |
| CYTOPLASM | 15.25 ± 2.22 | 17.40 ± 2.88 | 19.25 ± 3.30 | 14.00 ± 2.00 |
| CYTOCHELETON | 17.25 ± 6.70 | 28.00 ± 5.43* | 31.00 ± 5.60** | 47.33 ± 5.51*** |
| CHROMATIN | 15.00 ± 1.63 | 19.00 ± 2.65* | 18 ± 7.00 | 15.00 ± 2.00 |
| NUCLEUS | 15.30 ± 3.65 | 19.00 ± 4.53 | 20.33 ± 4.73 | 15.00 ± 4.00 |

**Fig. 5.** Panel A shows the TG2 expressions (western blot) after treatment (24 h) with PT digested gliadin. The band intensities were quantified by densitometry, normalized to the level of β-actin which was used as an internal control and calculated as percentage of the basal response. All data are shown as mean + SD of at least three independent experiments performed in triplicates. Panel B shows the TG2 activity percentage (versus the total activity of the same sample) in Caco-2 cells after treatment (24 h) with PT digested gliadin at 250, 500, 1000 µg/mL doses.
*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$ *vs.* CTRL.

### 2.11. Patients and immunohistochemical analysis

Twelve patients with a histologically proven CD (three males, mean age 42 years) and eleven non-CD controls (two male, mean age 50 years) with a normal duodenal mucosa and negative CD-specific serology, were enrolled at the Center for Prevention and Diagnosis of Celiac Disease of the Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico (Milan, Italy) between 2013 and 2014. Paraffin-embedded sections from duodenal biopsies of the CD and non-CD patients were used for immunohistochemical analysis. The study was approved by the Ethics Committee of the Fondazione IRCCS Ca' Granda.

The immunoreactivity of the duodenal biopsies for phosphorylated histone H2AX Ser139 (γ-H2AX, Abcam, Cambridge, UK), or 8-hydroxy-2′-deoxyguanosine (8-OHG) (8HG; clone N45.1, Abcam, Cambridge, UK) was analyzed in order to evaluate the DNA damage by immunohistochemistry.

Immunohistochemistry was performed using the BenchMark ULTRA automatic system (Ventana Medical Systems) and reactions were revealed using the UltraView Universal DAB, (Ventana Medical Systems). Immunoreactivity for γ-H2AX or 8-OHG was calculated as the percentage of positive cells out of the total number of cells separately for the epithelial (MUC) and lamina propria compartments (LP).

### 2.12. Statistical analysis

The data were analyzed using the statistics and graphic functions of the GraphPad Prism statistical software package rel. 6.0 (GraphPad Prism Software, Inc., California, USA). For the analysis of the results different statistical tests were used. The Anova test differences were considered as statistically significant when $p \leq 0.05$.

## 3. Results

### 3.1. Cytotoxicity and reactive oxygen species analysis

After a 24 h-long exposure to different concentrations of PT digested gliadin (from 125 µg/mL to 1000 µg/mL), the MTT assay did not show a significantly decreased viability of Caco-2 cells (data shown in Supplementary materials). Conversely, ROS production significantly increased (Fig. 1).

The results demonstrated that the high production of oxidizing species was induced by the different concentrations of PT digested gliadin and exposure times in a dose-dependent fashion. A significant increase in ROS production was found after the 12 h-long incubation with 500 µg/mL and 1000 µg/mL PT digested gliadin.

### 3.2. DNA damage

The potential effect of gliadin to induce single/double-strand DNA breaks was assessed with the comet assay. The cells were exposed to different concentrations of PT digested gliadin (from 250 µg/mL to 1000 µg/mL) for 24 h. The alkaline comet analysis showed DNA damage after exposure to 500 µg/mL PT digested gliadin for 24 h. In particular, the data obtained highlighted a significant increase of the DNA percentage in the tail at 500 µg/mL ($p < 0.05$) and 1000 µg/mL ($p < 0.001$) PT digested gliadin doses (Fig. 2D). The nucleoids were graded according to the degree of damage into 5 classes (from A to E): the number of nucleoids undamaged (A) decreased starting with the 250 µg/mL dose ($p < 0.001$) while the number of nucleoids with maximum damage (E) increased in a dose-dependent manner, at concentrations of 250 µg/mL, 500 µg/mL ($p < 0.05$) and 1000 µg/mL ($p < 0.01$). The number of nucleoids with some DNA damage (B, C and D) increased at a concentration of 250 µg/mL ($p < 0.01$) (Fig. 2A, B and C).

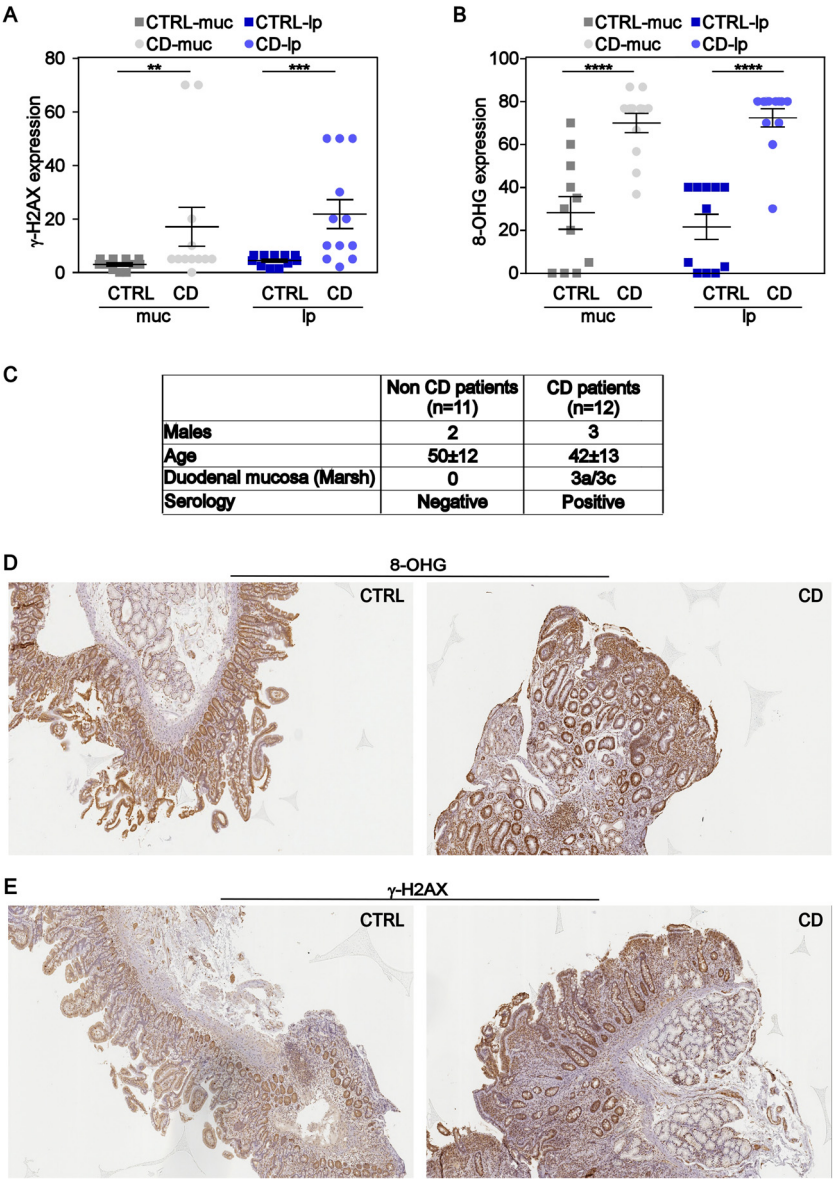



**Fig. 6.** Detection of DNA damage in the duodenal biopsies of celiac patients. Panel A and B show the immunohistochemical analysis of γ-H2AX and 8-OHG in the duodenal mucosa of celiac patients and non-celiac controls. The percentage of positive cells out of the total number of cells in the epithelial (MUC) and laminutesa propria compartments (LP) is reported. Panel C provides the patients' clinical and demographic characteristics. Panel D and E report immunohistochemical images (5× magnification) of: 8-OHG non-celiac control; 8-OHG celiac disease; γ-H2AX non-celiac control; γ-H2AX celiac disease.
**p < 0.0047; ***p < 0.0003, ****p < 0.0001 *vs.* CTRL.

We also performed an enzyme-modified alkaline comet assay to detect DNA modifications related to oxidative stress. In this case, the addition of Endonuclease III resulted in a significant induction of DNA breaks, in a dose-dependent manner. After 24 h-long treatment with PT digested gliadin at 1000 μg/mL, a significant increase in the Δ tail moment ($p < 0.01$) was noted (Fig. 2E). Panel F shows the Nucleoids classification: (a) undamaged, (b) lowly damaged, (c) moderately damaged, (d) damaged, (e) highly damaged (ghost).

DNA damaged was confirmed by γ-H2AX analysis. Fig. 3 shows the induction of foci after 24 h-long exposure to PT digested gliadin expressed as% number of cells with a different number of foci. We observed a low number of foci (0–5) in the controls (untreated cells). At 500 μg/mL and 1000 μg/mL doses we observed an increase in the number of foci >10 foci/cell ($p < 0.05$).

### 3.3. Apoptosis

An apoptotic effect, detected by means of annexin V, was clearly observed. After 24 h-long incubation, the PT digested gliadin induced an increased number of apoptotic cells (Fig. 4).

Moreover, after cells homogenization and electrophoresis in 10% SDS polyacrylamide gel, we observed a significant increase of TG2 expression after treatment with 1000 μg/mL PT digested gliadin, compared to the controls (Fig. 5A). In cellular compartments, the treatment with PT digested gliadin increased TG2 activity in chromatin and cytoskeleton compared to controls (Fig. 5B).

### 3.4. Detection of DNA damage in the duodenal mucosa of patients with celiac disease

We evaluated the DNA damage in the duodenal biopsies of 12 patients with a histologically proven CD (untreated, on a gluten containing diet) and 11 non-CD controls (Fig. 6). Results showed a significant increase in H2AX ($p = 0.0047$) in the duodenal mucosa of celiac patients compared to non-celiac subjects (Fig. 6A).

Oxidative damage significantly increased in celiac patients as demonstrated by 8-OHG levels ($p < 0.0001$) in the epithelial (MUC) and lamina propria compartments (LP) compared to non-CD subjects (Fig. 6B).

## 4. Discussion

The present study demonstrates that digested gliadin induces an increase of ROS production in Caco-2 cells with an alteration of the cellular redox state. Moreover, ROS concentration induces a pro-apoptotic process and DNA damage, as demonstrated here for the first time. This mechanism seems present also *in vivo* as demonstrated by the findings on the duodenal mucosa of CD patients.

ROS production usually occurs in a regulated manner in order to maintain the cellular homeostasis in healthy tissues. Free radicals play an important role as signaling molecules and they can act as second messengers to modulate signaling pathways [27]. In particular, free radicals are implicated in the pathogenesis of CD; in fact, CD patients present a severe reduction of antioxidant capacity (including antioxidant vitamins) [28].

Our results have demonstrated an increase of ROS production in Caco-2 cells induced by the PT digested gliadin in a dose-dependent manner. Interestingly, the highest concentration was reached after 12 h-long exposure, showing a mild reduction after 24 h, suggesting a premature oxidative event (Fig. 1). This phenomenon, together with the excessive amount of free radicals, may contribute to several cellular mechanisms, potentially leading to cell injury and/or cellular death. The ROS levels decrease after 24 h could be the expression of a defensive mechanisms induced by Caco-2 cells. The ROS and redox alteration, induced by gliadin treatment, potentially trigger an "oxidative stress" response in the Caco-2 intestinal cell line. This mechanism is linked to toxic effects driven by the production of peroxides and free radicals that damage different cell components, including proteins, lipids and DNA. Free radicals can attack the DNA and cause the formation of a large number of pyrimidine and purine lesions [29]. For such a reason, we decided to analyze the effect of PT digested gliadin on the DNA strings.

The present study shows that DNA damage occurs during PT digested gliadin treatment (500 μg/mL and 1000 μg/mL) as revealed by the standard alkaline comet assay. Interestingly, the classification of nucleotids has showed an increase of type-E nucleoids and suggests an apoptotic/necrotic phenomenon (as confirmed by the TG2 and annexin V findings).

In order to identify the subtype of DNA damage, the incubation of cells with enzymes recognizing specifically damaged bases, would help [30,31]. For this reason, we incubated Caco-2 cells with the DNA Endonucleases III, demonstrating a significant increase in Δ tail moment after treatment with 1000 μg/mL PT digested gliadin. This finding supports the conclusion that the observed damage is provoked by oxidative stress at the pyrimidine level.

The intracellular ROS accumulation has a number of direct and indirect consequences: it can interfere with gene expression, leading to changes in the post-translational protein modification. Specifically, an association between oxidative stress and TG2 up-regulation has been suggested. TG2 enzyme is localized in multiple cellular compartments, including nucleus, cytosol, mitochondria, endosomes, plasma membrane, cell surface and extracellular matrix; it interacts with a distinct subset of proteins and/or substrates and performs different tasks depending on its subcellular location [13,32]. Our study has showed an increased expression of TG2 in the cytoskeleton and chromatin, thus suggesting a probable apoptotic stimuli, highlighted by annexin V. The activation of intracellular TG2, which is mostly quiescent except during extreme stress conditions, may depend on the level of calcium influx. When various stimuli increase cytosolic [$Ca^{2+}$] above a certain threshold, the transamidating activity of TG2 is no longer inhibited by GTP and facilitates cell death processes. Multiple studies on cell death induced by oxidative stress have shown that high levels of ROS trigger $Ca^{2+}$ influx resulting in TG2 activation and, subsequently, cell death [33,34].

In our study, the *in-vitro* findings seem confirmed by the preliminutesary results obtained from the duodenal biopsies of celiac patients. The expression of 8-OHG and H2AX (which becomes phosphorylated on serine 139 as a reaction on DNA double-strand breaks) in celiac subjects, suggests that some mechanisms similar to those observed in Caco-2 cells are possibly present in the duodenal mucosa.

In conclusion, these findings add novel information about the biological properties of gliadin and the interaction between gliadin and enterocytes.

The alteration of the redox balance, the increase of ROS and apoptosis, and the DNA damage can be part of the initial steps of the CD pathomechanism and moreover, these findings suggest the potential use of antioxidant therapies in GRD.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dld.2018.06.020.

## References

[1] Catassi C, Alaedini A, Bojarski C, Bonaz B, Bouma G, Carroccio A, et al. The overlapping area of Non-Celiac Gluten Sensitivity (NCGS) and wheat-sensitive Irritable Bowel Syndrome (IBS): an update. Nutrients 2017:9.
[2] Sapone A, Bai JC, Ciacci C, Dolinsek J, Green PH, Hadjivassiliou M, et al. Spectrum of gluten-related disorders: consensus on new nomenclature and classification. BMC Med 2012;10:13.
[3] Elli L, Branchi F, Tomba C, Villalta D, Norsa L, Ferretti F, et al. Diagnosis of gluten related disorders: Celiac disease, wheat allergy and non-celiac gluten sensitivity. World J Gastroenterol 2015;21:7110–9.
[4] Sollid LM. Molecular basis of celiac disease. Annu Rev Immunol 2000;18:53–81.
[5] Luciani A, Villella VR, Vasaturo A, Giardino I, Pettoello-Mantovani M, Guido S, et al. Lysosomal accumulation of gliadin p31-43 peptide induces oxidative stress and tissue transglutaminase-mediated PPARgamma downregulation in intestinal epithelial cells and coeliac mucosa. Gut 2010;59:311–9.
[6] Dolfini E, Elli L, Dasdia T, Bufardeci B, Colleoni MP, Costa B, et al. In vitro cytotoxic effect of bread wheat gliadin on the LoVo human adenocarcinoma cell line. Toxicol In Vitro 2002;16:331–7.
[7] Dolfini E, Elli L, Roncoroni L, Costa B, Colleoni MP, Lorusso V, et al. Damaging effects of gliadin on three-dimensional cell culture model. World J Gastroenterol 2005;11:5973–7.

[8] Halliwell B. Reactive species and antioxidants. Redox biology is a fundamental theme of aerobic life. Plant Physiol 2006;141:312–22.
[9] Verbeek WH, Goerres MS, von Blomberg BM, Oudejans JJ, Scholten PE, Hadithi M, et al. Flow cytometric determinutesation of aberrant intra-epithelial lymphocytes predicts T-cell lymphoma development more accurately than T-cell clonality analysis in Refractory Celiac Disease. Clin Immunol 2008;126:48–56.
[10] Keshavarzian A, Banan A, Farhadi A, Komanduri S, Mutlu E, Zhang Y, et al. Increases in free radicals and cytoskeletal protein oxidation and nitration in the colon of patients with inflammatory bowel disease. Gut 2003;52:720–8.
[11] Dugas B, Dugas N, Conti M, Calenda A, Pino P, Thomas Y, et al. Wheat gliadin promotes the interleukin-4-induced IgE production by normal human peripheral mononuclear cells through a redox-dependent mechanism. Cytokine 2003;21:270–80.
[12] Maiuri MC, De Stefano D, Mele G, Iovine B, Bevilacqua MA, Greco L, et al. Gliadin increases iNOS gene expression in interferon-gamma-stimulated RAW 264.7 cells through a mechanism involving NF-kappa B. Naunyn Schmiedebergs Arch Pharmacol 2003;368:63–71.
[13] Ientile R, Caccamo D, Griffin M. Tissue transglutaminase and the stress response. Amino Acids 2007;33:385–94.
[14] Klöck C, Diraimondo TR, Khosla C. Role of transglutaminase 2 in celiac disease pathogenesis. Semin Immunopathol 2012;34:513–22.
[15] Rauhavirta T, Hietikko M, Salmi T, Lindfors k. Transglutaminase 2 and Transglutaminase 2 autoantibodies in celiac disease: a review. Clin Rev Allergy Immunol 2016 [Epub ahead of print] http://doi.org/10.1007/s12016-016-8557-4.
[16] Stojiljković V, Pejić S, Kasapović J, Gavrilović L, Stojiljković S, Nikolić D, et al. Glutathione redox cycle in small intestinal mucosa and peripheral blood of pediatric celiac disease patients. An Acad Bras Cienc 2012;84:175–84.
[17] Katar M, Ozugurlu AF, Ozyurt H, Benli I. Evaluation of glutathione peroxidase and superoxide dismutase enzyme polymorphisms in celiac disease patients. Genet Mol Res 2014;13:1030–7.
[18] Marnett LJ. Oxyradicals and DNA damage. Carcinogenesis 2000;21:361–70.
[19] Guyton KZ, Kensler TW. Oxidative mechanisms in carcinogenesis. Br Med Bull 1993;49:523–44.
[20] Halliwell B. Can oxidative DNA damage be used as a biomarker of cancer risk in humans? Problems, resolutions and preliminutesary results from nutritional supplementation studies. Free Radic Res 1998;29:469–86.
[21] Kawanishi S, Hiraku Y, Oikawa S. Mechanism of guanine-specific DNA damage by oxidative stress and its role in carcinogenesis and aging. Mutat Res 2001;488:65–76.
[22] Friis S, Dabelsteen E, Sjöström H, Norén O, Jarnum S. Gliadin uptake in human enterocytes. Differences between coeliac patients in remission and control individuals. Gut 1992;33:1487–92.
[23] Prosperini A, Juan-García A, Font G, Ruiz MJ. Reactive oxygen species involvement in apoptosis and mitochondrial damage in Caco-2 cells induced by enniatins A, $A_1$, B and $B_1$. Toxicol Lett 2013;222:36–44.
[24] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 1951;193:265–75.
[25] Collins AR, Oscoz AA, Brunborg G, Gaivão I, Giovannelli L, Kruszewski M, et al. The comet assay: topical issues. Mutagenesis 2008;23:143–51.
[26] Biancini GB, Moura DJ, Manini PR, Faverzani JL, Netto CB, Deon M, et al. DNA damage in Fabry patients: an investigation of oxidative damage and repair. Mutat Res Genet Toxicol Environ Mutagen 2015;784–785:31–6.
[27] Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol 2007;39:44–84.
[28] Diosdado B, van Oort E, Wijmenga C. "Coelionomics": towards understanding the molecular pathology of coeliac disease. Clin Chem Lab Med 2005;43:685–95.
[29] Olinski R, Gackowski D, Rozalski R, Foksinski M, Bialkowski K. Oxidative DNA damage in cancer patients: a cause or a consequence of the disease development? Mutat Res 2003;531:177–90.
[30] Collins AR. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 2004;26:249–61.
[31] Gielazyn ML, Ringwood AH, Piegorsch WW, Stancyk SE. Detection of oxidative DNA damage in isolated marine bivalve hemocytes using the comet assay and formamidopyrimidine glycosylase (Fpg). Mutat Res 2003;542:15–22.
[32] Elli L, Bergaminutesi CM, Bardella MT, Schuppan D. Transglutaminases in inflammation and fibrosis of the gastrointestinal tract and the liver. Dig Liver Dis 2009;41:541–50.
[33] Nurminutesskaya MV, Belkin AM. Cellular functions of tissue transglutaminase. Int Rev Cell Mol Biol 2012;294:1–97.
[34] Caccamo D, Currò M, Ferlazzo N, Condello S, Ientile R. Monitoring of transglutaminase 2 under different oxidative stress conditions. Amino Acids 2012;42:1037–43.