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Responses of peripheral blood mononucleated cells from non-celiac gluten sensitive patients to various cereal sources

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ABSTRACT

Non-celiac gluten sensitivity (NCGS) is still an undefined syndrome whose triggering mechanisms remain unsettled. This study aimed to clarify how cultured peripheral blood mononucleated cells (PBMC) obtained from NCGS patients responded to contact with wheat proteins. Results demonstrated that wheat protein induced an overactivation of the proinflammatory chemokine CXCL10 in PBMC from NCGS patients, and that the overactivation level depends on the cereal source from which proteins are obtained. CXCL10 is able to decrease the transepithelial resistance of monolayers of normal colonocytes (NCM 460) by diminishing the mRNA expression of cadherin-1 (CDH1) and tight junction protein 2 (TJP2), two primary components of the tight junction strands. Thus, CXCL10 overactivation is one of the mechanisms triggered by wheat proteins in PBMC obtained from NCGS patients. This mechanism is activated to a greater extent by proteins from modern with respect to those extracted from ancient wheat genotypes.
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1. Introduction

The consumption of wheat-based products is estimated to be very high in most Western countries (i.e. Europe, United States) and in continuous increase in Eastern countries as a consequence of a shift toward a Western lifestyle (Rubio-Tapia et al., 2009; Van den Broeck et al., 2010). Nowadays, most of the wheat products we consume are made from modern wheat varieties bred after the "Green Revolution" (in the 1970s). The introduction of dwarf genes led to the development of short straw lines with substantial gains in productivity and technological quality. The variation in dough rheology and bread-making performance among wheat varieties is largely determined by the differences in protein content and quality composition, depending on specific gliadins and high molecular weight glutenin subunits (Sliwinski, Kolster, Prins, & van Vliet, 2004). Not dwarf wheat genotypes, bred after the "Green Revolution" are defined as old. Generally, old wheat varieties have weaker rheological properties than the modern ones, as demonstrated by alveographic analyses (Guarda, Padovan, & Delogu, 2004). In recent years, scientists have shown increasing interest in investigating nutritional differences among wheat varieties, old genotypes and ancestors due to their diverse nutrient and phytochemical composition. Research studies concerned dietary fiber content (Marotti et al., 2012), phenolics and terpenoid composition (Di Silvestro et al., 2012; Shewry et al., 2011), but strong efforts recently focused on the study of gluten proteins, a major cause of celiac disease (CD) and gluten-related pathologies (Carroccio et al., 2011; Molberg et al., 2005; van den Broeck et al., 2010).

From an evolutionary point of view, gluten proteins were absent from the diet of hunter gatherers (Schnorr et al., 2014) and were introduced in human nutrition only about 10,000 years ago. This may explain the lack of a complete adaptation of humans to the ingestion of gluten proteins. Nevertheless, different studies demonstrate that the prevalence of CD has increased over the last 60 years (Lohi et al., 2007; Rubio-Tapia et al., 2009). Moreover, the increasing number of patients worldwide who are sensitive to

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dietary gluten but lack evidence of celiac disease or wheat allergy has contributed to the identification of a new gluten-related syndrome known as non-celiac gluten sensitivity (NCGS) (Volta, Caio, Tovoli, & De Giorgio, 2013). Double-blind placebo-controlled trials have confirmed that gluten proteins are involved in the development of this syndrome (Biesiekierski et al., 2011; Carroccio et al., 2012). The molecular mechanisms of NCGS remain for the most part unknown, even if it is widely accepted that the innate immune system plays a major role in the onset of NCGS (Catassi et al., 2013; Sapone et al., 2011). Even if the prevalence of NCGS in the western population is still debated, it seems clear that in recent years it has rapidly increased in adults (Sapone et al., 2012; Volta et al., 2014) and in children (Francavilla et al., 2014). Recently, modern wheat strains have been implicated in NCGS (de Lorgeril & Salen, 2014).

The present study evaluated the *in vitro* chemokine response of PMBC from NCGS patients to different cereals (including modern and ancient wheat genotypes) aiming to identify a possible marker for NCGS.

2. Materials and methods

2.1. Cereal samples

The investigated cereal samples consisted of a modern common wheat (*Triticumaestivum* L.) "Manitoba" variety, a modern durum wheat (*Triticumturgidum* spp. *durum*Desf. Husn.) "Claudio" variety, an old Italian durum wheat (*Triticumturgidum* spp. *durum*Desf. Husn.) "SenatoreCappelli" variety, an accession of KAMUT® khorasan wheat (*Triticumturgidum* spp. *turanicum*Jakubz), and a sample of rice (*Oryzasativa* L.) gluten-free flour as a negative control. KAMUT® is a registered trademark of KAMUT International Ltd. and KAMUT Enterprises of Europe bvba. Manitoba and rice samples were purchased at local supermarkets. The flours of Claudio, SenatoreCappelli and KAMUT® khorasan were obtained from the wheat germplasm of the Department of Agricultural Science of the University of Bologna (Italy) and were organically grown. Grains were milled using a stone mill (100% flour extraction).

2.2. Protein extraction

Proteins were extracted using the procedure described by Osborne (1907) and subsequently modified by Lookhart and Bean (1995) and van den Broeck et al. (2009). Proteins were extracted from three seed replicates for each accession. Briefly, 100 mg of flour was treated with 500 μl of distilled water (30 min, 160 rpm) vortexing for 1 min at 10-min intervals for the extraction of albumins. Samples were than centrifuged at 7000 rpm for 5 min and the supernatant collected. Albumin extraction was repeated with 400 μl of distilled water (10 min, 160 rpm), centrifuged and the second extract collected. The pellet was treated with 400 μl of NaCl 0.5 M (30 min, 160 rpm) vortexing for 1 min at 10-min intervals for the extraction of globulins. After centrifugation (3500 rpm, 5 min), the supernatant was collected and the globulin extraction repeated (400 μl of NaCl 0.5 M; 10 min, 160 rpm) to obtain the second globulin extract. Subsequently, the remaining pellet was used for the extraction of prolamins. Four hundred microliter of 50% (v/v) aqueous isopropanol were added to the test tube to allow the solubilization of gliadins (30 min, 160 rpm). After centrifugation (3500 rpm, 15 min), the first gliadin extract was collected and the extraction was repeated (10 min, 160 rpm). Glutenins were extracted using a solution of 50% (v/v) aqueous isopropanol containing 1% (w/v) DL-dithiothreitol for 30 min at 160 rpm and centrifuged at 10,000 rpm for 10 min. The supernatant was collected and the extraction repeated once. For the *in vitro* tests, all the extracts were pooled to obtain a total protein extract for each cereal variety.

2.3. Protein electrophoretic profiles (SDS–PAGE)

The protein profiles of the cereal samples investigated were obtained using the Bolt® Mini Gel Tank (Invitrogen™, Life Technologies) compatible with the Bolt® Bis–Tris Plus precast gels (10%). Electrophoretic patterns were obtained from three replicates for each cultivar. For the electrophoresis analysis the first supernatants of albumins, globulins and glutenins, and the second supernatant of gliadins were used. Extracts (30 μl) were diluted with 15 μl Bolt™ LDS sample buffer, 6 μl Bolt™ reducing agent, 9 μl distilled water and denatured at 70 °C for 10 min. The Bolt™ MOPS SDS running buffer was used for the run at 165 V (25 min). The protein patterns were compared with the Mark12™ unstained standard (2.5–200 KDa) for albumins and globulins, and the SeeBlue® Plus2 pre-stained standard (4–250 KDa) for gliadins and glutenins. The protein profiles were elaborated as presence/absence of the observed subunits for each sample.

2.4. Protein content and quantification of allergenic epitopes

The total protein content was measured using the Kjeldahl procedure ($N \times 5.7$) (AACC, 1983). Wheat flours were tested for the content of gluten allergenic epitopes using the RIDASCREEN®Gliadin competitive ELISA (R-Biopharm AG, Darmstadt, Germany). Flour samples were treated with the RIDA® Extraction Solution (R7099) to enable the solubilization of the gliadin fraction. The test is based on the antigen–antibody reaction between gluten epitopes and the R5 antibody. The absorbance values were obtained at 450 nm using a microplate reader (Multiskan EX 1.1 MTX Lab Systems, Virginia, USA). Results were compared with those of gliadin standard (5–80 ppb) and elaborated using the software RIDA®SOFT Win.net (R-Biopharm AG, Darmstadt, Germany). Epitope contents were expressed as percentage of total protein amount.

2.5. Patients

The study population included 48 NCGS patients (females 39, males 9, median age 41 years, range 20–67 years), diagnosed as having NCGS after a thorough evaluation in the tertiary referral centers of the SpedaliCivili (Brescia, Italy) and S.Orsola-Malpighi Hospital (Bologna, Italy). All these patients complained of one or more gastrointestinal (bloating, abdominal pain, diarrhea/constipation, nausea, epigastric pain, gastro-esopagheal reflux, aphthous stomatitis) and extra-intestinal (tiredness, headache, joint/muscle pain, arm numbness, 'foggy mind', dermatitis/skin rash, anxiety, depression, anemia) symptoms/manifestations with an early onset (a few hours or days) after gluten ingestion. As extensively stated in the literature (Catassi et al., 2013; Sapone et al., 2012), a fundamental prerequisite for NCGS diagnosis is the exclusion of both CD and wheat allergy (WA) on a gluten-containing diet. CD was ruled out in all 48 enrolled NCGS patients by negativity for anti-tissue transglutaminase (anti-tTG) and anti-endomysial antibodies (EmA) and the absence of villous atrophy in the duodenal biopsy, whereas WA was excluded by the negativity for specific IgE antibodies to wheat and/or skin prick tests. NCGS patients were put on a gluten-free diet obtaining a complete remission or a significant improvement of both gastrointestinal and extra-intestinal symptoms, thus confirming the clinical suspicion of NCGS. Thirty healthy volunteers, age- and sex-matched with the NCGS group, were enrolled among students and medical staff of the two referral centers (Bologna and Brescia). A blood sample was obtained from both NCGS patients and healthy volunteers. Before undergoing

blood sampling, NCGS patients were asked to switch from a gluten-free to gluten-containing diet for 6 weeks. The study was approved by the SpedaliCivili of Brescia and St.Orsola-Malpighi Hospital Independent Institutional Ethical Committees. Patients and healthy donors gave their written informed consent.

2.6. Isolation and culture of PBMC

Blood samples obtained from NCGS enrolled patients and healthy volunteers were collected in EDTA-containing vacuum tubes and kept under agitation on a roller mixer at room temperature for 4 h. PBMC separation was obtained using the kit-Mate™ Sep 15 (STEMCELL Technologies, Vancouver, BC, Canada), following the manufacturer's instructions. PBMCs were resuspended in 1 ml of complete RPMI-1640 (Life Technologies, CA, USA), supplemented with 25 mM HEPES and antibiotics (penicillin and streptomycin). After careful resuspension, 4×10^5 cells were seeded in each well of a 24-well plate. 40 μg of protein extracts of selected flours were resuspended in 200 μl of RPMI-1640 and added to PBMC cultures. Plates were incubated for 12 h at 37 °C and 5% CO_2 (Forma Series II Water Jacketed CO_2 Incubator), under mild shaking.

2.7. Determination of secreted CXCL10

After incubation, the well contents were transferred into eppendorf tubes and supernatant was collected after centrifugation (1000×*g* for 15 min at RT) and subjected to cytokine/chemokine determination using Luminex® MAP technology as previously described (Brigotti et al., 2013). Cytokine and chemokine levels were first determined using a multiplexed 27-plex human bead immunoassay kit (Bio-Rad, CA, USA). Then, a single plex for CXCL10 was used. Microsphere magnetic beads coated with monoclonal antibodies against cytokines and chemokines were added to the wells. After incubation for 30 min, the wells were washed and biotinylated secondary antibodies were added. After incubation for another 30 min, beads were washed and then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein, phycoerythrin (streptavidin/phycoerythrin). After washing, the beads (a minimum of 100 per analyte) were analyzed in the BioPlex 200 instrument (BioRad). Sample concentrations were estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor (Bio-Plex Manager software 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve. The intra-assay CV averaged 12%. This method is covered by patents owned by Alma Mater Studiorum, University of Bologna (WO 2014/037858; PCT/IB2013/058148).

2.8. Colonocyte cell culture and transepithelial electrical resistance (TEER) determination

Normal mucosa NCM-460 cells, kindly provided by Dr. Antonio Strillacci (University of Bologna, Italy) were grown in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 U^{-1} ml penicillin and 100 g^{-1} ml streptomycin at 37 °C in a humidified atmosphere of 95%, with 5% CO_2. For maximum viability, NCM460 cells were subcultured in fresh and spent growth medium in at a 1:1 ratio. All cell culture reagents were provided by Invitrogen (Milan, Italy). Differentiation of NCM460 cells to polarized monolayers was performed modifying the method reported by Dalpiaz et al. (2012). Briefly, after two passages, confluent NCM460 cells were seeded at a density of 25×10^4 cells/insert in the growth medium (fresh and spent medium 1:1) on 24-well Millicell inserts (Millipore, Milan, Italy) consisting of 1.0 μm pore size polyethylene terephthalate filter membranes, whose surface was 0.33 cm^2. Filters were presoaked for 24 h with fresh culture medium, and then the upper compartment (apical, A) received 0.2 ml of cells, whereas the lower (basolateral, B) received 1.25 ml of the cell-free growth medium. Half the volume of culture medium was replaced every 2 days with fresh medium to each of the apical and basolateral compartments. The integrity of the cell monolayers was monitored by measuring transepithelial electrical resistance (TEER) by means of a voltmeter (Millicell-ERS; Millipore, Milan, Italy). The measured resistance value was multiplied by the area of the filter to obtain an absolute value of TEER, expressed as $\Omega\,cm^2$. The background resistance of blank inserts not plated with cells was around 35 $\Omega\,cm^2$ and was deducted from each value. The homogeneity and integrity of the cell monolayer were also monitored by phase contrast microscopy. Based on these parameters, cell monolayers reached confluence and epithelial polarization after 7 days and monolayers with a TEER stable value around 200 $\Omega\,cm^2$ were used. Cell monolayers were treated with concentrations ranging from 0.01 to 10 ng/ml of CXCL10 (R&D Systems, Inc., Minneapolis, MN, USA) reconstituted at 100 μg^{-1} ml in sterile PBS containing 0.1% BSA and added to serum-free medium of the apical or the basolateral side. Corresponding amounts of BSA were also added to untreated cells. The integrity of the confluent polarized monolayers was verified by measuring TEER at different time intervals. 10 ng/ml human IL-1β (R&D Systems, Inc., Minneapolis, MN, USA) were also added to the apical or basolateral side of the appropriate inserts as positive control.

2.9. RNA extraction and real-time PCR

Total RNA from CXCL10-treated NCM460 cells was extracted using Trizol® reagent (Life Technologies, CA, USA) according to the manufacturer's instructions. Extracted RNA samples were treated with DNase I to remove any genomic DNA contamination using DNA-free kit (Ambion, USA) and reverse-transcripted using random hexamerprimers (Fermentas, Canada). E-cadherin, Zonulin-1, 2, 3 and β-actin mRNA levels were analyzed by real-time PCR using SYBR® Select Master Mix (Life Technologies, CA, USA) and StepOne Plus™ system (Applied Biosystems, CA, USA) according to the manufacturer's instructions. The melting curve data were collected to check PCR specificity. Each cDNA sample was analyzed in triplicate. Target mRNA levels were normalized against β-actin mRNA and relative expressions were calculated using the $2^{-2\Delta Ct}$ formula. Cadherin-1 primer pairs: 5′-AAGAAGGAGGCGGAGAAGAG-3′ and 5′-ACACGAGCAGAGAATCATAAGG-3′ (228 bp product); tight junctions protein 1 primer pairs: 5′-CCAGAGACCAACAAGGAACC-3′ and 5′-CCGACATCATTGCCACCAG-3′ (185 bp product); tight junctions protein 2 primer pairs: 5′-CACGCCGAGCAGA TTGTC-3′ and 5′-GTCTCCCTTCTTGAACCTTACC-3′ (226 bp product); tight junctions protein 3 primer pairs: 5′-CTCTTCACAGCCACCATCC-3′ and 5′-CCGTCCGTCTCGTAGTCG-3′ (200 bp product); β-actin primer pairs: 5′-ACCAACTGGGACGACATGGAG-3′ and 5′-GTGGTGGTGAAGCTGTAGCC-3′ (207 bp product).

2.10. Statistical analyses

One-way analysis of variance (ANOVA) was carried out to assess the significance of the differences among cereal samples for the protein and epitopes content, to assess the differences among CXCL10 secretion and to assess the differences among mRNA relative expression. Tukey's Honestly Significant Difference test was used to determine differences between means at $P < 0.05$. Principal component analysis (PCA) was performed on the standardized matrix of presence/absence of each protein electrophoretic band to compare the protein profiles of cereal sources. Two-way ANOVA

followed by Bonferroni's post-test was performed to determine the differences of TEER variations, at $P < 0.05$ and at $P < 0.01$. All statistical analyses were conducted using Statistica 6.0 software (StatSoft, Tulsa, OK, USA). GraphPad Prism 6 software (GraphPad Software, Inc., CA, USA) was also used to analyze differences in CXCL10 secretion.

3. Results

3.1. Protein and allergenic epitope content of cereal sources

The investigated cereal sources showed a broad variation in the total protein amount (Table 1). The lowest protein level was found for the rice flour (5.9 ± 0.1 g/100 g). Among the wheat samples, the commercial Manitoba flour had the lowest protein amount equal to 11.8 ± 0.1 g/100 g. Organically grown old wheat varieties KAMUT® khorasan flour and SenatoreCappelli had the highest protein content, with mean values of 16.8 ± 0.1 and 16.4 ± 0.2 g/100 g, respectively. The modern durum wheat genotype Claudio semolina showed a lower protein amount of 12.4 ± 0.2 g/100 g. The gluten extracts of cereal samples were also tested for the presence of T-cell stimulatory peptides using the RIDASCREEN®Gliadin competitive ELISA. The immunoassay is based on the use of R5 monoclonal antibody which recognizes specific sequences (QQPFP, QQQFP, LQPFP, QLPFP) occurring in gliadins (van den Broeck et al., 2009). Epitope content is reported in Table 1 and expressed as mg/kg of flour and as percentage of total protein. No epitopes were detected in the rice flour sample, confirming the "gluten-fee" label of the commercially purchased product. Considering the concentration of epitopes expressed in mg/kg, the old durum wheat variety SenatoreCappelli showed the highest amount (28.2 ± 1.2 mg/kg), while the other cereal samples had values ranging from 21.3 ± 0.3 to 22.3 ± 0.2 mg/kg. van den Broeck et al. (2009) reported that the epitope content of different wheat varieties ranged from 8.26 to 21.30 mg/kg and therefore our results align with the highest limit of that study. Since the cereal samples showed a broad variation in total protein content, the percentage of epitopes in the total protein can provide information on the quality composition of the protein fractions. Results showed that Claudio, Manitoba and SenatoreCappelli had similar percentage values (0.017–0.019%) while KAMUT® khorasan wheat showed the lowest percentage (0.013%) suggesting that, although it was the richest in proteins, KAMUT® khorasan flour had a slight but statistically significant lower epitope composition. This may be related to a lower concentration of gliadin proteins carrying allergenic epitopes among the total protein pattern.

3.2. Protein profiles of cereal sources

The protein profiles of the investigated wheat samples were characterized using SDS–PAGE electrophoresis. All the protein fractions (albumins, globulins, gliadins, glutenins) were analyzed and the patterns compared to determine the differences among cereal samples (Fig. 1). The matrix of presence/absence of each electrophoretic band detected was elaborated using PCA, and the distribution of the cases as a function of the first and second principal components is shown in Fig. 2 (explaining 66.84% and 19.24% of the total variability, respectively). Multivariate analysis of the electrophoretic data allowed a description of the differences detected among the protein profiles of the wheat genotypes. Manitoba flour had the most divergent protein profile and it was projected along the negative arm of the first component (PC1), at a great distance from the other cases located in the positive arm of PC1 (Fig. 2). The divergence of this sample was expected as it is the only hexaploid genotype among tetraploid varieties. The tetraploid wheats (Claudio, KAMUT® khorasan wheat, SenatoreCappelli) shared similar protein profiles for the albumin and globulin fractions, while Manitoba had 4 albumins (between 24 and 37 kDa) and 5 globulins (31–60 kDa) that were not found in the tetraploid genotypes. The tetraploid wheats also differed for the gliadin profile and had 6 subunits of ω-, αβ- and γ-gliadins (with molecular weight ranging from 44 to 65 kDa) not detected in the other samples. The KAMUT® khorasan sample was projected on the negative part of the second principal component (PC2), while Claudio and SenatoreCappelli were clustered together on the positive arm (Fig. 2). The main differences detected among these genotypes were related to the glutenin profiles. The KAMUT® khorasan sample lacked the high and low molecular weight glutenin subunits found in both Claudio and SenatoreCappelli flour (i.e. 32, 68 and 91 kDa subunits), and had 48 and 80 kDa subunits exclusively detected on the khorasan sample.

3.3. CXCL10 secreted by PBMC of NCGS patients and healthy controls

Significant differences were observed in the responses of PBMC from healthy donors stimulated with protein extracts from flour containing gluten compared to stimulation with rice gluten-free flour (Fig. 3 panel A). CXCL10 secretion was significantly higher ($P < 0.01$) for all four grains tested. This means that the secretion of this pro-inflammatory cytokine is somehow stimulated by protein extracts of cereals containing gluten. Using corn flour

Table 1
Protein and gluten epitope content (±standard deviation) of the cereal flours investigated.

	Proteins	Epitopes	
	g/100g	mg/kg	% on tot. proteins
Claudio	12.4 ± 0.2 (c)	21.3 ± 0.3 (b)	0.017 ± 0.001 (a)
Kamut®	16.8 ± 0.1 (a)	22.3 ± 0.2 (b)	0.013 ± 0.001 (b)
Manitoba	11.8 ± 0.1 (d)	22.0 ± 1.0 (b)	0.019 ± 0.001 (a)
SenatoreCappelli	16.4 ± 0.2 (b)	28.2 ± 1.2 (a)	0.017 ± 0.001 (a)
Rice	5.9 ± 0.1 (e)	N.d.	N.d.

Abbreviations: N.d., not detected.

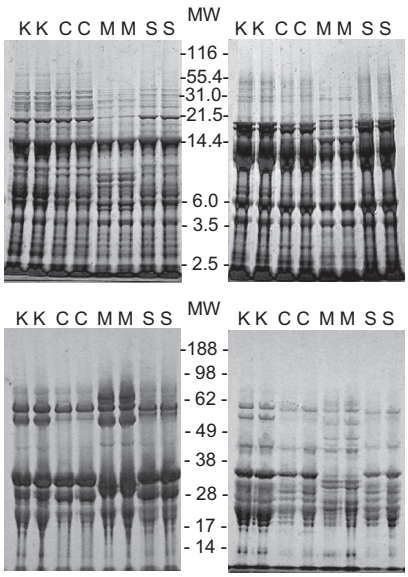

Fig. 1. Electrophoretic patterns of the investigated wheat varieties. The protein profiles were obtained by using SDS–PAGE electrophoresis for each protein fractions (A, albumins; B, globulins; C, gliadins; D, glutenins). *Abbreviations:* K, KAMUT® khorasan wheat; C, Claudio; M, Manitoba; S, SenatoreCappelli.

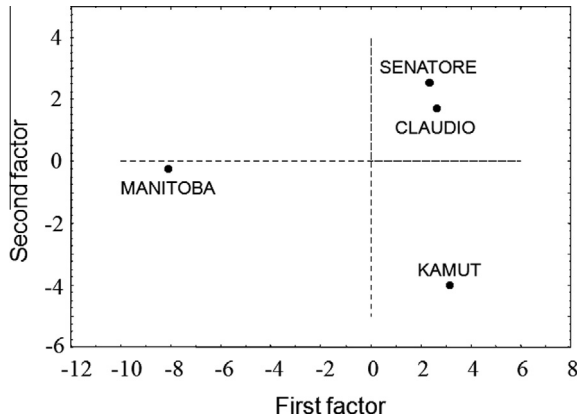

Fig. 2. Score plot of the first two principal components obtained from the electrophoretic patterns of the investigated wheat varieties. Claudio, KAMUT® khorasan wheat, Manitoba and SenatoreCappelli electrophoretic data were elaborated using the matrix of presence/absence of each detected protein band in the four protein fractions (albumins, globulins, gliadins, glutenins). PCA was carried out with three samples for each variety. In the Cartesian plan, the mean coordinates of each variety are shown.

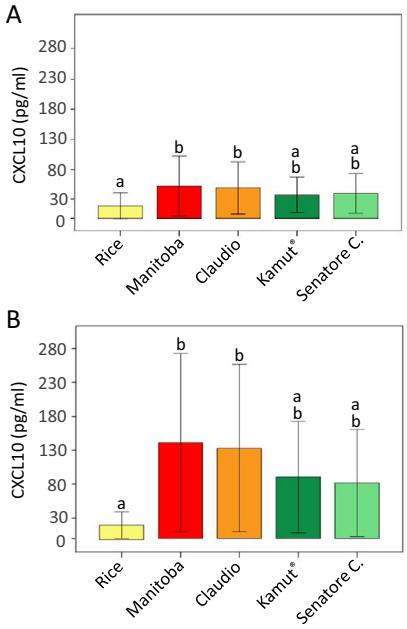

Fig. 3. CXCL10 secretion by cultured PBMC stimulated with different wheat protein extracts. PBMC obtained from healthy donors (A) or from NCGS patients (B) were stimulated for 24 h with total wheat protein extracts from different wheat cultivars. The amount of secreted CXCL10, measured by using Luminex® assay, is indicated as mean ± 1SD. a, $P < 0.001$ *vs.* Manitoba group. b, $P < 0.001$ *vs.* rice group.

instead of rice flour as a negative control, we obtained the same results (not shown), thereby confirming that the increased CXCL10 secretion seems to be due to gluten-containing cereal proteins. The responses of PBMC from NCGS patients stimulated with protein extracts from flour containing gluten compared to stimulation with gluten-free flours (rice) showed a significantly higher level of secreted CXCL10 ($P < 0.01$) for all four grains tested (Fig. 3 panel B). It is interesting that the responses of PBMC stimulated with protein extracts from gluten-containing flours significantly increased both in healthy controls and in NCGS patients. However, the levels of CXCL10 secretion under stimulation by gluten-containing flours were significantly higher in NCGS patients for all four grains tested ($P < 0.01$). When we analyzed separately the effect of the modern grains from that of the ancient grains, we found that modern dwarf varieties (Manitoba and Claudio) behave differently from the old varieties (SenatoreCappelli and khorasan) in terms of chemokine secretion. Analyzing the differences in terms of CXCL10 secretion stimulated by modern *vs.* ancient grains, we verified that these differences were always significant, both in NCGS patients and in healthy controls ($P < 0.01$). Analyzing the differences in CXCL10 secretion stimulated by the two modern grains (Manitoba *vs.* Claudio) we could not observe a significance either in healthy controls ($P > 0.33$) or in NCGS patients ($P > 0.17$). The differences between the two ancient grains used (SenatoreCappelli *vs.* khorasan wheat) showed no significance in healthy controls ($P = 0.22$) while in NCGS patients SenatoreCappelli seemed to stimulate CXCL10 secretion to a lesser extent than khorasan wheat ($P = 0.060$).

3.4. Proinflammatory chemokine decreased transepithelial electrical resistance (TEER) in NCM460 cells

To evaluate the effect of CXCL10 on intestinal barrier function in the *in vitro* model of the normal human colon cell line NCM460, we measured the TEER of cell cultures in Millicell systems. TEER of NCM460 cells increased in a time-dependent manner, achieving a plateau after 7 days, representing the establishment of tight monolayers (data not shown). NCM460 cell monolayers were therefore exposed to chemokine after barrier function was established. For determination of mucosal or serosal effects of CXCL10, cell monolayers were treated for up to 54 h with concentrations ranging from 0.01 ng/ml to 10 ng/ml of CXCL10 in the apical (mucosal) or basolateral (serosal) side and TEER was measured over time. The proinflammatory cytokine IL-1β (10 ng/ml) was also added on each of the two sides of separate inserts as a positive control, since IL-1β was previously reported to impair barrier functions in intestinal epithelia (Al-Sadi et al., 2013). Two-way ANOVA indicated that variations in TEER value significantly depended on cell treatment ($P < 0.0001$) and sampling time ($P < 0.0001$). As expected, a significant decrease in TEER values in comparison to control cells was obtained after exposure NCM460 cells in the apical (mucosal) side to 10 ng/ml IL-1β for 24 h ($P < 0.05$) up to 54 h, with a maximal effect after 48 h ($P < 0.001$) (Fig. 4 panel A). Further, addition of CXCL10 (0.01–10 ng/ml) to the apical side for up to 54 h resulted in a time- but not concentration-dependent decrease in TEER, with a significant effect starting after 48 h of exposure and similar for all the concentrations used when compared to the control group ($P < 0.001$), but without any significant difference from the IL-1β-induced TEER decrease at any concentration (Fig. 4 panel A). Exposure of the NCM460 cell monolayer basolateral (serosal) side to 10 ng/ml IL-1β induced a significant TEER decrease after 24 h ($P < 0.05$) reaching a maximal effect after 48 h ($P < 0.001$) in comparison to the control cells (Fig. 4 panel B). CXCL10 (0.01–10 ng/ml) added to the basolateral side (Fig. 4 panel B) induced both a time- and concentration-dependent decrease in TEER, with a significant effect starting at a concentration of 0.01 ng/ml and after 48 h of exposure when compared to the control group ($P < 0.001$). A broader TEER decrease against control cells ($P < 0.001$) was obtained when IL-1β and CXCL10 were present in the basolateral side, where the effects of the lower concentrations of CXCL10 were also significantly different from the IL-1β-induced TEER decrease.

The mechanism by which CXCL10, added in the basolateral (serosal) side, affected TERR was analyzed by real-time PCR, evaluating the expression of genes involved in tight junction formation. The basolateral side was chosen since it is where CXCL10 could be secreted by the intra-epithelial lymphocytes (IEL). CXCL10 determines the decreased intestinal permeability of NCM460 monolayer *via* the decreased expression of cadherin-1 (CDH1) mRNA ($P < 0.05$

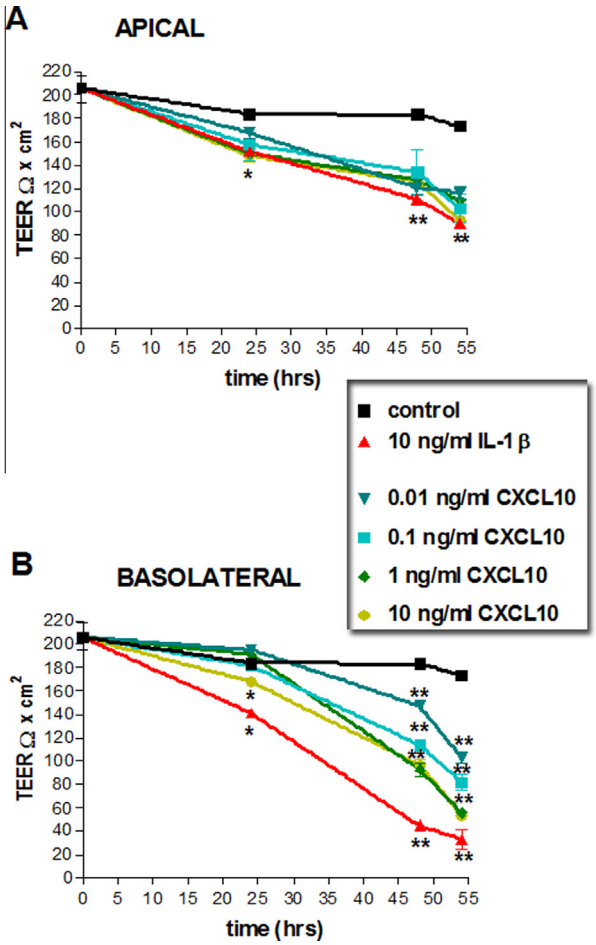

Fig. 4. Transepithelial electrical resistance (TEER) through NCM460 cells induced by CXCL10. CXCL10 and interleukin-1β (IL-1β) were added to the apical (A) or to the basolateral side of the Millicell insert monolayers (B). Increasing concentrations of CXCL10 were applied to the apical side (A) or to the basolateral side (B) of NCM460 monolayers. Data are the mean ± SEM of three experiments, performed in duplicate on individual NCM460 monolayers. $^{*}P < 0.05$ and $^{**}P < 0.001$ *vs.* control group.

at 10 ng/ml) and tight junctions protein 2 (TJP2) mRNA ($P < 0.05$ at 1 ÷ 10 ng/ml) (Table 2), which are all part of the tight junction complex (Lu, Ding, Lu, & Chen, 2013).

4. Discussion

Although awareness of NCGS has progressively increased, this diagnosis remains highly presumptive and based only on a clinical evaluation since no biomarker is yet available. As there is currently no serological test able to identify NCGS, antigliadin antibodies have been proposed as a possible indicator, being found in about 50% of NCGS patients (Carroccio et al., 2012; Sapone et al., 2012; Volta et al., 2012), but their usefulness is limited by their very low specificity (Volta et al., 2008). *In vitro* cytokine responses of PMBCs to gluten and rice have been studied in patients with IBS and gluten sensitivity, but this research failed to disclose a biomarker for NCGS (Vazquez-Roque et al., 2013). An overactive chemokine profile has recently been documented in the small intestinal mucosa of patients with untreated CD (Bondar et al., 2014), and this may explain the specific recruitment of the major cell populations that infiltrate the epithelium and lamina propria in CD. This finding prompted us to study the *in vitro* chemokine response of PMBC from NCGS patients to different cereal sources (including modern and ancient wheat genotypes). For this study we chose to use four different wheat grains, two modern and two ancient varieties, characterized by different protein profiles. Our previous studies disclosed that under organic agricultural management the old not dwarf wheat genotypes have the highest ability to accumulate proteins during grain filling (Di Silvestro et al., 2012). Although pattern analysis did not identify variety-specific protein profiles, it provided a description of variable qualitative composition of each wheat grain investigated. Protein variability may have different effects on digestibility and the immune-stimulatory activity of cereal products in the human body. Proteins extracted from the different flours were used undigested for stimulation of PBMC. Although these proteins are subjected to digestive processes in human gut, a fraction of these proteins may reach the small intestine undigested and be assimilated by transcytosis. Furthermore, *in vitro* methods of enzymatic digestion introduce an important experimental variable difficult to control, and do not guarantee comparable results with what really happens in the human gut. The decision to use all the protein fractions and not only the fractions containing gliadin and glutenin is due to the possibility that other wheat proteins, such as lectins, wheat germ agglutinin or α-amylase/trypsin inhibitors, activate innate immune responses involved in the mechanisms of gluten sensitivity (Dalla Pellegrina et al., 2009; Haas et al., 1999; Junker et al., 2012). In line with this hypothesis, many investigators prefer to use the term non-celiac wheat sensitivity instead of NCGS (Carroccio, Rini, & Mansueto, 2014).

The patented method used in this study for the assessment of CXCL10 secretion by the PBMCs of NCGS patients was deemed a possible biochemical test to help in the diagnosis of this disease. The responses of NCGS patients enrolled in this study, however, proved non-homogeneous. In particular, analyzing the differential secretion of CXCL10 ($CXCL10_{[Manitoba]} - CXCL10_{[Rice]}$) in NCGS patients we observed two different groups of patients: the first (44.7% of enrolled patients) showed differential responses higher or equal to 80 pg/ml, (threshold value to define positivity in the patented method) while the second showed differential responses lower than 80 pg/ml (55.3% of enrolled patients) even though on average higher compared to healthy controls. Although only a portion of NCGS patients can be defined as positive to this test, the method sheds light on a possible mechanism involved in the

Table 2
Expression of tight junction genes evaluated by real time PCR. mRNA expression was evaluated in NCM-460 cell monolayers 48 h after CXCL10 addition to the basolateral side of the Millicell insert monolayers and normalized against β-actin mRNA. Down arrows indicates statistically significant downregulations.

Gene	Fold-change relative expression ($2^{-2\Delta Ct}$)					
	CXCL10 1 ng/ml			CXCL10 10 ng/ml		
	Mean	SD	$P < 0.05$	Mean	SD	$P < 0.05$
Cadherin-1 (CDH1)	0.89	0.10	–	0.70	0.06	⇩
Tight junctions protein 1 (TJP1)	0.94	0.08	–	0.81	0.07	–
Tight junctions protein 2 (TJP2)	0.78	0.07	⇩	0.63	0.06	⇩
Tight junctions protein 3 (TJP3)	1.04	0.09	–	1.12	0.11	–

immune response to proteins of gluten-containing cereals. It is interesting that even a small percentage of healthy donors (10%) showed a differential secretion of CXCL10 higher or equal to 80 pg/ml. This suggests that the increased CXCL10 secretion by PBMC in response to contact with the proteins of gluten-containing cereals is a physiological mechanism but it tends to increase significantly in the NCGS population. This finding also indicates that modern grains are able to induce significantly more CXCL10 secretion by PBMC compared to the ancient grains. This is true both in healthy controls and in NCGS patients, although the differences in patients were much more evident in terms of chemokine amount.

CXCL10 is a chemokine produced mainly by neutrophils and macrophages but also by resident cells such as endothelial cells, able to recruit leukocytes (Ahmadi, Arababadi, & Hassanshahi, 2013). CXCL10 has been found overexpressed in colonic mucosa during active irritable bowel syndrome (IBS) (Ostvik et al., 2013), in the duodenal mucosa of untreated CD patients (Bondar et al., 2014), but also in various autoimmune diseases (Ahmadi et al., 2013) suggesting its active role in triggering tissue inflammation. Its over-expression in PBMC from NCGS patients, after wheat protein contact, if it happened also *in vivo* following wheat ingestion, could explain the peculiar distribution of IEL observed in NCGS duodenal biopsies (Villanacci, Lanzini, Lanzarotto, & Ricci, 2013). We analyzed the *in vitro* response of PBMC from 13 CD patients (of whom 8 on a gluten-free diet) to wheat protein, in terms of CXCL10 secretion. CD patients with active disease showed more than fivefold higher secretions of CXCL10 with respect to NCGS patients, while treated CD patients on a gluten-free diet showed a similar behavior to that of healthy controls (see Supplementary Fig. 1). Our data indicate that the chemokine CXCL10 actually contributed to the loss of intestinal epithelial barrier function associated with inflammation. Indeed, CXCL10 in the basolateral compartment of epithelial monolayers is also able to change TEER by decreasing both CDH1 and TJP2 mRNA expression. This may reduce tight junction strands, strand alterations and breaks (Lu et al., 2013). In the presence of increased intestinal permeability, undigested or partially digested wheat proteins could easily reach the immune cells in the intestinal wall and induce them to increase CXCL10 secretion on the serosal side, creating a sort of loop which tends to maintain the decreased intestinal permeability. Sapone et al. (2011) hypothesized the existence of decreased permeability in the intestine of NCGS patients. However, the indirect methods used and the overlap of intestinal disorders in NCGS and IBS patients, characterized instead by an increased intestinal permeability, shed some doubt on the existence of a decreased intestinal permeability in NCGS patients (Vazquez-Roque et al., 2013).

5. Conclusion

This study highlights a new wheat protein-induced mechanism triggering increased CXCL10 secretion in NCGS patients. For the first time, we stress that wheat proteins trigger pro-inflammatory mechanisms in a manner dependent on the wheat variety, but independent of both gluten content and gluten allergenic epitopes, as they are currently known. In other words, it is reasonable to assume that other hitherto unknown pro-inflammatory characteristics of wheat proteins exist and are much more prominent in the protein extracts of modern dwarf varieties, if compared to ancient wheat genotypes.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.12.061.

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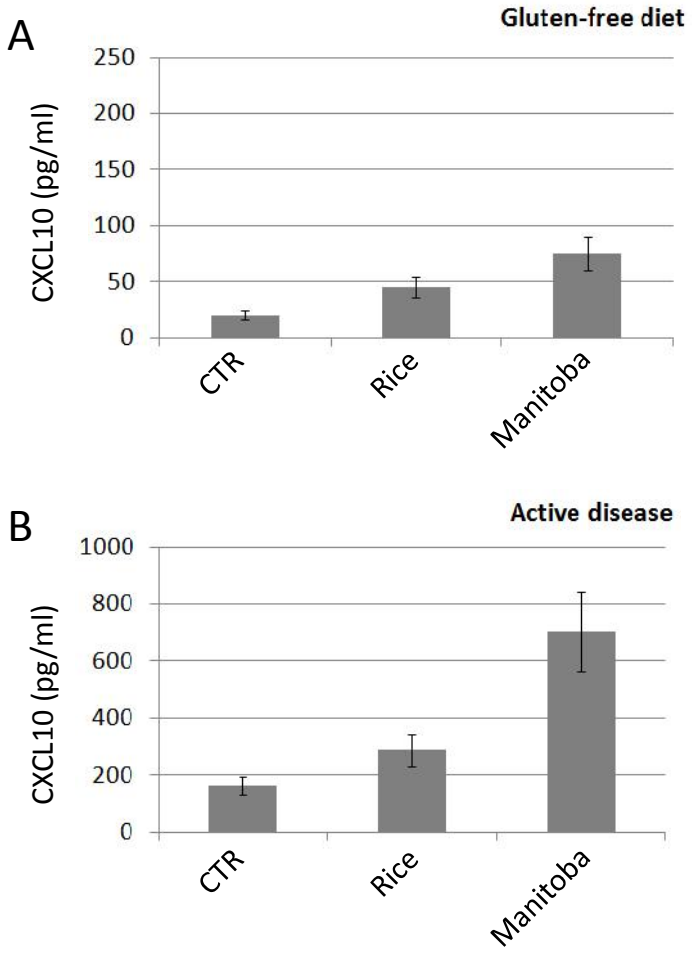
Supplementary Figure 1

In vitro response of PBMC, purified from CD patients, to wheat protein, in terms of CXCL10 secretion. CD patients were on a gluten-free diet for at least 12 months (8 patients, panel A) or had active disease (5 patients, panel B). Patients with active CD showed a significant increase in CXCL10 secretion respect to patients without active disease, for all the condition tested (*P*<0,05). In PBMC obtained from CD patients with active disease, CXCL10 secretions was very high even in basal condition (CTR), in the absence of cereal protein addition. In CD patients, we did not observe differences in the values of secreted CXCL10, by using different protein sources (Manitoba, Claudio, Kamut or SenatoreCappelli). Data are shown as mean ± SEM.