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Triticum monococcum amylase trypsin inhibitors possess a reduced potential to elicit innate immune response in celiac patients compared to *Triticum aestivum*

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ABSTRACT

Scope: Several studies reported a role of amylase/trypsin-inhibitors (ATIs) of common wheat species in promoting immune reactions. Here, we investigated in celiac disease (CD), the immunogenic properties of ATIs from diploid compared to common hexaploid wheats after an *in vitro* proteolytic hydrolysis.
Methods and Results: ATIs purified from two lines of diploid Triticum monococcum (TM), Monlis and Norberto-ID331, and from Triticum aestivum (TA), Sagittario, were digested with pepsin-chymotrypsin (PC) enzymes and analyzed using a proteomic approach, and subsequently their immune stimulatory properties were investigated on jejunal biopsies and T-cell lines from CD patients. No significant expression of IL-8 and TNF-α were detected on biopsies cultured with ATIs from TM in comparison with ATIs from TA. No significant IFN-γ production was observed in intestinal gliadin- raised T-cells in response to ATIs from both TM and TA wheats. Proteomic results revealed that both TM ATIs showed reduced stability to proteolytic enzymes compared to TA ones.
Conclusion: TM ATIs are substantially different from those of TA, showing a reduced ability to trigger the innate immunity in CD and a higher susceptibility to enzymatic hydrolysis.

1. Introduction

Amylase-trypsin inhibitors (ATIs) represent the bulk of salt-soluble wheat proteins (4% of the total wheat proteins) (Geisslitz, Longin, Koehler, & Sherf, 2020). ATIs are albumin proteins acting as plant defence proteins against insect pests and pathogen (Feng et al., 1996; Ryan, 1990) by inactivating their digestive enzymes (Franco et al., 2000). Because of a tightly folded conformation held together by five intrachain disulphide bonds, these proteins are highly resistant to protease activity (Schuppan & Zevallos, 2015). ATIs are a family of 17 structurally related proteins of 120–150 amino acids (Strobl et al., 1995); in modern hexaploid wheat species, with up to 19 different encoding genes for ATIs and similar protease inhibiting proteins have been identified by complete genome sequencing (Juhász et al., 2018). Conversely, the genome of diploid *T. monococccum* (TM) ATIs has not yet been fully sequenced, leading to limited knowledge of the proteome.

Within ATIs, the subtypes 0.19 and CM3, has been identified as strong inducers of innate immune responses in human and murine

Abbreviations: ATIs, amylase-trypsin inhibitors; CD, celiac disease; TM, *Triticum monococcum*; TA, *Triticum aestivum*; NCWS, non-celiac wheat sensitivity; TLR4, toll-like receptors 4; PC, pepsin-chimotrypsin; TCLs, gliadin-reactive T-cell lines; PBMCs, peripheral blood mononuclear cells; *cv*, cultivar; GD-BBM, gastric-duodenal and brush border membrane; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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monocytes, by triggering the toll-like receptors 4 (TLR-4), causing severe intestinal inflammations and symptoms (Cuccioloni et al., 2017; Junker et al., 2012; Zevallos et al., 2017). As a matter of fact, for their general *in vitro* and *in vivo* TLR4 stimulatory activity (Junker et al., 2012; Zevallos et al., 2017), ATIs are not only a long-sought nutritional trigger of innate immunity in celiac disease (CD), but were suspected to have more far-reaching pathogenic roles in the emerging epidemic of non-celiac wheat sensitivity (NCWS), and especially in autoimmune, chronic inflammatory intestinal and extraintestinal or metabolic disease (Aufiero, Fasano, & Mazzarella, 2018; Schuppan & Zevallos, 2015; Tilg, Koch, & Moschen, 2014).

Several studies have highlighted significant differences in ATIs content among different species of wheat (Reig-Otero, Manes, & Manyes, 2018; Wang et al., 2007). Interestingly, polyploid wheat species are known to have high concentrations of ATIs, compared with diploid wheat species (Aufiero et al., 2018; Geisslitz, Ludwig, Scherf, & Koehler, 2018; Zoccatelli et al., 2012), thus suggesting that the diploid wheat species could possess a lower toxicity for subjects suffering from NCWS.

We have previously investigated, in *in vitro* models, the immunological properties of gliadin protein from two monococcum *cvs*, Monlis and Norberto-ID331, in view of their possible use in CD patients (Gianfrani et al., 2012). We found that partially digested gliadin proteins extracted from both monococcum lines, Monlis and Norberto-ID331, induced adaptive immune response in CD patients, whereas the innate immune response could be elicited only by gliadin from Monlis *cv* (Gianfrani et al., 2012; Picascia et al., 2020). Subsequently, we have demonstrated, by proteomic analysis, that almost all immunotoxic gluten peptides from Monlis and Norberto-ID331, are *in vitro* degraded during digestion by gastric-duodenal and brush border membrane (GD-BBM) enzymes, whereas gluten immunogenic peptides from hexaploid *Triticum aestivum* (TA) resist intestinal digestion (Gianfrani et al., 2015). Similarly, recent result showed that gliadin of *Triticum durum*, was almost unaffected by the *in vitro* gastrointestinal digestion, while TM gliadin had a marked susceptibility to digestion (Di-Stasio et al., 2020).

It is noteworthy that the immunogenic potential of wheat gluten peptides depends on their resistance to gastrointestinal digestion (Shan et al., 2002).

Accordingly, in this study, by using organ cultures of jejunal biopsies and intestinal T-cell lines from CD patients, we evaluated, after an *in vitro* pepsin-chymotrypsin (PC) digestion, the immunogenic properties of ATIs obtained from two selected TM cultivars (*cvs*), Monlis and Norberto-ID331, and TA Sagittario cultivar (*cv*).

2. Material and methods

2.1. Sample preparation and pepsin-chymotrypsin digestion

ATIs from TA (Sagittario *cv*) and TM (Monlis and Norberto-ID331, *cvs*) were extracted from milled wheat seeds as described in Tundo et al. (2018). Briefly, 100 mg of flour was suspended in 1 mL of 50 mMTris-HCl pH 7.8, 100 mMKCl, 5 mM EDTA and shaked for 20 min. After centrifugation at 8000 g for 20 min, the supernatant was recovered and the procedure was repeated twice. Supernatant was mixed with five volumes of 0.1 M ammonium acetate in methanol and incubated overnight at −20 °C. After centrifugation at 8000*g* for 20 min, proteins were precipitated by addition of four volume of cold acetone, kept overnight at −20° and centrifuged at 8000*g* for 40 min. Finally, the pellet containing ATI fraction was washed twice with acetone and dried. Protein concentration was determined by Lowry method (Sigma Aldrich, Milan, Italy).

Subsequently, in order to perform the *in vitro* PC digestion, an aliquot of ATI-extracted samples (1 mg) was dissolved in 200 μL of 5% formic acid (pH3.0) and incubated at 37 °C with pepsin (1:50 enzyme/ substrate ratio) for 2 h. The sample was then dried by speed-vac and incubated with chymotrypsin (enzyme/substrate ratio of 1:50) in 400 μL of 100 mM sodium phosphate (pH 7.0), for 4 h at 37 °C. Immediately before tissue transglutaminase (TG)-mediated deamidation (only for T-cell assay), $CaCl_2$ and dithiothreitol were added to a final concentration of 10 mM and 20 mM, respectively; the pH was adjusted to 6.8, and sample was incubated with TG (at 1:10 ratio enzyme/substrate) at 37 °C for 6 h. Finally, sample was stored at −20 °C until further analysis.

2.2. Patients and organ culture

Jejunal biopsies were taken from 8 treated CD patients (8 women, median age 37 years, range: 23–54 years) on a gluten-free diet for at least two years with normal histology and negative serology (anti-endomysium antibodies) and without other gastrointestinal disorders (e.g. IBS and Crohn's disease).

Organ cultures were performed as previously reported (Gianfrani et al., 2015), in the presence of medium alone or PC-digest of gliadin from TA Sagittario *cv* (500 μg/mL), as negative and positive control, respectively, or PC-digest of ATIs (200 μg/mL) extracts from Sagittario, Monlis and Norberto-ID331.

Following 5 h of culture, the biopsy specimens were promptly processed for qRT-PCR analyses.

Patients undergone jejunal biopsy for routine checkup and they gave their full informed consent to the study. The protocol of the study was approved by the Ethical Committee of the S.G. Moscati Hospital, Avellino Italy (Register CECN/819 dated 03/21/2018).

2.3. RNA preparation and cytokine mRNA quantification by real-time RT-qPCR

After incubation biopsies were directly processed in the presence of 4U RNasin RNase inhibitor (Promega, Italy). Total RNA was isolated according to the manufacturer's instructions by using the TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Italy). Resulting RNA was recovered in DEPC water and the samples were stored at −80 °C until use. The overall quality of RNA preparation was evaluated by electrophoresis on denaturing agarose gel. RNA was quantified using the RNA HS Assay Kit on a Qubit® ver.3 instrument (Thermo Fisher Scientific, Italy). RNA (2 μg) were reverse transcribed in the presence of random hexamer primers by using the SuperScript® VILO cDNA Synthesis Kit (Life Technologies, Thermofisher Scientific, Italy) according to the manufacturer's instructions. Relative levels of IL-8, TNF-α and IFN-γ cDNAs were quantified by the PowerSYBR Green PCR Master Mix (Applied Biosystems, Thermofisher, Italy) employing an ABI-PRISM 7000 SDS instrument (Applied Biosystems, Thermofisher, Italy). RT-qPCR reactions were performed in triplicate in a final volume of 35 μL containing 2 μL of specific cDNAs. Primer sequences and cycling conditions for RT-qPCR have already been described (Iacomino et al, 2016, 2018). Gene expression was normalized to the level of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Serial dilutions of cDNA containing a known quantity of each transcript were used to generate a standard curve. The specificity of the amplification products was evaluated by melting curve analysis. The optimum dilution of cDNAs to obtain PCR products during the linear process of the amplification was determined in preliminary tests. Relative expression of transcripts was calculated by the ΔΔCT method using the Data Assist Software v3.01 (Applied Biosystems, Thermofisher, Italy). Results were reported as normalized mean expression ± SD.

2.4. Celiac gut T-cell lines

T-cell lines (TCLs) highly reactive to gliadin were established from duodenal biopsies of HLA-DQ2.5 positive CD patients (n = 5), as previously reported (Camarca et al 2009, 2017). Briefly, intestinal mucosa cells were cyclically stimulated with autologous peripheral blood mononuclear cells (PBMCs) and gliadin extracted from hexaploid wheat (Sagittario *cv*). More specifically, a tissue transglutaminase (TG) deamidated PC-digested gliadin was used as antigen. Long-term cultures

were obtained by cyclic stimulation with PHA (Roche) and IL-2 as growth factor (Roche). To evaluate the immunological properties of the ATIs preparations, the T-cells (3×10^4) were co-incubated with irradiated autologous EBV-transformed B cells (1×10^5) and PC-digested ATIs (100 μg/ml) purified from diploid TM wheat (Monlis and Norberto-ID331 *cvs*), and from TA wheat (Sagittario *cv*), in 200 μL of X-Vivo15/5% human serum (Lonza-Bio Whittaker, Verviers, Belgium). Incubation was run for 48 h, thereafter culture supernatants (50 μL) were collected for determination of IFN-γ, measured by a standard ELISA sandwich. Each antigen preparation was assayed in duplicates and in at least three independent experiments for each T-cell line. The sensitivity of the assay was 32 pg mL^{-1}.

This study with CD patients derived TCLs was approved by the ethical committee of S.G. Moscati Hospital, Avellino Italy (Register CECN/819 dated 03/21/2018), and Department of Pediatrics University Federico II of Naples (Register 343/17 dated 01/30/2018).

2.5. SDS–PAGE analysis

Proteins were loaded onto a precast 12% polyacrylamide gel (Bio-Rad, Mini-Protean) under non-reduction conditions, before and after PC digestion. After migration, the gel was stained in a water solution containing 0.05% (w/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol, 0.7% (v/v) acetic acid, and destained in water solution containing 10% (v/v) acetic acid and 40% (v/v) methanol. The gel was imaged with a scanner and processed using the LABScan software 3.00 (Amersham Bioscience). Protein bands were characterized according to Di Stasio et al. (2017) and analysed by LC-HR-MS/MS analysis, using the same parameter previously reported (Mamone & Iacomino, 2018). MS data were elaborated using ProteinProspector (http://prospector.ucsf.edu/prospector.htm) restricting the search to *Triticum* database extracted from the Uniprot (https://www.uniprot.org/) (updated to November 2019). Database searching parameters for identification of SDS-PAGE protein bands were the following: Met oxidation, pyroglutamic for N-terminus Gln; a mass tolerance value of 10 ppm for precursor ion and 0.01 Da for MS/MS fragments; no modification of cysteine residues; no proteolytic enzymes was selected for searching.

2.6. Sample preparation for shotgun proteomic analysis

The ATIs extract (100 μg) was dissolved in urea 7 M, TBS buffer containing 10 mM DTT for 1 h at 55 °C. After reduction, samples were centrifuged at 5000*g* for 30 min and 14 mM IAA was incorporated and incubated for 30 min in the dark. To quench the alkylation reaction, DTT was added up 10 mM final concentration and incubated for an additional 15 min at room temperature. Hydrolysed samples were diluted with ten volumes of 50 mM AMBIC pH 8.0, containing 1 mM $CaCl_2$ and incubated overnight at 37 °C with modified proteomic grade trypsin (Promega, Madison, WI, USA) using a 1:20 ratio (w/w, trypsin-protein). Resulting peptides from ATIs extract and PC digested ATIs were desalted using Sep-Pak® C18 cartridges (Waters, Milford, MA, USA) and finally dried in a Savant SpeedVac centrifuge, before LC-HR-MS/MS analysis.MS data elaboration and database searching parameters were the same described for SDS-PAGE analysis.

2.7. Statistical analysis

Data were expressed as mean ± SD. Differences between groups were determined using a one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparisons. A value of $*p < 0.01$ and of $**p < 0.05$ were considered to be statistically significant. Statistical analysis was executed using GraphPad Prism 6 Software (GraphPad, San Diego, USA).

3. Results

3.1. Digested amylase/trypsin-inhibitors from T. monococcum wheats were unable to activate both innate and adaptive immunity in CD.

The immunological properties of PC-digested ATIs purified from TM wheats, Monlis and Norberto-ID331 *cvs*, and from hexaploid wheat, TA Sagittario *cv,* were compared by using the organ culture system and gliadin reactive T-cell lines from celiac gut mucosa.

Following culture stimulation with PC-ATIs from TA, a significant increase in the levels of IL-8 ($p < 0.01$) and TNF-α ($p < 0.05$), were detected by qRT-PCR (Fig. 1A and B, respectively).

Likewise, PC gliadin (containing ATI) induced a statistically significant increase in IL-8 ($p < 0.01$) and TNF-α production ($p < 0.01$).

Conversely, no significant differences of IL-8 and TNF-α expression were noted in biopsy specimens cultured with PC-ATIs of both Monlis and Norberto-ID331 in comparison with in medium alone, as negative control (Fig. 1A and B, respectively), which indicated that PC-ATIs from both TM *cvs* failed to stimulate innate immunity in CD mucosa.

To further exclude any adaptive immune-stimulatory property of ATIs from TM wheats, we investigated the IFN-γ production in T-cells lines isolated from intestinal biopsies of CD patients in response to digested ATIs samples from both TM *cvs* (Monlis and *Norberto*-ID331) and TA *cv* (Sagittario).

All ATIs preparations, were assayed on T-cells either in wild type or deamidated forms, and were treated with tTG before being tested, as it is well known that deamidation by this enzyme increases the binding affinity of antigens to CD-associated HLA molecules, thus representing a key process to trigger T-cell mediated adaptive immune responses in CD mucosa.

As expected, high levels of IFN-γ were produced by gliadin-raised T-cells in response to PC-gliadin extracted from hexaploid wheat (median IFN-γ 582 pg/ml) (Fig. 2, panel A), used as the internal positive control.

Conversely, no significant IFN-γ detection was measured in response to PC digests of TM ATIs (both Monlis and Norberto-ID331). TM digested ATIs did not stimulate celiac gut T-cells, neither in wildtype (medianIFN-γ: 77 and 82.6 pg/ml, respectively) nor after deamidation (89.5 and 80 pg/ml, respectively). Similar results have been observed when T-cells were stimulated with PC ATIs from TA Sagittario *cv* (0.6 and 1.49) (Fig. 2, panel A).

In order to exclude any cell toxicity of the ATIs preparations, a cytotoxicity assay on healthy donor peripheral blood mononuclear cells (PBMCs) was performed with negative results (data not shown).

The *ex vivo* organ culture experiments further excluded the involvement of ATIs in evoking adaptive immune-stimulatory responses.

Following the incubation of CD mucosal explants with PC digested ATIs, from both modern and diploid wheat *cvs*, no significant increases of IFN-γ were documented. Conversely, a marked increase in IFN-γ level was obtained in response to PC-gliadin of TA, used as the positive control (Fig. 2, panel B).

Overall, our findings demonstrated that PC digested ATIs from either the two TM wheats or TA wheat were unable to stimulate celiac gut T-cells to produce the pro-inflammatory cytokine, so excluding their potential involvement in the adaptive immune response in CD.

3.2. SDS PAGE and LC-HR-MS/MS analysis of bands

ATI extracts from TA wheat (Sagittario *cv*) and TM (Monlis and Norberto-ID331 *cvs*) were evaluated by SDS-PAGE analysis. Samples were run before and after PC digestion (Fig. 3, panel A and B, respectively). Mass-spectrometry based analysis was employed for protein/band identification (labelled from 1 to 19 and from *a* to *f*, Fig. 3, panel A and B, respectively). Protein migrated in upper gel region of both TA and TM species were mainly identified as avenin-like protein and gliadins which were co-extracted with ATIs buffer (Supplementary table 1).

The bands between 7 and 20 kDa of TA Sagittario were assigned as

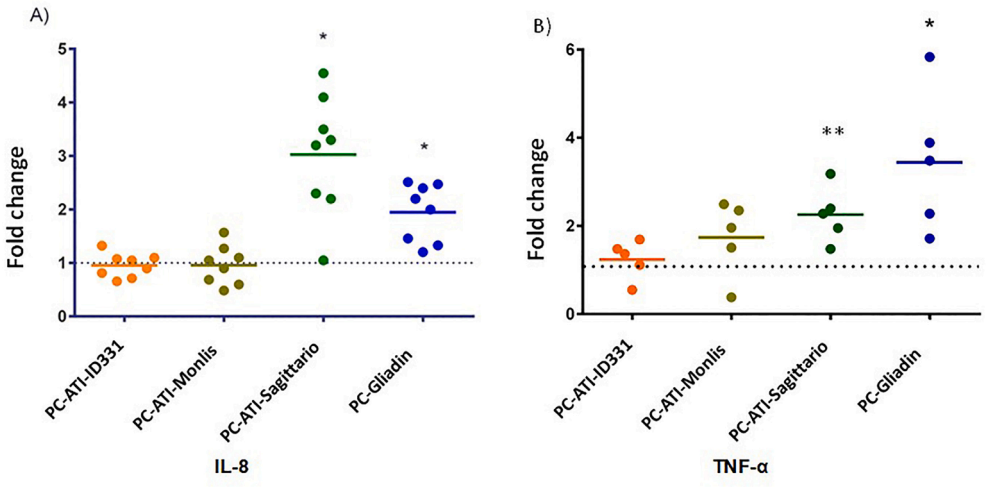

Fig. 1. Innate immune response of PC-ATIs from different wheat varieties. ATIs stimulus on biopsies of treated CD patients was evaluated by RT-qPCR following 5 h exposure in the presence of PC-ATIs extracts from different wheat varieties. A) In biopsies cultured in the presence of PC ATIs from SAG or PC gliadin, IL-8 levels (n = 8) resulted significantly higher than in biopsies cultured in medium alone (dotted line). B) Similarly, in the presence of PC ATIs from SAG or PC gliadin, TNF-α levels (n = 5) resulted significantly higher than in biopsies cultured in medium alone (dotted line). Each spot represents result from single patient. Dashes indicate the mean values. *p < 0.01;**p < 0.05.

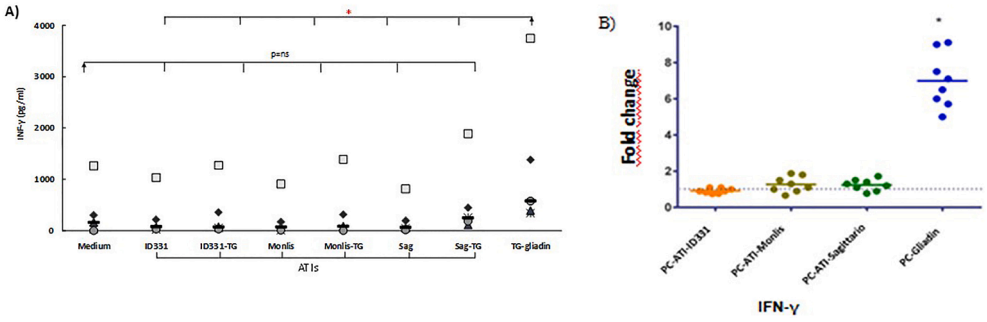

Fig. 2. Adaptive immune property of PC-ATIs from different wheat varieties. A) IFNγ responses obtained after *in vitro* stimulation of intestinal T-cell lines (TCLs) from (n = 5) CD patients with ATIs purified from diploid *T. monococcum* wheat, (Monlis and Norberto-ID331 *cvs*), and from hexaploid *T. aestivum* (Sagittario *cv*) wheat either in naïve and deamidated forms (100 μg/ml). IFN-γ production was detected in cell supernatants by ELISA. Each symbol represents the responses as mean of duplicates of each individual patient. Red line indicted the median values of IFN-γ released by T-cells. *p < 0.05, p = ns not significant. B) Determination of ATIs bioactivity evaluated by RT-qPCR following 5 h exposure of biopsies from treated CD patients (n = 8) in the presence of 200 μg/ml of PC ATIs extracts from different wheat cultivars. IFN-γ was induced only by PC-gliadin. Each spot represents result from single patient. Dashes indicate the mean values. *p < 0.01. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

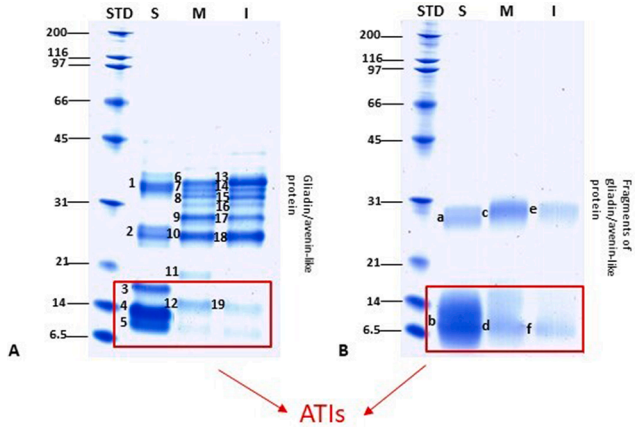

Fig. 3. SDS-PAGE. SDS-PAGE (12%) analysis carried out in non-reducing conditions of ATIs from *T. aestivum* (S = Sagittario cv) and *T. monococcum* cvs (M = Monlis and I = Norberto-ID331 cv). STD indicates the molecular marker (numbers reported are expressed in kDa). Before (panel A) and after PC digestion (panel B). Band excised from SDS gel (panel A), were analyzed by LC-HR-MS/MS and identified using ProteinProspector software (Supplementary Table 1).

ATIs subtype 0.28, 0.19, 0,53, CM1, CM2, CM3, CM16 and CM17, CMX1/CMX3 and WCI (Supplementary table 1). These findings were in line with a previous report (Dupont, Vensel, Tanaka, Hurkman, & Altenbach, 2011).

Conversely, electrophoretic profiles of TM showed faint bands in the ATIs region, demonstrating that ATIs from TM were significantly different from those of TA (Fig. 3 panel A and B, supplementary table 1). In addition, in both TM *cvs*, only an ATI protein isoform, assigned to accession number C5J3R4, was detected before PC digestion (Supplementary table 1).

Notably, an in-depth identification of TM ATIs by proteomics were also hampered by the poor knowledge of genome sequences. Interestingly, the SDS-PAGE analysis of PC digested ATIs from TA highlighted the high resistant to proteolytic enzymes since intense Comassie bands were still visible (Fig. 3, panel B). Conversely, PC digestion meaningfully affected ATIs from TM, which were no longer detected (Fig. 3, panel B).

In order to identify peptides released from PC digestion of ATIs samples was employed the gel-free proteomic (shotgun) analysis. The shotgun analysis demonstrated that most of ATIs from TA were not significantly affected by PC enzyme digestion, since a large number of their hydrolyzed peptides were detected by LC-HR-MS/MS, so confirming their high stability to proteases (Table 1). Conversely, few peptides arising from TM ATIs (C5J3R4) were detected (Table 1). These data confirmed the lower content in ATIs peptides in TM, Monlis and Norberto-ID331 *cvs*, resulting in greater susceptibility to proteolytic digestion (PC) compared to common wheat TA.

To improve our data, a detailed quantitative proteomic analysis to better characterize TM *cvs* is now in progress.

Table 1
ATIs proteins (expressed by more than one peptide) within TA (Sagittario *cv*) after PC digestion.

Accession	Protein name	Num. Unique	Protein Mw (Da)
Q19A44	Dimeric alpha-amylase inhibitor	19	13468.7
Q4U1985	Dimeric alpha-amylase inhibitor	16	15071.6
C3UWI4	Dimeric alpha-amylase inhibitor	2	15308.7
P16851	Alpha-amylase\trypsin inhibitor CM2	26	15459.9
P17314	Alpha-amylase\trypsin inhibitor CM3	54	18221.5
Q41540	CM17 protein	46	15989.7
P83207	Chymotrypsin inhibitor WCI	16	12944.1
P82977	Subtilysin-Chymotrypsin inhibitor WSCI	3	9325.8
P81713	Bowman-Birk type trypsin inhibitor	2	7962.5

ATIs proteins (expressed by more than one peptide) within TM (Norberto-ID331 *cv*) after PC digestion

Accession	Protein name	Num. Unique	Protein Mw (Da)
Q19A44	Dimeric alpha-amylase inhibitor	2	13468.7
P17314	Alpha-amylase\trypsin inhibitor CM3	2	18221.5
C5J3R4	Trypsin inhibitor	4	1614.9

4. Discussion

TM, the most ancient wheat in nature, without genetic improvement or variety hybridization, is the progenitor of hexaploid wheat, resulting for natural breedings of TM (AA genome) with other diploid wheat species (BB and DD genomes). From agronomical aspect, TM has a tiny crop and a reduced yield in weight at harvest but a higher herbicide and diseases resistance compared to hexaploid wheats (Løje, Møller, Laustsen, & Hansen, 2003).

Recently, it has been reported that wheat ATIs, a family of protease-resistant proteins, which activate innate immunity, are present in high concentrations in modern wheat, compared with diploid wheat (Zoccatelli et al., 2012). Particularly, a study by Junker et al. (2012) found that ATIs engage TLR-4/MD2/CD14 complex and release innate pro-inflammatory cytokines in myeloid cells of both patients with CD and non-diseased controls. Moreover, the authors also showed that the addition of exogenous ATIs to the organ culture of jejunal biopsies from treated CD induced an increase in IL-8 and TNF-α mRNAs levels compared with healthy controls. Subsequently, the same group found in TLR4-responsive mouse and human cell lines that older wheat variants, had lower bioactivity than modern wheat (Zevallos et al., 2017).

To date, nothing is known about the immunogenic properties of ATIs from TM varieties in CD.

Previously, we found that the great majority of gluten immunotoxic peptides from two selected TM *cvs*, Monlis and Norberto-ID331, were degraded during an *in vitro* digestion by human GD-BBM, whereas, TA immunotoxic gluten peptides resisted to intestinal digestion (Gianfrani et al, 2012, 2015; Picascia et al., 2020). Furthermore, a recent *in vivo* study confirmed the reduced immunogenicity of wheat gluten from TM, Norberto-ID331, compared to common wheat after a short oral wheat challenge (Picascia et al., 2020).

In this study, by using organ cultures of jejunal biopsies and intestinal T-cell lines from CD patients, we evaluated the immunogenic properties of ATIs obtained from two selected TM *cvs*, Monlis and Norberto-ID331, and TA Sagittario *cv* after an *in vitro* proteolytic digestion (PC).

We found that PC-digested ATIs purified from modern wheat induced IL-8 and TNF-α secretion in organ culture of jejuna mucosa of treated CD patients, confirming resistance of ATIs to enteric proteases and their stimulatory potential *in vivo* (Junker et al., 2012). In contrast, the capability of ATIs from TM to stimulate innate immunity was meaningfully affected by proteolytic digestion. Actually, following the incubation of CD mucosal explants with PC-digested ATIs from Norberto-ID331 and Monlis, IL-8 and TNF-α mRNA levels were significantly decreased in the duodenal mucosa, compared to the PC-digested ATIs from Sagittario.

It is well known that in CD both the innate and adaptive immune branches mediate the immune response against wheat gluten proteins. Accordingly, to exclude conceivable adaptive immune-stimulatory property of ATIs, we further investigated, for the first time, the IFN-γ production in T-cells isolated from intestinal biopsies of CD patients in response to PC digested ATI samples from both TM and TA wheats. Our findings demonstrated that native or PC-digested ATIs from TA *cv* and from both two TM *cvs*, were unable to stimulate celiac gut T-cells to produce the pro-inflammatory IFN-γ, excluding their potential involvement in the adaptive response.

Moreover, no IFN- γ expression was obtained in biopsy specimens cultured with PC-digested ATIs from TA *cv* and from both TM *cvs*, confirming the ATIs inability to induce IFN-γ production.

Besides, our study confirmed the resistance to protease digestion of ATIs from modern wheat compared to TM wheat. In detail, SDS-PAGE and shotgun analysis showed a different protein profile in modern wheat compared to ancient ones. We confirmed the presence of all the subtypes of ATIs which characterizes modern wheat, as previously reported (Dupont et al., 2011; Geisslitz et al., 2018; Zevallos et al., 2017). Conversely, according to previous data showing a poor representation of TM ATIs (Geisslitz et al, 2018, 2020), in both TM *cvs*, only one ATI subtype was detected. Interestingly, PC digestion dramatically affect the ATIs content from both TM *cvs* than TA, resulting in a decrease in protein yield and disappearance of typical ATIs SDS-PAGE bands, certainly supported by the lower ATIs content in diploid wheats.

The resistance to the gastrointestinal digestion process is a prerequisite for wheat proteins to reach the lamina propria intact and exert their harmful action on the immune competent T-cells in subjects suffering from wheat-related disorders. The stability to hydrolysis by human digestive enzymes of ATIs from hexaploid wheat, affects the activation of mucosal innate immune response, leading to the production of IL-8 and TNF-α. In contrast, ATIs from diploid wheat seems to be more susceptible to *enzymatic hydrolysis*. Therefore, the proteolytic digestion of ATIs from TM resulted in a failure to induce the innate immune response.

5. Conclusions

In the past decade, several studies have highlighted the central role of innate immunity in the induction of tissue damage in the CD mucosa.

We have here demonstrated that ATIs from TM are sufficiently different from those of common TA wheat, so to determine a reduced innate immune response in CD, as shown by the lower expression of IL-8 and TNF-α in an *in vitro* organ culture model. Moreover, we observed in both Monlis and Norberto-ID331 *cvs*, higher susceptibility to enzymatic hydrolysis of ATIs, compared to TA wheat.

Although clinical studies demonstrated that diploid wheat are not tolerated by CD patients, data herein reported are encouraging findings that point to a better tolerability of ancestral diploid TM for subjects suffering from NCWS, an emerging clinical entity triggered by an adverse reaction to certain wheat components and characterized by gastrointestinal and by extra-intestinal manifestations. Nowadays, the pathogenesis of NCGS is not yet well understood, but the activation of the innate immune system has been implicated. Therefore, clinical studies on NCWS subjects, to assess the effects of a TM wheat–based foods diet are warranted.

CRediT authorship contribution statement

Giuseppe Iacomino: Methodology, Investigation. **Vera Rotondi Aufiero:** Methodology, Investigation. **Luigia Di Stasio:** Methodology,

Investigation. **Stefania Picascia:** Methodology, Investigation. **Nunzia Iannaccone:** Methodology, Investigation. **Nicola Giardullo:** Methodology, Investigation. **Riccardo Troncone:** Supervision. **Carmen Gianfrani:** Conceptualization, Methodology, Investigation, Writing-original draft. **Gianfranco Mamone:** Conceptualization, Methodology, Investigation, Writing-original draft. **Giuseppe Mazzarella:** Conceptualization, Writing-original draft.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2021.110386.

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