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Tracking the Fate of Pasta (*T. Durum* Semolina) Immunogenic Proteins by *In Vitro* Simulated Digestion

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ABSTRACT

The aim of the present study was to identify and characterize the celiacogenic/immunogenic proteins and peptides released during digestion of pasta (*Triticum durum* semolina). Cooked pasta was digested using a harmonized *in vitro* static model of the oral-gastro-duodenal digestion. The course of pasta protein digestion was monitored by SDS-PAGE, and gluten proteins were specifically analysed by Western-blot using sera of celiac patients. Among the allergens, non specific-lipid transfer protein was highly resistant to gastro-duodenal hydrolysis, while other digestion stable allergens such as α -amylase/trypsin-inhibitors were not detected being totally released in the pasta cooking water. To simulate the final stage of the intestinal degradation, the gastroduodenal digesta were incubated with porcine jejunal brush border membrane hydrolases. Sixty-one peptides surviving the brush border membrane peptidases were identified by liquid chromatography-mass spectrometry, including several gluten-derived sequences encrypting different motifs responsible for the induction of celiac disease. These results provide new insights into the persistence of wheat-derived peptides during digestion of cooked pasta samples.

KEYWORDS: alpha-amylase/trypsin inhibitor; Brush Border Membrane; celiac disease; gluten; *in vitro* human digestion; nsLTP; pasta; wheat allergy;

INTRODUCTION

Pasta is a valuable example of a foodstuff in which the starting raw material (flour) undergoes a technological process and high temperatures in boiling water before being consumed. Pasta is among the most popular foods in the world because of its convenience, nutritional properties and palatability.¹ It is regularly consumed in most Mediterranean countries as well as in Europe and in the United States. In Italy, the name “pasta” can be used only for 100% *Triticum durum* wheat products (semolina).—Several components may influence pasta quality; in particular, the storage proteins of the wheat endosperm determine dough strength, extensibility and stability.² Based on their differential solubility, wheat proteins can be classified into water/salt-soluble albumins-globulins, and water/salt-insoluble gluten, accounting for about 20% and 80% of endosperm proteins, respectively. Wheat gluten comprises the monomeric gliadins, sub-grouped into α/β - γ - ω -gliadin, and the polymeric glutenins occurring as high (HMW) and low (LMW) molecular weight subunits.³

Wheat gluten and related proteins from oat, rye and barley are the main factor triggering celiac disease (CD), in genetically susceptible individuals.⁴ Due to their high content of proline residues, gluten proteins are relatively resistant to gastrointestinal digestion, so that large fragments reach sufficient concentrations at the gut epithelium to trigger immunological pathways in CD patients.⁵ Gluten and non-gluten wheat proteins are also immunogenic determinants of IgE-mediated allergic reactions in sensitized individuals, including respiratory allergies (bakers’ asthma and wheat-dependent exercise-induced anaphylaxis).⁶

During technology production, pasta proteins undergo modifications and establish chemical/physical inter-protein links and interactions with other compounds.⁷ Dough formation and drying temperature are key factors prompting protein aggregation *via* disulfides or other inter-chain covalent bonds. Cooking causes further structural changes while a significant portion of the proteins may be dispersed in the cooking water.⁸

By affecting structure and properties of the food matrix, all of these factors have a critical impact on digestibility, determining the survival or the release of components with positive (health-promoting activity) or adverse (allergy, intolerance, toxicity) effects on human health.⁹

The aim of this study was to investigating the fate of pasta proteins upon *in vitro* simulated gastrointestinal digestion, with special focus on the immunogenic potential of survived polypeptides. In spite of the previous attempts to track the fate of pasta subjected to gastrointestinal digestion¹⁰⁻¹² the current experimental workflow was devised according to conditions and parameters inferred from *in vivo*. Indeed, here we used the *in vitro* multi-compartmental model, including oral, gastric, and duodenal phases, recently harmonized in the framework of the EU Infogest Cost Action.¹³ This protocol was integrated with a simulation of the final intestinal degradation step using porcine jejunal brush border membrane (BBM) vesicles.

MATERIALS AND METHODS

Chemicals

Enzymes used for *in vitro* human digestion were purchased from Sigma (St Louis, MO, USA) in line with those indicated by the Infogest protocol.¹³ Urea, DTT, 2-(N-morpholino)ethanesulfonic acid (MES), Tris-HCl, *p*-toluene-sulfonyl-L-arginine methyl ester (TAME), sodium phosphate, ammonium bicarbonate, acetic acid, sulfosalicylic acid, trichloroacetic acid (TCA) and “modified Lowry assay kit” were from Sigma-Aldrich as well. Egg lecithin was from Lipid Products (Redhill UK). HPLC-grade solvents were provided from Merck (Whitehouse Station, NJ, USA). Electrophoresis reagents were all from Invitrogen (Carlsbad, CA, USA).

Pasta cooking

A sample of commercial brand of pasta (spaghetti type, 100% durum wheat semolina) was processed according to *Hoebler et al.*¹⁴ Pasta (100 g) was cooked in 1.0 L water (tap water) containing 3.5 g NaCl for 10 min.

Whole protein extraction

An aliquot of raw pasta and cooked (undigested) pasta was grounded and whole protein was extracted twice in 1 mL of 7 M urea, 80 mmol/L Tris-HCl, pH 7.5, under magnetic stirring overnight at 4°C. The sample was centrifuged at 10.000 g for 20 min. The surnatant was collected and desalted with Econo-Pac® 10 DG columns (Biorad, Milan, Italy), eluting with 25 mmol/L ammonium bicarbonate. “Modified Lowry methods assay”, determined a protein concentration of 6.42 mg/mL and 3.76 mg/mL for raw pasta and cooked pasta respectively. Finally, samples were lyophilized and stored at -20°C.

***In vitro* human digestion.**

After cooking, the pasta was immediately subjected to human simulated *in vitro* digestion using the static model system as developed in the framework of the Infogest COST Action.¹³ Simulated salivary fluid (SSF), simulated gastric fluid (SGF) and simulated intestinal fluid (SIF) were prepared according to the harmonized conditions.¹³

Oral phase: Mastication was simulated by using a common manual mincer. A 5g quantity of cooked pasta was mixed with 5 mL of SSF and minced for 2 min. SSF included 1500 U mL⁻¹ of human salivary amylase.

Gastric phase: The oral digesta was mixed with SGF (50:50 v:v), and added of gastric liposome (egg lecithin prepared in a vesicular form) up to 0.17 mmol/L final concentration, 2000 U/mL of SGF porcine pepsin and 80U/mL of SGF fungal lipase. The pH of the digesta was adjusted to 3, and the incubation was carried out for 2 hours in a shaking incubator at 37°C and 170 rpm. Samples were collected at different times (0, 30, 60, 90 and 120 min), the reaction stopped by raising the pH to 7.0 and proteolysis was monitored by SDS-PAGE analysis.

Duodenal phase: Gastric digesta (50:50 v:v) was added to the SIF containing 10 mmol/L bile salts (5 mmol/L sodium taurocholate, 5 mmol/L sodium glycodeoxycholate and 1.8 mmol/L egg lecithin final concentration) and pancreatin. The amount of pancreatin added was calculated based on the measured activity of trypsin up to 100 U of trypsin/mL final volume. Trypsin activity was determined by TAME assay.¹⁵ Sample was incubated for 2 hours in a shaking incubator at 37°C. Aliquots were collected at different times (0, 5, 15, 30, 60, 90, 120 min). The reaction was stopped by raising the pH to 10 and the digestion was monitored by SDS-PAGE analysis.

Intestinal brush border membrane (BBM) digestion.

BBM (650 mU/mg) enzymes were obtained from pig jejunum according to the method by Shirazi-Beechey *et al.*¹⁶ Triplicate aliquots of each sample after duodenal digestion were two-fold diluted with 0.1 mol/L sodium phosphate buffer, adjusted to pH 7.2 and incubated for up to 4 h after BBM

supplementation; the reaction was stopped by heating for 5 min in a boiling water bath. The sample (aliquots of 1 mL) was then chromatographed using an Econo-Pac® 10 DG size exclusion chromatography (SEC) pre-packed column (Bio-Rad Laboratories, Hercules, CA, USA) by eluting with 25 mmol/L Tris-HCl, pH 7.0, to separate the protein from the low MW peptide fraction. Effluents were collected in fractions of 1 mL and monitored by the UV-absorbance at 280 nm (Ultrospec 2100 pro, Amersham Biosciences). The peptide (MW<6 kDa) fractions were pooled and further desalted using Sep-Pak® C18 pre-packed cartridges (Waters, Milford, MA, USA) washed with aqueous 0.1% TFA (v/v) and eluted with 70% ACN (v/v)/0.1% TFA (v/v).

Analysis of protein recovered from the pasta cooking water.

After the pasta was cooked as described above, 250 mL of the cooking water was recovered and dialyzed overnight with a dialysis membrane (molecular weight cut-off=3 kDa) against 0.1 M acetic acid. The sample was then lyophilized and resuspended in 1 mL of 50 mmol/L Tris-HCl, pH 7.5. The sample was further freed from low molecular weight compounds by filtration through Econo-Pac® 10 DG columns (Biorad) by eluting with 25 mmol/L ammonium bicarbonate, pH 8.5.

***In vitro* digestion of salt soluble proteins.**

Salt soluble proteins (albumin/globulin) were extracted from raw and cooked undigested pasta, according to Mamone *et al.*¹⁷ Proteins were then digested by simulating gastric, pancreatic and BBM *in vitro* digestion.¹⁸ In order to confirm the stability of selected wheat albumins/globulins, in this case we used a simplified model of gastrointestinal digestion that minimized the presence of interfering compounds.¹⁷

SDS-PAGE analysis.

SDS-PAGE was performed on 15% or 4–20% gradient gels run for 35 min at 200 V in MES buffer. 1ml of oral-gastric and oral-gastric-duodenal aliquots were centrifuged at 4000xg for 20min. The

supernatant (75 μ L) was with diluted 25 μ L of NuPAGE LDS (Invitrogen) sample buffer (4X) containing DTT at final concentration 5 mmol/L. The pellet was resuspended in 100 μ L NuPAGE LDS sample buffer (1X) containing 5 mmol/L DTT. Samples were heated for 10 minutes at 70 °C, and 20 μ L of each gastric or duodenal fraction, respectively, was loaded into adjacent wells in the gel. Whole proteins (15 μ g) extracted from raw and cooked undigested pasta were also run.

The gels were fixed with 50 mL of sulfosalicylic acid/TCA in a 10.5:36 ratio in 300 mL of water for 30 minutes, washed twice with water and stained using colloidal Coomassie G250.

The protein bands of interest were excised from the gels with a scalpel, placed in Eppendorf tubes, washed twice with 50 μ L of Milli-Q-purified water and destained with 50 mmol/L ammonium bicarbonate in 50% (v/v) aqueous acetonitrile. The destained spots were dehydrated by submersion in acetonitrile, dried under vacuum and incubated in 20 μ L of 50 mmol/L ammonium bicarbonate containing 12 ng/ μ L of trypsin on ice. Gel pieces were allowed to rehydrate for 45 min; afterwards the excess of trypsin solution was removed and the protein bands were incubated overnight at 37°C. The tryptic digests were extracted in 40 μ L of 50% acetonitrile, 2.5% formic acid and concentrated to a tenth of the original volume in a vacuum centrifuge before mass spectrometry analysis.

Western blotting (WB) analysis.

The proteins separated by SDS-PAGE (15%) were electroblotted onto nitrocellulose membranes (Transblot nitrocellulose, Bio-Rad) at 120 V for 60 min. The membrane was blocked for 1 h at room temperature (RT) using 5% non-fat milk in TBS-T (Tris saline buffer plus 0.05% Tween-20) for 1 h. The membranes were then incubated overnight at 4 °C with pooled sera (N=8) of celiac subjects (range 5-36, average 24 years old) or from healthy individuals (N=4), as the negative controls, diluted 1/500 in TBS-T. Sera were pooled in order to average the individual variability of the immune response. After washing with TBS-T, monoclonal peroxidase-conjugated anti-human IgA antibody (Sigma) diluted in blocking solution (1/10,000) was applied to the membrane for 2 h

at RT. The membrane was extensively rinsed with TBS-T (3×10 min) and finally with TBS (1×10 min) before development. The immunoreactive spots were detected using a chemiluminescence reagent (ECL® Plus, GE Healthcare) and X-ray film (Kodak, Chalons/Saône, France).

Mass Spectrometry analysis.

Nanoflow LC-ESI MS/MS analysis was carried out using an Ultimate 3000 HPLC (Dionex, Sunnydale, CA, USA) coupled to a Q-STAR mass spectrometer (Applied BioSystems, Framingham, USA). The eluents were (A) 5% ACN in 0.1% FA and (B) 80% ACN in 0.08% FA. The peptides were loaded into a C₁₈ loading cartridge (LC Packings, USA) and separated with a C₁₈ PepMap100 column (15 cm length, 75 µm ID and 300 Å [LC Packings]) using a 5-40% linear gradient of B over 60 min at a constant flow rate of 300 nL/min. LC-MS/MS experiments were performed in the information-dependent acquisition (IDA) mode. The precursor ions were selected using the following MS to MS/MS switch criteria: ion m/z > 400, charge states 2 to 4, intensity exceeding 15 counts and ion tolerance of 50.0 mmu; former target ions were excluded for 30 s. CID was used to fragment the multiple-charged ions, and nitrogen was used as the collision gas. The raw spectra files were used to generate text files in mascot generic file format (.mgf), which were submitted to the Mascot ver. 2.3 (<http://www.matrixscience.com>) and Batch-tag (Protein Prospector, University of California San Francisco, USA) search engines.

Tryptic peptides from SDS-PAGE bands were identified by Mascot (version 2.3) search engine (<http://www.matrixscience.com>) using the following criteria: databases, NCBI and Uniprot; taxonomy, other green plants; type of search, MS/MS ion search; enzyme, trypsin; fixed modifications, carbamidomethyl; variable modifications, methionine oxidation and pyro-Glu formation through N-terminal loss of ammonia at Gln; mass values, monoisotopic; parent tolerance, 0.08 Da; ms/ms tolerance, 0.15 Da; number of maximum missed cleavages, 1.

Peptides released from oral-gastric-duodenal and BBM hydrolysis were identified using the Batch-Tag Web tool of Protein Prospector (<http://prospector.ucsf.edu>), taxonomically restricting the

188 search to *Triticum*. The same search criteria used above were utilized in this case. The resulting
189 peptide identifications were validated by manual inspection of the MS/MS spectra. The peptide
190 entries were considered identified when the measured molecular weight corresponded to the
191 expected value and a sequence of at least three consecutive b- or y-ions occurred in the spectrum.
192 For each of the protein/peptide fractions, nanoLC–MS/MS analyses were run in triplicate, and only
193 matches occurring in all the three analyses were included in the entry list.

194

3 RESULTS

Pasta proteins were digested using an *in vitro* static oral-gastro-duodenal (OGD) digestion model that mimics human physiological conditions.¹³ The digestion steps were carried out under simulated physiological conditions, including digestive enzymes and their activity, pH, lipid and salt concentrations. Digestion times were selected according to the average transit time of chyme in the respective compartments. A more exhaustive justification of the conditions used is given in Minekus *et al.*¹³ Our model also included an additional step with porcine intestinal BBM hydrolases that simulates peptide degradation at the level of the jejunum.¹⁶ Assessment of the peptide stability to the BBM peptidases has already demonstrated *in vivo* consistence, intended as correspondence *in vitro-in vivo*, since it supported the discovery of some of the most important determinants of CD.^{5,19}

SDS-PAGE analysis.

The kinetics of simulated gastric digestion was assessed by SDS-PAGE (Figure 1). The protein patterns arising from digestion of pasta at 0-120 min were compared to the intact proteins extracted from raw undigested pasta. The pattern of the uncooked sample showed the typical protein components of *Triticum* wheat flour, which can be grouped into HMW-GS (65-90 kDa), ω -gliadins (44-55 kDa), LMW-GS (30-40 kDa, respectively), and α/β - and γ -gliadins (30-45 kDa).²⁰ Bands of albumins/globulins were also detected, including β -amylase(s) (60 kDa) and alpha-amylase/trypsin inhibitor (ATI) (14-16 kDa).²¹

In cooked pasta, at the beginning of the gastric digestion, wheat proteins were not detected, except for a faint band, most likely because the proteins remained insoluble in the saline buffer or were still embedded in the starchy matrix of pasta (Figure 1, panel A). The main band at 35 kDa was identified as porcine pepsin by LC-MS/MS of tryptic derived peptides (not shown). Because of its high concentration in SGF, pepsin masked the pasta proteinaceous material with similar molecular weight and interfered with the data evaluation. The increased accumulation of low molecular weight polypeptides (7-17 kDa) indicated the progressive degradation of proteins during digestion from 30

to 120 min. These polypeptides were made soluble by the proteolytic action of pepsin on the insoluble parent proteins.

After a 2-h incubation, the simulated gastric digests were subjected to simulated duodenal digestion, and aliquots were sampled over the 0-120 min time course for SDS-PAGE analysis (Figure 1, panel B). The progressive appearance of newly formed low molecular weight polypeptides was the result of extensive hydrolysis by pancreatic enzymes. However, the complex protein pattern generated by the action of porcine pancreatin, used at such a high proteases-to-substrate ratio, could bias the clear detection of the products of digestion²².

Identification of proteins resistant to OGD digestion

A proteomic approach was employed for the analysis of the polypeptides that survived the OGD digestion model. The numbered bands of the D120 lane were excised from the gel and selected for proteomic analysis (Figure 1, panel B). Except for the broad band at ~5kDa that escaped identification, the protein components were characterized by LC-MS/MS analysis. The list of significant protein hits from the Mascot search is shown in Table 1. No gluten polypeptides were detected, while the main bands were from the pancreatic enzymes. The occurrence of multiple bands for a single enzyme is most likely due to autolysis or mutual digestion among pancreatin hydrolases. The large relative amount of pancreatin enzymes also complicated the identification of proteins survived to digestion.²²

Non specific-lipid transfer protein (nsLTP), one of the metabolic proteins (albumins and globulins), was the only wheat-derived gene product identified. No other protein belonging to the albumin/globulin fraction was identified, nor was gluten (gliadin and glutenin). These results indicated that, with the exception of nsLTP, the pasta proteins were digested relatively rapidly into small peptides that escaped detection by SDS-PAGE electrophoresis.

Analysis of IgA-reactive proteins in OGD digesta.

The immunoreactivity of the OGD digesta against IgA-antibodies from sera pool of CD patients was assayed (Figure 2). The OGD digesta effluents (both collected at 2 hours) were centrifuged, and the resulting soluble and insoluble fractions were run on SDS-PAGE for immunoblotting analysis. The total protein extracted from the cooked pasta before digestion was used as the positive control. The sera pool of health subjects did not bind to any band (not shown), confirming the specificity of the immunoreaction. As shown in Figure 2, the major IgA-reactive undigested proteins were detected between 30 and 97 kDa. The soluble gastric digesta (lane 3) showed a main immune-reactive band with a MW of 45 kDa. Conversely, the distribution of IgA-binding proteins in the insoluble gastric sample (lane 2) was compatible with the total protein patterns (lane 1), indicating that the majority of the immunoreactive pasta proteins, in all likelihood comprising the gliadins fraction, were not solubilized and were pelleted by centrifugation. This might be a result of a chemically reversible heat-induced protein aggregation that occurs during pasta processing. Such aggregation can decrease protein solubility in the acid gastric environment, as previously demonstrated.²³ However, after simulated OGD hydrolysis only a few faint bands were still detected in both the insoluble (lane 4) and soluble (lane 5) fractions, indicating that the majority of IgA-binding proteins were degraded by pancreatic proteases.

Identification of pasta-derived peptides.

To identify the most stable polypeptide sequences and in order to narrow the list of epitopes that are potentially involved in eliciting immune reactions, it is necessary to consider the action of intestinal BBM enzymes located on the polarized apical surface of the duodenal and jejunal enterocytes. The use of BBM is a fundamental step in assessing intestinal stability because BBM contains a series of endo- and exo-peptidases with a range of different cleavage specificity, which have significant effects in gradually shortening the surviving OGD peptides into di-, tri- and oligopeptides as well as free amino acids. The OGD digesta were incubated with BBM enzymes purified from the pig

intestinal jejunum and then fractionated by SEC, allowing the separation of the large pasta polypeptides from the >6 kDa fraction (here referred to as the “peptide” fraction). The peptide fraction was analyzed via LC-MS/MS followed by software-assisted database searching. A comprehensive list of the sixty-one peptides surviving the digestion of pasta is reported in Table S1 (Supporting Information). The highest number of peptides arose from the γ -gliadin and LMW-glutenin proteins, which presented several motifs associated with the induction of CD. For instance, QGQGIIQPQQPA, QQPQQPFPPQ, and PQQSFPQQQ fragments are generally classified among the main gliadin toxic motifs, commonly indicated as γ -II (or glia- γ 30),²⁴⁻²⁶ γ -VIIa²⁵ and γ -I,²⁵ respectively.

The peptide α -gli 31-55 (LGQQQPFPPQQPYQPQPFPSQQPY), together with its shortened form, namely α -gli 31-51, were also released in the digesta. This gliadin domain harbors the toxic motifs 31-43 and 44-55.²⁷ The potential stability of α -gli 31-55 to the GI degradation has been already demonstrated in our previous studies.^{18,28} The α -gli 56-68 peptide, largely used as a model peptide in CD investigations, was degraded into shorter fragments (i. e. QPFQPQLP and QPFQPQL), with lower immunogenicity compared to their parent peptide.^{29,30} A few fragments arising from HMW By8 and Bx7 glutenin were also found, while β -amylase peptides were the only non-gluten peptides identified. Most likely due to their prompt full digestion, no other peptides deriving from the soluble protein fraction (albumins and globulins) occurred in detectable amounts. Nevertheless, the possibility that the overabundant amount of gluten-derived peptides suppressed the detection of low-abundance fragments of metabolic proteins can not be ruled out.

A more comprehensive characterization of the peptide fractions was severely challenged by a series of concomitant factors, including the extreme heterogeneity of the parent proteins, the possible heat-induced modifications of proteins, the non-tryptic nature of the peptides and the not predictable cleavage specificity of protein hydrolysis due to the action of a pool of proteases and peptidases. Indeed, the faint MS/MS production of b- and y-series fragments and the abnormally intense

internal fragment ions, combined with the lack of amino acid constraints at the N- and C-terminal position, decreased the identification rate. Thus, although more than 200 peptides with MWs between 400 and 6000 were detected in the LC-MS chromatogram, two thirds of these peptides remained unidentified due to low database scores.

Identification of protein lost in the cooking water.

Because nsLTP was the only protein identified in the digested sample, we assumed that salt-soluble proteins might have leached into the water during the cooking process. To track the fate of expected and well established potential allergens,³¹ the proteins released into the cooking water were extracted and analyzed by SDS-PAGE (Figure 3). The protein profile was compared to the salt-soluble fractions isolated from the raw and cooked (undigested) pasta. The identity of each polypeptide was assessed by proteomic analysis (not shown). Minor changes were observed among the individual fractions, indicating that only a part of the salt-soluble proteins were dispersed in the water during cooking. The bands at 9 kDa of wheat nsLTP were detected in all samples. A noticeable difference was due to the bands at estimated Mr 14 kDa, which were missing in the cooked pasta pattern (lane 3). These proteins, identified as the ATIs -CM3 (gi|100834), -CM16 (P16851) and -CM2 (gi|123956), detected in the raw pasta sample (lane 2) were entirely retrieved in the cooking water (lane 1). This finding may explain why ATIs were not detected in the digesta either as whole proteins or as peptides, despite their high-abundance in flour and their relative stability to hydrolysis, functionally ascribable to their role as protease-inhibitors.

To confirm the digestion stability of ATIs, a simulated gastrointestinal digestion was performed specifically on the albumin/globulin fractions extracted from the raw and cooked pasta. In this case, proteins were digested using a simplified model on purified fraction¹⁸, to minimize the interferences due to both the matrix and high amount of pancreatin enzymes. SDS-PAGE analysis (Figure 4) showed the complete degradation of the albumins/globulins from raw pasta, with the exception of nsLTP and the ATIs, which exhibited high resistance to proteases (lane 3). In contrast, in digested

324 cooked pasta, nsLTP was the only salt soluble protein still detectable (lane 4), while ATIs were no
325 longer detected (lane 4). This experiment confirmed that ATIs, though resistant to digestion are lost
326 in the cooking water, so that nsLTP was the only salt soluble protein found in the digested pasta
327 (Figure 1).

328

DISCUSSION

The identification of the (poly)peptides surviving the *in vitro* model has important biochemical, immunochemical and potentially allergenic implications. Previous attempts in this sense did not take into account the effect of the matrix in a real food system,^{18,28, 32} which is expected to dramatically influence the accessibility of proteins to proteases as well as their susceptibility to hydrolysis, thereby contributing to determine the bioaccessibility and hence the bioavailability of wheat peptides. Alternatively, simplified digestion models did not adequately mirror the physiological process of protein hydrolysis have been carried out on several wheat based-systems and may result in a poor *in vitro-in vivo* correspondence.¹²

In the present study, accurately mimicking the human physiological conditions, we determined the effect of the simulated digestion on proteins from pasta, prepared according to the ordinary home cooking conditions. Although proteins are not affected by the oral phase hydrolysis, chewing and the action of human α -amylase are important to shear and break down the starch gel, so making the protein cleavage sites accessible to proteolysis. However, a consistent pellet containing particles of undigested pasta was still evident after gastric digestion. SDS-PAGE and WB analysis showed that the majority of wheat proteins were not solubilized in the acidic environment of the gastric juice and were resistant to pepsin action. This behavior is related to the heat-induced modifications of the wheat proteins.⁷ As demonstrated in previous studies, thermal modifications occurring during industrial dehydration (i.e., up to 110 °C) and domestic cooking processes induce the aggregation of wheat proteins by disulfide bond formation and through hydrophobic interactions, which combine with the intrinsically low water solubility of the gluten proteins.^{23,33} In support of these data, HPLC analysis was carried out on the ethanol-extracted gliadins before and after cooking the pasta, as shown in figure S1 (Supplemental Information). Ethanol buffer extracts only monomeric gliadins that do not contain covalent cross-links.³ Before cooking, the gliadin profile was somewhat similar to that of the gliadins extracted from flour, while the chromatogram dramatically changed after cooking. In particular, the peaks of ω -gliadins, which do not contain cysteine residues, were

practically unaffected by cooking, while those relevant to α/β - and γ -gliadins significantly decreased after cooking, suggesting that these proteins underwent massive thermally induced aggregation (Figure S1).

Following the action of duodenal enzymes, the digesta became liquid, though highly viscous, due to the partial hydrolysis of starch by pancreatic α -amylase. After 2 hours of simulated duodenal digestion the wheat gluten proteins were almost completely degraded. WB analysis showed that few soluble and insoluble IgA-reactive bands resisted to OGI digestion. These polypeptides were not identified by proteomic analysis, also as a consequence of the thermally induced protein modifications (i.e cross-linking, Maillard reaction, deamidation).

The OGD digestion process releases immunologically-active peptides that are able to reach the intestinal lumen and trigger immune responses in susceptible individuals. However, before crossing the gut epithelium, the peptides are further hydrolyzed by endo-/exopeptidases of BBM enterocytes, which typically degrade peptides to single amino acids or short di- or tri-peptides. Due to their high percentage of proline residues, many gliadin and glutenin peptides are resistant to BBM digestion. The α -gliadin derived 33-mer peptide, which is considered the most immunogenic and highly BBM-resistant peptide, was not detected because it is not expressed by *T. durum*, being characteristic of the D genome of *T. aestivum* (soft wheat).¹⁹ However, several peptides containing epitopes that can trigger adaptive responses in CD patients were identified in the digesta resulting from the complete digestion. For instance, peptide 31-55, harboring toxic sequences (31-43 and 44-55), was found to be stable to digestion.^{18,28} On the other hand, the repertoire of the pasta-derived OGD-BBM resistant peptides still remains partial, as only approximately one third of the peptides were clearly identified.

The incomplete identification can be primarily attributed to i) the formation of high molecular mass peptides not easily detectable with the MS-based approaches; ii) the incompleteness of wheat protein database annotation; iii) intrinsic technical challenges, as explained in the Results section, and iv) the high dynamic range of the components, which limits the identification of low-abundance

peptides, also due to ion suppression effects. It has to be underlined that in this research we used a representative commercial brand of pasta. The high genetic polymorphism of wheat cultivars might be a further source of heterogeneity and gluten derived peptides could diverge to some extent if pasta samples produced with different wheat accession are analyzed.³⁴

The nsLTP was the only protein virtually unaffected by OGD digestion. This protein can elicit IgE-mediated sensitization in predisposed allergic individuals.³¹ Previous reports demonstrated that nsLTP is highly resistant to proteolysis along with other albumin/globulin proteins, especially those functionally acting as proteases/inhibitors, such as ATIs.^{35,36} These latter proteins were not found in cooked pasta because they were almost completely dispersed in the cooking water. ATIs are known to be the major allergen responsible for baker's asthma.⁶ *In vitro* studies demonstrated that ATIs could also play a major role in triggering the innate immune response in CD. More recently, it has been suggested that ATIs may fuel inflammation and immune reactions in other intestinal and non-intestinal immune disorders, in particular "non-celiac gluten sensitivity".³⁷ This would indicate that eating pasta, as it is normally prepared, has a lower allergenic potency than raw flour and probably bakery products, and should be less "toxic" for "gluten-related disorder" patients who show symptoms after eating pasta. This finding is also in line with the results by De Gregorio *et al.*⁷ who observed that processing of wheat flour decreases the allergenic potency of the major salt-soluble wheat proteins (IgE binding-capacity) in the resulting food products.

In conclusion, we demonstrated that the proteins from cooked pasta are completely hydrolyzed during *in vitro* digestion, releasing gliadin and glutenin peptides, which contain amino acid sequences harmful to CD patients. Among the wheat IgE allergens, nsLTP is highly resistant to both cooking and hydrolysis, while important allergen proteins such as ATIs are lost during pasta cooking.

The application of the harmonized static digestion model that mimics *in vivo* physiology, provided new insights for establishing the evolution of wheat proteins during pasta processing, cooking and

digestion in humans. Thus, we also provide insights into the possible immunological implications and the possible effects of gluten resistant epitopes on human health.

ABBREVIATION. ATI, α -amylase/trypsin inhibitor; BBM, Brush Border Membrane; CD, Celiac disease; nsLTP, nonspecific lipid transfer protein; OGD, oral-gastro-duodenal; SGF, simulated gastric fluid; SIF, simulated intestinal fluid; SSF, simulated salivary fluid;.

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Supporting Information Available: Supporting materials comprise the supplementary Table S1 and Figure S1. Supplementary Table S1 displays a list of low molecular weight peptides identified by LC–ESI-MS/MS. Supplementary Figure S1 shows the HPLC analysis of gliadin extracted from

431 pasta before and after cooking. This material is available free of charge via the Internet at
432 <http://pubs.acs.org>.

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Figure legends:

Figure 1: SDS PAGE (4-20%) analysis of gastric (panel A) and pancreatic (panel B) digestion of cooked pasta. MW markers (lane STD) are indicate on the left-hand side of gel. Whole proteins from raw pasta were also run (lane 1). The number on bottom denote the incubation times in minutes for gastric (G) and duodenal (D) digestion. Bands from intestinal digestion (line D120min) were cut and digested with trypsin for proteomic analysis.

Figure. 2: WB analysis against sera of celiac disease of whole cooked pasta proteins (lane 1), and insoluble (lane 2 and 4) and soluble (lane 3 and 5) phase of gastric and intestinal digestion respectively.

Figure 3: SDS-PAGE (15%) analysis of cooking water protein (lane 1) and salt soluble proteins extracted from raw (lane 2) and cooked (lane 3) pasta. MW markers (lane STD) are on left. Arrows indicate the region of ATIs and nsLTP. ATIs were identified as ATIs -CM3 (gi|100834), -CM16 (P16851) and -CM2 (gi|123956). nsLTP was identified as P16851.

Figure 4: SDS-PAGE (15%) analysis of salt soluble before and after simulated GI digestion. Lane 1 and 3: albumin/globulin proteins extracted from raw pasta before (lane 1) and after digestion (lane 3). Albumin/globulin proteins extracted from cooked pasta before (lane 2) and after (lane 4) digestion. Arrows indicate the region of ATIs and nsLTP proteins. ATIs were identified as ATIs -CM3 (gi|100834), -CM16 (P16851) and -CM2 (gi|123956). nsLTP was identified as P16851.

Table I: Identification of protein bands extracted from the gel shown on Fig 1

Band label	Accession number	Protein name	Species	MW(Da)	Score	Matched peptides
1	AMYP_PIG	Pancreatic α -amylase	Sus scrofa	57791	431	17
2	CBPB1_PIG	Carboxypeptidase B	Sus scrofa	47750	337	14
3	CBPA1_PIG	CarboxypeptidaseA1	Sus scrofa	47320:	193	11
4	AMYP_PIG	Pancreatic α -amylase	Sus scrofa	57791	157	7
5	TRYP_PIG	Trypsin	Sus scrofa	25078	87	8
6	AMYP_PIG	Pancreatic α -amylase	Sus scrofa	57791	74	6
7	TRYP_PIG	Trypsin	Sus scrofa	25078	41	7
8	CBPB1_PIG	Carboxypeptidase B	Sus scrofa	47750	74	5
9	TRYP_PIG	Trypsin	Sus scrofa	25078	69	4
10	1AN1_E (gi 3318722)	Chain E, Leech-Derived Tryptase Inhibitor	Sus Scrofa	24142	61	4
11	P24296	nsLTP	Triticum aestivum	9494	101	5

Figure 1

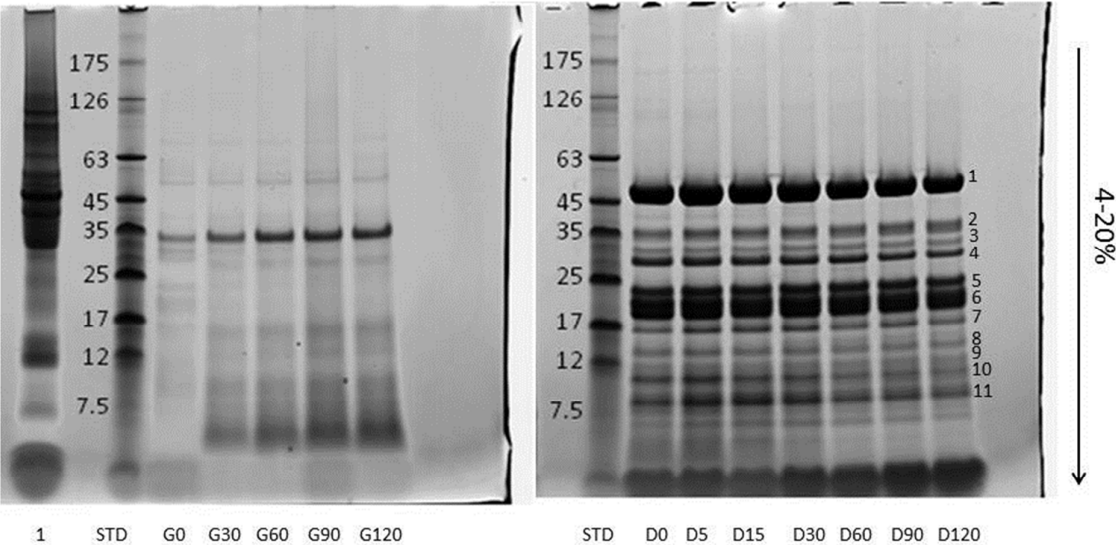


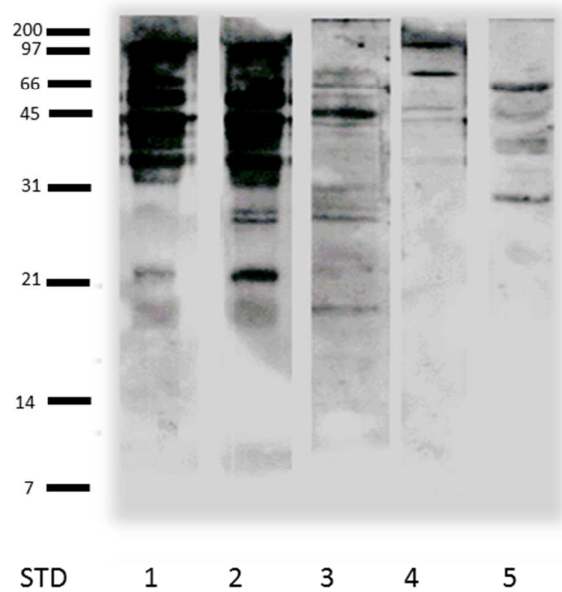
Figure 2

Figure 3

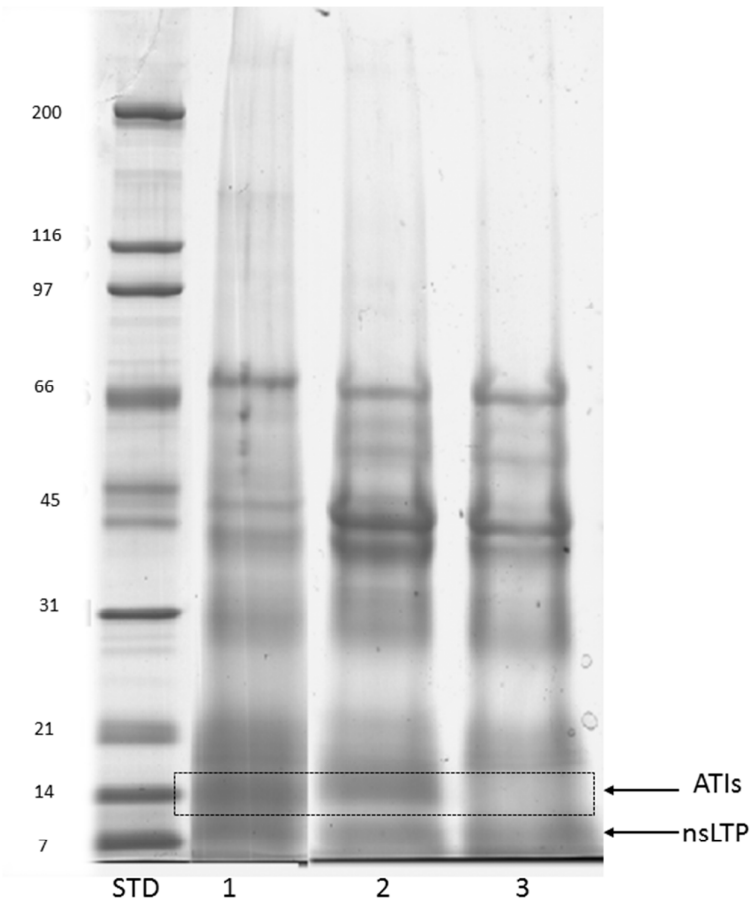


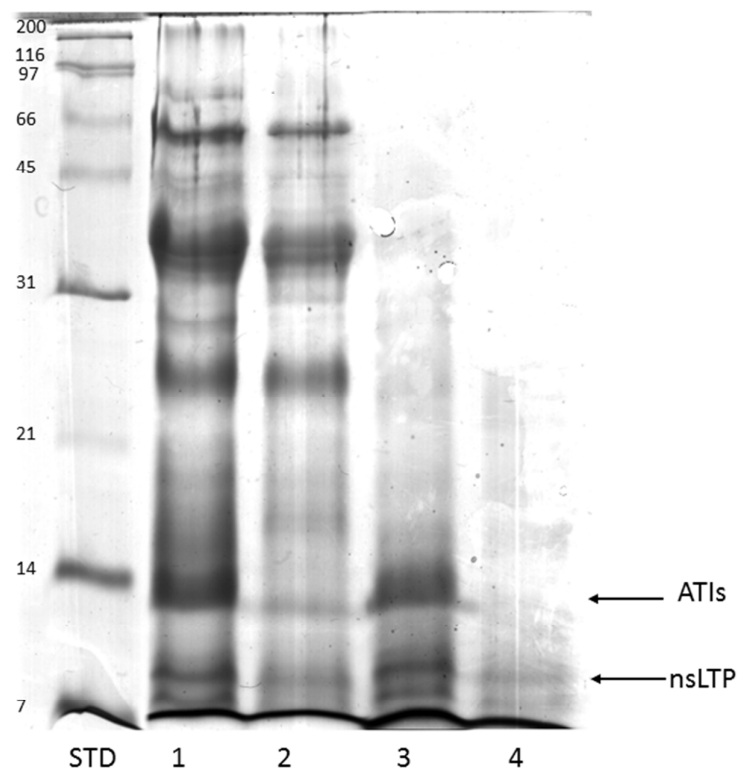
Figure 4

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