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Influence of wheat flour refinement on oxidative modifications and pro-inflammatory properties of amylase-trypsin inhibitors

Massimiliano Cuccioloni[1]✉, Luigi Moriconi[1], Helena Juricic[1], Filippo Cocchioni[1], Francesco Maria Grasselli[1], Ziqi Liu[1,2], Massimo Nabissi[3], Elena Vittadini[1], Anna Maria Eleuteri[1] & Mauro Angeletti[1]

Amylase/trypsin inhibitors (ATIs), cereal proteins that confer natural resistance against pest and pathogen digestive enzymes, have been implicated in TLR4-mediated innate immune activation and wheat-related intolerances in vitro, though their causal role and genetic susceptibility factors in non-coeliac wheat sensitivity remains unclear. Given the established influence of the oxidation state of triggering proteins on TLR4 binding and activation, this study investigated how common industrial milling methods (roller versus stone milling) affect the proinflammatory potential of ATIs from a single commercial wheat variety. Integrated immunometric and targeted proteomic analyses showed that milling technology and processing parameters are critical determinants of the inflammatory potential of wheat flour, as they shape ATI redox status; notably, proinflammatory cytokine secretion paralleled oxidative modification rather than ATI abundance.

Keywords ATI, Wheat processing, Protein oxidative modifications, Cytokines response

Wheat is a major staple food cultivated worldwide and represents a primary source of dietary calories, proteins, fibers, and vitamins. Regular consumption of wheat-based products has been associated with a reduced risk of nutrition-related diseases such as type-2 diabetes, cardiovascular disorders, and certain cancers[1–4]. In terms of protein composition, approximately 80% of total wheat grain proteins are gluten, a viscoelastic network of disulfide-cross-linked polypeptides that provides structural consistency to dough and enables the retention of carbon dioxide during fermentation[5–7].

Wheat intake can also trigger immune-mediated adverse reactions, including coeliac disease (CD)[8] and IgE-mediated allergies. Beyond gluten storage proteins, the soluble albumin/globulin fractions contain key allergens such as lipid transfer proteins (LTPs) and amylase-trypsin inhibitors (ATIs), which drive food allergy, baker's asthma, and other wheat-dependent manifestations[9]. This diversity of antigenic protein classes underlies the broad clinical heterogeneity in wheat-related intolerance and allergic disorders.

ATIs serve relevant physiological roles in the wheat grain, participating in structural and metabolic processes and acting as defense proteins against pests and microbial threats by inhibiting exogenous hydrolytic enzymes[10,11]. These inhibitors exhibit a stable, disulfide bond-rich architecture and are resistant to proteolysis and denaturation. ATI isoforms differ by aggregation state (monomeric, dimeric, or tetrameric) and by electrophoretic or solvent-partition properties. The monomeric 0.28, dimeric 0.19 and 0.53, and tetrameric CM2, CM3, and CM16 isoforms predominate in the starchy endosperm, accounting for most of the α-amylase and trypsin inhibitory activity in wheat. Targeted proteomic analyses show relatively constant ratios among these isoforms in hexaploid bread wheat, suggesting uniform defensive functionality across cultivars[11,12]. Conversely, the outer seed layers, including bran and aleurone tissues, contain a broader array of inhibitor families, such as α-amylase/subtilisin inhibitors, xylanase inhibitors, and other pathogenesis-related proteins. Their ATI content is lower and compositionally more diverse, reflecting adaptive protection toward a wider variety of enzymatic and microbial challenges[13,14]. Such differences across seed compartments support distinct ATI profiles among flour types, indicating that milling and refinement processes may selectively redistribute these protein species. In recent years, ATIs have been shown to activate Toll-like receptor 4 (TLR4)-dependent innate immune pathways

[1]School of Biosciences and Veterinary Medicine, University of Camerino, Camerino, MC, Italy. [2]College of Food Science and Engineering, Jilin Agricultural University, Jilin 130118, China. [3]School of Pharmacy, University of Camerino, Camerino, MC, Italy. ✉email: massimiliano.cuccioloni@unicam.it

and to exacerbate intestinal inflammation in experimental models of CD[11]. However, their causal involvement in human CD and non-coeliac wheat sensitivity (NCWS), an emerging condition with self-reported prevalence around 10% but lacking specific genetic markers or a defined pathogenic trigger, remains uncertain[15–19]. Evidence from in vivo human studies is still limited, and whether ATI-mediated immune activation contributes directly to symptom onset or aggravation in sensitive individuals is unresolved.

Several technological and breeding strategies have been investigated to reduce ATI bioactivity and its inflammatory impact. Sourdough fermentation involving selected lactic acid bacteria can degrade ATI tetramers and attenuate their TLR4-stimulating capacity, while specific thermal or enzymatic treatments further promote ATI denaturation or proteolysis. In parallel, developing genotypes or related cereals with intrinsically lower ATI expression offers promising approaches to modulate their immunogenic potential[20].

This study examines how wheat flour refinement may affect the redox state and pro-inflammatory properties of ATI-enriched proteins. In fact, stone and roller milling, the two dominant industrial processes, differ substantially in mechanical energy and shear forces, leading to variations in protein extractability, oxidation, and aggregation[21,22]. The comparative analysis of flour proteins produced by distinct milling technologies aims to elucidate how these physicochemical alterations influence ATI structure and activity, providing insights into their potential role in the immune reactivity associated with wheat consumption.

Results

Quantification of ATIs in protein extracts

ATI-enriched fractions were obtained from each flour sample with >60% purity (Supplemental Fig. S1), consistent with previous reports[19]. Total protein levels in ATI-enriched extracts ranged from 4.13 to 6.45 mg/g flour for roller-milled flours and 4.98–6.27 mg/g for stone-milled flours (Table 1). Minor variations in protein content among flours from the same mill, or between milling types, were generally attributed to whole-wheat processing differences (Supplemental Table S1).

Spectrophotometric assays of residual trypsin and α-amylase activities in the presence of individual protein extracts, with BPTI and acarbose as reference inhibitors, were used to derive ATIs' inhibitory concentrations. The levels of trypsin- and α-amylase-inhibitory ATIs were generally comparable, consistent with the dual inhibitory nature of these bifunctional proteins, which have previously been shown to inhibit both trypsin and α-amylase competitively with 1:1 stoichiometry[10,14,23]. Any small differences between the two measurements were attributed to variation in the ATI forms present in the sample: in fact, changes in the relative abundance of specific isoforms, such as CM-type tetramers, can modify the balance between trypsin and α-amylase inhibition without affecting the total amount of ATI.

Targeted MS/MS analysis

Protein extracts from four wheat flour types (F00, F0, F1, WGF) derived from the R1 mill were subjected to size-exclusion chromatography (Supplemental Fig.S2) to isolate fractions enriched in distinct ATI assembly states (tetramers, dimers, monomers). Individual fractions from each extract were then analyzed by targeted MS/MS to detect and quantify the unmodified principal ATI isoforms.

Nine peptides corresponding to distinct ATI isoforms were monitored (Supplemental Tables S2a and S2b), enabling specific assignment of unmodified amylase-trypsin inhibitors, predominantly present in dimeric and tetrameric forms. Quantitative results showed a progressive decline in unmodified ATIs with increasing processing refinement (F00 to WGF): tetramers from 1100 to 690 µg/g protein extract; dimers from 1190 to 170 µg/g; and monomers from 1580 µg/g to below LOD. This trend held across individual isoforms, with reduced peptide signal intensities for all ATIs, indicating that milling-induced oxidative modifications affected both chemical structure and assembly states (Fig. 1).

Mill code	Flour type	Total Proteins in ATI-enriched extract (mg/g flour)	Trypsin inhibitory ATI (mg/g protein extract)*	α-amylase inhibitory ATI (mg/g protein extract)*
R1	F00	4.13 ± 1.92^{d}	76.3 ± 11.9^{b}	70.8 ± 13.4^{c}
R1	F0	5.78 ± 1.86^{b}	76.3 ± 10.9^{b}	46.9 ± 6.7^{d}
R1	F1	$5.13 \pm 1.44^{b,c}$	89.9 ± 12.3^{a}	97.6 ± 10.5^{b}
R1	WGF	4.18 ± 1.17^{d}	80.5 ± 7.4^{a}	180.9 ± 10.5^{a}
R2	WGF	6.45 ± 2.02^{a}	63.4 ± 6.7^{c}	53.6 ± 3.8^{d}
S1	WGF	4.98 ± 1.81^{c}	82.6 ± 6.7^{a}	9.5 ± 0.7^{b}
S2	WGF	6.06 ± 1.93^{a}	81.6 ± 6.0^{a}	90.9 ± 6.7^{a}
S3	WGF	6.27 ± 1.77^{a}	55.3 ± 3.9^{d}	184.7 ± 13.4^{d}
S4	WGF	6.04 ± 1.89^{a}	85.4 ± 10.9^{a}	40.2 ± 13.4^{d}

Table 1. Protein and inhibitory ATI levels in extracts from differently processed flours. Distinct superscripts in each column indicate statistically significant differences ($p < 0.05$). *Inhibitory ATI concentrations calculated using 35 kDa weighted average MW.

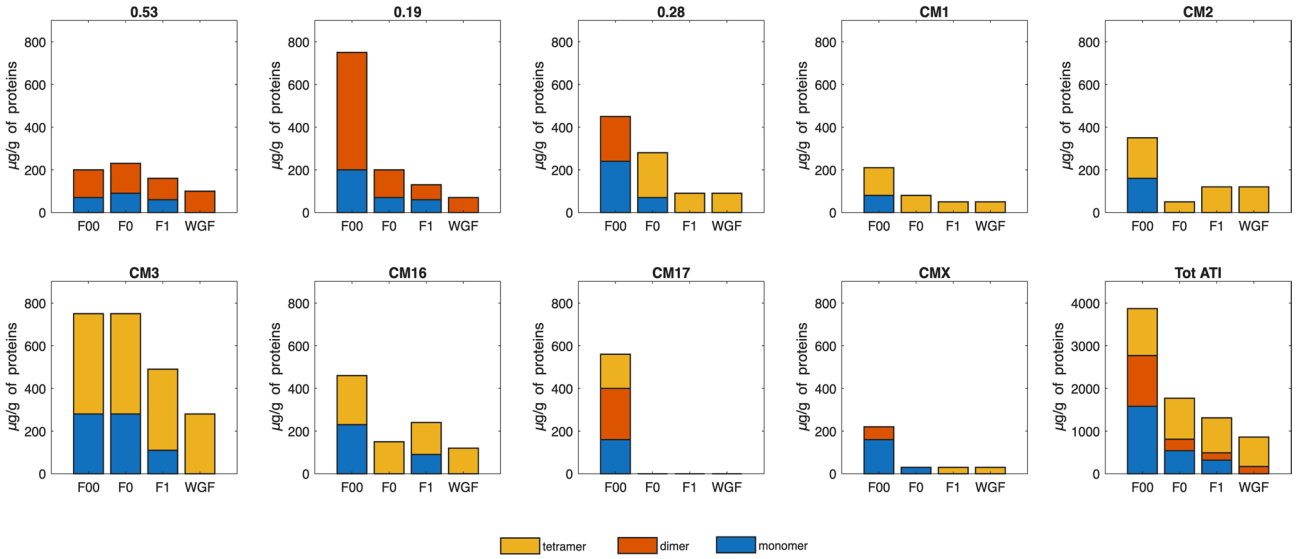


Fig. 1. Qualitative and quantitative analysis of unmodified ATI content in refined wheat flours (F00, F0, F1) and wholegrain flour (WGF) by targeted mass spectrometry. Isoforms quantified included 0.53, 0.19, 0.28, CM1, and CM2 in the upper panels, and CM3, CM16, CM17, CMX, and total ATIs in the lower panels. The distribution of individual isoforms across the three aggregation states is shown.

Flour extracts and protein oxidation

The effect of milling procedures on the redox modification of ATI-enriched wheat extracts was assessed by measuring protein carbonyls, 3-nitrotyrosines (3-NT), dityrosines (DT), and methionine sulfoxide (MSO) (Fig. 2).

Overall, oxidation patterns depended on the specific combination of processing conditions applied by each mill (Table S1), consistent with findings that environmental and processing factors modulate ATI bioactivity beyond total quantity[24]. In the R1 series, WGF and the bran-recombined flour (F1) generally showed higher levels of carbonyls, 3-NT, and DT, consistent with the more extensive processing they underwent[25]. Within the R1 flours, carbonyls, 3-NT, and DT followed an overall increase with processing intensity, whereas MSO showed a less uniform pattern. Among WGF samples, RM was associated with higher levels of 3-NT and intramolecular DT, particularly in monomeric and dimeric ATIs, while SM generally yielded lower carbonyl contents. Tempering also affected oxidation in SM flours, as WGF from S2, which did not include tempering prior to milling, showed the highest DT levels, this modification being likely dependent upon protein overoxidation[26]. By contrast, refined and bran-recombined flours produced by RM displayed lower carbonyl levels than those obtained by SM, reflecting both differences in mechanical and thermal/oxidative stresses between the two technologies[27] and their distinct impacts on flour composition and bran inclusion[28]. No major differences in carbonyl content were observed among WGF samples regardless of milling technology. Taken together, the immunodetection of protein oxidative modifications and the MS profiling of unmodified ATIs across differently processed flour extracts indicated that the progressive increase in oxidative damage was paralleled by a concomitant loss of non-modified ATI species from F00 to WGF, revealing two distinct yet concordant facets of the same processing-dependent phenomenon.

Regarding possible correlations among the observed oxidative modifications, only 3-NT and DT showed a significant linear association, consistent with a shared tyrosine-oxidation origin and their accumulation during thermal and mechanical processing of wheat proteins[29–31]. In contrast, carbonylation and methionine sulfoxidation did not correlate with the tyrosine-derived oxidation products, indicating that they likely reflect distinct oxidative pathways under these processing conditions (Supplemental Fig. S3).

ATI-induced cytokine responses in THP-1 macrophages

Wheat ATIs are nutritional activators of innate immunity via TLR4 signalling in vitro[32]. To assess whether wheat processing modulates the pro-inflammatory activity of ATIs, THP-1 macrophages were treated with ATI-enriched protein fractions obtained from differently processed flours. In line with previous studies[33], all ATI extracts significantly increased IL-1β production, a key pro-inflammatory cytokine involved in fever, lymphocyte activation, and acute-phase protein synthesis[34]. IL-1β release was accompanied by IL-6 induction, most notably in extracts from WGF flours, consistent with the role of this pleiotropic cytokine in immune and inflammatory responses associated with Crohn's disease and irritable bowel syndrome[35]. Conversely, IL-1α reached quantifiable levels only in extracts produced by the R1 mill (Table 2).

All ATI extracts also stimulated IL-8 and GM-CSF release. IL-8 is a major mediator of inflammation and neutrophil recruitment[36], whereas GM-CSF contributes to TLR4-linked inflammatory responses and regulates granulocyte and macrophage differentiation[37,38]. In addition, TNF-α was strongly induced, further supporting activation of a TLR4-associated pro-inflammatory program. Notably, the magnitude of the response generally

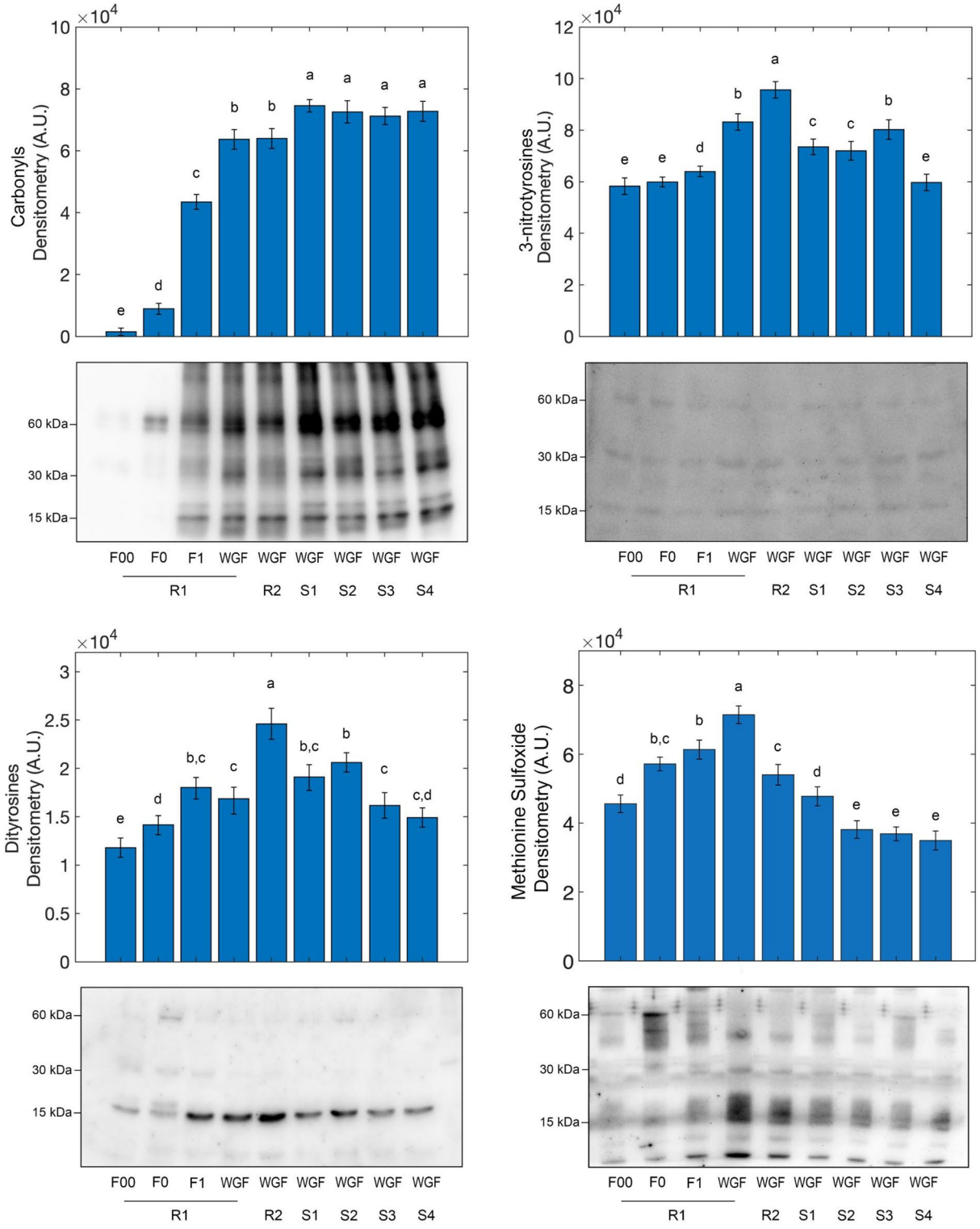


Fig. 2. Oxidative modifications in ATI-enriched protein extracts from differently processed wheat flours. Carbonyls, 3-nitrotyrosines (3-NT), dityrosines (DT), and methionine sulfoxide (MSO) were quantified by autoradiography and densitometric analysis. Distinct letters indicate statistically significant differences ($p < 0.05$). The corresponding uncropped blot images are shown in Supplementary Figure S10a–d.

increased with flour processing time and thus with the accumulation of oxidative modifications, including carbonyls, 3-NT, DT, and MSO. By contrast, WGFs produced by stone milling generally elicited lower IL-1β and TNF-α levels than those obtained by roller milling, in agreement with their lower oxidative burden. However, the relationship between oxidation and cytokine release was not strictly linear, indicating that ATI-

Mill	Flour	IL-1α	IL-1β	IL-6	IL-8	GM-CSF	IFN-γ	MCAF	TNF-α
Control		< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ	< LOQ
R1	F00	164.4 ± 19.4^{c}	< LOQ	33.5 ± 3.1^{d}	< LOQ	128.2 ± 12.7^{d}	< LOQ	98.4 ± 8.1^{c}	< LOQ
R1	F0	192.2 ± 16.1^{b}	149.5 ± 14.6^{c}	< LOQ	36.7 ± 3.1^{d}	388.1 ± 43.1^{a}	327.0 ± 33.4	100.2 ± 9.9^{c}	170.5 ± 15.9^{f}
R1	F1	268.6 ± 31.7^{a}	190.8 ± 15.5^{b}	66.8 ± 7.9^{c}	89.3 ± 10.4^{c}	183.2 ± 17.6^{b}	< LOQ	< LOQ	291.0 ± 30.3^{c}
R1	WGF	276.7 ± 24.1^{a}	242.3 ± 21.3^{a}	< LOQ	87.8 ± 7.9^{c}	140.6 ± 13.6^{c}	< LOQ	181.9 ± 17.3^{b}	369.9 ± 39.2^{a}
R2	WGF	< LOQ	181.5 ± 15.8^{b}	80.2 ± 7.3$^{b, c}$	55.8 ± 6.6^{d}	183.2 ± 14.7^{b}	< LOQ	370.0 ± 34.4^{a}	323.4 ± 32.0^{b}
S1	WGF	< LOQ	131.4 ± 12.2^{d}	106.9 ± 12.6^{b}	81.9 ± 9.3^{c}	400.1 ± 44.8^{a}	< LOQ	< LOQ	284.4 ± 33.6^{c}
S2	WGF	< LOQ	125.1 ± 15.0^{d}	158.7 ± 14.1^{a}	93.0 ± 10.0^{c}	93.9 ± 10.2^{e}	< LOQ	< LOQ	206.2 ± 19.8^{e}
S3	WGF	< LOQ	142.5 ± 33.2^{c}	104.7 ± 11.1^{b}	133.7 ± 12.7^{a}	82.4 ± 9.7^{e}	< LOQ	< LOQ	258.3 ± 22.2^{d}
S4	WGF	< LOQ	98.2 ± 11.8^{e}	104.7 ± 9.3^{b}	114.0 ± 11.7^{b}	196.6 ± 22.6^{b}	< LOQ	< LOQ	213.0 ± 25.0^{e}

Table 2. Levels of inflammatory cytokines (pg/mL) released upon treatment of THP-1 macrophages with equal protein levels of ATI-enriched extracts obtained from differently processed flours. Ammonium bicarbonate 10 mM, pH 7.8 was used as control. Distinct superscripts in individual data columns correspond to a statistically significant difference ($p < 0.05$).

driven inflammatory signalling likely depends on multiple structural features rather than on oxidation alone (Supplemental Figs. S4–S7).

Importantly, these pro-inflammatory effects were not attributable to LPS contamination. In fact, LPS levels remained below the threshold required for macrophage activation[39], and no correlation was observed between LPS abundance and cytokine release (Supplemental Fig.S8 and Table S3). These findings indicate that the enhanced cytokine responses arise from intrinsic properties of ATI-enriched extracts and their processing-dependent modifications, rather than from residual endotoxin.

Discussion

In summary, this study shows that wheat flour processing induces oxidative modifications of proteins, some of which appear to enhance TLR4 signalling and the pro-inflammatory activity of ATIs in a human intestinal cell model. Among the oxidative post-translational modifications that can alter protein structure and function, tyrosine nitration, dityrosine formation, and methionine oxidation have been linked to inflammatory signalling and chronic inflammatory diseases[40–43]. In our ATI-enriched extracts, nitrotyrosines, dityrosines, oxidized methionine residues, and protein carbonylation were detected at levels that depended on the specific combination of processing parameters, including wheat pre-treatment, tempering, milling time, and mechanical stress. In particular, in the R1 series, longer processing times progressively increased these oxidative modifications and were paralleled by higher secretion of TLR4-associated cytokines, including IL-1β and TNF-α.

The accumulation of dityrosine is consistent with the formation of intra- and intermolecular crosslinks that may stabilize ATI aggregation into dimers and tetramers. Such multimeric species could act as multivalent ligands and thereby facilitate TLR4 dimerization and downstream signalling[44]. Additionally, we show that extracts with the highest ATI content do not necessarily exhibit the strongest oxidative signal; rather, processing induces a decrease in native ATIs and a concomitant increase in oxidative modifications, two orthogonal readouts that collectively document ATI oxidation and are consistent with the higher cytokine-inducing activity associated with the oxidized fractions.

Beyond redox effects, milling also alters flour particle size distribution, bran inclusion, endosperm disruption, and damaged starch content, thereby modulating hydration behaviour, solvent accessibility, and protein extractability[45–48]. These microstructural effects may influence both the apparent recovery of ATI-enriched proteins in vitro and their accessibility during gastrointestinal digestion in vivo, a consideration particularly relevant for ATIs given that their biological effects depend not only on abundance but also on structural organization and resistance to proteolysis.

At the same time, although this work did not address upstream agronomic variables, such as pre-sowing[49,50] or pre-harvest[51,52] treatments, the data support a mechanistic link between technologically relevant processing conditions and the TLR4-mediated pro-inflammatory potential of wheat ATIs. This effect appears to be largely driven by oxidative modifications affecting tyrosine and methionine residues, and possibly by oxidation-dependent changes in ATI aggregation state. These findings suggest that processing-induced ATI carbonylation, tyrosine nitration, dityrosine crosslinking, and methionine oxidation may contribute to the inflammatory potential of wheat-based foods, while highlighting the need for further studies to disentangle the relative roles of ATI modification, extractability, and flour microstructure.

Materials and methods

Wheat sample collection and processing

A single batch of soft wheat (Bologna variety; thousand-kernel weight ≈ 30–35 g, high baking strength and protein content, classified as an improver bread wheat in Italy)[53] harvested in July 2020 was obtained from SIS (Bologna, Italy) and supplied to two roller mills (namely, R1, R2) and four stone mills (namely, S1, S2, S3, S4), where it was (pre)processed according to individual standard operating procedures (Supplemental Table S1). R1, the only mill producing all most common flour types, was selected to analyse intra-mill differences

between flours, whereas whole grain flour (WGF), the only flour type produced by all mills, was chosen to screen inter-mill differences. Flours were classified in compliance with the Italian limits for ash content in wheat flour labelling (Supplemental Table S4).

Isolation and characterization of amylase-trypsin inhibitors

ATIs were extracted according to the procedure described by Zevallos and colleagues[19]. Briefly, 15 g of flour were first defatted with a 1:1 methanol/diethyl ether mixture at RT, and extracted three times with ammonium bicarbonate 50 mM, H 7.8, at 4 °C. The protein fraction of interest was obtained from the resulting pooled supernatants by fractional precipitation with ammonium sulphate in the range of concentration 1.8-4 M. The pellet was resuspended and extensively dialyzed against ammonium bicarbonate 10 mM, pH 7.8, then analyzed for total protein content using the Bradford method[54].

The concentrations of inhibitory ATIs against human trypsin and α-amylase were quantified through spectrophotometric assays of standard enzymatic activity using N-α-benzoyl-DL-arginine-p-nitroanilide (L-BAPNA) and p-nitrophenyl-α-D-maltotrioside (CNP-G3) as synthetic substrates[10], respectively. Initial reaction velocities were measured following incubation of the enzymes with saturating concentrations of the corresponding substrates ([S] > K_m), both in the absence and in the presence of the protein extracts under analysis, and the reference inhibitors bovine pancreatic trypsin inhibitor (BPTI) and acarbose. Residual enzymatic activity curves obtained for trypsin and α-amylase in the presence of increasing concentrations of the reference inhibitors, respectively, were used to calculate the concentrations of inhibitory ATIs in each extract. Results were calculated as BPTI or acarbose equivalents and subsequently converted to milligrams of ATI, assuming an average molecular weight of 35 kDa for ATI polypeptides. Assays were performed in 50 mM Tris-HCl buffer, pH 8.0, for trypsin and in 20 mM phosphate buffer, pH 6.9, containing 6 mM NaCl for α-amylase. All reactions were carried out at 37 °C.

LPS contamination of ATI-enriched extracts was evaluated using a commercial LPS ELISA kit (Supplemental Fig.S7 and Table S3). Next, these ATI-enriched extracts were separated into their different assembly states by size exclusion chromatography using an AKTA FPLC system (Amersham Pharmacia Biotech, Milano, Italy) equipped with a Superdex 75 h 10/30 column by isocratic elution using a mobile phase consisting of 50 mM Na_2HPO_4, 150 mM NaCl, 0.01% NaN_3, pH 7.0; flowrate: 0.8 mL/min. The chromatograms were deconvoluted with Fityk software[55] using a multiple Gaussians fitting upon baseline subtraction; retention times of the peaks of interest: 14.10 (tetramer-enriched region), 15.16 (dimer-enriched region), 16.30 (monomer-enriched region) ± 0.1 min (Supplemental Fig. S8). These assignments were used operationally to collect SEC fractions for downstream targeted LC-MS/MS analysis; this does not strictly imply that every peptide detected within a given SEC window originates exclusively from a single native oligomeric ATI species, because partial co-elution and dissociation/re-association phenomena could likely occur during extraction and chromatography.

Immunodetection of ATI in protein extracts

Custom rabbit polyclonal antisera against wheat ATI were generated by Eurogentec (Liège, Belgium) as a commercial service, upon immunization of rabbits with purified ATIs obtained by size-exclusion chromatography of ATI-enriched extracts. All animal procedures were performed by Eurogentec under their institutional ethical approval in compliance with EU Directive 2010/63/EU. No animals were handled or experimented upon by the authors. Polyclonal antibodies were eventually purified as previously described[56] (see Supplemental Materials for details). ATI-enriched protein fractions from differently processed wheat samples (15 µg of total proteins) were separated by SDS-PAGE under reducing conditions, transferred to PVDF membranes, then immunodetected with anti-ATI Abs. ATI purity was assessed as the ratio between the immunoreactive ATI signal detected with polyclonal anti-ATI antibodies and the corresponding total protein signal visualized by Ponceau Red staining of the membrane. The ATI immunoreactive signal was acquired using a ChemiDoc MP imaging system, whereas the Ponceau-stained membrane was captured using a high-resolution digital camera.

Sample preparation and Identification of ATI by targeted LC-MS/MS

Samples were prepared according to the protocol of Geisslitz and colleagues[57], with some modifications. The fractions corresponding to tetrameric, dimeric and monomeric ATIs obtained by size exclusion chromatography from flours produced by R1 mill were evaporated to dryness and the residues were re-dissolved in a 2:1 mixture of 0.5 M Tris-HCl, pH 8.5 and isopropanol, then reduced with 50 mM DTE upon 30 min of incubation at 60 °C. Cysteine residues were alkylated with 0.5 M iodoacetamide for 45 min at 37 °C in the dark, and freeze-dried. Next, tryptic hydrolysis (1:50 enzyme-to-substrate ratio) using Proteomics Grade Trypsin (Merck-Sigma) was performed for 24 h at 37 °C in the dark. The reaction was stopped with 2 µL trifluoroacetic acid. The solutions were desalted using Sep-Pak C18 cartridges and eventually evaporated to dryness. ATIs in individual protein extracts were identified using a triple-quadrupole tandem mass spectrometer (Micromass Quattro micro–Waters Corp., Milford, MA, USA) equipped with electrospray ionization (ESI) interface. The ESI source was operated in positive ionization mode. The optimized ionization conditions were as follows: capillary voltage 3.0 kV, cone voltage 20 V, source temperature 120 °C, and desolvation temperature 250 °C. Nitrogen was used as desolvation and cone gas with the flow rate at 250 and 25 L/h, respectively. Argon was used as the collision gas at a pressure of 0.5 bar. The collision energy was 30 eV with the dwell time of 0.077 s. The MassLynx software (Version 4.1) was used to control the MS/MS system and process data. The targeted method monitored unmodified proteotypic peptides listed in Supplemental Table S2, including sequence-specific transitions for the major ATI isoforms detected in the extracts. Quantification was based on ATI marker peptides strategy as already proposed by Geisslitz and colleagues[57] using predefined precursor-to-product ion transitions acquired in MRM mode. The SEC-LC-MS/MS workflow provided a compositional readout of the tetramer-enriched, dimer-enriched and monomer-enriched SEC fractions.

Detection of oxidative modifications of ATIs

Protein oxidation modifications were immunodetected by western blot using specific antibodies. In detail, ATI-enriched extracts from differently processed wheat samples (15 µg of total proteins) were separated by SDS-PAGE under reducing conditions and transferred to PVDF membranes. Membranes were blocked overnight and then incubated independently with either anti-nitrotyrosine, anti-dityrosine, or methionine sulfoxide antibodies. Likewise, immunochemical detection of protein carbonylation was performed with the OxyBlot kit (Appligene-Oncor, Strasbourg, France). Briefly, ATI-enriched extracts (15 µg of total proteins) were incubated at room temperature with 2,4-dinitrophenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone (DNP-hydrazone). The resulting products were then separated by SDS-PAGE and electroblotted onto PVDF membranes. WB bands were acquired using ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Milano, Italy) on a ChemiDoc MP system (Bio-Rad Laboratories, Hercules, CA, USA). Coomassie blue staining was used to confirm equal sample loading, and the corresponding image was acquired using a high-resolution digital camera (Supplementary Figs. S9A–C). Standard acquisition and processing procedures were applied throughout, without altering the underlying data. Limited to nitrotyrosine modification, the immunometric semi-quantitative analysis was validated by MS/MS (Supplementary Table S5).

Pro-inflammatory effect of ATIs on macrophages

THP-1 cells were cultured in 10% foetal bovine serum-RPMI1640 (Merck-Sigma) in a humidified atmosphere of 5% CO_2 at 37 °C. Next, human monocytic cell line cells THP-1 were differentiated to macrophages by culturing the cells at a density of 1.0×10^5 cells/mL in the presence of 3.2×10^{-7} M PMA (phorbol-12-myristate-13-acetate) for 24 h[58]. Upon treatment with individual endotoxin-free ATI-enriched extracts (100 µg/mL in cell culture medium, with in assay LPS levels being always < 2.3 pg/mL), the levels of secreted IL-1α, IL-1β, IL-6, IL-8, GM-CSF, INF-α, MCAF, and TNF-α were quantified in cell supernatants using a multiplex ELISA kit according to the manufacturer's instructions (Arigo Laboratories - Hsinchu, Taiwan).

Statistical analysis

Analytical and biochemical data are expressed as mean values of 3 replicates ± S.D. Statistical analysis was performed with one way and n-way ANOVA, followed by the Bonferroni post hoc test using Matlab R2025b software and $p < 0.05$ was considered statistically significant.

Linear correlation analyses were performed in Matlab using *lmfit*; the R^2 values and corresponding p-values were used to assess the strength and significance of the relationships.

Data availability

All data supporting the findings of this study are available within the paper and its Supplementary Information.

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Author contributions

M.C. developed the methodology, performed formal analysis, supervised the project, and wrote the original draft and the revised manuscript. M.A. conceptualized the study, supervised the mass spectrometry analyses, and reviewed and edited the original and revised manuscripts. L.M. performed wheat sample processing, ATI extraction, and characterization. H.J., F.M.G., and Z.L. performed immunometric and cytokine assays. F.C. performed enzymatic and mass spectrometry analyses. M.N. contributed resources and expertise for experiments on macrophages. E.V. provided methodological input on flour processing. A.M.E. contributed resources and expertise for the analysis of enzymatic activity data. All authors reviewed the manuscript and approved the final version.

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Declarations

Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to M.C.

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