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Wheat proteins evolution and environmental triggers of Wheat-related diseases

Presentata da: Luigia De Fazio

Coordinatore Dottorato	**Supervisore**
Prof. Giulio Viola	Dott. Enzo Spisni

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Abstract

Cereals, and in particular wheat, have always been recognized as a fundamental food worldwide. In particular, the success of wheat is linked with unique properties of the gluten protein fraction used in bread making process to obtain products that are widely used in traditional and modern diets. The rapid increase in the world population let to a parallel increases in food production, particularly of wheat. Increasing yield potential and selection of cultivars much more resistant to plant disease and to environmental factors could have negatively affected the quality of the grain. Moreover, the "green revolution" was characterized by a widespread use of agricultural chemicals and by industrialization of food production that led to a huge rise in the consumption of refined products. Modern baking practices have shortened bread leavening, increased the use of chemical/yeast leavening agents and there is well-documented scientific evidence of the negative effects of ultra-processed food in human healthy.

All this changes profoundly modified the human diet and, as a result, may have affected Gluten-related disease (GRDs) that has arisen in the whole word populations. Gluten-related diseases (GRDs) are multifactorial pathologies in which environmental factors and genetic background contribute to a low-grade chronic inflammation of the gastrointestinal tract.

Here, I investigated the potential pro-inflammatory effect of different wheat varieties and whether bread making processing are involved in the onset or worsening of gut inflammation.

In vitro, ex vivo and in vivo studies conducted throughout my Phd period have shown a pro-inflammatory effect of wheat especially marked in modern varieties and a higher inflammatory response linked to the use of common raising agent as *Saccharomyces Cerevisiae* and to the addiction of chemical bakery improver substances.

Introduction

A brief overview of Wheat

Description and classification

Cereals are the edible seeds of specific grasses belonging to the Poaceae family, also known as Gramineae, including wheat (*Triticum aestivum*), oats (*Avena sativa*),rice (*Oryza sativa*),maize (*Zea mays*), barley (*Hordeum vulgare*), rye (*Secale cereale*), sorghum and millet. Inside these groups there are also varieties such as emmer and spelt which are all types of wheat as well as Triticeae tribe (tribus). Triticeae is considered a plant group with a high biological diversity. There are still major disagreements among taxonomists especially with regard to generic classification, moreover no comprehensive systematic review of Triticeae taxonomy has been conducted in recent years [1]. The widely ramified wheat family includes section that have up to three different chromosome sets and every sets has seven chromosomes:

- Diploid wheat (*Triticum Monococcum* section): AA genome, 2n (14 chromosomes)
- Tetrapolid Wheat (*T. turgidum* section) : AABB genome, 4n (28 chromosoms)
- Hexaploid wheat (*Triticum Aestivum* section): AABBDD genome, 6n (42 chromosomes)

Agronomically, wheat is a temperate plant that grows best in a moderate climate. The Italian classification systems is based on grain hardness: all wheat species are classified as hard (strong), more suitable for the preparation of bread and soft wheat used for the preparation of cakes and biscuits[2].

The most economically important species is *Triticum Aestivum* also known as soft or bread wheat. This species, which is mainly used for bakery products such as breads and cakes, contributes about 95% of the world wheat production. Durum wheat is one of the variety of *Triticum Turgidum*, a tetraploid species predominately cultivated in Mediterranean areas and semolina flour contributes about the 5% of the world wheat production covering the consumption of over 14 million tons of pasta in 2014 [2]. Other wheat species are only cultivated on small areas, these are einkorn (diploid *T.monococcum* var. *monococcum)*, emmer (tetraploid *T.turgidum* var.*dicoccum*), and spelt (*T. aestivum* var. *spelta*), the latter being a cultivated form of hexaploid wheat.

Wheat evolution

The oldest wheat evolutionary line is the diploid section comprising two wild forms of einkorn: *T. Monococcum* ssp. *Aegilopoides* (genome A^mA^m) and *T. urartu* (genome A^uA^u), genetically slightly different[3]. The first is the precursor of the earliest domesticated wheat, *Triticum monococcum* (genome AA). Many polyploidization processes have occurred involving *T. urartu* (genome A^uA^u) and the closely related extinct plant *Aegilops speltoides* (genome BB), which led to the emergence of an amphitetraploid species from which *T.dicoccum* (genome AABB), the progenitor of durum wheat, evolved about 0.5 million years ago[4]. This first speciation took place naturally and without any human intervention. The second polyploidization event involved the domesticated *T.dicoccum* and another wild form *T.tauschii* which led to the introduction of DD genome. From this spontaneous hybridization, originated 10,000 years ago, evolved the common wheat *T. aestivum* (AABBDD). [5]

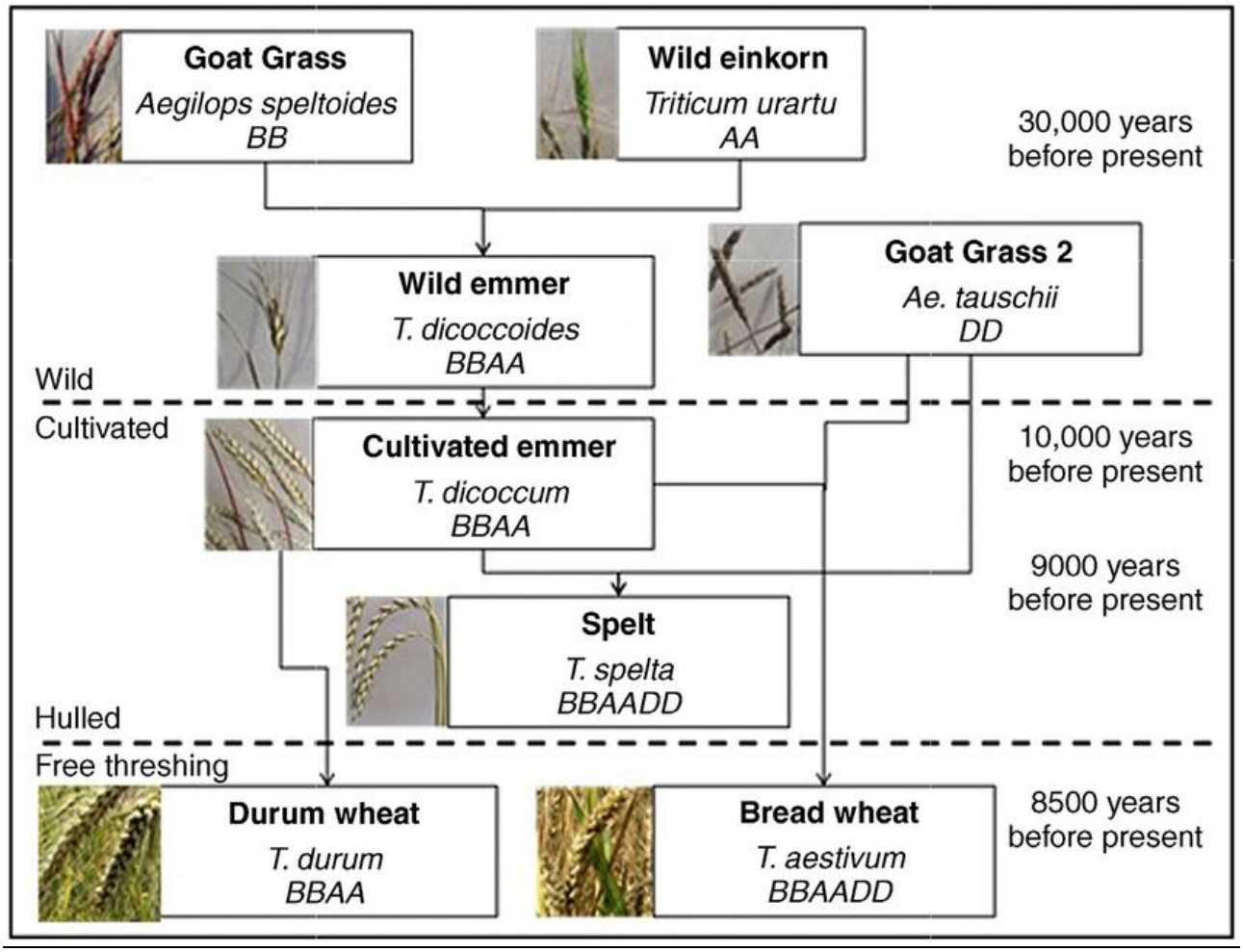


Figure 1: Origins and evolution of wheat.[6]

Ancient and Modern wheat

The term "Ancient grains" should be reserved for cereal types that are known to have originated through spontaneous hybridizations and remained unchanged over the last hundred years. They represent early forms of landrace, and usually ancient wheat is considered to include einkorn (Triticum monococcum), emmer (Triticum dicoccum) and spelt(Triticum aestivum). Starting from these varieties, there was a selection process in which genotypes were hybridized deliberately and the segregating progeny exposed to natural and guided selection at the sites where they are to be used. [7]
Ancient varieties selected until the green revolution are more correctly named heritage varieties.
"Green revolution" is considered as a boundary to divide "ancient and heritage wheats" from "Modern" ones. The term "Green Revolution" refers to a set of breeding program started by Norman Ernest Borlaug to increase wheat production. After the Second Word War, nutritional needs of a growing population was accompanied by war industries needs to reconvert the exceeding nitrates and phosphates in fertilizers, thus some of wheat varieties were used in many countries in breeding programs to develop high fertilizer responsive varieties improved in technological properties.[8]
Modern wheat cultivars are much more resistant to plant disease and are higher yielding, which satisfies the need for food supply for the word population using less lend and fewer pesticides.
Modern and Ancient wheat varieties are distinguished by yield, morphological and physiological characteristics such as the height of the plants, the length of the phenological cycle, susceptibility to biotic and abiotic stresses and grains quality[9].
Heritage varieties refers to popular and successful old varieties of plants maintained in different ways to retain their original characteristics. There are several heritage cultivars of *Triticum aestivum* and *Triticum durum* that remained unchanged over the years, namely Russello, Senatore Cappelli, Timilia or Tumminia and Urria (*Triticum durum*), as well as Autonomia B, Frassineto, Gentil Rosso, Inallettabile, Maiorca, Sieve, Solina, and Verna (*Triticum aestivum*) [10]. In the last decade, ancient and heritage grains have been reintroduced and the growing awareness regarding foods considered natural and healthy have further increased the interest in alternative cereals. This interest is also associated with the fact that some of them are reported to be better tolerated by individuals that suffer from intolerance or sensitivities to modern wheat [11].
Scientific panorama still debates about benefits of ancient or heritage wheats often supposed to have superior health-promoting properties than modern cultivars. It is possible to distinguish an anti-inflammatory effect of wheat derived from antioxidant compounds, and a pro-inflammatory effect related to protein contents.

The antioxidant activity of elicited by wheat may be related to by bioactive compounds, which belong to different groups of hydrophilic and lipophilic compounds such as polyphenols, carotenoids, phytosterols, and selenium[12] . Introduction of ancient whole wheats as a strategy to reduce the burden of non-transmissible chronic diseases has also been suggested, proposing old wheat varieties as raw material for developing wheat-derived foodstuffs with health promoting characteristics[13] .

Anyway, scientific data on antioxidant content in ancient and modern wheats are conflicting. Some studies have shown a higher nutraceutical value of ancient wheats respect to modern ones, in particular HPLC analysis have highlighted the particular polyphenolic composition of ancient varieties of soft wheat such as Verna and durum wheat such as Senatore Cappelli[14]. A relevant antioxidant activity was also detected by Lachman and coworkers, along with superior contents of proteins, tocols, carotenoids and polyphenols, of einkorn and emmer [15] . Some other studies instead found no differences in bioactive compounds content between ancient and modern wheats [16]. The commercial success of modern grains is related to content and quality of gluten proteins. Referring to general protein content some studies show in hybrid modern varieties the presence of 5% of proteins not found in the parental generations[17], approximately 14 new proteins belonging to high molecular weight gluteninic subunits in symmetric somatic hybrids were identified by SDS-Page and subsequent genetic analyses [18]. Germplasm screenings, which compare the thousands of lines in the world wheat collection, show a variation in protein content from 7 to 22% of dry weight[18] .

In contrast with more modern forms of wheat there is evidence that the gliadin protein of einkorn seems to be less toxic to sufferers of celiac disease. It has yet not to be recommended in any gluten-free diet, since einkorn wheat contain gluten, even if it is different from those present in tetraploid or exaploid wheats. Thus, einkorn gluten seems to be better tolerated in patients with gluten sensitivity[19]. On the other hand, it seems that modern grains have gluten more immunogenic than those contained in ancient varieties and it has been proposed that the genetic improvement may have increased the percentage of epitopes involved in GRDs[20].

Characterization of the grain

Wheat grain (also known as a seed or kernel) is the most economically important part of the wheat plant because it constitutes a major source of people food. Wheat is often considered primarily as a source of carbohydrate (energy), but it also contains significant amounts of other important nutrients including proteins, fiber, and minor components including lipids, vitamins, minerals, and

phytochemicals which may contribute to a healthy diet. Wheat (Triticum spp.) has been consumed by humans for over 10000 years, and currently supplies about 20% of global dietary proteins[21].

Grain anatomy

The wheat grain also known as a seed or kernel (botanically known as caryopsis) is a single-seeded fruit characteristic of the grasses. It is a composite organ with three distinct compartments, the bran, the embryo, and a prominent and persistent endosperm for which cereal species have been domesticated [22]. A longitudinal section of wheat grains is shown in Figure 1. Bran is the external layers of the grains, which provide support and protect the interior parts of grains during development from the external environment [23]. Bran comprise more than 80 % of fibers, mainly water-insoluble fibers, which serves as semi-permeable barrier. It also contains a small protein amount, B-vitamins and minerals. Bran is included in whole-wheat flour[24]. Proportionately, endosperm is the largest morphological portion of all cereal grains, in fact it makes up 80 – 84 % of the wheat grain[25]. It consists of the outer aleurone layer and the inner starchy endosperm. The aleurone is a uniform single layer of cells in wheat [26] grain and completely surrounds starchy endosperm. It responds to gibberellic acid during germination to produce hydrolytic enzymes that are secreted into the starchy endosperm for the mobilisation of grain reserves. Most of the aleurone layer is removed as part of the bran during roller milling. The embryo, a reproductive wheat organ, consist in the scutellum and an embryonic axis. The scutellum acts as a secretory and absorptive organ, leading to *de novo* synthesis and secretion of hormones and enzymes as well as absorption of solubilized nutrients during germination [27]. The embryonic axis is the plant of the next generation. Germ comprising about 2.5 % of the grain weight and is usually separated from the flour during most milling processes because of its fat content (about 10 %) that may limits the flour quality during the storage. Fats can easily go rancid, and this may limits the shelf life of the flour. Germ is present in whole-wheat flour. Besides lipids, germ further contains minimal quantities of proteins, and a greater share of B-vitamins and minerals.[21]

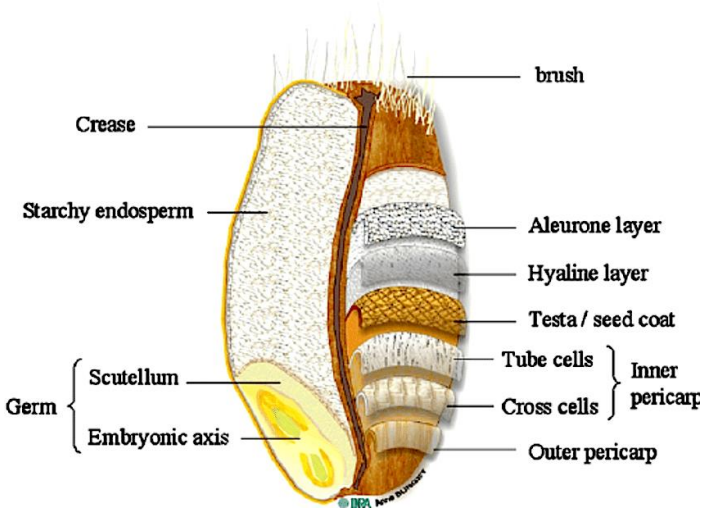


Figure 2: A longitudinal section of wheat grains

Grain composition

A grain of wheat is mostly composed of carbohydrates, proteins, lipids, and minerals: the major component of wheat grain are starch (60-78%), water (14%) and proteins (10–12%). Wheat proteins are essential for bread-making quality. According to their functionality, wheat grains proteins are divided in two types: gluten, which includes gliadins and glutenins, and non-gluten proteins, which includes albumins and globulins[28]. Non-starch polysaccharides (2–3%), in particular arabinoxylans and lipids (2%) are important minor flour constituents relevant for bread production and quality[29]. In addition to seed proteins, wheat also contains clinically relevant carbohydrates known as fructans. Fructans are fructose polymers with, or without, one glucose conjoined by β-glycosidic linkages[30] and are considered dietary fiber that can't be broken by human digestive enzymes. Fructans pass through the upper gastrointestinal tract without undergoing digestion and arrive in the large intestine, where *Bifidobacteria* and other gut bacteria can cleave the β-linkages [31]. Recently, fructans are grouped into a large family of dietary carbohydrates called fermentable oligosaccharides, disaccharides, monosaccharides, and polyols (FODMAPs), which can be fermented by bacteria in the large intestinal tract. In addition to fructans, FODMAPs includes sorbitol (stone fruits), raffinose (legumes, lentils, cabbage, Brussels sprouts), and lactose [32].

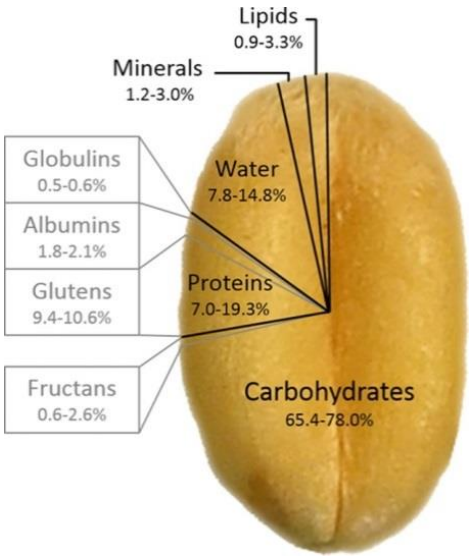


Figure 3: Grain components

Wheat grain proteins

The wheat seed contains 20% of the total grain proteins that correspond to non-gluten proteins, comprising albumins and globulins, which have metabolic and structural functions with a minor role in wheat quality. The non-gluten proteins, comprises 15-20% of total wheat flour proteins and mostly can be referred to enzymes that take part in plant growth. Many of these enzymes are involved in metabolic activity, development and response to environmental cues, but they also help in protecting embryo from insects and pathogens before germination[33]. For example, there is a family of Trypsin/alpha-amylase inhibitors (ATIs) implicated in plant defense[34]. Some albumins and certain globulins (called tricins) are considered to have a storage function as nutrient reserves for the germinating embryo [35] However, several other proteins have unknown functions and are still not well characterized. Non gluten-proteins are considered to have a nutritionally better amino acid compositions because of their higher lysine and methionine content as compared to the rest of the wheat grain proteins. Albumin and globulin proteins have not been reported to have large effects on grain hardness and dough rheological properties[35] . The remaining 80% of grain proteins are called prolammine because of their proline and glutamine amino acids content [36]. Prolammine proteins can be divided in gliadins and glutenins. Gluten was firstly isolated in 1728 by Jacopo Beccari, a professor of chemistry at University of Bologna. About 90 years later, gluten was separated in two distinct protein fractions named as follow: gliadins and Zymon [37],later renamed glutenin. The gliadin proteins as a whole compose the soluble part while the glutenins represent the insoluble fraction of the gluten. The unusual rheological and functional properties of gluten are dependent upon the ratio

of glutenins to gliadins, and the interactions of these structures. Gliadins contribute to the viscosity and extensibility of the dough, whereas hydrated glutenins are cohesive and contribute to dough strength and elasticity[38]

Osborne Fraction	**Solubility behaviour**	**Composition**	**Biological role**	**Functional role**
Albumin	Water and dilute buffers	Non-gluten proteins (mainly monomeric)	Metabolic and structural proteins	Protection from pathogens
Globulin	Dilute salt	Non-gluten proteins (mainly monomeric)	Metabolic and structural proteins	Providing food reserve to embry
Gliadin	Aqueous alcohols	Gluten proteins (mainly monomeric gliadins and low molecular weight glutenin polymers)	Prolamins-type seed storage proteins	Dough viscosity/plasticity
Glutenin	Dilute acetic acid	Gluten proteins (mainly HMW glutenin polymers)	Prolamins-type seed storage proteins	Dough viscosity/plasticity
Residue	Unextractable in water and dilute buffers but extractable with Urea+ DTT+SDS SDS+ Phosphate buffers+ sonication etc	Gluten proteins (high molecular weight polymers) and polymeric non-gluten proteins (triticins)	Prolamins-type (gluten) and globulin-type (triticin) seed storage protein	

Table 1:Classification of proteins according to their solubility[29]

Most of the gliadins are monomeric and are rich in proline and glutamine and have a low level of charged amino acids [36] . The molecular weight of gliadins is 30–80 kDa and they are classified into four groups of α, β, γ and ω on the basis of molecular mobility at low pH in acid polyacrylamide gel electrophoresis[39]. The α/β and γ- gliadins are the major components, whereas the ω-gliadins occur in much lower proportions of wheat varieties[40]. The composition of the gliadins in wheat differs from variety to variety. As a result of this extensive polymorphism, gliadins are used for the identification of the cultivar in hexaploid and tetraploid wheat[41].

Glutenins are polymeric proteins divided into high molecular weight glutenins (HMW-GS) ranging from 90 to 140 kDa and low molecular weight glutenins (LMW-GS) ranging from 30 to 75 kDa [40] by sodium dodecyl polyacrylamide gel electrophoresis under reducing conditions[36]. Glutenins and gliadins have very similar amino acid composition, thus glutenin have high levels of glutamine and proline and low levels of charged amino acids [36]. Glutenins appear to be largely responsible for gluten elasticity and strength. HMW-GS constitute no more than 10% of total flour protein; although they may be the most important determinants of bread-making quality because of their importance in forming the glutenin polymer [36]. Removal of the high-molecular weight glutenin had a negative effect on the quality of bread [42].

Glutenin subunits (HMW-GS and LMW-GS) are structurally related to monomeric α- and γ-gliadins: glutenin macropolymers intermingle randomly with individual particles of gliadins to form the aggregate, which is held together with non-covalent interactions. The arrangement of the gliadins in the aggregate has been demonstrated with immuno-localization transmission electron microscopy used with monoclonal and polyclonal antibodies selective for gliadins or the glutenin subunits[39] .

Both gliadins and glutenins form a viscoelastic network that traps the CO_2 released during fermentation, providing the typical texture characteristics of the wheat bread.

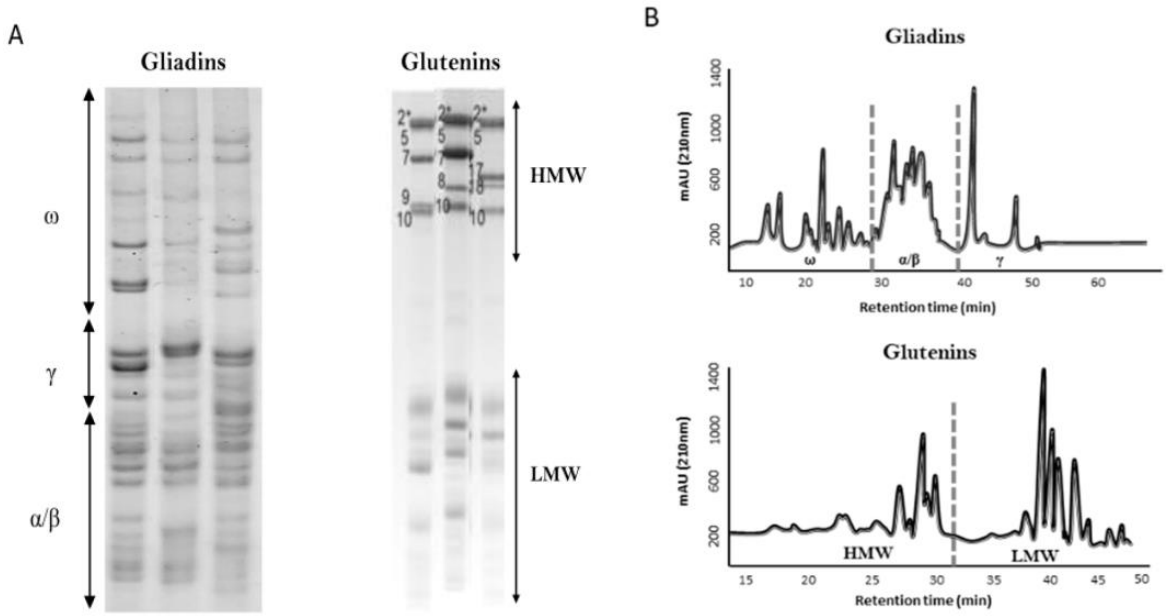


Figure 4: Gliadins and glutenins fractions revealed by using acid polyacrylamide gel electrophoresis (A-PAGE) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), respectively (A) and RP-HPLC (B).[43]

Group	**Subunit structure**	**Total fraction %**	**Molecular weight in Da**	**Amino acid composition %**
HMW subunits of glutenins	Polymeric	6-10	65 - 90000	30 - 35 Gln 10 - 16 Pro 15 - 20 Gly 0.5 - 1.5 Cys 0.7 - 1.4Lys
LMW Subunits of glutenins	Polymeric	70-80	30 - 45000	30 - 40 Gln 15 - 20 pro 2 - 3 Cys <1.0 Lys
α-Gliadins β-Gliadins γ -Gliadins	Monomeric Monomeric Monomeric	70-80	30 - 45000	30 - 40 Gln 15 - 20 pro 2 - 3 Cys

				<1.0 Lys
ω – Gliadins	Monomeric	10-20	40 - 75000	40 - 50 Gln 20 - 30 Pro 8 - 9 Phe 0 Cys 0 - 0.5 Lys

Table 2:. Classification and properties of wheat gluten proteins, adopted from [44] .

Wheat Flours

Wheat kernels are generally milled to separate the starchy endosperm from the outer layers and germ, thereby reducing the size of the starchy endosperm to obtain flour. One of the major wheat properties is its hardness, defined as the force needed to crush the kernels, used to differentiate 'soft' and 'hard' wheats[45].Common hexaploid wheat (Triticum aestivum L.) endosperm texture ranges from very soft to hard, whereas the tetraploid durum wheat (T. turgidum L. ssp. durum) presents the hardest kernels of all wheat cultivars [46]. In general, soft wheat flour, containing less protein about 8% to 11%, is used for producing cake and cookies. Hard wheat flour for bread, and durum wheat semolina from T. turgidum, containing about 10% to 14% protein, is used for pasta [47]. The quality of the flour depends on number of factors including gluten composition and particle size [48].

Wheat flour quality is directly related to the gluten formed by mixing the flour with water. The strength of the flour depends on the quality of the gluten that it is capable of developing and by its subsequent ability to absorb water. The index of bread making capacity (W) is used to classify the flour according to its gluten strength[49]. The flour with W between 90 and 160 is considered weak flour and absorbs 50% of its weight in water. Medium strength flour has W values between 160 and 250 and absorbs between 65% and 75% of its weight in water. The flour with W between 250 and 310 is considered strong flour and absorbs from 65% to 75% of its weight in water. Special flour has a W greater than 310, and absorbs up to 90% of its weight in water and is often used in mixes with other flour to increase strength.

Wheat grains can be milled into many different forms from the very finest flour (semolina or refined flour) to whole kernels (wholemeal flour). In Italy, just like many other European countries, flours are divided based on their rate of sifting or grinding yield. The wheat flour can be categorised into five types: "00", "0", "1", "2" and whole meal. "00" flour The flour "00" is the most refined flour

and is white, soft and rich in starch. Wholemeal flour is the most unrefined flour, is darker and contains more fibre, vitamins, proteins, fats and enzymes than refined ones. Manitoba flour is consider a flour with pro-inflammatory characteristics [50]. Also known as American flour gets its name from Manitoba, the south-western province of Canada where the production of this particular kind of wheat, highly resistant to the cold. Manitoba is classified as a special flour with a W index greater than 350. Currently, this term is used to refer to any flour that, irrespective of the variety of wheat used or production area, has resistant features similar to those of American flour.

Yeast and sourdough Bread and flour additives

Wheat is the most commonly used cereal for baked product and the main objective of bread making is to convert cereal flours into attractive, palatable, and digestible food. Bread is a key ingredient of the human diet, is consumed in large quantity in the world and makes up roughly 10% of the adult caloric intake[51]. Bread-products and bread-making techniques differ widely around the world. Based on the easy preparation techniques of wheat-derived food products, bread derived products underlie the human basic nutrition in many cultural cuisines. Essentially, the production of bread requires three important ingredients such as flour, leaving agents and water. The use of baker's yeast (Saccharomyces cerevisiae) as a leavening agent is a recent widely used addition to bread making[52]. In contrast, sourdough, which contains a culture of mostly wild yeast as well as lactic and acetic acid bacteria that naturally inoculate bread dough, has been used as a leavening agent since ancient times[53]. Yeast is an ubiquitous, unicellular, asexual eukaryote belonging to the kingdom Fungi, which is able to ferment, by enzymatic hydrolysis, sugars into alcohol and carbon dioxide. During fermentation yeast produces mainly carbon dioxide and ethanol, but the role of yeast goes much deeper than just gas production[54], concerning the production of other secondary metabolites, which have an impact on the final product quality.

Sourdough is "a mixture of flour and water, spontaneously fermented by lactic acid bacteria and yeasts, which after several refreshments are responsible for its capacity to leaven the dough[55]. Sourdough is composed of stable associations of lactobacilli and yeasts. Microbiological studies have revealed that more than 50 species of lactic acid bacteria, mostly species of the genus Lactobacillus, and more than 20 species of yeasts, especially belonging to the genera Saccharomyces and Candida, occur in mature sourdough [56]. Differently from other biological or chemical straight dough processes sourdough fermentation releases several compounds that are not found in modern yeast fermentation, and acidification, proteolysis, and activation of a number of enzymes as well as the synthesis of

microbial metabolites during sourdough fermentation cause influences all aspects of bread quality[57]. Two main factors difference sourdough processes from straight dough processes. Frist, the presence of lactic acid bacteria adds the metabolic potential of this heterogeneous group of organisms to the metabolic potential of yeast[58]. Second the fermentation time of sourdough processes ranges from 8h to over 72 h[59]. This long fermentation time compared to straight dough processes allows a substantial contribution of endogenous enzymes to biochemical conversions at the dough stage. The acidification of sourdough modulates the activity of cereal enzymes and the solubility of gluten proteins and consequently their susceptibility to enzymatic degradation[60]. Moreover, sourdough fermentation shifts the ambient pH to the optimum pH of aspartic proteases, the major proteinase in resting grains of wheat and rye[47]. Primary proteolysis is dependent on endogenous proteinases and the proteolysis of sourdough remains limited to degradation of less than 5% of the cereal proteins[61].

Furthermore, a recent study confirms the ATI-degrading capacity of *Lactobacllus* strains [62]. Moreover, different studies suggest sourdough breads increase mineral bioavailability of iron and zinc when compared with yeast bread[63-64] and induce advantageous effects on glucose metabolism[38]. Lastly, bakers mainly use different flour additives, such enzymes as amylases, hemicelluloses, vital wheat gluten and proteases to change and improve dough properties and/or bread quality. The introduction of additives such as food preservatives and emulsifiers, whose roles are to improve the shelf-life and texture of commercial bread. Some emulsifiers were suggested to alter the gut microbiome in mice in a manner that induces inflammation and obesity [65].

Overview of Gluten-related Disorders (GRDs)

Recently increasing attention has been paid to the pathologic role of food in gut inflammation. A number of *in vitro* studies have confirmed the cytotoxicity of some wheat components and their effect on intestinal permeability and in immune response. Gluten and other wheat protein are considered the main causative factor of GRDs [66-67].

The Gluten-related Disorders (GRDs) include three distinct disease entities, namely celiac disease, wheat-associated allergy and non-celiac gluten/wheat sensitivity (NCWS). Despite having in common the contact of the gastrointestinal mucosa with components of wheat and other gluten containing cereals as a causative factor, these clinical entities have distinct pathophysiological pathways. Coeliac disease (CD) and Wheat allergy (WA) have been better studied and characterised, while NCGS is the youngest member of the family of GRDs[68].

Intestinal Barrier Function

The intestinal epithelial barrier is the cellular covering of the intestinal wall and has a crucial role in protecting the organism against pathogens and possible harmful substances derived from the external environment. The gut barrier is organized as a multi-layer system, made up of two main components: a physical barrier surface, which prevents bacterial adhesion and regulates paracellular diffusion to the host tissues, and a deep functional barrier, that is able to discriminate between pathogens and commensal microorganisms, organizing the immune tolerance and the immune response to pathogens[69] .In the physical barrier surface the predominant cells are enterocytes, devoted to the absorption of nutrients and goblet cells are the main mucus-secreting cells. Mucus forms a protective layer covering the apical surface of the intestinal epithelium to avoid adherence and subsequent invasion by external pathogens[70]. In this layer cells are attached to each other by the apical junctional complex (the adherents junction and the tight junction). The intestinal epithelium mediates selective permeability via two major routes: transepithelial/transcellular and paracellular pathways[71]. Transcellular permeability is generally associated with solute transport through the epithelial cells and predominantly regulated by selective transporters for amino acids, electrolytes, short chain fatty acids and sugars[72]. Paracellular permeability is associated with transport in the space between epithelial cells, and is regulated by intercellular complexes localized at the apical-lateral membrane junction and along the lateral membrane[73]. Tight junctions (TJs) are localized to the most apical part of the lateral epithelial cell membrane. TJs are multi-protein complexes that function as a selective/semipermeable paracellular barrier, which facilitates the passage of ions and solutes through

the intercellular space, while preventing the translocation of luminal antigens, microorganisms and their toxins. Main constituents of TJs include the transmembrane proteins occluding and the family of claudins. Although occludin was the first TJ component to be identified, its role is not fully delineated; however. It was reported to be involved in the regulation of paracellular permeability, since loss of occludin may affect the integrity of the epithelial barrier. Saitou et al.[74]and Schulzke et al.[75]have shown that occludin knockout mice present normal TJ strand formation and normal barrier function. However there is evidence suggesting occludin is involved in cellular adhesion[76]. The family of claudins play a crucial role in TJ formation and the epithelial barrier, but they also have functions in cytoskeleton organization, transport of vesicles and signalling pathways directly associated with scaffold proteins such as zonulin-1 (ZO-1)[77]. Recent studies, with claudin-deficient mice also provide corroborative data supporting a role for claudins in the regulation of barrier function. Claudin-$^{1-/-}$ mice die within one day of birth due to significant trans epidermal water loss[76]. Alterations in the expression of claudins are related to disturbance in homeostasis and contribute to several gut diseases[76]. The adherens junctions (AJ) is composed of two protein complexes associated with cell–cell adhesion: The nectin–afadin and the cadherin–catenin complex. These protein complexes have an extracellular region responsible for adhesion of adjacent cells, while the intracellular component is involved in signalling, controlling of the AJ dynamic and interactions with the cytoskeleton[78]. Lamina propria is a supportive layer of conjunctive tissue and lies underneath the intestinal epithelium. Within this layer, immune cells, including macrophages, dendritic cells and lymphocytes play a crucial role in the defence against harmful substances and in maintaining the homeostasis of the intestinal epithelium forming the gut-associated lymphoid tissue [79]. Mononuclear phagocytes (macrophages and dendritic cells) have various functions including phagocytosis for antigen sampling and/or clearance of pathogenic material as well as cytokine production and maintenance of epithelial barrier function [80]. Additionally, monocytes contribute to the maintenance of the epithelial barrier by producing the lipid mediator prostaglandin E2, which controls the neutrophil response to various stimuli and thereby supports the homeostasis of the epithelial layer[81]. Innate lymphoid cells (ILCs) are innate immune cells contributing to intestinal homeostasis inducing T-cell tolerance and to protection against intestinal infection. Within the epithelial layer there is a population of T-cells known as intraepithelial lymphocytes (IELs). These cells, mostly $CD3^+$ and $CD8^+$T-cells, interact directly with enterocytes and are in close proximity to antigenic material in the gut lumen ready to initiate immune response[82]. Kuhn et al. have shown that interactions between commensal gut microorganisms and IELs promote the secretion of cytokines thereby enhancing epithelial barrier function[83].

Coeliac Disease (CD)

Coeliac disease is a chronic inflammatory condition affecting the gastrointestinal tract, in particular the small intestine and jejunum, result in an atrophy of the absorbent apparatus and consequent malabsorption of nutrients [84]. Gluten is recognized as the environmental trigger of celiac disease in genetically susceptible individuals. The immune-mediated enteropathy is genetically associated with the human leukocyte antigen (HLA) class II genes, known as HLA-DQ2 and HLA-DQ8, located on chromosome 6p21. Most CD patients express genes encoding the major histocompatibility complex (MHC) class II protein HLA-DQ2. The remaining patients are usually HLA-DQ8-positive[85]. In susceptible individuals, peptides derived from ingested gluten cross the epithelial barrier and are deamidated by the tissue transglutaminase 2 enzyme (TG2) in the lamina propria, thereby increasing their affinity to the HLA-DQ2/DQ8 molecules on the membrane of antigen presenting cells[86]. These HLA haplotypes play a key role in promoting the immune response by presenting the immunogenic gliadin peptides to gluten-specific CD4+ T-cells. Once activated, the CD4+ T-cells secrete various cytokines, including IFNγ and IL-21 leading to mucosal damage [87].

Clinical presentation of this pathology includes a largely wide of manifestations ranging from intestinal to extraintestinal symptoms. Typically GI manifestation are abdominal bloating, weight loss, diarrhoea, and malabsorption. Extraintestinal related symptoms and signs such as anaemia, impaired growth, decreased bone mineral density, micronutrient deficiencies were observed. The only current treatment to minimize the disease's complications is a gluten free diet. Diagnostic criteria is based on the detection of serologic markers and subsequent confirmation obtained through small bowel biopsy. Serological test including immunoglobulin A (IgA) antitissue transglutaminase (TTG) antibody is the favoured test for screening for CD with sensitivities ranging from 93 to 95% and specificities approaching 96% [88]. Patients must be on a normal gluten containing diet to maximize the sensitivity of the serological testing. Other serologic markers include antiendomysial-IgA, which is 90% sensitive and approaches 99% specificity [89]. Common endoscopic findings in CD include villous atrophy, elongated crypts, increased mitotic index in the crypts, increased intraepithelial lymphocytes (IELs), cellular infiltrate in the lamina propria, and cuboidal pseudostratified epithelium [90]. The worldwide prevalence of CD ranges between 1% and 2% in the general population [91], with most patients remaining undiagnosed due to the subtle or multiform clinical manifestations of the disease[92].

Allergic Responses

Wheat allergy is a disorder characterized by immune activation where T helper cells type 2 (Th2) mediate immunoglobulin E (IgE) and non-IgE reactions after exposure (usually dietary) to wheat [93]. In IgE-mediated allergies, wheat induces symptoms in predisposed individuals including urticaria, asthma, allergic rhinitis, gastrointestinal upset, and exercise-induced anaphylaxis (EIA) through mast cell and basophil activation[93]. In non-IgE-mediated allergies, lymphocytic activation induces eosinophilic infiltration of the upper gastrointestinal tract resulting in eosinophilic esophagitis (EoE) and eosinophilic gastritis (EG). Both processes can be managed with wheat avoidance as well as addition of immunomodulating agents [93].

Allergy skin testing remains the most commonly used diagnostic procedure. In certain cases, serum wheat-specific IgE (sIgE) can be a substitute. Its prevalence is estimated at <0.5% in the general population[93]. It is more common than other cereal grains, including barley, rye, oat, and rice, but not as common as milk and egg in children or as peanuts, tree nuts, and seafood in older ages [94] .

Non-celiac wheat sensitivity (NCWS)

Non-celiac gluten sensitivity or, more broadly, non-celiac wheat sensitivity (NCWS) is a disorder characterized by the onset of gastrointestinal as well as extraintestinal symptoms such as tiredness, "foggy mind", headache and anxiety following ingestion of gluten-containing food in individuals in whom CD and wheat allergy have been excluded[95].

The pathogenesis of NCWS is not well understood and its lack of any biomarker makes it difficult to verify[96]. Patients do not present villous atrophy nor produce specific antibodies or IgE in response to wheat ingestion [97] and antibodies against native gliadin (AGA), were sometimes associated with NCWS, but have insufficient diagnostic accuracy [98]. The genetic marker HLA-DQ2 is not associated with this disease [99]. Several studies have identified an altered expression of innate immune components in response to wheat consumption in wheat-sensitive individuals including peripherical blood mononuclear cell-derived Inteleukin-10[50,100] and mucosal Toll-like receptor 2 and 4 (TLR-2 ,TLR-4)[98]. Due to the lack of evidence for T-cell involvement and the apparent contribution from TLR, NCWS may be more of an innate rather than immune response. Gut inflammatory reaction[99] increase in intraepithelial $CD3^+$ T cells lymphocytes (IELs), release of IFN-γ in the rectal mucosa after oral wheat challenge and IL-8 release in response to α-gliadin fragment has been shown in

patients NCGS [81,101-102]. More recently, a pre-existing dysbiosis of the intestinal microbiome leading to a decrease in butyrate and altering the gut barrier against inflammatory stimuli has been described in these patients[101].

Many studies suggest that the intestinal barrier function is rather reduced in NCWS. Sapone et al. investigated the RNA expression of claudin-4, a barrier-forming claudin, in NGWS patients and found a higher level of claudin-4 whereas no changes were found for claudin-1 to claudin-3, ZO-1 and occluding[102]. Fritscher-Ravens et al. published a work on *in vivo* analysis on human barrier function in NCWS by using confocal endomicroscopy after intravenous injection of fluorescein. Here, small intestinal epithelial defects and luminal leakage of fluorescein occurred in NCWS patients only few minutes after luminal exposure to wheat and were associated with increased expression of the pore-forming claudin-2[103]. In addition to gluten, several other food-derived stimuli have been shown to have a role in NCWS pathology such as α-amylase trypsin inhibitors (ATIs)[104] and fermentable oligo-, di- and mono-saccharides and polyols (FODMAPs)[105]. ATIs are albumins proteins found in wheat representing up to 4% of total proteins in grains [92] and have an important role in metabolic processes during seed development as enzymes inhibition of common parasites[106]. *In vitro* and *in vivo* study have suggested that wheat ATIs induce release of pro-inflammatory cytokines from monocytes, macrophages and dendritic cells through activation of TLR-4 complex[67-107]. Feeding of ATIs to mice increased intestinal and systemic release of cytokines as TNF-α and CCL2 [67]. FOODMAPs are short-chain sugars with less than 10 carbon atoms in the molecule that seems has a potential contribution to pathogenesis of gastrointestinal disorders [108]. Compounds belonging to the FOODMAPs group are not digested nor absorbed in the gastrointestinal tracts. They have a strong osmotic effect and undergo rapid fermentation in the intestine, resulting in intestinal liquefaction, excessive gas production, bloating and sometimes abdominal pain. They may exacerbate symptoms in susceptible patients with inflammatory bowel disease (IBD)[109] and irritable bowel syndrome (IBS)[108].

The symptoms of NCWS can occur within hours to days following exposure to gluten- containing food and can then dissipate upon withdrawal of gluten. Frequent symptoms reported by NGWS patients include bloating, abdominal pain, epigastric pain, diarrhoea and constipation[98]. Extraintestinal manifestations include lack of well- being, tiredness, headache, anxiety, foggy mind or difficulty focusing[110].

The first step in NCWS diagnosis is the exclusion of CD and WA. For this purpose the patient needs to be on a Gluten-Containing Diet (GCD) for a period of six weeks during which wheat specific IgE, IgA-tTG, IgG-tTG and IGa-EMA test are performed. The second step consist of starting the patient on Gluten-Free Diet (GFD) for six week and monitoring for symptom response. Gastrointestinal symptom rating scale (GSRS) and numerical rating scale (NRS) is used to identify and quantify the symptoms. To confirm the diagnosis a reintroduction of GCD diet is request. During this step the patient is exposed to either GFD + Gluten or GFD + placebo for a week. A one week washout period of strict GFD is observed, followed by the crossover to GFD + Gluten or GFD + placebo[111].

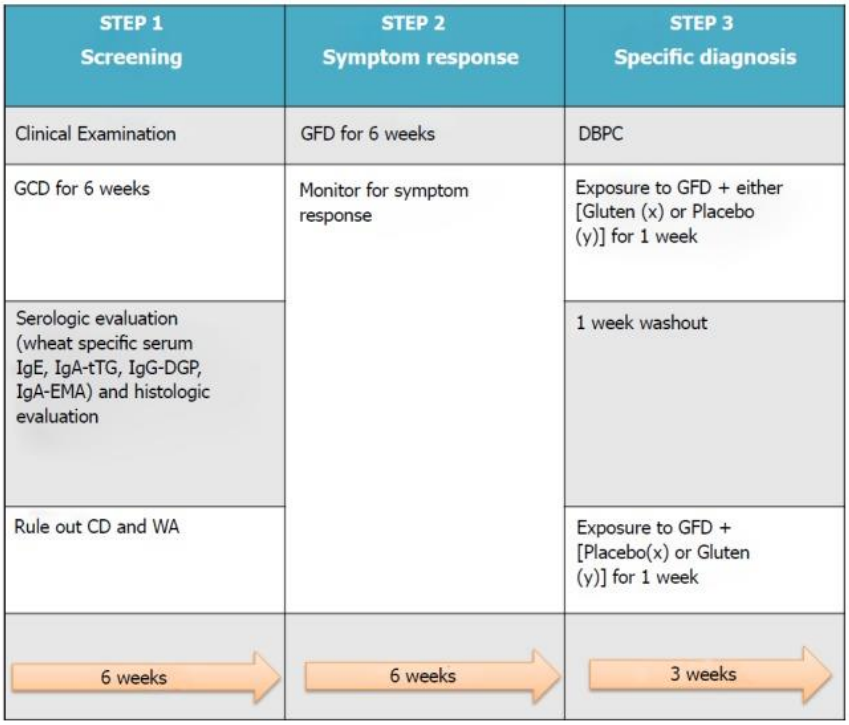


Figure 5: Diagnostic work up in NCWS [112]

The prevalence of NCWS is not yet clearly defined. Indirect evidence suggest that NCWS varied in different populations, with a range from 0.5% to 6% [43] .

Aims

In the last decades, it has been observed that the incidence of chronic non-communicable diseases is rising among industrialized countries. Several factors have been identified as possible responsible of this phenomenon, among these diet and lifestyle are considered the main factors affecting human health. During the last 100 years, human diet has radically changed with the introduction of industrial foods. In addition to the introduction of preservatives, flavorings, emulsifiers a radical change involved the main food consumed by humans. After the green revolution, the quality of the wheat has been completely distorted due to the needs of the food industries, and at the same time was recorded an increase in wheat-related disorders. It has been supposed that the daily consumption of wheat products can contribute to the manifestation of chronic inflammation and autoimmune diseases. Intestinal tissue (biopsies) from NCWS patients shows an increased expression of toll-like receptors (TLR2-TLR4) associated with an increased number of intraepithelial lymphocytes compared to healthy controls. These findings suggest a strong link between the immunogenic characteristics of the protein components of wheat (including gluten) and stimulation of the gut immune system.

This study aimed to investigate the relationship between wheat protein components, immune system activation and inflammatory response. Specifically, the present work is focused on the comparison between the ancient wheats (like *Triticum monococcum, T.Spelta and some heritage varieties*) and modern varieties of wheat (*Triticum aestivum* and *Triticum turgidum ssp. durum*) in one of the major disease related to wheat consumption (NCWS). To this aim, different in vitro, ex- vivo and in vivo studies have been planned and performed.

In chapter 1 we evaluated the pro-inflammatory properties of protein extracts from ancient and modern grain varieties in epithelial colon cells and in cells of immune system and the impact of wheat extracted proteins on the regulation of enzymes involved in chronic inflammation and tumorigenesis. Chapter 2 was focused on the immune cell response of stimulated PBMCs from paediatric subjects to protein extracts from different wheat cultivars. Moreover a serum of adult NCWS patients was used in Dot-blot analysis to analyse IgG response to different wheat varieties. The effect of different bread-based diets were tested on a model of mice gut inflammation and in an healthy mice model as shown in chapter 3. A randomized, single-blinded, crossover trial was conducted on semi-professional basketball players in order to mimic a model of mild systemic inflammation induced by physical activity. The inflammatory properties of ancient and modern wheat- based diets was reported in chapter 4.

Chapter 1 - Wheat proteins inflammatory profile: *in vitro* study of different wheat cultivars

1.1 Materials and methods

1.1.1 Cereal samples

The investigated cereal samples consisted of two modern common wheat (*Triticum Aestivum*) "Anapo" and "Sagittario" varieties, two ancient common wheat " Villagloria" and "Verna" varieties, two modern durum wheat (*Triticum turgidum* spp.*durum*) "Ciccio" and "Quadrato" varieties, two ancient Italian durum wheat (*Triticum turgidum* spp. *durum*) "Russello" and "Timilia" varieties. Manitoba and semolina flours were used as internal controls respectively for soft and durum wheat variety. The samples varieties are shown in Table 3. Flours, milled using a stone mill (100% flour extraction), were obtained from Department of Agricultural Science of the University of Bologna (Italy).

Name of Grains	**Type of Grains**	**Type Variety**	**Genome**
Manitoba	*Triticum aestivum*	Modern	Hexaploid
Anapo	*Triticum aestivum*	Modern	Hexaploid
Sagittario	*Triticum aestivum*	Modern	Hexaploid
Villagloria	*Triticum aestivum*	Ancient	Hexaploid
Verna	*Triticum aestivum*	Ancient	Hexaploid
Ciccio	*Triticum turgidum*	Modern	Tetraploid
Quadrato	*Triticum turgidum*	Modern	Tetraploid
Russello	*Triticum turgidum*	Ancient	Tetraploid
Timilia	*Triticum turgidum*	Ancient	Tetraploid
Semolina	*Triticum turgidum*	Modern	Tetraploid

Table 3: wheat samples.

1.1.2. Protein extraction

Proteins were extracted using the procedure described by Osborne (1907)[113] and subsequently modified by Lookhart and Bean (1995)[114] and van den Broeck et al. (2009)[20]. Albumin extractions: 100 mg of flour was extracted with deionized water (500 µl) for 30 min, vortexing for 1 min, every 10 min. The mixture was centrifuged for 5 min at 2,000 rpm. The supernatant (albumin extract) was decanted and stored. The procedure is repeated twice. Gliadin extraction: The insoluble pellet obtained from the globulin extraction was solubilized with 70% aqueous ethanol (400 pl) for 30 min, vortexed for 1 min every 10 min, and centrifuged for 5 min at 2,000 rpm on the mini-centrifuge. The supernatant (gliadin extract) was decanted and stored. The procedure is repeated twice. Glutenin extraction: Glutenins were extracted from the gliadin insoluble pellet with 400 pl of 50% 1-propanol + 1% dithiothreitol (DTT) for 30 min, vortexed for 1 min every 10 min. After centrifugation for 5 min at 2,000 rpm, the supernatant (glutenin extract) was decanted and stored. The procedure is repeated twice. For the ex vivo tests, all the extracts were pooled to obtain a total sample for each cereal variety. Protein samples were then precipitated at -20˚C overnight with 12:1 Acetone and Methanol mixture and centrifuged at 12,000 g for 45 min at 4˚C. Precipitated samples were then dried with vacuum and solubilized in 4M UREA over night at room temperature. The total protein content was measured using the colorimetric DC™ Protein Assay (Bio-Rad, CA, USA). The absorbance values were obtained at 700 nm using a microplate reader.

1.1.3. Protein electrophoretic profiles (SDS–PAGE)

The protein profiles of the cereal samples were detected by the sodium dodecyl sulfate-polyacyrlamide gel electrophoresis (SDS-PAGE) performed on 12% separating and 4% stacking gels in vertical electrophoretic unit. Prior to the electrophoresis, the proteins fractions were diluted 1:2 (v/v) with the sample buffer, heated at 90 °C for 5 min and cooled in ice. An equal amount of protein (40 µg) was loaded in each well. Gels were run at 20 mA for 30 minutes and then at 40 mA for 1 hour, fixed with methanol, glacial acetic acid, and stained with Comassie Brillant Blue (Biorad , Segrate, Italy). Destaining was performed with methanol, acetic acid and distilled water. Molecular weight of the polypeptides from protein flour sample were estimated by using known molecular weight marker (Precision Plus Protein™ Dual Color, Biorad). The protein profiles were elaborated as presence/absence of the observed subunits for each sample.

1.1.4. Cell culture

All the cell lines used were purchased from the American Type Culture Collection (ATCC). Routinely, cells were tested for Mycoplasma contamination and found to be free. Cell cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. CaCo-2 cells a human epithelial cells obtained by colorectal adenocarcinoma, CRL-1831, a human epithelial cells, and HL-60 a human promyeloblast cells, were grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen Corp.) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 100 U/ml penicillin/streptomycin and 4 mM glutamine (Sigma-Aldrich). Human THP-1 cells a human monocytic leukaemia cell line were grown in RPMI- 1640 medium (Invitrogen Corp.), supplemented with 10% heat-inactivated FBS (Sigma-Aldrich), 100 $U \cdot mL^{-1}$ penicillin, 50 $\mu g \cdot mL^{-1}$ streptomycin and 2 mM glutamine (Sigma-Aldrich).

1.1.5. Cell treatments

Each cell cultures was added to three T-75 culture flasks and grown to approximately 80% confluency. At 80% confluency, culture medium was removed and cells 60.000 cells were seeded in a 24 wells plate. Cells was treated with 40µg of protein extract of selected flours and incubated for 16h under mild shaking. Lipopolysaccharides (LPS Sigma-Aldrich) was used as positive control and a extracted protein from a modern soft wheat flour (Manitoba) and modern durum wheat (Semolina), with well-known inflammatory properties, were used as internal standard to evaluate the inflammatory potential of the different wheat proteins. After incubation, the well contents were transferred into 1,5 ml tubes, supernatant was collected after centrifugation (1000g for 15 min at RT) and stored at -80°C until cytokines and chemokines analysis. Cell number and viability were examined either by trypan blue exclusion or by MTT-test as described elsewhere. CaCo-2 cells were seeded at a density of 200,000 cells/well and TNF-α and IL-17 were from R&D Systems and were used at a concentration of 25 and 50 ng/ml respectively as inflammatory stimuli. 40 mg of Manitoba's extracted proteins and 40 mg of proteins obtained from blending flours of ancient wheat varieties was used . CaCo-2 cells were treated for 3–18 h.

1.1.6 Cell viability

Cell viability was determined by MTT (3-(4,5-dimethylimidazole-2-yl)-2,5-diphenyl tetrazolium bromide) colorimetric assay was used and the percentage of surviving cells were determined. Briefly,

a mitochondrial succinate dehydrogenase enzyme converts the yellow salt of MTT to the purple formazan crystalline, and the product colour is evaluated at the specified wavelength by ELISA reader. Cells were seeded into 96-well culture plate at density of 10,000 cells/cm^2 before the addition of the MTT reagent (1:10, 10% MTT), (Sigma) dissolved in PBS (5 mg/ml). Cells were incubated for 3,5 h at 37 C. After removing incubation medium, formazan crystals were dissolved in 200 µl solution of DMSO. MTT reduction was quantified by measuring the light absorbance at 570 nm using a microplate reader ELISA microplate reader VICTOR^3 1420 (PerkinElmer, Turku, Finland). Each test was repeated in triplicate wells. After treatment, cells were photographed with an Olympus CKX41 microscope (Olympus, Tokyo, Japan) with an Olympus DP20 digital camera.

1.1.7 Cytokine measurements

Cytokine levels were determined using Luminex technology (BioPlex Biorad). The IL-8 human kit was performed in 96-well magnetic plates following the manufacturer's instructions. After incubation, the well contents were transferred into 1,5mL tubes and supernatant was collected after centrifugation (1000×*g* for 15 min at RT). Microsphere magnetic beads coated with monoclonal antibodies against different target analytes were added to the wells. After 30 min incubation, the wells were washed and biotinylated secondary antibodies added. After further incubation for 30 min, the beads were washed and then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein, phycoerythrin (streptavidin/phycoerythrin). Samples were then analysed in the Bioplex 200 instrument (Biorad) and cytokine concentrations were estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/mL after adjusting for the dilution factor (Bio-Plex Manager software 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper quantification limit of the standard curves were assigned the highest value of the curve. The intra-assay coefficients of variability (CV) averaged 12%.

1.1.8. RNA extraction and real-time PCR

Total RNA from treated and untreated cells was purified using Trizol® reagent (Life Technologies, CA, USA) according to the manufacturer's instructions. Concentration and purity were determined using Nanodrop technology (BioPhotometer Plus, Eppendorf). All samples exhibited an OD ratio 260/280 between 1.8 and 2.1. One microgram of total RNA was converted to single-stranded cDNA by reverse transcription using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real Time PCR analysis of cDNA samples was performed using SYBR

Green (SSO Advanced, BioRad), with gene specific Forward and Reverse oligonucleotide primers. Primer pair sequences are listed in Table 2. All samples were run in triplicate, in 10 µl reaction volume using 100 ng of cDNA template. The thermal cycler (CFX96 TM Real Time System, BioRad) was programmed as follows: 30s at 95 °C; 40 cycles of 15s at 95°C; 30s at 60°C. The melting curve data were collected to check PCR specificity. Target mRNA levels were normalized against β-actin mRNA and relative expressions were calculated using the $2^{-2\Delta\Delta Ct}$ formula.

Gene	Forward primer	Reverse Primer
B-Actin	5′- GCACCACACCTTCTACAATG-3′	5′-TGCTTGCTGATCCACATCTG-3′
LDH-A	5′- GACCTACGTGGCTTGGAAGA-3′	5′-TCCATACAGGCACACTGGAA-3′
COX-2	5′-TTCTCTACAACAACTCCATCCTC-3′	5′-GCAGCCATTTCCTTCTCTCC-3′

Table 4: Real time PCR primer are reported as 5'-3' sequences

1.1.9 Data analysis

Data are presented as mean ± SD of at least three independent determinations. Statistical differences between groups were determined by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparison. All analyses were performed using GraphPad Prism software (version 6.0; La Jolla, CA, USA). $p<0.05$ was considered to indicate statistical significance. One-way analysis of variance (ANOVA) was carried out to assess the significance of the differences among cereal samples for the protein content, to assess the differences among IL-8 secretion and to assess the differences among mRNA relative expression. Tukey's Honestly Significant Difference test was used to determine differences between means at $P < 0.05$.

1.2 Results

1.2.1 Difference in durum and soft wheat are linked to HMW-GS and LMW-GS protein content

To perform a qualitative comparative analysis of protein samples from ancient and modern wheats, extracted proteins were separated by Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) and stained by colloidal Coomassie blue. Figure 1 shows the number and the molecular weight (MW) of the different proteins purified from different flour varieties. The stained gel shows that many proteins are located between 80-130 KDa (red boxes) corresponding to high molecular weight glutenin subunits (HMW-GS). This protein fraction seems to be more abundant in Triticum aestivum varieties (fig.6, F,G,H,I) than in Triticum durum varieties (fig.6 A,B,C,D).

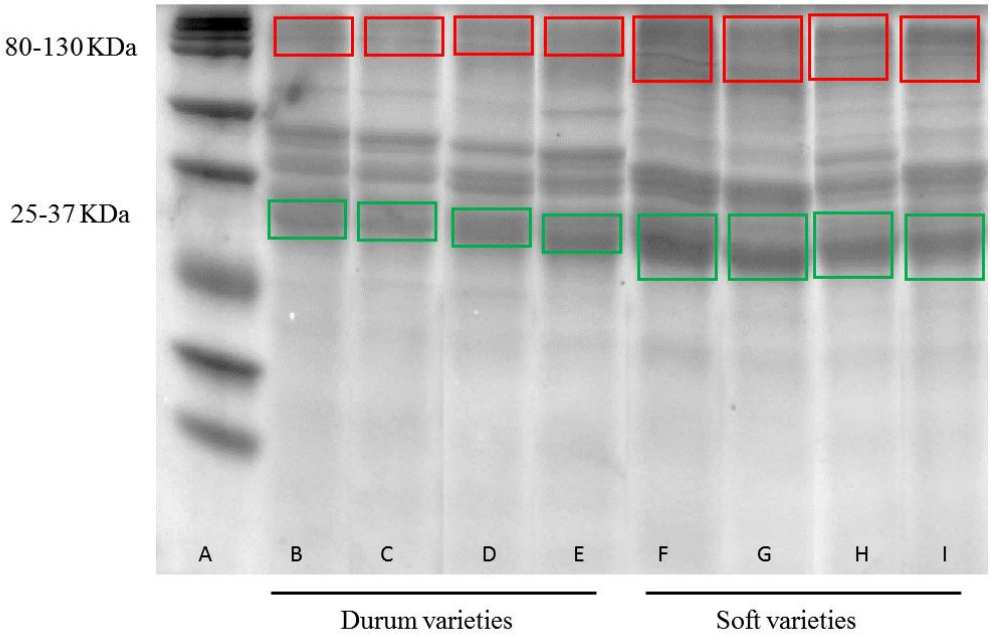


Figure.6 :Polyacrylamide gel electrophoresis of wheat variety: Ciccio (line B), Quadrato (line C), Russello (line D), Timilia (line E), Anapo (line F), Sagittario (line F), Villagloria (line H), Verna (line I).

A larger abundant protein group is visible between 25-37 KDa, (Fig.6, green boxes). These proteins were previously identified by LMW-GS as Gliadins and seems to be more abundant in Triticum aestivum varieties especially in modern varieties. No macroscopic differences in protein composition was detected between the modern and ancient varieties.

1.2.2 Wheat proteins induce no change in cells viability

The cytotoxic effect of different extracted wheat proteins has been evaluated by using the MTT assay. None of the wheat proteins tested turned out to be toxic by itself to Caco-2, HL-60, THP1 and CRL-1831 cells culture. Figures 7A and 7B show cell viability expressed as a relative percentage compared with the untreated control cells. Mann-Whitney analysis showed no significant difference in cells viability when comparing the negative control group and groups treated with different wheat proteins.

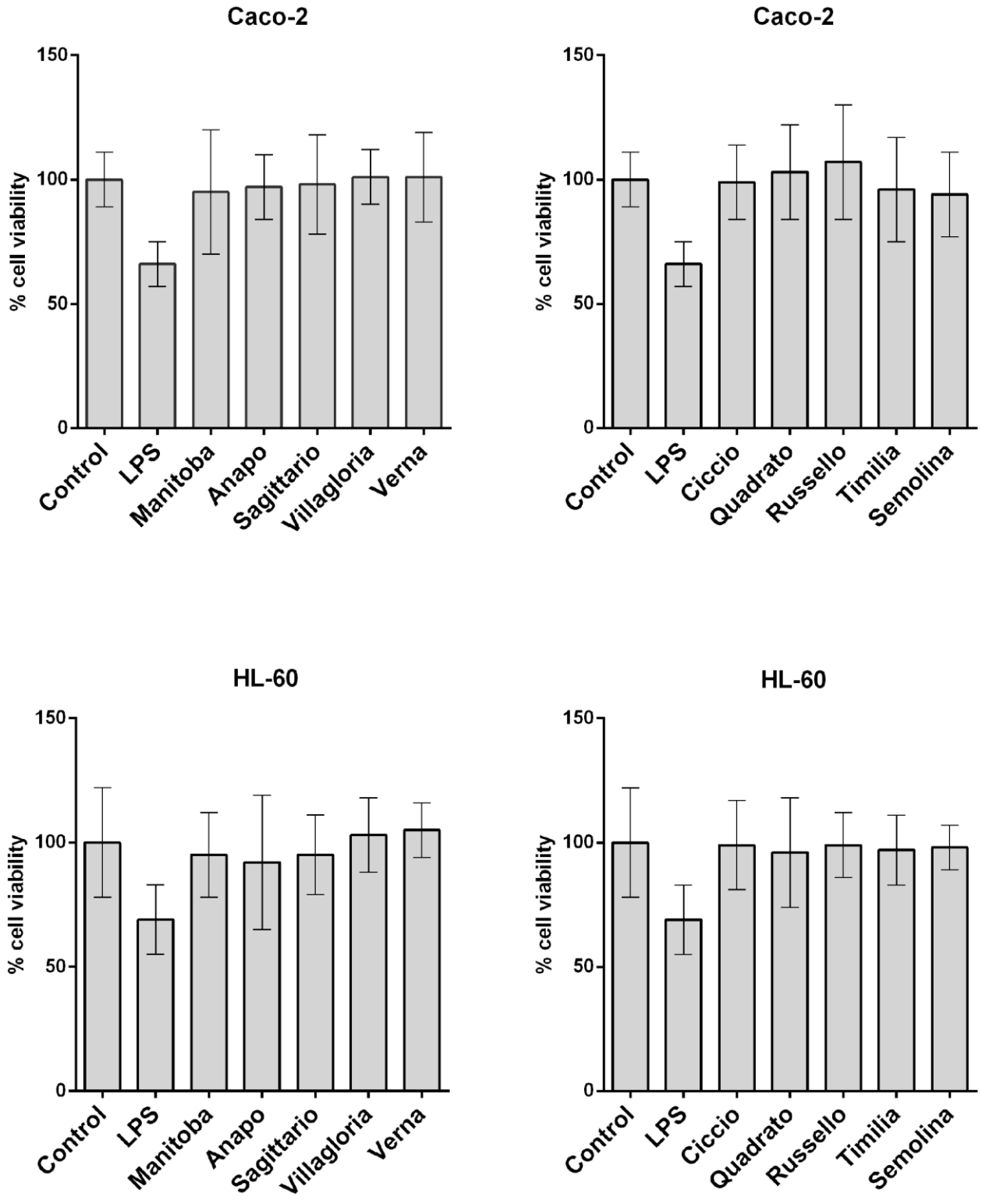


Figure. 7A Cell viability (%) of Caco-2 and HL-60 treated with soft and durum Modern and ancient wheat varieties.

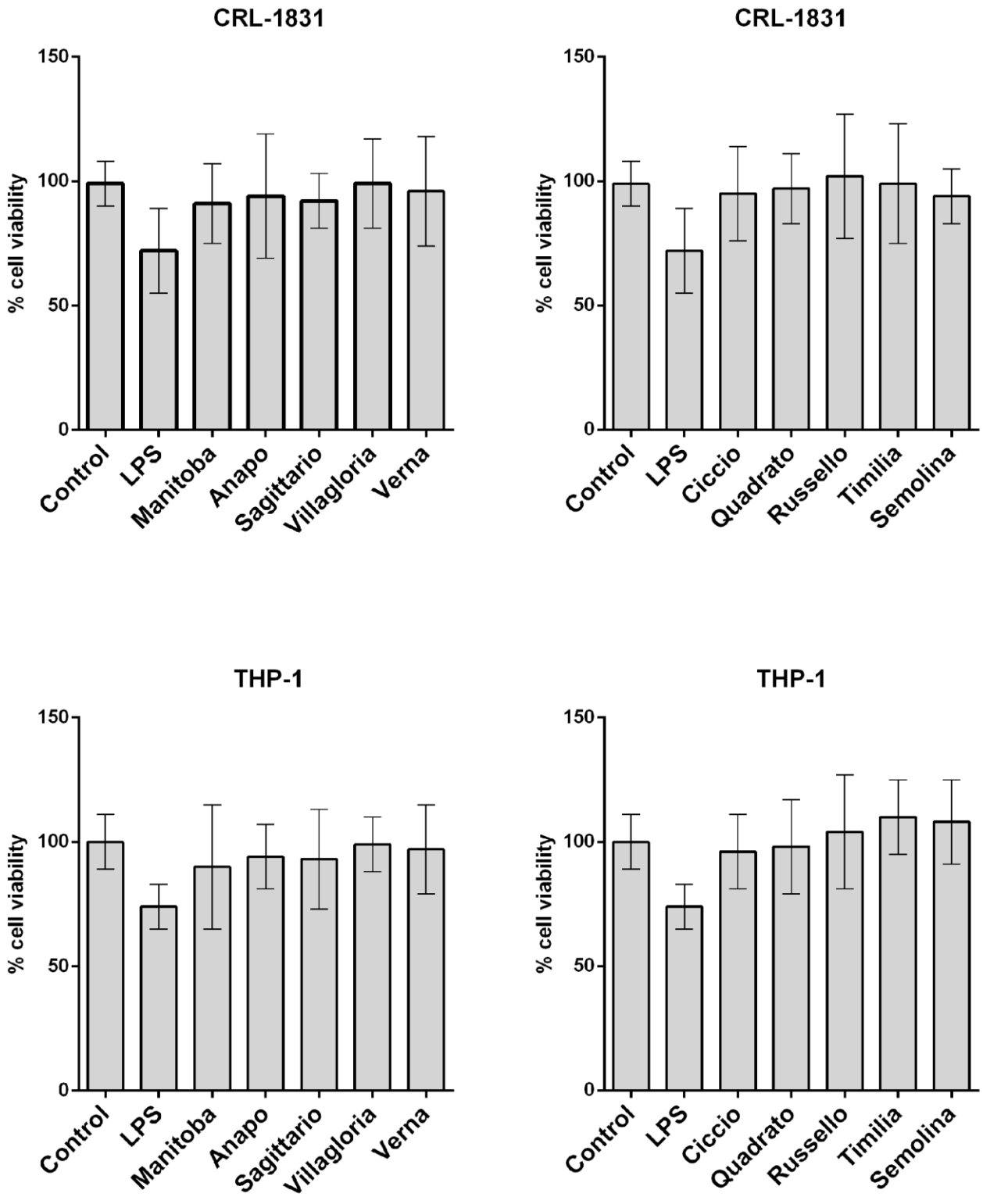
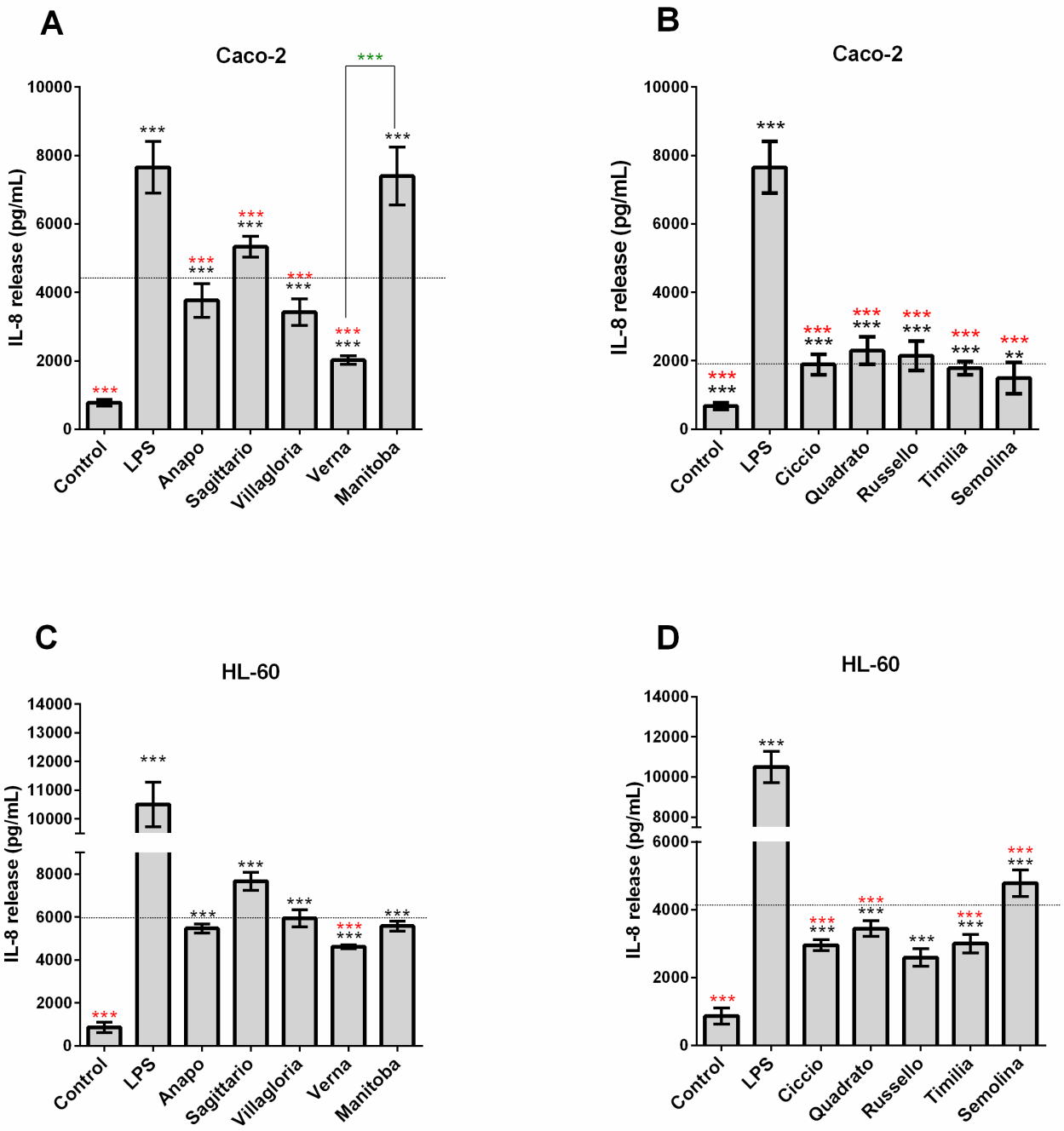


Figure. 7B Cell viability (%) of CRL-1831 and THP-1 treated with soft and durum Modern and ancient wheat varieties.

1.2.3 Pro-inflammatory effect of wheat proteins is more evident in soft wheat varieties

To evaluate the effect of wheat proteins on cytokines production, Caco-2, HL-60, THP1 and CRL-1831 cells were stimulated for 16 h with protein samples extracted from different varieties of Durum and Soft wheat. LPS was used a positive control and IL-8 Luminex analysis was performed on conditioned medium of treated cells. Results showed that treatment with soft wheat proteins induced a significant increase in IL-8 release in Caco-2 and HL-60 cells line if compared to control cells. In CTRL-1831 and THP1-cell line IL-8 release was found generally lower respect to Caco-2 cells and HL-60 cells. In CRL-1831 and THP1-cell lines no significant differences were observed between treated cells to control cells. In CaCo-2 cells, a pro-inflammatory effect was observed with protein samples from both ancient and modern soft wheats (figure 8, panel A and B). The increase of IL-8 release was found statistically significant all the samples compared to negative control cells (black asterisks), suggesting a generic stimulatory effect of wheat proteins on CaCo-2 cells. Interestingly, Manitoba treatment, led to a rise of IL-8 secretion similar to that induced by LPS treatment. No significant differences were observed between ancient and modern wheat varieties. Only Verna-treated cells, among soft varieties, resulted statistically different respect to Manitoba-treated cells and significantly lower respect to LPS treated cells (green asterisks). Durum wheats (Figure 8 panel B) seem to elicit significant lower inflammatory effect on CaCo-2 cell lines. In HL-60 cell line, we observed a greater basal IL-8 production (Figure 8 Panel C) in comparison to Caco-2 cells (Figure 8 Panel A). Proteins extracted from soft wheats (modern and ancient) led to a rise of IL-8 significantly higher respect to negative control (black asterisks) (Fig 8 Panel C). Moreover, almost all soft varieties seem to have an inflammatory effect similar to that of LPS. Notably, only in Verna-treated cells we found an IL-8 release significantly lower respect to LPS treated cells. The release of IL-8 in Manitoba-treated cells showed the same trend of the other treatments and no difference was found in cells treated with proteins extracted from modern or ancient varieties. Durum wheats (modern and ancient varieties) elicit an inflammatory effect on all the treated cell. In fact, we detected an increase of IL-8 production in all the wheat proteins treated cell lines if compared to the negative control (Fig. 8). We also detected a significant lower IL-8 release in all the wheat proteins treated cells if compared to LPS-treatment (Fig. 8). All wheat-treated cells showed no difference in IL-8 secretion respect to control cells. No significant differences between ancient and modern wheat varieties were found in these cell lines, both for soft and durum wheats.


A
Caco-2
IL-8 release (pg/mL)
10000
8000
6000
4000
2000
0
Control
LPS
Anapo
Sagittario
Villagloria
Verna
Manitoba
B
Caco-2
IL-8 release (pg/mL)
Control
LPS
Ciccio
Quadrato
Russello
Timilia
Semolina
C
HL-60
IL-8 release (pg/mL)
14000
13000
12000
11000
10000
Control
LPS
Anapo
Sagittario
Villagloria
Verna
Manitoba
D
HL-60
IL-8 release (pg/mL)
Control
LPS
Ciccio
Quadrato
Russello
Timilia
Semolina

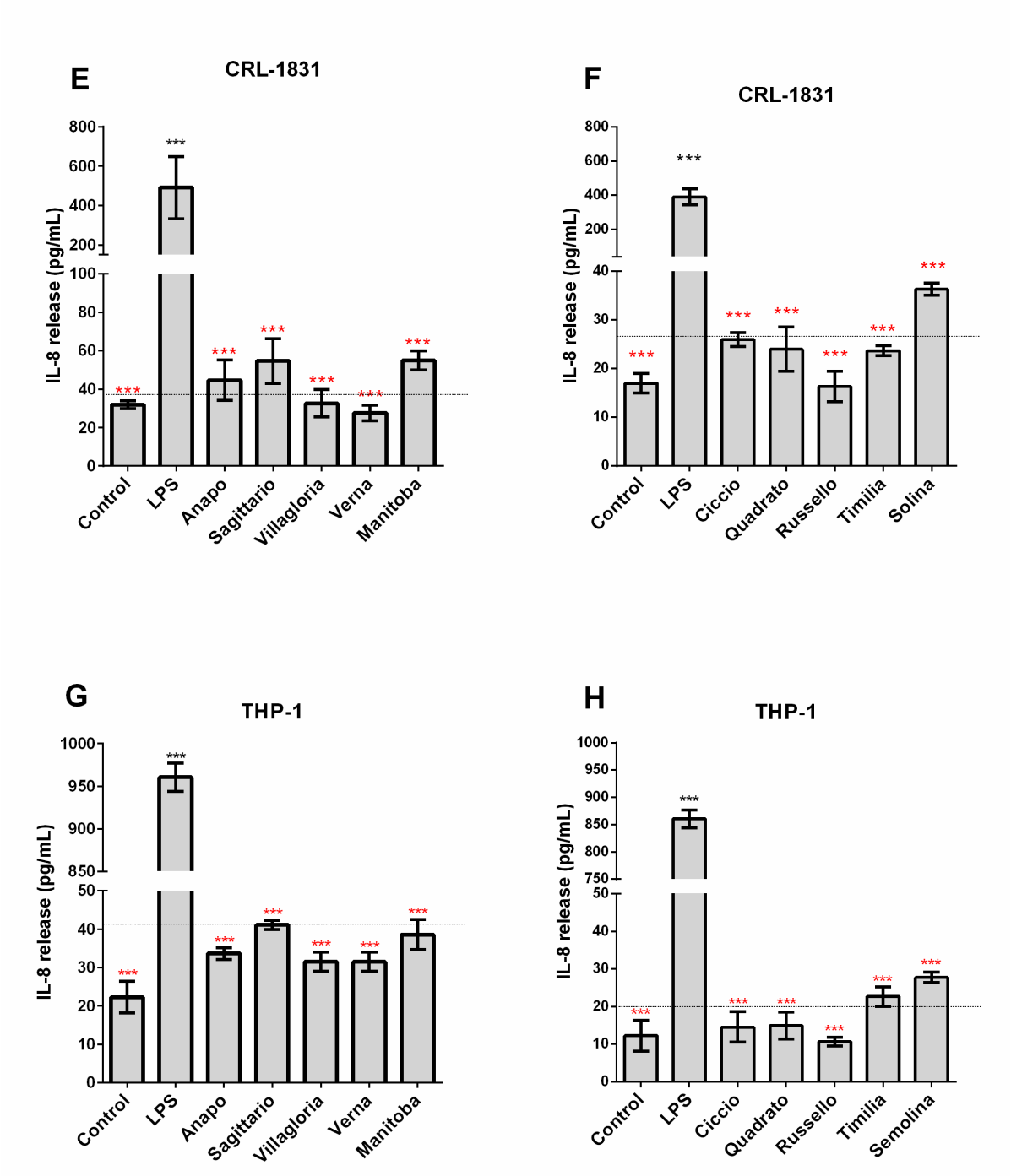


Figure. 8 Pro-inflammatory effects in Caco-2.(A and B), HL-60 (C and D) CRL-1831 (E and F) and THP-1 (G and H). Cells were stimulated with ancient and modern soft wheat proteins for 16h and IL-8 production was detected. Data are presented as mean ± SD (n=3). Asterisks indicate p-values calculated by one-way ANOVA as follow: *=<0.05, **=<0,005 and ***= < 0.0005. Black asterisks indicate statistical significance respect to the "Control" sample, red asterisks respect to "LPS" sample and green asterisks respect to "Manitoba" sample.

1.2.4 Expression of biomarkers related to chronic inflammation

In order to investigate a possible mechanism for the inflammatory effect elicited on colon mucosa by ancient and modern wheats, we stimulated Caco-2 cell line with proteins extracted from Virgo flour (a blend of Andriolo, Inallettabile, Verna, Gentil Rosso e Frassineto soft ancient wheat) and Manitoba, one of the most common soft modern variety. Cox-2 and LDH-A mRNA expression was measured by Real Time PCR at different time points after treatment (3, 6 and 18 h) and normalized on GAPDH expression (Figure 9). For these experiments, positive (inflamed) control was obtained by treating cells with TNF-α and IL-17 cytokines instead of LPS to induce an inflammation with features similar to a chronic disease[115]. Results shown in Fig. 9 panel A revealed a significant upregulation of Cox-2 expression in cytokines treated-cells compared with control, anyway the expression evaluated at the different time points strongly decrease over time. No significant difference of LDH-A expression was recorded at any time point .

In both modern and ancient wheat-treated cells (Fig.9 panel B and C) no significant difference in expression of Cox-2 gene was found compared to baseline at different time points. No significant changes in LDH-A mRNA expression was observed in Manitoba treated cells except for a slight increase observed after 18h of treatment. In Virgo treated cells only a slight decrease LDH-A mRNA expression over time was observed, suggesting an opposite trend respect to Manitoba treated cells although not significant.

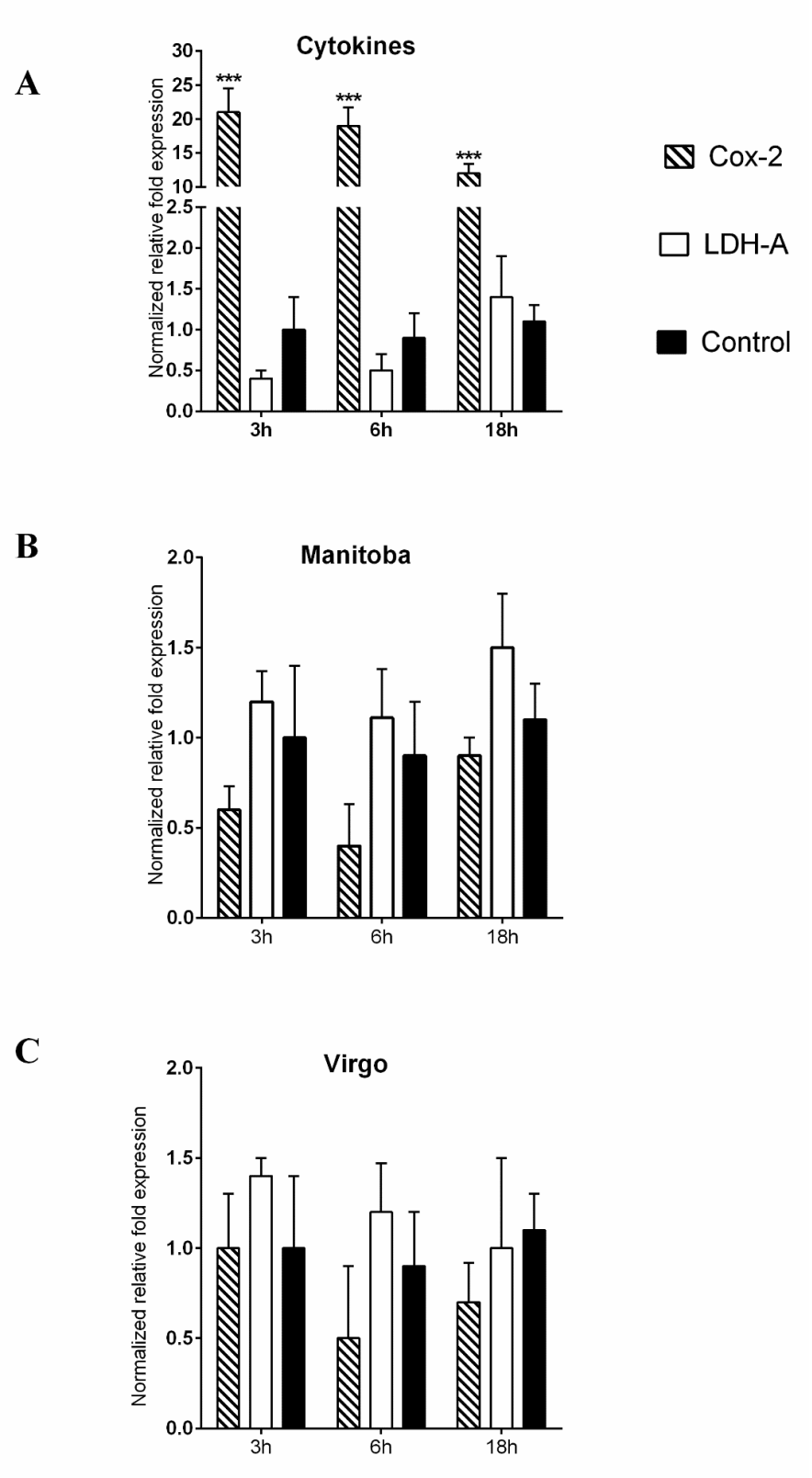


Figure 9. Real-time PCR of mRNA expression of Cox-2 and LDH-A in Caco-2 cells treated for 3,6 and 18h with cytokines(panel A), Manitoba (panel B) and Virgo (panel C). Graphs represent Real-time PCR analysis of Cox-2 (dashed bars), LDH-A (white bars) and Control (black bars) mRNA expression. Values are reported as Normalized fold expression (2^{-DDCT}) relative to untreated cells. B-Actin was used as housekeeping gene. Statistical analysis was performed by one-way ANOVA. *** = $p < 0,005$.

Chapter 2 – Evaluation of immune response to cereal sources in subjects with NCGS.

Two studies were performed to investigate the effect of wheat exposure on immune system. The first study[100] was aimed to verify a possible differential response of immune system in NCGS and healthy pediatric patients. The second was a pilot study aimed to validate a possible test to differentiate healthy subjects from NCGS patients. Both studies investigated in vitro the inflammatory proprieties of ancient and modern grain proteins.

2.1 Materials and methods

2.1.1 Cereals samples

For study conducted on paediatric NGWS patients the investigated cereal samples characteristics are reported in table 1. A sample of gluten-free rice (Oryza sativa) flour was used as negative control. Manitoba and rice samples were purchased at local supermarkets.

Name of Grains	**Type of Grains**	**Type Variety**	**Genome**
Manitoba	*Triticum Aestivum*	Modern	Hexaploid
Kamut	*Triticum Turanicum*	Ancient	Tetraploid
Senatore Cappelli	*Triticum Durum*	Ancient	Tetraploid

Table 5: cereals sample

2.1.2 Patients

We evaluated 11 children with NCGS, diagnosed according to the criteria proposed by the Expert Meeting held in Salerno[111]after a thorough evaluation at the Gastroenterology of Pediatric Unit, Maggiore Hospital, Bologna, Italy. All patients complained of one or more GI (bloating, abdominal pain, diarrhea/constipation, nausea, epigastric pain, gastro-esophageal reflux, aphthous stomatitis) and extraintestinal (tiredness, headache, joint/muscle pain, arm numbness, "brain fog", dermatitis/skin rash, anxiety, depression) symptoms/manifestations with an early onset, but with a normal height–weight growth. CD was excluded in all enrolled NCGS patients by negativity for anti-tTG and anti-EmA and the absence of histological alterations on duodenal biopsy (Marsh classification M0 or M1). WA was excluded by negativity for specific IgE antibodies to wheat and skin prick tests (SPTs). NCGS children showed an unusual gender distribution and comprised 9 males and 2 females ranging in age from 3.2years to 16years (median 12.8years, aver- age 11.3years). Family history and the medical history (remote and recent) were collected from all patients. All children were evaluated for inflammatory markers (ESR, CRP and fecal calprotectin), specific serology to exclude CD (total IgA, anti- tTG and anti-EmA), IgG antibodies to deamidated peptides and a gliadin IgG antibodies). They also underwent allergy tests (SPTs and specific IgE for foods including wheat and gluten and inhalants, patch testing for foods including wheat), endoscopy of the upper gastrointestinal tract under sedation and a search for human leukocyte antigen (HLA) haplotype predisposing to CD (DQ2–

DQ8). NCGS patients were initially put on a GFD, obtaining a complete remission of both GI and extraintestinal symptoms. Then they were placed on a 2weeks wheat challenge to confirm the clinical suspicion of NCGS. Eighteen CD patients on a GFD diet, age- and sex- matched with the NCGS group (ranging in age from 4 to 17years, median 14years, average 11.1years) were also enrolled. Sixteen healthy children without fever or GI symptoms history (ranging in age from 7 to 17years, median 12years, average 12.5years) admitted to the pediatric emergency ward of the same hospital were also enrolled as controls. A 2 ml blood sample was obtained from all patients. The study was approved by the Ospedale Maggiore Independent Institutional Ethical Committee (approval code GS-AS 2014). Children's parents gave their written informed consent.

2.2.3 Protein extraction

Proteins were extracted using the procedure described by Osborne (1907)[113] and subsequently modified by Lookhart and Bean (1995)[114] and van den Broeck et al. (2009)[20]. Albumin extractions: 100 mg of flour was extracted with deionized water (500 µl) for 30 min, vortexing for 1 min, every 10 min. The mixture was centrifuged for 5 min at 2,000 rpm. The supernatant (albumin extract) was decanted and stored. The procedure is repeated twice. Gliadin extraction: The insoluble pellet obtained from the globulin extraction was solubilized with 70% aqueous ethanol (400 pl) for 30 min, vortexed for 1 min every 10 min, and centrifuged for 5 min at 2,000 rpm on the mini-centrifuge. The supernatant (gliadin extract) was decanted and stored. The procedure is repeated twice. Glutenin extraction: Glutenins were extracted from the gliadin insoluble pellet with 400 pl of 50% 1-propanol + 1% dithiothreitol (DTT) for 30 min, vortexed for 1 min every 10 min. After centrifugation for 5 min at 2,000 rpm, the supernatant (glutenin extract) was decanted and stored. The procedure is repeated twice. For the ex vivo tests, all the extracts were pooled to obtain a total sample for each cereal variety. Protein samples were then precipitated at -20˚C overnight with 12:1 Acetone and Methanol mixture and centrifuged at 12,000 g for 45 min at 4˚C. Precipitated samples were then dried with vacuum and solubilized in 4M UREA over night at room temperature. The total protein content was measured using the colorimetric DC™ Protein Assay (Bio-Rad, CA, USA). The absorbance values were obtained at 700 nm using a microplate reader.

2.2.4 Isolation and culture of PBMC of paediatric patients

Blood samples from enrolled pediatric patients were collected in EDTA-containing vacuum tubes and kept under agitation on a roller mixer at room temperature for 4 h. PBMC were separated using the kit-MateTM Sep 15 (STEMCELL Technologies, Vancouver, BC, Canada) following the manufacturer's instructions. PBMC were resuspended in 1 ml of RPMI-1640 (Life Technologies, CA, USA), supplemented with 25 mM HEPES, antibiotics (penicillin and streptomycin), L-Glutamine and 10% FBS. After careful resuspension, $4x10^5$ cells were seeded in each well of a 24-well plate; 40 ug of protein extracts of selected flours were resuspended in 200 µl of RPMI-1640 and added to PBMC cultures. Plates were incubated for 12 h at 37°C and 5% CO_2 (Forma Series II Water Jacketed CO_2 Incubator) under mild shaking.

2.2.5 Determination of secreted CXCL10 in PBMC extracted from paediatric patients

After incubation, the well contents were transferred into1,5 ml tubes and supernatant was collected after centrifugation (1000g for 15 min at RT) and subjected to cytokine/chemokine determination using Luminex® MAP technology. Cytokine and chemokine levels were first determined using a multiplexed 27-plex human bead immunoassay kit (Bio-Rad, CA, USA). Then, a single plex for CXCL10 was used. Microsphere magnetic beads coated with monoclonal antibodies against cytokines and chemokines were added to the wells. After incubation for 30 min, the wells were washed and biotinylated secondary antibodies were added. After incubation for another 30 min, beads were washed and then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein phycoerythrin (streptavidin/phycoerythrin). After washing, the beads (a minimum of 100 per analyte) were analyzed in the BioPlex 200 instrument (BioRad). Sample concentrations were estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor (Bio-Plex Manager software 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper limit of quantification of the standard curves were assigned the highest value of the curve. The intra-assay CV averaged 12%. This method is covered by patents owned by Alma Mater Studiorum, University of Bologna (WO 2014/037858; PCT/IB2013/058148).

2.2.1 Cereals samples

A total of 5 flour samples were analysed for NCWS adult study. The samples varieties is shown in Table 2. The flours, milled using a stone mill (100% flour extraction), were obtained from Department of Agricultural Science of the University of Bologna (Italy).

Name of Grains	**Type of Grains**	**Type Variety**	**Genome**
Farro	*Triticum Monococcum*	Ancient	Diploid
Verna	*Triticum aestivum*	Ancient	Hexaploid
Sagittario	*Triticum aestivum*	Modern	Hexaploid
Timilia	*Triticum turgidum*	Ancient	Tetraploid
Ciccio	*Triticum turgidum*	Modern	Tetraploid

Table 6: cereals sample

2.2.2 Protein extraction

Proteins were extracted using the procedure described by Osborne (1907)[113] and subsequently modified by Lookhart and Bean (1995)[114] and van den Broeck et al. (2009)[20]. Albumin extractions: 100 mg of flour was extracted with deionized water (500 µl) for 30 min, vortexing for 1 min, every 10 min. The mixture was centrifuged for 5 min at 2,000 rpm. The supernatant (albumin extract) was decanted and stored. The procedure is repeated twice. Gliadin extraction: The insoluble pellet obtained from the globulin extraction was solubilized with 70% aqueous ethanol (400 pl) for 30 min, vortexed for 1 min every 10 min, and centrifuged for 5 min at 2,000 rpm on the mini-centrifuge. The supernatant (gliadin extract) was decanted and stored. The procedure is repeated twice. Glutenin extraction: Glutenins were extracted from the gliadin insoluble pellet with 400 pl of 50% 1-propanol + 1% dithiothreitol (DTT) for 30 min, vortexed for 1 min every 10 min. After centrifugation for 5 min at 2,000 rpm, the supernatant (glutenin extract) was decanted and stored. The procedure is repeated twice. For the ex vivo tests, all the extracts were pooled to obtain a total sample for each cereal variety. Protein samples were then precipitated at -20˚C overnight with 12:1 Acetone and Methanol mixture and centrifuged at 12,000 g for 45 min at 4˚C. Precipitated samples were then dried with vacuum and solubilized in 4M UREA over night at room temperature. The total protein content

was measured using the colorimetric DC™ Protein Assay (Bio-Rad, CA, USA). The absorbance values were obtained at 700 nm using a microplate reader.

For the *in vitro* tests, all the extracts were pooled to obtain a total protein extract for each cereal variety. Protein samples were precipitated at -20˚C overnight with 12:1 mix Acetone and Methanol and centrifuged at 12,000 g for 45 min at 4˚C. Concentrated samples were then dried with vacuum and solubilized in 4M UREA over night at room temperature. The total protein content was measured using the colorimetric DC™ Protein Assay (Bio-Rad, CA, USA). The absorbance values were obtained at 700 nm using a microplate reader.

2.2.3 Patients

First study was approved by the Ethics Committee of Spedali Civili di Brescia and the AOU S.Orsola-Malpighi di Bologna. 12 patients with NCWS diagnosis and 11 healthy controls were enrolled, mean age was 41 years, minimum age 20 years, maximum age 67, patients were on a free diet for at least 6 weeks. CD was ruled out in all enrolled NCGS patients by negativity for anti-tissue transglutaminase (anti-tTG) and anti-endomysial antibodies (EmA) and the absence of villous atrophy in the duodenal biopsy, WA was excluded by the negativity for specific IgE antibodies to wheat and/or skin prick tests. A blood sample was obtained from both NCGS patients, control subjects blood was anonymously provided by the transfusion centre (mean age 30 years). Patients gave their written informed consent.

2.2.4 Plasma Preparation

Whole blood was collected from each subject and centrifuged at 1000 g at 4℃ for 15 min, obtained plasma was collected and stored at -80℃ until use.

2.2.5 Dot-blot assay

Analysis of total protein from wheat flour was performed by dot blot as previously described [3,4]. For this, 6 ug of wheat protein were diluted in Tris-buffered saline (TBS)+UREA 2M+ DTT 50mM and each sample applied to a buffer-soaked nitrocellulose membrane using a Minifold I vacuum dot-blot system device (Whatman) with a 96-well top frame. Subsequently, the membrane was removed from the dot blot device and dried. Non-specific sites were blocked by soaking in 5% BSA in PBS-T (5 g BSA + 100 ml PBS, pH 7.2 + 30 µl Tween 20) then incubated with plasma samples diluted to 1:1000 in PBS-T for 2 hr at room temperature, washed 3 times with PBS-T 5min each. Then, it was

incubated with the anti-Human IgG 1:2500 in TBS-Tween solution at 2% BSA for 2h at RT. Secondary antibody conjugated with with CY5 and diluted to 1 µl: 1,250 µl in PBS-T followed by incubation 2 hr with shaking, washing 2 times with PBS-T 2 min each, and then once with PBS for 5 min. Membranes were acquired using a Pharos FX molecular imager, CY5 selected fluorescence, medium intensity, 50 m resolution. The images obtained was analyzed using the QuantityOne program (BioRad) using the Report Analysis Tool function. The method returns some parameters including the Adjusted (Adj.) Volume which was chosen as reference value as it indicates the intensity of the spot on the selected volume. The spot intensity was linearly correlated with the different total protein quantities used without saturation or latency.

2.3 Statistical analysis

Data are presented as mean ± SD of at least three independent determinations. Statistical differences between groups were determined by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparison. All analyses were performed using GraphPad Prism software (version 6.0; La Jolla, CA, USA).$p<0.05$ was considered to indicate statistical significance.

2.2 Results

2.1.2 CXCL10 secretion by PBMC of paediatric patients and controls

The main purpose of our ex-vivo studies was to investigate the immune cell response to protein extracts from ancient and modern grains.

CXCL10 secretion have shown significant differences ($p<0.01$) in PBMCs from pediatric NCWS patients stimulated with proteins extracted from wheat flour compared to stimulation with rice flour proteins (Figure 10 panel A). CXCL10 secretion was significantly higher, from 2- to 3.6-fold, for all three grains tested. These data suggest that the secretion of this proinflammatory cytokine by PBMCs is somehow stimulated by wheat proteins, including gluten. Analysing the effect of the modern dwarf variety (manitoba) in comparison with each single ancient grain, we found that this behaved differently from the two ancient varieties (Senatore Cappelli and khorasan wheat) in terms of chemokine secretion. Manitoba proteins-induced secretion reached values that were statistically higher than those obtained in the presence of ancient varieties proteins ($p<.01$ versus khorasan proteins and $p<.05$ versus Senatore Cappelli proteins), while no differences were observed between Senatore Cappelli and khorasan CXCL10-induced secretions. On the other side, the responses of PBMCs from paediatric controls did not significantly increase when wheat protein extracts were added (Figure 10 panel B). CXCL10 secretion in CD pediatric patients significantly increased under stimulation by gluten-containing flours, but at lower levels than those reached in NCGS patients (Figure 10 panel C).

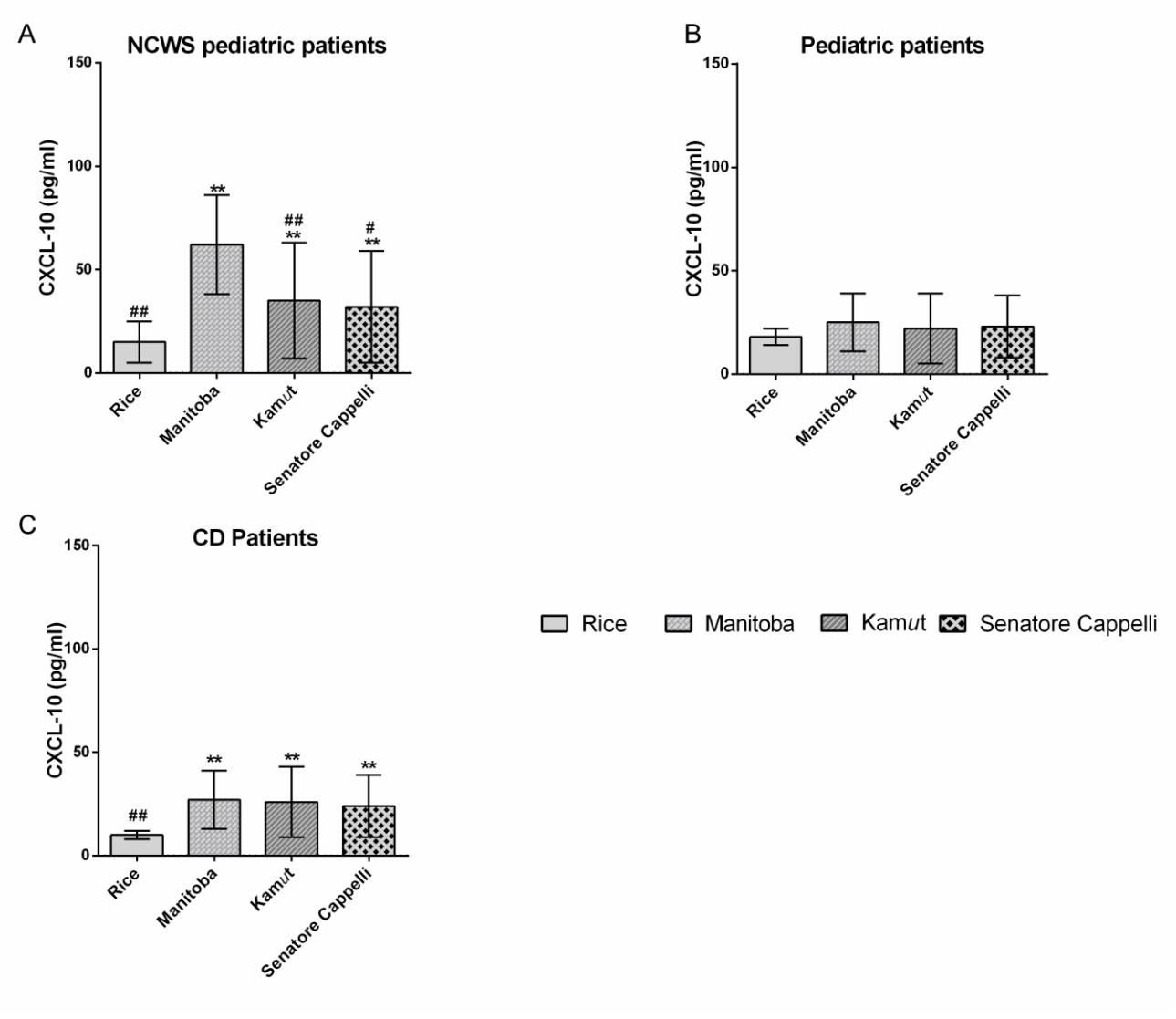


Figure 10: Detection of CXCL-10 release in PBMCs from paediatric NCGS patients (A), paediatric control patients (B) and paediatric CD patients (C) stimulated with proteins extracted from different wheat flours containing gluten. The levels of CXCL10 cytokine indicated by bars were obtained by differentially subtracting the baseline values of CXCL10 secretion of purified unstimulated PBMCs (Ctr value). ** indicates that CXCL10 secretion was statistically higher ($p < .0,01$) in comparison with that obtained by rice proteins . # and ## indicate that CXCL10 secretion was statistically different (#$p < .05$ and ##$p < .01$) in comparison with that obtained by manitoba proteins stimulation.

2.2.1 IgG response in adults patients and controls

Dot-Blot plasma analysis of NCWS patients and healthy controls showed a high interindividual variability in response to different extracted wheat proteins. Due to this variability was not possible to obtain confident analysable data. For this purpose we decided to use a cut-off method to obtain an adjusted value-layer population structure. In this case a cut-off of 50 was used.

The analysis revealed that in healthy population the 27.3% of subjects resulted positive to *Triticum Monococcum* proteins (figure 11). None of the subjects showed response to Verna proteins that remains the wheat *flour* characterized by lower *immunogenic potential* properties in this study. No difference was observed about Sagittario, Ciccio and Timilia proteins that seems induce no response in 9% of analysed population. The 63.7% of subjects no shows IgG response against wheat proteins (figure 11).

In NGWS population the 66.6% of subjects resulted positive to *Triticum Monococcum* proteins, 8.3% of subjects show IgG response against Verna. 12% NCWS patients show IgG response against Sagittario (12%) and protein from Durum varieties of wheat Timilia and Ciccio (12%) (figure 11). NSWS patients show a more higher effect of Triticum Monococcum comparing with the results from healthy population and in general their antibody responses seems more marked with regard to all wheat proteins.

Figures 12 and 13 show respectively adjusted volume values for each healthy control subjects and each NCWS patients.

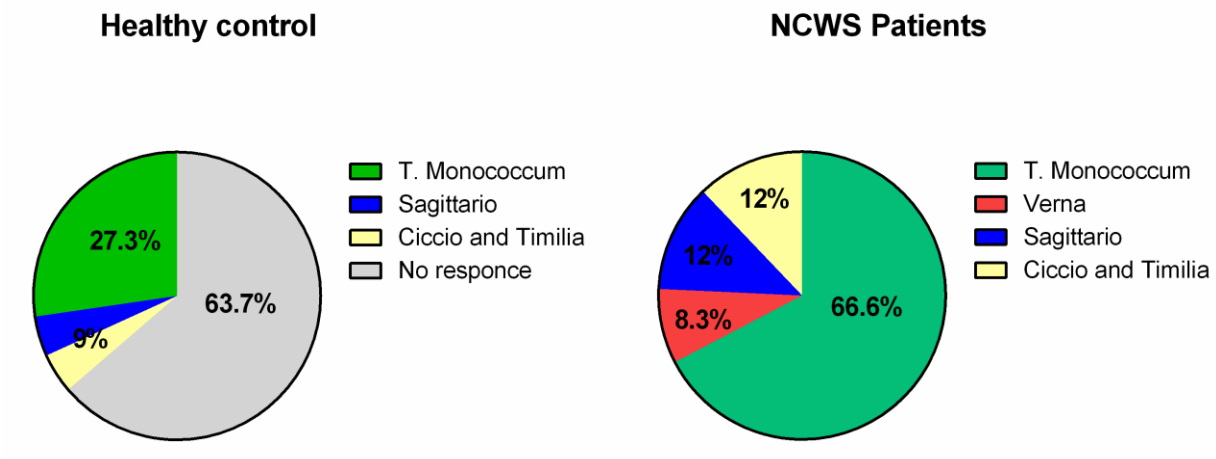
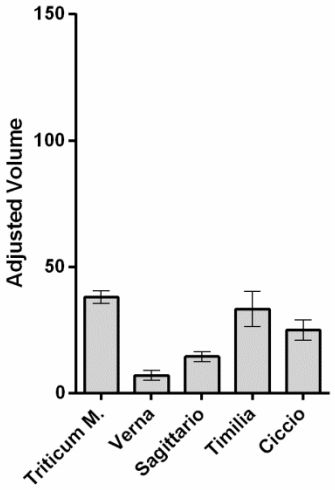
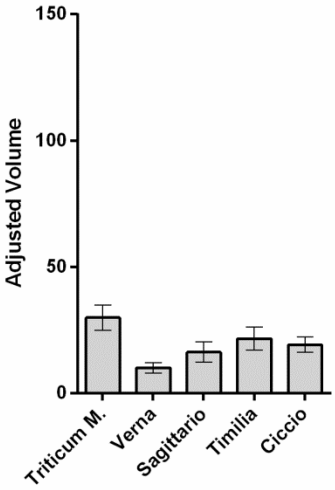


Figure 11: IgG mediated responses in heathy and NCWS patients.



Healty control 1



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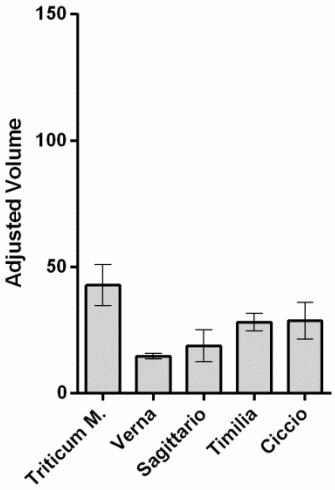

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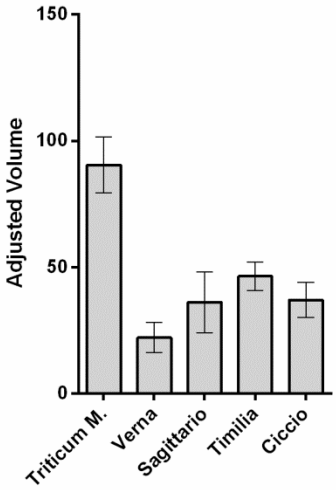

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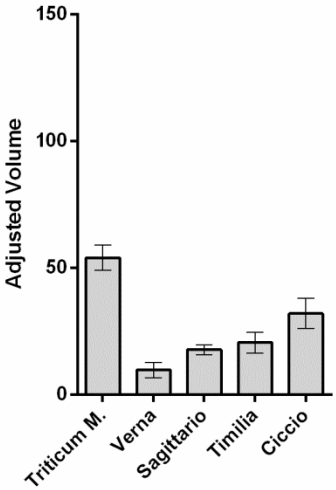

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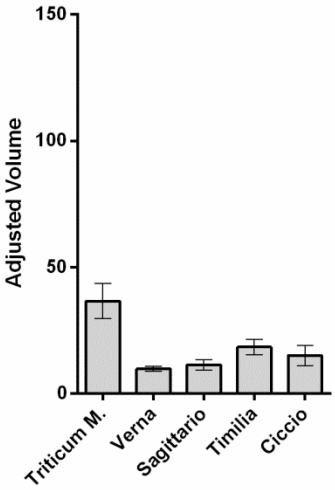

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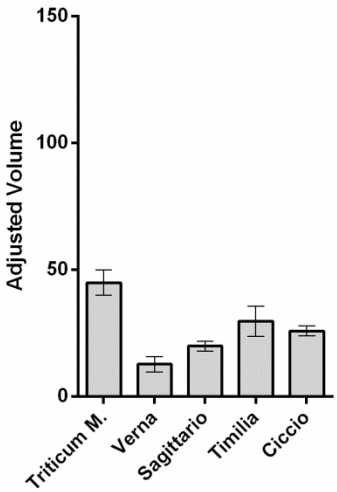

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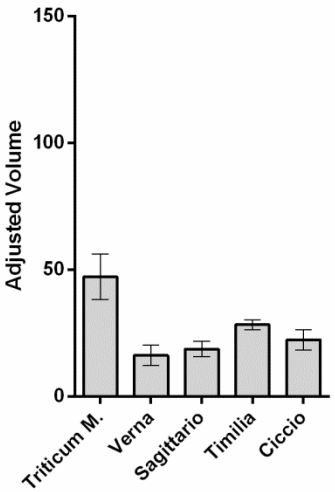
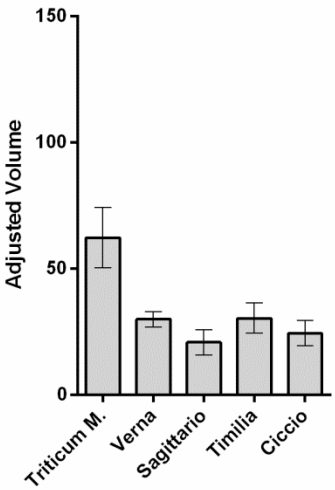


Healty control 8



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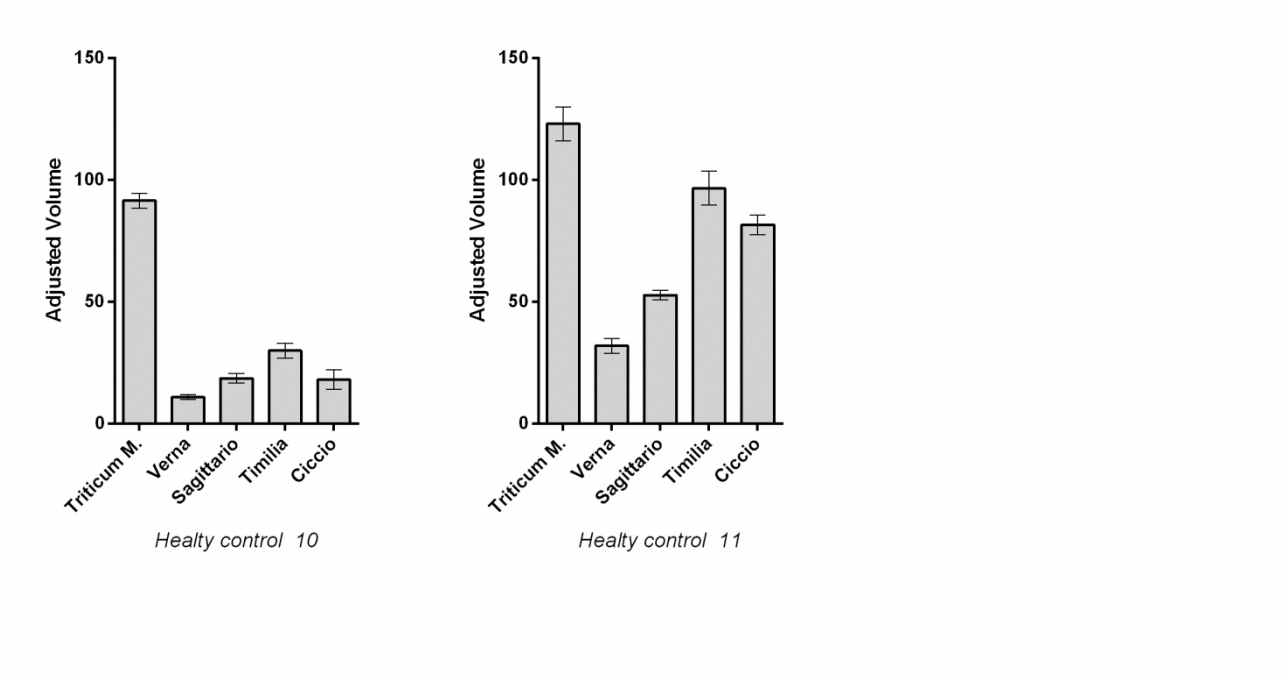
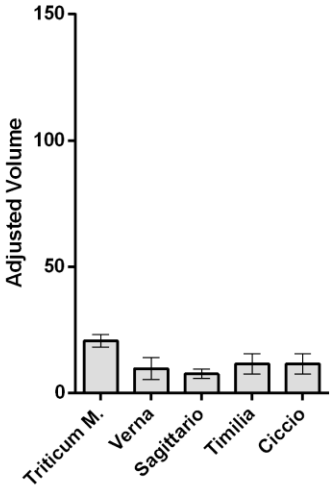
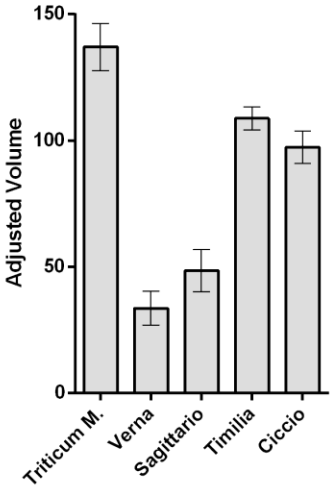


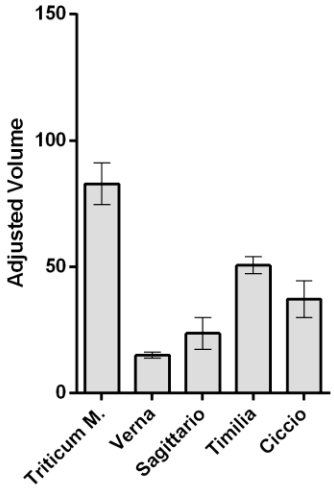
Figure 12: IgG mediated responses in each heathy patients.



Patient 1



Patient 2



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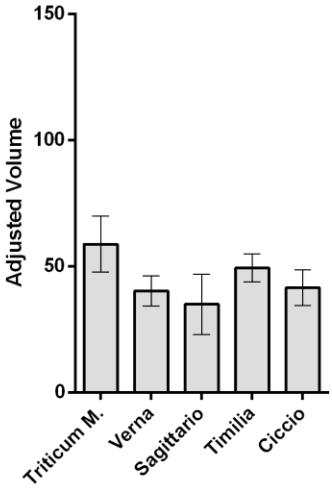

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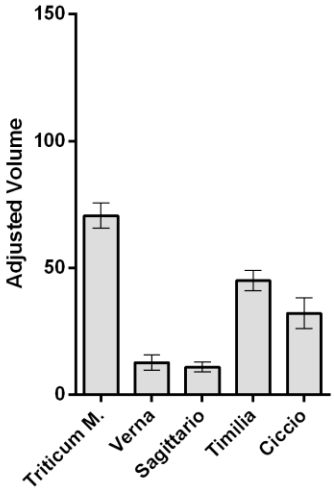
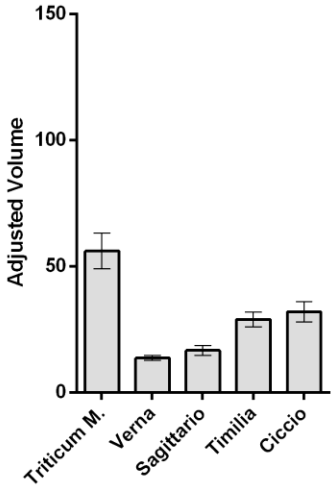


Patient 5



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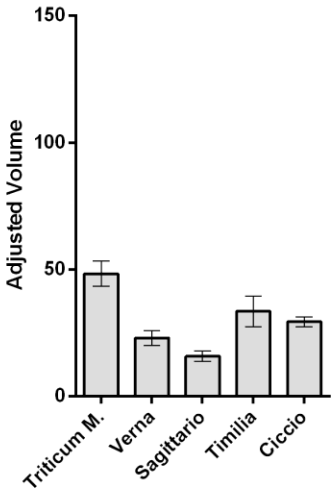
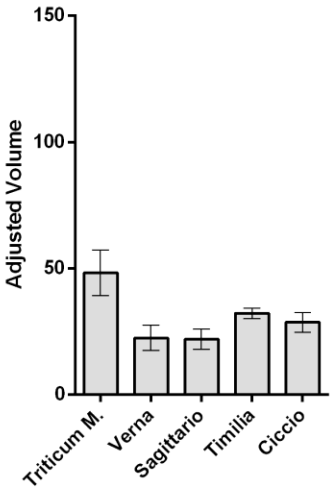
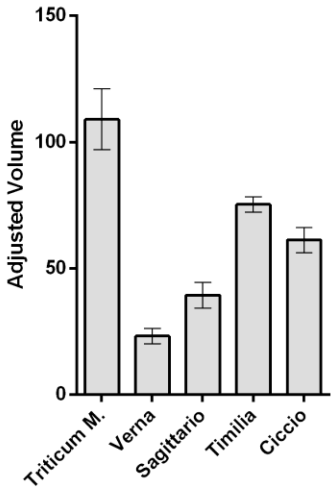


Patient 7



Patient 8



Patient 9

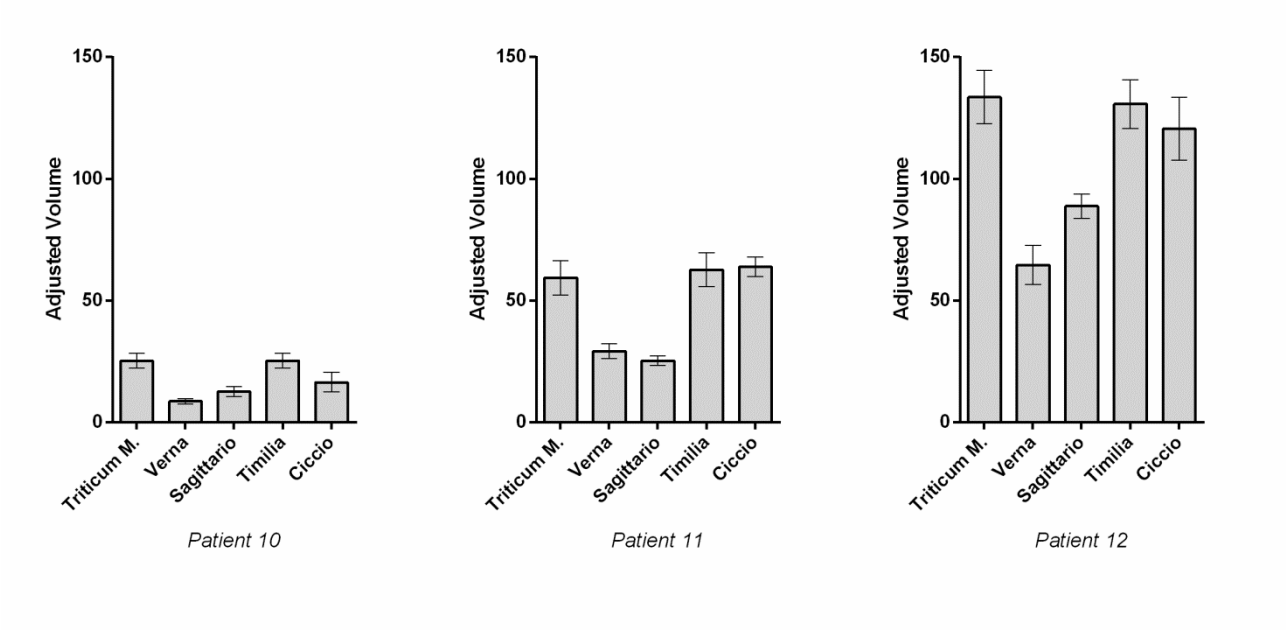


Figure 13: IgG mediated responses in each NCWS patients.

Chapter 3 – Inflammatory effect of different bread in mice model of gut disease

3.1 Material and methods

3.1.1 Animal study

64 six-week-old female with conditional deletion of caspase-8 in the intestinal epithelium ($Casp8^{\Delta IEC}$) and 64 $Casp8^{fl/fl}$ control littermates were provided by laboratory of Christoph Becker (Medical Clinic, Erlangen, Germany). Mice were housed in a specific pathogen-free (SPF) barrier facility with a fully controlled environment at 22 ± 2°C and 50% humidity, under a 12-h light/dark cycle accredited by the Association for Assessment and Accreditation for Laboratory Animal Care International. Animals were kept in collective cages containing two mice each. Before inclusion in experiments all mice received a gluten free breeding diet ad libitum. Experimental and animal handling protocols were approved by the local Animal Care and Use Committee (Regional Council Stuttgart, V314/18 EM).

3.1.2 Diet components

In this study two different superfine wheat flour varieties (*Triticum aestivum* and *Triticum aestivum subsp. Spelta* obtained from SchapfenMühle GmbH & Co. Ulm-Jungingen, Germany) were tested and two type of fermentation (yeast and sourdough) were used to obtain 4 type of bread: yeast-bread with modern variety of wheat flour (B1), sourdough-bread with modern wheat flour (B2), sourdough-bread with modern variety of wheat flour and backing improver (B3) and yeast-bread with Spelt flour (B4). Characteristics of bread production are reported in table 7.

	Bread 1	Bread 2	Bread 3	Bread 4
Ingredients [g]				
Flour	4848	3393	3382	4886
Yeast	50	-	-	50
Salt	100	100	50	100
Fat	50	50	100	50
Sugar	50	50	50	50
Water	2922	1377	50	2819
Ascorbic acid	0,2	0,2	1388	0,2
Sourdough	-	136,5	136,5	-
Bread making				
Total mixing time [min]	07:00	07:00	07:00	00:09
Intensive mixing [min]	06:00	06:00	06:00	00:06
Baking time [h]	02:36	02:36	02:36	02:36

Table 7: Bread composition.

Five different diets were designed in collaboration with the pellet manufacturers (Ssniff. Spezialdiäten GmbH) for experiments. Diets were produced by using a rice-based gluten-free mice diet added with one of the tested bread in a 1:1 ratio. Characteristics of mice diets are reported in table 8.

Ingredients (%)	*Gluten free*	*B1*	*B2*	*B3*	*B4*
Casein	20	16.55	16.5		
Rice	60	12.150	13.3		
Wheat gluten					
Bread (wheat) 1		50			
Bread (wheat) 2			50		
Bread (wheat) 3				50	
Bread (spelt) 4					50
Sucrose	4	2.9	3.25	2.7	3.65
Cellulose powder	4.3	7.7	6.6	5.9	7.7
L-Cysteine	0.2	0.2	0.2	0.2	0.2
Vitamin premix	1	1	1	1	1
Mineral premix	6	6	6	6	6

Choline CI (50%)	0.2	0.2	0.2	0.2	0.2
Soybean oil	4.3	3.3	2.95	2.55	3.75
Proximate contents (%)					
Crude protein	22.1	22.1	22.1	22.1	22.1
Crude fat	6	5.2	4.8	5.7	3.2
Crude fiber	5.9	8	6.9	8	3
Crude ash	6	6.7	6.8	6.9	6.8
Starch	45	45	45	45	45
Sugar	5.4	5.4	5.4	5.4	5.4

Table 8: Diet components

Before inclusion in experiments all mice received a gluten free breeding diet ad libitum. Mice were randomly assigned to 4 groups (n = 8/ group): **control group** fed with a gluten free diet (CTR); **B1 group** fed with a diet based on bread 1, **B2 group** fed a diet based on bread 2, **B3 group** fed with a diet based on B3, and **B4 group** fed with a diet based on B4. At the end of the 4 weeks, mice were anesthetized by intraperitoneal injection of ketamine-xylazine (100:16 mg/kg body weight). Blood was collected from the portal vein just before sacrifice. Gut tissue was collected and immediately frozen in dry ice or stored in neutral-buffered formalin.

3.1.3 Histological analysis of colon intestine section

Colon samples were collected and rinsed, fixed in 10% neutral-buffered formalin (Sigma-Aldrich) for 24 hours and embedded in paraffin. Paraffin sections (5 μm) were cut and de-waxed prior to staining. For inflammation scoring, sections were stained with hematoxylin/eosin (H&E; Merck, Darmstadt, Germany). Tissue sections were evaluated and images taken by standard light microscopy using an AxioImager Z1 microscope (Carl Zeiss MicroImaging, Jena, Germany). Mucosal integrity was evaluated by scoring the presence of: i) inflammatory cells infiltrate, ii) epithelial change as globet cell loss, hyperplasia, mucosal erosion, iii) mucosa architecture change as crypt loss, ulceration, granulation tissue, using table 3. Basing on this scores a Disease Activity Index (DAI) was calculated.

Category	*Criterion*	*Definition*	*Score Value*
I, Inflammatory cell infiltrate	Extent	Expansion of leukocyte infiltration:	
		Mucosal	1
		Mucosal and submucosal	2
		Mucosal, submucosal and transmural	3
		Increase in epithelial cell numbers in longitudinal crypts relative to baseline epithelial cell numbers per crypt; visible as crypt elongation:	
		Minimal: <25%	1
		Mild: 25-35%	2 or 3
		Moderate: 36-50%; mitoses in middle/upper third of crypt epithelium, distant from crypt base	3 or 4
	Hyperplasia	Marked: >51%; mitoses in upper third of crypt epithelium, distant from crypt base	4 or 5
		Reduction of goblet cell numbers relative to baseline goblet cell numbers per crypt:	
		Minimal: <20%	1 or 2
		Mild: 21-35%	2 or 3
	Goblet cell loss	Moderate: 36-50%	3 or 4
		Marked: >50%	4
	Cryptitis	Neutrophils between crypt epithelial cells	2 or 3
	Crypt abscesses	Neutrophils in crypt lumen	3 to 5
II, Epithelial changes	Erosion	Loss of surface epithelium	1 to 4
	Ulceration	Epithelial defect reaching beyond muscularis mucosae	3 to 5
	Granulation tissue	Connective tissue repair with new capillaries, surrounded by spindle-shaped fibroblasts, myofibroblasts, macrophages, neutrophils and mononuclear cells as well as cell debris; pseudopolyps: villiformous, hypertrophied areas projecting into the lumen	4 or 5
	Irregular crypts	Non-parallel crypts, variable crypt diameters, bifurcation and branched crypts	4 or 5
	Crypt loss	Mucosa devoid of crypts	4 or 5
	Villous blunting	Mild: villous-to-crypt-length ratio of 2:1 to 3:1	1 to 3
		Moderate: villous-to-crypt-length ratio of 1:1 to 2:1	2 to 4
III, Mucosal architecture		Villous atrophy	3 to 5

Table 9: DAI SCORE modified by Erben et al.[116]

3.1.4 Detection of inflammatory cytokines in mice plasma

Blood samples (200 µl) were taken from the portal vein and collected in Eppendorf tubes. Blood was centrifuged at 1000 rpm for 10 min, plasma was collected and stored at −80°C until BioPlex® analysis. Cytokine levels were determined using a multiplexed mouse bead immunoassay kit (Bio-Rad, CA, USA). The six-plex assay (IL-1β, IL-6, IL-10, IL-17A, IFNγ, TNFα) was performed in 96-well plates following the manufacturer's instructions. Microsphere magnetic beads coated with

monoclonal antibodies against the different target analytes were added to the wells. After a 30 min incubation, wells were washed and biotinylated secondary antibodies were added. After incubation for 30 min, beads were washed and then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein phycoerythrin (streptavidin/phycoerythrin). After washing, the plate (a minimum of 100 per analyte) was analyzed in the BioPlex 200 instrument (BioRad). Sample concentrations were estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/ml after adjusting for the dilution factor (Bio-Plex Manager software 5.0). The intra-assay CV was <14%.

3.1.5 RNA isolation and Real-time -PCR

Total RNA from colon samples were extracted using Trizol® reagent (Life Technologies, CA, USA) according to the manufacturer's instructions. Concentration and purity was determined using nanodrop technology (BioPhotometer Plus, Eppendorf, Rotselaar, Belgium). All samples exhibited an OD260/OD280 ratio between 1.8 and 2.1. RNA integrity was assessed using agarose gel electrophoresis. One microgram of total RNA was converted to single-stranded cDNA by reverse transcription using the iScript™ cDNA synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real Time PCR analysis of cDNA was performed using SYBR Green (SSO Advanced, BioRad), with specific Forward and Reverse primers. Primer pair sequences are listed in Table 1. All samples were run in triplicate, in 10 µl reaction volume containing 100 ng of cDNA. The thermal cycler (Bio-Rad iQ5 Real- Time System) was programmed as follows: 95 °C for 30 s; 40 cycles at 95°C; C for 5 s and 64 °C for 30 s. Melting curve data were collected to check PCR specificity. Target mRNA levels were normalized against β-actin mRNA and relative expressions were calculated using the $2^{-2\Delta Ct}$formula. Reactions for each sample were carried out in triplicate.

Gene	Forward primer	Reverse Primer
Claudin-2	5′-TTCTCTACAACAACTCCATCCTC-3′	5′- GCAGCCATTTCCTTCTCT CC -3′
Occludin	5′-ATGTCCGGCCGATGCTCTC-3′	5′- CTTTGGCTGCTGTTGGGTCTG -3′
Zonulin-1	5′- GAATGTGAGGCAGATGACAG-3′	5′-GTGTTCACCCATTGCTTCTC-3′
β-Actin	5′-CACCATTGGCAATGAGCGGTTC-3′	5′-AGGTCTTTGCGGATGTCCACGT-3′

Table 10: Real time PCR primer are reported as 5'-3' sequences.

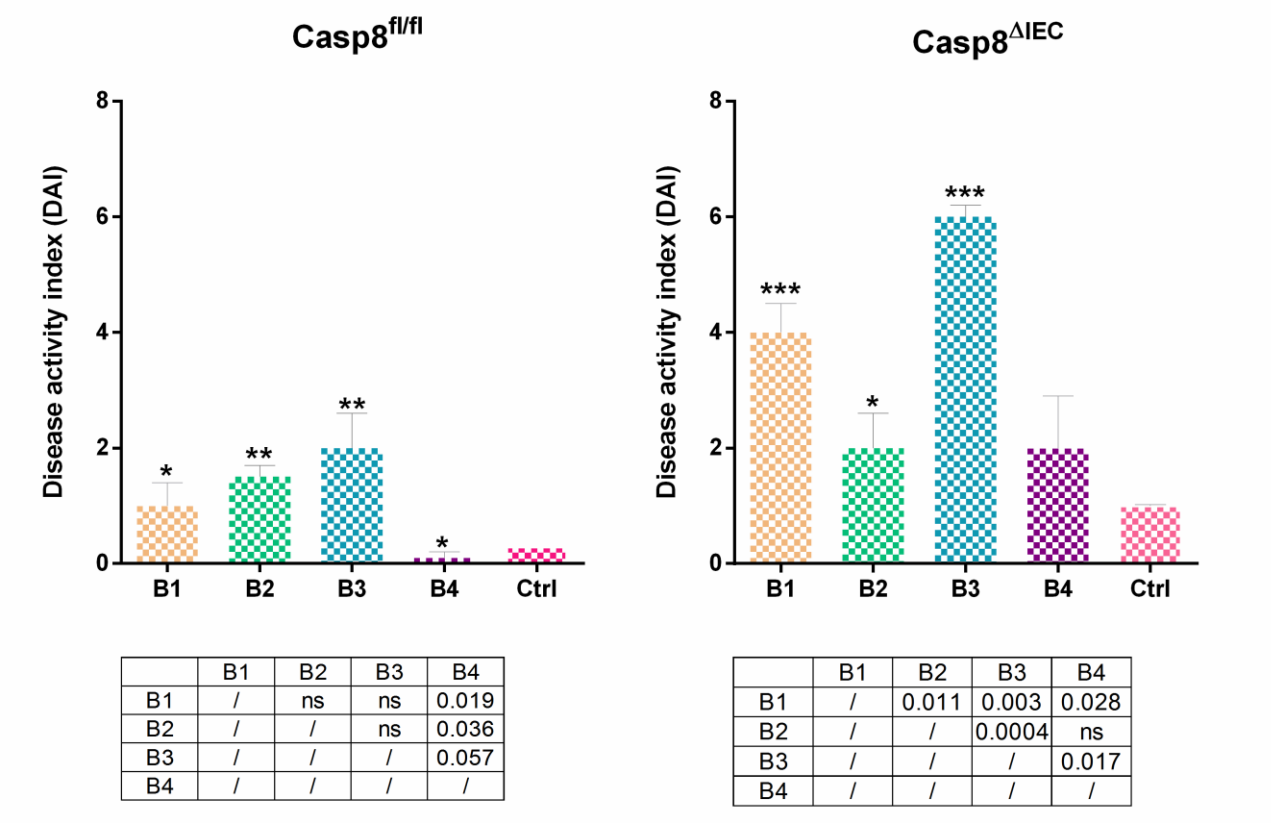
3.1.6 Statistical analysis

Data are presented as mean ± SD of at least three independent determinations. Statistical differences between groups were determined by one-way analysis of variance followed by Bonferroni's post-hoc test for multiple comparison. All analyses were performed using GraphPad Prism software (version 6.0; La Jolla, CA, USA). $p < 0.05$ was considered to indicate statistical significance.

3.2 Results

3.2.1 Bread-based diets induce different grades of inflammation

Histomorphology analysis remains a powerful standard methodology to evaluate intestinal inflammation in animal models. Three main categories reflects the severity of inflammation: inflammatory cell infiltrates, epithelial changes and mucosal architecture distortion. As reported in figure 14, Casp8$^{\Delta IEC}$ mice presented a worse DAI score respect to Casp8 $^{fl/fl}$.



	B1	B2	B3	B4
B1	/	ns	ns	0.019
B2	/	/	ns	0.036
B3	/	/	/	0.057
B4	/	/	/	/

	B1	B2	B3	B4
B1	/	0.011	0.003	0.028
B2	/	/	0.0004	ns
B3	/	/	/	0.017
B4	/	/	/	/

Figure. 14: DAI score of Casp8 $^{fl/fl}$ and Casp8$^{\Delta IEC}$ mice. P value for each group are reported in table.

A slight increase of submucosal and lamina propria thickness with immune cells infiltration was detectable in Casp8$^{\Delta IEC}$ mice (figure 15) but not in Casp8$^{fl/fl}$ Moreover, minimal hyperplasia, crypt distortion and loss of globet cells were observed in Casp8$^{\Delta IEC}$ mice. No changes in surface epithelial cell layer nor in muscular layer were observed in both mice strands.

Histological examination in colon of Casp8 $^{fl/fl}$ mice after 4 week of bread-based diets showed no signs of histological damage in treated groups, a slight raise in immune cells infiltration is visible in B1, B2 and B3 groups respect to control group, and slight increase of lamina propria thickness with immune cells infiltrate was observed in B1 and in B3. Macroscopic evaluation of Casp8$^{\Delta IEC}$ colon after 4 week of bread-based diets showed slight signs of immune cells infiltration in B2 group, anyway these features, similar to those found in control group mice, can be considered characteristic of the spontaneously developed colitis typical of this animal model. B1 mice showed an increased thickness of lamina propria with higher number of inflammatory cells and a mild hyperplasia respect to control group Casp8$^{\Delta IEC}$ mice. Moreover, crypts appeared dilated and globet cell depletion was observed. B3 mice showed a marked colitis characterized by high number of inflammatory cells in mucosa and submucosa, mild epithelial hyperplasia, dilated crypts and erosion of epithelial layer. No sign of histological damage was observed in B4 mice, only a slightly increased thickness of lamina propria and a slight immune cells infiltration in mucosa respect to control, group was observed.

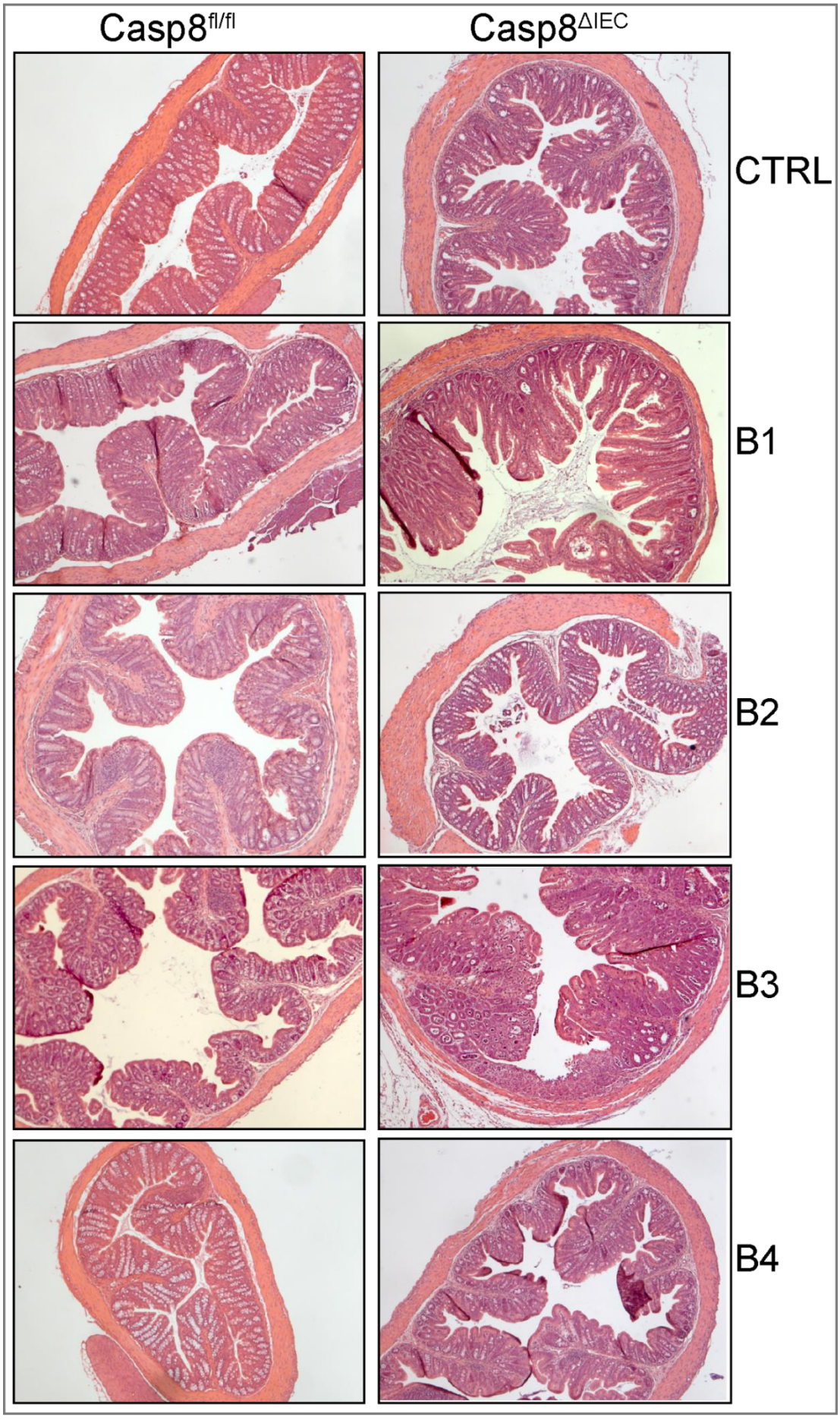
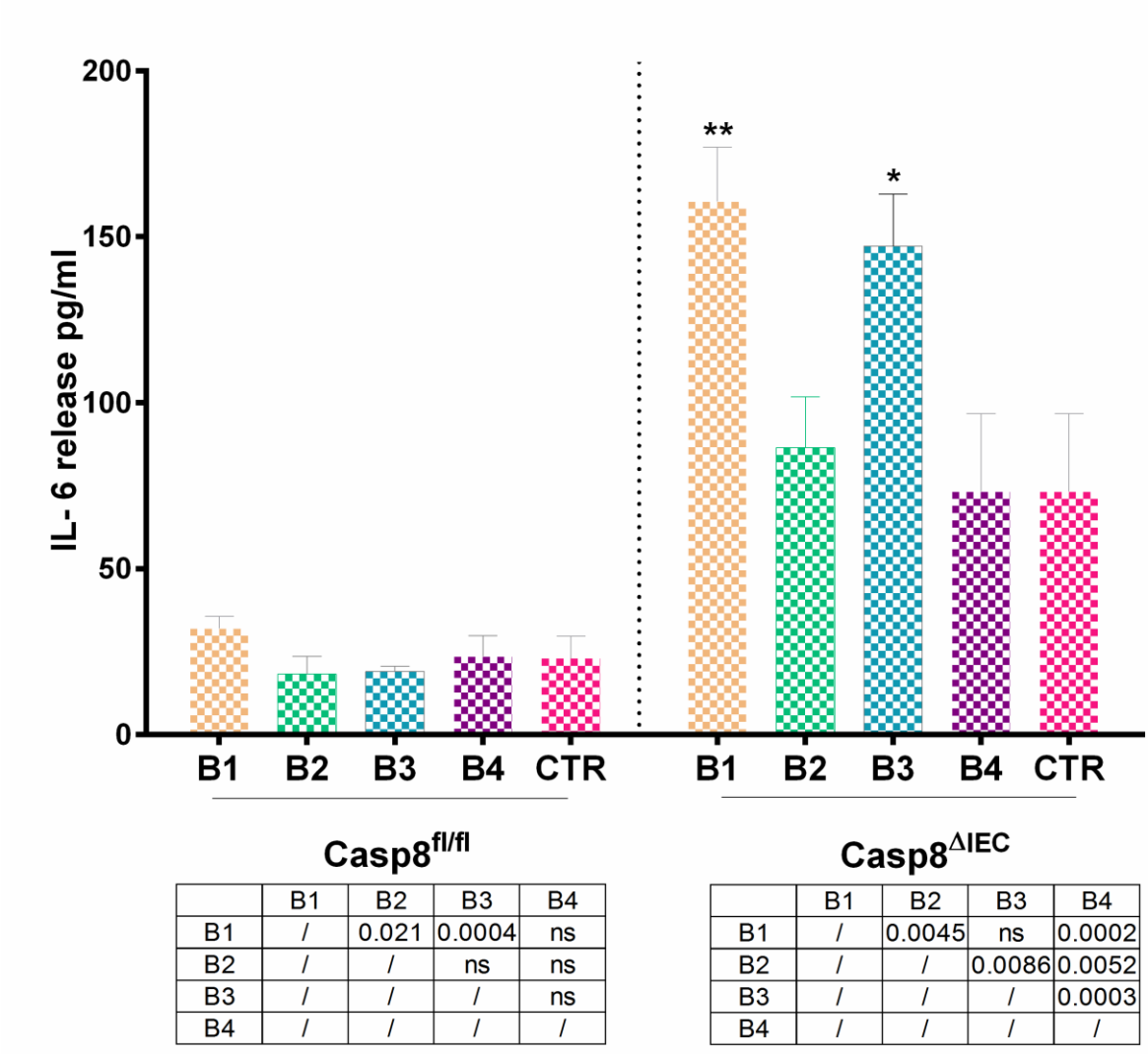

Casp8fl/fl
Casp8ΔIEC
CTRL
B1
B2
B3
B4

Figure 15: Histological feature of large intestine sections. On the left side and right side respectively Hematoxylin–eosin staining of colon longitudinal sections of section of Casp8 $^{fl/fl}$ and Casp8$^{\Delta IEC}$ mice. In the upper part of the figure is reported the strand of mice,On the right side are reported the experimental groups. Original magnification 5x.

3.2.2 Proinflammatory immune response in mice plasma

Secretion of proinflammatory cytokines IL-1β, IL-6, IL-10, IL-17A, IFNγ, and TNFα was quantified in mice plasma samples. In all analyzed sample, only IL-6 was detectable.



	B1	B2	B3	B4
B1	/	0.021	0.0004	ns
B2	/	/	ns	ns
B3	/	/	/	ns
B4	/	/	/	/

	B1	B2	B3	B4
B1	/	0.0045	ns	0.0002
B2	/	/	0.0086	0.0052
B3	/	/	/	0.0003
B4	/	/	/	/

Figure 16:detection of IL-6 release in mice plasma of to Casp8$^{fl/fl}$ and Casp8$^{\Delta IEC}$. Data are presented as mean ± SD (n=3). Asterisks indicate p-values calculated by one-way ANOVA as follow: *=<0.05, **=<0,005 and ***= < 0.0005. P value for each group are reported in tables.

Overall, as shown in figure 16, IL-6 levels were considerably higher in Casp8$^{\Delta IEC}$ compared to Casp8 $^{fl/fl}$ mice. No statistical difference was observed between control and treated groups in Casp8 $^{fl/fl}$ mice. Only in B1 group a slight increase of IL-6 was detected, though not significant.

In Casp8$^{\Delta IEC}$ B1 and B3 groups showed a significant rise of IL-6 secretion comparing to control-mice. No significant increase of IL-6 was observed in B2 group. Remarkably, IL-6 levels in B2 and B4 groups, were similar to control group. Overall, B4 diet showed to have a statistically significant decreased IL-6 inflammatory effects respect to the other tested diets.

3.2.3 Expression of biomarker related to intestinal permeability by Real-time PCR

Maintenance of intestinal homeostasis is regulated by epithelial barrier integrity. mRNA expression of Claudin-2, Zonulin-1 and Occludin genes, encoding for the main protein components of the Tight Junctions, were analyzed in colon tissue by Real-time PCR.

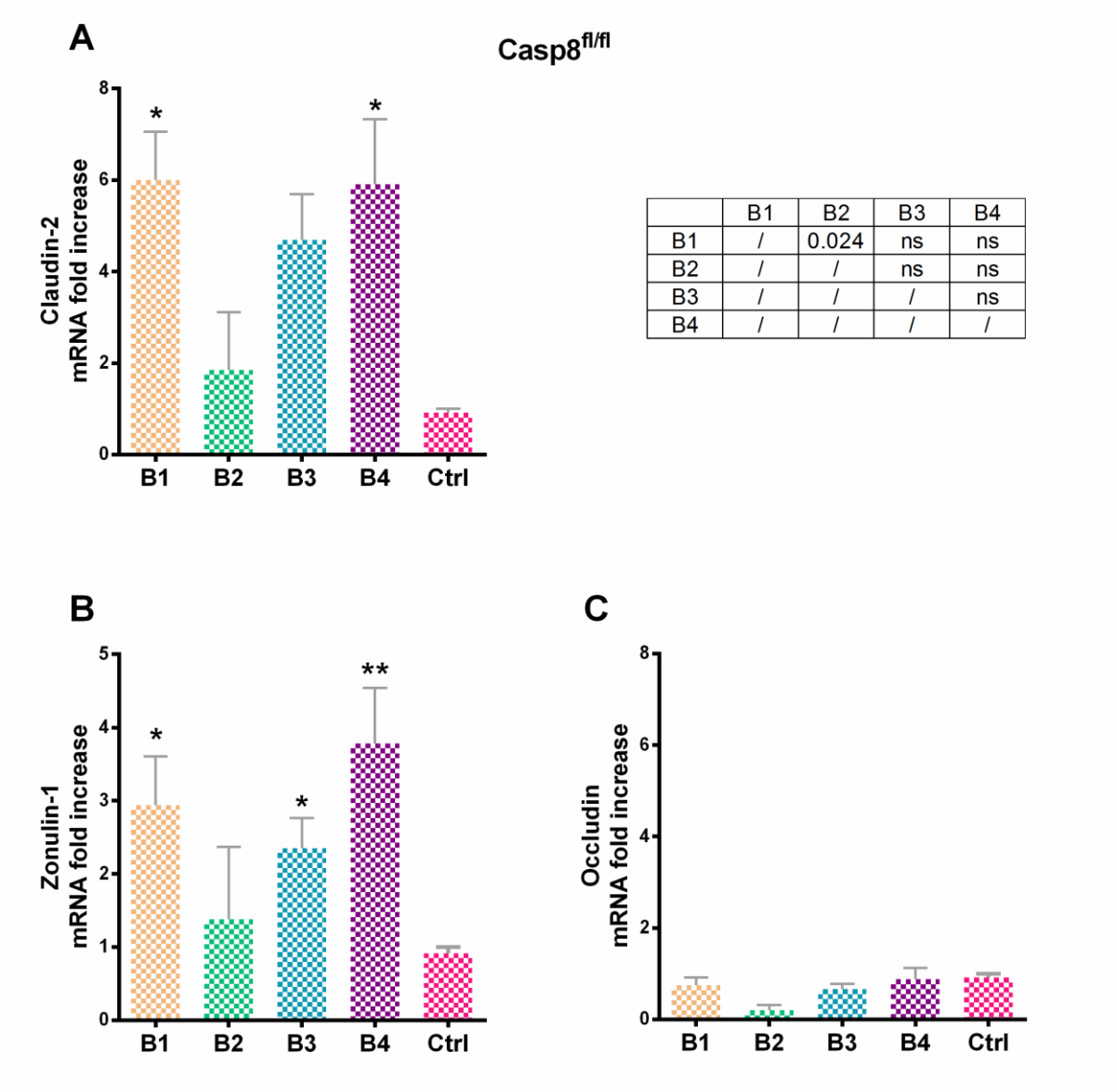
Results showed that in Casp8 $^{fl/fl}$ mice the 4 week exposure to bread-based diets significantly impacted on the expression level of Claudin-2 respect to control group (P value: B1 0.0014; B3 0.0036; B4 0.0111) and Zonulin-1 (P value: B1 0.0231; B3 0.0211; B4 0.0072) while no effect was detected on Occludin gene expression. In Casp8$^{\Delta IEC}$ mice a significantly expression level of Claudin-2 respect to control group (P value: B1 0.0108; B4 0.0105) was observed but no effect was detected on Zonulin-1 and Occludin genes expression.

In Casp8 $^{fl/fl}$ mice no significant difference was observed on Occludin mRNA level (figure. 17 panel C) between the different groups, suggesting that there is not a specific modulatory effect of wheat consumption on this protein. Similar result were observed in Casp8$^{\Delta IEC}$ mice (figure 18 panel C).

Claudin-2 expression in Casp8 $^{fl/fl}$ mice showed a significant increase in B1 (5 fold), B4 (5 fold) and B3 (4 fold) groups respect to control group (figure 17 panel A) . In B2 group a slight increase of Claudin-2 mRNA level was observed but it was not significant when compared to control group. In Casp8$^{\Delta IEC}$ mice Claudin-2 gene expression (figure 18 panel A) was increased in B1 and B4 mice groups, when compared with the control groups, while in B2 and B3 groups no difference was detected compared to control group.

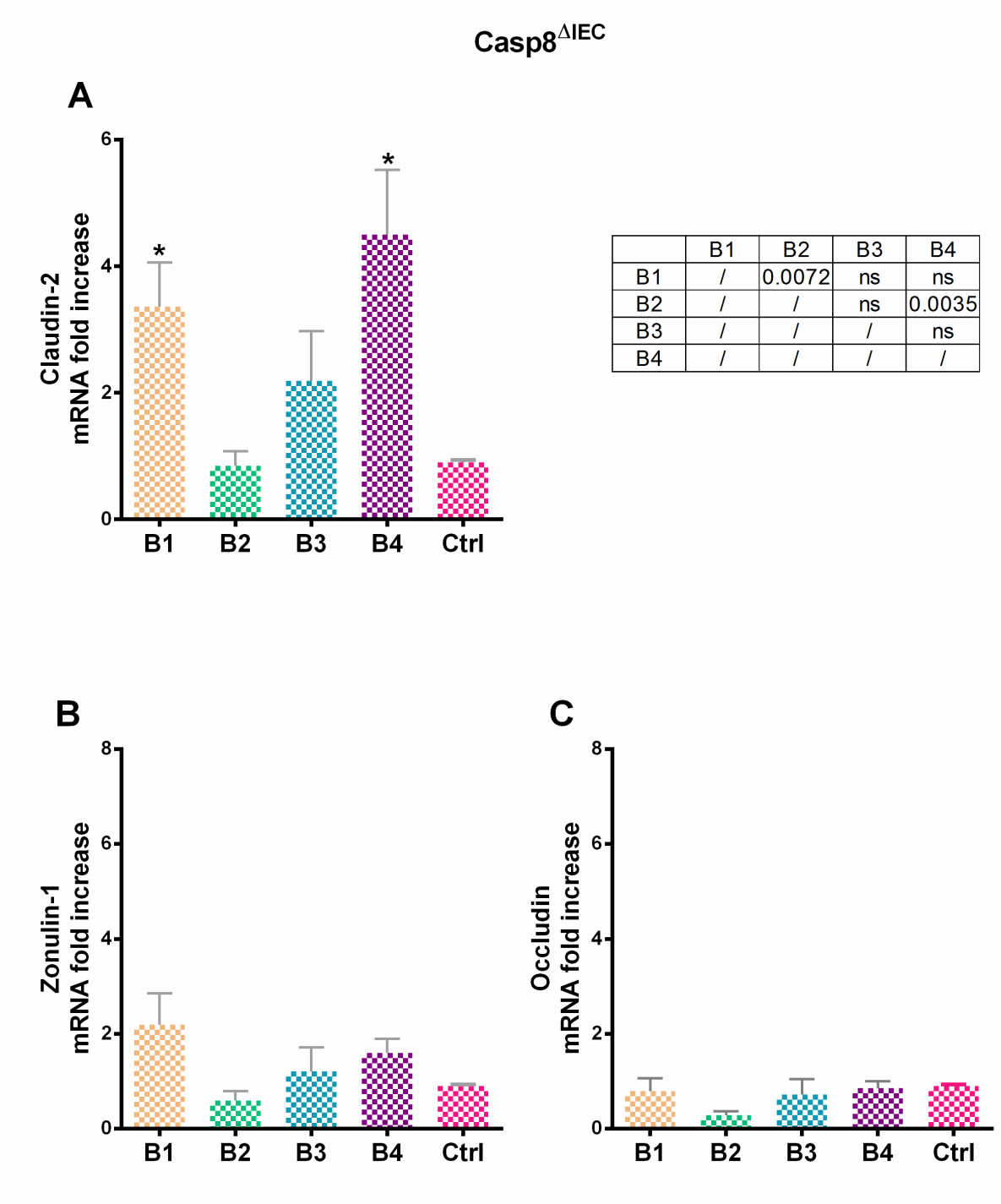
Zonulin-1 in Casp8 $^{fl/fl}$ expression (figure 17 panel B) showed a significant increase in B1 and B4 groups (3 and 4 fold respectively) and in B3 group a 2.5 fold increase was detected. No difference

B2 group was detected compared to control group. In Casp8$^{\Delta IEC}$ mice Zonulin-1 expression (figure 18 panel B) no showed difference in B2, B3, B4 groups when compared to control group. Only in B1 group a mild increase in mRNA level was observed but not statistically significant respect to control group.



	B1	B2	B3	B4
B1	/	0.024	ns	ns
B2	/	/	ns	ns
B3	/	/	/	ns
B4	/	/	/	/

Figure 17: Real-time PCR of mRNA expression of inflammatory marker in mice colon samples after four weeks of bread-based diet trietment. Graphs showed the fold increase for Claudin-2 (A), Zonulin-1 (B) and Occludin (C) in in Casp8 $^{fl/fl}$ mice. Values are reported as Normalized fold expression (2^{-DDCT}) relative to untreated cells. B-Actin was used as housekeeping gene. Statistical analysis was performed by one-way ANOVA as follow: *=<0.05, **=<0,005 and ***= < 0.0005. P value for each groups are reported in table shown in panel A.



	B1	B2	B3	B4
B1	/	0.0072	ns	ns
B2	/	/	ns	0.0035
B3	/	/	/	ns
B4	/	/	/	/

Figure 18: Real-time PCR of mRNA expression of inflammatory marker in mice colon samples after four weeks of bread-based diet trietment. Graphs showed the fold increase for Claudin-2 (A), Zonulin-1 (B) and Occludin (C) in Casp8$^{\Delta IEC}$ mice. Values are reported as Normalized fold expression (2^{-DDCT}) relative to untreated cells. B-Actin was used as housekeeping gene. Statistical analysis was performed by one-way ANOVA as follow: *=<0.05, **=<0,005 and ***= < 0.0005.. *** = p < 0,005. P value for each groups are reported in table shown in panel A.

Chapter 4 - A Khorasan wheat-based diet improves systemic inflammatory profile

Based on:

A Khorasan wheat-based diet improves systemic inflammatory profile in semi-professional basketball players: a randomized pilot study.

Enzo Spisni[1], Luigia De Fazio[1], Enrica Rotondo[1], Marcella Di Natale[1], Elisabetta Giovanardi[1], Giovanni Posabella[2], Valeria Bregola[3], Sara Bosi[3], Rocco Enrico Sferrazza[3], Giovanni Dinelli[3]*

1. Department of Biological, Geological and Environmental Sciences, University of Bologna, Bologna, Italy;
2. BioSportMed, Via Murri 45, Bologna
3. Department of Agricultural Sciences, University of Bologna, Bologna, Italy

4.1 Materials and methods

4.1.1 Study population

The study population was comprised of 20 semi-professional basketball players enrolled from the SALUS basketball team of Bologna, Italy. Written informed consent was obtained from each participant before the initial screening visit and before initiation of the experimental trial. Parental consent was obtained for those participants under 18 years old. Parents of underage boys were also involved and educated to support and supervise their sons during the study. The institutional review board at the University of Bologna approved the study protocol.

4.1.2 Study design

Inclusion criteria required written informed consent, age between 15 and 32 years, and membership of the SALUS basketball teams (senior and youth teams). Exclusion criteria were chronic use of non-steroidal anti-inflammatory drugs (NSAIDs) or anti-inflammatory steroids, celiac disease, non-celiac gluten sensitivity (NCGS), wheat allergy or chronic use of food supplements. Sporadic use of FANS was permitted, but volunteers were recommended to notify their use in the food frequency questionnaire. The study design was a randomized, single-blinded, crossover trial (Fig. 19). All basketball player volunteers followed a Mediterranean type balanced diet (calorie distribution: 60% carbohydrates, 15% protein and 25% fat) provided by their athletic trainer throughout the duration of the study. At the beginning and end of each diet period, the basketball players underwent sports medical evaluation that included the standard fitness tests. In addition, blood and saliva samples were collected for cytokines and cortisol determinations (Fig. 19).

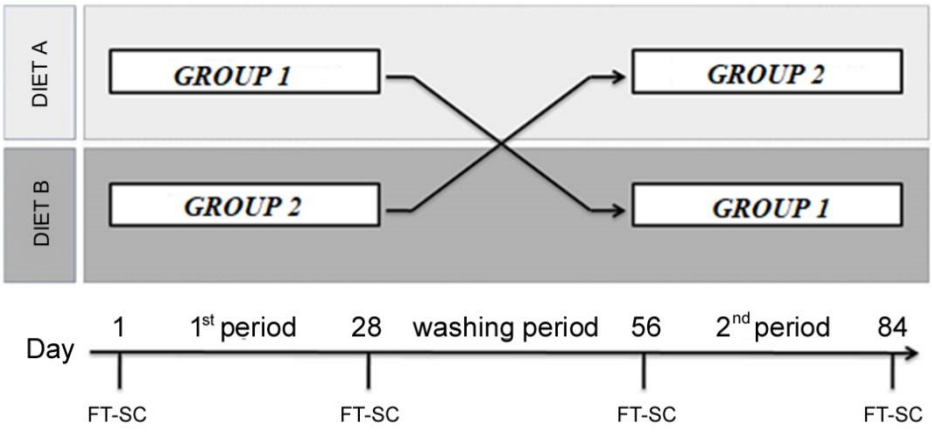


Figure 19. Experimental design of the basketball players study. FT = Fitness test, SC=Sample collection. Fitness test results are not shown.

The study protocol was approved by the Bioethical Committee of the University of Bologna Alma Mater Studiorum. Athletes were instructed not to alter their eating plan during the study periods, and to consume the same daily amount of pasta, bread, biscuits and crackers provided. They were asked not to change the quantity or the quality of the beverages they consumed, but only replace all sources of wheat with those provided from the University of Bologna. The only restriction imposed throughout the study was to avoid restaurant-prepared pizza or pasta, and fast foods, since different frequency patterns between the diet periods might have interfered with the validity of the results. Throughout the diet periods, each volunteer received an appropriate daily amount of wheat-derived carbohydrates, made of up to 300 g of pasta, up to 300 g of bread, up to 250 g of biscuits and up to 100 g of crackers. Diet products were consumed in blind, the packaging being identical for both Diet A (Khorasan) and Diet B (modern) wheat products. Athletes were randomized into two groups: 1 and 2. They followed the experimental design represented in Fig. 19. During the washout period, athletes ate non-organic and non-whole grain products bought autonomously in supermarkets. Blood and saliva samples were collected for cytokine and cortisol analysis.

4.1.3 Wheat samples and derived foodstuff products

All organic semi-whole flours and semolina were provided by Antico Molino Rosso (Verona, Italy). Pasta products were made from semi-whole wheat semolina obtained from a mix of Italian modern durum wheat (Triticum turgidum ssp. durum Desf. Husn) or from Khorasan wheat (Triticum turgidum ssp. turanicum), while bakery products were prepared using semi-whole wheat flour from a mix of Italian modern soft wheat (Triticum aestivum L.) or from Khorasan wheat. The pasta was

produced by La Romagnola Prodotti Alimentari S.r.l. (San Biagio di Argenta, Ferrara, Italy) and the Un Chicco S.r.l. Bakery (Bologna, Italy) supplied the bread, crackers and biscuits. The Khorasan wheat was provided by KAMUT® Enterprises of Europe (KEE), Oudenaarde, Belgium. KAMUT® is a registered trademark of Kamut International Ltd and Kamut Enterprises of Europe bvb.

4.1.4 Chemical composition of wheat-based foods

Protein content was determined with a CHN analyzer (Costech ECS 4010, CA, USA), multiplying the total nitrogen content detected by a factor of 5.7. Total starch (TS) was measured using the Total Starch assay kit (Megazyme Int. Ireland Ltd, Wicklow, Ireland) according to the AOAC 996.11 method. Lipid analysis was carried out with the standardized method.15 Free and bound phenolic compounds were extracted as described by Dinelli et al.[14]. Free and bound polyphenols were quantified following the colorimetric procedure based on the Folin–Ciocalteu reagent, as described by Singleton et al.[117], while free and bound flavonoids were determined by the spectrophotometric method previously described[14]Antioxidant activity was determined using two different analytical methods [DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing ability of plasma) assays]. The DPPH test was performed following the procedure described by Brand-Williams et al[118].; the FRAP assay was carried out according to the Benzie and Strain[119] protocol, with minor modifications.

4.1.5 Sample collection

Blood was collected (300 μL) from the basketball players by digital puncture and transferred to Eppendorf tubes containing ethylenediaminetetraacetic acid (EDTA). Blood and saliva were collected 1 h after the beginning of the fitness test (about 40 min after the end of the test). Blood samples were kept at 4 ∘C for 12 h then centrifuged at 1000 × g for 15 min. Plasma was collected and stored at −80 ∘C until the cytokine and chemokine analyses were performed. Saliva samples (1.5 mL) were collected in polypropylene tubes and kept at 4 ∘C for 12 h, then frozen at −20 ∘C until cortisol determinations.

4.1.6 Cytokine assays

The cytokines present in plasma were quantified in duplicate (plasma dilution 1:4) using a customized detection panel (IL-6, IL-8, IL-10, IL-1ra, MCP-1, IL-4) purchased by BioRad (Hercules, CA, USA). Immunoassays were performed in 96-well filter plates and the signal detected using a multiplexed Luminex® instrument following the manufacturer's instructions. Microsphere magnetic beads coated with monoclonal antibodies against the different target analytes were added to the wells. After 30 min incubation, the wells were washed and biotinylated secondary antibodies added. After further incubation for 30 min, the beads were washed and then incubated for 10 min with streptavidin-PE conjugated to the fluorescent protein, phycoerythrin (streptavidin/phycoerythrin). After washing, the beads (a minimum of 100 per analyte) were analyzed in the BioPlex 200 instrument (BioRad). Sample concentrations were estimated from the standard curve using a fifth-order polynomial equation and expressed as pg/mL after adjusting for the dilution factor (Bio-Plex Manager software 5.0). Samples below the detection limit of the assay were recorded as zero, while samples above the upper quantification limit of the standard curves were assigned the highest value of the curve. The intra-assay coefficients of variability (CV) averaged 12%.

4.1.7 Statistical analysis

Statistical analysis was performed using Statistica 6.0 software 2001 (StatSoft, Tulsa, OK, USA). All variables were checked for normal distribution and homogeneity of variance before data analysis. Data were expressed as arithmetic means ±SD. Differences in dietary products were assessed for significance by one-way analysis of variance (ANOVA).

4.2 Results

4.2.1 Characterization of wheat-based foods

The aim of our study was to evaluate inflammatory properties of ancient and modern wheat in a model of mild systemic inflammation induced by physical activity.

The major difference in the wheat flour used in the two diets was the protein content, ranging from 12.24% to 13.36% in the Khorasan products in comparison to the 10.10–10.65% range of modern wheat foodstuffs (Table 11). These differences were statistically significant both for bakery products and pasta. Differences in nutraceutical proprieties (polyphenol and flavonoid content) and antioxidant proprieties of administered food were also evaluated. In particular, we detected lower amounts of total flavonoids in bakery products made with Khorasan wheat but an increase of these in the pasta produced with the same wheat (Table 11). Antioxidant capacity measured with the DPPH assay was significantly higher in Khorasan bakery products, but not in pasta. These results can be explained by the fact that the bakery products made with modern wheat were obtained from soft wheat (Triticum aestivum L.), while the pasta produced from modern grains was made with hard wheat (Triticum durum ssp. durum Desf. Husn). Our results agree with those obtained by Sofi et al., 21 whereby the major differences in total flavonoids and antioxidant capacity were observed in bakery products made from Khorasan wheat.

		Bakery products[a]			Pasta		
Variable		Khorasan wheat	Modern wheat		Khorasan wheat	Modern wheat	
Protein	g/100 g	12.24	10.10	**	13.36	10.65	**
Lipid	g/100 g	10.03	9.92	ns	1.21	1.41	ns
Total starch	g/100 g	58.97	59.35	ns	69.37	68.34	ns
Resistant starch	g/100 g	0.78	1.00	ns	1.56	1.68	ns
Total dietary fiber	g/100 g	18.62	18.01	ns	14.09	16.11	ns
Insoluble dietary fiber	g/100 g	14.07	13.70	ns	10.18	11.47	ns
Soluble dietary fiber	g/100 g	4.56	4.31	ns	3.91	4.64	ns
Total polyphenols	mg/100 g (as gallic acid)	195.85	233.50	ns	194.48	235.73	ns
Total flavonoids	mg/100 g (as catechin)	105.18	198.09	*	86.36	69.45	**
DPPH	µmol Trolox/g	5.38	3.94	*	2.34	3.41	ns
FRAP	mmol Fe^{2+}/100 g	2.64	2.53	ns	1.09	0.98	ns

Table 11:[a]:Baked goods included bread, cracker and biscuits*=P<0.05; **=p<0.01; ***=p<0.001, ns=not statistically significant.

4.2.2 Cytokines related to pro-inflammatory activity

The IL-6, IL-10 and IL-4 levels in the blood were undetectable (below the lower point of the standard curve) in most of the athletes. For this reason, these data are not shown, nor were statistical significance calculated for these cytokines. We observed a very significant reduction in monocyte chemoattractant protein-1 (MCP-1) levels in blood collected after 4 weeks of the Khorasan wheat diet (P (P = 0.084) while IL-8 levels were not affected by the Khorasan diet (figure. 20B). As reported in figure. 20C, there was a slight decrease of Interleukin-1 receptor antagonist (IL-1ra) following the modern wheat diet, but it was not significant (P = 0.147). IL-1ra levels were not affected by the Khorasan-based diet.

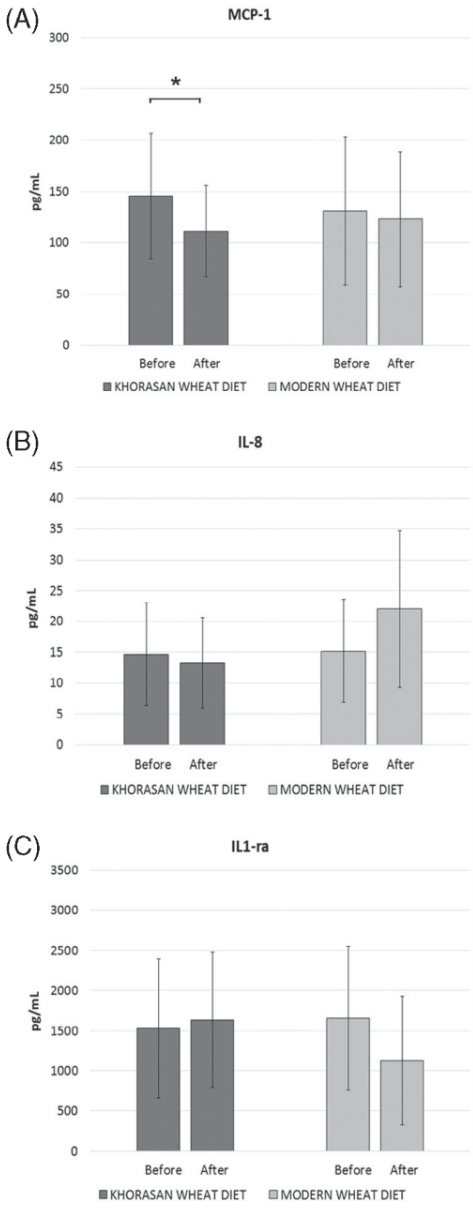


Figure20: (A) Blood levels of monocytechemoattractantprotein-1 (MCP-1) pro-inflammatory chemokine detected before anda fter dietary periods in basketball athletes enrolled in the trial. (B) Blood levels of Interleukin-8 (IL-8) detected before and after dietary periods in basketball athletes enrolled in the trial. (C) Blood levels of Interleukin-1 receptor antagonist (IL-1ra) detected before and after dietary periods in basketball athletes enrolledinthetrial.*P <0.05.

Discussion

Based on the increasing interest in the role of the diet in the increasing prevalence of non-communicable chronic disease, there is a rising focus on the gluten and wheat in particular, sometimes generally considered as pro-inflammatory foods[120]. For example, gluten-free diet has become one of the most popular diet for non-celiac healthy people in USA[121]. On the other hand, the increasing prevalence of GRDs, including NCWS, has led some scientist to propose that modern wheat cultivars may be co-responsible for this increase [122]. In this scenario, it is not surprising that a wide number of studies focuses on possible differences between ancient, heritage and modern wheat varieties. In particular, ancient and heritage varieties are gaining scientific and commercial interest since several studies suggested that they could represent a healthier choice respect to modern ones.

Our *in vitro* studies revealed that wheat proteins treatment do not show any cytotoxic activity and effect in cell viability regardless of genotypes exhibited or cultivar variety. These results are consistent with the observation of Zevallos and co-workers [104]. The protein wheat profile, obtained by SDS PAGE, revealed, as expected, a different protein composition between durum and soft wheat flour proteins. Quantitative differences were more marked in durum varieties and were particularly evident in bands representing HMW-GS and in LMW-GS. These are important seed storage proteins that determine wheat dough elasticity and processing quality especially in Triticum aestivum [123]. Although previous *in vitro* study has been showed that both HMW-GS and LMW-GS were able to trigger a stronger immune response in gluten-sensitive T cells obtained from CD patients [20]. In our *in vitro* study, soft wheat proteins showed a higher pro-inflammatory effects compared to durum wheat proteins. The pro-inflammatory effect of wheat proteins was assessed by measuring of IL-8, a chemokine that can be produced by epithelial cells and has been recognized as an indicator of the cellular inflammatory response , linked to different colon mucosa disease such as ulcerative colitis [124-125] and colorectal cancer development[126]. In our experiments, relevant data were obtained from HL-60 and Caco-2 colonocytes but not from THP-1 and CRL-1831 cell lines, after wheat proteins stimulation. In HL-60 and Caco-2, wheat proteins treatment induced a significant rise of IL-8 secretion respect to untreated cells. This increase was especially marked in HL-60 and Caco-2 cells line when stimulated with protein from soft wheat but no difference was observed between ancient and modern cultivars. A significant lower stimulation was elicited by durum wheats, with no detectable differences between ancient and modern varieties. This data might be partly explained by evidence that the D genome of bread wheat has more "toxic" gluten epitopes, active in Celiac Disease (CD), respect to the A and B genomes. Some of these epitopes linked to the D genome showed, at least in vitro, to be the most active in triggering inflammatory response in colonocytes[127].

Consequently, diploid and tetraploid wheats (T. monococcum for example), are likely to be less toxic than exaploid wheats. In order to investigate a possible mechanism by which wheat could trigger a pro-inflammatory response, we evaluated LDH-A and Cox-2 gene expression in colon adenocarcinoma cells (CaCo-2) cells stimulated with ancient and modern wheat proteins. Indeed, it is generally accepted that Cox-2 is upregulated under conditions of inflammation while LDH-A, a key enzyme in glycolysis, plays a critical role in tumorigenesis [115]. The RNA expression of Cox-2 is induced by cytokines and growth factors and in this study TNF-alpha and IL-17 co-treatment was used as positive control. TNF-alpha and IL-17 have been chosen because of their association with inflammation and cancer progression [128-129]. In wheat-treated cells no significant change in Cox-2 expression was found. These observation could be correlated with a low inflammatory environment led to wheat proteins stimulation. Interestingly, a not significant increase of LDH-A occurred.

The main purposes of our *ex vivo* studies was to investigate immune cell response to protein extracts from ancient and modern grains. Assays performed on PBMCs obtained from paediatric patients were part of a study that was initially addressed on adult patients. In this first study, we observed that modern wheat proteins triggered an increased CXCL10 secretion in PBMC obtained from NCGS patients respect to ancient wheat proteins [50]. Anyway, despite the diagnosis of NCWS is now quite standardized, we realized that the presence of co-morbidity and psychological factors often found in adult patients made difficult to obtain clean data. From this point of view, paediatric patients represented a more standardized population. Anyway, despite chemokine secretion by PBMCs purified from pediatric patients was generally lower compared to adult patients, our previous results were confirmed. Indeed, PBMC stimulation resulted in a higher secretion of the proinflammatory chemokine CXCL10 induced by proteins extracted from the modern hexaploid wheat respect to ancient wheats in these NGWS population. Since in our study total grain protein fraction was used, we cannot rule out that other protein components besides gluten could trigger PBMCs responses in-vitro and thus NCWS in vivo.

It was observed that in NCWS patient sera there is an increased level of AGA IgG antibodies directed against gluten [130]. Our hypothesis was that the IgG response of NCWS patients could be more specific for modern wheat proteins. Serum of adult NCWS patients was used in Dot-blot analysis to analyse IgG response to different wheat varieties. Unfortunately, results showed no significant differences between the amounts of anti-gluten IgG antibody detected in controls and NCWS patients. These test seemed unable to identify different IgG response to different wheat proteins obtained by modern or old varieties. Moreover, it was not possible to standardize the epitope exposition of protein bound to

the membrane, so the signal produced by interaction between IgG an wheat proteins was different in each single experiment. However, in Dot-blot, proteins have not been previously separated according to their molecular weight and a possible limitation of Dot-blot assay could be the accessibility of the proteins by the primary antibody. Moreover, a recent study on T. monococcum showed an higher solubility of its proteins[131] compared to those obtained from other wheat varieties. This higher solubility could increase the amount of proteins bound to the membrane. These evidences may suggest that the method developed for this study may produce false positives results. Limitation of this technique was recently also confirmed by Janset at al[132]. Moreover, since NCWS aetiology is still unknown, is possible that many gluten immunogenic epitopes may be tolerated by NCWS patients depending on the patients' sensitivity to the different single epitopes. In this scenario, it becomes very important to accurately detect which immunogenic protein epitopes are responsible for this disease[133].

Nowadays, scientific evidence support the immunological properties of wheat components [134]. It is well-known that refined and processed foods are relate to subclinical chronic inflammation [135] and to gastrointestinal disorders [136]. A general inflammatory effect of bread consumption was clearly showed in our *in vivo* experiments on mice. Histological analysis of Casp8 $^{fl/fl}$ mice revealed feature of mild colitis in all experimental groups except control group (gluten-free diet). This observation suggests that bread may have a pro-inflammatory effect in healthy animals which is not related to the type of wheat flour or raising agent used. Casp8$^{\Delta IEC}$ mice showed histologic features of spontaneously developed mild colitis, as expected, regardless of consumed diet. Interestingly, wheat consumption was able to induce a marked worsening of colitis in mice fed with yeast-bread from modern wheat and in mice fed with sourdough-bread from modern wheat and baking improver. This inflammatory effect was more attenuated in mice fed with sourdough bread from modern wheat . These data are consistent with the proposed role for Lactobacilli contained in sourdough in detoxifying ATI and gluten present in wheat-based foods, as previously described [62] . Furthermore, other studies showed that sourdough fermentation is able to modify macromolecules in the dough, especially to reduce digestibility of starch [137-138]. Sourdough fermentation has been shown to increase mineral bioavailability [63-139] and improve glucose metabolism[140] and release several compounds that are not detectable in yeast fermented products. Starting from the beginning of the twentieth century, sourdough was gradually replaced by baker's yeast (consisting of cell biomass mainly belonging to the yeast species *Saccharomyces cerevisiae*), a microbial starter culture produced at industrial level which add it at low percentages (0.5–2.5%) in bread dough to obtain leavening. The main

consequence of this revolution was a decreased of fermentation times. It has also been hypothesized that short fermentation may have contributed to bread intolerance through its effects on fermentation in the colon [141]. Comparing histologic features of B2 and B3 groups (modern wheat varieties) with B4 group (ancient wheat variety, Spelta) in both Casp8 $^{fl/fl}$ and Casp8 $^{\Delta IEC}$ we can suppose that diet based on bread from modern wheat variety may have a more marked inflammatory effect on gut. Since inflammation features appears more marked in B3 than in B2 group, we can also suppose that introduction of baking agents (B3 group) to improve the shelf-life and texture of commercial bread may alter gut homeostasis also in healthy subjects. These results are in line with other studies, which showed that additives might alter the gut microbiome inducing inflammation and obesity [65]. Moreover a number of studies have suggested that ancient wheats have health benefits compared with modern bread wheat. Valerii et al suggest that modern grains are able to induce significantly more CXCL10 secretion by PBMC compared to the ancient grains[50]. According with previous studies, our results suggest that in Casp8 $^{fl/fl}$ and Casp8 $^{\Delta IEC}$ mice fed to diet based on yeast-bread from ancient wheat Spelta seems induce a lower colon inflammation and no changes in colon structure comparing to mice group fet to diet based on yeast-bread from modern wheat. The impact of wheat consumption on systemic inflammation was confirmed by release of IL-6 in mice sera of Casp8 $^{\Delta IEC}$ mice, suggesting that the ancient wheat Spelta have a lower impact on colon homeostasis. On the other hand, modern wheats seems correlated with a mild colitis that appears less marked in sourdough leavening and more marked in backing agent. We can conclude that may sourdough reduce the inflammatory effect of modern wheat while the backing agent seems trigger an inflammatory effect. Moreover, bread-based diets alters barrier function in intestinal epithelium of Casp8$^{\Delta IEC}$ and Casp8$^{fl/fl}$ treated mice. Disruption of the intestinal TJ barrier, followed by permeation of luminal noxious molecules, induces a perturbation of the mucosal immune system and inflammation, which can trigger intestinal and systemic diseases. Modification of TJ barrier function and paracellular permeability is dynamically regulated by various extracellular stimuli and is closely associated with healthy and susceptibility to gut diseases[142]. This study highlights the increase of Claudins-2 mRNA levels in both Casp8$^{fl/fl}$ and in Casp8$^{\Delta IEC}$ mice compared to control group. The increase of Claudin-2 was found especially marked in B1 and B4 diets. In physiologic conditions claudin-2 expression is restricted to proliferative colonic crypt base epithelial cell. During mucosal inflammation its expression is extended beyond the crypt-base proliferative cell in the colon, causing colonic epithelial cell proliferation[143,144]. These observations are not in line with the histological results of these study that highlights hyperplasia in colon tissue only in B1 group. Indeed, previous *in vitro* study have linked upregulation of Claudin-2 in response to inflammatory cytokines exposure[145]. However, results of our experiments suggest that

wheat-induced cytokines release may disturb intestinal barrier function and subsequently increase intestinal permeability only in Casp8$^{\Delta IEC}$ mice . Previous studies suggest that IL-6 is associated to the increase of paracellular permeability to cations and the increase in pore-forming Claudin-2[146]. Since the upregulation of Claudin-2 might lead to alterations in TJP structure which were observed also in active CD patients[147], we can associate the slight changes detected in Claudin-2 expression to the impaired epithelial function observed in B1, B3 and B4 groups of both Casp8$^{fl/fl}$ and Casp8$^{\Delta IEC}$ mice. Occludin and Zonulin-1 are both important regulators in the tightness of the tight junction and have an important role in the regulation of TJ assembly. Occludin is ubiquitously expressed in TJ and has been shown to contribute to TJ functions, whereas Zonulin-1 is thought to regulated occlunin functions[148]. In this study no difference was observed in Occludin expression in Casp8$^{fl/fl}$ and Casp8$^{\Delta IEC}$ while results concerning Zonulin-1 mRNA expression are unclear. Fasano et al, demonstrated that binding of gliadin with the chemokine receptor CXCR3 at the lumial surface of epithelial cells trigger a MyD88-dependent luminal zonulin release[66]. In this study Casp8$^{fl/fl}$ a high expression of Zonulin-1 was detected in all mice groups excepted to B1, B3 and B4 group but not in Casp8$^{\Delta IEC}$ mice, where the expression of these gene seems in line with control results. It is likely that the upregulation of Claudin-2 might altered the component of TJP also modulating the expression patterns of other gene involved in this multicomplex proteins.

Our *in vivo* study on healthy subjects showed that a Korasan-based diet may reduce inflammatory profile in young athletes. Other clinical trials conducted on both healthy or diseased subjects showed that ancient or heritage wheat consumption was able to protect against metabolic disease, ameliorate metabolic profile in patients with metabolic syndrome[149] and cardiovascular diseases[150] and improve symptoms in patients with gastrointestinal diseases[151] . Since our subjects were already following a balanced diet, we were able to attribute specific effects to wheat consumption and avoid a "life-style" effect. The analysis of pro- and anti-inflammatory blood cytokines showed differences between athletes following the two diets. MCP-1 is a pro-inflammatory protein whose concentration increases significantly in plasma following intensive short-duration exercise[152].In our study, we observed that the Khorasan diet was linked to a significant reduction in blood MCP-1 (post-exercise) levels in athletes after 4 weeks. While the Khorasan diet did not significantly modify the blood concentration of IL-8, we found higher IL-8 levels in athletes after the modern wheat dietary period, even if this increase was not statistically significant. It is not to be excluded that in a study carried out on a greater number of athletes, this datum may reach statistical significance. Although changes in IL-1ra levels were not statistically significant, this anti-inflammatory cytokine showed an opposite

general trend to that observed for the pro-inflammatory IL-8. IL-8 is released into the circulation not only following prolonged, intense physical exercise but also after short-lived intensive exercise[152]. Interestingly, other clinical trials have demonstrated that diets based on ancient wheat cultivars were capable of lowering circulating IL-8 levels. In particular, a Khorasan wheat-based diet was found to cause a significant reduction in IL-8 (of 24%) in patients with non-alcoholic fatty liver disease (NAFLD)[153].

Conclusions

This study confirm that wheat may have a pro-inflammatory effect in healthy subject that could trigger a more severe inflammatory events on genetically predisposed subjects or on subjects with a gut environment already compromised (e.g. dysbiosis). Moreover, the ancient varieties of wheat seems associated with a lower inflammatory response in *in vivo* study but, unfortunately, it was not possible to detect clear differences using in-vitro test. Our results are in line with the recent literature suggesting that the progressive introduction of modern wheat varieties could be linked to an increased prevalence in chronic bowel diseases. It has been supposed that the rapid changes in wheat genome have not been followed by a complementary adaptation of human physiology and that the different quality of protein components in modern grains may alter the homeostasis of intestinal mucosa and trigger an inflammatory response. Our study confirm the positive effect of ancient and heritage wheat varieties on systemic inflammation as revealed by cytokines measurement. In fact, inflammatory molecules such as cytokines and chemokines may disturb intestinal barrier function probably by increasing intestinal permeability. Overall, our results demonstrate that wheat elicited an inflammatory effect on gut and that modern wheat varieties but also wheat transformation processes may further increase these pro-inflammatory activities. Animal study with gut system already compromised confirmed that the use of sourdough is associated with a lower inflammatory response comparing to the traditional and common raising agent Saccharomyces cerevisiae. Also in healthy mice gut, some features of inflammation were detected after wheat consumption. Reported to the human population, these data could explain the rising incidence of wheat related disorders of unknown etiology . Basing on our results, an inflammatory effect can be elicited by wheat consumption also in healthy people even when it is not linked to gastrointestinal symptomatology. This effect could become symptomatic in people with particular genetic backgrounds and worsened by modern wheat and baking agents, which are largely used by industries during wheat flour processing.

Unfortunately, the mechanisms by which gluten or other wheat components causes NCWS symptoms are still not understood and this makes difficult to identify which wheat components may be responsible of inflammatory effects probably elicited by wheat proteins. Also in healthy subjects, despite it is clear that there is a tendency to not tolerate wheat, it is difficult to understand which components are responsible of the low immune response observed. Moreover, is not yet entirely clear what could be the contribution of particular genetic backgrounds that could intervene in the response of the population to wheat components exposure. Further studies with larger populations should be conducted in order to definitively clarify these aspects. Moreover a larger number of wheat varieties

should be tested in order to understand if there is a common denominator that qualitatively differentiates ancient and modern wheat varieties.

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