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DIETARY METALLIC NANOPARTICLES: A NEW ENVIRONMENTAL FACTOR IN THE DEVELOPMENT OF CELIAC DISEASE?

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Chapter 1: General Introduction

DIETARY METALLIC NANOPARTICLES

NANOTECHNOLOGY, FOOD INDUSTRY AND REGULATION

Nanotechnology is a recent field of research born to study and exploit the peculiar properties of nanomaterials (NMs). All the material in the "nano" size present new exciting chemo-physical properties compared to their conventional bulk counterparties that can be useful in several applications. For this reason nanomaterials are today used in a wide range of applications, from agriculture and sustainable energy to food chain and medical applications[1-9].

Anyway, this large use of NMs in several fields potentially increases the exposure of our body to such materials. Therefore, along with many advantages, a big question about possible risks for the human health has raised.

According to the Nanotechnology Consumer Product Inventory (CPI) compiled in 2014, oral ingestion is one of the major exposure route for NMs contained into consumer products and the intake of nanoparticles (NP) is estimated around 10^{12} NP/person per day[7, 10, 11].

NP that reach the gastrointestinal tract (GIT) can be divided in two main groups: a) food additives intentionally added to food, beverage and their packages[12] or to pharmaceuticals (mainly to preserve aliments[13, 14] or to improve organoleptic properties and bioavailability of nutritional supplements[15]); b) NP non specifically directed to enter in contact with the GIT, but that can do so as

contaminants from the preparation and processing of food, from cosmetics (lipstick and toothpaste) and dental prosthesis debris, and also part of inhaled nanoparticles and nano-pesticide used to improve the agriculture field[16, 17]. This concern is also supported by scientific evidences that have linked food nanoparticles ingestion to intestinal bowel diseases (IBD) and colon neoplastic lesion development[18-21]. All these factors have raised questions about the increasing usage of nanomaterials into the food chain, leading to the worldwide efforts to regulate both their production and use[22].

In 2008 the Commission of the European Communities underlined the need to implement the legislation regulating nanomaterial use. Consequently, in 2011, the European Food Safety Authority (EFSA) provided the first "Guidance on the risk assessment of the application of nanoscience and nanotechnologies in food and feed chain". This document, regarding the definition of nanomaterial (2011/696/EU), states: *" Nanomaterial means a natural, incidental or manufactured material containing particles (i.e. minute pieces of matter with defined physical boundaries), in an unbound state or as an aggregate (i.e. particles comprising of strongly bound or fused particles) or as an agglomerate (i.e. collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components) and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm-100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50 %*

may be replaced by a threshold between 1 and 50 %.". In line with this definition, the more recent EFSA Guidance (2018; "Guidance on risk assessment of the application of nanoscience and nanotechnnologies in the food and feed chain: Part 1, human and animal health"[23]) suggested the use of a multi-method approach to assess whether a material is a nanomaterial (NM). Moreover, it stated the need to extend the definition of NM to materials that, although with size above 100 nm, retained properties characteristic of NM. Regarding the NM risk assessment, they stated that it needs to be performed on a case-by-case basis. Moreover, the Guidance delineates a structured pathway of tests for the risk assessment of NM exposure through the oral route.

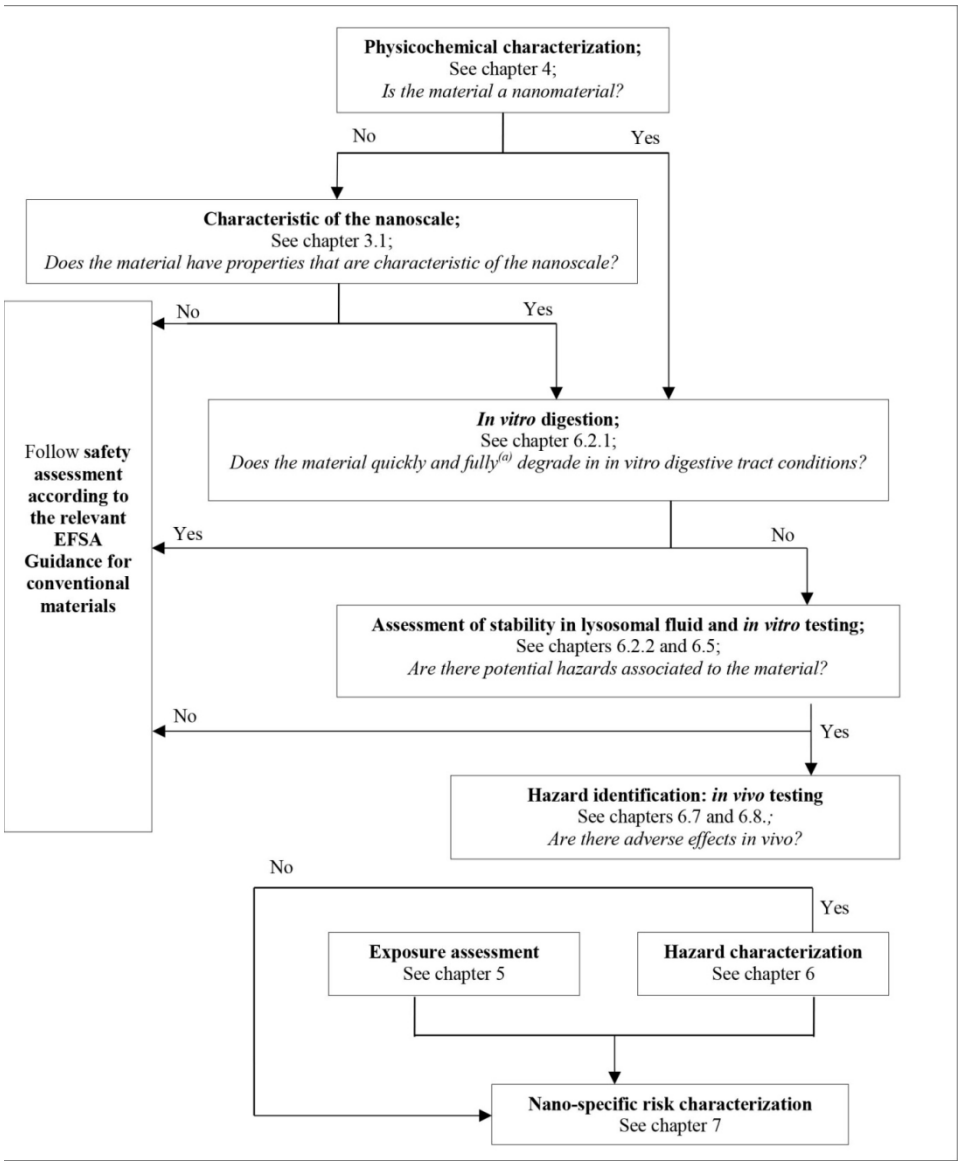

Figure 1: Scheme of EFSA guidelines for nanomaterial risk assessment. From EFSA Scientific Committee. Guidance of risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health. EFSA Journal 2018.

This document even suggests the best techniques to use in each step, and underlines the current lack of validated, common and suitable methods to study all the NMs aspects. However, there is not a "gold standard" technique for the characterization of NMs into complex matrices, as well as a common method for NMs dispersion[24-26]. Along with this guidance, the European Union (and Switzerland) translated these concerns in legislation. Anyway, it has to be underlined that actions so stringent have not been taken yet in other countries[22]. Considering U.S. and Canada governments, they decided to establish a common regulatory approach for the NM risk assessment (in both human and environment) under the US-Canada Regulatory Cooperation Council (RCC)[27]. Although the definition of NM results similar to the European one, it has to be considered that it has not legal validity.

Even if a case by case risk assessment is today the only possible scenario, several efforts have been made to find a common NM risk determinant. This would allow a more efficient approach, suitable with the still increasing nanotechnology use and evolution.

NANOPARTICLES IN THE GASTROINTESTINAL TRACT

CPI reported that nanomaterials present in commercial food and/or food-related products are mainly under form of metallic nanoparticles (mNP), which we will mainly address hereafter.

Although several data about food mNPs are today available, few studies have taken into consideration the interactions between mNP and gastrointestinal tract (GIT). mNPs are composed of substances usually slowly- or not- dissolved during digestion. Their fate and toxicity into the GIT is affected by several factors:

- Core material: NP core composition determines their chemical reactivity that, in turn, can differently induce substances adsorption on NP surface. Lichtenstein et al.[28] suggested that the core material influences the epithelial translocation route of mNP. In addition, depending on this feature, mNP can be left unchanged or partially, fully digested/dissolved by GIT fluids. Consequently, the propensity of NP to release heavy metals can change, affecting NP toxicity. In this sense AgNP, ZnONP and CuONP are regarded as the most dangerous ones[29, 30].

- NP surface composition: The composition of the surface of mNP primary depends on the physiochemical properties of the nude surface itself, such as charge, chemical reactivity, core material and hydrophobicity. All these factors, together with the characteristics of the GIT environment, drive the adsorption of surface-active components, mainly proteins, on the mNP surface[31, 32]. This process occurs over time, forming the so called "protein corona" on the NP surface. The protein corona is formed by two layers: the "hard corona" composed of permanently adsorbed proteins near to the surface; and the "soft corona", a more distal and dynamic layer where proteins are reversibly adsorbed[33]. Although the protein corona has been useful in drug delivery and medical applications,

dietary mNP protein corona formation (which occurs during their transit into the GIT) may cause unwanted physiological consequences and toxic effects. Particularly important, in this sense, it's the capacity of mNP to interfere with protein folding and enzymatic activity[32, 34], enhancing their cross-linking and fibrillation, but also exposing new, previously cryptic, antigens (fact which could promote autoimmune diseases)[35, 36]. Dietary mNP protein corona is formed upon the initial contact with the food matrices and with the GIT thereafter. Each GIT- compartment presents specific physiochemical characteristics such as ionic strength and pH, digestive enzymes, mechanical forces, osmotic concentration, mucus layer and commensal microbiota. Therefore, before being absorbed, mNP surface composition could completely change along the way into the GIT[31], thus affecting their fate[37].

-NP size: several studies reported that the size of food mNP might influence their uptake from intestinal cells[38-40]. Since their size can be variable, from a few to hundreds nanometers, this parameter appears crucial for mNP fate. The smaller the mNP the faster and easier will be its passage into the mucosa either by transcellular or paracellular transport.

- Aggregation/agglomeration state: NP can reach the GIT as single entities or in cluster (agglomerates or aggregates[41]). This feature depends on the NP composition, but also on the physiochemical properties of the environment. Peters et al.[42], Walczak et al.[43] and Sieg et al.[44] reported that the degree of aggregation/agglomeration of SiO_2-, Ag- and aluminium-NP can change in artificial mouth, gastric

and intestinal conditions. At the same time this factor can also affects NP uptake and toxicity, as demonstrated by McCracken et al.[45], Albanese et al.[46] and Huang et al.[47]

- Gastrointestinal environment and food: As mentioned above, the physiochemical features of either the aliment matrices or the GIT are crucial to NP stability, size, surface composition and aggregation/agglomeration state[31]. Really few studies about the characterization of mNP within the initial food matrix have been performed, also due to the fact that researchers have not agreed yet on the best technique to study it[23]. Some studies about the interaction of mNP with digested food have instead been done; Wang et al.[48] and Cao et al.[49] demonstrated an higher oxidative stress-related toxicity exerted by ZnONP when associated with Vitamin C and palmitic oil, respectively; on the contrary, the presence of flavonoids or quercetin seems to protect against AgNP toxicity[50, 51]. Few studies have also been performed considering the gastrointestinal digestive fluids demonstrating, as above described, changes in mNP uptake, toxicity and aggregation/agglomeration state[42, 44, 45, 52]. Anyway, from the best of our knowledge, only two works took into consideration both digestion fluids and food presence showing an increase in both uptake and translocation of the mNP into the mucosa[53, 54].

It appears clear that the interactions between NPs and the GIT are quite complex. Another point to consider is that most of the performed studies mimic an acute insult, whereas in reality a chronic (long term) exposition of dietary mNP should be assessed. As already

suggested by the European Food Safety Authority in the "Guidance on risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health"[23], it is necessary to use complex systems (taking in consideration all the above explained factors) and, once confirmed an acute insult, also perform long-term exposition studies.

METALLIC NANOPARTICLES TRANSCYTOSIS MECHANISMS

The principal district of the gastrointestinal tract involved in the mNP uptake mechanisms is the small intestine. Its main function is the absorbance of nutrients, this is why the wall surface is structured in villi and crypts, thus reaching a surface area of about $30m^2$ [55]. At the same time, intestine behaves as a barrier for protection from pathogens and immunogenic molecules and it can be regarded as a system composed of two main functional layers: a continuous and tight epithelium and the gut-associated lymphoid tissue (GALT). Nevertheless, for mNP to be absorbed it has also to be considered the mucus layer, composed of glycoproteins and antimicrobial agents such as secretory IgA[31, 56].

What makes the epithelium a selective barrier is the presence of highly dynamic intercellular junctions, being adherent junctions (AJ) and tight junctions (TJ) the most representative ones[57]. AJ are composed of transmembrane proteins cadherins, which are connected between themselves extracellularly, but bind catenin proteins intracellularly. Catenins are, in turn, linked to the actin-

myosin complex. TJ are formed by occludins (OCLN), claudins and JAM-A proteins that interact with zonula occludens proteins (ZO-1 and ZO-2) and catenins in the intracellular space. All these components form a ring-like complex that can regulate the permeability (paracellular route) of the intestinal barrier, following intracellular or extracellular signals[58].

The other important component in the intestinal barrier, manly involved in the protection from harmful molecules and microbes is the GALT. It is composed by a complex network of immune cells, mainly organized in isolated or aggregated lymphoid follicles named Peyer's patches[59], that represent about the 70% of the immune cells of all the human body[60]. It makes contact with the luminal side through specialized cells (dendritic cells and M-cells), whereas presenting antigens to T cell on the sierosal side, either inducing tolerance versus the commensal bacteria or immune reactions against pathogens.

Due to the increasing use of mNP, several efforts have been made to understand their uptake, transport and fate within the cells. Mainly two routes can be used by mNP: trancellular transport (through epithelial cells) and paracellular transport (between adjacent epithelial cells). The paracellular route is mainly regulated by the TJ and AJ complexes that in the small intestine create a paracellular pore diameter of approximately 1 nm. Therefore, in normal conditions, this route is not accessible to mNP. Several evidences, instead, pointed out that transcytosis is the mainly process by which mNP enter in the body. Both clathrin- and caveolin-dependent

endocytosis, driven by NP interaction with membrane receptors and involving M-cells (lining intestinal Peyer's patches) or enterocytes, have been reported[31, 61]. It must be underlined, however, that data are lacking about the interaction/absorption of mNP with less differentiated cells such as those present in the crypts. Last but not least, the difficulty in characterizing the specific involved endocytotic mechanism is linked to the complex nature of these mechanisms, influenced by both the physiochemical properties of the mNP (such as protein corona and size) and the type and differentiation state of the cells[62].

BIOLOGICAL EFFECTS OF METALLIC NANOPARTICLES

Although studies are scanty and sometimes controversial, mNPs have been reported to alter intestinal homeostasis through several mechanisms: they can alter the intestinal barrier increasing the paracellular transport[6] or inducing cytotoxic damage in the epithelium layer[5] (mainly through oxidative stress but also impairment of the autophagic/endocytotic pathways), but they can also affect the immune response.

Intestinal barrier damage

A growing number of diseases has recently been associated with intestinal barrier alterations, particularly related to TJ dysfunctions. This finding can be easily explained: gastrointestinal barrier permeability alterations can increase the cut-off of molecules passing

into the submucosa (in physiological conditions only small molecules with molecular weight of about 600Da can pass the barrier), resulting in the passage of more complex and possibly immunogenic molecules, leading in turn to the activation of the immune system and the establishment of an inflammatory state. Since inflammatory mediators are also known to affect the intestinal barrier, a mild inflammatory status could eventually lead to a more severe disruption of the barrier itself[63]. Particularly important in this sense is the association of a leaky barrier with IBD and other autoimmune diseases, such as CeD[63 16, 64]. To develop CeD, gluten peptides have to pass into the submucosa, be deamidated by tissue transglutaminase (TG2) and recognized by the immune system. Therefore, any factor able to alter the intestinal barrier permeability and allowing a higher passage of these peptides into the submucosa, may increase the number of predisposed subjects developing the disease.

In this context, Lerner and Matthias[65] observed that the increase in the incidence of autoimmune diseases (considering also CeD among others) paralleled the increasing food additive use in the industry. They therefore postulated that the permeability alterations induced by food additives could be associated with the increment in incidence of autoimmune diseases. The authors did not refer directly to the mNP, but several studies have demonstrated mNP impact on the GI barrier. In fact, mNP can affect the intestinal permeability both directly, altering the TJ or inducing epithelial cell death[38, 66-68]; or indirectly, inducing inflammation or oxidative stress that in turn can impair TJ and permeability[63, 69]. In this context, it is interesting the

work of Ruiz et al.[18] that studied the impact of TiO_2NP both in vivo (mice with DSS-induced ulcerative colitis model) and in vitro (intestinal epithelial cells and macrophages). TiO_2NP oral administration worsened the already established colitis through inflammasome activation. In addition, in vitro stimulations with TiO_2NP induced IL-1β and IL18 increment as well as higher epithelial permeability.

Epithelial mechanisms of toxicity

As above reported, mNP are principally internalized by endocytic mechanisms. All the endocytic vesicles containing the internalized NP are considered a common pool of apical early endosomes (AEE). They can follow several routes inside the cells: recycled to the apical membrane; transcytosis to the basolateral side; transport into late endosome (LE) and lysosome (LY) for their proteolytic degradation; fusion with autophagosomes and formation of amphisomes[70]. Anyway, the mechanisms involved in determining NP fate at this point are poorly understood[61]. Even so, it is recognized that, once into the cells, mNP exert deleterious effects on several organelles. Noteworthy it's the strong oxidative stress induction by mNP, that in turn leads to endoplasmic reticulum stress (ER stress), DNA damage, mitochondrial dysfunction and eventually cellular death[71]. Another widely recognized mechanism of mNP toxicity is their impact on the lysosome functionality, which often leads to lysosome membrane permeabilization, consequent oxidative stress and cellular death[72, 73].

A new cellular pathway that has also been recently associated with mNP toxicity is the autophagic process[74]. Autophagy can be subdivided into three main groups: microautophagy, chaperone-mediated autophagy and macroautophagy. Particularly, macroautophagy (from now referred as to autophagy) involves the sequestration of several cargos (such as damaged organelles, but also endocytic vesicles, aggregated proteins and pathogens) into double-membrane structures called autophagosomes, which are formed by various membrane reservoirs. They later fuse with lysosomes forming the so-called autolysosome, resulting in the breakdown of the encapsulated cargos, either to discard them or to reutilize their components into the cells.

The molecular signals and consequent pathways that can trigger the autophagic process are many.

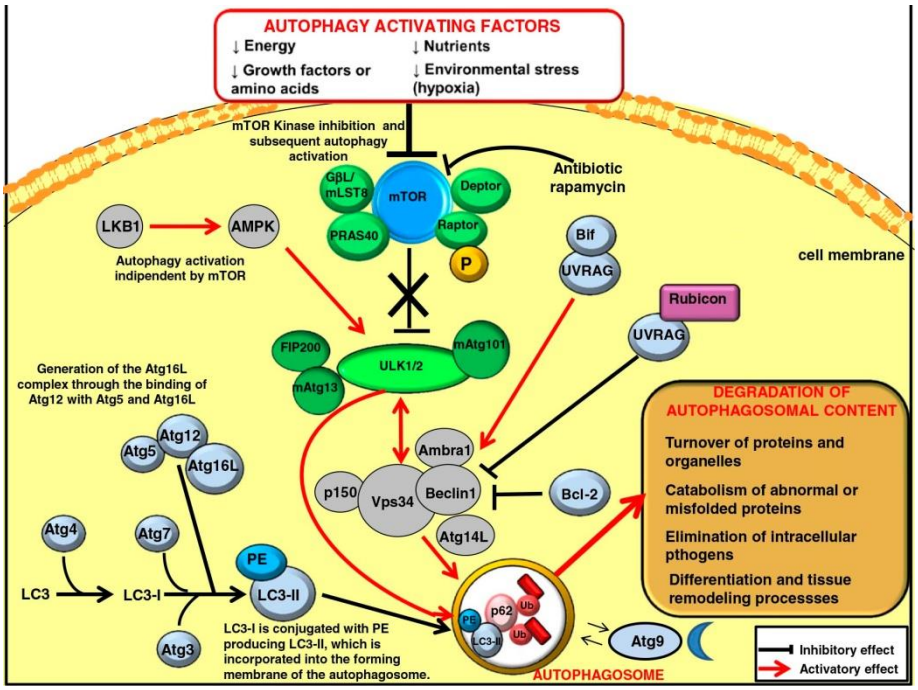

Figure 2: The autophagic pathway in mammalian cells. From Gianchecchi et al., 2014.

They all lead to the nucleation and the assembly of the phagophore membrane. This first step occurs through the involvement of the phosphatidylinositol 3-kinase/vacuolar protein sorting 34 (PI3K3/Vps34) complex, which is positively regulated by autophagy-related 14 (Atg14L), endophilin B1/UV radiation resistance-associated gene (Bif-1/UVRAG) and autophagy/Beclin-1 regulator 1 (Ambra 1), whereas it is suppressed by Rubicon/UVRAG. Autophagy activation implies the dissociation of Ambra 1 complex and of PI3K3 complex from the cytoskeleton and their translocation into the ER, where the

phagofore formation begins. Atg9, Atg2 and Atg18 are found at the edges of the expanding phagophore, while the members of the small ubiquitin-like Atg8/LC3 protein family have been implicated as possible closure factors. The next step involves the maturation of the phagofore into autophagosome, which is linked to the conjugation of Atg8/LC3 (known as LC3-I) with PE (forming LC3-II) by Atg4 protease[75]. After fusion with the lysosome, most of the Atg proteins involved in the autophagosome formation and maturation are recycled.

Although autophagy was principally known as a non selective process during cellular stress conditions, it has recently been proved its role in selective removal of damaged organelles, pathogens, and ubiquitinated proteins. There are two main protein degradation systems in eukaryotic cells: the proteasome and autophagy. Several studies have been recently performed to better understand their specific role. Results seem to point at an activation of the autophagic system when the proteasome capacity is exceeded, after strong ER stress, and when the misfolded proteins tend to polymerize and form aggregates. This mechanism is driven by the p62/SQSTM1 protein, which works as an adaptor molecule, recognizing both the polyubiquinated misfolded proteins and LC3-II protein on the autophagosome[76].

A growing amount of evidences suggest that nanomaterials can be selectively compartmentalized by autophagic vesicles and this may play an important role in NP toxicity[74]. Anyway, it is still not clear the precise mechanism by which NP perturb (both for induction and

blockage of the flux) the autophagic process. Particularly, NMs could activate autophagy through an oxidative mechanism (accumulation of oxidatively damaged proteins and consequent ER stress)[77]; through direct ubiquitination of nanomaterials or their co-localization with ubiquitinated proteins; or due to the fact that NM are perceived as foreign or aberrant by the cell (like it happens for pathogens)[74]. Particularly meaningful in this context is the work of Huang et al.[47], that demonstrated how iron oxide nanoparticles strongly elicit autophagy when aggregated, but not when dispersed. Their results could be explained as a direct autophagic activation driven by aggregated material (in this case NP, but probably also proteins) into the cells.

Therefore, even if the endolysosome/autophagic pathways have the potential to dispose of the internalized mNP, they could in turn be negatively impacted by the nanomaterial toxicity mechanisms.

NP and immune system

The use of engineering mNP for application in the immune system is today an exciting and emerging field. It is in fact widely accepted that mNP can interact, both positively and negatively, with both innate and adaptive immunity[78, 79].

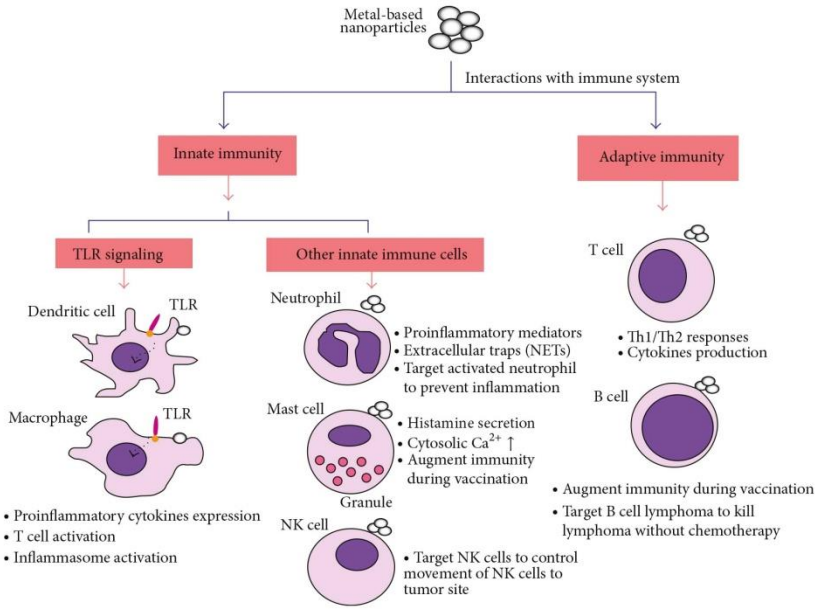

Figure 3: Metallic nanoparticles mechanisms of immune modulation. From Luo et al., 2015.

The innate immunity is known as the first line of defense, its response is nonspecific and it consists of different cells. In this sense, together with immune cells such as phagocytic cells (macrophages/monocytes and dendritic cells), mast cells, eosinophilis, basophils and natural killer cells, also physical epithelial barriers, such as the intestinal one, play an important role. The principal role of the innate immunity is characterized by the

recognition of conserved molecular patterns on pathogens (PAMPs) and the consequent proinflammatory response. In this sense the toll-like receptors (TLR) are known as the main molecules involved in PAMPs recognition, but several evidences have shown that they can be activated by mNP, even if the mechanisms are not clear yet. For this reason mNP can be used as powerful adjuvants enhancing TLR activity and inflammatory response (as vaccine, allergy or infection adjuvant or in antitumor therapy)[78, 80]. mNP are in general taken up by phagocytic cells, therefore another possible application could be antigen or drug delivery in both tumor-associated and vaccine-related dendritic cells (DC). Dendritic cells are particularly important mediators between innate and adaptive immunity. Their main role is the continuous examination of the microenvironment; this task involves taking up antigenic materials, processing them, and presenting the epitopes to the T cells, with subsequent adaptive immune system activation. DC can activate both CD8+ or CD4+ T-cells, eliciting different responses. In DC, the processing of the antigenic molecules is strictly linked to the endolysosomal system[81], which can be altered by the mNP presence. Other important immunemodulatory effect of mNP is related to their capacity to trigger naive T cell activation and proliferation[82].

It is therefore clear that, together with their potential medical applications, mNP can also elicit immune toxic effects when delivered to the body through the diet. In this sense it is not yet well understood the relation between mNP physiochemical properties and immune effects.

GOLD, SILVER, TITANIUM DIOXIDE AND ZINC OXIDE NANOPARTICLES

In food products it has been observed an higher presence of silver (AgNP or E174), Titanium dioxide (TiO_2NP or E171), Zinc oxide (ZnONP) and gold (AuNP or E175) nanoparticles[7].

AgNP

Since few decades ago the use of mNP (inorganic material) as antibacterial agents has been preferred to the classical organic agents. This choice was due to mNP stronger activity at low concentration and good resistance to pressure and high temperature (typical conditions in the industrial processes)[83, 84]. Among them, silver nanoparticles show exceptional anti-microbial properties (anti-fungal, anti-viral and antibiotic), which is why they are mostly used in aliment/beverage packaging materials, but also into the food itself[5, 12, 85]. AgNP migration from the packages has been estimated in several works and it depends from various parameters such as temperature, pH, time of contact with food and release area. In most cases, the size of the released NP is mainly around 40-60 nanometers of diameter[86-88]. Even though their use is increasing, several works reported that AgNP can induce undesirable and hazardous interactions with biological systems, thereby generating toxicity. Yang et al. [2] reported that AgNPs can also induce nutrients alteration, such as changes in the molecular structures of proteins and fatty acids. Anyway, since the results are often controversial, the EU Panel

was unable to reach a definitive conclusion and decide a maximum permitted level. Therefore, in the EU, AgNP are authorized at "quantum satis", meaning as much as it needs to achieve the goal of its use[89]. Recently their use is also increased in the agriculture field, to improve the growth of crops[90]. Particularly interesting for our work are some evidences about their positive effect on triticum aestivum (also known as the common wheat), even if some studies also found some toxic effects[91, 92].

AuNP

Although AuNP are mainly studied for medical applications, they are also used to improve crop protection, growth and germination. In general, the use of NP as agrochemicals permits to reduce the amount of pesticides used, sometimes even increasing the performance[93]. The consequent risks, even if mostly linked to the environment, involve also human health, since contaminants can potentially be present on harvest. Judy et al. reported that AuNP can be taken up by tobacco and wheat plants, even if this depends on size and surface features. AuNP are particularly used for ameliorating crop growth and as nanosensor to detect soil quality and nutrients[1, 94]. Other sources of AuNP are food packaging (used as anti-bacterial agent), beverage and external food colouring and decoration. Since AuNP main application is in the medical field, there are few studies about the dietary NP and their possible impact on the gastrointestinal tract[95].

TiO_2NP

Titanium dioxide exists in nature in three different crystalline structures: rutile, anatase and brookite. It also contains impurities such as vanadium, sirconium or iron. The forms used in the food sector are anatase and rutile, either alone or mixed in different percentages, even if the anatase is the most frequently utilized[96-98]. In the food chain TiO_2NP are used as food colorant in several aliments such as pastries, coffee creamer and candies, but also to confer brightness (for example in the toothpastes) and for clearing beverages (such as wine)[25, 99]. Although the primary size of TiO_2NP in the food is around 200-300 nanometers, it has been reported that inevitably (as a consequence of production processes) it also contains 17-36% of nano-sized NP[97, 99]. What worries the most is that TiO_2 dietary mNP consumption is really high in children. It has been estimated that in children 3-9 year old, the intake of TiO_2NP is around 0,9-8,8 mg/kg body weight per day, against 0,2-0,7 mg/kg/day in the other age groups[99, 100]. For this reason few studies have employed young laboratory animals, and the results suggested that TiO_2NP may be more prone to cause deleterious effects in younger subjects than in adults[101, 102]. In addition it has been proved that these mNP induce a low inflammation in the intestinal mucosa, which could, in turn, help to initiate preneoplastic lesions or to develop IBD[103]. Although the data regarding the TiO_2NP fate and toxicity in the GIT are many, the EFSA Panel was unable to reach a definitive conclusion. As for the

AgNP, the Panel did not decide a maximum permitted level, suggesting its use as "quantum satis"[100].

ZnONP

Zinc oxide nanoparticles are principally used as antibacterial agent in food/beverage packaging. Their antibacterial properties are linked to the capacity to induce ROS formation and toxicity through release of Zn^{2+} [104-106]. Moreover, antibacterial activity is dependent on the migration rate from the package to the food and on NP physiochemical characteristics such as size and morphology. They can be found in different material such as PVC, glasses, chitosan and polyurethane[107, 108]. In addition to the antibacterial activity, Li et al.[109] also reported that ZnO improved the mechanical strength of polyurethane films. Many data showed that ZnONP does not migrate from the packages into the food, therefore the EU consider it safe. Following this directive, in 2016 EFSA recommended that risk assessment should be focused on migration of Zn ions and NP[110].

CELIAC DISEASE AND ITS PATHOGENESIS

Definition

Celiac disease (CeD) is a multifactorial autoimmune disorder that develops in genetically predisposed subjects, particularly involving the small intestinal tract. The principal genetic variants associated with the disease are the HLA-DQ2 and HLA-DQ8 haplotypes, but several other non-HLA risk loci have been identified[111, 112]. CeD develops in response to the ingestion of gluten-containing food and, to confirm it, the only effective therapy for this disorder is a strict gluten-free diet. Briefly, to develop the disease, gluten peptides have to pass the gastrointestinal barrier and enter into the lamina propria, where they can activate the immune response against themselves as well as against TG2, triggering all the processes involved in CeD pathogenesis.

Although the primary site of damage is the small intestine, the involved districts, as well as the clinical manifestations are many. CeD patients can suffer from the more common enteropathy form (chronic diarrhoea and malabsorption) to dermatitis herpetiformis[113], iron deficiency[114], headache, osteoporosis and neurological dysfunctions[115].

Diagnosis

Since the symptoms are various, this should be taken into account when making diagnosis of CeD. The classical symptoms, such as malabsorption and weight loss, are caused by strong architectural changes in the small intestine mucosa which present several grades of severity and can lead to the complete villous flattening and crypts hyperplasia. Therefore a classification based on these different immuno-histopathological phases has been introduced by Marsh and co-workers[116-118] and it is used for the diagnosis of the disease. This classification particularly refers to four interrelated lesions: preinfiltrative (Marsh 0 or normal mucosa), infiltrative (Marsh 1), hyperplastic (Marsh 2), destructive and hypoplastic (Marsh 3).

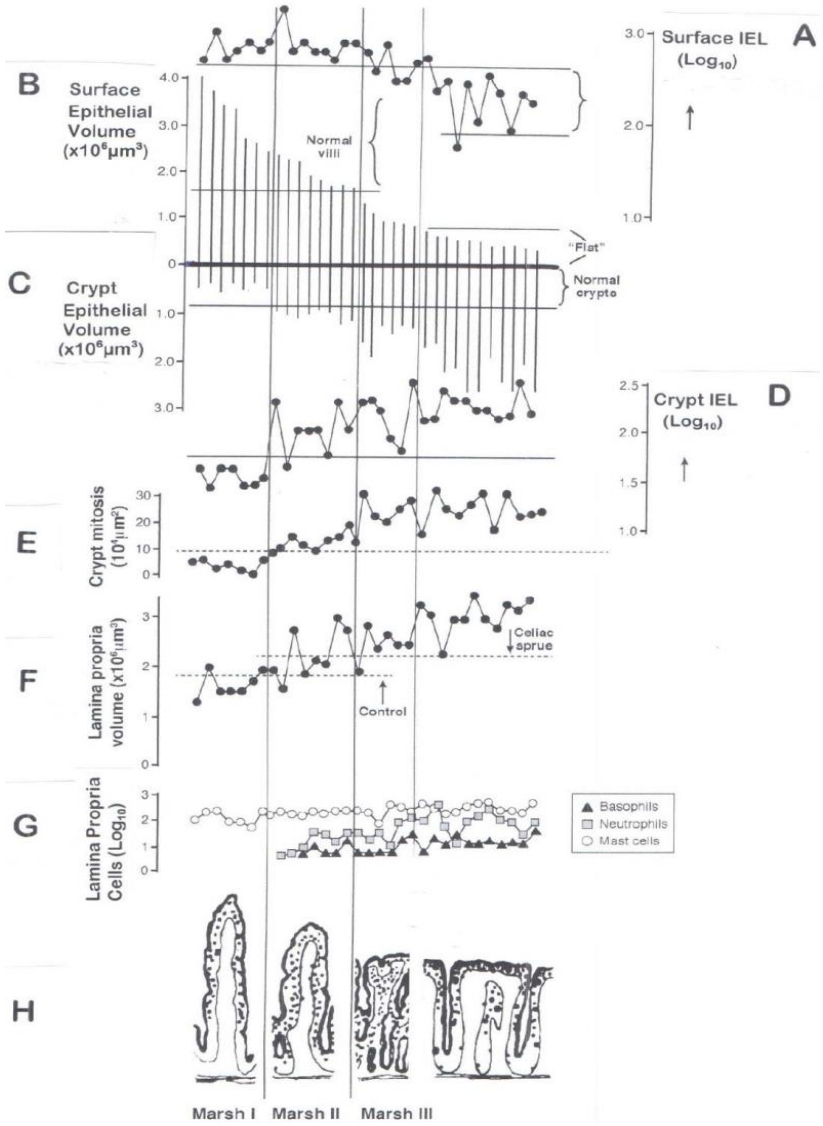

Figure 4: Mucosal transformation from "normal" to "flat" in CeD pathogenesis. From Marsh et al., 2017.

The lesions are interpretable as the response to several mechanisms such as cell-mediated immunologic activation and enzymatic dissolution of the mucosa by metalloproteinases (MMP) activity.

Nevertheless, these histological features are usually patchy in the duodenum, therefore a minimum of 4 biopsies are necessary for the correct diagnostic interpretation. In addition, several other requirements have to be met to avoid misinterpretation of the results[119]. Since obtaining a biopsy needs a gastroscopy, serology tests are at first performed for the diagnosis of the disease. The principal markers evaluated are autoantibodies IgA anti-tissue transglutaminase, anti-endomysial and anti-deamidated gliadin peptide (the latter mainly in small children). Based on the results of these pre-tests the decision of whether to proceed with a biopsy will be made. Controversial is the importance of the HLA genotyping (not required as routine test), since it could only exclude the presence of celiac disease. It is in fact known that, even if the genetic predisposition (HLA-DQ2) is present in about 30% of the general population[120] (and the gluten in a common component of the western diet), only 2-5% of this population develops the disease.

Incidence

It appears therefore clear that gluten and genetic predisposition alone are not sufficient for the developing of the disease, suggesting that other etiological factors may be involved. In line with this, the age of presentation of the disorder is really random, often affecting subjects only in adult age[121]. Nowadays, CeD is considered one of the most common worldwide disorders, affecting about the 1% of the population[122, 123], with an increased incidence observed in the last few years[119, 124, 125]. Herein the importance of searching for additional

etiological factors. Progresses have been recently made in this research area and several possible factors have been suggested: viral infections[126, 127], impaired commensal homeostasis[128, 129], increasing permeability[130] and a leaky gastrointestinal barrier[16]. It's also worth mentioning the Swedish epidemic of CeD in the mid-1980, where the number of children affected increased 4-fold times in few years and then returned to normal in the mid-1990[131]. Several studies were performed to understand what additional etiological factors caused this transient shift in CeD incidence, concluding that the infant feeding pattern, such as the age and amount of gluten introduction in their diet played an important role. However, two large studies more recently published support the idea that the age of gluten introduction does not change the incidence of the disease[132, 133]. It has also been postulated that modern food additives, increasingly used in food and beverage industries, may be environmental factors for the development of autoimmune diseases. As Lerner et al.[65] demonstrated, food additives can induce intestinal epithelial barrier dysfunction, thus increasing the entry of foreign immunogenic molecules in the mucosal side and the consequent autoimmune system activation. This mechanism could be relevant in the pathogenesis of celiac disease, considering that the passage of gluten peptides into the lamina propria is a crucial event. Besides, in CeD, it has already been documented an intestinal barrier defect, although it isn't still clear its role. Although barrier defects may appear as a consequence of the disease development, recent studies have suggested that increased intestinal permeability, resulting in

excessive activation of mucosal immune cells, could play a role in initiating and also developing CeD. In this sense, as above specified, mNP can be a perfect candidate for confirming this hypothesis.

Main etiopathogenic factors

Genetic components

The involvement of the genetic component in CeD pathogenesis is well documented. First grade relatives show 20-fold higher risk to develop the disease, moreover an high concordance between identical twins has been observed (about 75%)[123, 134]. CeD is strongly associated to specific variants of the human leukocyte antigen (HLA) related genes: HLA-DQA1*05-DQB1*02 (DQ2) and HLA-DQA1*03-DQB1*0302 (DQ8) encoding for HLA class II heterodimeric proteins. These molecules are expressed on the surface of the antigen presenting cells (APC) and their peculiarity is to strongly bind the deamidated gluten-peptides. A study performed on the Caucasian population showed that the majority of the celiac subjects carry the DQ2 or DQ8 heterodimer, while some only encodes for one chain[135]. Importantly, not all the subject carrying the genetic predisposition develop the disease, indicating that other genetic or environmental risk factor may play a role. Several studies have already associated the risk of developing CeD to other 42 non-HLA loci[111, 112].

Environmental component: gluten

Gluten is an heterogeneous and complex aggregate of proteins[136, 137] present in several types of cereals such as wheat, barley, rye and oats. Particularly, wheat is globally one of the most consumed cereal, used for making both bread and pasta and accounts for 20% of calories consumed by humans. Gluten can be separated into two fractions that differ for their alcohol/water solubility and tend to reaggregate when in water[137]: glutenins (insoluble fraction) and gliadins (alcohol soluble fraction). While glutenin is mostly an aggregate of proteins linked by disulphide bonds, gliadin can be divided in 4 different types: ω5-, ω1,2-, α/β- and γ-gliadins[136]. In the past, gliadin was considered the only fraction able to trigger the CeD[138]. Instead, recently, other gluten components have been related to the disease[139, 140]. Independently from the different structures they can possess, the high proline and glutammine content appear to be a common and important property. Several studies have in fact demonstrated that this characteristic makes the gluten peptides highly resistant to intra-luminal and intestinal brush border enzymes (BBM), explaining why most of them reach, almost undigested, the intestinal lamina propria. Particularly, Perez-Gregorio et al.[141] followed the in vitro gastrointestinal digestion of several gluten sources demonstrating that their different compositions have variable resistance to digestive and BBM digestion. The amount and composition of the formed immunogenic oligopeptides is, in turn, variable. Moreover, they showed that the most resistent epitopes

belonged to gliadins, being PFPQPQLPY the main one. To sustain this finding it should be noted that gliadins, more than glutenins, are characterized by high content of proline (15%) and glutamines (35%). In addition, their structure contains several cysteines that, forming intramolecular disulphide bonds, further reduce the enzyme access. Notably, two peptides, named 33-mer and p31-43, have been found highly resistant to degradation[142] and therefore capable to reach the intestinal lumen and trigger, respectively, the adaptive[143] and innate immune responses[144, 145] in CeD patients.

Since gliadin peptides are rich in glutammines, they are also an ideal substrate for the intestinal tissue transglutaminase. TG2 is an ubiquitous enzyme found in both the intracellular and extracellular compartment. Into the cells it is primary involved in apoptosis and autophagy pathway, whereas it plays a role in cell signalling and adhesion, matrix assembly and wound healing in the extracellular matrix. In the small intestine it is normally inactive, but under stress condition, as inflammation, TG2 became active as deamidation- and crosslinking-enzyme[146]. TG2 role in CeD pathogenesis is principally linked to the deamidation of specific glutammine residues of gliadin peptides, which strongly increase their affinity for the HLA-DQ molecules on the antigen presenting cells (APC)[147, 148]. Moreover, it has been reported that TG2 can be intracellularly activated by p31-43 peptide. P31-43 alters the endo-lysosomal pathway[149], thus inducing cellular stress and a pro-oxidative environment, ideal for TG2 activation. The active enzyme can lead to the downregulation of the anti-inflammatory peroxisome proliferator-activated receptor and

contribute to the development of gut inflammation, as shown by Luciani et al.[150]. Since TG2 plays also a role in both the ubiquitination-proteasome and autophagy system[151-153], its intracellular activation could also interfere in these pathways.

It has to be noted that the possible involvement of autophagy in the CeD pathogenesis has been recently enlightened. Barone et al.[149] reported that p31-43 sequence resembles the one of hepatocyte growth factor-regulated substrate kinase (Hrs/Vps27), necessary for the maturation of both early endosome and auphagosome vesicles[154, 155]. Comincini et al.[156, 157] found that ATG7 and BECN1 genes and their regulatory miRNA (miR-30a; miR-17) are differently expressed by celiac and healthy patients and also demonstrated a dysregulation of the autophagosome apparatus after gliadin stimulation of Caco-2 cells. Moreover, the role of autophagy in autoantigens formation has been suggested for CeD dendritic cells[158].

Immune response

As previously explained, deamidated gliadin peptides show high affinity for APC HLA molecules and can easily be presented to the intestinal CD4 T cell, activating the adaptive immune response. Anyway, also the innate immune system plays a role in CeD pathogenesis, particularly in the epithelial compartment where natural killer T lymphocytes are strongly activated (know as intraepithelial lymphocytes-IEL)[159]. Even if these processes can explain the immune response at the small intestinal level, they cannot really be the initial cause of the autoimmune response

developed in celiac subjects. One possible mechanism could involve the transdamidation (cross-linking) activity of TG2. It has been proved that TG2 can cross-links gliadin oligopeptides with other proteins including the TG2 itself, starting the autoimmune response[160]. In this sense, the main autoantigen in celiac disease is the TG2. The consequent circulating autoantibodies are also probably responsible for the broad range of clinical manifestations present in CeD.

Innate immunity

Epithelial cells, dendritic cells and intra-epithelial lymphocytes (IEL), together with cytokines and chemokines, play the major role in the innate response into the gut. As above mentioned, p31-43 has been reported to be mainly involved in innate immunity activation. Anyway, it is not still clear the mechanism behind its action. Many studies suggested that it enters the epithelial cells via endocytosis, even if it has not been possible until now to isolate the specific receptor[161-163]. Anyway, p31-43 localizes in early endosome interfering with the endosomal trafficking of several other cargos[149] and escaping the degradation through lysosomal system[161]. This mechanism triggers a cascade of biological effects leading to cell stress and innate immune response, with interleukin-15 as a major mediator[164]. IL15, in turn, causes the expansion and the switch toward the natural-killer phenotype of IEL, thus inducing epithelial apoptosis and IFN-γ production[145]. Moreover it increases the paracellular permeability, favouring the gliadin passage into the

mucosa and thus amplifying all the involved mechanisms[165, 166]. Other cytokines reported to be important in the innate response to the gluten are IL-8, IL-18, IL-21 and TNF-α[167, 168].

Several studies have also suggested that toll-like receptors (TLRs) may play a role in the CeD pathogenesis, although their precise function has not been found yet. TLRs activation normally induces production of pro-inflammatory cytokines and chemokines that can help maintaining the innate immune response. Moreover, it alters the epithelial barrier permeability, thus increasing immunogenic molecules passage. Increased expression of TLR2, TLR4 and TLR9 have been observed in the duodenal mucosa of patients with CeD[169, 170].

Adaptive immunity

Although several T-cell stimulatory gluten epitopes have been identified, the 33-mer peptide is considered the most immunogenic, since its sequence includes six overlapping epitopes[171]. Interestingly, 33-mer peptide is only partially degraded during intracellular transport , passing into the sierosal compartment almost intact[161]. Once in the lamina propria, after being deamidated by TG2, it binds HLA-DQ2/DQ8 molecules on APC and activate the CD4+ T-lymphocytes. This step is crucial for the activation of the adaptive immune response toward the T-helper 1 pattern[172]. It has also been postulated the role of epithelial cells in the antigenic presentation of gliadin peptides to the T cell, fact sustained by the DQ-gliadin

complexes found into the late endosomes compartment[173, 174]. T-cell activation drives, in turn, the production of autoantibodies against deamidated gliadin itself and TG2. Moreover, activated T-cells produce several cytokines, particularly interferon-γ (IFN-γ) which is considered the key cytokine in CeD[172]. IFN-γ plays several roles in CeD pathogenesis: it induces alteration of the epithelial barrier[175]; together with TNF-α stimulates intestinal fibroblasts to proliferate and secrete metalloproteases (MMP) increasing the mucosal damage; it increases TG2 expression[176].

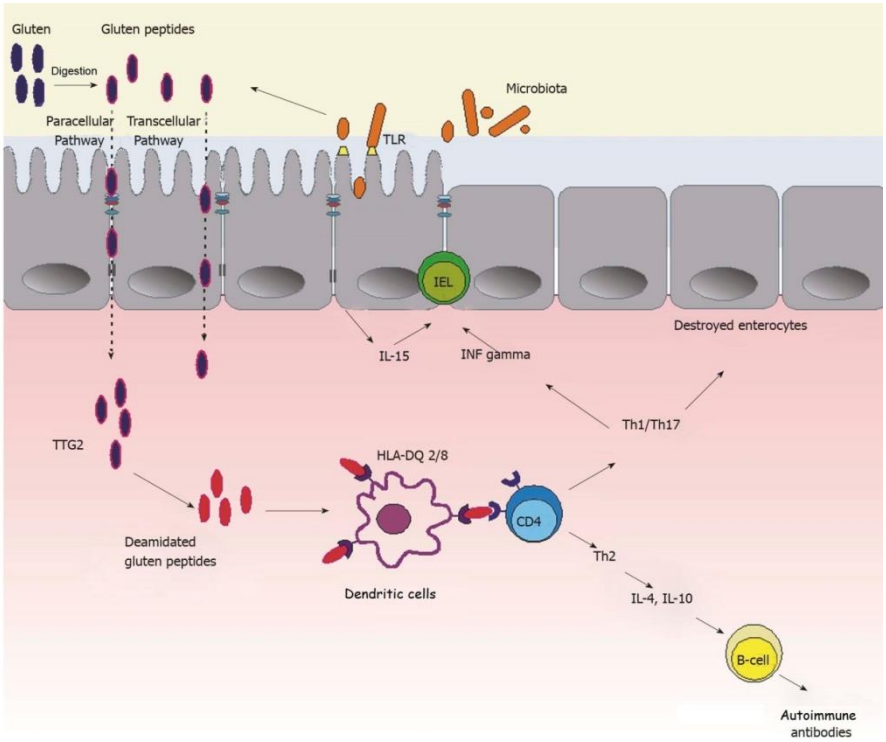

Figure 5: Schematic illustration of Celiac Disease pathogenesis. Modified from Cukrowska et al., 2017.

Role of the intestinal barrier in CeD pathogenesis

Despite their resistance to proteolytical enzymes, gliadin peptides have to cross the epithelial barrier to activate the immune cells located in the lamina propria. As above described, TJs principal role is to act as a gate for the paracellular transport of molecules. When they are functionally intact, only small molecules with a molecular weight (MW) of approximately 600Da can diffuse through them, while the majority passes through the transcellular pathway (transported by cellular mechanisms).

Association between genetic polymorfisms in region encoding for TJs-associated protein and CeD as well as alteration of the TJs complexes in CeD subjects have been reported[16, 64]. These findings raised the hypothesis that a defect in the gastrointestinal barrier and the consequent leakage of gliadin into the mucosa, might be the initial step in CeD pathogenesis. To explain the mechanism, Fasano at al.[177] proposed the action of the zonulin protein (now identified as prehaptoglobin-214), a eukaryotic analogue of the Vibrio cholerae toxin. They reported that this protein can reduce the transepithelial electrical resistance (TEER) in intestinal cells, increase the permeability to the lactulose-mannitol molecule (marker of paracellular transport) and that it is overexpressed in CeD patients[130, 178]. However, the MW of p31-49 and 33-mer peptides (2245 and 3900 Da) are approximately 10-fold higher than that of the paracellular marker (342 Da) used by these authors. Therefore, some doubts about the route of entry still remain, and they are sustained

by the well documented capacity of gliadin peptides to cross the intestinal barrier through the transcellular way[162, 179].

Anyway, several other factors may induce a primary gastrointestinal barrier dysfunction. It has in fact been postulated that an imbalance of the gut permeability in genetically predisposed subjects, caused by external factors, could anticipate the onset of the CeD[65, 180].

SCOPE OF THE THESIS

Lerner et al.[65] reported a parallel increment of both food additives use in the agro/food chain and autoimmune diseases frequency in the recent years. This finding made them think that the food additives could have a role in the increasing incidence of these disorders (particularly referring to intestinal epithelial barrier impairment).

Celiac Disease is today considered a common autoimmune intestinal disorder and its rate is expected to increase in the next years. A possible reason is the presence of new environmental factors, that have not yet been well identified. In this sense, as I described in the introduction, dietary metallic nanoparticles may act at several levels in the CeD pathogenesis:

- they could directly interact with the gliadin peptides and change their molecular structures;
- mNP could induce intestinal barrier dysfunction, both directly (effects on TJ expression and rearrangements) and indirectly (enterocytes cytotoxic effects);
- they could alter the immune (both innate and adaptive) response to the gliadin peptides.

Given these premises, the scope of my project was to assess, for the first time, the possible role that dietary mNP could play in the development of Celiac Disease.

To reach this aim, the work performed during this PhD course was:

- to assess possible molecular interactions between digested gliadin and the most used dietary mNP (AuNP, AgNP, ZnONP, TiO_2NP);
- to study the effects of dietary mNP, combined or not with the digested gliadin, on the intestinal Caco-2 cell line. We particularly evaluated both cytotoxic effects and possible alterations of the intestinal barrier;
- to also use an ex vivo model to evaluate mNP effects in presence of the complexity of the intestinal mucosa in vivo. We used duodenal biopsies from both healthy and celiac (under gluten-free diet from at least 1 year) patients and assessed the expression of various genes, including the main cytokines involved in CeD pathogenesis.

In the literature it is widely reported that mNP can affect cells in a different way according to their differentiative status. We therefore decided to use both post-confluent/differentiated and undifferentiated Caco-2 cells. Although post-confluent Caco-2 cells are well representative of normal differentiated enterocytes, undifferentiated Caco-2 cells are significantly different from normal crypt-like intestinal cells[181]. For this reason:

- we also performed experiments with the normal human intestinal epithelial crypt-like (HIEC) cell line, recently isolated by Menard et al.[182];
- we used small fetal intestine (mid-gestation) in organ culture to better represent the villous intestinal architecture. In this

latter case we particularly focused on the possible architectural changes induced by our stimulations.

References

1. Arora S, Sharma P, Kumar S, Nayan R, Khanna PK, Zaidi MGH. Gold-nanoparticle induced enhancement in growth and seed yield of Brassica juncea. Plant Growth Regulation 2012;66:303-310.
2. Yang J, Jiang F, Ma C, Rui Y, Rui M, Adeel M, Cao W, Xing B. Alteration of Crop Yield and Quality of Wheat upon Exposure to Silver Nanoparticles in a Life Cycle Study. Journal of Agricultural and Food Chemistry 2018;66:2589-2597.
3. Kumar V, Guleria P, Kumar V, Yadav SK. Gold nanoparticle exposure induces growth and yield enhancement in Arabidopsis thaliana. Science of The Total Environment 2013;461-462:462-468.
4. Prosa M, Tessarolo M, Bolognesi M, Margeat O, Gedefaw D, Gaceur M, Videlot-Ackermann C, Andersson MR, Muccini M, Seri M, Ackermann J. Enhanced Ultraviolet Stability of Air-Processed Polymer Solar Cells by Al Doping of the ZnO Interlayer. ACS Applied Materials & Interfaces 2016;8:1635-1643.
5. Verleysen E, Van Doren E, Waegeneers N, De Temmerman PJ, Abi Daoud Francisco M, Mast J. TEM and SP-ICP-MS Analysis of the Release of Silver Nanoparticles from Decoration of Pastry. Journal of Agricultural and Food Chemistry 2015;63:3570-3578.
6. Athinarayanan J, Alshatwi AA, Periasamy VS, Al-Warthan AA. Identification of Nanoscale Ingredients in Commercial Food Products and their Induction of Mitochondrially Mediated Cytotoxic Effects on Human Mesenchymal Stem Cells. Journal of Food Science 2015;80:N459-N464.
7. Vance ME, Kuiken T, Vejerano EP, McGinnis SP, Hochella MF, Rejeski D, Hull MS. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein Journal of Nanotechnology 2015;6:1769-1780.
8. Fogli S, Montis C, Paccosi S, Silvano S, Michelucci E, Berti D, Bosi A, and PA, P R. Inorganic nanoparticles as potential regulators of immune response in dendritic cells. Nanomedicine 2017;12.

9. Ren X, Chen Y, Peng H, Fang X, Zhang X, Chen Q, Wang X, Yang W, Sha X. Blocking Autophagic Flux Enhances Iron Oxide Nanoparticles Photothermal Therapeutic Efficiency in Cancer Treatment. ACS Applied Materials & Interfaces 2018.
10. Lomer MCE, Thompson RPH, Powell JJ. Fine and ultrafine particles of the diet: influence on the mucosal immune response and association with Crohn's disease. Proceedings of the Nutrition Society 2002;61:123-130.
11. Buzea C, Pacheco II, Robbie K. Nanomaterials and nanoparticles: Sources and toxicity. Biointerphases 2007;2:MR17-MR71.
12. Groh KJ, Geueke B, Muncke J. Food contact materials and gut health: Implications for toxicity assessment and relevance of high molecular weight migrants. Food and Chemical Toxicology 2017;109:1-18.
13. Yang FM, Li HM, Li F, Xin ZH, Zhao LY, Zheng YH, Hu QH. Effect of Nano-Packing on Preservation Quality of Fresh Strawberry (Fragaria ananassa Duch. cv Fengxiang) during Storage at 4 °C. Journal of Food Science 2010;75:C236-C240.
14. Kim JS, Kuk E, Yu KN, Kim J-H, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang C-Y, Kim Y-K, Lee Y-S, Jeong DH, Cho M-H. Antimicrobial effects of silver nanoparticles. Nanomedicine: Nanotechnology, Biology and Medicine 2007;3:95-101.
15. Pereira DIA, Bruggraber SFA, Faria N, Poots LK, Tagmount MA, Aslam MF, Frazer DM, Vulpe CD, Anderson GJ, Powell JJ. Nanoparticulate iron(III) oxo-hydroxide delivers safe iron that is well absorbed and utilised in humans. Nanomedicine: Nanotechnology, Biology and Medicine 2014;10:1877-1886.
16. Schumann M, Siegmund B, Schulzke JD, Fromm M. Celiac Disease: Role of the Epithelial Barrier. Cellular and Molecular Gastroenterology and Hepatology 2017;3:150-162.
17. Nawrocka A, Cieśla J. Influence of silver nanoparticles on food components in wheat. International Agrophysics 2013;27:49-55.
18. Ruiz PA, Morón B, Becker HM, Lang S, Atrott K, Spalinger MR, Scharl M, Wojtal KA, Fischbeck-Terhalle A, Frey-Wagner I, Hausmann M, Kraemer T, Rogler G. Titanium dioxide

nanoparticles exacerbate DSS-induced colitis: role of the NLRP3 inflammasome. Gut 2017;66:1216-1224.
19. Powell JJ, Harvey RSJ, Ashwood P, Wolstencroft R, Gershwin ME, Thompson RPH. Immune Potentiation of Ultrafine Dietary Particles in Normal Subjects and Patients with Inflammatory Bowel Disease. Journal of Autoimmunity 2000;14:99-105.
20. Evans SM, Ashwood P, Warley A, Berisha F, Thompson RPH, Powell JJ. The role of dietary microparticles and calcium in apoptosis and interleukin-1β release of intestinal macrophages. Gastroenterology 2002;123:1543-1553.
21. Bettini S, Boutet-Robinet E, Cartier C, Coméra C, Gaultier E, Dupuy J, Naud N, Taché S, Grysan P, Reguer S, Thieriet N, Réfrégiers M, Thiaudière D, Cravedi J-P, Carrière M, Audinot J-N, Pierre FH, Guzylack-Piriou L, Houdeau E. Food-grade TiO2 impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. Scientific Reports 2017;7:40373.
22. Amenta V, Aschberger K, Arena M, Bouwmeester H, Botelho Moniz F, Brandhoff P, Gottardo S, Marvin HJP, Mech A, Quiros Pesudo L, Rauscher H, Schoonjans R, Vettori MV, Weigel S, Peters RJ. Regulatory aspects of nanotechnology in the agri/feed/food sector in EU and non-EU countries. Regulatory Toxicology and Pharmacology 2015;73:463-476.
23. EFSA Scientific Committee. Guidance of risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain: Part 1, human and animal health. EFSA Journal 2018.
24. Contado C. Nanomaterials in consumer products: a challenging analytical problem. Frontiers in Chemistry 2015;3.
25. Peters RJB, van Bemmel G, Herrera-Rivera Z, Helsper HPFG, Marvin HJP, Weigel S, Tromp PC, Oomen AG, Rietveld AG, Bouwmeester H. Characterization of Titanium Dioxide Nanoparticles in Food Products: Analytical Methods To Define Nanoparticles. Journal of Agricultural and Food Chemistry 2014;62:6285-6293.

26. Calzolai L, Gilliland D, Rossi F. Measuring nanoparticles size distribution in food and consumer products: a review. Food Additives & Contaminants: Part A 2012;29:1183-1193.
27. RCC RCC. Nanotechnology Initiative- Final Report- Work Element 3: Risk Assessment/Risk Management. http://science.gc.ca/eic/site/063.nsf/vwapj/nano3_e.pdf/$file/nano3_e.pdf 2014.
28. Lichtenstein D, Ebmeyer J, Meyer T, Behr A-C, Kästner C, Böhmert L, Juling S, Niemann B, Fahrenson C, Selve S, Thünemann AF, Meijer J, Estrela-Lopis I, Braeuning A, Lampen A. It takes more than a coating to get nanoparticles through the intestinal barrier in vitro. European Journal of Pharmaceutics and Biopharmaceutics 2017;118:21-29.
29. Karlsson HL, Cronholm P, Hedberg Y, Tornberg M, De Battice L, Svedhem S, Wallinder IO. Cell membrane damage and protein interaction induced by copper containing nanoparticles—Importance of the metal release process. Toxicology 2013;313:59-69.
30. McShan D, Ray PC, Yu H. Molecular toxicity mechanism of nanosilver. Journal of Food and Drug Analysis 2014;22:116-127.
31. Bellmann S, Carlander D, Fasano A, Momcilovic D, Scimeca JA, Waldman WJ, Gombau L, Tsytsikova L, Canady R, Pereira DIA, Lefebvre DE. Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2015;7:609-622.
32. Nel AE, Mädler L, Velegol D, Xia T, Hoek EMV, Somasundaran P, Klaessing F, Castranova V, Thompson M. Understanding biophysicochemical interactions at the nano–bio interface. Nature Materials 2009;8.
33. Lynch I, Dawson KA. Protein-nanoparticle interactions. Nano Today 2008;3:40-47.
34. Xu Z, Liu X-W, Ma Y-S, Gao H-W. Interaction of nano-TiO2 with lysozyme: insights into the enzyme toxicity of nanosized particles. Environmental Science and Pollution Research 2009;17:798-806.

35. Linse S, Cabaleiro-Lago C, Xue WF, Lynch I, Lindman S, Thulin E, Radford SE, Dawson KA. Nucleation of protein fibrillation by nanoparticles. Proceedings of the National Academy of Sciences 2007;104:8691-8696.
36. Mirsadeghi S, Dinarvand R, Ghahremani MH, Hormozi-Nezhad MR, Mahmoudi Z, Hajipour MJ, Atyabi F, Ghavami M, Mahmoudi M. Protein corona composition of gold nanoparticles/nanorods affects amyloid beta fibrillation process. Nanoscale 2015;7:5004-5013.
37. Abdelkhaliq A, van der Zande M, Punt A, Helsdingen R, Boeren S, Vervoort JJM, Rietjens IMCM, Bouwmeester H. Impact of nanoparticle surface functionalization on the protein corona and cellular adhesion, uptake and transport. Journal of Nanobiotechnology 2018;16.
38. Yao M, He L, McClements DJ, Xiao H. Uptake of Gold Nanoparticles by Intestinal Epithelial Cells: Impact of Particle Size on Their Absorption, Accumulation, and Toxicity. Journal of Agricultural and Food Chemistry 2015;63:8044-8049.
39. Hanley C, Thurber A, Hanna C, Punnoose A, Zhang J, Wingett DG. The Influences of Cell Type and ZnO Nanoparticle Size on Immune Cell Cytotoxicity and Cytokine Induction. Nanoscale Research Letters 2009;4:1409-1420.
40. Imai S, Morishita Y, Hata T, Kondoh M, Yagi K, Gao J-Q, Nagano K, Higashisaka K, Yoshioka Y, Tsutsumi Y. Cellular internalization, transcellular transport, and cellular effects of silver nanoparticles in polarized Caco-2 cells following apical or basolateral exposure. Biochemical and Biophysical Research Communications 2017;484:543-549.
41. Sokolov SV, Tschulik K, Batchelor-McAuley C, Jurkschat K, Compton RG. Reversible or Not? Distinguishing Agglomeration and Aggregation at the Nanoscale. Analytical Chemistry 2015;87:10033-10039.
42. Peters R, Kramer E, Oomen AG, Rivera ZEH, Oegema G, Tromp PC, Fokkink R, Rietveld A, Marvin HJP, Weigel S, Ad A.C.M. P, Bouwmeester H. Presence of Nano-Sized Silica during In Vitro Digestion of Foods Containing Silica as a Food Additive. ACS Nano 2012;6:2441-51.

43. Walczak AP, Fokkink R, Peters R, Tromp P, Herrera Rivera ZE, Rietjens IMCM, Hendriksen PJM, Bouwmeester H. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology 2012;7:1198-1210.
44. Sieg H, Kästner C, Krause B, Meyer T, Burel A, Böhmert L, Lichtenstein D, Jungnickel H, Tentschert J, Laux P, Braeuning A, Estrela-Lopis I, Gauffre F, Fessard V, Meijer J, Luch A, Thünemann AF, Lampen A. Impact of an Artificial Digestion Procedure on Aluminum-Containing Nanomaterials. Langmuir 2017;33:10726-10735.
45. McCracken C, Zane A, Knight DA, Dutta PK, Waldman WJ. Minimal Intestinal Epithelial Cell Toxicity in Response to Short- and Long-Term Food-Relevant Inorganic Nanoparticle Exposure. Chemical Research in Toxicology 2013;26:1514-1525.
46. Albanese A, Chan WCW. Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity. ACS Nano 2011;5:5478.
47. Huang D, Zhou H, Gao J. Nanoparticles modulate autophagic effect in a dispersity-dependent manner. Scientific Reports 2015;5.
48. Wang Y, Yuan L, Yao C, Ding L, Li C, Fang J, Sui K, Liu Y, Wu M. A combined toxicity study of zinc oxide nanoparticles and vitamin C in food additives. Nanoscale 2014;6:15333-15342.
49. Cao Y, Roursgaard M, Kermanizadeh A, Loft S, Møller P. Synergistic Effects of Zinc Oxide Nanoparticles and Fatty Acids on Toxicity to Caco-2 Cells. International Journal of Toxicology 2015;34:67-76.
50. Martirosyan A, Bazes A, Schneider Y-J. In vitrotoxicity assessment of silver nanoparticles in the presence of phenolic compounds – preventive agents against the harmful effect? Nanotoxicology 2014;8:573-582.
51. Martirosyan A, Grintzalis K, Polet M, Laloux L, Schneider Y-J. Tuning the inflammatory response to silver nanoparticles via quercetin in Caco-2 (co-)cultures as model of the human intestinal mucosa. Toxicology Letters 2016;253:36-45.

52. Walczak AP, Kramer E, Hendriksen PJM, Helsdingen R, van der Zande M, Rietjens IMCM, Bouwmeester H. In vitrogastrointestinal digestion increases the translocation of polystyrene nanoparticles in anin vitrointestinal co-culture model. Nanotoxicology 2015;9:886-894.
53. Lichtenstein D, Ebmeyer J, Knappe P, Juling S, Böhmert L, Selve S, Niemann B, Braeuning A, Thünemann AF, Lampen A. Impact of food components during in vitro digestion of silver nanoparticles on cellular uptake and cytotoxicity in intestinal cells. Biological Chemistry 2015;396.
54. Di Silvio D, Rigby N, Bajka B, Mackie A, Baldelli Bombelli F. Effect of protein corona magnetite nanoparticles derived from bread in vitro digestion on Caco-2 cells morphology and uptake. The International Journal of Biochemistry & Cell Biology 2016;75:212-222.
55. Helander HF, Fändriks L. Surface area of the digestive tract – revisited. Scandinavian Journal of Gastroenterology 2014;49:681-689.
56. Atuma C, Strugala V, Allen A, Holm L. The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. Am J Physiol Gastrointest Liver Physiol 2001;280:G922-G929.
57. Schneeberger EE, Lynch RD. The tight junction: a multifunctional complex. Am J Physiol Cell Physiol 2004;286:C1213-1228.
58. Hartsock A, Nelson WJ. Adherens and tight junctions: Structure, function and connections to the actin cytoskeleton. Biochimica et Biophysica Acta (BBA) - Biomembranes 2008;1778:660-669.
59. Cornes JS. Number, size, and distribution of Peyer's patches in the human small intestine. Part I The development of Peyer's patches. Gut 1965;6.
60. Pearson C, Uhlig HH, Powrie F. Lymphoid microenvironments and innate lymphoid cells in the gut. Trends in Immunology 2012;33:289-296.
61. Lundquist P, Artursson P. Oral absorption of peptides and nanoparticles across the human intestine: Opportunities,

limitations and studies in human tissues. Advanced Drug Delivery Reviews 2016;106:256-276.
62. Sahay G, Alakhova DY, Kabanov AV. Endocytosis of nanomedicines. Journal of Controlled Release 2010;145:182-195.
63. Turner JR. Intestinal mucosal barrier function in health and disease. Nature Reviews Immunology 2009;9:799-809.
64. Wapenaar MC, Monsuur AJ, van Bodegraven AA, Weersma RK, Bevova MR, Linskens RK, Howdle P, Holmes G, Mulder CJ, Dijkstra G, van Heel DA, Wijmenga C. Associations with tight junction genes PARD3 and MAGI2 in Dutch patients point to a common barrier defect for coeliac disease and ulcerative colitisAn unusual case of ascites. Gut 2007;57:463-467.
65. Lerner A, Matthias T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. Autoimmunity Reviews 2015;14:479-489.
66. Brun E, Barreau F, Veronesi G, Fayard B, Sorieul S, Chanéac C, Carapito C, Rabilloud T, Mabondzo A, Herlin-Boime N, Carriére M. Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia. particle and Fibre Toxicology 2014;11.
67. Williams KM, Gokulan K, Cerniglia CE, Khare S. Size and dose dependent effects of silver nanoparticle exposure on intestinal permeability in an in vitro model of the human gut epithelium. Journal of Nanobiotechnology 2016;14.
68. Koeneman BA, Zhang Y, Westerhoff P, Chen Y, Crittenden JC, Capco DG. Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. Cell Biology and Toxicology 2010;26:225-238.
69. Sheth P, Basuroy S, Li C, Naren AP, Rao RK. Role of Phosphatidylinositol 3-Kinase in Oxidative Stress-induced Disruption of Tight Junctions. Journal of Biological Chemistry 2003;278:49239-49245.
70. Berg TO, Fengsrud M, Strømhaug PE, Berg T, Seglen PO. Isolation and Characterization of Rat Liver Amphisomes: EVIDENCE FOR FUSION OF AUTOPHAGOSOMES WITH BOTH

EARLY AND LATE ENDOSOMES. The Journal of Biological Chemistry 1998;273:21883-21892.
71. Fu PP, Xia Q, Hwang H-M, Ray PC, Yu H. Mechanisms of nanotoxicity: Generation of reactive oxygen species. Journal of Food and Drug Analysis 2014;22:64-75.
72. Yang E-J, Kim S, Kim JS, Choi I-H. Inflammasome formation and IL-1β release by human blood monocytes in response to silver nanoparticles. Biomaterials 2012;33:6858-6867.
73. Zhang J, Zou Z, Wang B, Xu G, Wu Q, Zhang Y, Yuan Z, Yang X, Yu C. Lysosomal deposition of copper oxide nanoparticles triggers HUVEC cells death. Biomaterials 2018;161:228-239.
74. Stern ST, Adiseshaiah PP, Crist RM. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Particle and Fibre Toxicology 2012;9:20.
75. Reggiori F, Ungermann C. Autophagosome Maturation and Fusion. Journal of Molecular Biology 2017;429:486-496.
76. Ding W-X, Yin X-M. Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. Autophagy 2014;4:141-150.
77. Li JJ, Hartono D, Ong C-N, Bay B-H, Yung L-YL. Autophagy and oxidative stress associated with gold nanoparticles. Biomaterials 2010;31:5996-6003.
78. Luo Y-H, Chang LW, Lin P. Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications. BioMed Research International 2015;2015:1-12.
79. Borgognoni CF, Kim JH, Zucolotto V, Fuchs H, Riehemann K. Human macrophage responses to metal-oxide nanoparticles: a review. Artificial Cells, Nanomedicine, and Biotechnology 2018:1-10.
80. Cui Y, Liu H, Zhou M, Duan Y, Li N, Gong X, Hu R, Hong M, Hong F. Signaling pathway of inflammatory responses in the mouse liver caused by TiO2 nanoparticles. Journal of Biomedical Materials Research Part A 2011;96A:221-229.
81. Turley SJ, Inaba K, Garret WS, Ebersold M, Unternaehrer J, Steinman RM, Mellman I. Transport of Peptide–MHC Class II Complexes in Developing Dendritic Cells. Science 2000;288:522-27.

82. Schanen BC, Karakoti AS, Seal S, III DRD, Warren WL, Self WT. Exposure to Titanium Dioxide Nanomaterials Provokes Inflammation of an in Vitro Human Immune Construct. ACS Nano 2009;3:2523-2532.
83. Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnology Advances 2009;27:76-83.
84. Wang ZL. FUNCTIONAL OXIDE NANOBELTS: Materials, Properties and Potential Applications in Nanosystems and Biotechnology. Annual Review of Physical Chemistry 2004;55:159-196.
85. Chaudhry Q, Scotter M, Blackburn J, Ross B, Boxall A, Castle L, Aitken R, Watkins R. Applications and implications of nanotechnologies for the food sector. Food Additives & Contaminants: Part A 2008;25:241-258.
86. Artiaga G, Ramos K, Ramos L, Cámara C, Gómez-Gómez M. Migration and characterisation of nanosilver from food containers by AF4-ICP-MS. Food Chemistry 2015;166:76-85.
87. Echegoyen Y, Nerín C. Nanoparticle release from nano-silver antimicrobial food containers. Food and Chemical Toxicology 2013;62:16-22.
88. von Goetz N, Fabricius L, Glaus R, Weitbrecht V, Günther D, Hungerbühler K. Migration of silver from commercial plastic food containers and implications for consumer exposure assessment. Food Additives & Contaminants: Part A 2013;30:612-620.
89. EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food).Scientific opinion on the re-evaluation of silver (E 174) as food additive. EFSA Journal 2016;14:4364.
90. Mehmood A. Brief overview of the application of silver nanoparticles to improve growth of crop plants. IET Nanobiotechnology 2018;12:701-705.
91. Razzaq A, Ammara R, Jhanzab HM, Mahmood T, Hafeez A, Hussain S. A Novel Nanomaterial to Enhance Growth and Yield of Wheat. Journal of Nanoscience and Technology 2016;2:55-58.

92. Karimi J, Mohsenzadeh S. Physiological Effects of Silver Nanoparticles and Silver Nitrate Toxicity in Triticum aestivum. Iranian Journal of Science and Technology, Transactions A: Science 2017;41:111-120.
93. Duhan JS, Kumar R, Kumar N, Kaur P, Nehra K, Duhan S. Nanotechnology: The new perspective in precision agriculture. Biotechnology Reports 2017;15:11-23.
94. Dasary SSR, Rai US, Yu H, Anjaneyulu Y, Dubey M, Ray PC. Gold nanoparticle based surface enhanced fluorescence for detection of organophosphorus agents. Chemical Physics Letters 2008;460:187-190.
95. EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food).Scientific Opinion on the re-evaluation of gold (E 175) as a food additive. EFSA Journal 2016;14:4362.
96. Commission Regulation (EU) No 231/2012 of 9 March 2012 laying down specifications for food additives listed in Annexes II and III to Regulation (EC) No 1333/2008 of the European Parliament and of the Council (Text with EEA relevance). https ://publi catio ns.europ a.eu/s/etMb.
97. Yang Y, Doudrick K, Bi X, Hristovski K, Herckes P, Westerhoff P, Kaegi R. Characterization of Food-Grade Titanium Dioxide: The Presence of Nanosized Particles. Environmental Science & Technology 2014;48:6391-6400.
98. Rompelberg C, Heringa MB, van Donkersgoed G, Drijvers J, Roos A, Westenbrink S, Peters R, van Bemmel G, Brand W, Oomen AG. Oral intake of added titanium dioxide and its nanofraction from food products, food supplements and toothpaste by the Dutch population. Nanotoxicology 2016;10:1404-1414.
99. Weir A, Westerhoff P, Fabricius L, Hristovski K, von Goetz N. Titanium Dioxide Nanoparticles in Food and Personal Care Products. Environmental Science & Technology 2012;46:2242-2250.
100. EFSA ANS Panel (EFSA Panel on Food Additives and Nutrient Sources added to Food). Re-evaluation of titanium dioxide (E 171) as a
food additive. EFSA Journal 2016;14:e04545. .

101. Chen Z, Wang Y, Ba T, Li Y, Pu J, Chen T, Song Y, Gu Y, Qian Q, Yang J, Jia G. Genotoxic evaluation of titanium dioxide nanoparticles in vivo and in vitro. Toxicology Letters 2014;226:314-319.
102. Wang Y, Chen Z, Ba T, Pu J, Chen T, Song Y, Gu Y, Qian Q, Xu Y, Xiang K, Wang H, Jia G. Susceptibility of Young and Adult Rats to the Oral Toxicity of Titanium Dioxide Nanoparticles. Small 2013;9:1742-1752.
103. Winkler HC, Notter T, Meyer U, Naegeli H. Critical review of the safety assessment of titanium dioxide additives in food. Journal of Nanobiotechnology 2018;16.
104. Jalal R, Goharshadi EK, Abareshi M, Moosavi M, Yousefi A, Nancarrow P. ZnO nanofluids: Green synthesis, characterization, and antibacterial activity. Materials Chemistry and Physics 2010;121:198-201.
105. Xie Y, He Y, Irwin PL, Jin T, Shi X. Antibacterial Activity and Mechanism of Action of Zinc Oxide Nanoparticles againstCampylobacter jejuni. Applied and Environmental Microbiology 2011;77:2325-2331.
106. Padmavathy N, Vijayaraghavan R. Enhanced bioactivity of ZnO nanoparticles—an antimicrobial study. Science and Technology of Advanced Materials 2016;9:035004.
107. Emamifar A, Kadivar M, Shahedi M, Soleimanian-Zad S. Evaluation of nanocomposite packaging containing Ag and ZnO on shelf life of fresh orange juice. Innovative Food Science & Emerging Technologies 2010;11:742-748.
108. Anitha S, Brabu B, Thiruvadigal DJ, Gopalakrishnan C, Natarajan TS. Optical, bactericidal and water repellent properties of electrospun nano-composite membranes of cellulose acetate and ZnO. Carbohydrate Polymers 2013;97:856-863.
109. Li JH, Hong RY, Li MY, Li HZ, Zheng Y, Ding J. Effects of ZnO nanoparticles on the mechanical and antibacterial properties of polyurethane coatings. Progress in Organic Coatings 2009;64:504-509.
110. EFSA. Safety assessment of the substance zinc oxide, nanoparticles, for use in food contact materials. EFSA Journal 2016;14.

111. Trynka G, Hunt KA, Bockett NA, Romanos J, Mistry V, Szperl A, Bakker SF, Bardella MT, Bhaw-Rosun L, Castillejo G, de la Concha EG, de Almeida RC, Dias K-RM, van Diemen CC, Dubois PCA, Duerr RH, Edkins S, Franke L, Fransen K, Gutierrez J, Heap GAR, Hrdlickova B, Hunt S, Izurieta LP, Izzo V, Joosten LAB, Langford C, Mazzilli MC, Mein CA, Midah V, Mitrovic M, Mora B, Morelli M, Nutland S, Núñez C, Onengut-Gumuscu S, Pearce K, Platteel M, Polanco I, Potter S, Ribes-Koninckx C, Ricaño-Ponce I, Rich SS, Rybak A, Santiago JL, Senapati S, Sood A, Szajewska H, Troncone R, Varadé J, Wallace C, Wolters VM, Zhernakova A, Thelma BK, Cukrowska B, Urcelay E, Bilbao JR, Mearin ML, Barisani D, Barrett JC, Plagnol V, Deloukas P, Wijmenga C, van Heel DA. Dense genotyping identifies and localizes multiple common and rare variant association signals in celiac disease. Nature Genetics 2011;43:1193-1201.
112. Coleman C, Quinn EM, Ryan AW, Conroy J, Trimble V, Mahmud N, Kennedy N, Corvin AP, Morris DW, Donohoe G, O'Morain C, MacMathuna P, Byrnes V, Kiat C, Trynka G, Wijmenga C, Kelleher D, Ennis S, Anney RJL, McManus R. Common polygenic variation in coeliac disease and confirmation of ZNF335 and NIFA as disease susceptibility loci. European Journal of Human Genetics 2015;24:291-297.
113. Taylor TB, Schmidt LA, Meyer LJ, Zone JJ. Transglutaminase 3 Present in the IgA Aggregates in Dermatitis Herpetiformis Skin Is Enzymatically Active and Binds Soluble Fibrinogen. Journal of Investigative Dermatology 2015;135:623-625.
114. Freeman HJ. Iron deficiency anemia in celiac disease. World Journal of Gastroenterology 2015;21:9233.
115. Hadjivassiliou M, Aeschlimann P, Sanders DS, Mäki M, Kaukinen K, Grünewald RA, Bandmann O, Woodroofe N, Haddock G, Aeschlimann DP. Transglutaminase 6 antibodies in the diagnosis of gluten ataxia. Neurology 2013;80:1740-45.
116. Marsh MN, Johnson MW, Rostami K. Mucosal histopathology in celiac disease: a rebuttal of Oberhuber's sub-division of Marsh III. Gastroenterol Hepatolo Bed Bench 2015;8:99-109.
117. Marsh M, Heal C. Evolutionary Developments in Interpreting the

Gluten-Induced Mucosal Celiac Lesion: An Archimedian Heuristic. Nutrients 2017;9:213.
118. Marsh MN. Gluten, major histocompatibility complex, and the small intestine: A molecular and immunobiologic approach to the spectrum of gluten sensitivity ('celiac sprue'). Gastroenterology 1992;102:330-54.
119. Lebwohl B, Ludvigsson JF, Green PHR. The Unfolding Story of Celiac Disease Risk Factors. Clinical Gastroenterology and Hepatology 2014;12:632-635.
120. Megiorni F, Mora B, Bonamico M, Barbato M, Nenna R, Maiella G, Lulli P, Mazzilli MC. HLA-DQ and risk gradient for celiac disease. Human Immunology 2009;70:55-59.
121. Rampertab SD, Pooran N, Brar P, Singh P, Green PHR. Trends in the Presentation of Celiac Disease. The American Journal of Medicine 2006;119:355.e9-355.e14.
122. Mustalahti K, Catassi C, Reunanen A, Fabiani E, Heier M, McMillan S, Murray L, Metzger M-H, Gasparin M, Bravi E, Mäki M. The prevalence of celiac disease in Europe: Results of a centralized, international mass screening project. Annals of Medicine 2010;42:587-595.
123. Fasano A, Berti I, Gerarduzzi T, al. e. Prevalence of Celiac Disease in At-Risk and Not-At-Risk Groups in the United States: A large multicenter study. Arch Intern Med 2003;163:286-92.
124. Rubio-Tapia A, Kyle RA, Kaplan EL, Johnson DR, William P, Erdtmann F, Brantner TL, Kim WR, Phelps TK, Lahr BD, Zinsmeister AR, Melton LJ, Murray JA. Increased Prevalence and Mortality in Undiagnosed Celiac Disease. Gastroenterology 2009;137:88-93.
125. Lohi S, Mustalahti K, Kaukinen K, Laurila K, Collin P, Rissanen H, Lohi O, Bravi E, Gasparin M, Reunanen A, MÄKi M. Increasing prevalence of coeliac disease over time. Alimentary Pharmacology & Therapeutics 2007;26:1217-1225.
126. Lerner A, Arleevskaya M, Schmiedl A, Matthias T. Microbes and Viruses Are Bugging the Gut in Celiac Disease. Are They Friends or Foes? Frontiers in Microbiology 2017;8.
127. Stene LC, Honeyman MC, Hoffenberg EJ, Haas JE, Sokol RJ, Emery L, Taki I, Norris JM, Erlich HA, Eisenbarth GS, Rewers M.

Rotavirus Infection Frequency and Risk of Celiac Disease Autoimmunity in Early Childhood: A Longitudinal Study. The American Journal of Gastroenterology 2006;101:2333-2340.
128. Caminero A, Nistal E, Herrán AR, Pérez-Andrés J, Ferrero MA, Vaquero Ayala L, Vivas S, Ruiz de Morales JMG, Albillos SM, Casqueiro FJ. Differences in gluten metabolism among healthy volunteers, coeliac disease patients and first-degree relatives. British Journal of Nutrition 2015;114:1157-1167.
129. Caminero A, Galipeau HJ, McCarville JL, Johnston CW, Bernier SP, Russell AK, Jury J, Herran AR, Casqueiro J, Tye-Din JA, Surette MG, Magarvey NA, Schuppan D, Verdu EF. Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. Gastroenterology 2016;151:670-683.
130. Drago S, El Asmar R, Di Pierro M, Clemente MG, Tripathi A, Sapone A, Thakar M, Iacono G, Carroccio A, D'Agate C, Not T, Zampini L, Catassi C, Fasano A. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. Scandinavian Journal of Gastroenterology 2006;41:408-419.
131. Ivarsson A, Persson L, Nystrom L, Ascher H, Cavell B, Danielsson L, Dannaeus A, Lindberg T, Lindquist B, Stenhammar L, Hernell O. Epidemic of coeliac disease in Swedish children. Acta Paediatrica 2000;89:165-71.
132. Lionetti E, Castellaneta S, Francavilla R, Pulvirenti A, Tonutti E, Amarri S, Barbato M, Barbera C, Barera G, Bellantoni A, Castellano E, Guariso G, Limongelli MG, Pellegrino S, Polloni C, Ughi C, Zuin G, Fasano A, Catassi C. Introduction of Gluten, HLA Status, and the Risk of Celiac Disease in Children. New England Journal of Medicine 2014;371:1295-1303.
133. Vriezinga SL, Auricchio R, Bravi E, Castillejo G, Chmielewska A, Crespo Escobar P, Kolaček S, Koletzko S, Korponay-Szabo IR, Mummert E, Polanco I, Putter H, Ribes-Koninckx C, Shamir R, Szajewska H, Werkstetter K, Greco L, Gyimesi J, Hartman C, Hogen Esch C, Hopman E, Ivarsson A, Koltai T, Koning F, Martinez-Ojinaga E, te Marvelde C, Pavic A, Romanos J, Stoopman E, Villanacci V, Wijmenga C, Troncone R, Mearin

ML. Randomized Feeding Intervention in Infants at High Risk for Celiac Disease. New England Journal of Medicine 2014;371:1304-1315.
134. Kuja-Halkola R, Lebwohl B, Halfvarson J, Wijmenga C, Magnusson PKE, Ludvigsson JF. Heritability of non-HLA genetics in coeliac disease: a population-based study in 107 000 twins. Gut 2016;65:1793-1798.
135. Karell K, Louka AS, Moodie SJ, Ascher H, Clot F, Greco L, Ciclitira PJ, Sollid LM, Partanen J. Hla types in celiac disease patients not carrying the DQA1*05-DQB1*02 (DQ2) heterodimer: results from the european genetics cluster on celiac disease. Human Immunology 2003;64:469-477.
136. Wieser H. Chemistry of gluten proteins. Food Microbiology 2007;24:115-119.
137. Urade R, Sato N, Sugiyama M. Gliadins from wheat grain: an overview, from primary structure to nanostructures of aggregates. Biophysical Reviews 2017;10:435-443.
138. De Re V, Caggiari L, Tabuso M, Cannizzaro R. The versatile role of gliadin peptides in celiac disease. Clinical Biochemistry 2013;46:552-560.
139. Junker Y, Zeissig S, Kim S-J, Barisani D, Wieser H, Leffler DA, Zevallos V, Libermann TA, Dillon S, Freitag TL, Kelly CP, Schuppan D. Wheat amylase trypsin inhibitors drive intestinal inflammation via activation of toll-like receptor 4. The Journal of Experimental Medicine 2012;209:2395-2408.
140. Donnelly SC, Šuligoj T, Ellis HJ, Ciclitira PJ. Identification of coeliac disease triggering glutenin peptides in adults. Scandinavian Journal of Gastroenterology 2016;51:819-826.
141. Perez-Gregorio MR, Días R, Mateus N, de Freitas V. Identification and characterization of proteolytically resistant gluten-derived peptides. Food & Function 2018;9:1726-1735.
142. Mamone G, Ferranti P, Rossi M, Roepstorff P, Fierro O, Malorni A, Addeo F. Identification of a peptide from α-gliadin resistant to digestive enzymes: Implications for celiac disease. Journal of Chromatography B 2007;855:236-241.
143. Shan L, Molberg Ø, Parrot I, Hausch F, Filiz F, Gray GM, Sollid LM, Khosla C. Structural Basis for Gluten Intolerance in Celiac Sprue. Science 2002;297:2275-79.

144. Maiuri L, Ciacci C, Ricciardelli I, Vacca L, Raia V, Auricchio S, Picard J, Osman M, Quaratino S, Londei M. Association between innate response to gliadin and activation of pathogenic T cells in coeliac disease. The Lancet 2003;362:30-37.
145. Maiuri L, Ciacci C, Auricchio S, Brown V, Quaratino S, Londei M. Interleukin 15 mediates epithelial changes in celiac disease. Gastroenterology 2000;119:996-1006.
146. Klöck C, DiRaimondo TR, Khosla C. Role of transglutaminase 2 in celiac disease pathogenesis. Seminars in Immunopathology 2012;34:513-522.
147. Molberg Ø, Mcadam SN, Korner R, Quarsten H, Kristiansen C, Madsen L, Fugger L, Scott H, Norén O, Roepstorff P, Lundin KEA, Sjostrom H, Sollid LM. Tissue transglutaminase selectively modifies gliadin peptides that are recognized by gut-derived T cells in celiac disease. Nature Medicine 1998;4:713-17.
148. Sollid LM, Jabri B. Celiac disease and transglutaminase 2: a model for posttranslational modification of antigens and HLA association in the pathogenesis of autoimmune disorders. Current Opinion in Immunology 2011;23:732-738.
149. Barone MV, Nanayakkara M, Paolella G, Maglio M, Vitale V, Troiano R, Ribecco MTS, Lania G, Zanzi D, Santagata S, Auricchio R, Troncone R, Auricchio S. Gliadin Peptide P31-43 Localises to Endocytic Vesicles and Interferes with Their Maturation. PLoS ONE 2010;5:e12246.
150. Luciani A, Villella VR, Vasaturo A, Giardino I, Pettoello-Mantovani M, Guido S, Cexus ON, Peake N, Londei M, Quaratino S, Maiuri L. Lysosomal accumulation of gliadin p31-43 peptide induces oxidative stress and tissue transglutaminase-mediated PPAR downregulation in intestinal epithelial cells and coeliac mucosa. Gut 2009;59:311-319.
151. Rossin F, D'Eletto M, Macdonald D, Farrace MG, Piacentini M. TG2 transamidating activity acts as a reostat controlling the interplay between apoptosis and autophagy. Amino Acids 2011;42:1793-1802.

152. D'Eletto M, Farrace MG, Rossin F, Strappazzon F, Giacomo GD, Cecconi F, Melino G, Sepe S, Moreno S, Fimia GM, Falasca L, Nardacci R, Piacentini M. Type 2 transglutaminase is involved in the autophagy-dependent clearance of ubiquitinated proteins. Cell Death & Differentiation 2012;19:1228-1238.
153. D'Eletto M, Grazia Farrace M, Falasca L, Reali V, Oliverio S, Melino G, Griffin M, Fimia GM, Piacentini M. Transglutaminase 2 is involved in autophagosome maturation. Autophagy 2009;5:1145-1154.
154. Tamai K, Tanaka N, Nara A, Yamamoto A, Nakagawa I, Yoshimori T, Ueno Y, Shimosegawa T, Sugamura K. Role of Hrs in maturation of autophagosomes in mammalian cells. Biochemical and Biophysical Research Communications 2007;360:721-727.
155. Manil-Segalén M, Lefebvre C, Culetto E, Legouis R. Need an ESCRT for autophagosomal maturation? Communicative & Integrative Biology 2014;5:566-571.
156. Comincini S, Manai F, Meazza C, Pagani S, Martinelli C, Pasqua N, Pelizzo G, Biggiogera M, Bozzola M. Identification of Autophagy-Related Genes and Their Regulatory miRNAs Associated with Celiac Disease in Children. International Journal of Molecular Sciences 2017;18:391.
157. Manai F, Azzalin A, Gabriele F, Martinelli C, Morandi M, Biggiogera M, Bozzola M, Comincini S. The In Vitro Effects of Enzymatic Digested Gliadin on the Functionality of the Autophagy Process. International Journal of Molecular Sciences 2018;19:635.
158. Rajaguru P, Vaiphei k, Saikia B, Kochhar R. Increased accumulation of dendritic cells in celiac disease associates with increased expression of autophagy protein LC3. Indian journal of Pathology and Microbiology 2013;56:342-48.
159. Abadie V, Discepolo V, Jabri B. Intraepithelial lymphocytes in celiac disease immunopathology. Semin Immunopathol 2012;34:551-66.
160. Skovbjerg H, Koch C, Anthonsen D, Sjöström H. Deamidation and cross-linking of gliadin peptides by transglutaminases and the relation to celiac disease. Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease 2004;1690:220-230.

161. Ménard S, Lebreton C, Schumann M, Matysiak-Budnik T, Dugave C, Bouhnik Y, Malamut G, Cellier C, Allez M, Crenn P, Schulzke JD, Cerf-Bensussan N, Heyman M. Paracellular versus Transcellular Intestinal Permeability to Gliadin Peptides in Active Celiac Disease. The American Journal of Pathology 2012;180:608-615.
162. Barone MV, Zimmer KP. Endocytosis and transcytosis of gliadin peptides. Molecular and Cellular Pediatrics 2016;3.
163. Paolella G, Lepretti M, Martucciello S, Nanayakkara M, Auricchio S, Esposito C, Barone MV, Caputo I. The toxic alpha-gliadin peptide 31-43 enters cells without a surface membrane receptor. Cell Biology International 2018;42:112-120.
164. Barone M, Troncone R, Auricchio S. Gliadin Peptides as Triggers of the Proliferative and Stress/Innate Immune Response of the Celiac Small Intestinal Mucosa. International Journal of Molecular Sciences 2014;15:20518-20537.
165. Clemente MG, De Virgiliis S, Kang JS, Macatagney R, Musu MP, Di Pierro M, Drago S, Congia A, Fasano A. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. Gut 2003;52:218-223.
166. Matysiak-Budnik T, Candalh C, Dugave C, Namane A, Cellier C, Cerf-Bensussan N, Heyman M. Alterations of the intestinal transport and processing of gliadin peptides in celiac disease. Gastroenterology 2003;125:696-707.
167. Garrote JA, Gçmez-Gonzàlez E, Brenardo D, Arranz E, Chirdo F. Celiac Disease Pathogenesis: The Proinflammatory Cytokine Network. Journal of Pediatric Gastroenterology and Nutrition 2008;47:S27-S32.
168. Jelínková L, Tučková L, Cinová J, Flegelová Z, Tlaskalová-Hogenová H. Gliadin stimulates human monocytes to production of IL-8 and TNF-α through a mechanism involving NF-κB. FEBS Letters 2004;571:81-85.
169. Szebeni B, Veres G, Dezsofi A, Rusai K, Vannay A, Bokodi G, Vasarhelyi B, Korponay-Szabó IR, Tulassay T, Arato A. Increased Mucosal Expression of Toll-like Receptor TLR2 and TLR4 in Coeliac Disease. Journal of Pediatric Gastroenterology and Nutrition 2007;45:187-93.

170. Ghasiyari H, Rostami-Nejad M, Amani D, Rostami K, Pourhoseingholi MA, Asadzadeh-Aghdaei H, Zali MR. Diverse Profiles of Toll-Like Receptors 2, 4, 7, and 9 mRNA in Peripheral Blood and Biopsy Specimens of Patients with Celiac Disease. Journal of Immunology Research 2018;2018:1-8.
171. Silano M, Vincentini O, De Vincenzi M. Toxic, Immunostimulatory and Antagonist Gluten Peptides in Celiac Disease. Current Medicinal Chemistry 2009;16:1489-98.
172. Nilsen EM, Lundin KEA, Krajci P, Scott H, Sollid LM, Brandtzaeg P. Gluten specific, HLA-DQ restricted T cells from coeliac mucosa produce cytokines with Thl or ThO profile dominated by interferon y. Gut 1995;37:766-776.
173. Zimmer K-P, de Laffolie J, Barone MV, Naim HY. Endocytosis in enterocytes. Wiener Medizinische Wochenschrift 2016;166:205-210.
174. Hatano R, Yamada K, Iwamoto T, Maeda N, Emoto T, Shimizu M, Totsuka M. Antigen presentation by small intestinal epithelial cells uniquely enhances IFN-γ secretion from CD4+ intestinal intraepithelial lymphocytes. Biochemical and Biophysical Research Communications 2013;435:592-596.
175. Du L, Kim JJ, Shen J, Dai N. Crosstalk between Inflammation and ROCK/MLCK Signaling Pathways in Gastrointestinal Disorders with Intestinal Hyperpermeability. Gastroenterology Research and Practice 2016;2016:1-9.
176. Bayardo M, Punzi F, Bondar C, Chopita N, Chirdo F. Transglutaminase 2 expression is enhanced synergistically by interferon-γ and tumour necrosis factor-α in human small intestine. Clinical & Experimental Immunology 2012;168:95-104.
177. Fasano A, Not T, Wang W, Uzzau S, Berti I, Tommasini A, Goldblum SE. Zonulin, a newly discovered modulator of intestinal permeability, and its expression in coeliac disease. The Lancet 2000;355:1518-1519.
178. Tripathi A, Lammers KM, Goldblum S, Shea-Donohue T, Netzel-Arnett S, Buzza MS, Antalis TM, Vogel SN, Zhao A, Yang S, Arrietta MC, Meddings JB, Fasano A. Identification of human zonulin, a physiological modulator of tight junctions,

as prehaptoglobin-2. Proceedings of the National Academy of Sciences 2009;106:16799-16804.
179. Matysiak-Budnik T, Moura IC, Arcos-Fajardo M, Lebreton C, Ménard S, Candalh C, Ben-Khalifa K, Dugave C, Tamouza H, van Niel G, Bouhnik Y, Lamarque D, Chaussade S, Malamut G, Cellier C, Cerf-Bensussan N, Monteiro RC, Heyman M. Secretory IgA mediates retrotranscytosis of intact gliadin peptides via the transferrin receptor in celiac disease. The Journal of Experimental Medicine 2008;205:143-154.
180. Fasano A. Leaky Gut and Autoimmune Diseases. Clinical Reviews in Allergy & Immunology 2012;42:71-78.
181. Tremblay E, Auclair J, Delvin E, Levy E, Ménard D, Pshezhetsky AV, Rivard N, Seidman EG, Sinnett D, Vachon PH, Beaulieu J-F. Gene expression profiles of normal proliferating and differentiating human intestinal epithelial cells: A comparison with the Caco-2 cell model. Journal of Cellular Biochemistry 2006;99:1175-1186.
182. Ménard D, Arsenault P. Explant Culture of Human Fetal Small Intestine. Gastroenterology 1985;86:691-700.

Chapter 2

Deleterious combined effect of dietary mNP and gliadin: the potential role in Celiac Disease pathogenesis

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Abstract

Background & Aims: The introduction of metallic nanoparticles (mNP) in the diet is a matter of concern for the human health. Particularly, their gastrointestinal toxicity and immunomodulatory properties may play a role in the worldwide increasing celiac disease (CeD) incidence. We evaluated the potential synergistic effects that the peptic-tryptic digested gliadin (PTG) and the most used mNP in the food sector may induce on the gastrointestinal tract.

Methods: UV-Vis spectra and TEM analyses were performed to assess the interaction between mNP and PTG**.** Cytotoxity (MTT and apoptosis assay) and epithelial integrity alterations (TEER, permeability of either [14C]-sucrose and [3H]-propranolol, TJ mRNA expression and protein distribution) caused by 4 different mNP (TiO_2-, ZnO-, Ag- and AuNP) with and without PTG were evaluated on Caco-2 intestinal cells. Inflammatory response to the same stimulations were assessed on both healthy/celiac human duodenal biopsies (IFN-γ, IL-15, IL-8, TLR2 and TLR4).

Results: PT-gliadin interaction with mNP and their consequent aggregation were observed, particularly with Ag- and Au-NP. In vitro experiments proved a synergistic cytotoxic effect of PTG and mNP, particularly with Ag- and TiO_2NP. Similar effects were obtained in the monolayer integrity experiments. A significant cytokines expression increment was induced only in duodenal biopsies from celiac patients, particularly with the combination of Au-, Ag- and TiO_2-NP with the PTG.

Conclusion: Our data proved an interaction and a synergistic effect of PTG with mNP. The results suggest that the mNP, recently introduced in the food sector, may represent an important environmental risk factor for the development of the CeD.
Keywords: Celiac Disease, metal nanoparticles, food additives, nanotoxicity,

Introduction

An increasing interest in the nanomaterial world has been recently observed in several fields. These materials, like nanoparticles and their aggregates, present new exciting chemo-physical properties compared to their macro-counterparts that are useful in a wide range of applications. Nanomedicine[1, 2] is certainly the principal field of application, but they are also used to create sustainable energy[3], improve agriculture[4-6] and in the cosmetic and food sector[7-9]. Therefore, consciously or not, a large amount of nanoparticles comes into contact with our body.

Although the literature regarding the toxicity and safety of NP has exponentially increased in the recent years, results are often controversial and a complete understanding of the NP effects on the human health is still lacking. It is in fact known that the interactions between NP and biological systems are quite complex involving several factors such as the size[10], shape and surface properties of NPs, cell types[11, 12], but also the physico-chemical properties of the surrounding environment[13]. Thus, NP theoretically safe could became dangerous in a complex environment like the gastrointestinal tract, leading to unwanted physiological consequences[14]. Here the necessity to perform studies in which the NP are associated with food components, situation more representative of the use of NP as food additives[15, 16].

Metallic nanoparticles were enlisted as the most abundant category of nanomaterials entering through the ingestion route in the

Nanotechnology Consumer Product Inventory[9] .Different types are used in the food sector, but mostly TiO_2NP, a food colouring agent, ZnO- and Ag-NP used for their antimicrobial effects in food contact materials, and AuNP to improve plant yield and growth. Although studies on the gastrointestinal tract are scanty, it has been shown that mNP can alter intestinal homeostasis and permeability[17-20], fact that could increase the passage of immunogenic molecules in the lamina propria and in turn, trigger the immune system. This could be relevant in patients with autoimmune disorders, such as those with intestinal bowel disease[21] (IBD) or celiac disease (CeD)[22]. Celiac disease is a common chronic enteropathy[23] and its incidence is rising worldwide[24]. It develops in genetically predisposed individuals (HLA-DQ2 or HLA-DQ8 haplotypes) after the ingestion of gluten, the principal environmental trigger factor present in wheat and other cereals. The gluten peptides have to reach the lamina propria, where they are deamidated by the tissue transglutaminase 2, loaded onto antigen-presenting cells and then recognized by T cells. Even if the HLA-DQ2 is present in about 30% of the Caucasian population and the gluten is a common component of the western diet, only 2-5% of these subjects develop the disease, indicating that these factors alone are not sufficient. Therefore, in the recent years, several additional etiological factors have been proposed, like viral infections[25, 26], impaired commensal homeostasis[27, 28], increasing permeability[29] and a leaky gastrointestinal barrier. The introduction of mNP into the human diet may thus represent one of the

environmental factors increasing the percentage of genetically susceptible individuals developing this disease.

In this paper, we demonstrated that mNP (Au-, Ag- ZnO- and TiO_2-NP) can interact with gliadin and affect the intestinal barrier homeostasis in a in vitro system. We also detected their capacity to activate the immune system ex vivo, in duodenal biopsies from celiac (under gluten free diet) patients.

Methods

Peptic-tryptic digested gliadin

The digestion procedure was performed as described by Frazer et al.30, with some modifications. In brief, gliadin (Sigma-Aldrich, Milan, Italy) was incubated with pepsin in 0.1 M HCl, pH 1.8, and stirred at 37°C for 4 h. pH was adjusted to 7.8 and trypsin digestion (substrate/enzyme ratio of 200:1 for both reactions) was performed at 37°C for 4 h with vigorous agitation. Adjustment of the pH to 4.5 resulted in a precipitate, which was removed by centrifugation. To inhibit the residual enzymatic activity both N-tosyl-l-phenylalanine chloromethyl ketone and N- α-tosyl-l-lysine chloromethyl ketone hydrochloride (Sigma-Aldrich) were used. Then, the peptic-tryptic digested gliadin (PTG) was dialyzed against 10 mM ammonium carbonate, pH 7.8 (molecular mass exclusion 1000 Da) overnight, sterile filtered, and lyophilized. The resultant powder was dissolved in sterile water and stored at -20° C.

UV-Vis spectra and Transmission Electronic Microscope (TEM)

AgNP (40nm diameter), TiO_2NP (<25nm diameter, anatase) and ZnONP (<100nm diameter) were purchesed from Sigma-Aldrich. AuNP (15nm diameter) were obtained from Cytodiagnostics (Burlington, Canada). E171 whitening agent was purchased from an Italian commercial supplier of food colouring. UV-Vis spectra were acquired after 30 minutes of mNP incubation with/without PTG or BSA with Spectra max PLUS 384 spectrophotometer (Molecular

Devices, San Jose, CA). The measured spectral range was initially 200-700 nm with an interval of 10 nm, then a shorter range, based on the characteristic SPR wavelength of each mNP, was measured using 2 nm intervals. For TEM analyses, Ag- and Au- NP were sonicated for 1 minute, then incubated with/without PTG or BSA for 30 minutes. A small drop of their suspension was deposited onto carbon-coated copper grids and allowed to dry at room temperature. Images were obtained using HITACHI H-7500 transmission electron microscope (Tokyo, Japan) and analyzed using ImageJ software. The surface area of NP/aggregate was used to calculate the radius, diameter and the three dimensional area. The number of NP in every cluster was obtained dividing these values for the three dimensional area of one NP, considering 15 nm as AuNP's diameter, and 40nm the AgNP's diameter (as datasheet specifics). Aggregates were considered all the clusters with 2 or more NP. For each conditions, three separate experiment were performed, and at least 3 images for each experiment analyzed with Image J.

Cell viability and apoptosis assays

Caco-2 cells (Istituto Sieroterapico, Bergamo, Italy) were cultured in complete DMEM supplemented with 100 IU/ml penicillin/ 2mM L-Glutamine and 10% fetal bovine serum at 37°C (Euroclone, Milan, Italy) in a 5% CO2 atmosphere. For all the viability assays, undifferentiated cells were grown until 80-90% confluence and post confluent cells until domes were formed, then stimulations with mNP+PTG were performed. For MTT assay, after 6/24h of

stimulation, 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenytetrasolium bromide (Sigma) was added at 0,5mg/ml concentration and incubated for 2h at 37°C in a 5% CO2 atmosphere. The formed formazan salts were dissolved in EtOH 100% and the plates read at 570 nm with Microplate Reader 550 (Bio-Rad, Segrate, Italy). At least 8 separate experiments were performed for each condition. CellTox™ Green Cytotoxicity Assay (Promega, Milano, Italy) was used to determine the appropriate time for measuring the transient caspase activity. For the Caspase-Glo® 3/7 assay (Promega), cells were treated for just 6h at 37°C in a 5% CO2 atmosphere, the luminogenic substrate was added, the plate incubated at room temperature for 2h and read with the Tecan Infinite® 200 PRO plate reader (Mannendorf, Switzerland).

Transepithelial electrical resistence (TEER) and Apparent Permeability

For the permeability experiments, Caco-2 cells were seeded on 12-well transwell polycarbonate inserts (0,4um porous) with a density of 16,5 × 10^3 cells/insert. Cells were periodically inspected and the transepithelial electrical resistance (TEER) monitored with STX2 electrode Epithelial Volt-Ohm meter (World Precision Instruments, Sarasota, FL). Only inserts with TEER ≥ 250Ω/cm^2 were used. The quality of the monolayer was also tested by measuring the ratio between the apparent paracellular permeability (P_{App}) of [14C]-sucrose and the transcellular P_{App} of [3H]-propranolol. The transport of these probes from the upper to lower chamber was determined by

adding 0.045 µCi of [14C]-sucrose and 0.045 µCi [3H]-propranolol to the upper chamber. Then, after 150 minutes, 100 µL samples were taken from the lower chamber and the P_{App} was determined as: "$Papp=dQ/dt \cdot 1/C_0A$" , where *dQ/dt* is the transport of the probes as a function of time, C_0 is the initial probes concentration and *A* the superficial area of each insert. For each conditions, at least three separate experiments were performed.

Immunofluorescence

Caco-2 cells were plated on 35 mm collagen-coated glass-bottom dishes, and treated at 4 days postconfluence. After 24h of stimulation, cells were fixed in methanol for 10 min at −20°C, washed three times with high salt buffer (10 min each) and incubated overnight at 4°C with Anti-ZO1 (Cat. 402200, INVITROGEN) or Anti-OCLN (Cat. 331588, INVITROGEN) primary antibodies in BSA 1X or BSA 1X with 0,1% saponine, respectively. Cells incubated with Anti-ZO1 Ab, were then washed and treated with the secondary antibody goat anti-rabbit Alexa Fluor 568 (Abcam, Cambridge, UK) diluted in GDB, at room temperature for 1 h. One micromolar of 4',6-diamidino-2-phenylindole (DAPI) in PBS was used to stain cell nuclei (5 min). Images were acquired by an inverted confocal microscope (ZEISS).

Patients

Duodenal biopsy specimens were collected from healthy subjects (n.15) and celiac patients (n.26 on gluten-free diet from at least 1

year), treated in vitro with mNP and/or PTG for 4 h at 37 °C and at 5% CO_2, frozen in liquid nitrogen and maintained at −80 °C until RNA extraction. Only data from biopsies with histological Marsh score 0-1 were considered. The study was approved by the ethics committee of the IRCCS-Ospedale Maggiore, Milan, Italy and informed consents were obtained from all patients.

RNA extraction and RT-qPCR

Total RNA was extracted from cells and biopsies using the MiRcury RNA Isolation Kit (Exiqon, Vedbaek, Denmark) following the manufacturer's instructions. RNA quality analyses and quantification was performed by NanoDrop 1000 Spectrophotometer (Thermo Scientific, Waltham, MA). Reverse Transcription Reagents kit (Applied Biosystems) was used with random primers to obtain cDNAs, and TaqMan Gene Expression Assay (Applied Biosystem) were used for gene expression studies. Probes were chosen to be intron spanning to avoid co-amplification of genomic DNA, and no signal was detected when un-retrotranscribed RNA was used as template. qPCR was performed using 7900HT Fast Real-Time PCR System (Applied Biosystems-Life Technologies, Carlsbad, CA, USA). For all analyses, each sample was examined in triplicate. All data were normalized using HPRT1(Hypoxanthine Phosphoribosyltransferase 1) and relative expression assessed by the $2^{-\Delta\Delta Ct}$ method using an external control.

Statistical analyses

ANOVA and Student's t paired test was performed for the comparison of the data. ANOVA on ranks followed by Dunn post hoc test was used when data failed the equal variance test. The significance level was set at p value<0.05. Statistical evaluation was performed with the SYSTAT software package (SPSS, Chicago, IL).

Results

Food nanoparticles interactions with digested PT-gliadin

UV-Vis spectra

All the mNP exhibit a characteristic maximum absorbance, called surface plasmon resonances (SPR), which changes depending on the size, shape and surface alterations of the NP such as when an interaction with proteins (protein corona formation) occurs[31]. We analyzed the UV-Vis spectra of the mNP either alone or after 30 minute of incubation with PTG or BSA protein (known for its interaction with several NPs). As expected, we observed a shift in the maxima SPR absorbance after 30min incubation of the BSA protein with Ag-, ZnO- and TiO_2-NP, but not with Au-NP (Figure 1). Interestingly our data showed a stronger interaction between all the NP and PTG, as indicated by the red shift of 48nm, 29nm, 9nm and 22nm obtained after 30 minutes incubation with Ag-, Au- and ZnO- and TiO_2-NP, respectively. The strong shift, additional maximal absorbance and broadening of the curves are also consistent with NP aggregation. In addition, after Ag- and Au-NP incubation with PTG,

we observed a color change (data not shown), further indication of mNP aggregation[32]. To be sure that the interaction involved the gliadin peptides and not the inactivated trypsin and pepsin still present in the preparation, we also employed PTG filtered with a 10kDa cut-off membrane (able to separate the peptides from the enzymes), confirming our results (data not shown).

TEM analyses

Since we observed the biggest shift for the Au- and Ag-NP when combined with the PT-gliadin, we used the transmission electron microscope (TEM) technique to better characterize the suggested aggregation of these mNPs. As expected, the mean particles diameter measured was 15,371±3,025 nm and 44,358±8,061 nm for Au- and Ag-NP, respectively. We only observed an high rate of aggregation, i.e.> 90%, after 30 minutes of incubation of both Au- and Ag-NP with PTG (92 and 99%, respectively), but not with BSA (aggregation rate 49% for Au-NP and 41% for Ag-NP). We also used mNP alone, and they shown a basic rate of aggregation of about 25%. These data confirmed a strong interaction between Au- and Ag-NP with PTG, leading to the formation of big aggregates with diameters up to 248 nm and 569 nm respectively (Figure 2).

Cellular viability

Regarding the cytotoxic effects, we first tested several mNP concentrations at both 6h and 24h, on 80-90% confluent CaCo2 cells. MTT assay was used to evaluate the dose-response effect (Figure S1); for further experiments only mNP concentrations that didn't induce

more than 40% of mitochondrial dysfunction (AuNP: 12,5-25ug/ml; AgNP: 2,5-5ug/ml; TiO_2NP: 50-100ug/ml; ZnONP: 10-25ug/ml) were used, in order to assess the possible additive effect of PT-gliadin (0,5-1 mg/ml). No effects were observed after 6h stimulations (Figure S2-Panel A), therefore 24h incubation time was chosen for further experiments. Since a different sensibility to the mNP, based on cell differentiation, had been demonstrated[33, 34], both undifferentiated and post confluent Caco-2 intestinal cells were evaluated using MTT assay. The obtained data showed a concentration-dependent cell viability reduction, more evident when 80-90% confluent cells were employed. In the undifferentiated Caco-2 cells (Figure 3- Panel A) a significant reduction in cell viability was observed after stimulations with the higher concentrations of either gliadin or Au- and Ag-NPs alone ($P<0,01$ vs control), but worse effects were detected when NPs were combined with gliadin ($P<0,001$ vs control). Only TiO_2NP did not induce a response either alone or in combination. When post-confluent cells were analyzed (Figure 3-Panel B), a significant reduction in the cell viability was observed after Ag- and TiO_2NP treatments in combination with PTG ($P<0,05$ vs control). Different results were observed after stimulations with ZnONP, that seems to have opposite effects on undifferentiated and post-confluent cells showing a reduction and an increment, respectively, of the cell mitochondrial activity. Since it has been demonstrated that mNP can interfere with colorimetric assays[35], noncellular tests[36] were also run, with null or negligible signal (data not shown).

After this, we evaluated if the apoptosis mechanism was involved in the cell viability reduction. Caspases activation is a transient event, therefore we first investigated changes in the cell membrane integrity occurring as a result of cell death. Since data showed the beginning of cell death 6 hours after stimulations (Figure S2-Panel B), we used this time point to assess apoptosis activation. We observed a significant increment of the caspases 3/7 activation in both undifferentiated (Figure 3- Panel C) and post-confluent Caco-2 cells (Figure 3- Panel D) after stimulations with the PTG at the higher concentration (P-value<0,01 vs ctr). Moreover, data showed a more profound apoptosis activation when gliadin was combined with Ag- and TiO_2NP (P-value<0,001 vs ctr).

Collectively, the data obtained suggest that the food mNP have an additive effect on the toxicity exerted by the PTG on both undifferentiated and post-confluent Caco-2 cells.

Gastrointestinal barrier impairment

In the development of celiac disease, an important role could be played by agents able to cause an intestinal barrier dysfunction. Caco-2 cells were seeded on transwell filters until their differentiation (17 days) and the monolayer integrity assessed by measuring both the transepithelial electrical resistance (TEER) and the permeability of either ^{14}C-sucrose and ^{3}H-propranolol, probes for the paracellular and transcellular pathways, respectively. Data showed a significant TEER reduction (P<0,05 vs control) after 4h and 6h of stimulation with both PTG and mNP alone or in combination

(Figure 4- Panel A). Anyway only the combination of AgNP with PTG induced an increment(P<0,05 vs control) of the paracellular permeability (Figure 4- Panel B). We also measured the transcellular passage of radiolabeled propranolol, but no alterations were observed (unpublished data).

To assess if tight junction alterations could play a role in the proved gastrointestinal barrier impairment, we evaluated the mRNA level of occludin (OCLN) and zonula occludens-1 (ZO-1) in post-confluent Caco-2 cells. Cells were treated for 24h with the higher concentration of mNP with or without PT-gliadin, but no alterations were detected (Figure 4- Panel C). We also studied the possible protein rearrangements through Immunofluorescence. The signals of both Zonula Occludens-1(Figure 5) and Occludin (Figure 6) showed an initial membrane ruffling after all the stimulations compared to the untreated cells (control). Furthermore, we observed an alteration of the intracellular trafficking of the occludin protein, particularly after stimulations with Ag- and TiO_2-NP either alone or in combination with PT-gliadin .

E171 food colouring agent vs TiO_2 NPs

Several crystalline forms of TiO_2 exist in the food colouring agent E171[37]. Thus, to confirm our findings, we also investigated the potential toxicity exerted by the commercial food colouring agent E171.

UV-Vis spectra

As expected, the UV-Vis spectra of the commercial E171 showed a different peak compared to the TiO_2NP (Figure S3-Panel E), suggesting a different composition. It must be considered that we didn't observe a really well defined peak with both TiO_2NP and E171 alone, making the considerations about the red shift not accurate. Anyway the broadening of both curves clearly indicates the tendency to aggregate of both TiO_2NP and E171 only when combined with PTG.

Cell viability assays

Results obtained by MTT assay on undifferentiated cells indicate a similar cell viability reduction after stimulations with TiO_2 or E171 (Figure S3- Panel A), despite their different composition. Interestingly an even stronger toxicity, exerted by E171, was detected on post-confluent Caco-2 cells (Figure 6 - Panel B), in fact data showed a significant cell viability reduction after stimulations with E171 either alone or combined with gliadin (P-value < 0,05 vs control). Even the apoptosis assay revealed that E171 induced a stronger toxicity than TiO_2NP, particularly on post-confluent Caco-2 cells (Figure S3 - Panel C and D). Here again, we observed a profound effects when the PTG was combined with the colouring agent.

TJ protein rearrangements

Lastly, we assessed the capacity of E171 to induce OCLN and ZO-1 protein rearrangements, like we observed after TiO_2NP + PT-gliadin stimulations. Results revealed an even worse effect after gliadin combination with E171, than with TiO_2NP. In fact, the data obtained suggest a disintegration of the occludin junctions after E171 plus

gliadin treatment (Figure S3 - Panel E), effect confirmed by the ZO-1 signal showing an initial separation of the cells (Figure S3 - Panel F).

Ex vivo analyses

In addition to the in vitro experiments, we also evaluated the potential effect of the interaction between mNPs and gliadin ex vivo, on duodenal biopsies from both celiac (under gluten-free diet) and healthy patients.

Apoptosis evaluation and TJ expression

The activation of the apoptosis pathway was assessed evaluating the ratio of the mRNA expression of BCL-2 (anti-apoptotic) and BAX (pro-apoptotic) genes. We observed a reduction, although not statistically significant, of the ratio BCL-2/BAX (index of apoptosis activation) after stimulations with the PTG alone or in combination with Ag- and Au-NP, but not with TiO_2- and ZnO-NP (Figure S4).

We also investigated the expression of ZO-1 and OCLN genes and, as expected, no alterations of their mRNA levels were induced after 4h of treatments (data not shown).

Cytokines and TLRs expression (Immune response evaluation)

To assess if mNP could also modify the immune system response to gliadin, we evaluated the mRNA amount of the most representative cytokines involved in the development of the celiac disease lesions; IFN-γ for the adaptive immune system, IL-15 and IL-8 for the innate immunity. We observed their increment only in duodenal biopsies from celiac patients under gluten-free diet, but not in those from healthy individuals. Particularly, results showed a significant increase

of IFN-γ expression after stimulations with PTG, Ag- and AuNP alone (P-value < 0,05 vs control), but an even stronger response was induced with their combination (P-value < 0,01 vs control) (Figure7-Panel A-B). Moreover, we observed a significant increment of the IL-15 mRNA amount after stimulations with Ag ± gliadin and when AuNPs were combined with PTG (P-value < 0,05 vs control). No alteration of the IL-8 level was detected after Au- or AgNP stimulation, while a significant increment was observed after treatments with PT-gliadin and TiO2-NP either alone or in combination (P-value < 0,05 vs control) (Figure7-Panel C). No alterations of cytokines expression were detected after 4h ZnO-NP stimulations (Figure7-Panel D).

To further evaluate innate immunity, TLR2 and TLR4 mRNA expression was investigated. Interestingly, results showed a statistically significant increase of TLR2 and TLR4 after treatments with TiO_2NP+PT and AgNP+PT, respectively (Figure S5, panel D and C, respectively). No alterations of their expression was observed after treatments with both Au- and ZnO-NPs alone or in combination with the gliadin.

Discussion

Several studies showed that the mNP can alter the intestinal microbiota composition[38], impair the gastrointestinal barrier permeability[10, 39] and induce immune modulation [17, 18, 20], variations which could have an important effects on patients with inflammatory bowel diseases[19, 21, 40]. Herein we continue in that direction, pointing to CeD as another possible intestinal disorder where the dietary intake of mNP could play a significant and deleterious role. Moreover our work is one of the few testing the mNP toxicity when associated with food components[7, 15, 16, 40-42].

We initially evaluated the potential interaction between mNP and PTG measuring the SPR of the nanoparticles after 30 minutes of incubation with and without PTG. The observed strong red shift proving a dielectric change at the surface of the NP is consistent with protein corona formation. Moreover, the broadening of the curves suggests that aggregation is occurring and an additional confirmation about AgNP and AuNP aggregation in presence of PTG was given by the solution colour change, even if further studies should be assessed to understand the nature of the chemical interactions involved. TEM experiments were performed to better characterize aggregates formation. We observed a fourfold increase in NP aggregation in presence of PTG compared to Au- and Ag-NP alone. To confirm that the interaction is specific for gliadin peptides and it doesn't occur with other proteins, we performed parallel experiments using bovine serum albumin, highly present in several culture media, but we didn't

observe such a strong interaction. Then, our results suggest that gliadin peptides can bind the surfaces of the mNP herein studied and induce their aggregation. Whether these aggregates remains, either in presence of other food components or digestion juices[43, 44] and if they pass the gastrointestinal barrier is something that needs to be estimated. Hypothetically, the binding of gluten peptides to NPs may bring an higher amount of PTG into the lamina propria[32]; NP can bind immunogenic molecules on their surfaces, carry them to the immune cells and increase their responsiveness[21] (hypothesis of the "Trojan horse"[22]).

The formation of a protein corona as well as the aggregation states of the mNP can change the cytotoxic effects of the mNP, as observed by Wang et al.[15] and Albanese et al.[32]. Our data confirmed these findings demonstrating that the single mNP induce a mild toxicity on Caco-2 cells, while a synergistic effect is clearly observed when they interact with the PTG. Particularly AgNP, at the lower concentration, induced a reduction of metabolic activity only when combined with the PTG in both undifferentiated and post-confluent Caco-2, AuNP induced the same effects but only in undifferentiated cells. TiO_2NP only induced some effects on post-confluent Caco-2 when combined with PTG, whereas stimulations with ZnONP showed a reduction of cell viability in undifferentiated cells and the opposite in post-confluent cells. These results are also in line with Hanley et al.[33], showing a different toxicity based on the cell proliferative status. This could be important not only considering the normal mucosa, but also in the case of celiac disease, since exposure of duodenal mucosa to gluten leads to a

situation of deepening of the crypts and hyperproliferation in an attempt to restore the normal intestinal architecture, thus changing the ratio of completely differentiated/immature cells.

Apoptosis is the main death mechanism involved in the celiac disease pathogenesis and it is induced by both the extrinsic and intrinsic pathway[45]. Therefore we evaluated caspases 3/7 activation (in vitro) and the Bcl2/Bax mRNA ratio (ex vivo), markers of the common apoptosis pathway.

Significant activation of apoptosis was observed in vitro after stimulations with Ag-, TiO_2- and ZnO-NP only when combined with the PTG. It must be underlined that also the single PTG induced a slight decrease in the cell viability, as demonstrated in others works[45, 46]. Conversely, we did not observe a significant variation in Bax/Bcl2 ratio ex vivo. This discrepancy could be explained by the different methodology or by the short incubation that was not enough to fully induce apoptosis. Anyway the absence of apoptosis corroborates the obtained results on the cytokines production confirming they are linked to the immune response and not to the tissue destruction.

From the literature it is clear that both gliadin[29, 47] and mNP[10, 39, 48] alone can impair the gastrointestinal barrier. Since this alteration may facilitate the gliadin passage from the intestinal lumen to the lamina propria, thus increasing the immune response, we evaluated whether the combination of PTG-mNP could get worse effects than the single component. Since Caco-2 cells at 17 days postconfluence could be not homogeneously polarized and differentiated[49], we based our experiments on the presence of TEER values above

250Ωcm^2 (regarded as an index of tight junction formation) before performing our stimulations. Moreover in all the experiments performed the ratio "P_{app} ^{3}H-propranolol/ P_{app} ^{14}C-sucrose" was > 1 in the untreated cells, value proving the integrity of the monolayer.

TEER values were significantly decreased starting from 4h after treatments, but no increased effect was observed when PTG and mNP were combined. Instead a significant increment of [14C]-sucrose paracellular permeability was induced only after stimulation with AgNP combined with PTG. The data obtained on paracellular permeability are not in line with the other works where the gliadin alone induces a much higher dysfunction of the barrier, such as in Sander et al.[47] work, in which a six-fold increase in the paracellular transport of the 4kDa FITC Dextran marker was registered after stimulation with the only digested gliadin.

To assess the role of TJ alterations we evaluated the mRNA level of the key proteins OCLN and ZO-1, but no alterations were detected, whereas TJ proteins redistribution was observed by immunofluorescence, with both proteins showing membrane ruffling after all the stimulations. Moreover we observed an increase of cytosolic punctate staining, consistent with an alteration of the intracellular vesicular trafficking of the occludin protein, particularly after treatment with Ag- and TiO_2NP both alone or combined with PTG. This pattern was slightly present after stimulation with the PTG alone, which is in line with the findings of Sander et al.[47] Our results on the monolayer integrity could appear somewhat controversial, anyway it has to be considered that while TEER was measured at 4

and 6h after stimulation, permeability, TJ expression and distribution were evaluated at 24h.

mNP size, composition and concentration are often controversial for E171[37, 50]. E171 composition is a mixture of anatase (usually the most elevated component) and rutile with P, Si and Al contaminants, moreover it presents an high distribution in size. Therefore we performed experiments also with the commercial food grade E171, to confirm our findings on TiO_2NP. Although UV-Vis spectra proved a different composition between the commercial E171 and TiO_2NP, both cytotoxic effects and TJ protein rearrangements after E171 exposure were even worse than the ones induced by TiO_2NP.

Metal nanoparticles can interact with the immune system[51], which represents a considerable potential in biomedical application, but could be harmful for NP present in the food. Various studies proved that dietary particles, particularly E171, could have a role in the IBD development. Ruiz et al.[40] performed the studies on DSS-induced ulcerative colitis mice, Powell et al.[21] and Evans et al.[19] on biopsy specimens from IBD patients showing an increased activation of the innate immune system in this condition. On the contrary, Bettini et al.[52] suggested that the E171 intake does not trigger IBD, but only induces a mild inflammatory response. However it has to be considered that they used healthy rats without biological potentiality to develop IBD . However, Crohn disease, Ulcerative Colitis and CeD are driven by an interplay of genetic and environmental factors, thus is possible that only models that mimic this complex situation needs to be used. Therefore we make sure to use an ex vivo system that

mimic subjects with the predisposition for developing CeD. Cytokines[53] and TLR[54] were selected as representative on the CeD; IFN-γ for evaluating the adaptive response, IL-15, IL-8, TLR2 and TLR4 to assess the innate response. Interestingly, an increment was observed only after stimulations of duodenal biopsies from CeD patients, but not from healthy subjects. Moreover the worse effects were induced after stimulation with the combination of mNP and PTG. AuNP specifically induced IL-15 and IFNG expression, Ag-NP treatments IL-15,IFNG and TLR4 while TiO_2NP increased IL-8, TLR2, but also slightly the IFNG levels. No alterations after ZnONP stimulation with or without PTG were observed. Particularly the IFNG increment, directly linked to the adaptive immune response, leads to hypothesize that the mNPs may promote the antigenic presentation of gliadin to the dendritic cells. Several works support our hypothesis: Schanen et al.[55] demonstrated that TiO2 nanoparticles induce dendritic cell (DC) maturation and prime naïve T cell activation and proliferation, Fogli et al.[1] showed as different NP core and coating can induce different responses in DC; Galbiati et al.[56] proved an immunostimulatory effect on THP-1 line cells and pheripheral blood monocytes. Therefore studies regarding dendritic cell and T cell activation and proliferation with the combination of mNP and PTG are recommended.

To the best of our knowledge, our work appears to be the first to document the synergy between food mNP and gliadin peptides, leading to the hypothesis that mNP could be one of the unknown factors playing a role in the increasing CeD incidence. What the

specific mechanisms involved are, whether the mNP lead to an higher gliadin passage into the lamina propria (representing the "Trojan horse[57]" and/or inducing a leak gastrointestinal barrier[14]) or they increase the immune cell responsiveness to the gliadin[58], needs to be further evaluated.

Conclusions

Despite the necessity of further studies, the diet ingestion of mNP could be deleterious for subject predisposed to develop the celiac disease. We observed a synergistic effect on both cytotoxicity and impairment of the epithelial barrier in vitro when cells were treated with the combination of PTG and mNP. More important, cytokines amount was strongly increased ex vivo after the same stimulations only in celiac patients, suggesting that although mNP could be not a risk for healthy patients, they probably are for celiac ones. Particularly mNP may be one of the cause of the increasing percentage of individuals developing this pathology among genetically susceptible ones.

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Figure Legends

Figure 1: *UV-Vis spectra of mNP*. The graphs report the Absorbance on the ordinate axis and the wavelength on the abscissas. **A)** AuNP spectra **B)** AgNP spectra **C)** TiO_2NP spectra **D)** ZnO spectra **E)** E171 spectra

Figure 1: *Nanoparticles TEM pictures, scatter plots and histograms of frequency, based on the NP diameter*. **A)** AuNP **B)** AuNP combined with BSA **C)** AuNP combined with PTG **D)** AgNP **E)** AgNP combined with BSA **F)** AgNP combined with PTG

Figure 2: *MTT and Caspases 3/7 activation Assay graphs*. The reported values are % of the average of the CTR of all the experiments performed. *p < 0,05; # p < 0,01; §p < 0,001. **A)** MTT on undifferentiated cells **B)** MTT on post-confluent cells **C)** Apoptosis assay on undifferentiated cells **D)** Apoptosys Assay on post-confluent cells.

Figure 4: *TEER, paracellular passage of ^{14}C-sucrose and TJ expression on CaCo2 cells.* **A)** Trans-epithelial electrical resistance (Ωcm^2) measured after 0h, 4h and 6h of stimulations. *p < 0,05; # p < 0,01; §p < 0,001. B) Apparent paracellular permeability of ^{14}C-sucrose after 24h of treatments. The values are expressed as % of the untreated cells. **C)** Box plots showing the mRNA levels of occludin (OCLN gene) and zonula occludens-1 (TJP1 gene) evaluated by qPCR.

Figure 5: *Immunofluorescence of Zonula Occludens-1.* For every condition we reported the overlay with DAPI (left panels) and the single ZO-1 signal. All the images were taken at 63x magnification.

Figure 6: *Immunofluorescence of Occludin.* For every condition we reported the overlay with DAPI (left panels) and the single OCLN signal. All the images were taken at 63x magnification.

Figure 7: *Cytokines expression after 4h stimulation of duodenal biopsies from healthy (right part of each graph) and CD patients under gluten-free diet (left part of each graph). *p < 0,05; # p < 0,01; §p < 0,001.* **A)** IFNG, IL15 and IL8 expression after stimulations with PT 1mg/ml ± AuNP 12,5/25 ug/ml **B)** IFNG, IL15 and IL8 expression after stimulations with PT 1mg/ml ± AgNP 2,5/5 ug/ml **C)** IFNG, IL15 and IL8 expression after stimulations with PT 1mg/ml ± TiO_2NP 50/100 ug/ml **D)** IFNG, IL15 and IL8 expression after stimulations with PT 1mg/ml ± ZnONP 10/25 ug/ml.

Supplementary Figure Legends

Figure S1: *Initial MTT Assay to test different mNP concentrations. The reported values are % of the average of the CTR of all the experiments performed.* **A)** 24h stimulations with AuNP ± PTG **B)** 24h stimulations with AgNP ± PTG **C)** 24h stimulations with ZnONP ± PTG **D)** 24h stimulations with TiO_2NP ± PTG.

Figure S2: **A)** MTT after 6h of treatments. The reported values are % of the average of the CTR of all the experiments performed. **B)** The membranes leakage at several time points was evaluated using CellTox™ Green Cytotoxicity Assay. To normalize the value we decided to use the t=0h measurements.

Figure S3: *E171 vs TiO_2NP*. A) MTT performed on undifferentiated CaCo2 cells. B) MTT performed on post-confluent CaCo2 cells. C)

Apoptosis Assay on undifferentiated CaCo2 cells D) Apoptosis Assay on post-confluent CaCo2 cells. E and F) Immunofluorescence of occludin and zonula oclludens-1, respectively. We only reported images of cells treated or not with the combinations: TiO_2NP (100ug/ml) +PTG (1mg/ml) and E171 (100ug/ml) + PTG (1mg/ml).

Figure S4: *Apoptosis index reported as ratio of the Fold Change* $2^{-\Delta\Delta Ct}$ *of BCL-2/BAX genes. The data referred to experiments performed on biopsy samples from CD patients under gluten free diet.* **A)** 4h stimulations with AuNP ± PTG **B)** 4h stimulations with AgNP ± PTG **C)** 4h stimulations with ZnONP ± PTG **D)** 4h stimulations with TiO_2NP ± PTG.

Figure S5: *TLR2 (panels on the left) and TLR4(panels on the right) genes expression (qPCR). The data referred to experiments performed on biopsy samples from CD patients under gluten free diet*. **A)** 4h stimulations with AuNP ± PTG **B)** 4h stimulations with AgNP ± PTG **C)** 4h stimulations with ZnONP ± PTG **D)** 4h stimulations with TiO_2NP ± PTG.

Figure 1

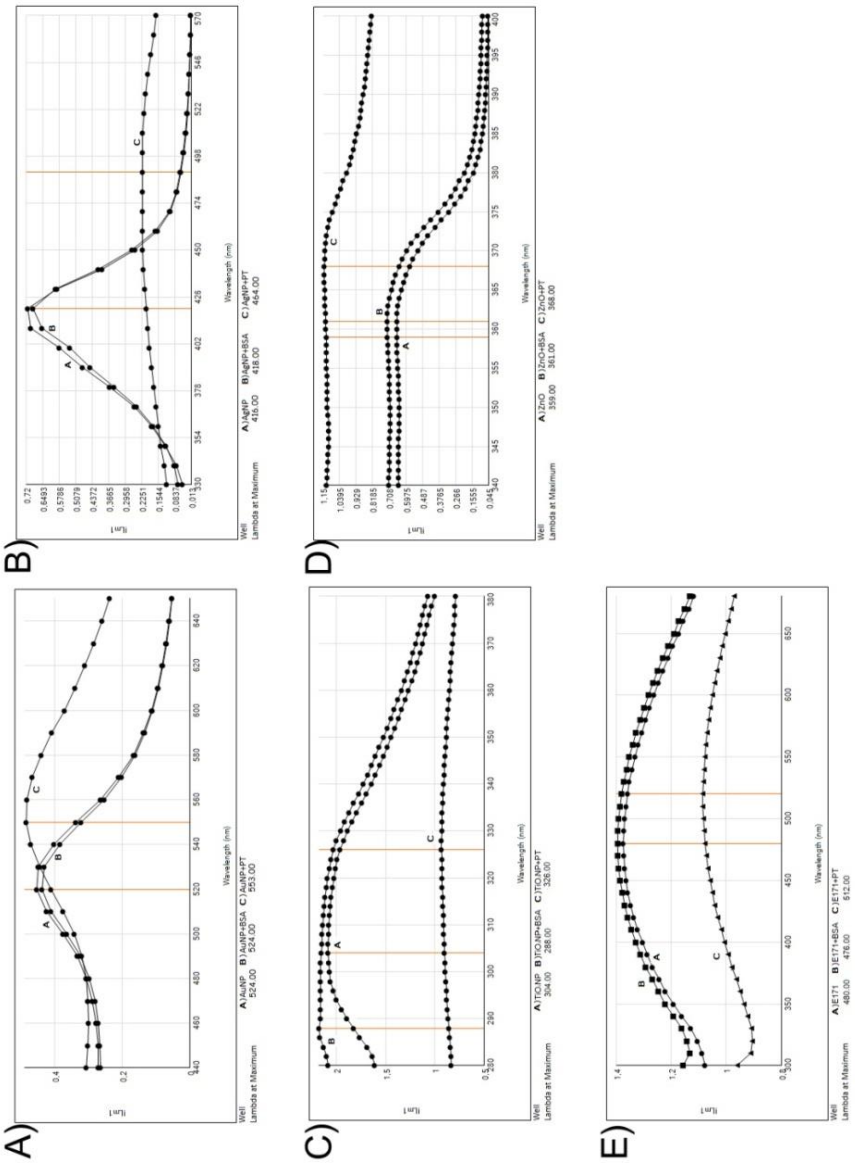


Figure 2

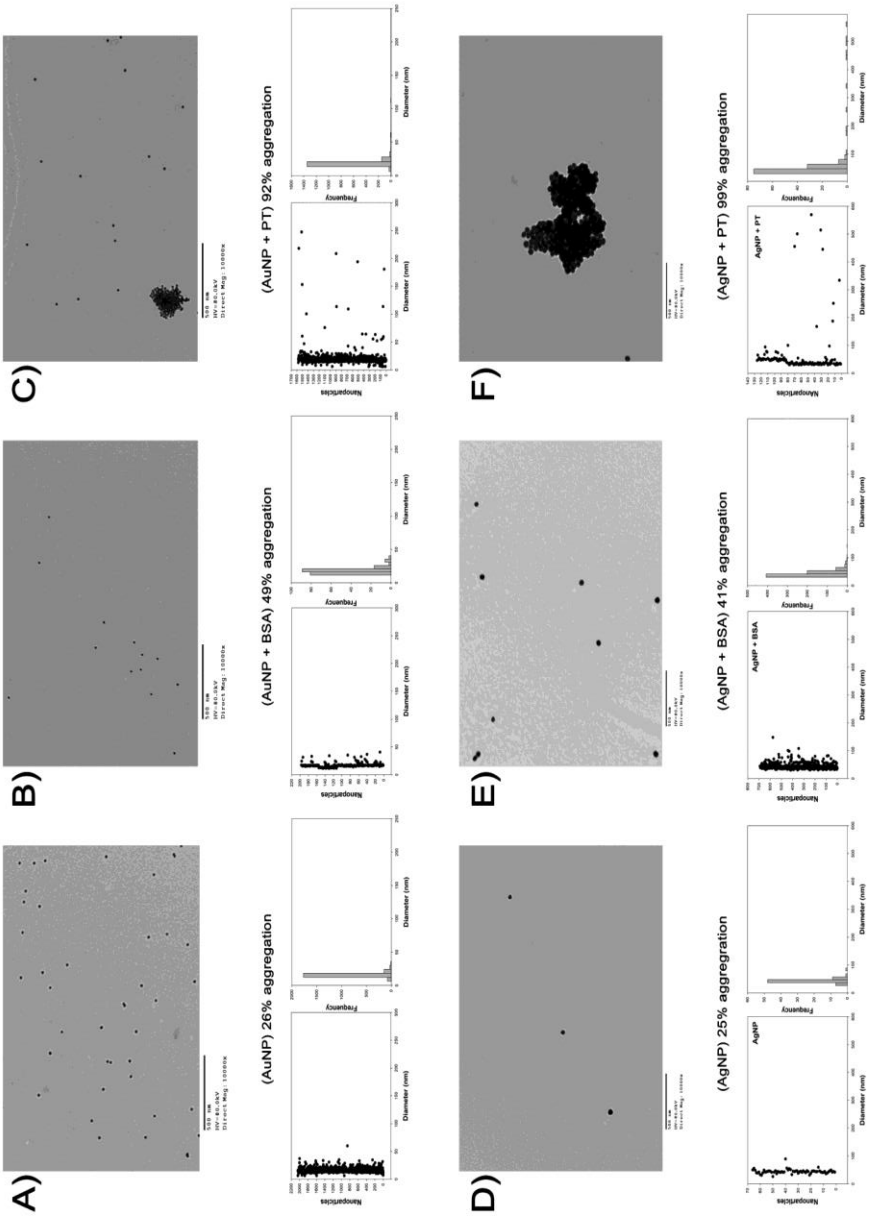

A)
B)
C)
D)
E)
F)
(AuNP) 26% aggregation
(AuNP + BSA) 49% aggregation
(AuNP + PT) 92% aggregation
(AgNP) 25% aggregation
(AgNP + BSA) 41% aggregation
(AgNP + PT) 99% aggregation
Nanoparticles
Frequency
Diameter (nm)
AgNP
AgNP + BSA
AgNP + PT

Figure 3

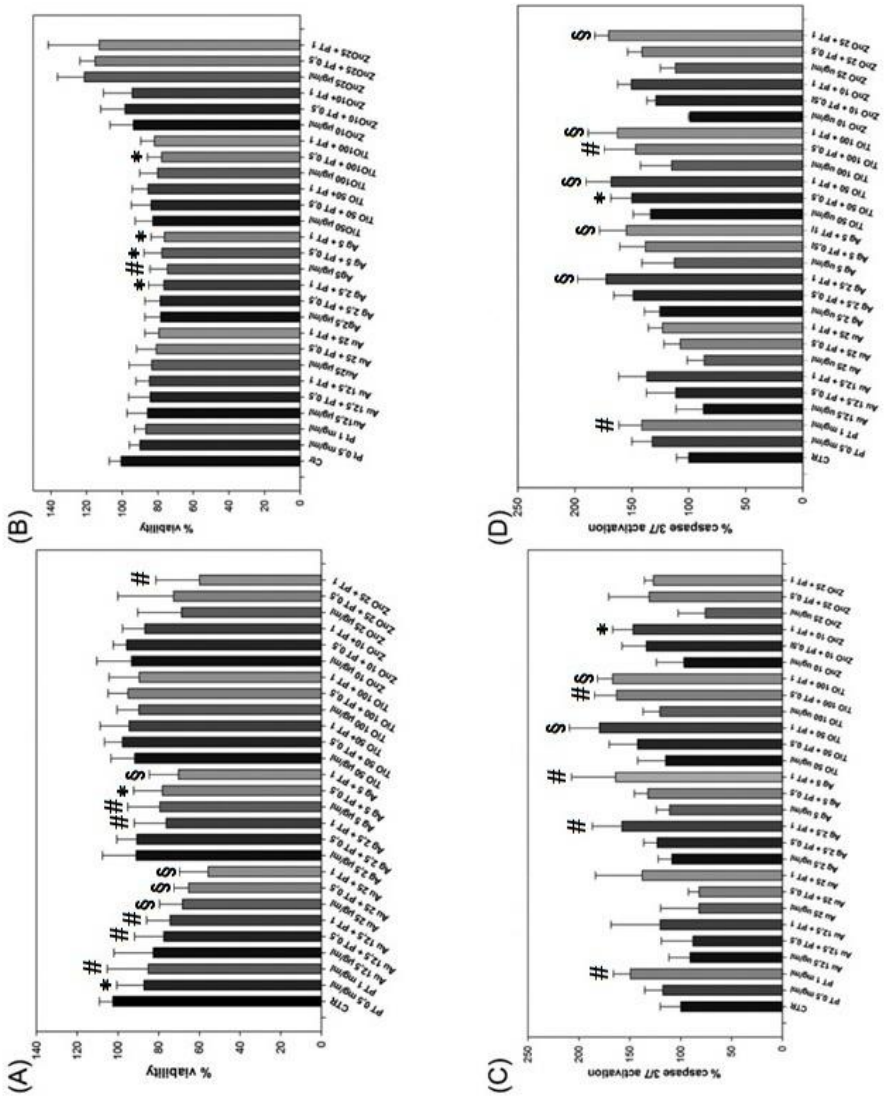
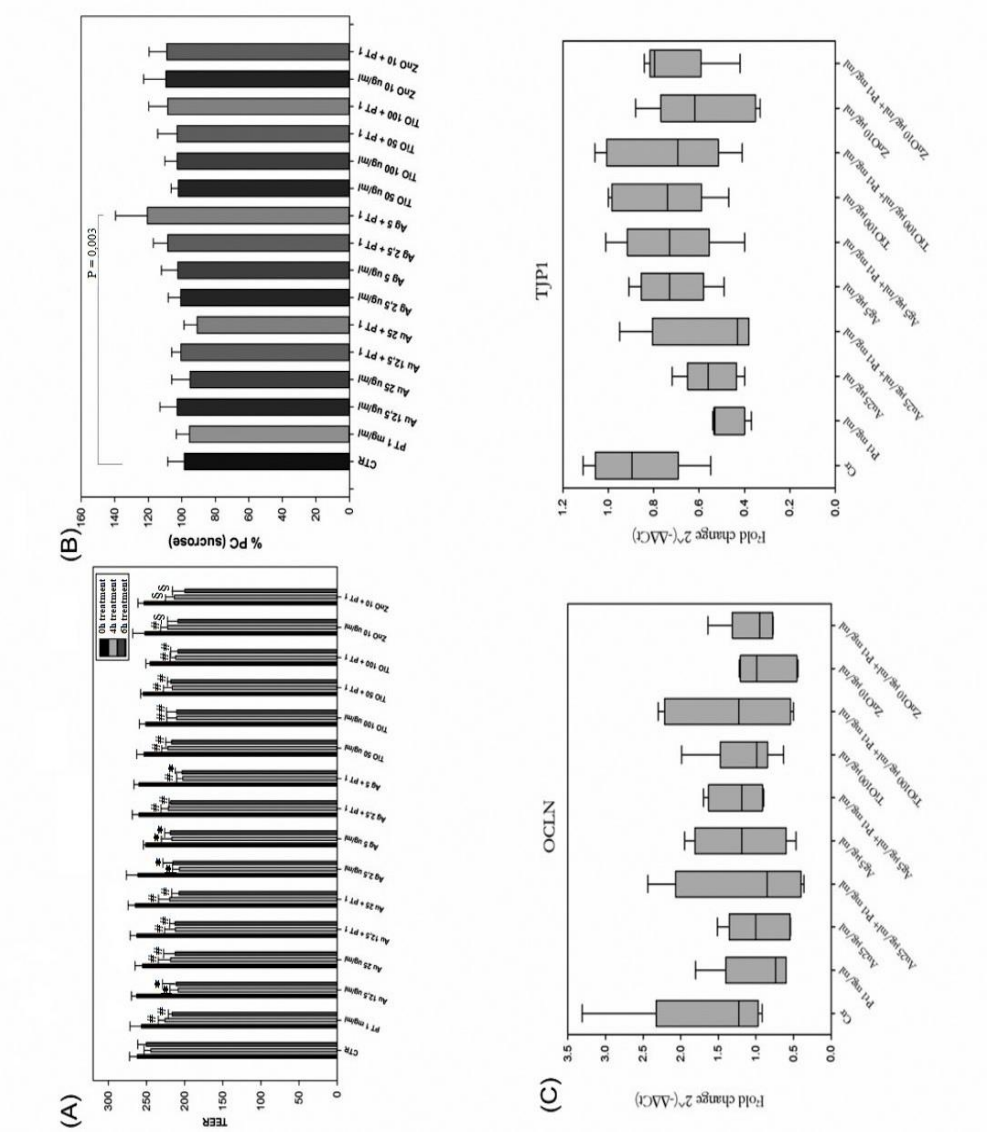

(A)
% viability
(B)
% viability
(C)
% caspase 3/7 activation
(D)
% caspase 3/7 activation

Figure 4


(A)
TEER
0h treatment
4h treatment
6h treatment
CTR
PT 1 mg/ml
Au 12,5 ug/ml
Au 25 ug/ml
Au 12,5 + PT 1
Au 25 + PT 1
Ag 2,5 ug/ml
Ag 5 ug/ml
Ag 2,5 + PT 1
Ag 5 + PT 1
TiO 50 ug/ml
TiO 100 ug/ml
TiO 50 + PT 1
TiO 100 + PT 1
ZnO 10 ug/ml
ZnO 10 + PT 1
(B)
% PC (sucrose)
P = 0,003
CTR
PT 1 mg/ml
Au 12,5 ug/ml
Au 25 ug/ml
Au 12,5 + PT 1
Au 25 + PT 1
Ag 2,5 ug/ml
Ag 5 ug/ml
Ag 2,5 + PT 1
Ag 5 + PT 1
TiO 50 ug/ml
TiO 100 ug/ml
TiO 50 + PT 1
TiO 100 + PT 1
ZnO 10 ug/ml
ZnO 10 + PT 1
TJP1
Fold change 2^(-ΔΔCt)
Ctr
Pt1 mg/ml
Au25 ug/ml
Au25 ug/ml + Pt1 mg/ml
Ag5 ug/ml
Ag5 ug/ml + Pt1 mg/ml
TiO100 ug/ml
TiO100 ug/ml + Pt1 mg/ml
ZnO10 ug/ml
ZnO10 ug/ml + Pt1 mg/ml
(C)
OCLN
Fold change 2^(-ΔΔCt)
Ctr
Pt1 mg/ml
Au25 ug/ml
Au25 ug/ml + Pt1 mg/ml
Ag5 ug/ml
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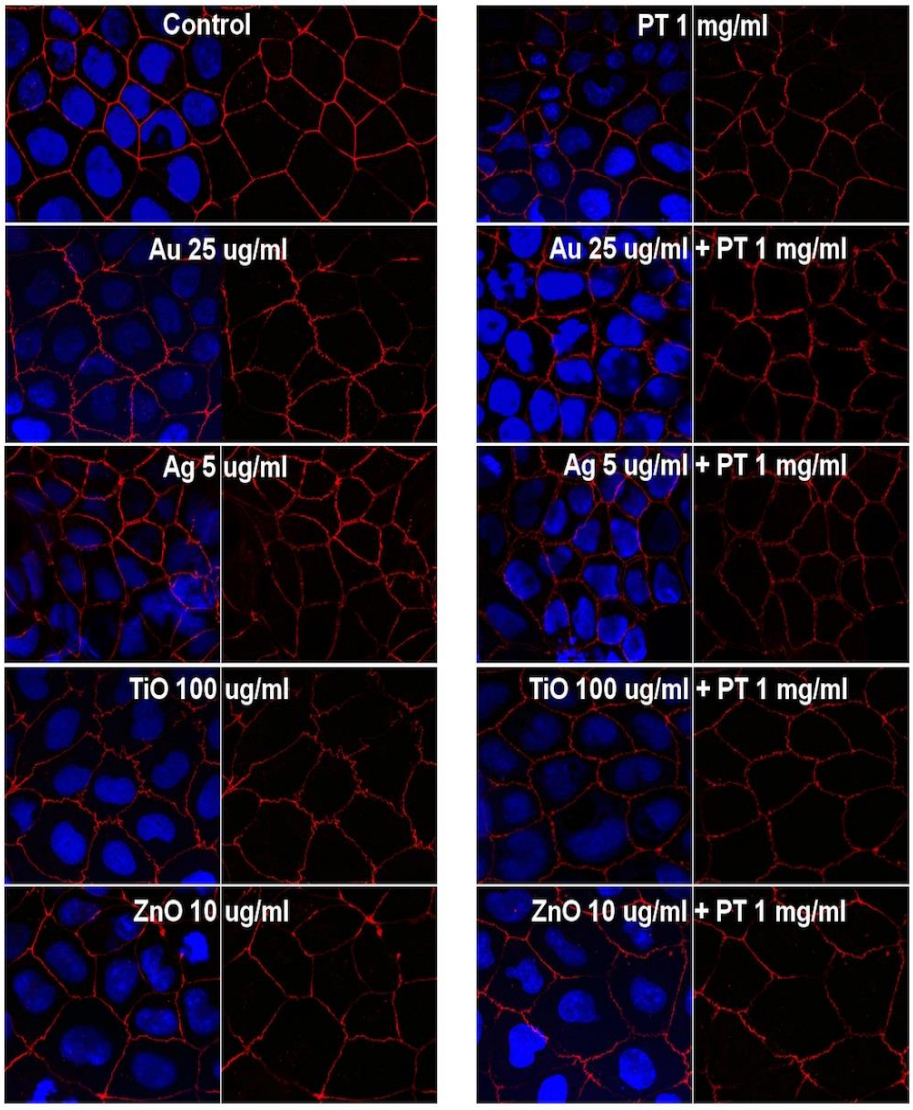
ZnO10 ug/ml
ZnO10 ug/ml + Pt1 mg/ml

Figure 5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Au 25 ug/ml
Au 25 ug/ml + PT 1 mg/ml
Ag 5 ug/ml
Ag 5 ug/ml + PT 1 mg/ml
TiO 100 ug/ml
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ZnO 10 ug/ml
ZnO 10 ug/ml + PT 1 mg/ml

Figure 6

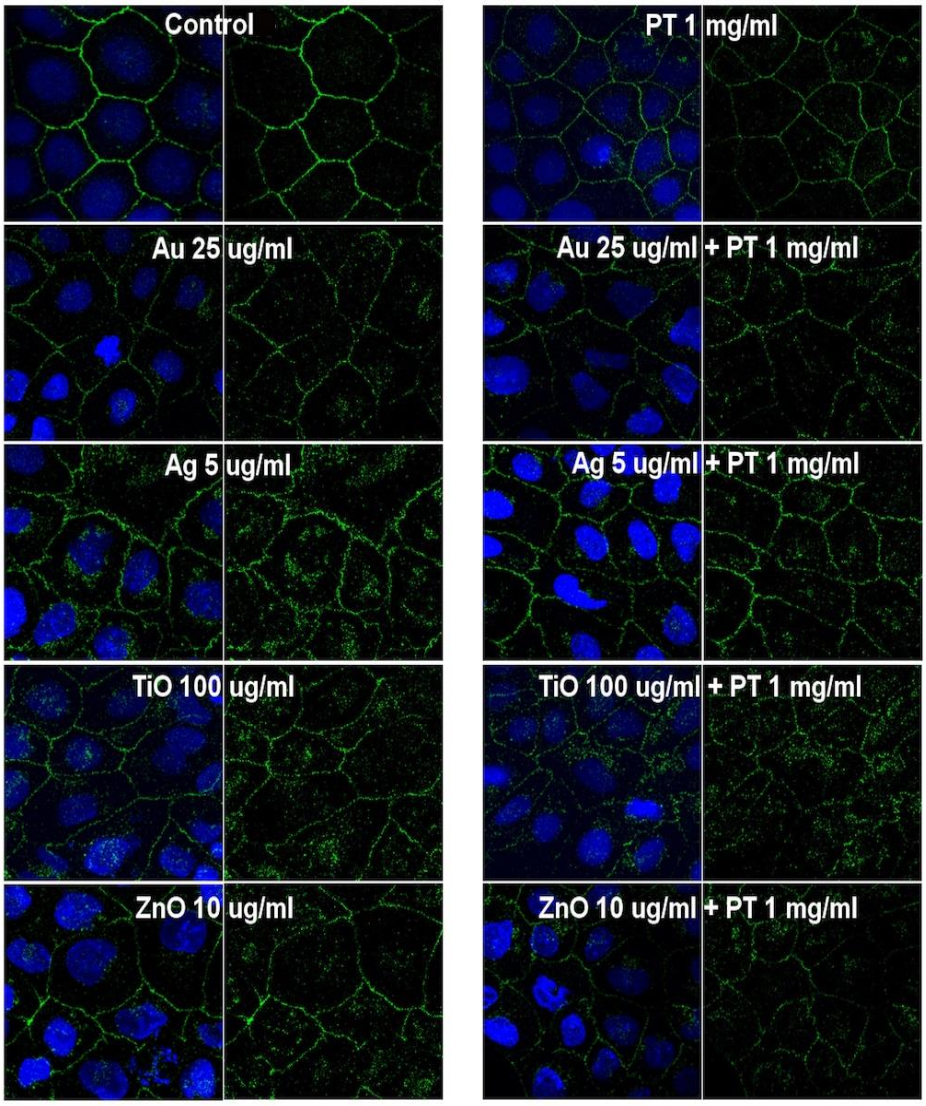
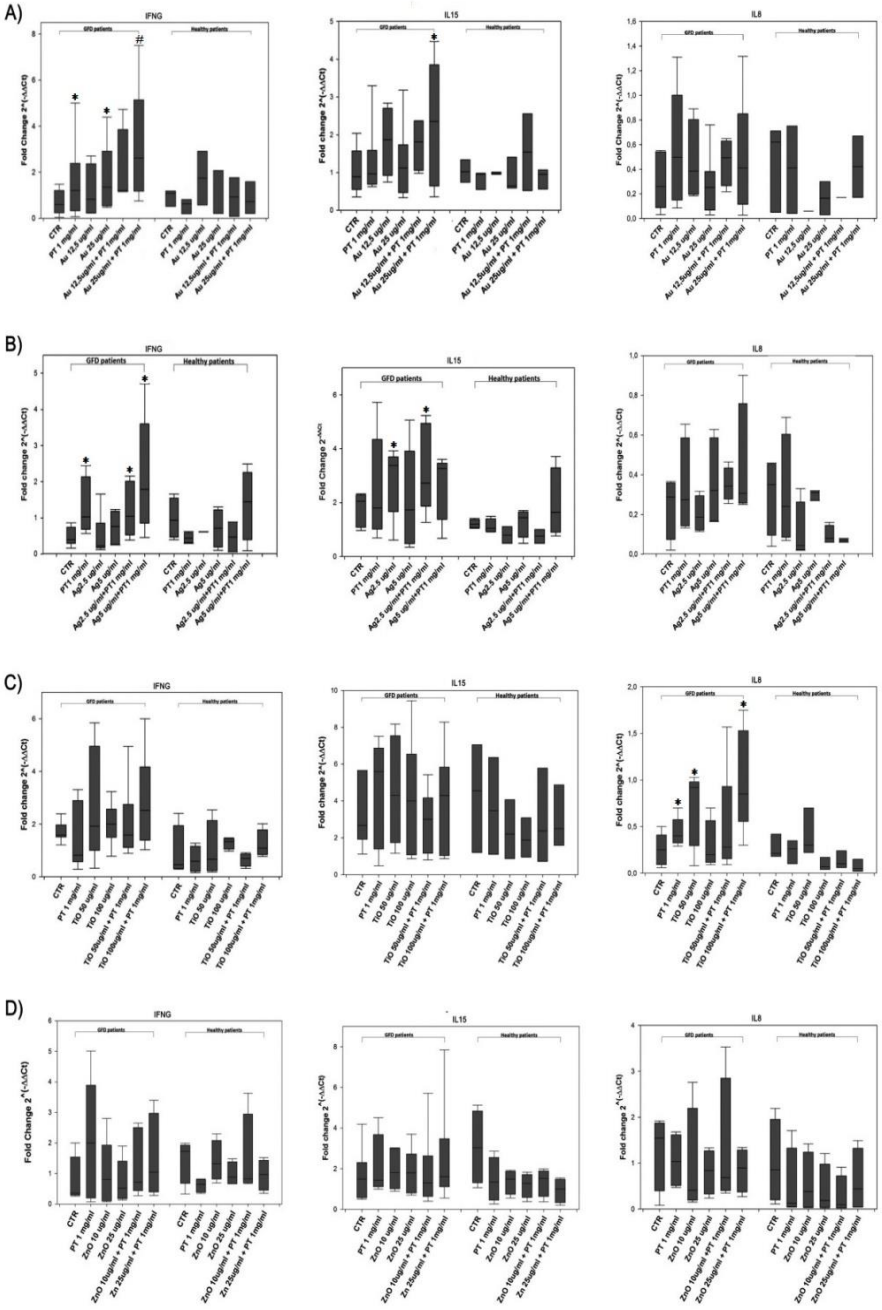
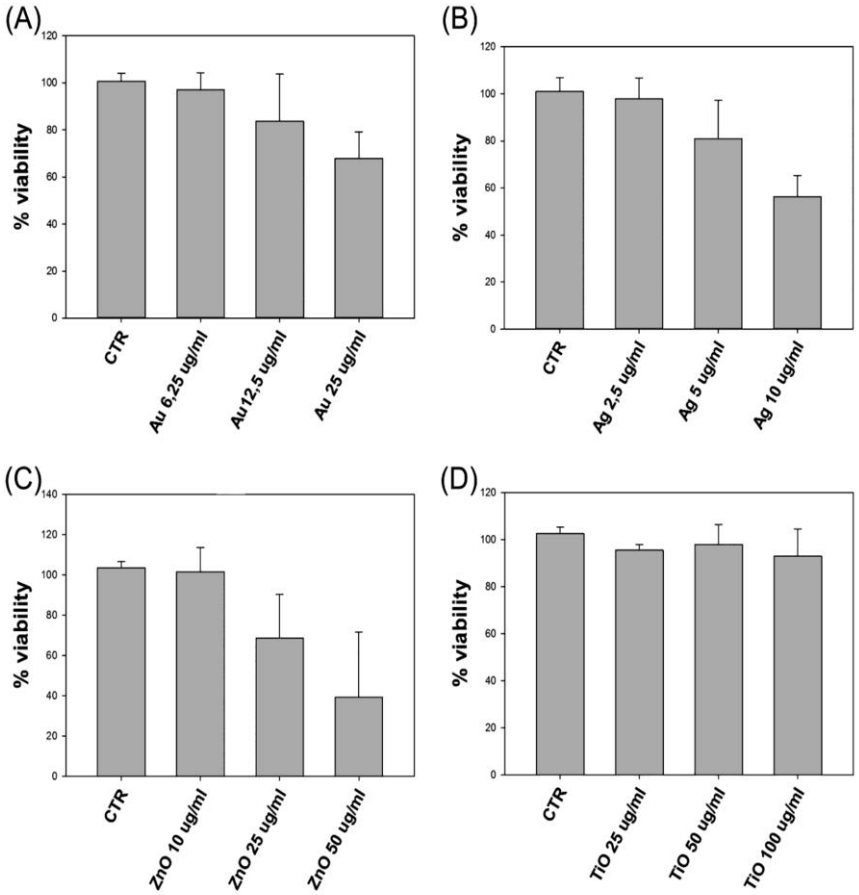

Control
PT 1 mg/ml
Au 25 ug/ml
Au 25 ug/ml + PT 1 mg/ml
Ag 5 ug/ml
Ag 5 ug/ml + PT 1 mg/ml
TiO 100 ug/ml
TiO 100 ug/ml + PT 1 mg/ml
ZnO 10 ug/ml
ZnO 10 ug/ml + PT 1 mg/ml

Figure 7



Supplementary Figure S1



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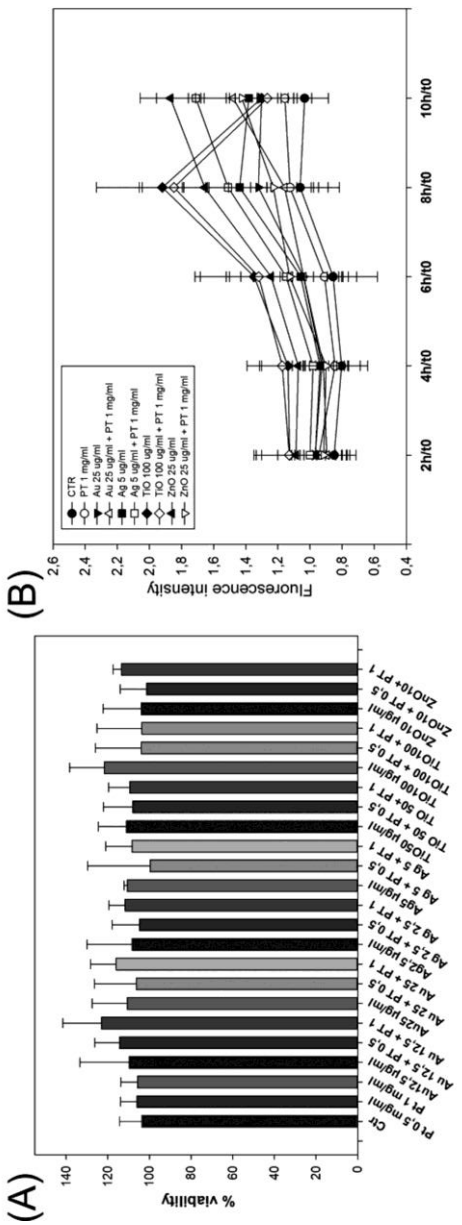

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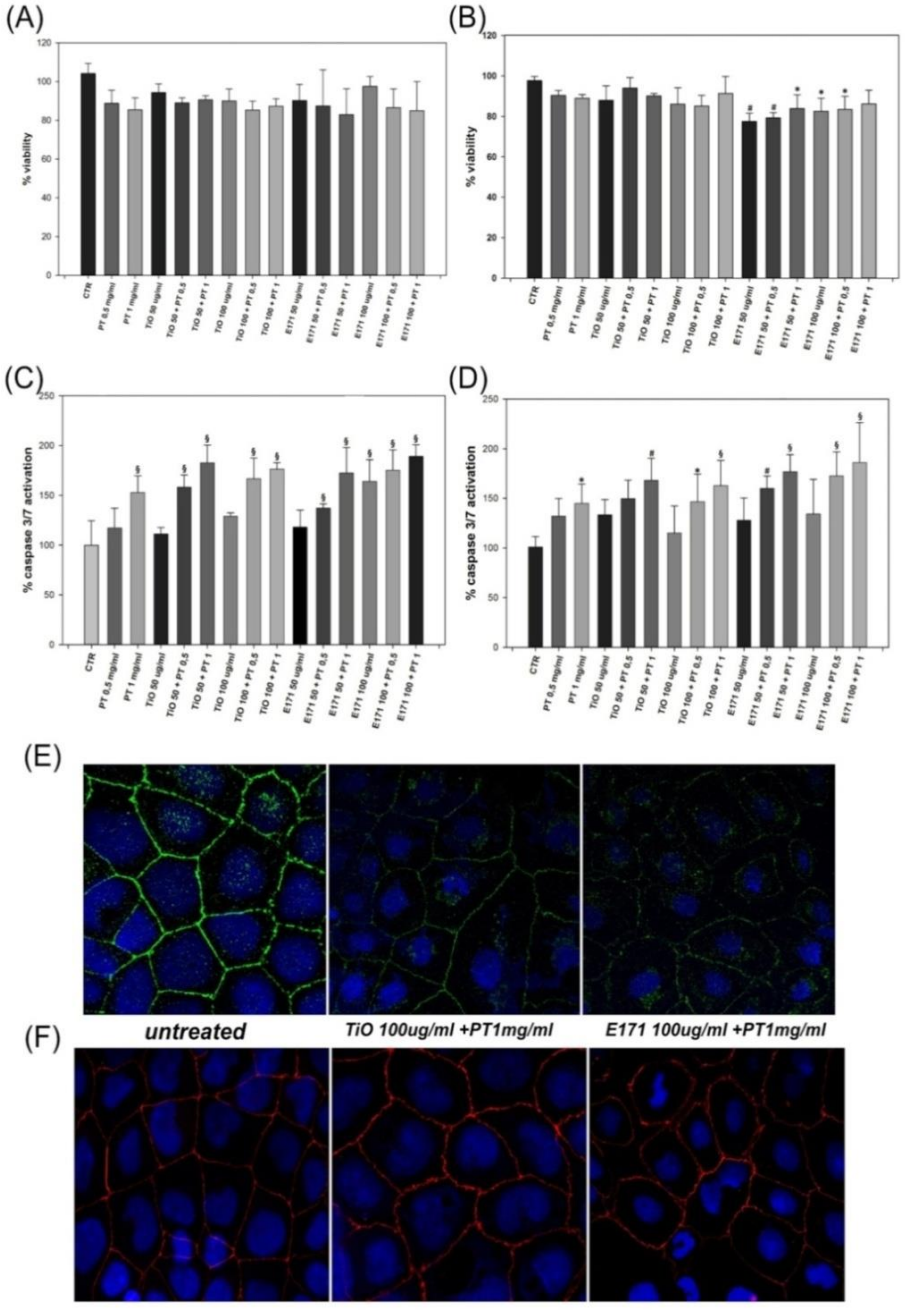

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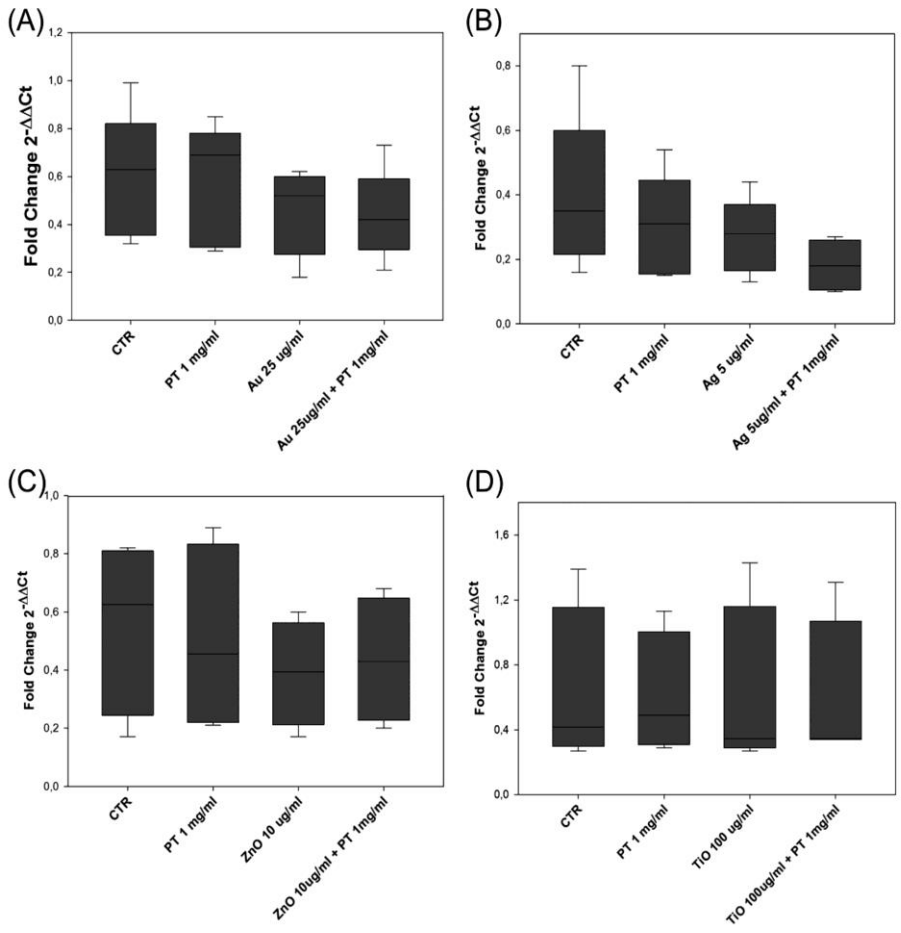

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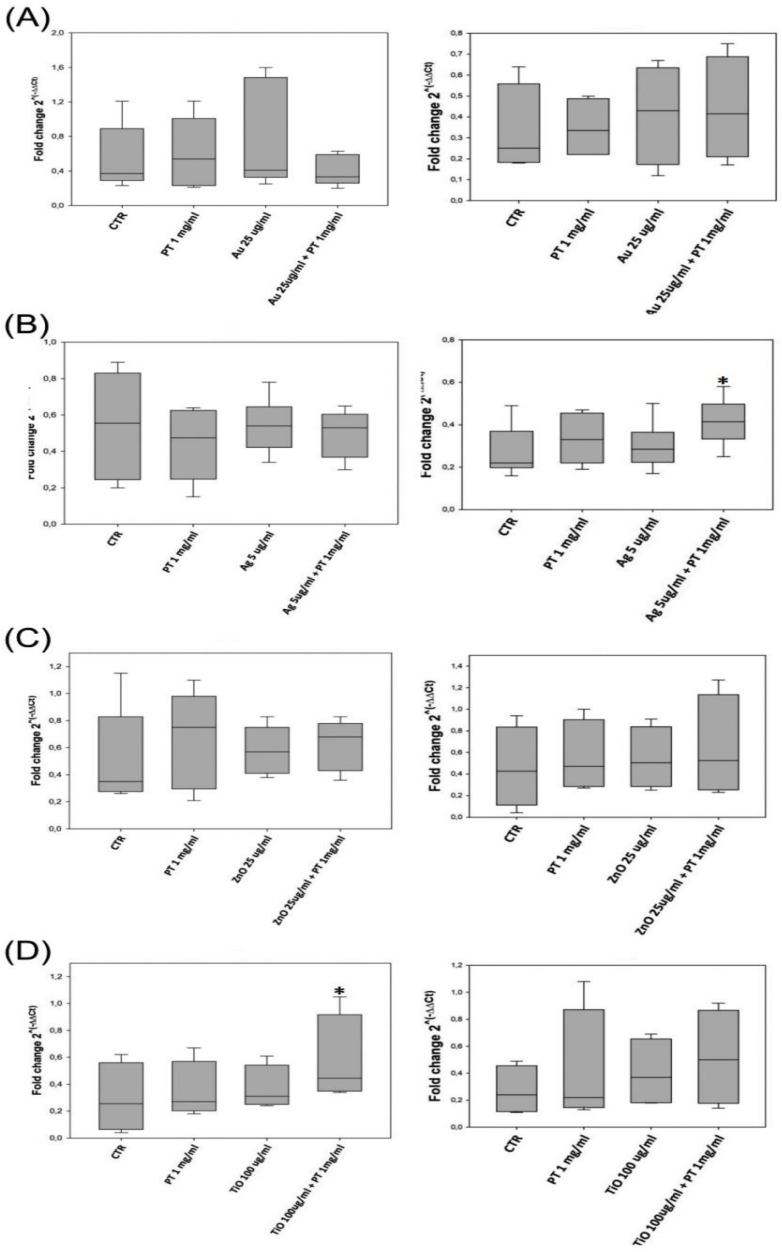

References

1. Fogli S, Montis C, Paccosi S, Silvano S, Michelucci E, Berti D, Bosi A, and PA, P R. Inorganic nanoparticles as potential regulators of immune response in dendritic cells. Nanomedicine 2017;12.

2. Ren X, Chen Y, Peng H, Fang X, Zhang X, Chen Q, Wang X, Yang W, Sha X. Blocking Autophagic Flux Enhances Iron Oxide Nanoparticles Photothermal Therapeutic Efficiency in Cancer Treatment. ACS Applied Materials & Interfaces 2018.

3. Prosa M, Tessarolo M, Bolognesi M, Margeat O, Gedefaw D, Gaceur M, Videlot-Ackermann C, Andersson MR, Muccini M, Seri M, Ackermann J. Enhanced Ultraviolet Stability of Air-Processed Polymer Solar Cells by Al Doping of the ZnO Interlayer. ACS Applied Materials & Interfaces 2016;8:1635-1643.

4. Arora S, Sharma P, Kumar S, Nayan R, Khanna PK, Zaidi MGH. Gold-nanoparticle induced enhancement in growth and seed yield of Brassica juncea. Plant Growth Regulation 2012;66:303-310.

5. Yang J, Jiang F, Ma C, Rui Y, Rui M, Adeel M, Cao W, Xing B. Alteration of Crop Yield and Quality of Wheat upon Exposure to Silver Nanoparticles in a Life Cycle Study. Journal of Agricultural and Food Chemistry 2018;66:2589-2597.

6. Kumar V, Guleria P, Kumar V, Yadav SK. Gold nanoparticle exposure induces growth and yield enhancement in Arabidopsis thaliana. Science of The Total Environment 2013;461-462:462-468.

7. Verleysen E, Van Doren E, Waegeneers N, De Temmerman PJ, Abi Daoud Francisco M, Mast J. TEM and SP-ICP-MS Analysis of the

Release of Silver Nanoparticles from Decoration of Pastry. Journal of Agricultural and Food Chemistry 2015;63:3570-3578.

8. Athinarayanan J, Alshatwi AA, Periasamy VS, Al-Warthan AA. Identification of Nanoscale Ingredients in Commercial Food Products and their Induction of Mitochondrially Mediated Cytotoxic Effects on Human Mesenchymal Stem Cells. Journal of Food Science 2015;80:N459-N464.

9. Vance ME, Kuiken T, Vejerano EP, McGinnis SP, Hochella MF, Rejeski D, Hull MS. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein Journal of Nanotechnology 2015;6:1769-1780.

10. Yao M, He L, McClements DJ, Xiao H. Uptake of Gold Nanoparticles by Intestinal Epithelial Cells: Impact of Particle Size on Their Absorption, Accumulation, and Toxicity. Journal of Agricultural and Food Chemistry 2015;63:8044-8049.

11. Hanley C, Thurber A, Hanna C, Punnoose A, Zhang J, Wingett DG. The Influences of Cell Type and ZnO Nanoparticle Size on Immune Cell Cytotoxicity and Cytokine Induction. Nanoscale Research Letters 2009;4:1409-1420.

12. Chueh PJ, Liang R-Y, Lee Y-H, Zeng Z-M, Chuang S-M. Differential cytotoxic effects of gold nanoparticles in different mammalian cell lines. Journal of Hazardous Materials 2014;264:303-312.

13. Bellmann S, Carlander D, Fasano A, Momcilovic D, Scimeca JA, Waldman WJ, Gombau L, Tsytsikova L, Canady R, Pereira DIA, Lefebvre DE. Mammalian gastrointestinal tract parameters

modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology 2015;7:609-622.

14. Lerner A, Matthias T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. Autoimmunity Reviews 2015;14:479-489.

15. Wang Y, Yuan L, Yao C, Ding L, Li C, Fang J, Sui K, Liu Y, Wu M. A combined toxicity study of zinc oxide nanoparticles and vitamin C in food additives. Nanoscale 2014;6:15333-15342.

16. Fang X, Jiang L, Gong Y, Li J, Liu L, Cao Y. The presence of oleate stabilized ZnO nanoparticles (NPs) and reduced the toxicity of aged NPs to Caco-2 and HepG2 cells. Chemico-Biological Interactions 2017;278:40-47.

17. Vamanu C, Hol P, Allouni Z, Elsayed S, Gjerdet N. Formation of Potential Titanium Antigens Based on Protein Binding to Titanium Dioxide Nanoparticles. Int J Nanomed 2008;3:69-74.

18. Khan HA, Abdelhalim MAK, Alhomida AS, Al Ayed MS. Short Communication Transient increase in IL-1β, IL-6 and TNF-α gene expression in rat liver exposed to gold nanoparticles. Genetics and Molecular Research 2013;12:5851-5857.

19. Evans SM, Ashwood P, Warley A, Berisha F, Thompson RPH, Powell JJ. The role of dietary microparticles and calcium in apoptosis and interleukin-1β release of intestinal macrophages. Gastroenterology 2002;123:1543-1553.

20. Borgognoni CF, Kim JH, Zucolotto V, Fuchs H, Riehemann K. Human macrophage responses to metal-oxide nanoparticles: a review. Artificial Cells, Nanomedicine, and Biotechnology 2018:1-10.

21. Powell JJ, Harvey RSJ, Ashwood P, Wolstencroft R, Gershwin ME, Thompson RPH. Immune Potentiation of Ultrafine Dietary Particles in Normal Subjects and Patients with Inflammatory Bowel Disease. Journal of Autoimmunity 2000;14:99-105.

22. Fasano A. Leaky Gut and Autoimmune Diseases. Clinical Reviews in Allergy & Immunology 2012;42:71-78.

23. Mustalahti K, Catassi C, Reunanen A, Fabiani E, Heier M, McMillan S, Murray L, Metzger M-H, Gasparin M, Bravi E, Mäki M. The prevalence of celiac disease in Europe: Results of a centralized, international mass screening project. Annals of Medicine 2010;42:587-595.

24. West J, Fleming KM, Tata LJ, Card TR, Crooks CJ. Incidence and Prevalence of Celiac Disease and Dermatitis Herpetiformis in the UK Over Two Decades: Population-Based Study. The American Journal of Gastroenterology 2014;109:757-768.

25. Lerner A, Arleevskaya M, Schmiedl A, Matthias T. Microbes and Viruses Are Bugging the Gut in Celiac Disease. Are They Friends or Foes? Frontiers in Microbiology 2017;8.

26. Stene LC, Honeyman MC, Hoffenberg EJ, Haas JE, Sokol RJ, Emery L, Taki I, Norris JM, Erlich HA, Eisenbarth GS, Rewers M. Rotavirus Infection Frequency and Risk of Celiac Disease Autoimmunity in Early Childhood: A Longitudinal Study. The American Journal of Gastroenterology 2006;101:2333-2340.

27. Caminero A, Nistal E, Herrán AR, Pérez-Andrés J, Ferrero MA, Vaquero Ayala L, Vivas S, Ruiz de Morales JMG, Albillos SM, Casqueiro FJ. Differences in gluten metabolism among healthy volunteers, coeliac disease patients and first-degree relatives. British Journal of Nutrition 2015;114:1157-1167.

28. Caminero A, Galipeau HJ, McCarville JL, Johnston CW, Bernier SP, Russell AK, Jury J, Herran AR, Casqueiro J, Tye-Din JA, Surette MG, Magarvey NA, Schuppan D, Verdu EF. Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. Gastroenterology 2016;151:670-683.

29. Drago S, El Asmar R, Di Pierro M, Clemente MG, Tripathi A, Sapone A, Thakar M, Iacono G, Carroccio A, D'Agate C, Not T, Zampini L, Catassi C, Fasano A. Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. Scandinavian Journal of Gastroenterology 2006;41:408-419.

30. Frazer A, Fletcher R, Roaa C, Shaw B, Sammons H, Schneider R. Gluten-induced enteropathy: the effect of partially digested gluten. Lancet 1959;2:252-255.

31. Mirsadeghi S, Dinarvand R, Ghahremani MH, Hormozi-Nezhad MR, Mahmoudi Z, Hajipour MJ, Atyabi F, Ghavami M, Mahmoudi M. Protein corona composition of gold nanoparticles/nanorods affects amyloid beta fibrillation process. Nanoscale 2015;7:5004-5013.

32. Albanese A, Chan WCW. Effect of Gold Nanoparticle Aggregation on Cell Uptake and Toxicity. ACS Nano 2011;5:5478.

33. Hanley C, Layne J, Punnoose A, Reddy KM, Coombs I, Coombs A, Feris K, Wingett D. Preferential killing of cancer cells and activated human T cells using ZnO nanoparticles. Nanotechnology 2008;19:295103.

34. Gerloff K, Pereira DIA, Faria N, Boots AW, Kolling J, Förster I, Albrecht C, Powell JJ, Schins RPF. Influence of simulated gastrointestinal conditions on particle-induced cytotoxicity and interleukin-8 regulation in differentiated and undifferentiated Caco-2 cells. Nanotoxicology 2012;7:353-366.

35. Kroll A, Pillukat MH, Hahn D, Schnekenburger J. Interference of engineered nanoparticles with in vitro toxicity assays. Archives of Toxicology 2012;86:1123-1136.

36. Lupu AR, Popescu T. The noncellular reduction of MTT tetrazolium salt by TiO2 nanoparticles and its implications for cytotoxicity assays. Toxicology in Vitro 2013;27:1445-1450.

37. Yang Y, Doudrick K, Bi X, Hristovski K, Herckes P, Westerhoff P, Kaegi R. Characterization of Food-Grade Titanium Dioxide: The Presence of Nanosized Particles. Environmental Science & Technology 2014;48:6391-6400.

38. Williams K, Milner J, Boudreau MD, Gokulan K, Cerniglia CE, Khare S. Effects of subchronic exposure of silver nanoparticles on intestinal microbiota and gut-associated immune responses in the ileum of Sprague-Dawley rats. Nanotoxicology 2014;9:279-289.

39. Brun E, Barreau F, Veronesi G, Fayard B, Sorieul S, Chanéac C, Carapito C, Rabilloud T, Mabondzo A, Herlin-Boime N, Carriére M. Titanium dioxide nanoparticle impact and translocation through ex

vivo, in vivo and in vitro gut epithelia. particle and Fibre Toxicology 2014;11.

40. Ruiz PA, Morón B, Becker HM, Lang S, Atrott K, Spalinger MR, Scharl M, Wojtal KA, Fischbeck-Terhalle A, Frey-Wagner I, Hausmann M, Kraemer T, Rogler G. Titanium dioxide nanoparticles exacerbate DSS-induced colitis: role of the NLRP3 inflammasome. Gut 2017;66:1216-1224.

41. Martirosyan A, Grintzalis K, Polet M, Laloux L, Schneider Y-J. Tuning the inflammatory response to silver nanoparticles via quercetin in Caco-2 (co-)cultures as model of the human intestinal mucosa. Toxicology Letters 2016;253:36-45.

42. Cao Y, Roursgaard M, Kermanizadeh A, Loft S, Møller P. Synergistic Effects of Zinc Oxide Nanoparticles and Fatty Acids on Toxicity to Caco-2 Cells. International Journal of Toxicology 2015;34:67-76.

43. Peters R, Kramer E, Oomen AG, Rivera ZEH, Oegema G, Tromp PC, Fokkink R, Rietveld A, Marvin HJP, Weigel S, Ad A.C.M. P, Bouwmeester H. Presence of Nano-Sized Silica during In Vitro Digestion of Foods Containing Silica as a Food Additive. ACS Nano 2012;6:2441-51.

44. Walczak AP, Fokkink R, Peters R, Tromp P, Herrera Rivera ZE, Rietjens IMCM, Hendriksen PJM, Bouwmeester H. Behaviour of silver nanoparticles and silver ions in an in vitro human gastrointestinal digestion model. Nanotoxicology 2012;7:1198-1210.

45. Shalimar DM, Das P, Sreenivas V, Gupta SD, Panda SK, Makharia GK. Mechanism of Villous Atrophy in Celiac Disease: Role of

Apoptosis and Epithelial Regeneration. Archives of Pathology & Laboratory Medicine 2013;137:1262-1269.

46. Giovannini C, Sanchez M, Straface E, Scazzocchio B, Silano M, Vincenzi MD. Induction of apoptosis in Caco-2 cells by wheat gliadin peptides. Toxicology 2000;145:63-71.

47. Sander GR, Cummins AG, Powell BC. Rapid disruption of intestinal barrier function by gliadin involves altered expression of apical junctional proteins. FEBS Letters 2005;579:4851-4855.

48. Song Y, Guan R, Lyu F, Kang T, Wu Y, Chen X. In vitro cytotoxicity of silver nanoparticles and zinc oxide nanoparticles to human epithelial colorectal adenocarcinoma (Caco-2) cells. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis 2014;769:113-118.

49. Vachon PH, Beaulieu J-F. Transient mosaic patterns of morphological and functional differentiation in the Caco-2 cell line. Gastroenterology 1992;103:414-423.

50. Peters RJB, van Bemmel G, Herrera-Rivera Z, Helsper HPFG, Marvin HJP, Weigel S, Tromp PC, Oomen AG, Rietveld AG, Bouwmeester H. Characterization of Titanium Dioxide Nanoparticles in Food Products: Analytical Methods To Define Nanoparticles. Journal of Agricultural and Food Chemistry 2014;62:6285-6293.

51. Luo Y-H, Chang LW, Lin P. Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications. BioMed Research International 2015;2015:1-12.

52. Bettini S, Boutet-Robinet E, Cartier C, Coméra C, Gaultier E, Dupuy J, Naud N, Taché S, Grysan P, Reguer S, Thieriet N, Réfrégiers

M, Thiaudière D, Cravedi J-P, Carrière M, Audinot J-N, Pierre FH, Guzylack-Piriou L, Houdeau E. Food-grade TiO2 impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. Scientific Reports 2017;7:40373.

53. Garrote JA, Gçmez-Gonzàlez E, Brenardo D, Arranz E, Chirdo F. Celiac Disease Pathogenesis: The Proinflammatory Cytokine Network. Journal of Pediatric Gastroenterology and Nutrition 2008;47:S27-S32.

54. Ghasiyari H, Rostami-Nejad M, Amani D, Rostami K, Pourhoseingholi MA, Asadzadeh-Aghdaei H, Zali MR. Diverse Profiles of Toll-Like Receptors 2, 4, 7, and 9 mRNA in Peripheral Blood and Biopsy Specimens of Patients with Celiac Disease. Journal of Immunology Research 2018;2018:1-8.

55. Schanen BC, Karakoti AS, Seal S, III DRD, Warren WL, Self WT. Exposure to Titanium Dioxide Nanomaterials Provokes Inflammation of an in Vitro Human Immune Construct. ACS Nano 2009;3:2523-2532.

56. Galbiati V, Cornaghi L, Gianazza E, Potenza MA, Donetti E, Marinovich M, Corsini E. In vitro assessment of silver nanoparticles immunotoxicity. Food and Chemical Toxicology 2018;112:363-374.

57. Lomer MCE, Thompson RPH, Powell JJ. Fine and ultrafine particles of the diet: influence on the mucosal immune response and association with Crohn's disease. Proceedings of the Nutrition Society 2002;61:123-130.

58. Lacerda SHDP, Park JJ, Meuse C, Prostinskt D, Becker ML, Karim A, Douglas JF. Interaction of Gold Nanoparticles with Common Human Blood Proteins. ACS Nano 2009;4:365-79.

Chapter 3

Dietary metallic nanoparticles-gliadin complexes induce autophagic alterations in intestinal crypt-like cells

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ABSTRACT

Although the use of metallic nanoparticles (mNP) in the food industry is increasing, it is not yet clear if they represent a risk for our health. mNP toxic effects on the intestine have been reported, but most of the studies have been performed on model representative of differentiated intestinal cells, while few took into consideration the potential effect of mNP in crypt-like cells. Crypt cells play an essential role in intestinal homeostasis and attempt to restore the mucosa after damage, such as in Celiac Disease, a multifactorial autoimmune disease triggered by the ingestion of gluten peptides. We therefore decided to evaluate the possible alterations induced by AuNP and AgNP (widely used in the food sector), alone or combined with the gluten peptides, in the HIEC cell model, representative of undifferentiated crypt-like cells. TEM analyses suggested an alteration of the autophagic pathway, finding that was further confirmed by molecular approaches (increase in LC3 II and p62 expression). Immune modulation and intestinal architectural remodelling, important aspects in the Celiac Disease pathogenesis, were also assessed in organ culture (small intestinal fetal specimens). Results showed a significant increment ($p<0.05$) of the innate immune-related cytokines IL-8 and IL1-5 only after treatment with AgNP-PTG complexes at 36h. The obtained results demonstrated that gluten peptides and mNP form stable complexes which can be internalized by HIEC cells, inducing alteration in the autophagic pathway. The induced alterations resulted worse than the effects

elicited by the single components both in vitro and in organ cultures, suggesting a combined effects of gluten and mNP. Since their co-presence in several food products, our results enlighted a potential negative role of dietary mNP in the Celiac Disease pathogenesis.

INTRODUCTION

Celiac disease (CeD) is a multifactorial autoimmune disorder that develops in subjects carrying the specific genetic HLA-DQ2 and/or -DQ8 background. It is triggered by the ingestion of gluten, the main environmental factor involved. Gluten is composed of a heterogeneous complex of proteins rich in glutamine and proline, and it is contained in several types of cereals such as wheat, rye, barley and oats[1]. Even though gluten is a common component of the western diet and the HLA-DQ2 polymorphism is present in about 30% of the general population[2], CeD affects only 2-5% of these subjects, suggesting the presence of other environmental factors for its aetiology. CeD is now considered to be one of the most common digestive disorders (affecting 1% of the global population) and its increasing incidence is raising concerns[3]. In this sense, several efforts have recently been made to identify possible new etiological factors. Many have been suggested such as viral infections[4], increased intestinal permeability[5 6], impaired commensal homeostasis[7] and the growing food additive use in the industry[8].

In this context, the use of metallic nanoparticles (mNPs) in the food chain appears particularly worrysome since their ingestion has been linked to intestinal permeability alteration, cytotoxic effects and immune system impairment[6, 9] as well as to intestinal bowel disease and colon neoplastic lesions[10]. However, the potential involvement of dietary mNP in the increasing incidence of CeD is still poorly documented.

Nanotechnology is a recent field of research that takes advantage of the new exciting properties that nanomaterials possess compared to their bulk counterparts. Their applications are numerous ranging from sustainable energy, to medical applications and the agro/feed/food chain[11]. Particularly in this last field they are used as dyes or additive to improve organoleptic properties, as antimicrobial agents to preserve foods and improve crop growth[12] and to increase the bioavailability of nutritional supplements[13]. Silver (AgNPs) and gold (AuNPs) metal nanoparticles are among the most commonly used mNPs in agro-food applications[11g]. Although data about their toxicity on differentiated intestinal cells are increasing, no studies have yet been performed about their potential effects on intestinal crypt-like cells. Moreover, the need to study dietary mNP effects in systems taking into consideration the presence of food components has been recently enlightened [14].

Given these premises, we focused on the possible combined effect of digested gliadin (the main active gluten component) with Ag- or Au-NPs on the normal human intestinal epithelial crypt-like (HIEC) cell line[15], a cell model that has been used for various purposes from the mechanisms underlying intestinal stemness[16] and differentiation[17] as well as cytotoxicity[18]. Based on our preliminary data, we particularly focused on the potential effects of mNP-gliadin complex on the macroautophagic process (hereafter referred as autophagy). Indeed, autophagy is an important intracellular pathway involving the sequestration of different cargos, such as damaged organelles, aggregated proteins and pathogens, into double-membrane

autophagic vacuoles and their consequent lysosomal-mediated degradation[19]. Furthermore, its activity involves crosstalk with several other cellular pathways, playing a critical role in the maintenance of cellular homeostasis. Incidentally, it has been recently reported that the autophagic pathway may have a role in both mNP toxicity mechanisms and in CeD pathogenesis[9b,20]. Moreover, Groulx et al.[21] showed that autophagy is active in intestinal proliferative/undifferentiated cell populations (e.g. the HIEC cell line). Herein, autophagic alterations in response to mNP and gliadins were assessed using transmission electronic microscopy (TEM) and evaluation of LC3, p62 and Beclin-1 expression at both the protein (Western blot and immunofluorescence assays) and transcript (qPCR) levels. We also used fetal intestine organ culture to further mimic the complex intestinal environment in vivo.

RESULTS AND DISCUSSION

TEM is an informative method for monitoring autophagy and the only technique that shows autophagic vacuoles (AV) in their complex cellular environment. For these experiments, HIEC cells were treated for 24h with mNP or peptic tryptic-digested gliadin (PTG) alone or in combination, and then processed for TEM visualization. Control cells showed a typical appearance of undifferentiated cells with a poorly organized cytoplasm and only a few intracellular vacuoles (Fig. 1A). Cells treated with either PTG (Fig. 1B) or mNP (Fig. S1 A and B) displayed a similar appearance as the controls. However, a significant increase in the number of vacuoles was observed in cells treated with 50 nM bafilomycin, used as positive control (Fig.1S C). Bafilomycin is an inhibitor of the vacuolar H+ ATPase, which in turn inhibits the fusion between AV and lysosome causing a block in the normal autophagic flux[22].

We have already demonstrated (submitted paper) that both AgNP and AuNP alone are mostly dispersed, while they form highly aggregated complexes when incubated with PTG. In this sense our results showed a large amount of aggregated NP, particularly AuNP (Fig.1 C), inside the cells and strictly associated with the intracellular vacuoles. On the contrary, in cells stimulated with mNP alone a low % of vacuoles was seen, as well as a reduced number of aggregated or isolated mNP in the endosomal vesicles (see supplementary data Fig. 1). It is noteworthy that the ultrastructural characteristics of the intracellular vacuoles observed, which include double-membrane

vesicles containing cell debris together with endocytosed mNP aggregates, are consistent with the description of autophagic vacuoles (particularly amphisomes)[23]. This suggested that aggregated mNP (and consequently the gliadin peptides adsorbed on their surface) were internalized by HIEC cells through endocytotic mechanisms to then be engulfed in autophagic vacuoles. Along with this hypothesis, and in line with the findings of Huang et al.[24], our results suggest that aggregated NP (in cells treated with the combination of mNP and PTG) stimulate the autophagic process, a phenomenon not observed with dispersed nanoparticles (in cells treated with mNP or gliadin alone). To test this hypothesis we then evaluated several autophagy-related molecular markers.

Microtubule-associated protein light chain 3 (LC3) is the most used molecular marker for the evaluation of autophagy. It exists in two separated forms, LC3-I (16-18 kDa) and its PE-conjugated form LC3-II (that although larger in mass, shows faster electrophoretic mobility in SDS-PAGE gels). Particularly, LC3-II is a protein marker associated with complete autophagosomes[25]. Therefore, measuring the transformation of LC3-I to the LC3-II form, can give information about autophagic alterations[26]. Our results showed a significant increase ($p<0.05$) in LC3-II protein levels after stimulation with AgNP-PTG complexes, while no alteration was observed under other tested conditions (Fig.2B). To further characterize a possible alteration in autophagic flux, we also evaluated p62 protein levels. p62 is a multiadaptor protein that interacts with both polyubiquitinated proteins and LC3-II on the AV for engulfment[19b]. At the same time it

remains incorporated into the autophagosome and degraded only after fusion with the lysosome, thus serving as an index of autophagic degradation[27]. Although other signals may induce an increase in p62, it is usually used as a marker for a block in autophagic flux. We observed a slight increase in its protein level that was as significant after stimulation with the AgNP-PTG complexes ($p<0.05$) as in the positive control($p<0.05$) (Fig.2C). Accumulation of p62 protein can also be linked to accumulation of misfolded proteins into cells, a mechanism that could be induced by mNP exposure[28]. To confirm that the higher p62 protein level was related to a block in autophagic flux, we also evaluated its transcript through qPCR (SQSTM1 gene). Results only indicated a slight increase of its transcript level after stimulation with the AgNP-PTG complex (Fig. S2A), supporting our hypothesis.

We then measured levels of ATG6/ Beclin-1, a component involved in the initial formation of AV[29]. A slight increase in the protein level after stimulation with AgNP-PTG complexes was seen ($p=0.0631$) (Fig.2D). On the contrary, we observed a significant reduction of its transcript level in cells treated with the AuNP-PTG complex (Figure S2B). This difference between transcript and protein levels is not clear. One possible explanation is that, when intracellular homeostasis becomes irremediably compromised, both an inhibition of autophagy and an induction of cell death mechanisms are induced[30].

Evaluation of LC3 II, p62/SQSTM1 and ATG6/Beclin-1 levels, only confirmed in part the TEM observation regarding the potential

involvement of an autophagic-related mechanism in response to mNP-PTG complexes in intestinal crypt cells. However, observation of cell cultures by phase-contrast microscopy revealed that mNP-PTG complexes are heterogeneous and do not superimpose evenly on cell monolayers. Indeed, as shown in Fig 3, AuNP and AgNP are under a mostly dispersed form when added alone onto cells (thus not visible at low power microscope magnification)(Fig. 3 A-B) while they form large and visible aggregates when added under the form of AuNP-PTG and AgNP-PTG complexes that persist over the 24 h period of culture (Fig 3C-D). This observation can be important considering that ingested mNP and gliadin are usually subjected to the presence of other food/beverage components[31]. It is noteworthy that these aggregates can be observed in only a subset of cells under both types of mNP-PTG complexes (Fig. 3 C-D).

We thus re-evaluated the expression of autophagic markers by indirect immunofluorescence in cells treated with mNPs and PTG alone and in combination. Cells were stained with both anti-p62 and anti-LC3 antibodies (Fig.4). Since the anti-LC3 antibody identifies both the LC3-I and LC3-II forms, we decided to overlay the signal with p62 staining, that would more precisely indicate mature autophagosomes[23a]. Our results are consistent with the WB data obtained. For both proteins evaluated, a diffuse pattern was observed in the negative control (untreated cells; Fig. 4A), and under stimulation with PTG (Fig 4B) or mNPs alone (not shown). However, a more punctate pattern was observed in cells stimulated with mNP and PTG, even if the average number of puncta per cell was less than

in the positive 50nM bafilomycin-control (Fig. 4E), as expected from the TEM observations. Indeed, following stimulation with the AgNP-PTG complex, a higher co-localization of p62 and LC3 signals, compared to the negative control or PTG alone, was clearly observed (Fig. 4D), thus confirming an alteration in the autophagic flux. The overlaid spots were not well distributed in the monolayer of cells. This phenomenon was more evident after stimulation with the AuNP-PTG aggregates, where the signal was very patchy (Fig. 4C). This last finding, together with the WB data obtained, could be explained by the fact that in the treatment with the AuNP-PTG complex, the large aggregates only targeted a few cells leading to a lower percentage of cells exhibiting-autophagic alterations as well as molecular changes in the autophagic markers.

Although further studies are necessary to better characterize the mechanisms involved, our results show that aggregates formed by AuNP and AgNP with PTG are internalized by intestinal cells. In parallel, alterations of the autophagic process were also observed in cells treated with the mNP-PTG complexes. The phenomenon appeared heterogeneous, affecting only a portion of the cells as a consequence of mNP-PTG aggregation in vitro, especially for the AuNP-PTG complexes.

It has been shown that at least one component of PTG, the specific p31-49 gliadin oligopeptide, can interfere with the endocytic and autophagic pathways, since it has a similar molecular structure as the hepatocyte growth factor-regulated substrate kinase (Hrs/Vps27), a protein involved in the maturation of both early endosome and

autophagosome vesicles[32]. Moreover, it has recently been demonstrated by Manai et al. that PTG tend to aggregate and be internalized into large autophagosome[20b]. The combined effect that we observed could, therefore, be explained by the more efficient internalization of PTG-mNP complexes, compared to PTG alone, their localization into AV and the consequent autophagic pathway impairment. An additional contribution can also result from mNP uptake under the same conditions, since mNP also have been associated with autophagic alterations[33]. It is noteworthy that even though alteration in autophagic flux can lead to mitochondrial dysfunction[34], we did not observe a difference in mitochondrial activity using the MTT assay (Fig. S2C).

Immune modulation and intestinal tissue remodelling are important factors in the celiac disease pathogenesis[35]. IL-15 as well as IL-8 are among the main cytokines of the innate immune response associated with CeD development[36]. IL-8 expression was not significantly modified (Fig. 5A), but a decrease in IL-15 was observed after exposure to both AgNP-PTG and AuNP-PTG aggregates ($p<0.05$) (Fig. 5B). We also evaluated the expression of matrix metalloproteinases 2 (MMP2 or gelatinase A) and 7 (MMP7 or matrilysin), also involved in the tissue remodelling of CeD[37]. MMP2 and MMP7 have been linked to the intestinal tissue injury mediated by T cells in an in vivo model of CeD[38]. Results indicated a significant ($p<0.05$) increase of MMP7 levels after treatment with PTG or AgNP alone as well as with AuNP- and AgNP-complexes ($p<0.05$) (Fig. 5D). For MMP2, a significant reduction of its level was only observed after stimulation with AuNP

alone (Fig. 5C). This last finding is in line with Wu et al.[39] who demonstrated a reduction of MMP9 in the SGC-7901 human gastric cancer cell line after stimulation with AuNP. This could indicate that AuNPs have the potential to reduce the expression of some MMP.

We then used organ culture of the mid-gestation fetal intestine to further document the interactions of the mNP-PTG complexes with intestinal cells. The interest of this system is that the 3D structure of the intestine is preserved as well as epithelial interactions with the stromal and immune cells of the lamina propria. At first, experimental conditions were set. Then, RNA extraction and qPCR were performed to evaluate the expression of cytokines (IL-8, IL-15, IFNγ), MMP (MMP-2 and 7) and specific markers of epithelium/ECM (sucrase-isomaltase, E-cadherin, tenascin). We also used some explants to visualize the possible tissue remodelling/damage induced by our stimulations. We decided to use 2 time points, 4h and 36h, to better understand the dynamic of the effects.

The light microscope appearance of the explants after stimulation was evaluated. Considering the 4h time point, the untreated tissue showed a normal appearance, with a well defined villous architecture and the presence of inflammatory cells in the lamina propria (Fig. 6A). After stimulation with PTG alone we observed a slight distortion of the villous architecture, while the surface epithelial cells seemed to suffer in a major measure (Fig. 6B). As expected, the explants stimulated with the combination of digested gliadin and NPs, showed more severe changes. They appeared oedematous with a very distorted villous architecture (Fig. 6 C-D). Principally after stimulation

with AgNP plus PTG, we also observed a marked infiltration of inflammatory cells into the lamina propria. Although our consideration are based on few aspects of the tissue remodelling, they seem to be in line with the characteristic lesions induced in celiac disease[40 35b]. We decided not to show the appearance of the explants harvested at 36h, since the tissue was completely disrupted, except for the negative control (untreated explants). We also evaluated several markers (qPCR) linked to tissue remodelling and damage, such as MMP2 and 7, SI, TNC and E-Cad. Considering the low number of specimens, we preferred to not show the data at this time. For the same reason we are not showing the results of the treatment with the mNP alone.

We previously evaluated the transcriptional level of the cytokines IL-15, IL-8 and IFNγ on adult duodenal biopsies from both healthy and celiac subjects. After 4h of stimulation, an increase of all cytokines, particularly IL-15 and IFNγ, was registered with the combination of NP plus digested gliadin, but only in celiac specimens (submitted paper). The organ culture method gave us the possibility to evaluate the cytokine response at a longer time point (36h). Results showed a significant increase ($p<0.05$) of the innate immune-related cytokines IL-8 and IL-15 only after treatment with AgNP-PTG complexes at 36h (Fig. 7). However, a significant increase ($p<0.05$) of IFNγ (main cytokine of the CeD adaptive immunity response) was seen after stimulation with digested gliadin at 4h (Fig. 7). It should be noted that we could not have information concerning the possible genetic predisposition (HLA-DQ2/8) of the specimens. We expected an

increase of the IL-8 and IL-15 cytokines at the longer time period, considering that AgNP are known to possess several immune modulating properties and that fetal tissue is usually more sensitive to external insult. This last reason could also explain the strong increase of IFNγ induced by PTG at 4h.

CONCLUSION

Our studies show that mNP form complex and stable aggregates with digested gliadin and that these mNP-PTG complexes can be internalized by intestinal crypt-like cells inducing alterations in the autophagic pathway. While the phenomenon was found to be patchy throughout the monolayer, because of the nature of the mNP-PTG aggregates themselves, it is speculated that this may be relevant to CeD pathology since only affecting a subset of epithelial cells in the monolayer may be sufficient to trigger a pathological response in the submucosa. Overall, one key element of our results is the demonstration that the combination of mNP with gliadin induces a more deleterious effect than with the single components.

METHODS

Enzymatic digested gliadin

Commercial wheat gliadin was subjected to peptic-tryptic digestion. The procedure has been previously described (submitted paper). Briefly, commercial gliadin was incubated with pepsin and trypsin for 4h under intestinal physiochemical conditions. Enzyme activity was then inhibited chemically. The solution was dialyzed, filtered and lyophilized. The powder was dissolved in sterile water at 50 mg/ml, aliquoted and stored at -80°C.

HIEC cell culture, stimulations and viability (MTT)

The crypt-like human intestinal epithelial cells (HIEC) were grown as described previously[15]. The commercial AgNP (730807; Sigma Aldrich) and AuNP (CG-15-XX; Cytodiagnostics) size was chosen according to the literature[41]. The concentration of the treatments performed was chosen according to previously obtained data (submitted paper). AgNPs were used a concentration of 5µg/ml, AuNPs at 25µg/ml, while PTG was used at 1mg/ml. Cells were seeded at a density of 156 x $10^3/cm^2$ in all assay performed, except for the MTT. After reaching confluence, cells were stimulated for 24h. For the MTT assay, cells were seeded onto 96-well plate with a density of 50 × 10^3 /well in 100ul medium. After stimulation, 3-(4,5-dimethylthiazolo-2-yl)-2,5-diphenytetrasolium bromide (Sigma) was added at 0.5mg/ml and incubated for 2h at 37°C in a 5% CO2 atmosphere. The formed formazan salts were dissolved in EtOH 100%

and the plates were read at 570 nm with a Spectra Max Plus 384 spectrophotometer (Molecular Devices).

Transmission Electronic Microscope (TEM)

Cell cultures were rinsed with PBS, fixed for 30 min with freshly prepared 1.5% glutaraldehyde sodiumcacodylate (0.1M, pH 7.4) at room temperature, and over night with 2.5% glutaraldehyde sodiumcacodylate (0.1M, pH 7.4) at 4°C. After they were washed twice with sodiumcacodylate (0.1M, pH 7.4) and post-fixed in uranyl acetate 1% overnight at 4°C (in darkness). At this point the HIEC cells were rinsed twice with distilled water and dehydrated with a growing percentage of EtOH solutions (from 40% to 100%). Cells were then epon-embedded and put on carbon-coated copper grids. Staining with uranyl acetate 2% was performed and photographs were taken using a HITACHI H-7500 transmission electron microscope.

Fetal specimens were directly put into 1.5% glutaraldehyde sodiumcacodylate (0.1M, pH 7.4) and fixed for at least 24h at 4°C. They were then washed twice and post-fixed in OsO_4 1%-sodiumcacodylate (0.1M, pH 7.4). After rinsing with distilled water, samples were dehydrated with a growing percentage of EtOH solutions (from 70% to 100%). They were then epon-embedded and stained with uranyl acetate 2% and lead citrate. Semi-thin sections were observed with a light microscope.

Protein extraction and Western Blot (WB)

Total proteins were extracted in Laemmli 1X buffer. 30 ug of proteins were loaded for Western blot analyses, and run on SDS-PAGE gels under denaturing conditions. Proteins were separated on 12% gels and electro-transferred onto a nitrocellulose membrane. Nonspecific protein binding was blocked using 10% Blotto-0.1% Tween PBS followed by incubation with primary antibodies (anti-LC3, L8918 Sigma-Aldrich; anti-p62, 610832 BD Biosciences; anti-Beclin1, ab207612 Abcam) diluted in the blocking solution, overnight at 4°C. Horseradish peroxidase-conjugated secondary antibodies (anti-mouse (LNA931V, GE Healthcare), anti-rabbit (LNA934V, GE Healthcare) were used to detect the primary antibodies and the signal was developed using the Immobilon Western1 kit (Millipore, WBKLSO100).

Indirect immunofluorescence and phase contrast assay

For immunofluorescence, HIEC cells were fixed in MeOH for 10 min at −20°C and nonspecific sites were blocked for 1 h at room temperature with 10% Blotto-PBS (pH 7.4). Primary antibodies were diluted 1:1000 in blocking solution containing 0.05% azide and incubated overnight at 4°C. The secondary antibodies were used at 1:400 dilution in the blocking solution and incubated at room temperature for 1 h. Nuclei were stained with DAPI 1:3000 in PBS for 3minutes at room temperature. The slides were viewed with a DMRXA microscope (Leica) equipped for epifluorescence and digital imaging (RTE/CCD Y/Hz-I300 cooled camera). Images were acquired

using MetaMorph software (Universal Imaging Corporation) with 20x and 40x objectives and images were modified using Photoshop software (Adobe).

Organ culture

Small intestinal jejunum was obtained from four fetuses ranging from 17 to 19 weeks following legal or therapeutic pregnancy termination with informed patient consent. Samples were prepared as previously described[42]. Briefly, the small intestine was cleansed of mesentery, split longitudinally, washed in culture medium (LeibovitzL-15 supplemented with amphotericin at 40µg/ml and mycostatin at 40µg/ml), and cut into explants. The explants were then transferred, with the mucosal side up, onto lens paper that covered a stainless steel grid lying over the central well of a Falcon organ culture dish. Two culture dishes containing approximately 6–8 explants (3X7 mm) were prepared for each experimental condition. AgNP were added a concentration of 5ug/ml, AuNP at 25ug/ml and PTG at 1mg/ml and they were maintained in culture (incubator at 37°C, 5% CO_2-95% air and saturated water vapour) for 4h and 36h, respectively. To eliminate possible residual activity of digestive enzymes, PTG was incubated 30 minutes at 100° C, before being used. Although the number of obtained fetuses was four, we only have n=2 for the experiments, since the others were used to fine-tune the experimental conditions. Studies were approved by the Institutional Review Committee for the use of human material from the “Centre

Hospitalier Universitaire de Sherbrooke/Faculté de Médecine et des Sciences de la Santé".

RNA extraction, RT and quantitative PCR

After the stimulations, HIEC cells were lysed with RiboZol (Amresco).Total RNA from the fetal small intestine specimens was instead extracted with the RNeasy Mini Kit (74104 Qiagen). The extracted RNA was treated with DNAse (18068-015) and RT-PCR was performed using SuperScript II (18064-014 Invitrogen) according to the manufacturer's instruction. Real-time PCR amplification was performed using Mx3000P qPCR system (Stratagene). Differences in gene expression were evaluated by comparing untreated vs treated samples for a given intestinal segment using the Pfaffl equation[43]. HPRT1 and PPIA genes were used for normalization. All samples were run in duplicate and the no template control did not show an amplification product.

Statistical analyses

Except for the fetal specimens, independent experiments were repeated at least 3 times. Kruskal-Wallis statistical test was performed to compare the different conditions. Statistical analyses were performed using SigmaPlot 12.5 or Prism 7 (GraphPad) software.

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Figure Legends

Figure 1: TEM photographs of HIEC cells. A) Untreated cells B) 24h treatment with digested gliadin 1 mg/ml C) 24h treatment with AuNP 25ug/ml + PTG 1 mg/ml D) 24h treatement with AgNP 5ug/ml + PTG1 mg/ml

Figure 2: WB analyses. A) Representative WB analysis of p62 (MW 62 kDa), Beclin1 (MW 52 kDa), LC3-I (MW 16-18 kDa) and LC3-II (MW 14-16 kDa) proteins after stimulation of HIEC cells for 24h. B) LC3-II protein relative quantification C) p62 protein relative quantification D) Beclin1 protein relative quantification

Figure 3: Phase-contrast microscopy (20X magnification). A) HIEC cells treated with AuNP 25ug/ml B) HIEC cells treated with AgNP 5ug/ml C) HIEC cells treated with AuNP 25ug/ml + PTG 1mg/ml D) HIEC cells treated with AgNP 5ug/ml + PTG 1mg/ml

Figure 4: Representative immunofluorescence staining of LC3 (left panel), p62 (central panel) and their overlay (right panel). A)untreated cells B) 24h treatment with digested gliadin 1 mg/ml C) 24h treatment with AuNP 25ug/ml + PTG1 mg/ml D) 24h treatment with AgNP 5ug/ml + PTG1 mg/ml, and E) 2h treatment with 50nM bafilomycin (positive control).

Figure 5: Quantitative RT-PCR HIEC cells. Data are expressed according to the Pfaffl ratio (HPRT1 and PPIA housekeeping genes) and are normalized against the negative control (untreated cells). A) IL8 transcript level, B) IL15 transcript level, C) MMP2 transcript level, D) MMP7 transcript level.

Figure 6: Light microscopy of fetal jejunum explants. A) untreated B) 4h treatment with PTG 1mg/ml C) 4h treatment with AuNP 25ug/ml + PTG 1 mg/ml D) 4h treatment with AgNP 5ug/ml + PTG 1 mg/ml.

Figure 7: Quantitative RT-PCR fetal samples 4h and 36h. Data are expressed according to the Pfaffl ratio (HPRT1 and PPIA housekeeping genes) and are normalized against the negative control at each time-point. From above: IL-8, IL-15 and IFNγ transcript levels.

Supplementary Figure Legend

Figure S1: TEM photographs of HIEC cells. A) 24h treatment with AuNP 25ug/ml B) 24h treatment with AgNP 5ug/ml C) 2h treatment with 50nM Bafilomycin (positive control)

Figure S2: A) Quantitative RT-PCR HIEC cells: SQSTM1 transcript level B) Quantitative RT-PCR HIEC cells: BECN1 transcript level C) MTT assay. Data are expressed as % of the negative control (untreated HIEC cells)

Figure S3a: Enlargement of Figure 4. Representative immunofluorecence staining of the p62-LC3 protein overlay after 24h

of stimulation with AgNP 5ug/ml plus PTG 1mg/ml. Arrow: cells with protein co-localization dots.

Figure S3b: Enlargement of Figure 4. Representative immunofluorecence staining of the p62-LC3 protein overlay after 24h of stimulation with AuNP 25ug/ml plus PTG 1mg/ml. Arrow: cells with protein co-localization dots.

Figure 1

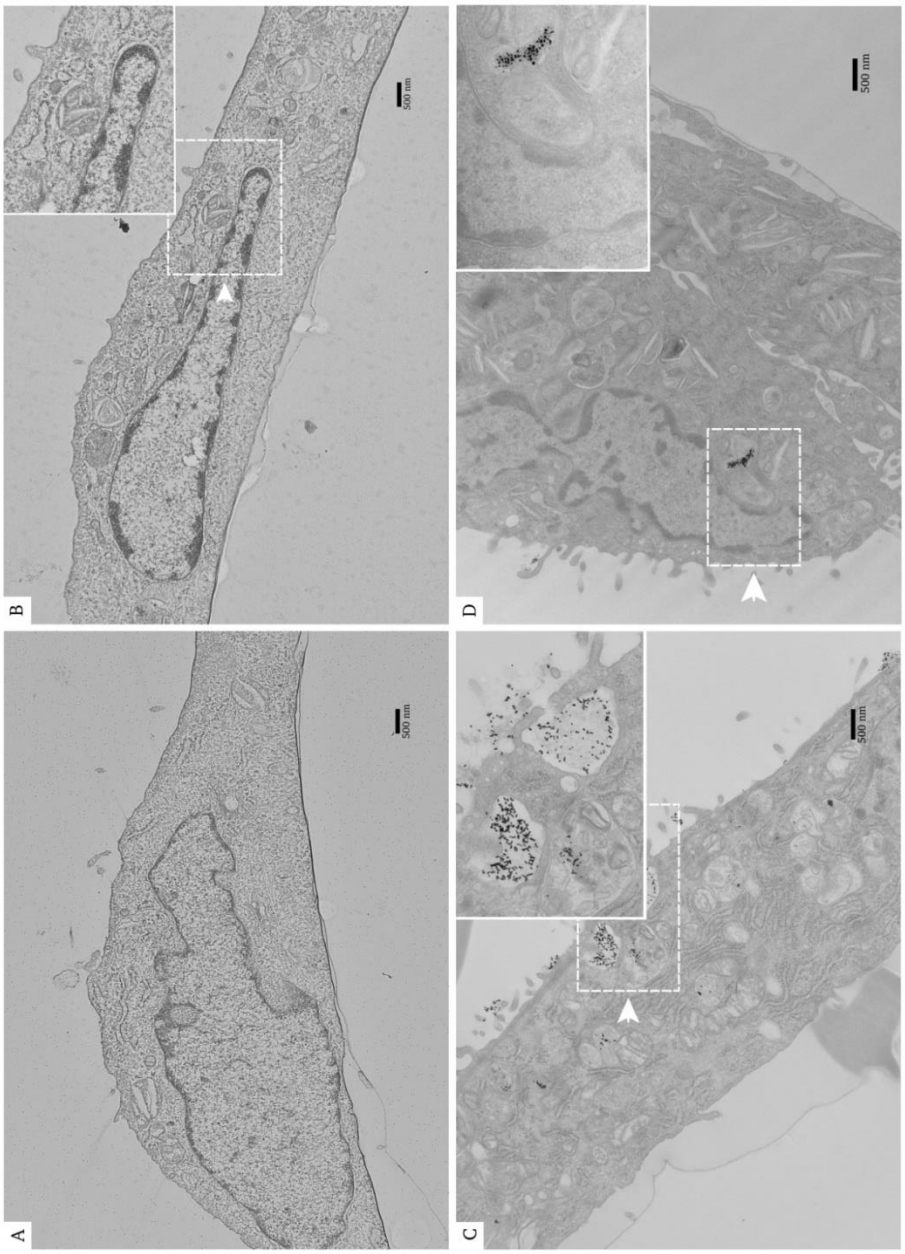


Figure 2

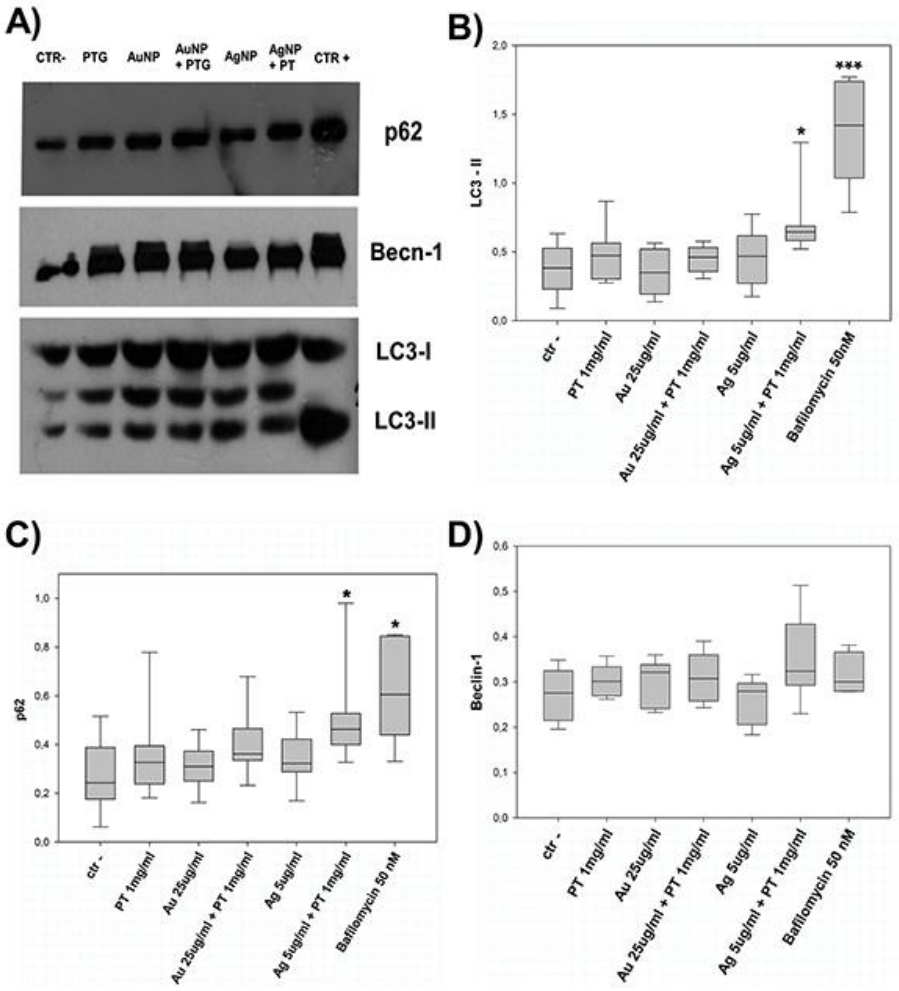

A)
CTR-
PTG
AuNP
AuNP + PTG
AgNP
AgNP + PT
CTR +
p62
Becn-1
LC3-I
LC3-II
B)
LC3 - II
ctr -
PT 1mg/ml
Au 25ug/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml
Ag 5ug/ml + PT 1mg/ml
Bafilomycin 50nM
C)
p62
ctr -
PT 1mg/ml
Au 25ug/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml
Ag 5ug/ml + PT 1mg/ml
Bafilomycin 50 nM
D)
Beclin-1
ctr -
PT 1mg/ml
Au 25ug/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml
Ag 5ug/ml + PT 1mg/ml
Bafilomycin 50 nM

Figure 3

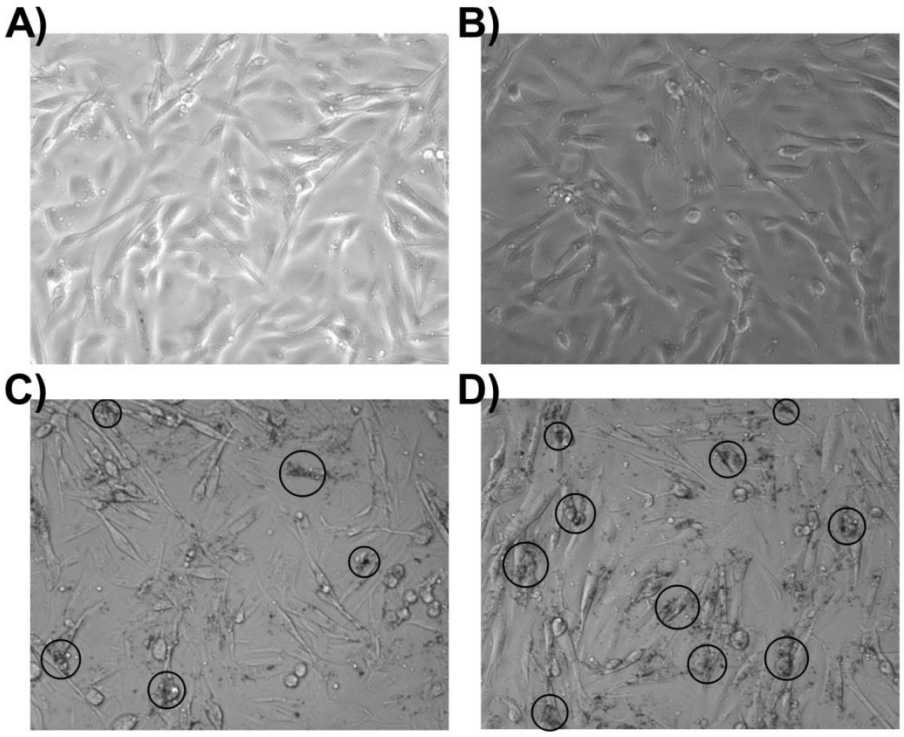


Figure 4

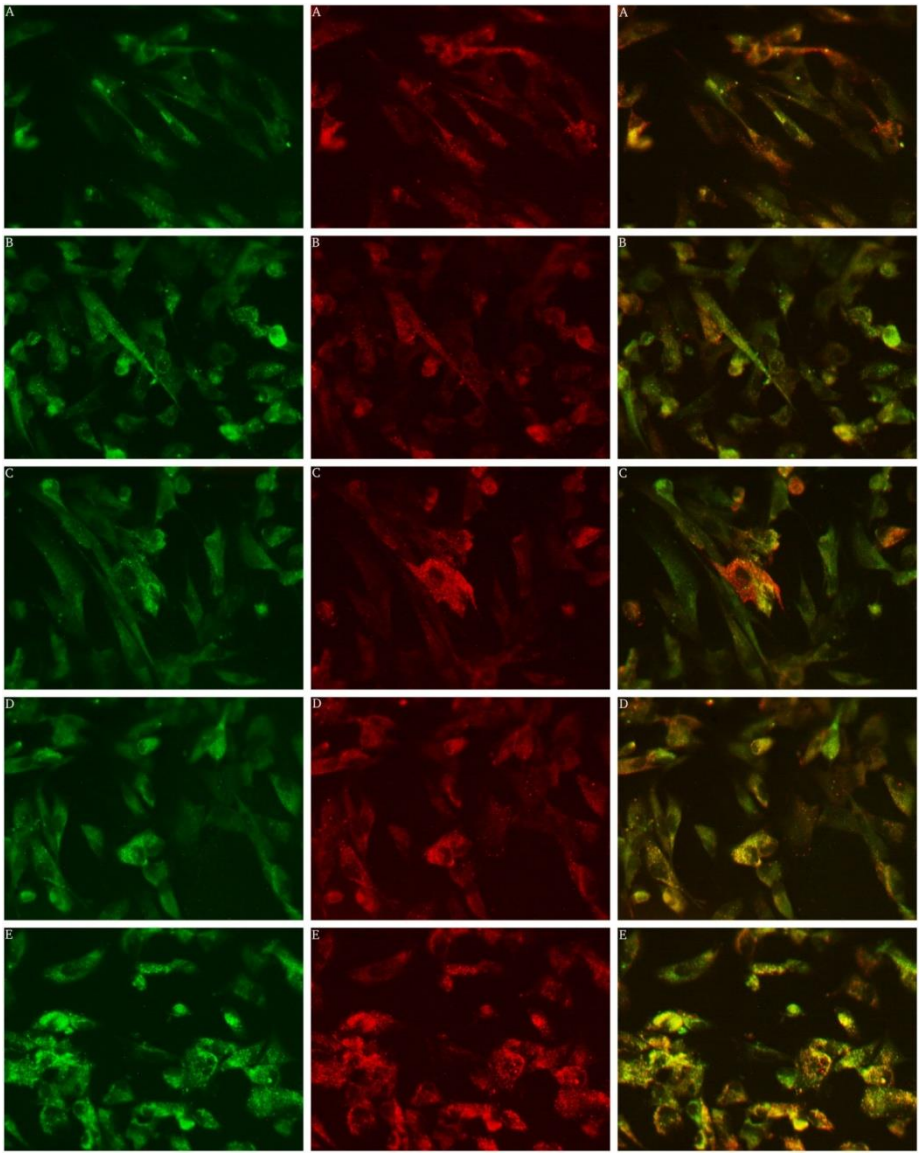


Figure 5

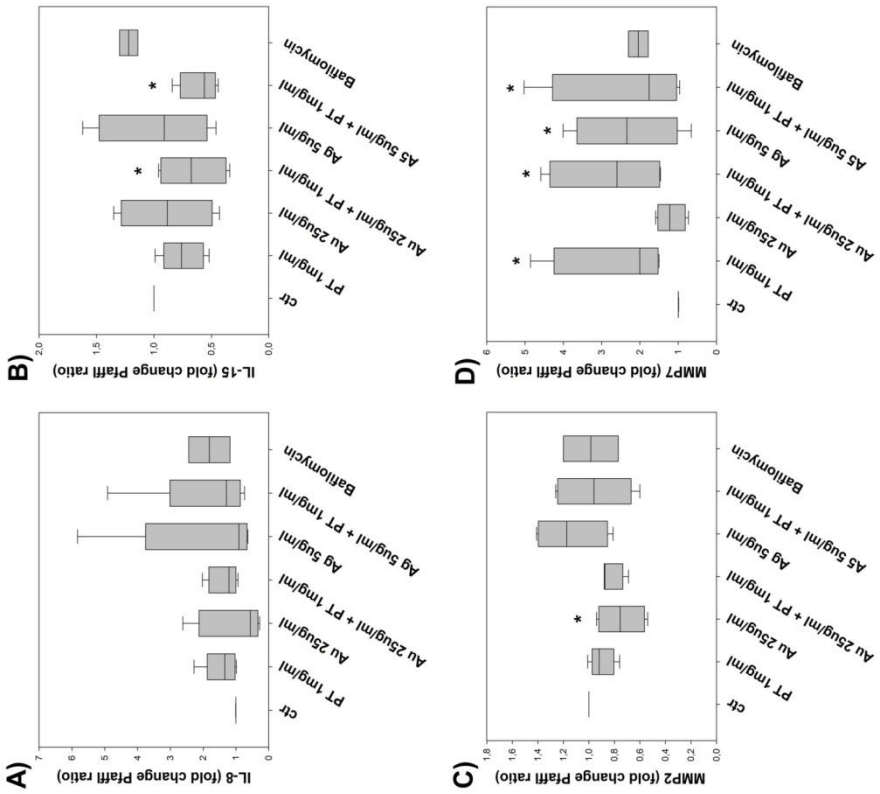

A)
IL-8 (fold change Pfaffl ratio)
B)
IL-15 (fold change Pfaffl ratio)
C)
MMP2 (fold change Pfaffl ratio)
D)
MMP7 (fold change Pfaffl ratio)
ctr
PT 1mg/ml
Au 25ug/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml
As 5ug/ml + PT 1mg/ml
Bafilomycin

Figure 6

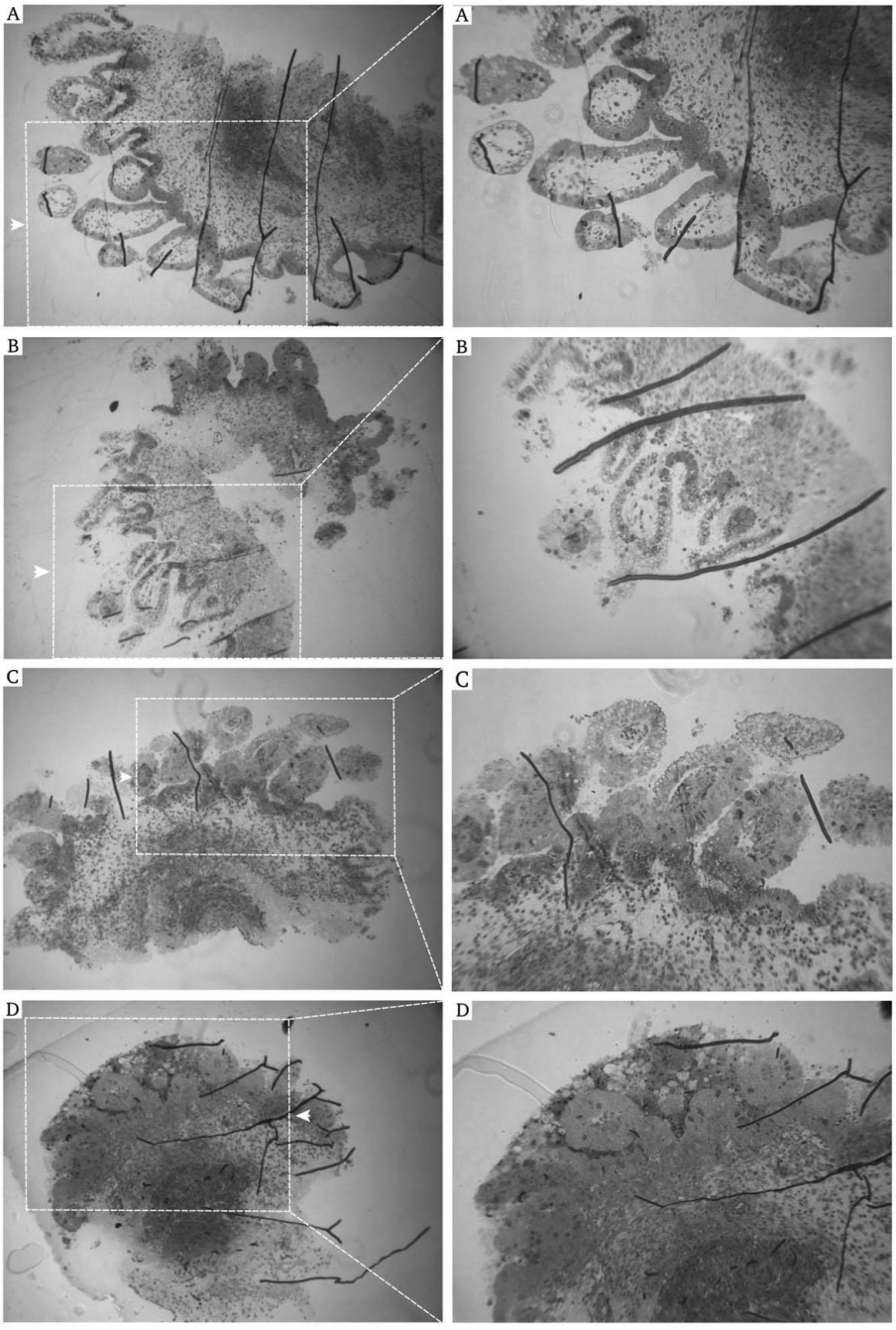
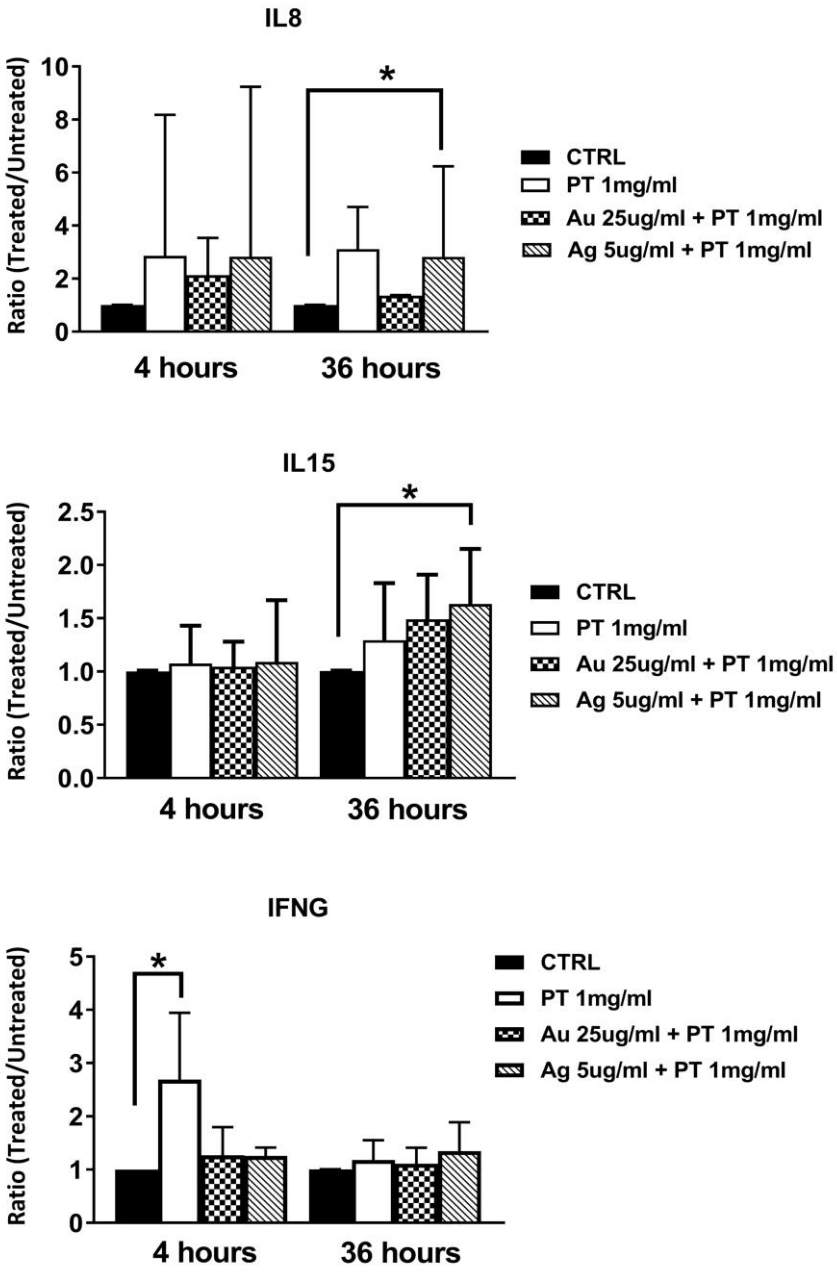
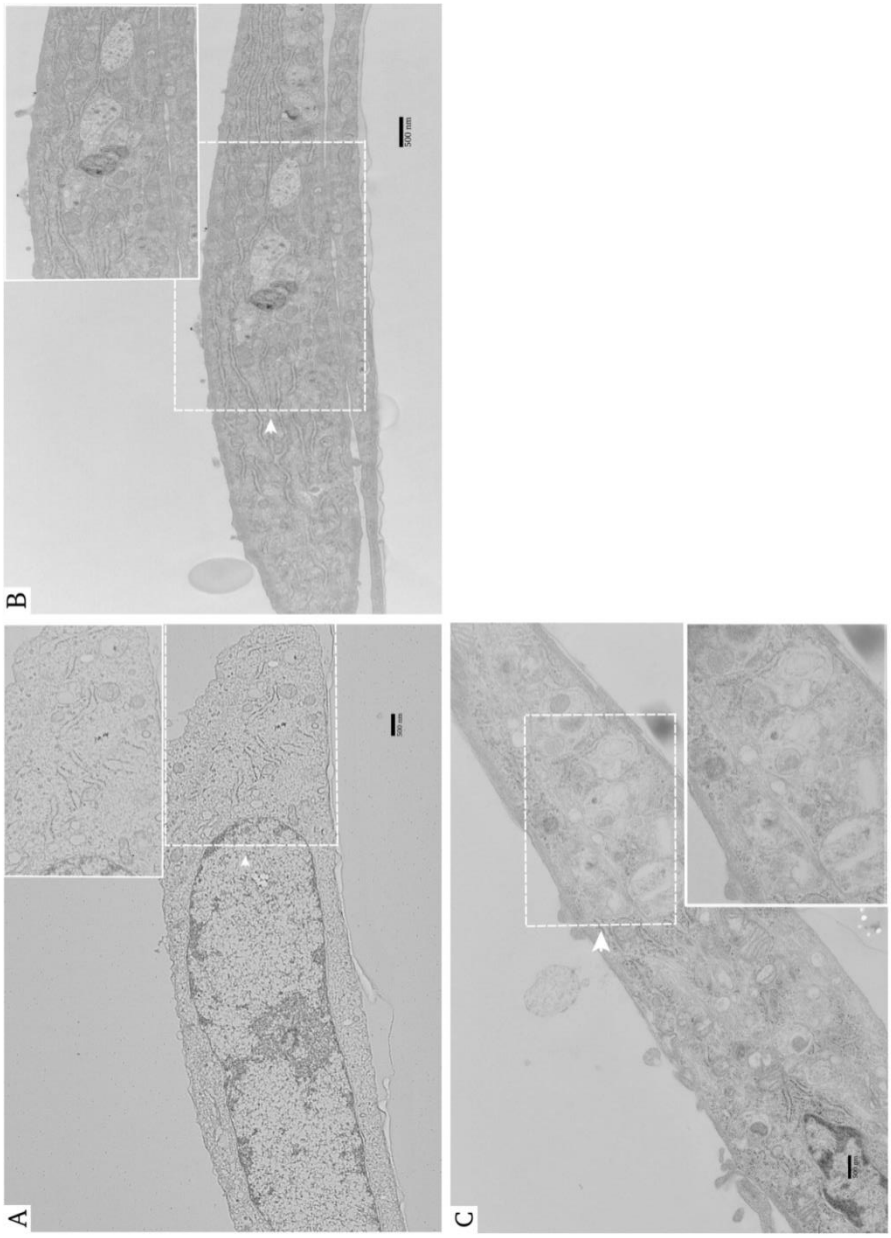


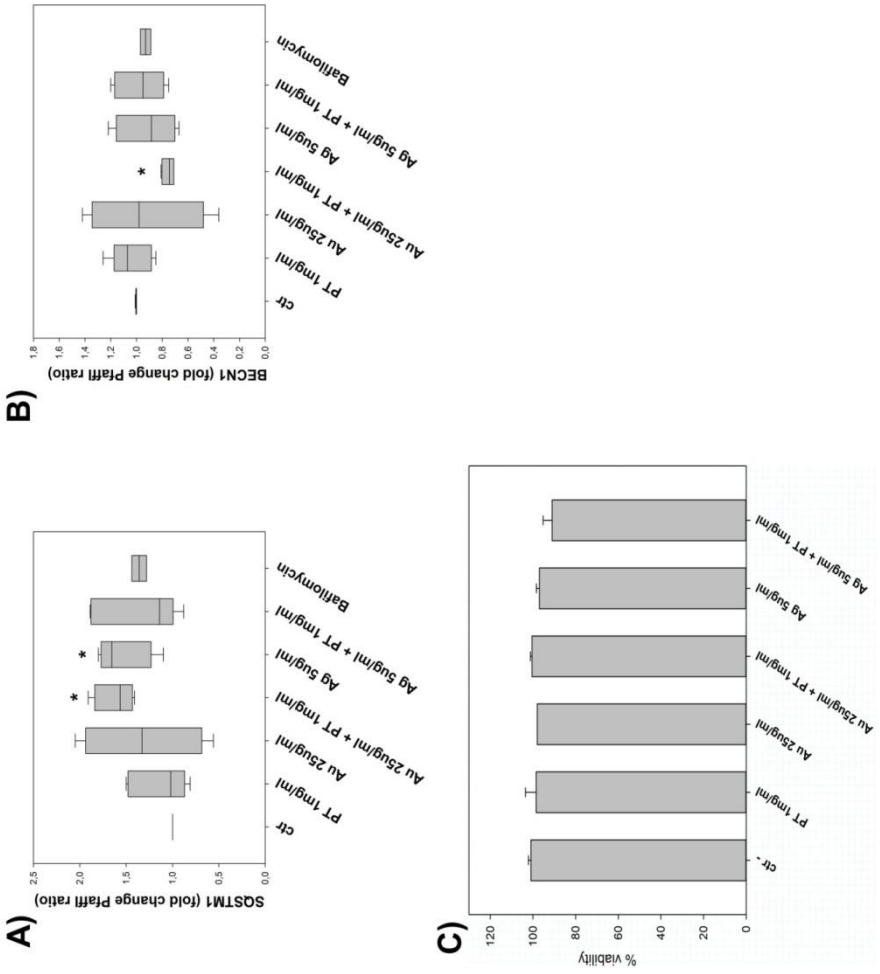
Figure 7


IL8
Ratio (Treated/Untreated)
10
8
6
4
2
0
*
4 hours
36 hours
CTRL
PT 1mg/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml + PT 1mg/ml
IL15
Ratio (Treated/Untreated)
2.5
2.0
1.5
1.0
0.5
0.0
*
4 hours
36 hours
CTRL
PT 1mg/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml + PT 1mg/ml
IFNG
Ratio (Treated/Untreated)
5
4
3
2
1
0
*
4 hours
36 hours
CTRL
PT 1mg/ml
Au 25ug/ml + PT 1mg/ml
Ag 5ug/ml + PT 1mg/ml

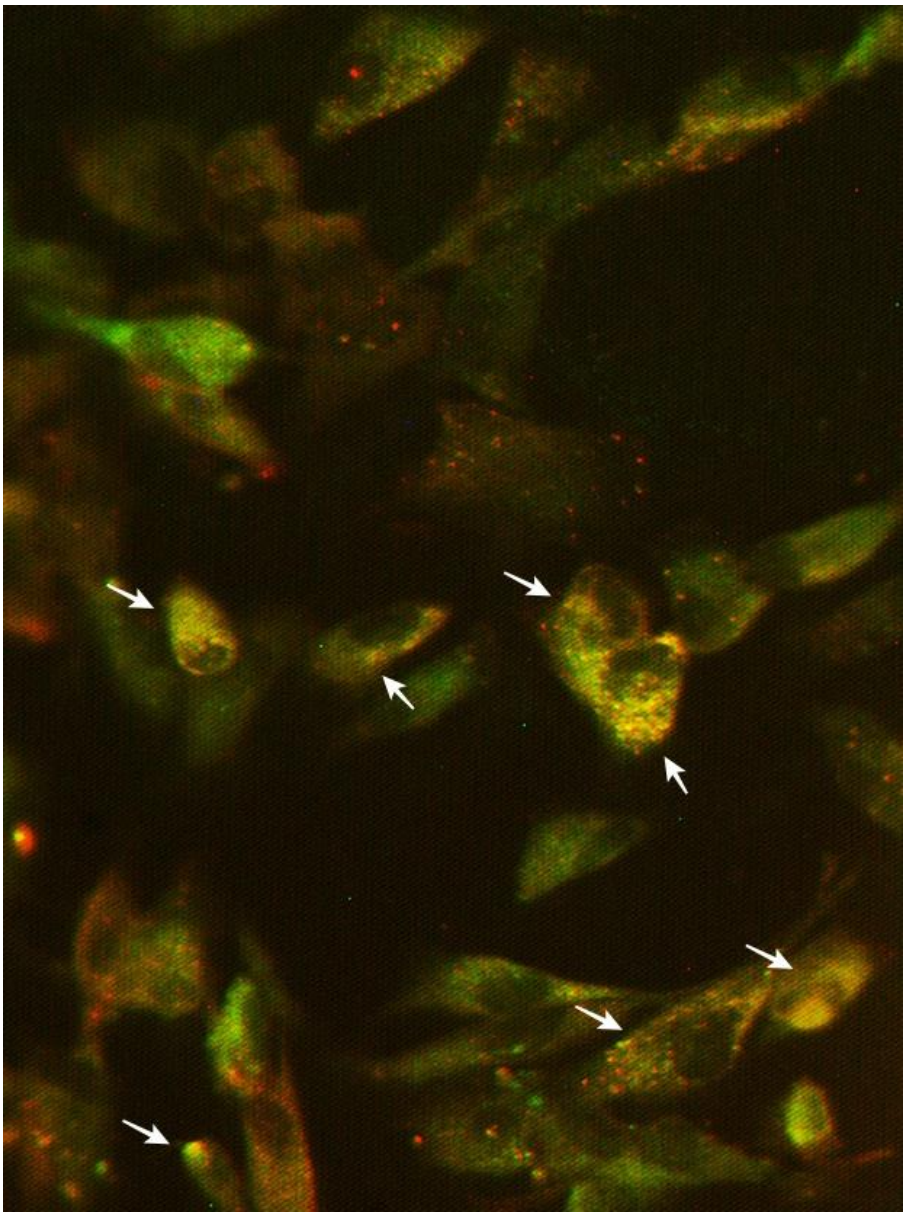
Supplementary Figure S1



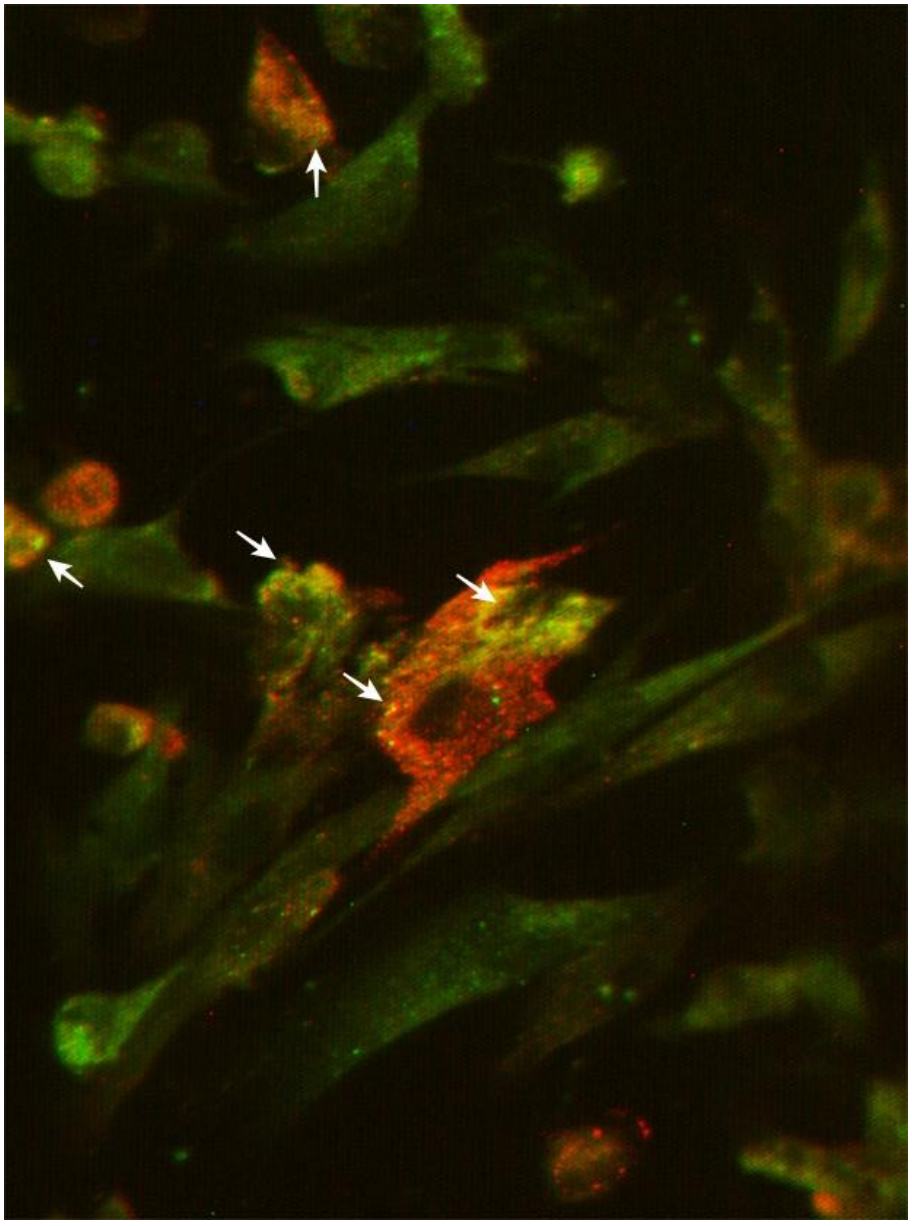
Supplementary Figure S2



Supplementary Figure S3a



Supplementary Figure S3b



References

1. Urade, R.; Sato, N.; Sugiyama, M., Gliadins from wheat grain: an overview, from primary structure to nanostructures of aggregates. *Biophysical Reviews* **2017,** *10* (2), 435-443.

2. Megiorni, F.; Mora, B.; Bonamico, M.; Barbato, M.; Nenna, R.; Maiella, G.; Lulli, P.; Mazzilli, M. C., HLA-DQ and risk gradient for celiac disease. *Human Immunology* **2009,** *70* (1), 55-59.

3. (a) Mustalahti, K.; Catassi, C.; Reunanen, A.; Fabiani, E.; Heier, M.; McMillan, S.; Murray, L.; Metzger, M.-H.; Gasparin, M.; Bravi, E.; Mäki, M., The prevalence of celiac disease in Europe: Results of a centralized, international mass screening project. *Annals of Medicine* **2010,** *42* (8), 587-595; (b) Fasano, A.; Berti, I.; Gerarduzzi, T.; al., e., Prevalence of Celiac Disease in At-Risk and Not-At-Risk Groups in the United States: A large multicenter study. *Arch Intern Med* **2003,** *163* (3), 286-92.

4. (a) Lerner, A.; Arleevskaya, M.; Schmiedl, A.; Matthias, T., Microbes and Viruses Are Bugging the Gut in Celiac Disease. Are They Friends or Foes? *Frontiers in Microbiology* **2017,** *8*; (b) Stene, L. C.; Honeyman, M. C.; Hoffenberg, E. J.; Haas, J. E.; Sokol, R. J.; Emery, L.; Taki, I.; Norris, J. M.; Erlich, H. A.; Eisenbarth, G. S.; Rewers, M., Rotavirus Infection Frequency and Risk of Celiac Disease Autoimmunity in Early Childhood: A Longitudinal Study. *The American Journal of Gastroenterology* **2006,** *101* (10), 2333-2340.

5. Drago, S.; El Asmar, R.; Di Pierro, M.; Clemente, M. G.; Tripathi, A.; Sapone, A.; Thakar, M.; Iacono, G.; Carroccio, A.; D'Agate,

C.; Not, T.; Zampini, L.; Catassi, C.; Fasano, A., Gliadin, zonulin and gut permeability: Effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. *Scandinavian Journal of Gastroenterology* **2006,** *41*, 408-419.

6. Schumann, M.; Siegmund, B.; Schulzke, J. D.; Fromm, M., Celiac Disease: Role of the Epithelial Barrier. *Cellular and Molecular Gastroenterology and Hepatology* **2017,** *3* (2), 150-162.

7. (a) Caminero, A.; Nistal, E.; Herrán, A. R.; Pérez-Andrés, J.; Ferrero, M. A.; Vaquero Ayala, L.; Vivas, S.; Ruiz de Morales, J. M. G.; Albillos, S. M.; Casqueiro, F. J., Differences in gluten metabolism among healthy volunteers, coeliac disease patients and first-degree relatives. *British Journal of Nutrition* **2015,** *114* (08), 1157-1167; (b) Caminero, A.; Galipeau, H. J.; McCarville, J. L.; Johnston, C. W.; Bernier, S. P.; Russell, A. K.; Jury, J.; Herran, A. R.; Casqueiro, J.; Tye-Din, J. A.; Surette, M. G.; Magarvey, N. A.; Schuppan, D.; Verdu, E. F., Duodenal Bacteria From Patients With Celiac Disease and Healthy Subjects Distinctly Affect Gluten Breakdown and Immunogenicity. *Gastroenterology* **2016,** *151* (4), 670-683.

8. Lerner, A.; Matthias, T., Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. *Autoimmunity Reviews* **2015,** *14* (6), 479-489.

9. (a) Koeneman, B. A.; Zhang, Y.; Westerhoff, P.; Chen, Y.; Crittenden, J. C.; Capco, D. G., Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. *Cell Biology and Toxicology* **2010,** *26* (3), 225-238; (b) Stern, S. T.; Adiseshaiah, P. P.;

Crist, R. M., Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. *Particle and Fibre Toxicology* **2012,** *9* (1), 20; (c) Luo, Y.-H.; Chang, L. W.; Lin, P., Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications. *BioMed Research International* **2015,** *2015*, 1-12.

10. (a) Ruiz, P. A.; Morón, B.; Becker, H. M.; Lang, S.; Atrott, K.; Spalinger, M. R.; Scharl, M.; Wojtal, K. A.; Fischbeck-Terhalle, A.; Frey-Wagner, I.; Hausmann, M.; Kraemer, T.; Rogler, G., Titanium dioxide nanoparticles exacerbate DSS-induced colitis: role of the NLRP3 inflammasome. *Gut* **2017,** *66* (7), 1216-1224; (b) Bettini, S.; Boutet-Robinet, E.; Cartier, C.; Coméra, C.; Gaultier, E.; Dupuy, J.; Naud, N.; Taché, S.; Grysan, P.; Reguer, S.; Thieriet, N.; Réfrégiers, M.; Thiaudière, D.; Cravedi, J.-P.; Carrière, M.; Audinot, J.-N.; Pierre, F. H.; Guzylack-Piriou, L.; Houdeau, E., Food-grade TiO2 impairs intestinal and systemic immune homeostasis, initiates preneoplastic lesions and promotes aberrant crypt development in the rat colon. *Scientific Reports* **2017,** *7*, 40373.

11. (a) Arora, S.; Sharma, P.; Kumar, S.; Nayan, R.; Khanna, P. K.; Zaidi, M. G. H., Gold-nanoparticle induced enhancement in growth and seed yield of Brassica juncea. *Plant Growth Regulation* **2012,** *66* (3), 303-310; (b) Yang, J.; Jiang, F.; Ma, C.; Rui, Y.; Rui, M.; Adeel, M.; Cao, W.; Xing, B., Alteration of Crop Yield and Quality of Wheat upon Exposure to Silver Nanoparticles in a Life Cycle Study. *Journal of Agricultural and Food Chemistry* **2018,** *66* (11), 2589-2597; (c) Kumar, V.; Guleria, P.; Kumar, V.; Yadav, S. K., Gold nanoparticle exposure

induces growth and yield enhancement in Arabidopsis thaliana. *Science of The Total Environment* **2013,** *461-462*, 462-468; (d) Prosa, M.; Tessarolo, M.; Bolognesi, M.; Margeat, O.; Gedefaw, D.; Gaceur, M.; Videlot-Ackermann, C.; Andersson, M. R.; Muccini, M.; Seri, M.; Ackermann, J., Enhanced Ultraviolet Stability of Air-Processed Polymer Solar Cells by Al Doping of the ZnO Interlayer. *ACS Applied Materials & Interfaces* **2016,** *8* (3), 1635-1643; (e) Verleysen, E.; Van Doren, E.; Waegeneers, N.; De Temmerman, P. J.; Abi Daoud Francisco, M.; Mast, J., TEM and SP-ICP-MS Analysis of the Release of Silver Nanoparticles from Decoration of Pastry. *Journal of Agricultural and Food Chemistry* **2015,** *63* (13), 3570-3578; (f) Athinarayanan, J.; Alshatwi, A. A.; Periasamy, V. S.; Al-Warthan, A. A., Identification of Nanoscale Ingredients in Commercial Food Products and their Induction of Mitochondrially Mediated Cytotoxic Effects on Human Mesenchymal Stem Cells. *Journal of Food Science* **2015,** *80* (2), N459-N464; (g) Vance, M. E.; Kuiken, T.; Vejerano, E. P.; McGinnis, S. P.; Hochella, M. F.; Rejeski, D.; Hull, M. S., Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. *Beilstein Journal of Nanotechnology* **2015,** *6*, 1769-1780; (h) Fogli S; Montis C; Paccosi S; Silvano S; Michelucci E; Berti D; Bosi A; and, P. A.; P, R., Inorganic nanoparticles as potential regulators of immune response in dendritic cells. *Nanomedicine* **2017,** *12* (14); (i) Ren, X.; Chen, Y.; Peng, H.; Fang, X.; Zhang, X.; Chen, Q.; Wang, X.; Yang, W.; Sha, X., Blocking Autophagic Flux Enhances Iron Oxide Nanoparticles Photothermal Therapeutic Efficiency in Cancer Treatment. *ACS Applied Materials & Interfaces* **2018**.

12. (a) Yang, F. M.; Li, H. M.; Li, F.; Xin, Z. H.; Zhao, L. Y.; Zheng, Y. H.; Hu, Q. H., Effect of Nano-Packing on Preservation Quality of Fresh Strawberry (Fragaria ananassa Duch. cv Fengxiang) during Storage at 4 °C. *Journal of Food Science* **2010,** *75* (3), C236-C240; (b) Kim, J. S.; Kuk, E.; Yu, K. N.; Kim, J.-H.; Park, S. J.; Lee, H. J.; Kim, S. H.; Park, Y. K.; Park, Y. H.; Hwang, C.-Y.; Kim, Y.-K.; Lee, Y.-S.; Jeong, D. H.; Cho, M.-H., Antimicrobial effects of silver nanoparticles. *Nanomedicine: Nanotechnology, Biology and Medicine* **2007,** *3* (1), 95-101; (c) Nawrocka, A.; Cieśla, J., Influence of silver nanoparticles on food components in wheat. *International Agrophysics* **2013,** *27* (1), 49-55.

13. Pereira, D. I. A.; Bruggraber, S. F. A.; Faria, N.; Poots, L. K.; Tagmount, M. A.; Aslam, M. F.; Frazer, D. M.; Vulpe, C. D.; Anderson, G. J.; Powell, J. J., Nanoparticulate iron(III) oxo-hydroxide delivers safe iron that is well absorbed and utilised in humans. *Nanomedicine: Nanotechnology, Biology and Medicine* **2014,** *10* (8), 1877-1886.

14. (a) Cao, Y.; Roursgaard, M.; Kermanizadeh, A.; Loft, S.; Møller, P., Synergistic Effects of Zinc Oxide Nanoparticles and Fatty Acids on Toxicity to Caco-2 Cells. *International Journal of Toxicology* **2015,** *34* (1), 67-76; (b) Martirosyan, A.; Bazes, A.; Schneider, Y.-J., In vitrotoxicity assessment of silver nanoparticles in the presence of phenolic compounds – preventive agents against the harmful effect? *Nanotoxicology* **2014,** *8* (5), 573-582.

15. Perreault, N.; Beaulieu, J.-F., Use of the Dissociating Enzyme Thermolysin to Generate Viable Human Normal Intestinal Epithelial Cell Cultures. *Experimental Cell Research* **1996,** *224*, 354-64.

16. Guezguez, A.; Paré, F.; Benoit, Y. D.; Basora, N.; Beaulieu, J.-F., Modulation of stemness in a human normal intestinal epithelial crypt cell line by activation of the WNT signaling pathway. *Experimental Cell Research* **2014,** *322* (2), 355-364.

17. Benoit, Y. D.; Parè, F.; Francoeur, C.; Jean, D.; Tremblay, E.; Boudreau, F.; Escaffit, F.; Beaulieu, J.-F., Cooperation between HNF-1α, Cdx2, and GATA-4 in initiating an enterocytic differentiation program in a normal human intestinal epithelial progenitor cell line. *Am J Physiol Gastrointest Liver Physiol* **2010,** *298*, G504-517.

18. Ferron, P.-J.; Dumazeau, K.; Beaulieu, J.-F.; Le Hégarat, L.; Fessard, V., Combined Effects of Lipophilic Phycotoxins (Okadaic Acid, Azapsiracid-1 and Yessotoxin) on Human Intestinal Cells Models. *Toxins* **2016,** *8* (2), 50.

19. (a) Reggiori, F.; Ungermann, C., Autophagosome Maturation and Fusion. *Journal of Molecular Biology* **2017,** *429* (4), 486-496; (b) Ding, W.-X.; Yin, X.-M., Sorting, recognition and activation of the misfolded protein degradation pathways through macroautophagy and the proteasome. *Autophagy* **2014,** *4* (2), 141-150.

20. (a) Comincini, S.; Manai, F.; Meazza, C.; Pagani, S.; Martinelli, C.; Pasqua, N.; Pelizzo, G.; Biggiogera, M.; Bozzola, M., Identification of Autophagy-Related Genes and Their Regulatory miRNAs Associated with Celiac Disease in Children. *International Journal of Molecular Sciences* **2017,** *18* (2), 391; (b) Manai, F.; Azzalin, A.; Gabriele, F.; Martinelli, C.; Morandi, M.; Biggiogera, M.; Bozzola, M.; Comincini, S., The In Vitro Effects of Enzymatic Digested Gliadin on the Functionality of the Autophagy Process. *International Journal of*

Molecular Sciences **2018,** *19* (2), 635; (c) Rajaguru, P.; Vaiphei, k.; Saikia, B.; Kochhar, R., Increased accumulation of dendritic cells in celiac disease associates with increased expression of autophagy protein LC3. *Indian journal of Pathology and Microbiology* **2013,** *56* (4), 342-48.

21. Groulx, J.-F.; Khalfaoui, T.; Benoit, Y. D.; Bernatchez, G.; Carrier, J. C.; Basora, N.; Beaulieu, J.-F., Autophagy is active in normal colon mucosa. *Autophagy* **2014,** *8* (6), 893-902.

22. Yamamoto, A.; Tagawa, Y.; Yoshimori, T.; Moriyama, Y.; Masaki, R.; Tashiro, Y., Bafilomycin A1 Prevents Maturation of Autophagic Vacuoles by Inhibiting Fusion between Autophagosomes and Lysosomes in Rat Hepatoma Cell Line, H-4-II-E Cells. *Cell structure and function* **1998,** *23*, 33-42.

23. (a) Klionsky, D. J.; et al, Guidelines for the use and interpretation of assays for monitoring autophagy (3rd edition). *Autophagy* http://www.tandfonline.com/loi/kaup20 **2015**; (b) Berg, T. O.; Fengsrud, M.; Strømhaug, P. E.; Berg, T.; Seglen, P. O., Isolation and Characterization of Rat Liver Amphisomes: EVIDENCE FOR FUSION OF AUTOPHAGOSOMES WITH BOTH EARLY AND LATE ENDOSOMES. *The Journal of Biological Chemistry* **1998,** *273* (34), 21883-21892.

24. Huang, D.; Zhou, H.; Gao, J., Nanoparticles modulate autophagic effect in a dispersity-dependent manner. *Scientific Reports* **2015,** *5* (1).

25. Kabeya, Y.; Mizushima, N.; Ueno, T.; Yamamoto, A.; Noda, T.; Kominami, E.; Ohsumi, Y.; Yoshimori, T., LC3, a mammalian

homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO Journal* **2000,** *19* (21), 5720-28.

26. Kimura, S.; Fujita, N.; Noda, T.; Yoshimori, T., Chapter 1 Monitoring Autophagy in Mammalian Cultured Cells through the Dynamics of LC3. **2009,** *452*, 1-12.

27. Evans, T.; Button, R.; Anichtchik, O.; Luo, S., Visualization and Measurement of Multiple Components of the Autophagy Flux. **2018,** *1854*, 1-12.

28. (a) Shaid, S.; Brandts, C. H.; Serve, H.; Dikic, I., Ubiquitination and selective autophagy. *Cell Death & Differentiation* **2012,** *20* (1), 21-30; (b) John, T.; Gladytz, A.; Kubeil, C.; Martin, L. L.; Risselada, H. J.; Abel, B., Impact of nanoparticles on amyloid peptide and protein aggregation: a review with a focus on gold nanoparticles. *Nanoscale* **2018**.

29. He, R.; Peng, J.; Yuan, P.; Xu, F.; Wei, W., Divergent roles of BECN1 in LC3 lipidation and autophagosomal function. *Autophagy* **2015,** *11* (5), 740-747.

30. (a) Gonzalez-Polo, R. A., The apoptosis/autophagy paradox: autophagic vacuolization before apoptotic death. *Journal of Cell Science* **2005,** *118* (14), 3091-3102; (b) Boya, P.; Gonzalez-Polo, R. A.; Casares, N.; Perfettini, J. L.; Dessen, P.; Larochette, N.; Metivier, D.; Meley, D.; Souquere, S.; Yoshimori, T.; Pierron, G.; Codogno, P.; Kroemer, G., Inhibition of Macroautophagy Triggers Apoptosis. *Molecular and Cellular Biology* **2005,** *25* (3), 1025-1040.

31. Bellmann, S.; Carlander, D.; Fasano, A.; Momcilovic, D.; Scimeca, J. A.; Waldman, W. J.; Gombau, L.; Tsytsikova, L.; Canady, R.; Pereira, D. I. A.; Lefebvre, D. E., Mammalian gastrointestinal tract parameters modulating the integrity, surface properties, and absorption of food-relevant nanomaterials. *Wiley Interdisciplinary Reviews: Nanomedicine and Nanobiotechnology* **2015,** *7* (5), 609-622.

32. (a) Barone, M. V.; Nanayakkara, M.; Paolella, G.; Maglio, M.; Vitale, V.; Troiano, R.; Ribecco, M. T. S.; Lania, G.; Zanzi, D.; Santagata, S.; Auricchio, R.; Troncone, R.; Auricchio, S., Gliadin Peptide P31-43 Localises to Endocytic Vesicles and Interferes with Their Maturation. *PLoS ONE* **2010,** *5* (8), e12246; (b) Manil-Segalén, M.; Lefebvre, C.; Culetto, E.; Legouis, R., Need an ESCRT for autophagosomal maturation? *Communicative & Integrative Biology* **2014,** *5* (6), 566-571.

33. (a) Lee, Y.-H.; Cheng, F.-Y.; Chiu, H.-W.; Tsai, J.-C.; Fang, C.-Y.; Chen, C.-W.; Wang, Y.-J., Cytotoxicity, oxidative stress, apoptosis and the autophagic effects of silver nanoparticles in mouse embryonic fibroblasts. *Biomaterials* **2014,** *35* (16), 4706-4715; (b) Mishra, A. R.; Zheng, J.; Tang, X.; Goering, P. L., Silver Nanoparticle-Induced Autophagic-Lysosomal Disruption and NLRP3-Inflammasome Activation in HepG2 Cells Is Size-Dependent. *Toxicological Sciences* **2016,** *150* (2), 473-487; (c) Li, J. J.; Hartono, D.; Ong, C.-N.; Bay, B.-H.; Yung, L.-Y. L., Autophagy and oxidative stress associated with gold nanoparticles. *Biomaterials* **2010,** *31* (23), 5996-6003.

34. Wu, H.; Che, X.; Zheng, Q.; Wu, A.; Pan, K.; Shao, A.; Wu, Q.; Zhang, J.; Hong, Y., Caspases: A Molecular Switch Node in the

Crosstalk between Autophagy and Apoptosis. *International Journal of Biological Sciences* **2014,** *10* (9), 1072-1083.

35. (a) du Pré, M. F.; Sollid, L. M., T-cell and B-cell immunity in celiac disease. *Best Practice & Research Clinical Gastroenterology* **2015,** *29* (3), 413-423; (b) Marsh, M.; Heal, C., Evolutionary Developments in Interpreting the Gluten-Induced Mucosal Celiac Lesion: An Archimedian Heuristic. *Nutrients* **2017,** *9* (3), 213.

36. (a) Barone, M.; Troncone, R.; Auricchio, S., Gliadin Peptides as Triggers of the Proliferative and Stress/Innate Immune Response of the Celiac Small Intestinal Mucosa. *International Journal of Molecular Sciences* **2014,** *15* (11), 20518-20537; (b) Garrote, J. A.; Gçmez-Gonzàlez, E.; Brenardo, D.; Arranz, E.; Chirdo, F., Celiac Disease Pathogenesis: The Proinflammatory Cytokine Network. *Journal of Pediatric Gastroenterology and Nutrition* **2008,** *47*, S27-S32.

37. Ciccocioppo, R.; Di Sabatino, A.; Bauer, M.; Della Riccia, D. N.; Bizzini, F.; Biagi, F.; Cifone, M. G.; Corazza, G. R.; Schuppan, D., Matrix metalloproteinase pattern in celiac duodenal mucosa. *Laboratory Investigation* **2004,** *85* (3), 397-407.

38. (a) Pender, S. L. F.; Tickle, S. P.; Docherty, A. J. P.; Howie, D.; Wathen, N. C.; MacDonald, T. T., A Major Role for Matrix Metalloproteinases in T Cell Injury in the Gut. *The Journal of Immunology* **1997,** *158*, 1582-90; (b) Salmela, M. T.; MacDonald, T. T.; Black, D.; Irvine, B.; Zhuma, T.; Saarialho-Kere, U., Upregulation of matrix metalloproteinases in a model of T cell mediated tissue injury in the gut: analysis by gene array and in situ hybridisation. *Gut* **2002,**

51, 540-47; (c) MacDonald, T. T.; Spencer, J., EVIDENCE THAT ACTIVATED MUCOSAL T CELLS PLAY A ROLE IN THE PATHOGENESIS OF ENTEROPATHY IN HUMAN SMALL INTESTINE. *J. Exp. Med.* **1988,** *167*, 1341-49; (d) D'Arienzo, R.; Stefanile, R.; Maurano, F.; Luongo, D.; Bergamo, P.; Mazzarella, G.; Troncone, R.; Auricchio, S.; David, C.; Rossi, M., A deregulated immune response to gliadin causes a decreased villus height in DQ8 transgenic mice. *European Journal of Immunology* **2009,** *39* (12), 3552-3561.

39. Wu, Y.; Ahang, Q.; Ruan, Z.; Yin, Y., Intrinsic effects of gold nanoparticles on proliferation and invasion activity in SGC-7901 cells. *Oncology report* **2016,** *35*, 1457-1462.

40. Marsh, M. N., Gluten, major histocompatibility complex, and the small intestine: A molecular and immunobiologic approach to the spectrum of gluten sensitivity (‘celiac sprue’). *Gastroenterology* **1992,** *102* (1), 330-54.

41. Artiaga, G.; Ramos, K.; Ramos, L.; Cámara, C.; Gómez-Gómez, M., Migration and characterisation of nanosilver from food containers by AF4-ICP-MS. *Food Chemistry* **2015,** *166*, 76-85.

42. Ménard, D.; Arsenault, P., Explant Culture of Human Fetal Small Intestine. *Gastroenterology* **1985,** *86*, 691-700.

43. Pfaffl, M., A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001,** *29* (9).

Chapter 4: Summary, Conclusions and Future Perspectives

Summary

Nanotechnology is a recent area of research and its applications range from sustainable energy and medicine to feed/agro/food sector and cosmetic industry[1-5]. Despite the use of nanomaterials has brought big improvements, a big question about their safety has raised. Particularly worrisome is the use of a great amount of metallic nanoparticles in the agro/food sector to improve organoleptic properties and preservation of the food[6-8]. Previous studies have demonstrated that dietary mNP can impair the intestinal homeostasis through several mechanisms: cytotoxic effects (endoplasmatic reticulum stress, DNA damage, lysosome membrane permeabilization, induction of autophagy and cellular death), alteration of the intestinal homeostasis and immune modulation[9-13]. Several works have also reported the metallic nanoparticles (particularly E171 food additive) role in inflammatory bowel diseases[14-16] (Crohn Disease and Ulcerative Colitis) pathogenesis, but no studies have been yet performed about their possible role in CeD. Celiac Disease is a multifactorial enteropathy caused by dietary gluten ingestion in genetically susceptible subjects (HLA-DQ2/DQ8 aplotypes)[17, 18]. To develop the disease, gluten peptides have to pass the gastrointestinal barrier, reach the lamina propria and thus be recognized and activate the immune system, which in turn will determine a strong architectural change in the small intestinal mucosa. In this context, altering the intestinal homeostasis, mNPs

could represent one of the new environmental factors involved in the increasing incidence of CeD[19-21].

We therefore assessed the potential molecular interaction and combined effects of the most used mNPs (TiO_2-NP used as food coloring agent, ZnO- and Ag-NP used for their antimicrobial effects and AuNP used in the agro-food sector) with the peptic-triptic digested gliadin (PTG), using several intestinal models.

The molecular interaction between digested gliadin and mNP was assessed by UV-Vis spectrophotometer and TEM analyses. Cytotoxic effects and permeability alterations were evaluated in Caco-2 intestinal cells, which in their post-confluent/differentiated status represent the absorptive intestinal enterocytes. We also perfomed experiments ex vivo, using duodenal specimens from both healthy and celiac (under gluten free-diet) subjects. This model better reproduces the complexity of the intestinal mucosa, particularly regarding the immune components. To assess the activation of the immune system the transcript levels of the main cytokines involved in the CeD (IFNγ for the adaptative immune system and IL-15/IL-8 for the innate response) were evaluated by qPCR.

Results revealed that gliadin peptides can absorb on mNPs surface inducing an high rate of aggregation, particularly when incubated with AgNP and AuNP. A combined deleterious effect of mNPs and PTG was observed in vitro (on both cytotoxic and permeability alteration). Particularly important were the results obtained ex vivo: a significant increment in cytokines production, after treatment with

the combination of Au-, Ag- and TiO_2-NP with PTG, was detected, but only on biopsies from celiac patients.

We also performed experiments on undifferentiated Caco-2 cells, and results showed (as already reported in literature[22]) a different sensibility depending on the differentiation status of the cells. We therefore used a model representative of the intestinal crypt-like cells[22]; the HIEC cell line (primary crypt-like proliferative and undifferentiated cells). Initially, TEM analyses were performed to assess the overall effect of our stimulations (Ag- and Au-NP combined or not with PTG). Cell aspect suggested an alteration of the autophagic pathway after internalization of the mNPs-PTG aggregates, but not in cell exposed to the mNPs or the PTG alone. Interestingly, autophagy has been recently linked to both CeD pathogenesis and mNP mechanisms of toxicity[9, 23-25]. Thus, we confirmed this hypothesis evaluating autophagic molecular markers (Beclin1, LC3 and p62) by qPCR, WB and immunofluorescence. We also performed experiments on organ culture of fetal small intestine, particularly assessing the architectural changes in the villus structure. Results obtained up to now further support our initial findings although organ culture results are still too preliminary and need further evaluation.

Overall, our results suggest that the combined presence of mNPs and gliadin into the food may induce a deleterious effect on the intestinal mucosa. This could be particularly dangerous in subjects susceptible to develop CeD or other intestinal inflammatory disorders.

Conclusion and application to translational medicine

Our studies demonstrated a molecular interaction of gliadin peptides with dietary mNPs, which leads to the formation of complexes (UV-Vis spectra and TEM). The presence of complexes in a multi-component environment (as detected by phase contrast analyses), suggests that the molecular interactions are quite strong. Should further analyses (mass spectrometry) confirm these initial findings, i.e. the fact that interactions between mNPs and gliadin are strong enough to remain even into complex food matrices, these data would become an important aspect for mNP risk assessment.

Our in vitro results proved a combined deleterious effect of gliadin and food mNP in the context of the intestinal tract. The peculiarity of our studies was to observe a combined effect in two different models, representative of both differentiated enterocytes (Caco-2 cell line) and undifferentiated crypt-like cells (HIEC cell line). Regarding Caco-2 cells, we demonstrated that gliadin-mNP aggregates induce an alteration of the intestinal permeability, both altering TJ complexes and increasing cell death through apoptosis mechanisms. In HIEC cells, instead, we observed an alteration of the autophagic pathway, although further studies are necessary to better characterize the molecular mechanisms involved. Thus, gliadin-mNP aggregates can impair the intestinal homeostasis affecting both differentiated and undifferentiated intestinal cells, determining a

worse effect compared to gliadin peptides alone on different cells essential for maintaining a normal intestinal structure.

To confirm this potential deleterious effect, we used intestinal fetal specimens in organ culture. Despite the number of specimens needs to be increased, the combination of mNP and gliadin induced more severe architectural villous changes than the single gliadin treatments, changes that resembles those present in CeD enterophathy, in particular the villous flattening[26]. These data were obtained on pre-term intestines, thus immature; although gluten exposure in infants usually occurs between 6-12 months of life and Lionetti et al.[27] have demonstrated that gluten introduction age does not alter CeD incidence at 5 years, our findings raise the issue about the necessity of strict controls, in order to prevent a precocious exposure to gluten in association with mNPs and its damaging effects.

The damage of the intestinal mucosa could involve several mechanisms, including the activation of the immune system. The ex vivo studies we performed on duodenal biopsies obtained from control or celiac patients showed an increased production of cytokines involved in innate or adaptive immune response mainly in CeD samples after the exposure to gliadin combined with mNP. This higher immune response could be explained by the fact that mNP may promote the antigenic presentation of gliadin to the immune system, property that has already been reported[13]. In this context, one of our future perspectives is to evaluate dendritic cell activation and their ability to present gliadin as an antigen to T cells after

stimulation with the combined mNP-gliadin, and to compare these results with those obtained with gliadin alone. It must be underlined that we obtained a response only in biopsies from celiac patients, but not from heathy subjects. This suggests that, although the use of mNP in the food industry induces really slight toxic effect on healthy people, it could be harmful for subjects predisposed to develop intestinal disorders. The higher passage of gliadin peptides into the lamina propria, as well as the increased immune response, suggest that dietary mNP may really represent one of the new environmental factors linked to the increasing incidence of CeD.

To the best of our knowledge, our studies appear to be the first to document the molecular interaction between dietary mNPs and gliadin peptides as well as to report their deleterious combined effect on the intestinal tract. Since these findings may be really important in the context of the increasing CeD incidence (as already demonstrated for the intestinal bowel diseases[14-16]), they should be taken into account to the competent regulative agencies (EFSA and RCC).

References

1. Kumar V, Guleria P, Kumar V, Yadav SK. Gold nanoparticle exposure induces growth and yield enhancement in Arabidopsis thaliana. Science of The Total Environment 2013;461-462:462-468.

2. Yang J, Jiang F, Ma C, Rui Y, Rui M, Adeel M, Cao W, Xing B. Alteration of Crop Yield and Quality of Wheat upon Exposure to Silver Nanoparticles in a Life Cycle Study. Journal of Agricultural and Food Chemistry 2018;66:2589-2597.

3. Athinarayanan J, Alshatwi AA, Periasamy VS, Al-Warthan AA. Identification of Nanoscale Ingredients in Commercial Food Products and their Induction of Mitochondrially Mediated Cytotoxic Effects on Human Mesenchymal Stem Cells. Journal of Food Science 2015;80:N459-N464.

4. Vance ME, Kuiken T, Vejerano EP, McGinnis SP, Hochella MF, Rejeski D, Hull MS. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein Journal of Nanotechnology 2015;6:1769-1780.

5. Fogli S, Montis C, Paccosi S, Silvano S, Michelucci E, Berti D, Bosi A, and PA, P R. Inorganic nanoparticles as potential regulators of immune response in dendritic cells. Nanomedicine 2017;12.

6. Yang FM, Li HM, Li F, Xin ZH, Zhao LY, Zheng YH, Hu QH. Effect of Nano-Packing on Preservation Quality of Fresh Strawberry (Fragaria ananassa Duch. cv Fengxiang) during Storage at 4 °C. Journal of Food Science 2010;75:C236-C240.

7. Kim JS, Kuk E, Yu KN, Kim J-H, Park SJ, Lee HJ, Kim SH, Park YK, Park YH, Hwang C-Y, Kim Y-K, Lee Y-S, Jeong DH, Cho M-H. Antimicrobial effects of silver nanoparticles. Nanomedicine: Nanotechnology, Biology and Medicine 2007;3:95-101.

8. Pereira DIA, Bruggraber SFA, Faria N, Poots LK, Tagmount MA, Aslam MF, Frazer DM, Vulpe CD, Anderson GJ, Powell JJ. Nanoparticulate iron(III) oxo-hydroxide delivers safe iron that is well absorbed and utilised in humans. Nanomedicine: Nanotechnology, Biology and Medicine 2014;10:1877-1886.

9. Stern ST, Adiseshaiah PP, Crist RM. Autophagy and lysosomal dysfunction as emerging mechanisms of nanomaterial toxicity. Particle and Fibre Toxicology 2012;9:20.

10. Brun E, Barreau F, Veronesi G, Fayard B, Sorieul S, Chanéac C, Carapito C, Rabilloud T, Mabondzo A, Herlin-Boime N, Carriére M. Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia. particle and Fibre Toxicology 2014;11.

11. Williams K, Milner J, Boudreau MD, Gokulan K, Cerniglia CE, Khare S. Effects of subchronic exposure of silver nanoparticles on intestinal microbiota and gut-associated immune responses in the ileum of Sprague-Dawley rats. Nanotoxicology 2014;9:279-289.

12. Williams KM, Gokulan K, Cerniglia CE, Khare S. Size and dose dependent effects of silver nanoparticle exposure on intestinal permeability in an in vitro model of the human gut epithelium. Journal of Nanobiotechnology 2016;14.

13. Luo Y-H, Chang LW, Lin P. Metal-Based Nanoparticles and the Immune System: Activation, Inflammation, and Potential Applications. BioMed Research International 2015;2015:1-12.

14. Ruiz PA, Morón B, Becker HM, Lang S, Atrott K, Spalinger MR, Scharl M, Wojtal KA, Fischbeck-Terhalle A, Frey-Wagner I, Hausmann M, Kraemer T, Rogler G. Titanium dioxide nanoparticles exacerbate DSS-induced colitis: role of the NLRP3 inflammasome. Gut 2017;66:1216-1224.

15. Powell JJ, Harvey RSJ, Ashwood P, Wolstencroft R, Gershwin ME, Thompson RPH. Immune Potentiation of Ultrafine Dietary Particles in Normal Subjects and Patients with Inflammatory Bowel Disease. Journal of Autoimmunity 2000;14:99-105.

16. Evans SM, Ashwood P, Warley A, Berisha F, Thompson RPH, Powell JJ. The role of dietary microparticles and calcium in apoptosis and interleukin-1β release of intestinal macrophages. Gastroenterology 2002;123:1543-1553.

17. Megiorni F, Mora B, Bonamico M, Barbato M, Nenna R, Maiella G, Lulli P, Mazzilli MC. HLA-DQ and risk gradient for celiac disease. Human Immunology 2009;70:55-59.

18. De Re V, Magris R, Cannizzaro R. New Insights into the Pathogenesis of Celiac Disease. Frontiers in Medicine 2017;4.

19. Lohi S, Mustalahti K, Kaukinen K, Laurila K, Collin P, Rissanen H, Lohi O, Bravi E, Gasparin M, Reunanen A, MÄKi M. Increasing prevalence of coeliac disease over time. Alimentary Pharmacology & Therapeutics 2007;26:1217-1225.

20. Lebwohl B, Ludvigsson JF, Green PHR. The Unfolding Story of Celiac Disease Risk Factors. Clinical Gastroenterology and Hepatology 2014;12:632-635.

21. Lerner A, Matthias T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. Autoimmunity Reviews 2015;14:479-489.

22. Hanley C, Layne J, Punnoose A, Reddy KM, Coombs I, Coombs A, Feris K, Wingett D. Preferential killing of cancer cells and activated human T cells using ZnO nanoparticles. Nanotechnology 2008;19:295103.

23. Comincini S, Manai F, Meazza C, Pagani S, Martinelli C, Pasqua N, Pelizzo G, Biggiogera M, Bozzola M. Identification of Autophagy-Related Genes and Their Regulatory miRNAs Associated with Celiac Disease in Children. International Journal of Molecular Sciences 2017;18:391.

24. Manai F, Azzalin A, Gabriele F, Martinelli C, Morandi M, Biggiogera M, Bozzola M, Comincini S. The In Vitro Effects of Enzymatic Digested Gliadin on the Functionality of the Autophagy Process. International Journal of Molecular Sciences 2018;19:635.

25. Huang D, Zhou H, Gao J. Nanoparticles modulate autophagic effect in a dispersity-dependent manner. Scientific Reports 2015;5.

26. Marsh M, Heal C. Evolutionary Developments in Interpreting the Gluten-Induced Mucosal Celiac Lesion: An Archimedian Heuristic. Nutrients 2017;9:213.

27. Lionetti E, Castellaneta S, Francavilla R, Pulvirenti A, Tonutti E, Amarri S, Barbato M, Barbera C, Barera G, Bellantoni A, Castellano E, Guariso G, Limongelli MG, Pellegrino S, Polloni C, Ughi C, Zuin G, Fasano A, Catassi C. Introduction of Gluten, HLA Status, and the Risk of Celiac Disease in Children. New England Journal of Medicine 2014;371:1295-1303.

Publications

"miRNA-regulated gene expression differs in celiac disease patients according to the age of presentation".

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