Dietary Protein Oxidation: A Silent Threat to Human Health?

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ABSTRACT

Protein oxidation has become a topic of great scientific interest in the field of Food Science and Nutrition. Food proteins are known to be preferential targets of radical species and protein oxidation has relevant consequences on protein functionality and food quality. Current trends in this field call attention to the nutritional and health dimensions of oxidized foods. Both lipid and protein oxidation products are accumulated in the food during processing and storage and also upon food intake, during the subsequent digestion phases. The gastrointestinal tract and internal organs are exposed to the cytotoxic and mutagenic potential of these species. While the molecular basis of the pathogenesis of particular dietary lipid oxidation products is well known, the impact of dietary oxidized proteins on human health has been largely ignored. The well-established association between *in vivo* protein oxidation and aging and age-related diseases urges scientists to investigate the contribution of dietary protein oxidation to particular pathological conditions. Recent reports indicate the involvement of dietary protein oxidation species on particular health disorders which emphasizes the link between dietary and *in vivo* protein oxidation.

KEYWORDS

Cancer; dietary antioxidants; inflammatory bowel disease; oxidative stress; oxidized proteins.

1. Protein oxidation: From the 'poor cousin' to the hot topic.

From a medical standpoint, protein oxidation (PROTOX) has been covered for more than a century by numerous scientists. The studies carried out by Henry D. Dakin (*1880–†1952) at the beginning of the 20th century (Dakin, 1906, 1908a,b) originally reported the oxidative degradation of particular amino acids during digestion and introduced the potential biological consequences of such biochemical reactions. The impact of PROTOX on human health was, at that moment, wholly unknown. During the succeeding decades, PROTOX has been in the focus of medical scientists owing to the association between the oxidative damage to proteins and aging and age-related diseases (Berlett & Stadtman, 1997). Yet, Michael J. Davies, one of the pioneers and most recognized experts in the field, accepted the role of '*poor cousin'* played by PROTOX throughout the 20th century (Davies, 2003). In opposition to lipid and DNA oxidation, PROTOX was scarcely studied during the first half of the century despite the wake-up call by Dakin (1906). Lipid oxidation (LOX), in particular, has been subject of assorted multidisciplinary scientific studies for several decades. Profuse literature has illustrated the mechanisms and consequences of LOX in food systems and the impact of LOX products on the pathogenesis of serious vascular diseases (Esterbauer et al., 1993; Morrissey et al., 1998). Davies (2003) identified some potential reasons for the lack of coverage of PROTOX, including the complexity of the underlying chemistry and the necessity of precise methodologies to detect specific PROTOX markers. Earl R. Stadtman (*1919–†2008), a renowned biochemist of the 20th century and mentor of various Novel-prized scientists, was one of the pioneers in unveiling the chemistry and biological consequences of PROTOX. From the elucidation of mechanisms whereby the rates of metabolic reactions match to the necessities of the living cell, he identified

the connection between unbalanced oxidative metabolism (≈ oxidative stress) and impaired physiological processes (Stadtman, 1990). Being aware of the key role played by proteins as modulators and executors of most biological functions, he investigated whether the oxidative damage to proteins led to pathological conditions. In some of his early breakthroughs, Stadtman described that cell aging may be related to an impaired protein turnover due to the inactivation of key metabolic enzymes by oxidative reactions (Fucci et al., 1983). Some years later, the accumulation of oxidized proteins in the brain was subsequently recognized as a neuropathological hallmark of Alzheimer's disease (Smith et al., 2000). Some of his achievements, including the comprehension of the chemistry behind PROTOX (Stadtman, 1993) and the detection of specific PROTOX products (Levine et al., 1990), encouraged many other scientists to study the biological significance of PROTOX. The studies that deepen into the involvement of PROTOX on the Alzheimer's disease and other neurological disorders (Smith et al., 1994; Hensley et al., 1995) boosted the interest on this topic during the mid-nineties. Following these first reports, more than thousand papers specifically devoted to expose the association between PROTOX and various health disorders have been published in the last 10 years. While some of the underlying mechanisms of the connection between *in vivo* PROTOX and disease are still to be clarified, it is accepted that PROTOX plays a role in aging and age-related diseases such as Alzheimer's, Parkinson's, inflammatory Bowel's (IBD), rheumatoid arthritis, diabetes, muscular dystrophy, and cataractogenesis, among others (Berlett & Stadtman, 1997). On account of the effort of brilliant scientists, the 'poor cousin' of lipid oxidation is now extolled as a topic of the utmost scientific interest.

2. Protein oxidation in Food Science and Nutrition

From the perspective of Food Science & Nutrition, oxidative reactions have always been a hot topic owing to the influence of LOX on the deterioration of processed oils, meat and dairy products. In line with the medical field, PROTOX has been for decades disregarded as a major cause of food deterioration despite the fact that proteins play a major role in foods from nutritional, sensory and technological points of view. During all this time, the striking impact of lipid oxidation on food flavor eclipsed the apparently plain short-term effects of protein oxidation in foods. In line with the depiction made by Davies (2003), Youling L. Xiong recently described PROTOX as '*the less appreciated sibling of lipid oxidation'* (Xiong & Foegeding, 2015). Before the impact of the *silent* PROTOX was recognized in food systems, protein denaturation and proteolysis accounted for most of the changes undergone by proteins during food processing and storage. It was, in fact, Xiong who pioneered PROTOX research in food science and discovered that muscle proteins were susceptible to oxidative reactions leading to deleterious effects on the functionality of myofibrillar protein and on the quality of meat and fish (Decker et al., 1993; Xiong et al., 1993; Xiong & Decker, 1995). After the influential review by the same author (Xiong, 2000), numerous subsequent studies shed light on the oxidative modifications undergone by muscle proteins during handling, processing and storage of muscle foods. Following the trail of medical researchers, food scientists aimed to identify the significance of PROTOX in food systems. As for medical research, the progress in this field necessarily required an in-depth comprehension of the chemistry behind food PROTOX and the application of advanced methodologies for the detection of particular PROTOX products. In such a manner, the application of accurate chromatographic (Estévez et al., 2009; Jongberg et al., 2011; Koivumäki et al., 2012), spectroscopic (Frederiksen et al., 2008; Estévez et al., 2008;

Utrera & Estévez, 2012a), spectrometric (Pazos et al., 2011), proteomic (Lametsch et al., 2008; Promeyrat et al., 2011; Pazos et al., 2013; Di Luccia et al., 2015), and microscopic (Astruc et al., 2007) methodologies have enabled recent progresses in the comprehension of biochemistry fundamentals. Among the recent progresses we highlight, i) the clarification of PROTOX initiation by physical factors –light (Dalsgaard et al., 2012) and irradiation (Wang et al., 2015) – and chemical species –radicals (Chen et al., 2016), transition metals (Estévez & Heinonen, 2010), heme pigments (Frederiksen et al., 2008) and reducing sugars (Villaverde & Estévez, 2013)–, ii) the description of precise oxidation routes and chemical pathways (Salminen & Heinonen, 2008; Estévez et al., 2011; Utrera & Estévez, 2012b) and iii) the detection and quantification of specific PROTOX markers in food systems (Estévez et al., 2009; Utrera & Estévez, 2013). It is also worth mentioning the recent efforts in adapting and improving routine methods for assessing protein oxidation in food systems such as the dinitrophenylhydrazine (DNPH) method (Mesquita et al., 2014; Soglia et al., 2016) and an improved procedure for the simultaneous detection of free thiols and disulphide bonds (Rysman et al., 2014).

It is currently known that the functionality and digestibility of meat and dairy proteins are affected by oxidative reactions (Santé-Lhoutellier et al., 2007; Feng et al., 2015). PROTOX has also been proven to impair the nutritional value and sensory attributes of muscle foods such as tenderness (Bao & Ertbjerg, 2015) and flavor (Villaverde et al., 2014). Further information about the chemistry behind food PROTOX, the occurrence and consequences of PROTOX during food storage and processing and the strategies aimed to control PROTOX in food systems, can be found in recent review articles (Bekhit et al., 2013; Estévez, 2015; Soladoye et al., 2015).

Given the proven occurrence and significance of PROTOX in food systems, a natural step forward in this emerging topic leads to hypothesize whether these oxidative reactions have consequences upon food intake. It is actually well-established that the composition of food and the dietary habits have physiopathological consequences (Figure 1). This new horizon is in line with current trends in Food Science and Nutrition aimed to assess the occurrence of biochemical reactions during the digestion phases and the impact of such reactions on nutrition and health. The investigation of postprandial events enables a more realistic approach to investigate the impact of food intake on nutrition and health as food components are severely modified during the digestion phases. Likewise, the understanding of the chemistry fundamentals behind oxidized proteins and PROTOX products is essential to comprehend the molecular basis of their potential pathogenesis. In the following sections, these points are addressed considering consolidated knowledge and future challenges.

3. Relevance of the molecular fundamentals

3.1. Consolidated knowledge

As redox-active biomolecules, proteins actively act as targets, sources and scavengers of reactive species. According to the reaction constants calculated by Davies (2005), reactive oxygen species (ROS), such as the hydroxyl radical, may react faster with proteins (albumin, 8×10^{10} L/mol s) or particular protein residues (cysteine, 3.4×10^{10} L/mol s; tryptophan, 1.3×10^{10} L/mol s) than with unsaturated lipids (linoleic acid, 9×10^{9} L/mol s). The early and preferential oxidation of these protein residues is described as a ‘sacrificial protection’, by which amino acid residues with antioxidant potential and irrelevant biological significance would scavenge ROS and hence,

protect valuable protein residues and other biomolecules against oxidation. As soon as the antioxidant capacity of proteins and of other redox-active compounds in the environment is exceeded, proteins, lipids and other susceptible molecules may undergo oxidative deterioration (Stadtman, 1993; Davies, 2005). PROTOX is a complex phenomenon as the pathways and the nature of the oxidation products depend on the targets in the protein and how the oxidative reactions commence. The chemical modifications caused to specific amino acid side chains and/or to the peptide backbone can lead to changes in the physical properties of the proteins, including fragmentation, aggregation, loss of solubility and functionality and decreased susceptibility to proteolysis (Xiong, 2000). In food systems, PROTOX has been assessed through several of its multiple chemical manifestations including loss of sulfhydryl groups, loss of tryptophan fluorescence, gain of carbonyl derivatives and formation of intra- and intermolecular cross-links (revised by Soladoye et al., 2015). Among the aforementioned changes, the formation of carbonyl compounds has been highlighted as one of the most remarkable modifications in oxidized food proteins (Estévez, 2011). Carbonylation is an irreversible modification in oxidized proteins induced by oxidative stress and other mechanisms (Shacter, 2000). In particular, carbonyls can be formed in proteins by three different pathways: i) direct oxidation of the side chains of alkaline amino acids such as lysine, threonine, arginine and proline (Schuessler and Schilling, 1984); ii) the reaction of the δ-amino group of an alkaline amino acid with reducing sugars or their oxidation products (Akagawa et al., 2005) and iii) the oxidative cleavage of the peptide backbone via the α-amidation pathway or the oxidation of glutamyl side chains (Requena et al., 2001). Among the three mechanisms, the Maillard-mediated pathway has been recently established as a relevant carbonylation pathway in both food and living systems (Villaverde &

Estévez, 2013; Trnková et al., 2015). The main protein carbonyl α-aminoadipic semialdehyde (AAS) and its end-product the α-aminoadipic acid (AAA) have been used as markers of PROTOX in food and living systems (Sell et al., 2007; Akagawa et al., 2005; Estévez et al., 2009; Timm-Heinrich et al., 2013). The accumulation of carbonyls in proteins has been directly related to a number of health disorders (Dalle-Donne et al., 2006) and is also believed to be responsible for the loss of protein functionality and food quality (Estévez, 2011; Ganhão et al., 2010a; Utrera & Estévez, 2012b).

3.2. Future challenges

Despite of all the efforts exerted in revealing the chemistry behind protein oxidation in food systems, the knowledge on this issue has been left far behind the information gathered by medical scientists. The identification of the chemistry fundamentals of PROTOX and the relationship with other biochemical reactions (LOX and the Maillard Reaction –MR–) is crucial to comprehend the potential implication of specific dietary PROTOX products on particular cellular signaling pathways, physiological processes and pathological conditions. This lack of knowledge on basic biological chemistry compromises the understanding of the molecular basis of numerous health disorders in which diet and dietary components may play a relevant role. For instance, the consumption of cured and processed red meat has been described as a 'convincing cause' of colorectal cancer (CRC) by the World Cancer Research Fund/American Institute for Cancer Research and more recently labelled as *carcinogenic* by the World Health Organization (IARC, 2015). The relationship between the intake of particular foods and the risk of suffering pathological conditions is typically established from cohort and case-control studies. The

aforementioned link between the consumption of red meat and the increased risk of suffering CRC is based on several meta-analyses of case-control studies. To ascertain the role of particular food components and consumption habits on specific pathological conditions requires a thorough analysis of the food, the fate of its components upon ingestion and the mechanisms of the physiopathological effects at a molecular level. The identification of the molecular interactions occurred between food constituents under physiological conditions may contribute to the comprehension of the pathological pathways of this and many other health disorders in which dietary components may have an influence.

The fulfilment of these objectives requires facing some future challenges. First, it is of great interest to study the initiation of PROTOX by ROS, reactive nitrogen species (RNS) and the interaction mechanisms between both groups of chemical species. The direct attack of ROS to protein bound-amino acids has been described as the most common and severe oxidative modification of oxidized proteins (Stadtman, 1993). As aforementioned, protein carbonylation is generally ascribed to the ROS-mediated oxidation of the ε-amino groups located in the side chains of alkaline amino acids. The oxidative degradation of other amino acid residues such as tryptophan and sulphur-containing ones may follow similar radical-mediated mechanisms (Lund et al., 2011; Ehrenshaft et al., 2015). In addition to ROS, RNS such as nitric oxide and peroxynitrite, are currently on the focus of scientists since they may be implicated in various physiopathological processes. A facile conversion between ROS and RNS has been reported in the literature (Skibsted, 2011) and these species may be able to initiate both PROTOX and protein nitration. Both reactions are regarded as typical chemical manifestations in degenerative and aged-related disorders (Berlett & Stadtman, 1997; Smith et al., 1997). As for PROTOX, the

chemistry involved in protein nitration needs further investigation in various disciplines including Food Science, Nutrition and Medicine. The formation, fate and hence, chemical modifications induced by RNS in proteins depends on the presence of other redox-active compounds, composition of the environment (O_2/CO_2) and nature of the targets in the protein. These factors have been scarcely investigated for their influence on the oxidation and nitration of particular food and human proteins.

Other challenges involve the molecular interaction mechanisms between PROTOX, LOX and MR products and the interconnections between these complex biological reactions. The interaction between proteins and oxidizing lipids has been a recurring topic of study. However, most of the precedent studies are limited to establish timely interactions based on significant correlations (Estévez, 2011). The chemical basis of such interactions is currently being studied. Whereas in medical research, specific lipid oxidation products such as malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) have been described as binding to proteins (Esterbauer, 2003), in food proteins such interactions have been poorly studied. MDA and hexanal are common LOX products in food systems and the formation of these lipid carbonyls takes place concomitantly with PROTOX. Both aldehydes may interact with oxidizing proteins (Zhao et al., 2012; Gurbuz & Heinonen, 2015) but the nature of these interactions, the conditions under which the interactions would take place and their consequences on food and human proteins are still a subject of numerous studies (Aldini et al., 2015). On the other hand, sugars with reactive carbonyl moieties are also able to induce protein carbonylation in both food and human proteins as an alternative pathway to that initiated by ROS. This Maillard-type mechanism, aforementioned, has been proposed to induce the formation of AAS in plasma proteins from

diabetic rats (Akagawa et al., 2005). Whereas both LOX and the MR are known to be implicated in the impairment of physiological processes and LOX and MR products are used as biomarkers of disease (Esterbauer, 2003; Trnková et al., 2015), the impact of these species on the oxidative stability of proteins is poorly understood. Reducing sugars are naturally present in multiple foods, in the gut and in living tissues, where they are used as fuel molecules. To which extent the oxidative deamination of alkaline amino acids via the Maillard pathway contributes to the overall protein carbonylation occurred in foods, during digestion or *in vivo*, is currently unknown. Finally, reactive species and oxidizing agents are also known to affect the DNA. DNA mutation is a critical step in carcinogenesis and elevated levels of oxidative DNA lesions (i.e. 8-OH-G) have been found in various tumours, strongly incriminating such damage in the aetiology of cancer (Cooke et al., 2003). In addition to the direct attack of ROS and RNS to DNA, specific oxidation products may also play a role in carcinogenesis via epigenetic modifications owing to their potential influence on the transcription of genes involved in cell growth regulatory pathways (i.e. Ref-1, NF-κB, AP-1) (Valko et al., 2006). The ability of particular LOX and PROTOX species to induce chemical damage to DNA molecules and impair signal transduction pathways and hence, gene expression, need to be ascertained.

It is worth mentioning that the understanding of the molecular basis of the pathogenesis would allow i) to outline efficient prevention strategies through, for instance, rational dietary recommendations to the population or individuals to avoid the potential health risks linked to dietary LOX and PROTOX products, ii) to identify reliable and meaningful biomarkers of oxidative stress and disease for an early diagnosis of the pathological process, and iii) to design targeted treatments against particular pathological processes once this has commenced.

4. Relevance of postprandial events in the gastrointestinal tract (GIT)

The understanding of the occurrence, severity and consequences of oxidative reactions during the three digestion phases (gastric, intestinal and colonic) is a topic of growing interest between food scientists and nutritionists (Figure 2). The assessment of the health risk linked to a particular food or dietary component is incomplete without having information on the fate of their constituents during the postprandial events in the gastrointestinal tract. To this regard, it is critical to identify the formation (amount and nature) of LOX and PROTOX products during the digestion phases. LOX, is known to be stimulated considerably during the gastric phase (Larsson et al., 2012) and later during the intestinal digestion (Kenmogne-Domguia et al., 2014), increasing the health risk associated with the intake of oxidized lipids (Goicoechea et al., 2011). In fact, it is proven that the occurrence of LOX in the lumen of the gastrointestinal tract has an influence on the postprandial oxidative stress *in vivo* (Ursini & Sevanian, 2002). On the contrary, up to now, only few studies have covered the fate of dietary proteins upon ingestion in terms of oxidative damage. Van-Hecke et al. (2014a) recently reported a remarkable increase of LOX and PROTOX in the colonic phase during a simulated digestion of red meat. A further study from the same authors (Van Hecke et al., 2014b) emphasized that the extent of intestinal PROTOX was promoted by other food components such as heme-iron and dietary fat. The interaction of oxidizing lipids and proteins with other food components (i.e. heme pigments) and food additives (i.e. nitrite, ascorbate, phytochemicals) may affect the nature and consequences of the oxidative reactions. It is poorly understood whether dietary sources of RNS (potassium and sodium nitrite; E-249/E-250) have an impact on the occurrence of PROTOX and protein

nitration in the food system, during digestion or *in vivo*. The biological significance of such reactions is also a subject to current interest given the alleged association between cured meat (≈ added nitrite) and CRC (IARC, 2015).

The fate of dietary phytochemicals during postprandial events and their role as potential modulators of luminal oxidative stress have been scarcely covered in the literature. Goupy et al. (2007) studied the ability of α-tocopherol, quercetin and β-carotene as inhibitors of metmyoglobin-induced peroxidation of linoleic acid during a simulated gastric digestion. Other antioxidant additives and phytochemicals such as lycopene (Carail et al., 2013), rutin and chlorogenic acid (Lorrain et al., 2012), grape seed extracts (Martínez et al., 2014) and olive oil polyphenols (Seiquer et al., 2015) have also been evaluated in gastrointestinal model systems. To our knowledge just a handful of recent articles cover the redox consequences of the molecular interactions between dietary phytochemicals and food proteins in simulated gastrointestinal systems. Wang et al. (2014) investigated the interactions between cloudberry ellagitannins with tryptic digests of β-lactoglobulin while Raes et al. (2015) assessed the impact α -tocopherol and carnosic acid on the extent of protein carbonylation during simulated gastric digestion of a standard diet.

A deeper understanding of these events is capital to identify the potential beneficial and/or detrimental effects of plant phenolics in the gut and their availability and potential biological effects upon absorption. In addition to the realistic assessment of the potential toxicity of dietary LOX and PROTOX, this innovative approach may serve to predict how a food system may behave during the digestion and its impact on human individuals in terms of nutritional value and health status. Nevertheless, most of the few available studies fail to use realistic digestion models

or an accurate chemical approach. Therefore, the occurrence, extent and nature of PROTOX and protein nitration during the digestion phases (including connections with LOX and the MR) require a detailed examination. It is also worth looking at the molecular interactions of food constituents (protein, lipids, carbohydrates, heme pigments) and their degradation products with other reactive food ingredients and additives (nitrite, phytochemicals…) as those determine the pool of chemical species in contact with intestinal cells and theoretically available for absorption. The latter include both potentially harmful (oxidation products) and beneficial compounds (the original phytochemicals or derivatives formed during digestion). The clarification of these postprandial events is essential to accurately identify i) the real loss of nutritional value as a result of oxidative reactions, ii) the role of particular food components (or combinations of them) as sources of potentially harmful compounds iii) the ability of dietary phytochemicals in controlling postprandial oxidative reactions and their negative consequences. The relevance of the issues involved justifies undertaking comprehensive and more challenging studies using realistic model systems and precise chemical analyses.

5. The silent threat is revealed

The evidence that *in vivo* oxidation is a source of aging and disease calls to elucidate to which extent dietary oxidative stress contributes to aggravating *in vivo* oxidative stress and its harmful consequences. In addition, particular dietary oxidation products may directly be able to induce or contribute to some pathological process in targeted cells or tissues through the induction of specific molecular responses (i.e. gene expression regulation). Numerous experimental documentation and comprehensive reviews have reported the role of dietary oxidized lipids as

external sources of *in vivo* oxidative stress and disease. The intake of oxidized lipids raises blood oxidation markers, leads to cell damage and increases the risk of suffering health disorders such as coronary-heart diseases, neurodegenerative disorders and certain types of cancer (Esterbauer et al., 1992, 1993; Sies et al., 2005; Awada et al., 2012). The role played by LOX products in the pathogenesis is usually linked to the cytotoxicity and mutagenicity potential of these species on the gastrointestinal tract or in internal organs upon absorption (Esterbauer et al., 1993). While the potential toxicity of oxidized food components is typically ascribed to lipids, proteins are regarded as targets for post-translational changes. In fact, the molecular basis of these processes commonly involves the interaction of primary and secondary LOX products (i.e. alkyl radicals, peroxides, hexanal, 4-HNE, MDA) with proteins of biological significance (formation of adducts) and other biomolecules such as DNA (Esterbauer et al., 1991; Awada et al., 2012). Atypical post-translational modification of proteins mediated by ROS or oxidized lipids may lead to malfunction and disease (Esterbauer et al., 1991). Other radical and non-radical species such as reducing sugars (Maillard-type reactions) and reactive nitrogen species (RNS) may be able to induce modifications (glycation/nitration, respectively) in proteins with biological consequences (Gong et al., 2005). The cellular responses to these molecular changes usually imply the activation of particular signalling pathways that involves gene expression and/or suppression (Figure 3). Numerous studies point out to the nuclear transcription factor NF-κB as a major pathway for the molecular mechanisms that underlie inflammatory, autoimmune and proliferative disorders (Aw, 1999). Until recently, the fact that dietary oxidized proteins and PROTOX products would, themselves, be active executors of specific pathological processes

was mostly unknown. The following sections provide scientific evidence to this silent health risk.

5.1. Pathogenesis of dietary protein oxidation products in the GIT

The oxidation of food proteins during processing and storage leads to the inexorable accumulation of oxidation products that will be primary exposed to the gastrointestinal tract. As aforementioned, food PROTOX also occurs during consumption and gastrointestinal digestion increasing the concentration of oxidation products in the lumen. Scientific evidences support the impact of dietary oxidized proteins on intestinal flora disturbance, the redox state of intestinal tissues and the onset of local pathological conditions (Keshavarzian et al., 2003; Fang et al., 2012; Xie et al., 2014).

Pierre et al. (2004), among others, already provided reasonable arguments to support the impact of luminal oxidative stress on cytotoxicity, genotoxicity and apoptosis in cells from colonic mucosa. More specifically, oxidative stress has been found to play a relevant role in the onset of carcinogenic processes, including CRC (Polyak et al., 1997; Valko et al., 2006). Interestingly, some clinical studies emphasize the extent of plasma protein carbonylation as a reliable marker of the risk of suffering CRC (Yeh et al., 2010; Chang et al., 2008). Chang et al. (2008) in particular, found altered protein carbonyl levels in CRC patients while LOX products remained at low levels. Others implicate the oxidative damage to proteins in the pathogenesis of CRC. This is the case of Nedic et al. (2013) who indicated the potential role of the carbonylation of insulin-like growth factor-binding proteins in CRC growth. Mehrabi et al. (2015) recently suggested that oxidative stress could be involved in the modification of oxidatively carbonyl

proteins in the pre-cancer stages, leading to increased aggressiveness of colorectal polyps. Trying to shed light on the role of luminal PROTOX on this process, Van-Hecke et al., (2014a) recently found a remarkable increase of LOX and protein carbonylation during digestion of red meat and demonstrated the formation of a genotoxic DNA adduct in a simulated colonic environment. In further studies, Van Hecke et al. (2014b, 2015) confirmed that severe lipid and PROTOX occurred concomitantly with an intense formation of O6-carboxy-methylguanine in a simulated colonic environment, strongly implicating not only LOX but also PROTOX products in the DNA damage that would initiate the carcinogenic process. These studies also supported previously reported evidence that heme iron may be involved in the epidemiological association between red meat consumption and CRC (Pierre et al., 2006). These *in vitro* studies are also supported by other recent works in which diets rich in readily oxidized lipids and heme iron facilitates a cancer promoting environment in the colon of rats (Guéraud et al., 2015). The implication of heme iron in CRC was comprehensively reviewed by Bastide et al (2011). Beyond the classical association between heme iron and CRC based on epidemiological data, the authors aimed to analyse the potential underlying molecular mechanisms. Studies in experimental animals showed that dietary hemoglobin and red meat dependably induced aberrant crypt foci, an alleged pre-carcinogenic injury (Pierre et al., 2003). The catalytic effect of heme iron on (i) the endogenous formation of carcinogenic N-nitroso compounds and (ii) the formation of cytotoxic and genotoxic lipid-derived aldehydes were proposed to be implicated in the pathogenesis (Bastide et al., 2011). A recent original research study from the same group confirms the central role of heme and its pro-oxidant potential on the association between dietary red meat and colon carcinogenesis (Bastide et al., 2015). It is worth mentioning that heme iron has been recurrently described as an efficient

promoter of food and luminal PROTOX (Lund et al., 2008; Estévez & Heinonen, 2010; Van-Hecke et al., 2014b, 2015). While both lipid and protein carbonyls may be implicated in the pathogenesis, the attempt to unveil the molecular basis of their mutagenic/carcinogenic actions has only been made for the former. Leuratti et al. (2002) found higher levels of the MDA-DNA adduct 1,N^2-malondialdehyde-deoxyguanosine (M1dG) in subjects with adenoma compared with adenoma-free subjects ($P < 0.005$) and diet seemed to be a highly influential factor. However, M1dG was also detected in colorectal biopsies from normal mucosa of 162 participants in the United Kingdom Screening Trials (Leuratti et al., 2002). There are no equivalent studies on particular protein carbonyls although they have been proved to be intensively formed in processed foods and during postprandial oxidative stress and their reactivity has been recurrently reported (Estévez, 2011; Chevion et al., 2000; Dalle-Donne et al., 2003).

The implication of intestinal oxidative stress on the redox imbalance of the intestinal epithelium and the onset of inflammatory disorders has also been extensively documented for dietary lipids (Aw, 1999; Monnier, 2007). Recent reports consistently signify a similar harmful impact of dietary oxidized proteins. Keshavarzian et al. (2003) reported that the oxidative injury (carbonylation and nitrotyrosination) to tissue and cytoskeletal proteins of colonic mucosa from IBD patients correlated with the severity of the disease. Intensively oxidized actin (>50%) was only detected in inflamed mucosa suggesting that cytoskeletal disruption by oxidative reactions is required for tissue injury, mucosal disruption, and IBD outbreak (ulcerative colitis, Crohn's disease and specific colitis). The authors emphasized the necessity of a cumulative oxidative damage on the colonic mucosa and that the injury may be exerted by luminal (≈ dietary) pro-oxidants. In fact, the same authors (Keshavarzian et al., 1990) were able to significantly decrease

the severity of the inflammation by the intra-colonic administration of species with antioxidant potential to experimental rats. More recently, Xie et al. (2014) reported that advanced oxidation protein products (AOPPs) would be involved in IBD progression by inducing tissue injury. The intraperitoneal administration of oxidized proteins to rats raised the level of AOPPs in the local intestine tissue and in blood inducing intestine epithelial death through a redox-dependent pathway. These results proven that PROTOX products may be implicated in the transfer of oxidative stress from the luminal phase to the lamina propia of the intestinal mucosa facilitating the process of IBD (Figure 4). The molecular mechanisms of this pathological effect involved NADPH oxidase-mediated ROS generation, JNK phosphorylation, and poly (ADP-ribose) polymerase-1 (PARP-1) activation. Hence, the occurrence of PROTOX (actin carbonylation) in the injured tissues as reported by Keshavarzian et al. (2003) could have been induced by external PROTOX products which may play an active role in the pathogenesis. Expanding on the pathological effects of AOPPs, Wu et al. (2015) recently reported a decreased expression of calcium transport channels via the p44/42 MAPK signaling pathway in small intestinal epithelium exposed to oxidized proteins. The authors hypothesized whether this molecular mechanism may contribute to IBD-associated osteoporosis. Interestingly, diets rich in readily oxidized components (polyunsaturared fatty acids) and meat proteins are believed to increase the risk of suffering various forms of IBD such as Crohn's disease and ulcerative colitis (Hou et al., 2011). It is reasonable to hypothesize that such diets may contribute considerable oxidized proteins given the close association between LOX and PROTOX in food systems and in the gastrointestinal tract (Soladoye et al., 2015; Van-Hecke et al., 2015). Some mechanistic studies carried out in cell lines has reported the harmful effect of luminal oxidants (iron/ascorbic acid)

from ingested foods on intestinal cell malfunction, DNA damage and apoptosis (Taha et al., 2010). Conversely, scientific evidences support that the intake of foods with antioxidant potential such as wine polyphenols alleviates the symptoms (Nunes et al., 2013). The ability of antioxidants (i.e. BHT) to prevent the occurrence of oxidative stress and its pathological consequences in cultured intestinal cells has also been proven (Taha et al., 2010).

5.2. Pathogenesis of dietary protein oxidation products in internal organs and functions

The occurrence of oxidized proteins in the gut may also have consequences in internal organs upon intestinal uptake of oxidized amino acids/small peptides (Figure 5). The intake of oxidized proteins has been found to increase PROTOX markers in blood and internal organs of experimental animals, hence contributing to promoting *in vivo* PROTOX (Li et al., 2013, 2014; 2015). Oxidized amino acids/peptides may induce homeostasis impairment and cell toxicity through various mechanisms. Gurer-Orhan et al. (2006) already hypothesized that oxidized amino acids may be misincorporated into proteins such as enzymes and structural element in cells, potentially contributing to malfunction, cell apoptosis and disease. These authors emphasized that post-translational oxidative modification of proteins may not be the only factor that contributes to *in vivo* PROTOX suggesting that external (dietary) sources of oxidized amino acids may cause direct toxic effects by being used for *de novo* synthesis of proteins. To similar conclusions came succeeding studies carried out by Dunlop et al. (2008; 2011). The absorption and subsequent deleterious effects of unnatural oxidized amino acids such as *meta*-tyrosine and 3,4-dihydroxyphenylalanine (L-DOPA) are known to occur in animals and humans leading to dysfunctional proteins and toxicity (Dunlop et al., 2015). These species may not only be formed

in foods as a result of tyrosine oxidation, they are also natural components of edible plants and beans (Siddhuraju & Becker, 2001; Davies, 2003; Dunlop et al., 2015). Chan et al. (2012) demonstrated that substitution of L-tyrosine residues in proteins with L-DOPA causes protein misfolding, promotes protein aggregation and stimulates the formation of autophagic vacuoles in SH-SY5Y neuroblastoma cells. Other oxidized forms of tyrosine, such as the *ortho*-tyrosine, contribute to the impairment of the insulin-induced arterial relaxation through the attenuation of endothelial nitric oxide synthase (eNOS) phosphorylation (Szijártó et al., 2014).

The neurotoxicity of tryptophan oxidation products (kynurenines) and their involvement in the pathogenesis of gastrointestinal diseases is also well documented (Chen & Guillemin, 2009; Keszthelyi et al., 2013). This amino acid is particularly sensitive to ROS and processing and storage of meat and dairy products has been described to significantly affect tryptophan stability (Leclere et al., 2002; Ganhão et al., 2010b; Utrera et al., 2012). Tryptophan oxidation products such as kynurenic acid are present in particular food products (Turski et al., 2009) and various kynurenine species have been found in processed and stored milk (Dalsgaard et al., 2007; Meltretter et al., 2014). The accumulation of kynurenines in processed foods in addition to the recognized formation and absorption of these species upon food digestion (Keszthelyi et al., 2009; Turski et al., 2013) illustrates the potential risk associated to the intake of tryptophan oxidation products. However, it is currently unknown to which extent dietary kynurenines could contribute to specific pathological disorders.

Lysine is another sensitive amino acid and its oxidation products (AAS and AAA) are commonly found in considerable quantities in dairy and muscle foods (Utrera et al., 2012; Timm-Heinrich et al., 2013). The hypothesis that lysine oxidation products may cause toxic effects upon absorption

received strength by findings from Wang et al. (2013). These authors confirmed the absorption and negative biological effects of the end oxidation product of lysine, the AAA. In a 12-years long metabolomic study with human patients, this compound was found to be the most reliable indicator of diabetes risk and postulated as a potential modulator of glucose homeostasis in humans. After oral administration, AAA levels are specifically increased in pancreas while its concentration in other tissues such as liver or skeletal muscle is not affected (Wang et al., 2013). The fact that AAA induces insulin secretion from pancreatic BTC6 cells in a dose- and time-dependent fashion support the targeted biological effect of this compound in pancreas. Of note, AAA carbonyl precursor, AAS, can be formed in both food and plasma proteins by a Maillard-mediated pathway in the presence of reducing sugars (Akagawa et al., 2005). This observation may provide the link between glucose metabolism, the formation of AAA and its potential biological effect on pancreatic cells. The contribution of dietary lysine oxidation products to this and other disorders, as well as the underlying mechanisms of their potential pathogenesis, remain indefinite.

Oxidized forms of sulphur-containing amino acids are also found at high quantities in processed milk and dairy products. Up to 74% of the original methionine content in milk is oxidized into methionine sulfoxide after processing (Baxter et al., 2007). Methionine sulfoxide has shown toxic potential as is responsible for the impairment of the endogenous antioxidant defenses in freshly isolated mouse hepatocytes (Dever & Elfarra, 2008). Dietary tyrosine oxidation products (i.e. dityrosine) have also recently found to display hepatotoxicity in experimental animals (Li et al., 2015). According to this study, livers from rats fed oxidized tyrosine suffered an increase in aspartate aminotransferase and alanine aminotransferase activities, total bilirubin content, and led

to oxidative damage and fibrotic degeneration. A detailed study of the underlying molecular mechanisms revealed that dietary oxidized tyrosine specifically increased the phosphorylation of p38 and ERK2 MAPKs and enhanced fibrosis-related TGF-β1 and Smad2/3 antibodies levels. Other studies from the same authors corroborate the susceptibility of liver and kidney to the dietary intake of oxidized proteins. In particular, the intake of oxidized casein has been found to cause i) redox stress in mice after short-term gavage (Li et al., 2013), ii) increase PROTOX markers (AOPPs, carbonyls and dityrosine) in blood and iii) up-regulation of Nrf2, GPX3, GPX4 and HO-1 genes in the injured tissues (Li et al., 2014). These studies proven the injurious impact of dietary oxidized proteins on internal organs through specific signaling pathways (Table 1) as already done by lipid scientists more than a decade ago (Aw, 1999).

It is worth highlighting that most of the recent scientific evidences of the toxicity of dietary oxidized proteins were made on acute treatments to observe short-term effects on experimental animals or cultured human cells. Given the proven link between *in vivo* PROTOX and age-related diseases, a prolonged exposure to dietary oxidized proteins may have some long term consequences on lifespan shortening. This hypothesis is worth investigating since a cellular redox imbalance is a typical feature in individuals with premature aging (Ishii et al., 1998; Finkel & Holbrook, 2000). Furthermore, recent studies support that a moderately protein-restricted diet protects against aging and cancer in humans by modulating the IGF-1 signalling pathway (Fontana et al., 2008). Interestingly, Youngman et al. (1992) already suggested a reduction in PROTOX and associated aging by dietary restriction of proteins. Regardless of their origin (*in vivo* or dietary) the underlying mechanisms of the pathological effects of oxidized proteins and PROTOX products are not fully clear and hence, requires further investigation.

6. Concluding remark

Food intake and dietary habits has a straightforward impact on health status. However, the understanding of the biology that governs the beneficial/detrimental effects of certain dietary components needs to be fully clarified. As oxidation has been recognized as a cause of aging and disease, recent years have witnessed a growing interest in dietary strategies aimed to enhance the health status, slowing the aging process, and prolonging functional lifespan. Proteins are major components of most foods (particularly animal-source) and their inclusion in a healthy diet is indisputable. While the discussion about dietary proteins is typically centered in the quantity, quality (≈ amino acid profile; biological value) and bioavailability upon digestibility, the impact of PROTOX on nutritional and health aspects are typically overlooked. The present review collects scientific data that support the hazards related to the intake of oxidized proteins and amino acids. This silent threat, largely ignored by food scientists, nutritionists and medical doctors, may be seriously considered in coming years as further scientific evidence becomes available. Furthermore, the current increase of the intake by population of highly processed animal-based foods with high protein content and presumably high oxidation rates predicts the raise of health disorders already associated to *in vivo* or dietary oxidative stress.

Abbreviations

AAA: aminoadipic acid;

AAS: α-aminoadipic semialdehyde;

DNPH: dinitrophenylhydrazine;

GGS: γ-glutamic semialdehyde;

PROTOX: protein oxidation;

LOX: lipid oxidation;

ROS: reactive oxygen species;

RNS: reactive nitrogen species;

AOPPs: advanced oxidation protein products;

MR: Maillard reaction;

IBD: Inflammatory bowel disease;

CRC: colorectal cancer;

MDA: malondialdehyde;

4-HNE; 4-hydroxynonenal

7. References

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Table 1. Impact of dietary PROTOX products on specific pathological processes.

Target Cell/Tissue	Dietary oxidation product	Cellular/Tissue response	Signalling pathways/Genes Affected	Pathological Process	Author
IEC-6/Intestine	AOPPs	Apoptosis	NADPH oxidase, JNK, PARP-1	IBD	Xie et al (2014)
Intestine	Oxidized casein	Intestinal flora & redox state disturbance	Nrf-2	IBD	Fang et al. (2012)
Colon	AOPPs	Cytoskeletal disruption	-	IBD	Keshavarzian et al. (2003)
Small intestine	AOPPs	Decreased Ca transport channels	p44/42, MAPK	IBD	Wu et al. (2015)
BTC6/Isolated murine islets	AAA	Oxidative stress, Insuline Secretion	POX-1, MafA	Diabetes	Wang et al. (2013)
Liver	Oxidized casein	Oxidative stress, Apoptosis	Nrf-2, TGF-β, ET-1	Fibrosis	Li et al. (2013)
Kidney, Liver	Oxidized casein	Oxidative stress, Apoptosis	Nrf-2, TGF-β, ET-2	Fibrosis	Li et al. (2014)
Liver	Tyrosine oxidation products	Oxidative stress	TGF-β1	Fibrosis	Li et al. (2015)

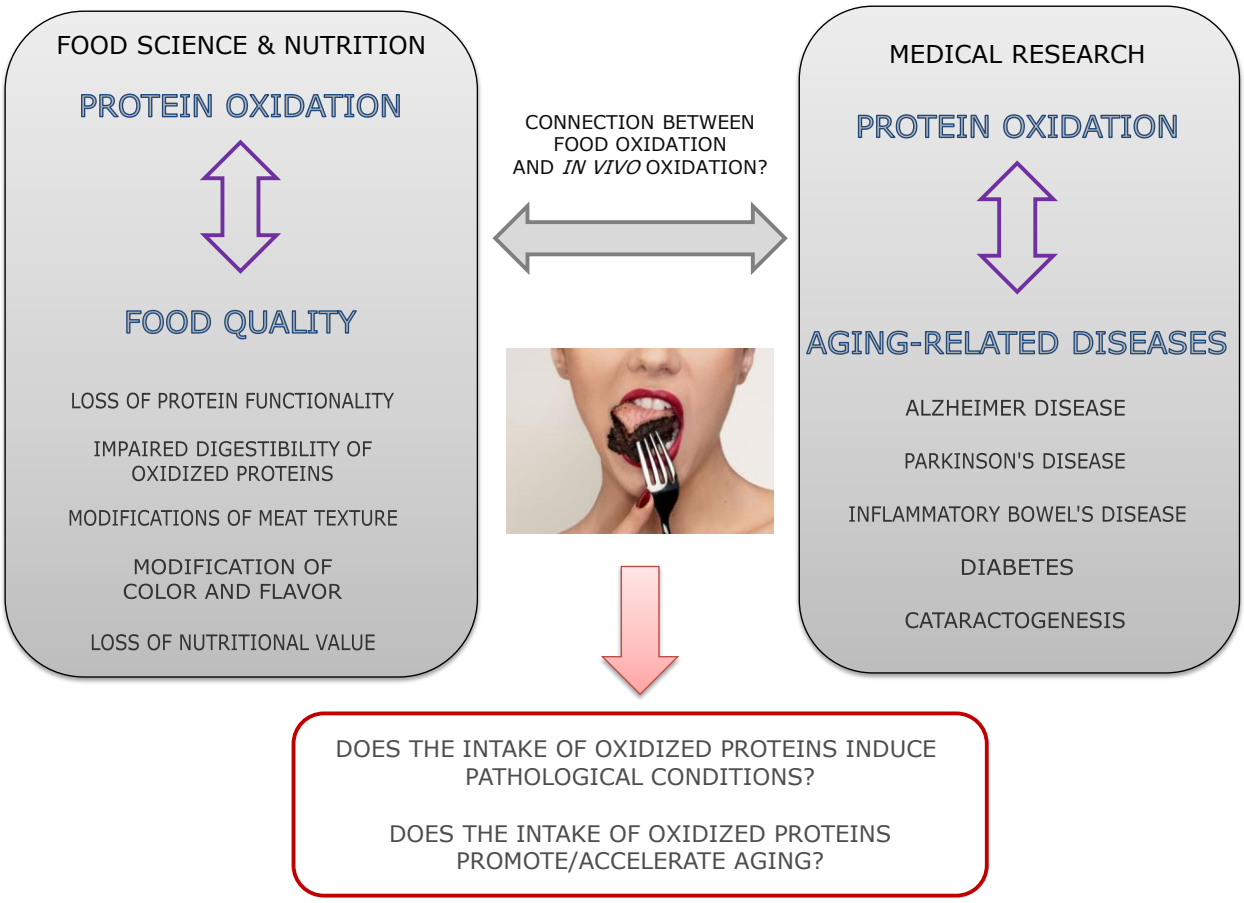

FIGURE 1. Hypothesis of the influence of dietary protein oxidation on *in vivo* oxidative stress and pathological conditions.

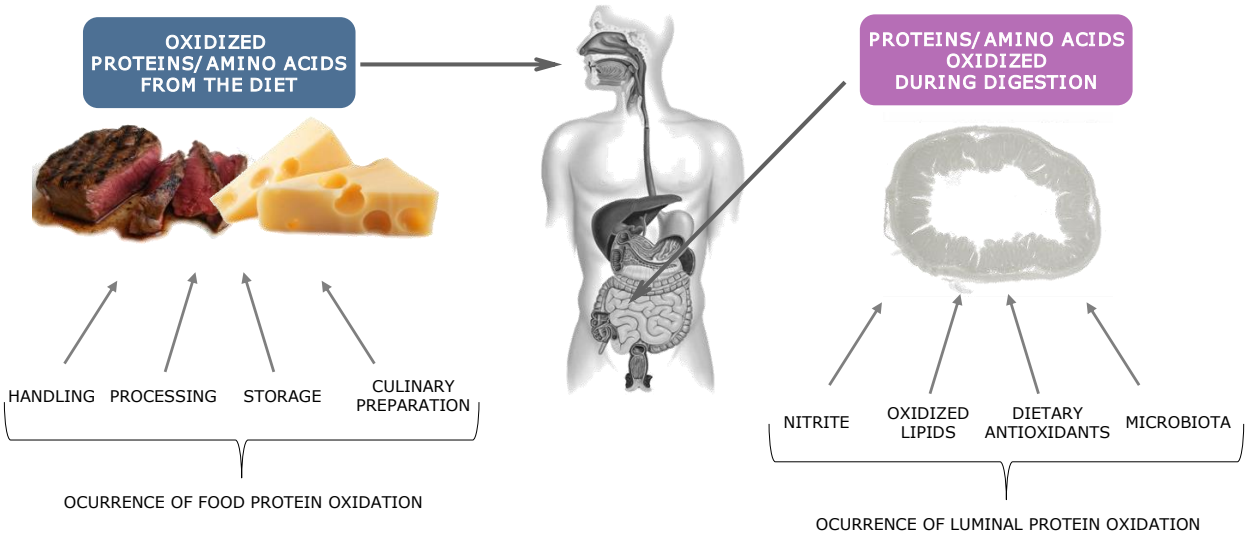

FIGURE 2. Dietary sources of protein oxidation products to humans: accumulation in food systems and formation in the GIT during subsequent digestion.

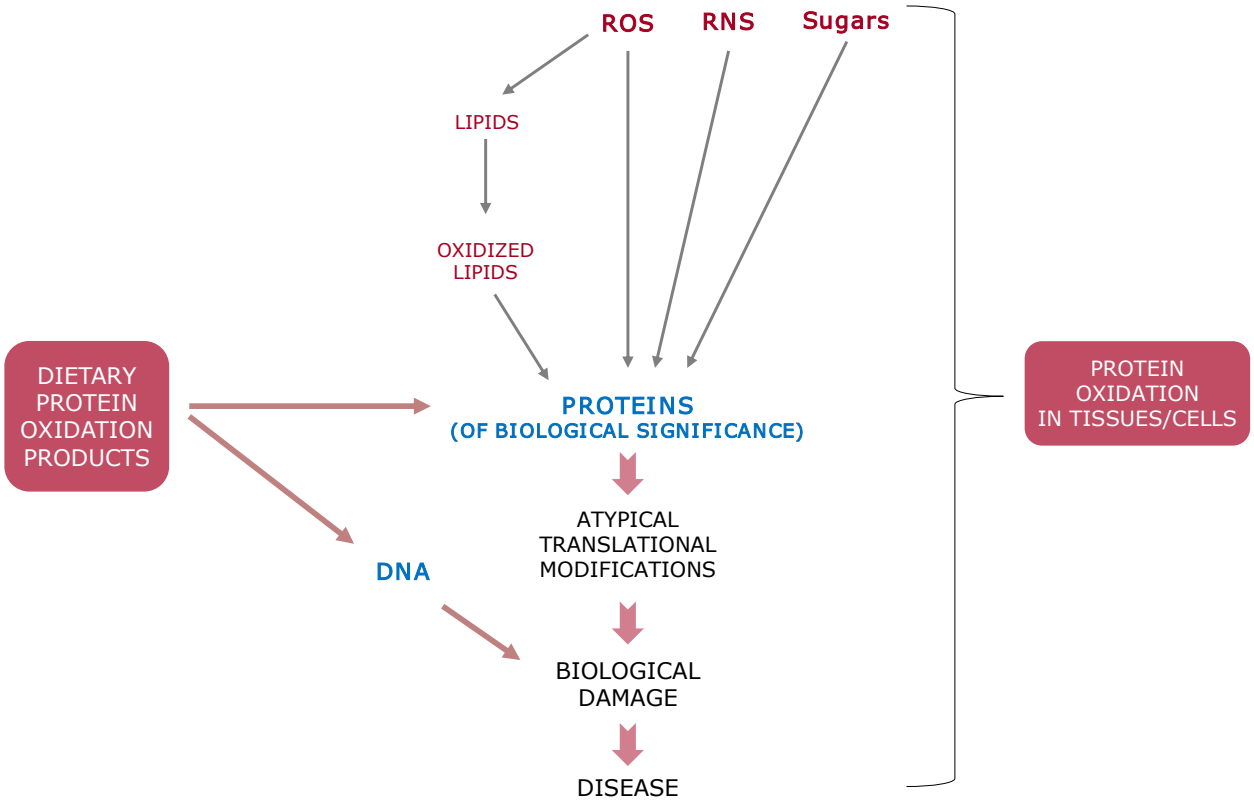

FIGURE 3. Proposed mechanisms of pathogenesis exerted by dietary protein oxidation products.

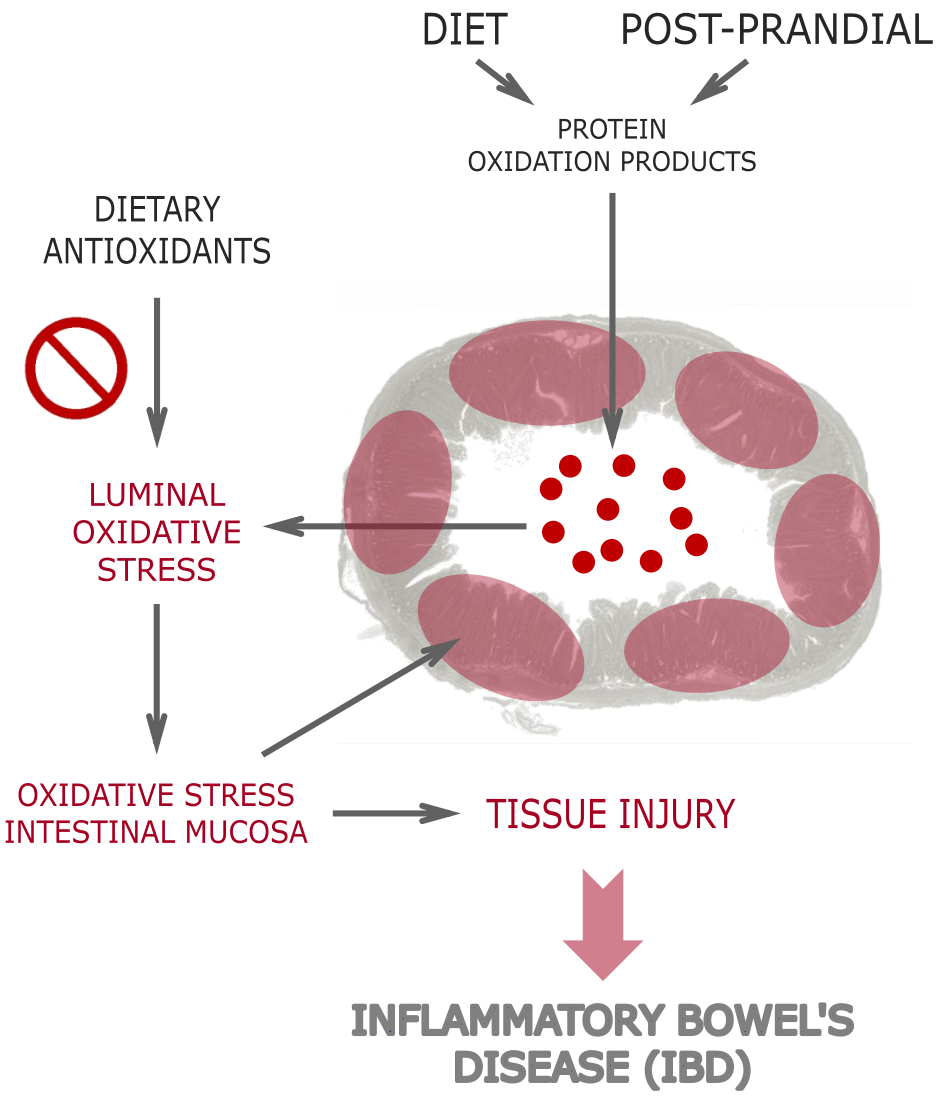

FIGURE 4. Pathogenesis of dietary protein oxidation products in the GIT: transfer of oxidative stress from lumen to intestinal mucosa, tissue injury and inflammatory disease.

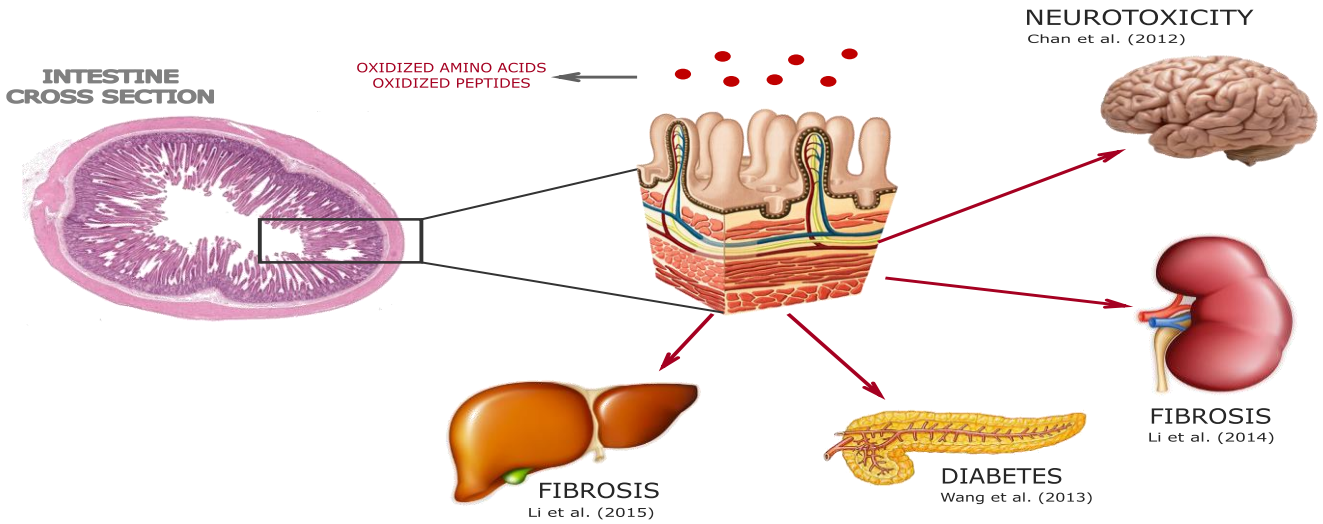

FIGURE 5. Absorption and subsequent pathological effects of dietary protein oxidation products in targeted tissues.