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The Role of Protein Carbonylation in Various Diseases: A Review

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Abstract

Protein carbonylation is a post-translational modification that involves the addition of a carbonyl group (-C=O) to a protein, resulting in the formation of a carbonylated protein. This modification can occur through various mechanisms, including oxidative stress, enzymatic reactions, and non-enzymatic reactions. Protein carbonylation can have significant consequences, including loss of protein function, protein aggregation, and cellular stress and damage.Recent studies have highlighted the importance of protein carbonylation in understanding the pathogenesis of various diseases, including neurodegenerative disorders, cancer, and metabolic disorders. Protein carbonylation has been implicated in the development of cancer, where it can contribute to the promotion of cell growth and survival. This review provides a comprehensive overview of the mechanisms, consequences, and detection methods for protein carbonylation. We discuss the various mechanisms by which protein carbonylation can occur, including the role of reactive oxygen species (ROS) and other oxidants. We also review the role protein carbonylation, including the loss of protein function, protein aggregation, and cellular stress and damage in several diseases. Therapeutic interventions and protein carbonylation are also considered. Furthermore, we discuss the various methods that have been developed to detect and quantify protein carbonylation, including the use of 2,4-dinitrophenylhydrazine (DNPH) and mass spectrometry. Finally, we highlight the advantages and limitations of the different methods used in the measurement of protein carbonylation.Understanding the mechanisms, role in disease, and detection methods for protein carbonylation is essential for the development of therapeutic strategies to prevent or treat diseases associated with protein carbonylation.

Keywords: Protein Carbonylation, Post-translational Modification, Oxidative Stress, Protein Aggregation, Quality Control

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Introduction

Protein carbonylation is a result of protein oxidation (post-translational modification) that is irreversible and precipitated by reactive oxygen species. This can occur in both animal and plant cells and is non-enzymatic, leading to the proteasomal breakdown of proteins by the proteasome system [1-2]. Protein carbonylation can result from metal-catalyzed oxidation (MCO) of Lys, Arg, Pro, and The side chains or through the Michael incorporation of lipid peroxidation-derived α,β-unsaturated aldehydes and ketones into the side chains of cysteine, lysine, and histidine residues [1].Reducing sugars have also been implicated in protein carbonylation [3]. The role of protein carbonylation spans the growth and development of animals and plants, oxidative stress, associated pathologies such as cellular damage, aging, age-related disorders, and cell signaling transduction [1,2,4-5]. The exact mechanisms and implications of protein carbonylation are still unclear, as an increase in carbonylated proteins is observed in conditions of stress, gradual decline in functional characteristics in human aging, ultimately leading to cell death [1].

The first proximal step of protein carbonylation involves hydroxyl radicals (HO), which largely originate from the Fenton reaction or the Haber-Weiss reactions of superoxide radicals (O_2), H_2O_2, and a transition metal such as iron (Fe) or copper (Cu). This suggests that the presence of Fe may determine the prevalence of protein carbonylation in living organisms [1,6-9]. Respiratory surge produces reactive oxygen species (ROS) such as superoxide anions, hydroxyl radicals, singlet O_2, and hydrogen peroxide in large

quantities. This increased production of inducible nitric oxide synthetase and pro-inflammatory cytokines leads to elevated levels of reactive nitrogen species (RNS) such as nitric oxide, nitrites, nitrates, and pro-inflammatory cytokines. ROS and RNS play a crucial role in eliminating microbes and have a beneficial effect on the host. However, excessive amounts of ROS and RNS can damage host tissues [10-11]. Protein carbonyl content in blood and tissues is a valuable indicator of protein oxidation due to its long-lasting stability under appropriate storage temperatures, specifically -80 degrees Celsius. It is the most frequently used and common indicator of oxidative protein damage [10,12-13]. However, it appears to be undervalued and underutilized in general practice settings to the best of our knowledge.

Importance of Studying Protein Carbonylation

Protein carbonylation evaluates the state of oxidative stress by measuring the extent of protein carbonyls present. This process is important for biomarking in disease and aging, as it can impact protein structure, function, and contribute to the development of various pathologies and age-related changes in the human body [14]. Carbonylation can disrupt the conformation of polypeptide chains, leading to partial or complete inactivation of proteins. It can also affect the chemical and biological activities of enzymes and other functional proteins, such as their ability to attract DNA transcription factors. Proteins involved in insulin signaling may also be weakened, disrupting the insulin signaling pathway. Additionally, protein carbonylation can slow down proteasomal activity, which is responsible for breaking down cellular proteins and recycling amino acids, serving as a form of protein quality control14. There is need to also understand the irreversible and reversible products of protein oxidation (Figure 1).

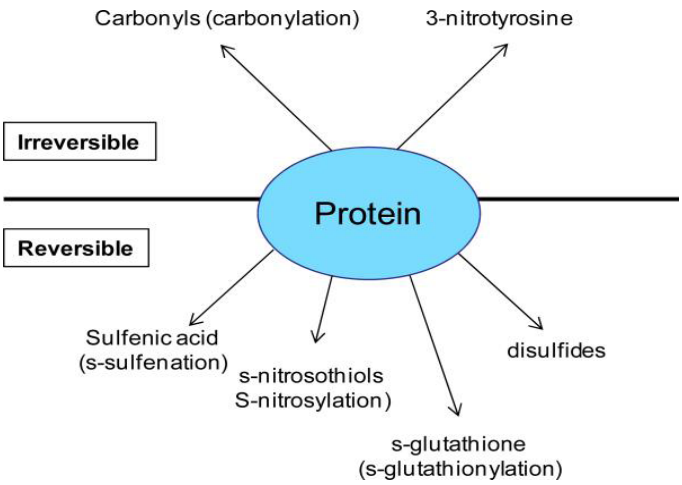

Figure 1: Irreversible and reversible protein oxidation products as described [29].

Protein carbonylation can be a useful tool for evaluating certain diseases, such as pre-eclampsia (a pregnancy-related hypertensive disorder), neurological diseases like Parkinson's and Alzheimer's, hematological cancers such as multiple myeloma, multiple sclerosis, myasthenia gravis, sarcopenia, and renal disease [14,15-16]. In terms of storage, protein carbonylation has been used for evaluations, particularly in stored blood samples [17-18]. With respect to dietary and nutrition practices, concerns have been raised about the toxic effects of consuming oxidized lipids. Lipid-derived carbonyls, like malondialdehyde (MDA), possess mutagenic and cytotoxic properties that can disrupt cellular equilibrium and contribute to health issues. Studies have explored these concerns and their implications on anatomical structures using animal models [19].

Role of Iron in Protein Carbonylation

All forms of life require iron as a vital element. The productivity of photosynthetic organisms is dependent on iron, even though it may not be easily attained and can also be toxic [1,20]. Cellular processes such as respiration, cellular differentiation, and photosynthesis rely on iron as helper molecules. In fauna populations, there is a balance between iron absorption, storage, utilization (effective use), and movement across cell membranes as a result of cellular iron metabolism. Cellular iron metabolism in fauna populations is affected by: (1). Soil acidity or alkalinity and the presence of iron - The optimal pH values for iron absorption by plants is 5.6, which improves iron solubility in the soil. Also required are iron concentrations that promote iron absorption by plants. (2). Iron chelators and transporters such as FRO3 and IRT1 facilitate iron absorption from the soil into the root cells of non-graminaceous plants like tomatoes and peas. In graminaceous plants like maize, iron-chelating phytosiderophores are released into the rhizosphere to form Fe^{3+}MA (Mugineic acid) complexes absorbed by a unique high-affinity transport mechanism. (3). Phytohormones such as auxin, ABA, and ethylene also influence iron metabolism. The association of these phytohormones has been well described [1,21-25].

When iron is in surplus, it can become harmful or toxic to plants, leading to oxidative stress through the Fenton reaction. Available mechanisms to mitigate this effect involve the effective usage of various antioxidant systems, which could be enzymatic and non-enzymatic. The enzymatic antioxidants are catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), which prevent the Fenton reaction by mopping up ROS and transforming it into less harmful molecules. The non-enzymatic antioxidants are glutathione, tocopherol (vitamin E), and ascorbate, which directly counteract the negative effects of ROS, thereby preventing oxidative stress.Other mechanisms of preventing free Fe^{2+} from initiating the Fenton reaction include: (1) Production of metal chelating agents. (2) Induction of iron storage proteins such as ferritins, which eliminate the Fenton reaction by binding Fe and sequestering it into a secure, readily absorbable form. Large amounts of Fe can be sequestered in this manner, not exceeding 4500 atoms, improving release to the plant cell matrix as needed [1,26-27].In humans, the role of iron in protein carbonylation is somewhat similar. Diseases such as hematological malignancies have found Fe chelators useful. Iron homeostasis plays a crucial role in

the treatment of various hematological tumors, especially myelodysplastic syndrome (MDS). In this context, patients with MDS experience transfusion dependence, which does not eliminate elevated oxidative stress parameters. Iron chelation, mainly by deferasirox (DFX), appears to improve the lifespan of patients with low-risk MDS and in stem cell transplant settings. Additionally, it can reduce mortality and cytopenia while enhancing the hematological response [15].

Deferasirox is an iron chelator commonly used as a therapy for patients with MDS who rely on blood transfusions. It is a powerful NF-kB inhibitor in myelodysplastic cells, acting independently of cell iron starvation through chelation and ROS scavenging. It eliminates the production of free radicals by suppressing the active redox forms of iron. Iron chelation therapy in myeloid leukemia promotes the differentiation of leukemia blasts and normal bone marrow precursors into monocytes/macrophages, requiring the regulation of ROS expression. However, the cytotoxic effects of iron chelation treatment on myeloid blast cells have been observed in vitro, in vivo, and ex vivo, showing synergy with acute myeloid leukemia drugs like decitabine and 5-azacitidine [15]. In multiple myeloma, intracellular iron chelation leads to cell death in myeloma cells. Deferasirox also induces apoptosis in myeloma cells by targeting the oncogenic Pyk2/β-Catenin signal transduction pathway. It is important to note that oxidative stress is a major factor in the development of multiple myeloma. For over two decades, there has been an imbalance in oxidant/antioxidant parameters in the disease, creating a persistent inflammatory environment in the tumor microenvironment. This oxidative state increases the likelihood of genetic mutations, leading to the acquisition of a malignant phenotype and cancer progression [15]. On the other hand, iron overload inhibits cell proliferation in multiple myeloma and enhances the effectiveness of bortezomib, as iron promotes lipid oxidation and inhibits proteasome function [28].

Cellular Sources of Oxidants

The mitochondria is a major production site for reactive oxygen species aside other systems localized within the cell. Moreso, there are several enzymes that are capable of producing ROS and are NADPH oxidase, xanthine oxidase , α-ketoglutarate dehydrogenase complex , d –amino acid oxidases and dihydrolipoamide dehydrogenase [29]. For nitric oxide production invivo, this is made successful by nitric oxide synthase even though deoxygenated myoglobin or xanthine oxido reductase or cytochrome C oxidase can be utilized in nitric oxide (NO) release in very specific conditions which presumably could be in hypoxic conditions or during infections [29]. NO is a gaseous molecule , water soluble with very significant role in signaling processes and has a very short half life with a physiological relevance that is concentration dependent much more restricting its role to a target site [30]. Its production, signaling and roles in human physiology and diseases are as described [30].

Protein carbonylation and Diseases

Protein carbonylation commonly occurs as a result of reactive species-induced protein modification. As mentioned earlier, it specifically targets proteins for degradation, leading to a loss of protein function [31]. Various reports by researchers have linked protein carbonylation to different disease conditions, including neurological and metabolic disorders [32]. Some of these conditions include pre-eclampsia, hypertension, Parkinson's disease, Alzheimer's disease, diabetes mellitus, sickle cell disease (SCD), and malaria infection. These diseases and their effects are often associated with oxidative stress, either directly or indirectly. In this article, we will explore the implications of protein carbonylation in some of these diseases.

Protein Carbonylation and Pre-eclampsia

In pre-eclampsia, when levels of reactive oxygen species (ROS) are elevated and antioxidant concentrations are reduced, it contributes to the progression of the condition. In this context, protein carbonyl could serve as a useful diagnostic tool for assessing protein damage caused by ROS [33]. A study investigating protein carbonyl levels in the decidua and placenta of pre-eclamptic women as markers of oxidative stress found that levels were higher in pre-eclampsia with HELLP (haemolysis, elevated liver enzymes, low platelets) compared to normal pregnancy. This suggests the presence of disturbances mediated by ROS in this disorder. Additionally, urinary protein carbonyl concentration was found to be elevated in pre-eclamptic women, although it was only partially associated with protein misfolding [34].

Also, as described by Ayub et al., their investigation concluded that elevated serum protein carbonyl levels and diminished antioxidant capacity indicate high levels of oxidative stress in women with pre-eclampsia, leading to endothelial dysfunction and the development of preeclampsia. Serum protein carbonyl levels were assessed using the 2,4-dinitrophenylhydrazone (DNPH) assay, which showed a significant association in pre-eclamptic women compared to normal pregnant women. The use of adjunct antioxidant therapies could potentially slow the progression of pre-eclampsia [35]. Pre-eclampsia has an unknown etiology, with some authors suggesting it is a disease based on theories but characterized by hypertension (>140/90 mmHg), proteinuria (>300 mg/d), and edema [35]. The presence of reactive oxygen species (ROS) in pregnancy arises from basal oxygen consumption, leading to mitochondrial stress and ROS production, which is associated with the development of oxidative stress and maternal vasculopathy [35]. Initially, the human placenta is hypoxic, creating an environment that supports maternal homeostasis. However, as the placenta vascularizes, an oxygen-rich environment is established, promoting the generation of ROS [35].

The presence of pre-eclampsia in developed countries is less than 3% of all pregnancies, while developing countries have a higher occurrence, with about 8,500,000 cases globally. This is associated with very high maternal and fetal mortality and morbidity. Protein-linked carbonyls

are identifiers of worldwide protein oxidation caused by various reactive oxygen species in blood, tissues, and cells. This leads to the formation of numerous products due to alterations in a large number of amino acids, damaging both sulfur-containing aromatic and aliphatic amino acids. It is important to note that oxidants disrupt the normal structure of amino acids in proteins, resulting in the formation of both protein-linked and liberated carbonyl groups. The presence of carbonyl proteins is responsible for cellular damage in the placenta [35].

Protein Carbonylation and Malaria

Malaria is a disease caused by five main species of Plasmodium, a vector-transmitted parasite that has been present for the past 70,000 years. It has had a unique influence on the human genome, resulting in erythrocyte polymorphisms that are both quantitative and structural, particularly in its haemoglobinopathies [36-37]. Vital proteins, including enzymes within the parasite, are mostly alkylated by endoperoxidases, specifically the cysteine residue of cysteine proteases. This activity within the parasite enables the uptake and digestion of hemoglobin, supporting the disintegration of red blood cells. Related concerns are as described [36]. Malaria is a communicable disease and a significant threat to life, particularly in tropical and subtropical countries where it is prevalent, leading to high morbidity and mortality rates. Factors contributing to the burden of malaria include population movement of non-immune individuals to endemic areas, lack of access to healthcare, gaps in the medication supply chain, and loss of human capital [38].

Oxidative stress is a significant immune defense system in malaria infection that affects its progression and clinical outcomes [37]. During the acute phase of infection, there is an increase in the rapid production of reactive oxygen species (ROS), leading to a decrease in parasitemia. Additionally, oxidative stress induced by antimalarial agents like artemisinin and chloroquine helps in clearing the malaria parasite. However, excessive ROS production can be harmful to host tissues, resulting in red cell breakdown, metabolic acidosis, and respiratory distress [36,37,39-40]. A pro-oxidant-rich environment favors the survival of the malaria parasite but also makes it susceptible to oxidative stress. Maintaining redox equilibrium is crucial for the parasite's survival. Studies on oxidation using Plasmodium yoelii-infected blood from mice showed reduced protein carbonylation compared to uninfected cells [41]. In a pediatric study on the oxidant and antioxidant status of severe malaria, it was observed that protein carbonyl levels, along with other oxidant and antioxidant markers, were significantly elevated ($p < 0.001$). The increased protein carbonyl levels indicate the extent of oxidative stress, providing valuable insights into the changes in these markers and their implications in the pathogenesis of severe malaria in children. Relevant associations have been elucidated [42].

Protein Carbonylation and Diabetes Mellitus

Diabetes mellitus is a metabolic disease identified by elevated blood glucose concentration. The increased blood glucose concentration, along with heightened levels of glucose-associated reactive products, accelerates the progression of diabetic complications, which can affect the kidneys, nerves, blood vessels, and eyes43. It is projected that 1.31 billion people worldwide will be affected by 2050 [44]. Currently, there is no cure for diabetes, but with proper clinical management, it can be successfully controlled. Sedentary lifestyle, poor diet, obesity, smoking, and excessive alcohol consumption are modifiable risk factors for type 2 diabetes, which increases the likelihood of developing diabetes mellitus [44]. It is important to note that diabetes is caused by either the lack of insulin secretion, insulin action, or both [45-46]. The origins of diabetic complications have been linked to various factors, including oxidative stress, pseudohypoxia, true hypoxia, carbonyl stress, activation of the polyol pathway, increased activity of protein kinase C, advanced glycation end products, changes in lipid metabolism, and alterations in cytokine or growth factor availability [45,47]. The impact of protein carbonylation in metabolic systems and cell signaling, reactive carbonyl stress, and the interaction of glucose with free radicals are explained in detail [43, 45, 47].

Protein Carbonylation and Sickle Cell Disease

The elements of oxidative damage in individuals with sickle cell disease include damage induced by free radicals during vaso-occlusion induced ischemia-reperfusion injury, as well as reduced antioxidant capacity in erythrocytes and in the circulation [48]. It has been established that plasma protein modification can be evaluated by measuring protein carbonyl levels, which have a wide range of associations with different disease conditions and have been identified as a factor that can contribute to disease pathology. In sickle cell disease, carbonyl-modified plasma proteins have been shown to cause endothelial disturbances, which are reported to be a concern in the progression of the disease. Elevated protein oxidation through carbonyl modification has been reported in sickle cell disease [48]. Additionally, carbonyl levels have been found to correlate with plasma iron and hemolysate zinc concentrations [48]. While post-translational modifications due to oxidative stress have been identified, the effects on protein function and quantity are not yet certain.

Protein Carbonylation and Hypertension

Hypertension is a leading cause of morbidity and premature death worldwide. It can affect two or more organs in the body and has a complex and multifactorial etiology [49-50]. The guideline for defining hypertension is a systolic blood pressure of > 130mmHg and/or a diastolic blood pressure of > 80mmHg. Children may have varying blood pressure numbers, and ambulatory measurements throughout the day seem to provide more accurate results than office-based measurements [50,51-52]. Various gender-based classifications have been developed and stratified 50. The etiology of hypertension has been identified in 5% of individuals (secondary hypertension), while 95% of individuals have no known cause (primary or essential hypertension) [53]. Risk factors are diverse and include environmental factors, genetics, and interactions among physiological organs [50,51].

Lifestyle factors can be modified in conjunction with the use of appropriate medication to lower blood pressure. Identifying risk factors early could lead to necessary interventions. Approximately one million people worldwide

are determined to suffer from hypertension, with 9 million deaths attributed to it annually. This number continues to rise at an alarming rate. Projections suggest that 1.56 billion people will be suffering from hypertension by the year 2025 [54]. Urgent and strict interventions are needed as soon as possible.

Vascular dysfunction, cardiovascular remodeling, immune system disturbances, and other issues are associated with hypertension and have been summarized [50]. Oxidative stress has been implicated in these disturbances through redox-reactive and responsive mechanisms, leading to vascular damage [50,55]. In a study using animal models to assess protein carbonylation using the oxyblot method, levels of protein carbonyl were found to be increased. Various concerns are described [56]. Similarly, Wong and Suzuki [57] presented noteworthy results, including mechanisms and approaches. It is important to note that there has been limited research on protein carbonylation in hypertension of different classes. The existing research could serve as a foundation for further insights into the role of carbonylation in hypertension.

Protein carbonylation and liver disease

The liver is the largest internal organ of the body and is exposed to reactive oxygen species activity [58]. The disequilibrum between production of free radicals and its elimination by the liver antioxidant defence system is what is responsible for the extensive damage. Some of the outcome from the extensive damage are induction of irreversible changes in cell ligands including proteins and DNA and these directly affect metabolic mechanisms that regulate appropriate biological functions [58]. Some key aspects of liver disease are alcoholic liver disease, non alcoholic fatty liver disease and alcoholic steatosis and liver cirrhosis which is the final stage and so far no pharmaceutical or nutrient based treatment for managing individuals with alcoholic liver disease [59]. Major and minor alcoholic liver process is described (Figure 2). In a publication by Pomacu et al. [60] pathological features that are useful in liver cirrhosis are varied and those include hepatocyte degradation and neo-fibrotic crosis, substitution of liver parenchyma by fibrotic tissues and nodules capable of regeneration , then loss of liver function. In any case, exposure of the liver to increase quantity of ethanol undergoes structural and functional changes due to oxidative stress and inflammation. Ethanol (Ethyl alcohol) elevates the release of reactive oxgen species with an attendant production of pro –fibrotic cytokines and the liberation of multiple inflammatory markers and collagen production when liver fibrosis progresses [60]. Depending on the type of liquor, the amount of ethyl alcohol varies between 20 -30%. It should be noted that the liver receives blood from the stomach and small intestine through the portal vein and its volume of ethyl alcohol is much more with only 2 – 5 % of it lost unchanged through urine and breath with the majority of its excretion taking place through hepatic metabolism61. The parenchymal cells of the liver which makes up more than 70 % of the liver tissue metabolises alcohol and has the largest number of enzymes that oxidizes ethanol. Chief among these enzymes are alcohol dehydrogenase and aldehyde dehydrogenase alongside cytochrome p4502E (CYP2E1) and catalase . In chronic alcohol consumption , oxidative stress leads to elevated peroxidation of polyunsaturated fatty acids to form highly reactive electrophillic α/β unsaturated aldehydes that brings about post translational protein modification altering capability [62]. In a study by Shearn et al. [62] using immunohistochemistry and western blotting, there was an elevated protein carbonylation in end stage alcoholic liver disease which occurred basically in the hepatocytes . Of the 1224 carbonyalted proteins in normal hepatic and end stage alcoholic cirrhosis , 411 have been identified to be unique to cirrhotic alcoholic liver disease, 261 unique to normal hepatic tissue and 552 common to both groups. Bioinformatic pathway analysis of these carbonylated proteins within the liver are as documented [62]. In a related study, age realted increases in reactive oxygen species and protein carbonylation have been identified in the liver but no report on the functional impact has been described and research that showed a functional effect of the oxidative stress did not identify targets of carbonylation. Moreso, elevated carbonylation of endoplasmic reticulum resident proteins takes place in the liver with age. These resident proteins are endoplasmic reticulum chaperone protein GRP78 and calreticlin . The reactivity of these proteins by carbonylation in the liver could interfere with protein folding and quality assurance [63]. Liver disease associated with elevated proetein carbonylation are alcoholic liver disease, non alcoholic fatty liver disease, non alcoholic steatohepatitis with fatty liver supplying the much needed ecosystemfor the generation of reactive lipid species and the protein modification that follws afterwards [63]. Also, alcohol down regulates antioxidant enzymes such as glutathione peroxiadse and upregulates hydrogen peroxidase [64].

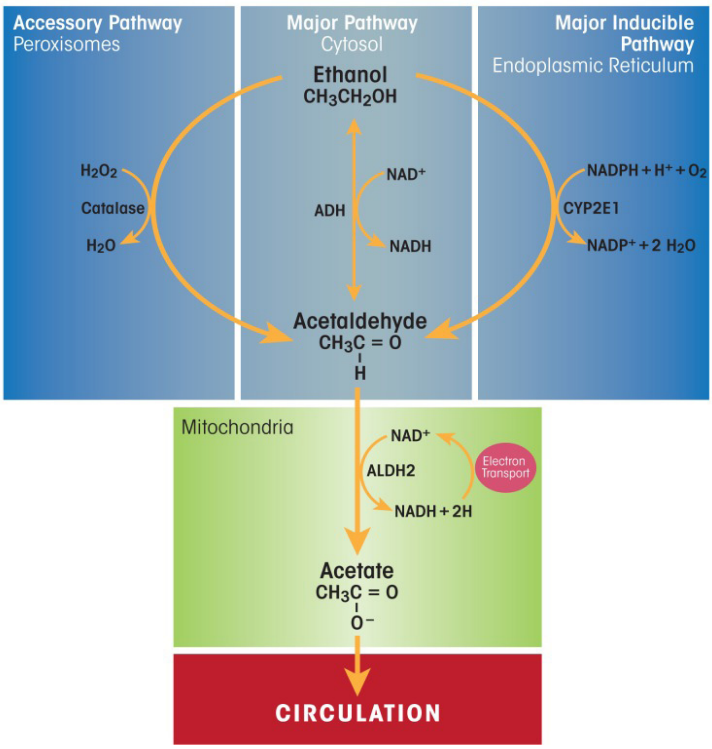

Fig 2: Major and Minor Alcohol Metabolic Process in the Liver [59].

Protein Carbonylation and Peptic Ulcer Disease

Peptic ulcer disease (PUD) is a gastro intestinal disease that is influenced by gastric acid or pepsin secretion . There is a discontinuation in the interior mucosal linning of the gastrointestinal tract (GIT). This alteration continues into the muscularis propria layer of the gastric epithelial surface. The stomach and the proximal duodenum are target sites. However, the lower eosophagus , distal duodenum or jejunum may be affected [65]. Pain within the epigastric region of the abdomen is symptomatic within half an hour following a meal in individuals with agastric ulcer. Also, pain with a duodenal ulcer becomes evident 2 – 3 hours after a meal . Besides , gastric acid secretion, *Helicobacter pylori* infection has been implicated in peptic ulcer disease alongside non steroidal anti-inflammatory drug accounting for most of the disease origin [65]. Rarer causes of PUD are Zollinger–Ellison syndrome, malignancy , stress , viral infection , vascular insufficiency, radiation treatment, chron disease and chemotherapy. Other agents that may be involved in peptic ulcer disease are smoking and alcohol consumption. Some of the complications that could arise from from PUD are upper gastrointestinal bleeding, gastric outlet obstruction, perforation , penetration and gastric ulcer. A 2015 report by Ashrafrezaei et al. [66] 68 out of 150 cases showed that protein carbonyl levels were significantly elevated in cases of H. pylori infection compared to controls. This therefore confirms oxidative damage by H. pylori. The pathogenesis of H. pylori infection is the production of alkaline mileu by urease enzymes and oxidative stress [66]. However, more work needs to be done in respect of carbonylation given the dearth of information on protein carbonylation associated with peptic ulcer disease.

Protein carbonylation and vitamin B12 deficiency

Vitamin B12 or cobalamin is a B group water soluble vitamin and lready implicated in neuronal health and haem production. It is mostly present in animal tissues and generally not available in plants and this may be the reason why vegetarians are a risk group for vitamin B12 deficiency [67-68]. In developed countries, clinical B12 deficiency is limited and primarily as a result of genetic aberrations and often leads to myeloneuropathy or megaloblastic anaemia, a disease where there is presence of megaloblast). Megaloblast becomes apparent when restriction of DNA production causes asynchronous maturation between the nucleus and the cytoplasm with symptoms being properly identified neurological symptoms [69]. B12 could possess antioxidant properties with subclinical B12 deficiency contributing to oxidative stress and the start of age associated diseases68. Subclinical B12 deficiency has been indicated to be serum B12 levels between 119 – 200 pmol/L with the possibility of long term destruction to nucleic acids, proteins and lipids even though individuals may be symptom free [68]. Three general factors have been identified to cause subclinical B12 deficiency and are inadequate intake, increased demand and malabsorption [68]. It is already established that the imbalance between pro-oxidant such as ROS and antioxidant lead to oxiadative stress. Eukaryotic cells constantly eliminate free radicals via endogenous antioxidant . The nuclear factor – erythroid -2-related factor (NrF2) is a major modulator of endogenous antioxidant defenses [68]. Once oxidative stress occurs , excess of the ROS promotes inflammation and subsequent cytokine production which brings about more ROS production. Inflammatory responses mediates tissue repair in response to aggressors but also with a deleterious effects if it continues longer than necessary [68]. Also ROS can destroy functional molecules and tissues through the transformation of carbohydrate, proteins , lipids, DNA and these activity alongside antioxidant properties of B12 have been summarized [68] (Figure 3). Currently, there is limited work done on investigations between protein carbonylation and vitamin B12 deficiency. This, most likely remains an area to be examined explicitly to further push the frontiers in vitamin B12 related conditions.

Protein Carbonylation and Psoriasis

Psoriasis is a chronic dermal disease that is not contagious and impacts various populations [70]. There is presence of cutaneous aggregation of neutrophils liberating reactive oxygen species [71]. The prevalence rate stands at less than 11.8% across populations. Majority of persons under the age bracket of 35 years are heavily impacted by psoriasis. Genetic, epigenetic and environmental effects contribute to the development of psoriasis [70]. The contributions by these effects lead to altered interactivity between immune elements, cell signaling proteins and dermal cells givig rise to psoriasis. The import of protein carbonylation and its

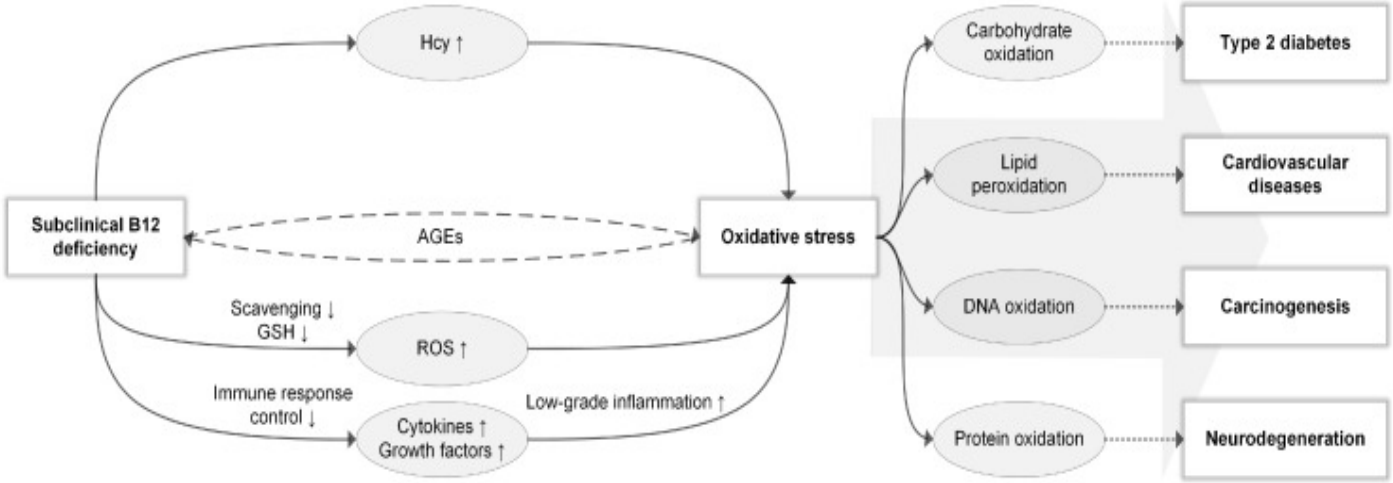

Figure 3: Subclinical B12 Deficiency in relation to Oxiadative Stress [68].

outcome between amino acid residues of proteins and reactive oxygen species or reactive nitrogen species (RNS) are already established and this leads to oxidative stress. Recently , it is established that persons with psoriasis presents with increased level of reactive oxygen species in the dermal stratum [70]. The attack of proteins by reactive oxygen species is a continuous process and this impacts protein molecular structure, net charges, folding and hydrophobicity/hydrophillicity and this certainly produces protein carbonyl groups. Much more, myeloperoxidase present in activated neutrophils releases hypochlorous acid, chloraminated oxidants and chloramines which results in advanced oxidation protein products (AOPPS) which has crosslinked dityrosine. The dual presence of carbonyl groups and AOPPS are identified as early determinants of oxidative stress and can be utilized to assess protein damage caused by oxidation [70]. Myloperoxidase is a basic modulator of anti tumor action of neutrophils [72]. Main functions of activated neutrophils are phagocytosis, liberation of granule contents, neutrophil extracellular traps, antigen presentation , production of reactive oxygen species, role in inflammatory responses and ability for tissue damage especially in chronic inflammatory conditions [72,73]. In psoriasis, those between the ages of 41-55 years, those who are obessed and females with waist circumference greater than 88 cm had significant levels of protein carbonyl and with a negative correlation but not significantly between protein carbonyl with age, duration of psoriasis, BMI and waist circumference in the studied population [70]. In a related study by Yacizi et al. [71] protein carbonyl were found to be higher in psoriasis patients with oxidative stress with findings such as inflammation, phagocytic cells oxidation, MPO–hypochlorous acid oxidation reactions which is shown by increased total/differential leucocytes counts, CRP, ESR, MPO, neopterin, AOPP, Protein carbonyl compound, pyrolised protein, lipid hydroperoxidases (LPH) and reduced thiol levels.

Protein Carbonylation and Therapeutic Interventions

A number of strategies and interventions have been deployed to mitigate the effect of oxidative stress as a result of reactive oxygen species . some of these interventions are enzymatic and non enzymatic in nature. Also, chelators, reducing agents scavengers but non of these have shown impactful delivery in the reduction of oxidative stress with great therapeutic impact [64]. For diabetes mellitus , carbonyl stress can be managed by redox mutation , RCO texofication and inhibition of carbonyl stress. Hypotensive agents such as angiotensin – converting enzyme inhibitor and angiotensin II receptor antagonist are identified as useful therapies because of non production of side effects such as vitamin B6 deficiency and neurotoxicity which have been associated with first generation of carbonyl stress inhibitors such as amino guadine that behaves as RCO trapping agents [12]. For antioxidant therapy, limitations exist and one of such limitation is the significant destruction to macromolecules and tissue injury which afterall leads to cell death In effect , antioxidant therapy can only rescue undamaged macromolecules and surviving cells, a concern that will not be satisfactory to limit symptoms . There is also the concern in utilizing antioxidant therapy which is that clinical experts cannot identify individuals who might benefit from particular antioxidant therapy . So far, optimal daily requirements of usual antioxidants such as α- tocopherol and vitamin C are still being debated and guidelines have not been for less usual though necessarily less significant antioxidants such as γ-tocopherol . It has been identified also that certain groups of individuals might react adversely to specific antioxidants as found in some living cancer patients utilizing β-carotene. In view of these , clinical experts will determine an appropriate method for the type of anti-oxiadant supplementation appropriate for certain individuals and the responsiveness of patients to the prescribed treatment [12].

Protein Carbonylation Procedures

The standard method for quantifying carbonyl groups involves their reaction with 2,4-dinitro-phenylhydrazine (DNPH) to form 2,4-dinitrophenylhydrazone, a stable product. Various techniques can be used to analyze the dinitrophenyl group (DNP) adduct. The interaction of the DNP group with ultraviolet light enables the spectrophotometric quantification of total carbonyl levels in proteins or protein complexes. To shift the absorption maximum of DNP from 370nm (UV) to 450nm (visible region), the medium must be alkalized [14,74-75].The primary protein carbonylation reaction involves the direct oxidation of lysine, arginine, proline, threonine, and other amino acid side chains, resulting in detectable protein products with DNPH. Secondary protein carbonylation reactions can also occur through the addition of aldehydes, such as those generated from lipid peroxidation chemistry [2].

A. Spectrophotometric and HPLC estimations of protein carbonyl following dinitrophenyl hydrazine modification

The original procedure for derivatizing carbonyl groups with various agents to estimate carbonyl availability was described by Levine in 1990. The content of the published document evaluates the reactivity of carbonyls with borohydride , DNPH, fluorescein thiosemicarbazide and fluorescein amine76. This procedure describes how oxidatively modified proteins greater than 0.0005g of protein are processed with 10mM DNPH for 60 minutes. This is followed by a precipitation step using 20% trichloroacetic acid (TCA), then washed with ethanol-ethyl acetate at a ratio of 1:1, and dissolved in 6M guanidine. The process is finalized by measuring at 360-390 nm 76,77. Other methods of derivatization with DNP followed by high performance liquid chromatography (HPLC) estimation and /or immunoblotting have also been described [78].

Limitations of the spectrophotometric procedure

1. It is complex and labour intensive and takes a lot of time.
2. It can't meet up for high throughput estimations.
3. Protein and volume needs are high.
4. Loss of acid soluble protein during wash is between 10 -15% (Result must be expressed in terms of the loss and protein level computed for.
5. Additional carbonyl groups may be added due to the acidic state.

6. DNP may be held in the protein pellet and making it soluble again may be defective which could lead to false reports [76,79].

Other identified concerns that are common include nucleic acid interference because it contains carbonyl groups. It may be advisable to consider a depletion protocol in this regard. Additionally, interference by biologically active compounds such as haemoglobin, myoglobin, and retinoids, which absorb within the visible spectrum of 370nm, can lead to elevated background readings. The lack of commercially available and accessible protein standards and controls makes it challenging to compare results using spectrophotometric measurements between laboratories [76].These limitations can be overcome by using HPLC determination, which offers the advantage of detecting DNP absorbance at 366nm, equivalent to protein absorbance at 280nm. In this case, DNP derivatization should be carried out using sodium dodecyl sulfate (SDS) instead of guanidine-HCL, as the latter is unsuitable for most columns [76].

B. Enzyme linked immunosorbent assay (ELISA) for the estimation of protein carbonyl

In 1971, Peter Perlmann and Eva Engvall developed the first ELISA, using an enzyme-linked antibody to detect rheumatoid factor. In 1997, it was further developed using anti-DNP antibody as described by Buss et al. [80] A standard curve was created using HOCl-oxidized (hypochlorous acid, a weak acid identified by French chemist Antoine Jerome Balard in 1834) and sodium borohydride-reduced BSA. Reduced BSA is used for the blocking step [76]. To proceed with the ELISA procedure, plasma from severely ill patients and healthy patients must be diluted to 4mg/ml and processed with 10mM DNPH for three-quarters of an hour at room temperature. The volume ratio of sample to DNPH is 1:4 (20% dilution). The variable concentration of carbonyl for both severely ill patients and healthy patients is documented [81]. A strong linear correlation (r = 0.70) was determined between the absorbance of the spectrophotometric assay (375nm) and the ELISA for plasma samples (n = 26) [76].

The protocol by Buss et al. [80] has been modified by Alamdari [82]. This procedure requires protein samples to be diluted in phosphate-buffered saline, which are then adsorbed to wells of an ELISA plate and reacted with DNPH. The protein-conjugated DNPH is analyzed by commercially produced anti-DNPH antibody, followed by the addition of a second antibody conjugate with horseradish peroxidase for estimation. Calibration of this procedure necessitates oxidized albumin and 5µg of protein. The valuable aspect of this modification is the elimination of the need to concentrate protein in samples, whether experimental or clinical, with minute amounts of protein. This modification also eliminates the effect of TCA on carbonyl measurement. The standard curve linearity was found to be in the range of 0 – 3.4 nmol carbonyls/mg protein. Aqueous humor and diluted plasma samples have been analyzed for carbonyl content using the modified protocol [82].

Studies utilizing anatomical portions of animal models..... (organ homogenate) as outlined by Augustyniak et al. [83] identified four distinct variants of the protein carbonyl assay across different laboratories. Reports from these laboratories varied in terms of the increase in protein oxidation status over time of irradiation. Reasons for these discrepancies include differences in assays, protein aggregation, and the disappearance of nearly all healthy oxidized proteins during the experiment [83]. Additional concerns that could contribute to the observed differences include dilutions, temperature variations, blocking agents used, washing requirements, sample diluents, and primary antibody requirements [83]. To ensure accurate and reliable results, it is essential for research experts to address these concerns in their technical approaches. The differences in incubation temperature for the ELISA and spectrophotometric methods are well explained [76].

ELISA is an enzyme immunoassay (EIA) procedure with a heterogeneous background used for clinical analysis, and is a modified form of the radioimmunoassay (RIA). Tagged antigens and antibodies are conjugated with enzymes instead of radioactive iodine [84]. In the ELISA method, a reactivity component is adsorbed nonspecifically or covalently bound to the surface of a solid stage. Examples of the solid stage include a microtiter well, plastic bead, or magnetic particle, which aids in easier segregation of the bound and free labeled components in the reaction [84]. The general steps for an ELISA procedure are coating, blocking, detection, and final read, and the four main types of ELISA techniques are as described [84].

Benefits of the ELISA protocol

1. Large number of samples can be estimated.
2. Aliquots of samples can be utilized.
3. Plasma, tissue and cell culture samples can be utilized.

Limitations of the ELISA protocol

1. Estimation of protein concentration is mandatory before executing the assay since assay takes 2 days for sample adsorption to the plate overnight.
2. The wash steps in-between may bring about loss of sample. ELISA plates with varied binding capacities for various requirements are being deployed by companies depending on the hydrophobic, hydrophilic or mixed domain of the molecules involved for estimation.
3. The various monoclonal and polyclonal antibodies present a problem as well since they potentially have a reactivity with different epitopes.
4. There is an absence of available uniform and recognized protein standards which leads to insufficient comparison [76].

There is no information about the molecules being oxidized or the identity of the carbonylation , whether primary or secondary [83].

C. Determination of Protein Carbonyl by Immunoblotting

The immunoblotting method described by Shacter et al. [81], Levine et al. [78] and Keller et al. [85] may not offer

the much-needed accuracy. Additionally, some of the identified problems include issues with reproducibility. If reproducibility is affected, reliability may not be guaranteed at any level of testing. Another concern is the performance of pre- and post-electrophoresis derivatization, specifically the use of nitrocellulose membrane which is not satisfactory for incubation in highly acidic solutions. The pre- and post-electrophoresis derivatization described by Keller et al. [85] is limiting due to lengthy wash steps, making the procedure time-consuming [76,78,81,85]. The results of the immunoblotting method cannot be determined with specific concentration due to its semi-quantitative nature [76]. To address this issue, the utilization of appropriate controls and good laboratory practices should be prioritized. Various variants of immunoblotting (western blot) for cell lines and protein extracts have been described [83]. Currently, carbonyl western blot using the trade name OxyBlot is widely used in educational experiments [3]. Immunoblotting, also known as western blot, was developed in 1979 and is a popular technique routinely used in research, molecular diagnostics, and proteomics studies in laboratories worldwide. It is used for the detection and semi-quantification of proteins with specific amino acids that have undergone post-translational modifications due to physiological changes in both diseased and healthy states. The identified mechanisms for amino acid modifications include phosphorylation, ubiquitination, biotinylation, glycosylation, methylation, acetylation, sumoylation, nitration, oxidation/reduction, nitrosylation, and other variations [86]. The protocol and issues associated with immunoblotting are extensively described [86].

D. Determination of Protein Carbonyl by Gel Electrophoresis

Gel electrophoresis using polyacrylamide for the determination of protein carbonyls has high resolution for protein evaluation and could eliminate impurities of low molecular mass. The reason for utilizing gel electrophoresis for the detection of protein carbonyls is the availability of unreacted DNPH and non-protein carbonyls [79]. The four significant steps using gel electrophoresis for protein carbonyl studies are DNPH derivatization of carbonyl groups at acidic pH (1M HCl), gel electrophoresis, electrotransfer to PVDF membrane, and antibody-based determination [79]. The beauty of the gel electrophoresis methodology, as described by Rogowska-Wrzesinska et al. [79] is the possibility to quantify the extent of carbonylation of each protein in association with its total quantity. It should be noted that various chemical probes for determining protein carbonyls in polyacrylamide gel have been invented and advanced [79]. The specific approaches for when the DNPH derivatization is carried out have been detailed alongside other associated procedures [79].

E. Determination of Protein Carbonyl by Chromatography

Notably, gas chromatography (GC), high performance liquid chromatography (HPLC), and liquid chromatography coupled with tandem mass spectrometry (LC-MSMS) can be used for the determination of protein carbonyls. This provides quantitative knowledge about protein carbonylation and the site of carbonylation. Released protein carbonyls such as formaldehyde, acetone, isobutyraldehyde, and glyoxylic acid, liberated from oxidized amino acids such as alanine, valine, leucine, and aspartic acid, have shown appreciable results following the deployment of reverse phase (RP-HPLC). The differential concerns regarding the use of chromatographic analysis for protein carbonyl determination are properly elucidated [79].

F. Detection of Protein Carbonyl by Mass Spectrometry

This method provides the opportunity to identify protein modifications without the need to understand the specific type of modification present. High-throughput investigations of complex protein mixtures may not be feasible with this procedure due to the labor requirements. Mass spectrometry offers an ideal methodology for evaluating modified proteins because the covalent incorporation or depletion of a chemical moiety from an amino acid results in an increased or decreased molecular mass of that residue. This phenomenon is demonstrated by the oxidation of a methionine residue, which increases the mass from 13Da to 147Da through the incorporation of a single oxygen atom [79]. Different proteins that have undergone oxidative modifications exhibit varying properties, therefore specific approaches tailored to a particular type of modification are necessary and have been clearly explained [79].

Quality Control, Standardisation and Safety Requirement in Protein Carbonyl Analysis

The work by Rogowska-Wrzesinska et al. [79] on the analysis of protein carbonylation is extensive and has indeed highlighted the challenges, assurances, and potentials in commonly used methods. As with any research and clinical investigations, the importance of quality control and standardization cannot be overlooked in order to achieve reliable, relevant, and reproducible results (3R's). In this context, their recommendations for quality control primarily focus on the analytical phase and include the following: 1) Measurements should be conducted promptly. 2) Experimental steps should be minimized to the essentials. 3) Derivatization should be carried out early in the process. 4) Primary chemicals from reputable vendors should be utilized. 5) Working solutions should be freshly prepared to prevent contamination. 6) Optimization and standardization of the entire procedure should be implemented. 7) Control samples and steps should be included to eliminate background noise and signal interference. Additionally, adherence to good laboratory practices and the adoption of a global quality assurance system incorporating ISO 15189, ISO 9001, and ISO 17025, with clearly defined rules for establishing and maintaining quality systems in the laboratory, should be taken into account [87-88].

Reference materials, internal reference samples, and participation in external quality assurance programs define the success of analytical accuracy. Additionally, in light of safety concerns in protocol handling, technical documents should be carefully reviewed to identify various risks, safe handling instructions, properties of reagents,

contact information for vendors and manufacturers, and the date the technical document was detailed according to Occupational Safety and Health Administration guidelines [87]. Pre-analytical considerations in quality control requirements include the sample matrix, buffer composition, quality of chemicals, acidity or alkalinity levels, temperature requirements, atmospheric oxygen, number of steps, stabilizers, presence of other oxidized molecules, elimination of excess reagents or interfering substances, storage conditions, and enrichment protocols. Guidance on modifying any aspect of the procedure should also be provided [79]. Effective healthcare delivery and expert team collaboration are essential. This involves establishing valuable connections between laboratories and in-vitro diagnostic (IVD) manufacturers to test the applicability and standardization of different methods for various sample matrix types and considerations. Interlaboratory evaluations will provide insight into the strengths, weaknesses, opportunities, and threats associated with different methods (reproducibility concerns) and allow participants to assess their competence and status in handling protein carbonyls [79,87].

Conclusion

Protein carbonylation is an intriguing aspect of oxidative protein chemistry. The shared developmental concerns between plants and humans demonstrate that oxidative stress is a persistent issue in all living organisms and must be effectively managed. This paper has presented the progression and outcome trends of protein carbonylation in several common clinical and pathological conditions. By utilizing the methods outlined, the degree of protein carbonylation can be accurately determined, providing insight into which proteins have been modified and where these modifications have occurred. It is recommended that user-friendly methodologies be implemented and that these technologies be advanced to different institutions and hospitals with the necessary expertise.

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