CHAPTER 1

INTRODUCTION

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And God has created you from the earth, growing (gradually), and in the end he will return you into the earth, and raise you forth (again at the resurrection). Meanwhile, the recent researches have proved that, the constituents of human corps are the same as earth especially minerals and metals. Metals play important roles in a wide variety of biological processes of living systems. Homeostasis of metal ions, maintained through tightly regulated mechanisms of uptake, storage and secretion is therefore critical for life and is maintained within strict limits [(1)]. Metal ion transporters participate in maintaining the required levels of the various metal ions in the cellular compartments [(2)]. It is also known that several essential transition metals, such as zinc, iron, copper, cobalt and manganese participate in the control of various metabolic and signaling pathways. However, their rich coordination chemistry and redox properties are such that they are capable of escaping out of the control mechanisms such as homeostasis, transport, compartmentalization and binding to the designated tissue and cell constituents. Breakdown of these mechanisms can lead to the metal binding to protein sites other than those tailored for that purpose or displacement of other metals from their natural binding sites. A growing amount of results provide evidence that toxic and carcinogenic metals are capable of interacting with nuclear proteins and DNA causing oxidative deterioration of biological macromolecules [(3)].

Metals are known to modulate gene expression by interfering with signal transduction pathways that play important roles in cell growth and development [(4)]. Deregulation of cell growth and differentiation is a typical characteristic of the cancer phenotype. Actions of metals interfere with deregulation of cell proliferation by activating various transcription factors, controlling cell cycle progression and apoptosis [(5)]. The most important involve the nuclear factors NF-κB (nuclear factor- kappa B), AP-1 (activator protein 1), NFAT (nuclear factor of activated T-cells) and the tumor suppressor protein p53 [(6)].

It is well established that organic life depends on inorganic elements for carrying out many vital processes. Life metal ions play a crucial role in the human body and small deviations from normal levels of concentration are recognized as symptoms of malfunctions or diseases. They are essential for several cell reactions and varied metabolic and physiological functions. No one can deny the importance of Na^+, K^+, Mg^{2+} and Ca^{2+} ions in cells and as neurotransmitters. Trace and ultra trace metal ions control essential biological processes of living cells and without their catalytic presence many biological reactions would not take place. The appearance of several diseases may be related to metal ion depletion. For instance, deficiency of iron, magnesium or calcium causes anemia, cardiovascular diseases or osteoporosis, respectively [(7)]. However, they become toxic to cells when their concentrations surpass certain optimal (natural) levels. When there is excess of metals, such as, Cu (II) and Fe (II) in Wilson's and thalaseamia diseases, correspondingly, then chelating agents may be used to reduce their concentrations [(7, 8)]. Metal ions exist as 'free' or 'bound' in the body. They 'free' hexahydrated when they can move around in the body liquids and they are 'bound' when they form complexes with covalent bonds [(7)].

1. Essential Metals

1.1. Iron

Iron occurs in the oxidation states (+2) and (+3). The ferrous ions are soluble in biological fluids and generate in the presence of oxygen damaging hydroxyl radicals. The ferrous ions are unstable in aqueous media and tend to react with molecular oxygen to form ferric ions and superoxide anion radical. The oxidized form of iron is insoluble in water at neutral pH and precipitates in the form of ferric hydroxide [(9)]. Paradoxically, despite the fact that both iron ions, ferrous and ferric are so inaccessible, iron is the key catalytic site of many of the enzymes and oxygen-transporting proteins in cells [(6)].

Although iron is vital for life, it can be toxic when it is present in excess [(10)]. Iron homeostasis is a complex process, as there are many different proteins that respond not only to the total body burden of iron, but also to stimuli such as hypoxia, anemia and inflammation [(6)]. About 65% of iron is bound to hemoglobin, 10% is a constituent of myoglobin, cytochromes and iron-containing enzymes, and 25% is bound to the iron storage proteins, ferritin and hemosiderin. About 0.1% of body iron circulates in the plasma as an exchangeable pool, essentially all bound to transferrin. The process of chelation not only facilitates the transport of iron into cells, but also prevents iron-mediated free radical toxicity [(6)].

1.2. Copper

The most oxidation numbers of copper in living organisms are Cu (II) and Cu (I). The essential trace element copper is a cofactor of many enzymes involved in redox reactions, such as cytochrome c oxidase, ascorbate oxidase, or superoxide dismutase. In addition to its enzymatic roles, copper is used in biological systems for electron transport [(3)]. The blue copper proteins that participate in electron transport include azurin and plastocyanin [(6)]. Copper is readily absorbed from the diet across the small intestine (~ 2 mg/day) and stored in the liver. The major excretory route of copper stored in liver is via the biliary pathway (~ 80%) [(11)]. Copper is bound to either serum albumin or histidine and trafficked through the bloodstream for delivery to tissues or storage in the liver. Copper is imported into the hepatocytes via the high-affinity human copper transporter, hCtr1 localized on the plasma membrane. hCtr1 also participates in the intracellular compartmentalization of this metal [(12)]. Once inside the cell, copper is escorted to *(i)* metallothionein pool, or *(ii)* transported to the mitochondria for cytochrome c oxidase incorporation, or *(iii)* for delivery to emerging Cu, Zn-SOD or *(iv)* transported to the Wilson disease P-type ATPase in the trans-golgi network for subsequent incorporation to the ceruloplasmin [(13)]. Ceruloplasmin contains about 95% of the copper found in serum [(6)].

Copper can catalyze reactive oxygen species (ROS) formation via Fenton and Haber–Weiss chemistry and therefore under physiological conditions, free copper very rarely exists inside cells. In the process of the investigation of copper chaperone for superoxide dismutase (SOD), Rae et al. (1999) [(14)] explored that the upper limit of so-called “free pools of copper”

was far less than a single atom per cell. This finding is of great importance, especially when considering other physiologically important trace metal ions [6].

1.3. Zinc

Zinc is a ubiquitous trace element found in plants and animals. The adult human body contains approximately (1.5–2.5 grams) of zinc, present in all organs, tissues, fluids and secretions [6]. Zn not only enhances the action of insulin and manages blood glucose concentration, but also plays an essential role in the development and maintenance of the immune system [15]. In contrast, Zn deficiency causes growth retardation and hypogonadism, loss of appetite, dermatitis, reduced taste acuity, delayed wound healing, impaired reproduction, and poor immune function [16]. Zn deficiency is related to poor dietary intake, excessive dietary phytate intake, chronic illness, or over-supplementation with for Cu [17].

Protective role

Zn plays an essential role in cell membrane integrity, and is a component of more than 300 different enzymes that function in many aspects of cellular metabolism, involving metabolism of proteins, lipids and carbohydrates [18]. The functions of Zn comprise the stabilization of conformation in transcription factors. Zn also modulates cellular signal transduction processes [19].

In numerous systems Zn can antagonize the catalytic properties of the redox-active transition metals (Fe and Cu) with respect to their abilities to promote formation of $^{\bullet}OH$ from H_2O_2 and superoxide [20]. Some studies have reported on the ability of Zn to interact with Cu and Fe, decreasing the metal content in tissues and retarding oxidative processes [21]. For instance, in LEC rats, Zn acetate increases tissue concentrations of Zn and metallothionein and decreases that of Cu and Fe by reducing Cu and Fe transport from the mucosal to serosal intestinal sides through competitive mechanisms [22, 23]. In a fibroblast cell line exposure to both Zn and Fe caused Zn accumulation with a concomitant decrease in Fe content. The reduction of Fe content in Fe–Zn cells compared to Fe cells has been attributed to competitive inhibition of Fe uptake by the divalent metal transporter in the presence of Zn [24, 25].

In addition, the role of Zn in protecting biological structures from free radical damage may be due to several possibilities: *i)* maintaining an adequate level of metallothioneins (MTs), which are also free radical scavengers; *ii)* as an essential component of Cu, Zn-SOD; *iii)* as a protective agent for thiols and other chemical groups [19]. Santon et al. (2003) [26] have shown that the maintenance on basal levels of the antioxidant enzymes activities, of GSH content and other serum biochemical parameters in Zn-treated LEC rats strongly support the hypothesis that Zn can retard oxidative mechanisms, suggesting a role in modulating ROS.

1.4. **Magnesium**

Magnesium is the second most abundant element in cellular systems [27]. It is an important cation that plays significant role as a cofactor for more than 350 enzymes in the body, especially those utilizing high energy phosphate bonds such as ATPases [28-30]. Besides being involved in maintaining the genomic stability, processes of synthesis, transcription, and translation, it also regulates activity of several ion channels [30, 31]. Magnesium homeostasis is closely linked with calcium, sodium and potassium homeostasis and therefore any disturbances in magnesium homeostasis are bound to be associated with calcium, sodium and potassium homeostasis and vice versa [32]. It exerts a large variety of biological functions, ranging from structural roles by complexing negatively charged groups, i.e. phosphates in nucleic acids, catalytic roles in enzyme activation or inhibition, and regulatory roles by modulating cell proliferation, cell cycle progression and differentiation. Even though less understood as compared to calcium homeostasis, the intracellular magnesium content appears to be regulated by Mg^{2+} uptake, efflux, and intracellular compartmentization, also in response to external stimuli. With respect to genomic stability, several aspects are of major importance. They include the role of magnesium in DNA replication and protein synthesis, its function as cofactor in DNA repair proteins, its role in maintaining the anti-oxidative status of the cell and finally its effect on cell cycle regulation and apoptosis. Magnesium deficiency or the displacement of Mg^{2+} by other toxic divalent metal ions leads to an increased genomic instability, as evident by inhibited DNA repair, oxidative stress, aging, and carcinogenicity [27].

Magnesium is transported across the plasma membrane in both directions, most likely via Na^{+}/Mg^{+} exchange. Whether influx and efflux of Mg^{2+} are mediated by the same mechanism operating in opposite directions or whether there are distinct pathways is still not known. Both influx and efflux are hormonally controlled and regulated by modifications in intracellular cAMP levels and in protein kinase C activity. Concerning the intracellular distribution of magnesium, major intracellular compartments are mitochondria, the nucleus, and the endoplasmatic reticulum. Bound magnesium can be mobilized from these compartments to increase the concentration of free intracellular Mg^{2+} if required, for example for cell cycle progression or apoptosis. Thus, the distribution of magnesium within cells is highly regulated, and many enzymes in different biochemical pathways are activated or inhibited by changes in free Mg^{2+}, some of which are relevant for maintaining genomic stability [27].

1.5. **Calcium**

Calcium (Ca) is the most abundant mineral in the human body 99% of total body Ca is contained in the bones; approximately 1% of this Ca is freely exchangeable with the extracellular fluid. Three hormones - parathyroid hormone, vitamin D, and calcitonin - act in concert to maintain serum Ca concentration at a nearly constant level. These hormones direct intestinal Ca absorption, renal reabsorption, Ca excretion, and use of Ca stores in the bone [33]. Bone Calcium accretion or accumulation is positive during the first 18 years of life, with the highest rate of accumulation occurring in the first year. When growth stops, bone mineral status decreases to a very low level. Peak bone mass is genetically determined. Between 20

and 30 years of age, bone mass accumulation peaks and then slowly declines throughout life [33].

Since the discovery of calcium as a second messenger, it has been implicated in an increasingly number of biological functions [34]. Indeed, calcium regulates basic cell processes such as proliferation, protein synthesis, and differentiation, but is also engaged in more specialized cell functions such as muscle contraction, bone growth, blood clotting, cardiac function, neurotransmitter release, electrical excitability, and synaptic plasticity [35]. This implication in ever increasing cell functions reveals the amazing versatility in signaling properties of this second messenger. Moreover, Ca^{2+} is not only a trophic factor, but is also involved in programmed cell death [36]. Ca^{2+} differs from other second messengers in that, as all the elements of the periodic table, it cannot be metabolized. The second original property of Ca^{2+} is that it is present in relatively high concentration in the extracellular space (1.0 to 2.0 mM). Consequently, a strict spatiotemporal control of intracellular Ca^{2+} concentration is essential for its involvement in a wide variety of cell functions. To this end, Ca^{2+} homeostasis is managed by a wealth of ion channels localized at both the plasma and the intracellular organelle membranes that permit elevation in cytosolic Ca^{2+} concentration, as well as by Ca^{2+}-binding proteins and Ca^{2+}-pumps that restrict cytoplasmic Ca^{2+} rise and organize its propagation in the cytosol and nucleus [37]. One amazing and unique property of Ca^{2+} as a second messenger is its ability to control cellular events that develop on a large time scale, from milliseconds to hours [35]. The millisecond scale gives the full measure of the importance of fast gating Ca^{2+} channels, unique pathways that promote localized Ca^{2+} elevation. For instance, electrical excitability and neurotransmitter release entirely rely on this rapid signaling [38]. Due to the intricate balance between import, sequestration and export pathways, intracellular Ca^{2+} signals are generally transient. Long-term Ca^{2+} effects consequently occur thanks to the effective recruitment of various cytosolic and/or nuclear signaling pathways whose lifetimes are of longer durations than Ca^{2+} signals themselves. Ca^{2+} signal integration indeed explains how Ca^{2+} can be involved in development, cell differentiation and synaptic plasticity. The ability of Ca^{2+} to control both short- and long-term processes links cell differentiation and cell specialization to Ca^{2+}-regulated cell activity. As an example, late-phase of long-term potentiation in hippocampal CA1 neurons requires protein synthesis under the control of N-methyl-D-aspartate (NMDA) receptors and voltage-dependent calcium channels [39]. Many long-term effects of Ca^{2+} as a second messenger imply a direct or indirect effect of Ca^{2+} on gene regulation [40]. Recent development of high-through put analyses methods has provided a vertiginous list of candidate genes whose expression is under the control of Ca^{2+} homeostasis [41, 42]. For instance, 70% of the gene expression modifications (up- or down regulation of 111 genes) observed in T-lymphocytes from severe combined immunodeficiency patients results from a defect of Ca^{2+} entry [42]. In neuronal cells as well, exon expression profiling revealed modifications in the expression of several thousand transcripts in response to depolarization-induced Ca^{2+} influx [41].

1.6. **Manganese**

Manganese (Mn) is an essential element for humans, animals, and plants, and is required for growth, development, and maintenance of health. There are inorganic and organic manganese compounds, with the inorganic forms being the most common in the environment [43]. Manganese is naturally present in food, with the highest concentrations

typically found in nuts, cereals, legumes, fruits, vegetables, grains, and tea; it is also present at low levels in drinking water [43]. Typical, daily intakes range from 2-9 mg/day for adults and approximately 3-5 per cent is absorbed from the gastrointestinal tract [44]. Absorption of manganese from the diet occurs in the divalent and tetravalent state [45]. Manganese balance studies and excretion data indicate that low gastrointestinal absorption and rapid elimination of manganese limits the toxicity of the manganese following the ingestion of high doses [45]. Chronic inhalation exposure to relatively high levels of manganese has been associated with adverse neurological effects and a few studies have reported the same following the ingestion of high levels or chronic exposure to manganese in drinking water [44, 46]. Clinical manganese neurotoxicity has been reported in patients receiving long-term parenteral nutrition and in patients with chronic liver dysfunction or renal failure, as a result of their inability to eliminate and clear manganese from the blood [47-49].

Manganese ions (Mn^{3+}) bind to the same location as ferric ions (Fe^{3+}) on the large glycoprotein molecule mucin, which is known to stabilize the ions preventing precipitation in the lumen of the gastrointestinal tract. Both metals are known to have an affinity for the intercellular metal binding molecule transferrin. Absorption of metal ions into enterocytes is known to take place via transmembrane transporters [50]. Gunshin et al. (1997) [51] cloned the divalent metal transporter1 (DMT1) from proximal small bowel, which avidly binds Fe^{2+} ions, but also has an affinity for Mn^{2+} and other cations. In this regard, it is important to mention that dietary Fe^{3+} is firstly reduced to Fe^{2+} by ascorbate or surface ferrireductases before being transported via DMT1 into the enterocytes [52]. During iron deficiency the number of transporters in enterocyte membranes is increased in order to maximize iron absorption. This will inevitably result in increased Mn absorption, particularly in the absence of Fe. Iron has a strong influence on Mn homeostasis as both metals share the transporter, transferrin (Tf), binding and uptake via the Tf transporter and the divalent metal transporter, DMT1/ NRAMP2 [50].

1.7. **Chromium**

Chromium is the 24th element of the Periodic table. Chromium exists in a series of oxidation states with a valence from (-2) to (+6); the most important stable states are (0) "elemental metal", (+3) "trivalent", and (+6) "hexavalent". Trivalent Cr (III) and hexavalent Cr (VI) compounds are thought to be the most biologically significant. Cr^{+3} is an essential dietary mineral in low doses. It is required to potentate insulin and for the normal glucose metabolism [53]. Chromium deficiency has been associated with impaired glucose tolerance, fasting hyperglycemia, glucosuria, elevated percent body fat, decreased lean body mass, maturity-onset diabetes, cardiovascular disease, decreased sperm count, and impaired fertility [3]. The US National Academy of Science has established a safe and adequate daily intake for chromium in adults of 50–200 (µg/day) [54]. Cr^{+3} is found in most fresh foods and drinking water. Dietary sources rich in chromium include bread, cereals, spices, fresh vegetables, meats and fish. Other significant sources of chromium are mineral supplements and brewer's yeast. Of interest were comparative studies on two Cr (III) popular dietary supplements; chromium (III)-picolinate and chromium (III)-niacin [104]. This study revealed that Cr (III)-picolinate produces significantly more oxidative stress and DNA damage. Studies have implicated the toxicity of chromium picolinate in renal impairment, skin blisters, anemia, haemolysis, tissue edema, liver dysfunction, neuronal cell injury, depletion of

antioxidant enzymes (SOD, GPx, and GSH) and DNA damage. Oxidative stress has therefore been proposed as a major pathway of Cr (III)-picolinate induced toxicity. Niacin-bound chromium (III) has been demonstrated to be more bioavailable and efficacious with almost no toxicity. This study demonstrated that the toxicity of chromium (III) compounds is largely dependent on the ligand [(55)].

1.8. **Cobalt**

Cobalt is the 27th element of the Periodic table. The most common oxidation numbers of cobalt are +3 "Co (III)", and +2 "Co (II)" which form a number of organic and inorganic salts. Cobalt is an element that occurs naturally in many different chemical forms throughout our environment. It is a natural earth element and is present in trace amounts in soil, plants and in our diets. In pure form it is a steel-grey to black shiny hard metal. Cobalt usually occurs in association with other metals such as copper, manganese, nickel and arsenic. Small amounts are found in most rocks, soil, surface and underground water, plants and animals. Cobalt is also released to the environment from burning coal and oil, from automotive/airplane exhausts and from industrial processes that use the metal or its compounds. Cobalt and its salts are used in a variety of processes e.g. as a paint drier, as an ingredient of colored pigments and others. Some radioactive isotopes of cobalt, such as Cobalt 60, are used in treating patients in nuclear medicine and in research. Natural cobalt is air stable for a few days but it is more stable in water and soil [(3)].

The discovery, in 1948, that Vitamin B12 contains 4% cobalt proved this element was essential to man although it was known to be essential for ruminant animals much earlier. Even before 1935 it was known that a lack of cobalt, from deficiency in the soil, produced a wasting disease in animals [(3)]. Cobalt is considered an essential element, it is readily absorbed in the small intestine, but the retained cobalt serves no physiological function since human tissues cannot synthesize B12 in the intestine by *E. coli* [(3)]. Most of the consumed cobalt is excreted in the urine with very little being retained, mainly in the liver and kidneys. Cobalt's only known function is its integral part of Vitamin B12. A cobalt deficiency has never been produced in humans. Signs and symptoms of one are actually those of a B12 deficiency. Interestingly, recent studies show that cobalt may be linked with iodine in the formation of thyroid hormones [(56)].

1.9. **Vanadium**

Vanadium is the 23rd element of the Periodic table. Vanadium may be beneficial and possibly essential in humans, but certainly essential for some living organisms [(57)]. The usual oxidation states of the vanadium are V (III), V (IV) and V (V). The vanadium ions play a role in biology as counter ions for various proteins, DNA and RNA. Vanadium is widely distributed on Earth; however, its role as a micronutrient in humans is not yet established. Humans are exposed to vanadium mainly through the polluted atmosphere from combustion products of vanadium bearing fuel oils, fumes and dust. Food contains a very low content of vanadium, usually below 1.0 ng/g. Vanadium enters the organism by inhalation, skin and

gastrointestinal tract and accumulates mainly in the liver, kidney, spleen, bones and to a lesser extent also in lungs [3]. Vanadium (V), dominant at the cellular level at physiological pH, enters the cell through the anion transport mechanism. Inside the cell vanadate (V) is reduced to vanadyl (IV) by biological reductants, such as intracellular glutathione or ascorbic acid. Vanadium in cells is distributed in the nucleus and the supernatant fractions. Almost 90% of vanadium is in the supernatant fractions, the rest is in the mitochondria, microsomes and the nuclear and cell debris. Vanadyl and vanadate is bound to carboxyl and amino groups of the amino acids of proteins and phosphates. Brain cells contain high levels of ascorbate which also bind to V (IV) [3].

Vanadium as a Therapeutic Agent

Vanadium compounds may exert preventive effects against chemical carcinogenesis in animals by modifying xenobiotic enzymes, thus inhibiting carcinogenic active metabolites. Research on the activity of anti-tumor metal compounds received strong attention after the discovery of the anticancer effects of *cis*-$PtCl_2(NH_3)_2$ (*cis*-platinum). Vanadium may also exert inhibitory effects on cancer cell metastatic potential through modulation of cellular adhesive molecules and reverse antineoplastic drug resistance [58]. Due to its relatively low-toxicity, vanadium was established as an effective non-platinum, metal anti-tumor agent. However, many problems remain which require both basic and applied research in this area [3].

1.10. **Molybdenum**

Molybdenum (Mo) occurs in a wide range of metalloenzymes in bacteria, archaea, fungi, algae, plants and animals where it forms part of the active sites of these enzymes. However, in order to gain biological activity Mo requires the coordination by a pyranopterin, thus forming a prosthetic group named molybdenum cofactor (Moco). Mo has a versatile redox-chemistry that is used by the enzymes to catalyze diverse redox reactions. This redox-chemistry is controlled both by the different ligands at the Mo atom and the enzyme environment [59]. Mo-containing enzymes are essential for life, since they hold key positions both in the biogeochemical redox cycles of nitrogen, carbon and sulfur on Earth [60] and in the metabolism of the individual organism. Hitherto more than 50 enzymes are known to be Mo-dependent. The vast majority of them are found in bacteria while in eukaryotes only seven have been identified [61]. Mo belongs to the group of trace elements, i.e. the organism needs it only in minute amounts. Uptake of too high amounts of Mo however results in toxicity symptoms [62]. On the other hand unavailability of Mo is lethal for the organism. However, even if Mo is available for the cell, it is biologically inactive until it becomes complexed to form molybdenum cofactor [59].

1.11. **Selenium**

Selenium is a metalloid element with the atomic number 34. It is one of the elements that determine the normal functioning of an organism; it has antioxidant properties and protects the organism against the actions of free radicals and carcinogenic factors. Selenium is an element that fulfills an important physiologic function, but there is a fine line between the concentration that still has beneficial effects on an organism and that at which selenium begins exerting toxic effects. Selenium is involved in the metabolism of hydrogen peroxide and lipid hydroperoxides. It constitutes an integral part of some enzymes, including the glutathione peroxidase (GPx), deiodinase iodothyronine, and thioredoxin reductase (TRxR) which protects cells from the noxious effects of free radicals formed during oxidation processes. Selenium is found in relatively unfamiliar active proteins, generally designated selenoproteins. The significance of the newly identified type SelO, SelT, SelV, and SelI selenoproteins (Sel) still has not been completely elucidated. Studies clearly indicate that selenium-containing yeast can be an effective, safe, and natural source of dietary selenium [(63)].

Selenium has been shown to be an essential component of all living organisms [(64-66)]. It was discovered in 1817 by the Swedish chemist and doctor Jöns Jakob Berzelius [(67, 68)]. Selenium is present in 25 identified selenoproteins that commonly occur in the human organism as well as in 12 selenoproteins in yeast cells. Selenoproteins play an important part at a cellular level in many metabolic processes [(69-72)].

Selenium is characterized by a narrow safety range between deficiency and toxic doses [(73, 74)]. The recommended selenium intake varies according to geographic regions. The World Health Organization recommends a daily selenium dose of 30 μg to 40 μg for adults, for adult Egyptians (49 μg/day) and also emphasizes that a selenium dose of 400 μg/day is harmless. The Food and Nutrition Board of the National Academy of Science states that, the daily selenium requirement according to age varies in men (40–70 μg) and in women (45–55 μg) [(74-76)]. Daily selenium doses of 100 μg to 200 μg suppress genetic damages. Clinical studies have shown that selenium also may protect against prostatic carcinoma [(77)]. The selenium antineoplastic mechanism of action is associated mainly with its antioxidant action. Apart from the substantial function of the anti-free radical mechanism in the defense against neoplasmas, the considerable effect of this element on the cytotoxic activity of natural killer cells is emphasized. Nevertheless, the mechanism that visibly mediates against the occurrence of neoplastic effects is not yet fully understood [(70, 78, 79)]. Selenium doses above 400 μg/day to 700 μg/day may exert toxic actions [(74, 76, 80)]. Doses exceeding the recommended dietary allowances are certainly required to suppress genetic damage and neoplasma occurrence. However, there was the hypothesis that excessive selenium intake may cause oxidative damage, which leads to genome instability [(77)].

Selenium activates antineoplastic factors, prevents heart diseases, has anti-proliferative and anti-inflammatory properties, stimulates the immune system, and exerts antagonistic action in relation to heavy metals [(78, 81, 82)]. In research carried out by Nakajima et al (2001) [(83)], an influence was found of selenium on the accumulation of heavy metals, that is, uranium, mercury, zinc, and herbicide paraquat by *Candida utilis* and *Saccharomyces cerevisiae* yeast. Thus, as the selenium concentration in yeast increased the levels of heavy metals (uranium, mercury, and zinc) absorbed by *C. utilis* yeast cells also increased. Protein complexes with selenium contain stoichiometric amounts of these elements and block bound metal involvement in biological reactions [(77, 83)].

Selenium also shows a protective action in persons infected with the hepatitis B or C virus. Studies carried out in recent years have shown that selenium plays a substantial part in diabetes prevention [70, 82]. Selenium deficiency may result not only from poor nutrition, but also genetically disturbed selenoprotein synthesis and abnormal transport of selenium [63].

Sources rich in selenium are eggs, fish (87.6–737 ng/g), corn (wheat, maize, and rice), meat (chicken liver), fruits, vegetables (garlic bulbs, onion, and broccoli), yeast, bran, coconut fruits, Brazil nuts, seafood, and young barley seedlings [138,158,159]. Selenium in its inorganic form, that is, selenite (IV) or selenate (VI) is absorbed by plants from the soil and then converted into organic forms, mainly Selenocysteine (SelCys) (Figure 1) and Selenomethionine (SelMet) (Figure 2) [75]. The other form dominates in products coming from animals. Humans consume this form of selenium. In human organisms, it undergoes further conversions by binding to proteins and its incorporation into the amino acids SelCys. Most plants have no possibility for the accumulation of selenium in large amounts (the concentration rarely exceeds 100 mg/g of dry substance). However, certain plant species, such as garlic (*Allium sativum*), mustard greens (*Brassica juncea*), rape (*B. napus*) and certain fungi are characterized by their special ability to bind selenium. According to data in the literature [84], selenium bioaccumulation levels can exceed 1000 mg/kg without negative consequences. This probably causes a decrease in intracellular concentration of selenium from the SeCys and SeMet forms during the digestion process [85].

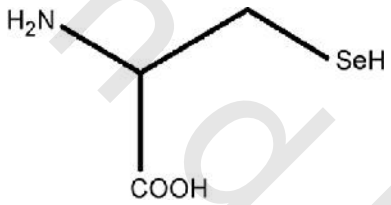

Figure 1: Selenocysteine [75].

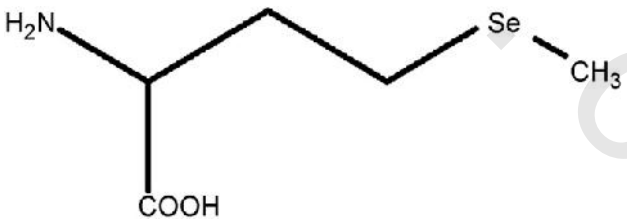

Figure 2: Selenomethionine [75].

Metabolism of selenium

In an organism, compounds containing selenium are metabolized along two pathways [69]. The first involves the reduction of selenium combined with its methyl group. Starting from compounds in oxidized state (+VI). Selenates (+VI) are reduced to selenites (+IV) and, probably via elemental selenium (±0), further to selenides (-II). Selenates (+VI) may undergo enzymatic activation with adenosine-5'-triphosphate to adenosine-5'-selenophosphate, which then is reduced to (+IV) selenite in the presence of glutathione. Non-methylated forms of

selenium are converted to monomethyl, dimethyl, and trimethyl forms. The trimethylselenonium ($TMeSe^+$) ion is excreted in urine. The other dimethyl form of selenium (DMeSe) is an intermediate metabolite that is excreted through breathing, but only when its formation rate exceeds the further methylation to $TMeSe^+$ ion rate. The selenium monomethyl form (MMeSe) is usually excreted through SelMet metabolism. Metabolic conversions taking place within this pathway are represented in (figure 3) [69].

$$Na_2SeO_4 \rightarrow Na_2SeO_3 \rightarrow Se_2(CH_3)_2$$

Figure 3: Selenium metabolic conversions associated with its reduction and methylation [69].

Biomethylation processes tend toward organism detoxification because DMeSe and $TMeSe^+$ are less dangerous compared with other selenium compounds [63]. The inorganic form of selenium becomes reduced to an intermediate product (i.e. HSe^- selenide). Selenium-containing amino acids are converted into selenides through hydrolysis of the C–Se bond in the SeCys β-position, which is either a direct product of selenoprotein hydrolysis or is formed in the SelMet-conversion pathway resembling the sulphur pathway for methionine and cysteine. Furthermore, methylselenoamino acids are converted into methylselenol by hydrolysis of the C–Se bond in the SeMet γ-position and β-position of Se-methylselenocysteine (MeSeCys). Then, the formed methylselenol is converted into a selenide [86].

The active form in selenium supplements is methylselenol for the biological use of SelCyc and SelMet [63]. Thus, organic or inorganic forms of selenium are converted into the same intermediate product (i.e. selenide). Any excess is converted into methylated metabolites (e.g. 1-β-methylseleno-N-acetyl-D galactosamine "selenosugar") and urea [87]. Hydrogen selenide (an intermediate metabolite formed during the conversion of all selenium forms in cells) can be used for selenoprotein synthesis or further metabolized in the methylation process pathway [86].

The SelCys remnants in selenoproteins that come from food products are a main source of selenium for humans. SelCys is present in most cases as a single remnant in selenoproteins that form active sites in selenoenzymes, except for selenoprotein P in blood plasma [88]. SelCys is used for the synthesis of selenoproteins after digestion to the intermediate product (i.e. selenide). SelCys is formed by synthesis and not hydrolyzed in the selenoprotein synthesis process [87].

The second metabolic pathway for selenium compounds is direct selenium incorporation or binding by proteins in which selenium replaces sulphur mainly in the amino acids cysteine and methionine. Antagonism in the selenium–sulphur relationship can both reduce selenium toxicity and intensify selenium deficiency in an organism. L-SelMet is the major selenium form and is well assimilated by the organic form of selenium. It is absorbed into the protein polypeptide chain more easily than any other form of this element. The assimilation of D-SelMet enantiomers is five times lower than that of L-SelMet and they are decomposed to inorganic selenium (figure 4) [89, 90].

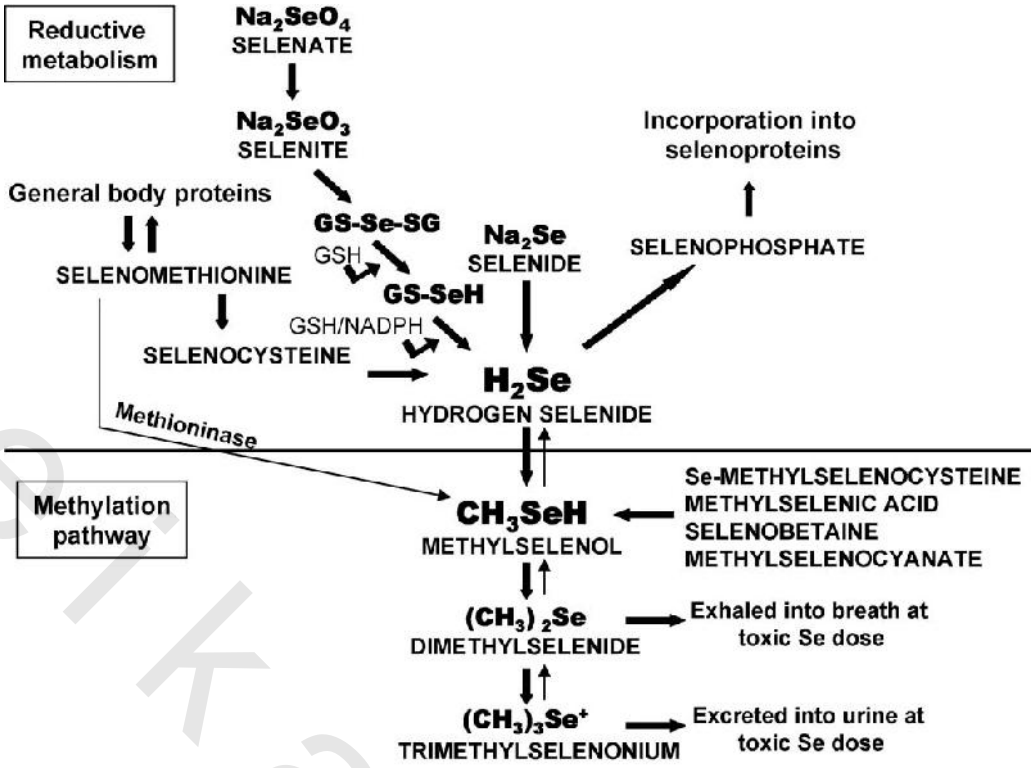

Figure 4: Schematic representation of the Se metabolic pathway [90].

2. Essential nonmetals:

2.1. Phosphorus

Phosphate plays a major role in cellular metabolism. It is a constituent of membrane phospholipids and is used by the mitochondria to form adenosine triphosphate. Additionally, Pi is involved in both glycogenolysis and glycolysis. 85 % percent of total body Pi is in the skeleton and 14% is in soft tissues. Less than 0.3% of total Pi is in the blood. The concentration of Pi in the serum of human adults is maintained between (3.5 and 4.5 mg/dl). There is a diurnal variation of serum Pi with a nadir in the morning, a rise in early afternoon, and a peak at night. The maintenance of phosphate homeostasis in the face of variations in dietary phosphorus supply is achieved primarily by the kidneys and to a lesser degree by the intestine. Both the intestinal absorption step and the renal tubular reabsorption of Pi are altered in response to acute and chronic changes in dietary phosphorus intake. Phosphate availability can also be markedly reduced by the administration of antacids and other phosphate binders that are commonly prescribed to patients with chronic renal insufficiency [91].

2.2. **Sulfur**

Sulfur is an essential component of all living cells. It is the seventh or eighth most abundant element in the human body by weight, being about as common as potassium and a little more common than sodium or chlorine. A 70 kg human body contains about 140 grams of sulfur. In plants and animals, the amino acids cysteine and methionine contains most of the sulfur. Sulfur is present in all polypeptides, proteins and enzymes that contain these amino acids. In human, methionine is an essential amino acid that must be ingested. However, save for the vitamins biotin and thiamine, cysteine and all sulfur-containing compounds in the human body can be synthesized from methionine. Disulfide bonds formed between cysteine residues in peptide chains are very important in protein assembly and structure. These covalent bonds between peptide chains confer extra toughness and rigidity. For example, the high strength of feathers and hair is in part due to their high content of disulfide bonds and their high content of cysteine and sulfur. Homocysteine and taurine are other sulfur-containing amino acids that are similar in structure, but not coded by DNA, and are not part of the primary structure of proteins. Many important cellular enzymes use prosthetic groups ending with -SH moieties to handle reactions involving acyl-containing biochemicals: two common examples from basic metabolism are coenzyme A and α-lipoic acid. 2 of the 13 classical vitamins, biotin and thiamine contain sulfur, with the latter being named for its sulfur content. Sulfur plays an important part, as a carrier of reducing hydrogen and its electrons, for cellular repair of oxidation. Reduced glutathione, a sulfur-containing tripeptide, is a reducing agent through its sulfhydryl (-SH) moiety derived from cysteine. The thioredoxins, a class of small protein essential to all known life using neighboring pairs of reduced cysteines to act as general protein reducing agents, to similar effect [(92)].

Analogous to carbon-based molecules, metals are crucial for the maintenance of cell homeostasis and preservation of life. They display important structural, regulatory and catalytic functions in different types of proteins, such as enzymes, receptors and transporters [(50)]. Among the 23 elements with known physiological functions, 12 are metals (sodium, magnesium, potassium, calcium, vanadium, chromium, manganese (Mn), iron (Fe), cobalt, copper, zinc, and molybdenum) [(93)]. Nutritional deficiencies in specific trace-element metals (Fe) [(94)], zinc [(95)] and Mn [(96)], as well as genetic disorders leading to altered metal homeostasis [(97, 98)], culminate in human diseases. At the other spectrum, exposures to toxic levels of essential metals, such as Mn [(99)], Fe [(100)] and zinc [(101)], may lead to pathological conditions. Of particular importance, oxidative stress and neurodegeneration have been reported as consequences of toxic exposures to essential metals, along with dyshomeostasis in essential metal metabolism [(102-104)].

Xenobiotic metals with no physiological functions, such as aluminum, cadmium, lead and mercury, are present in measurable concentrations in living organisms [(93)]. Such metals often enter organisms by molecular mimicry, utilizing inherent transporters for essential metals [(105)]. Environmental, occupational or intentional exposures to xenobiotic metals are frequently related to the development of toxicity and pathological conditions [(3)].

The best evidence supporting the hypothesis of the oxidative nature of metal-induced genotoxic damage is provided by the wide spectrum of nucleobase products typical for the oxygen attack on DNA in cultured cells and animals exposed to carcinogenic metals. Detailed studies in the past two decades have shown that metals like iron, copper, cadmium, chromium, mercury, nickel and vanadium possess the ability to produce reactive radicals,

resulting in DNA damage, lipid peroxidation, depletetion of protein sulfhydryls and other effects. Reactive radical species include a wide range of oxygen-, carbon-, sulfur- radicals, originating from the superoxide radical, hydrogen peroxide, and lipid peroxides but also in chelates of amino acids, peptides, and proteins complexes with the toxic metals. The toxic effects of metals involve hepatoxicity, neurotoxicity and nephrotoxicity [3].

Most carcinogenic metals have been shown to produce the superoxide anion radical ($O^{\bullet}_{2}^{-}$) and hydroxyl radical ($^{\bullet}OH$) mostly via the Fenton reaction [106]. Metal-induced ROS production has also been implicated in the initiation of cellular injury and the stimulation of inflammatory processes, which can lead to cancer development [107]. Further, oxidative stress causes genetic and epigenetic changes, uncontrolled cell growth, and abnormal cellular signaling, all of which are primary mechanisms involved in metal-mediated carcinogenesis [17].

3. **Nonessential metals**

3.1. **Cadmium**

Cadmium is the 48th element and a member of group 12 in the Periodic table of elements. The most common oxidation number of cadmium is (+2). Cadmium is a heavy metal; roughly 13,000 tons of cadmium is produced, worldwide, each year for nickel-cadmium batteries, pigments, chemical stabilizers, metal coatings and alloys. The toxicity of cadmium relates to smelting where the main route of exposure is through the lungs. In contaminated areas, there is evidence to suggest increased body burdens of cadmium among a proportion of the exposed population, with some evidence of increased urinary excretion of β-2-microglobulin and some loss of bone density among people with the highest urinary cadmium concentrations [3]. There are also areas where extensive mining operations have led to contamination, particularly affecting the soil. Soluble cadmium salts accumulate and result in toxicity to the kidney, liver, lungs, brain, testes, heart, and central nervous system. Cadmium is listed by the US Environmental Protection Agency as one of 126 priority pollutants. In most studies, the half-life in humans is estimated to be between 15 and 20 years [3]. Cadmium can cause osteoporosis, anemia, non-hypertrophic emphysema, irreversible renal tubular injury, eosinophilia, anosmia and chronic rhinitis. Cadmium is a potent human carcinogen and has been associated with cancers of the lung, prostate, pancreas, and kidney. Because of its carcinogenic properties, cadmium has been classified as a #1 category human carcinogen by the International Agency for Research on Cancer of USA [108].

Cadmium itself is unable to generate free radials directly however, via indirect mechanisms, it can cause free radical-induced damage to the gene expression. It has been reported that cadmium can cause activation of cellular protein kinases (protein kinase C), which result in enhanced phosphorylation of transcription factors and consequently lead to the transcriptional activation of target gene expression. These conclusions are based on experiments involving inhibitors that are regarded as specific for various kinases [3].

New findings in the explanation of cadmium-induced carcinogenicity have been reported [109]. It has been reported that E-cadherin, a transmembrane Ca (II)-binding glycoprotein

playing an important role in cell-cell adhesion, can bind cadmium to Ca (II)-binding regions, changing the glycoprotein conformation. The disruption of cell-cell adhesion caused by cadmium binding to the E-cadherin could play an important role in the tumor induction and promotion. Calcium was found to inhibit colorectal carcinogenesis, and thus may act as an anticarcinogen [(110)].

Cadmium and DNA Repair Mechanism

The results indicate that cadmium primarily causes toxicity by deactivating an essential DNA repair activity. The direct inhibition of DNA mismatch repair by cadmium provides a molecular mechanism for cadmium toxicity. There exist many mutation avoidance systems that correct damaged DNA. These include direct damage reversal, base excision repair, nucleotide excision repair, and double strand break repair and mismatch repair (MMR). Cadmium seems to inactivate only one of them the MMR system. During the past decade, results from human systems and yeast have showed that genetic disruption of MMR by mutations in the Mut S homolog (MSH) family of proteins leads to substantial increases in genome instability and greatly increases the incidence of several kinds of human cancer [(3)]. Jin et al (2003) [(111)] found that cadmium-induced inhibition of MMR in human cell extracts leaves about 20 – 50% of DNA mismatches unrepaired. Inhibition of MMR leads to the propagation of cellular errors, thus the toxic effects of cadmium can be amplified in cells by creating mutations in genes that induce further faulty functions. As cadmium binds to protein sites with high occupancy when it has multiple protein ligands and typically replaces zinc, these results suggest an unidentified zinc site for MMR function that is specifically disrupted by cadmium [(3)].

Cadmium and Free Radicals

Cadmium itself is unable to generate free radicals directly; however, indirect generation of various radicals involving the superoxide radical, hydroxyl radical and nitric oxide has been reported [(112)]. Some experiments also confirmed the generation of non-radical hydrogen peroxide which itself in turn may be a significant source of radicals via Fenton chemistry [(113)].

Various studies have been made on the cadmium-induced testicular toxicity in rat models. Yang and coworkers (2003) [(114)] reported significantly higher contents of malondialdehyde (MDA) and glutathione peroxidase (GSH-Px) in exposed groups. Glutathione was found to scavenge intracellular oxygen radicals either directly or via the GSH peroxidase/GSH system. The activity of superoxide dismutase (SOD) in the tested animals was lowered. This study also revealed that the number of cells with DNA single strand breaks and the levels of cellular DNA damage were significantly higher in exposed groups than in controls [(3)].

An interesting mechanism explaining the indirect role of cadmium in free radical generation was presented some years ago. In this mechanism it was proposed that cadmium can replace iron and copper in various cytoplasmic and membrane proteins (e.g. ferritin, apoferritin), thus increasing the amount of unbound free or chelated copper and iron ions participating in oxidative stress via Fenton reactions [(115)]. Displacement of copper and iron by cadmium can explain the enhanced cadmium-induced toxicity, because copper, displaced

from its binding site, is able to catalyze breakdown of hydrogen peroxide via the Fenton reaction [3].

3.2. **Lead**

Lead is a non-essential element that occurs naturally in the environment. However, the highest concentrations found in nature are the result of human activities. Many of its physical and chemical properties such as softness, malleability, ductility, poor conductibility and resistance to corrosion, have favored that man uses lead and lead compounds since ancient times for a great variety of applications. It is well documented that lead can cause adverse health effects that include neurotoxicity, nephrotoxicity, and deleterious effects on the hematological and cardiovascular systems [116]. It has also been found to be capable of eliciting a positive response in a wide range of biological and biochemical tests, which include tests for enzyme inhibition, fidelity of DNA synthesis, mutation, chromosome aberrations (CA), cancer and birth defects [116]. Nevertheless, data related to the mutagenic, clastogenic and carcinogenic properties of inorganic lead compounds are still conflicting. The International Agency for Research on Cancer (IARC) classified lead as possible human carcinogen (group 2B) and inorganic lead compounds as probable human carcinogens (group 2A) [117]. In some epidemiological studies exposure to lead has been linked to an increased incidence of some cancers such as stomach, lung and bladder cancers [116]. There are several proposed mechanisms to better understand the carcinogenic properties of lead and the conditions required for this purpose. These mechanisms include mitogenesis, alterations in gene transcription, oxidative damage and several indirect genotoxicity mechanisms [118].

Lead and oxidative stress

Similar to other persistent toxic metals such as arsenic, cadmium and mercury, lead damages cellular components via elevated levels of oxidative stress. The pathogenetic effect of lead is multifactorial since it directly interrupts the activity of enzymes, competitively inhibits absorption of important trace minerals and deactivates antioxidant sulfhydryl pools [119].

Free radical-induced damage by lead is accomplished by two independent, although related mechanisms [120]. The first involves the direct formation of ROS including singlet oxygen, hydrogen peroxides and hydroperoxides and the second mechanism is achieved via depletion of the cellular antioxidant pool. Interrelations between these two mechanisms exist so that the increase in ROS on one side simultaneously leads to depletion of antioxidant pools on the other [121]. Glutathione represents more than 90% of the non-tissue sulphur pool of human body and the major effect of lead is on glutathione metabolism [122]. In addition, glutathione is important substrate acting in the metabolism of specific drugs and toxins via glutathione conjugation in the liver. The sulfhydryl groups of glutathione bind effectively toxic metals such as arsenic and mercury. Therefore an organism exposed to lead has significantly lowered levels of glutathione, with respect to the control groups, which may in turn enhance the toxicity of other metals [6].

There are two specific enzymes, glutathione reductase (GR) and δ-aminolevulinic acid dehydrogenase (ALAD) that are both inhibited by lead [123]. An epidemiological survey of lead exposure among children (lead concentration >10μg/dL) in India has shown significantly suppressed levels of ALAD with respect to children with lead concentration (<7μg/dl) [124]. A direct correlation between blood lead levels, ALAD activity and erythrocyte levels of MDA has been observed among workers exposed to lead. Inhibition of ALAD by lead increases levels of the substrate δ-aminolevulinic acid (ALAD) which is known to stimulate the formation of ROS substantiated by the elevated levels of MDA [6].

Glutathione reductase (GR) is an enzyme responsible for recycling of oxidized glutathione (GSSG) to reduced glutathione (GSH) and lead has been shown to interfere with this cycle resulting in depressed GSH levels. Both trends, elevated and suppressed blood levels of catalase, SOD and glutathione peroxidase have been observed [6].

3.3. **Nickel**

Nickel is the 28th element of the Periodic table. It is a silver-white metal found in several oxidation states, ranging from (-1) to (+4). However, the (+2) oxidation state "Ni (II)" is the most common form of nickel in biosystems. Chemical and physical forces (e.g., erosion, leaching, and precipitation) constantly redistribute nickel between land, water, and air. Depending on the soil type and pH, nickel is highly mobile in soil [3]. Based on laboratory studies, nickel probably does not accumulate in fish and there is little evidence for the bio-magnification of nickel in the food chain. Occupational exposure occurs in mining, alloy production, electroplating, refining and welding. Epidemiological studies revealed an increased risk of respiratory tract and nasal cancers in miners and workers in nickel refineries [125].

Nickel Homeostasis, Essentiality and Toxicity

Nickel can enter body via inhalation, ingestion and dermal absorption. The amount of nickel absorbed by the gastrointestinal tract depends on the type of nickel species in the food, the content and the absorptive capacity. Normally, only 1-2 % of ingested nickel is absorbed. The daily intake of nickel has been estimated to be in the range (35–300 μg/day). The chemical form of nickel determines the route by which nickel enter the cells. Soluble nickel, for example nickel carbonyl is fat soluble and can freely cross cell membranes, most probably by diffusion or through calcium channels. Some authors in fact suggested absorption of nickel by trans-membrane diffusion, whilst others proposed absorption of Ni (II) via Ca (II) channels [3].

Several studies related toxic and carcinogenic effect of nickel with changes in calcium metabolism. Soluble nickel compounds are known to enter the cell through the calcium ionophore channel ionomycin, which increases the uptake of nickel by a factor of (4-5). Nickel is known to be a calcium channel blocker, thus the decrease in transport of Ca (II) to intracellular space is compensated by increase of free Ca (II) from intracellular stores. These changes of intracellular concentrations of Ca (II) have been shown to signal gene expression changes associated with cell growth, differentiation and apoptosis. The mechanism of nickel

invoked release of stored intracellular calcium involves a cell surface receptor [3]. Absorption of nickel carbonyl during inhalation has been confirmed by numerous studies. On the other hand, it has been reported that insoluble nickel particles enter the vertebrate cells by phagocytosis [3].

The main transport protein of nickel in blood is albumin, although a nickel containing (α_2-macroglobulin), called nickeloplasmin, also transports nickel. Following exposure to nickel carbonyl, the highest concentrations of nickel appear in the lung, brain, kidney, liver, and adrenals. The biological half-life of nickel oxide in the lung depends, in part, on particle size and ranged from (11-21 months) in animal studies. Nickel is not a cumulative metal; it is excreted well via urine and feces. The urinary excretion of nickel is rapid and the elimination appears to follow first order kinetics without evidence of dose-dependent excretion of nickel. Following absorption, the kidney is the primary route of elimination. Excretion of nickel also occurs in the saliva and sweat, which may contribute significantly to the elimination of nickel in hot environments. Based on the data obtained two mechanisms for nickel-induced oxidative DNA damage have been proposed: (*i*) all the nickel compounds used induced indirect damage through inflammation, and (*ii*) Ni_3S_2 also showed direct oxidative DNA damage through H_2O_2 formation. This double action may explain the relatively high carcinogenic risk of Ni_3S_2. This implies that a high content of nickel and its clearance from tissue is directly proportional to nickel carcinogenic activity [3].

Nickel-Induced Activation of Transcription Factors

Nuclear factor-kappa B (NF-κB) is an important transcription factor in both apoptosis and the inflammatory process and was found to be activated by nickel. For example nickel-induced allergic response and skin hypersensitivity are connected with activation of NF-κB [3]. Also the ATF-1 transcription factor was found to be activated in nickel treated cells. The ATF-1 transcription factor belongs to an ATF/CREB family that was originally identified as a target of the cAMP signaling pathway [126]. As described above, nickel is known to affect calcium homeostasis. An increase of intracellular calcium after nickel administration in turn leads to activation of a protein kinase cascade that mediates ATF/CREB phosphorylation. Some studies suggest that the activation of the ATF-1 transcription factor by nickel played the role of a negative regulator of TSP I. The TSP I protein is a potential regulator of tumor development *in vivo* since the increased levels of this protein suppress growth of blood vessels into the tumor body. Thus, the diminution of TSP I expression in tumors enhances angiogenesis which in turn stimulates the growth of tumors [3].

The hypoxia-inducible factor I (HIF-1) was found to be elevated in nickel treated cells [127]. HIF-1 is very sensitive to hypoxia stimulus and precise regulation of oxygen homeostasis. HIF-1 is composed of two β HLH proteins, HIF-1α and HIF-1 β. HIF-1a is expressed and HIF-1 β accumulated only in hypoxic cells [128]. One explanation of the nickel-induced activation of the HIF-1 transcription factor is based on the assumption that nickel replaces iron in the oxygen carrier, Fe (II)-hybrid hemoglobin. Substitution of iron by nickel switches signal to permanent hypoxia, which in turn activates the HIF-1 factor. HIF-1 is also involved in the regulation of numerous genes involving also glucose transport and glycolysis [128]. Several experiments on various cells confirmed activated glucose metabolisms and glycolysis after nickel exposure [3].

P53 is an important tumor suppressor gene and transcription factor involved in the regulation of apoptosis and cell proliferation. The *p53* gene was reported to be mutated in human kidney epithelial cells exposed to nickel. Mutations in *p53* are where most of the transformations were observed, *e.g.* these are the common genetic alterations found in human cancers, and however, several conflicting results have been reported. It has been found that altered *p53* gene structure and expression in human epithelial cells after exposure to nickel, however, in contrast, a low incidence of point mutations were detected in the *p53* tumor suppressor gene from nickel induced rat renal tumors [(3)].

Oxidative Stress and Nickel-Induced Carcinogenicity

Nickel produces rather low, but measurable levels of free radicals in cells. Fluorescent methods revealed that both, soluble $NiCl_2$ and insoluble Ni_3S_2 evoked formation of free radicals. Many studies also revealed depletion of glutathione (GSH), representing another marker of oxidative stress [(129)].

The oxidative effect in human lymphocytes after acute nickel treatment was evaluated [(130)]. The levels of intracellular reactive oxygen species, lipid peroxidation and hydroxyl radicals and also the potential effects of antioxidants were examined. The level of hydroxyl radical in the Ni-treated group was much higher than in control. Also the levels of thiobarbituric acid-reactive substances (TBARS) in human lymphocytes *in vitro* in a concentration-dependent manner were detected [(3)]. Catalase partially reduced the $NiCl_2$-induced elevation of oxidants, whereas superoxide dismutase (SOD) enhanced the level of oxidants and TBARS. Both $NiCl_2$-induced lipid peroxidation was prevented significantly by glutathione (GSH) and mannitol. $NiCl_2$-induced increase in generation of hydroxyl radical was prevented significantly by catalase, GSH and mannitol, but not by SOD. These results suggest that $NiCl_2$-induced lymphocyte toxicity may be mediated by oxygen radical intermediates. Catalase, GSH and mannitol each provides protection against the oxidative stress induced by Ni. [(3)].

Nickel has been shown to inhibit DNA repair in a way that may play a role in its toxicity. Since nickel treatment increases cellular reactive oxygen species (ROS), Lynn et al. (1997) [(131)] investigated the involvement of ROS in nickel inhibition of DNA repair. Inhibition of glutathione synthesis or catalase activity increased the enhancing effect of nickel on the cytotoxicity of ultraviolet (UV) light. Inhibition of catalase and glutathione peroxidase activity also enhanced the retardation effect of nickel on the rejoining of DNA strand breaks accumulated by hydroxyurea plus cytosine-β-D-arabinofuranoside in UV-irradiated cells. In addition, glutathione could completely recover the inhibition by nickel or H_2O_2 alone but only partially recover the inhibition by nickel plus H_2O_2. Therefore, it has been proposed that nickel may bind to DNA-repair enzymes and generate oxygen-free radicals which cause protein degradation *in situ*. This irreversible damage to the proteins involved in DNA repair, replication, recombination, and transcription could be important for the toxic effects of nickel [(3)].

3.4. **Aluminium**

Aluminium is a ubiquitous element, comprising approximately 8% of the earth's crust, exceeded only by oxygen (47%) and silicon (28%). The almost ubiquitous presence of this element has so heavily contaminated the environment that exposure to it is virtually inescapable. The elemental aluminium does not occur in its pure state but is always combined with other elements such as hydroxide, silicate, sulphate and phosphate. The wide distribution of this element ensures the potential for causing human exposure and harm [(132)]. It has been suggested that there is a relationship between high levels of aluminium and increased risk of a number of neurodegenerative disorders including dialysis encephalopathy, Alzheimer's disease (AD) and Parkinson's disease (PD) [(133)]. The effect of renal failure on aluminium accumulation in different organs and the subsequent systemic toxicity is well known. It has been shown clearly that aluminium accumulates in various mammalian tissues such as brain, bone, liver and kidney and is accompanied by renal failure or associated with age [(134)].

Mounting evidence in the recent years has suggested aluminium to have severe toxic manifestations on the central nervous system. The neurotoxic symptoms associated with the use of dialysis fluids containing a high concentration of aluminium, usually above 200µg/L are speech disturbances, dysparaxia, tremors, partial paralysis and marked decline in learning and memory [(134)]. Many researchers have shown aluminium to result in changes in acetyl cholinesterase (AchE) activity. [(135)].

Aluminium has been shown to accumulate in all regions of rat brain following chronic exposure [(136)]. Becaria et al (2001) [(133)] described aluminium as a toxicant when present in high doses in circulation and suggested that a prolonged exposure to relatively low levels of aluminium may be responsible for the observed neurotoxic effects. It has been detected in both senile plaques and neurofibrillary tangle (NFT) bearing neurons in the brains of AD patients, which suggests the role of this metal in AD [(134)]. Walton (2007) [(137)] showed increased oxidative damage, neuronal degeneration and hyperphosphorylated tau in rat model of AD, further linking it to AD. The learning and memory deficits following aluminium exposure can be explained on the basis of this being a cholinotoxic agent. Besides, aluminium also has a strong prooxidant activity inspite of its non-redox status. Strong evidence is there that aluminium complexes with superoxide anion forming aluminium superoxide anion, which is a more potent oxidant than superoxide anion on its own and promotes the formation of hydrogen peroxide and hydroxyl radicals that contribute to an oxidizing environment. However, despite the abundance of reports regarding possible involvement of aluminium in a variety of human maladies, the exact mechanism by which aluminium exerts its toxic effects in the brain is yet to be completely understood [(138)].

Metabolism: incorporation into body

It has been reported that despite the quantity of this nonessential metal that people inhale and ingest daily, the respiratory system and gastrointestinal tract are felt to be relatively less permeable to aluminium. The average oral aluminium bioavailability from the diet has been estimated to be 0.1–0.3% [(134)]. Although, the main route of elimination of ingested aluminium is the alimentary tract, considerable evidence is consistent with the view that some aluminium is absorbed and enters the circulation. The presence of phosphate prevents absorption of aluminium just as aluminium hydroxide prevents phosphate uptake. It has been reported that aluminium binds to low molecular weight compounds, predominantly citrate. The absorption

of aluminium is enhanced by the presence of citrate. In fact, aluminium citrate is the major species in the brain extracellular fluid (approximately 60%) and transported out of the brain. Therefore, taking antacids along with fruit juice can pose a serious threat [134]. Aluminium administration has been demonstrated to decrease the iron content of intestinal cells. It is speculated that aluminium shares iron transporting mechanism for its absorption. In plasma, aluminium is bound to transferrin presumably to the same site as Fe^{3+} and shares this iron transporter protein for its transport [134]. It has been reported that up to 90% of aluminium in the plasma is in a complex with transferrin, and the remainder (up to 11%) is predominantly associated with citrate. In addition, studies have shown that aluminium transport out of brain extracellular fluid occurs by monocarboxylate transporter or transferring mediated endocytosis and glutamate transporter [139]. It has been observed significant decreases in duodenal uptake of aluminium in the presence of verapamil, a calcium channel blocking agent, revealing the importance of calcium channels in aluminium absorption, thereby hypothesizing that calcium channels might also be an entry site for aluminium [134].

Aluminium exposure and oxidative stress

In isolated systems aluminium can potentiate the ROS production by iron (Fe) and copper (Cu). Fe and Cu, which are present in most cell compartments, are known to be pro-oxidant metals and aluminium potentiates the capability of these transition metals to produce oxidative stress. It is hypothesized that colloidal aluminium may bind these metals and thus modulate their ability to promote metal-based oxidative events [140]. Exposure of cells to aluminium sulphate increased ROS formation, accompanied with elevated mitochondrial activity and glutathione depletion, in glial but not neuronal cell lines [140]. Because aluminium salts, complexed to strong acids such as sulphate and chloride, have a tendency to form colloidal particles as they age, it is possible that the glial-specific aluminium-induced increase in oxidative parameters is due to activation of these cells by extracellular aluminium complexes [134].

Increased lipid peroxidation has been reported after long-term low-level aluminium exposure [141]. Very recently, the products of lipid oxidation; malondialdehyde (MDA) have been quantified in rat brain exposed to lead, aluminium and phenolic antioxidants [142]. It seems that aluminium might exert its toxic effects by interfering with pathways involved in normal iron metabolism and homeostasis [139]. There are several studies which show that aluminium promotes iron-dependent lipid peroxidation in model membranes, such as phospholipid liposomes and membranes. These results suggest that oxidative stress and/or membrane lipid peroxidation linked with ROS generation in cells may be an important mechanism for aluminium toxicity development. Thus, aluminium ions may exert their toxic effects by oxidative damage to brain cell components and thus mediate neurotoxicity [134].

4. **Metals, toxicity and oxidative stress**

Carcinogenic metals present in occupational and general environments are believed to be critical factors involved in the increased incidence of cancers over the last half century [143]. Potential sources of metal exposure include groundwater contamination, metal working, leather tanning, and mining. In addition to environmental and occupational settings, a variety

of uses in medicine can result in exposure to different forms of metals [143]. Many metals, such as arsenic (As), beryllium (Be), cadmium (Cd), chromium (Cr), cobalt (Co), lead (Pb), mercury (Hg), Nickel (Ni), and vanadium (V), are toxic even at low levels of exposure [106,143]. These metals are known to induce cellular damage, inflammation, and cancers mainly in the kidney, liver, lung, prostate, and skin [106]. Even though metals, such as copper (Cu), iron (Fe), selenium (Se), and zinc (Zn), are essential to living organisms in trace amounts, chronic and extensive exposure causes detrimental effects to tissues and organs, eventually resulting in carcinogenesis [106,144,145]. Although the molecular mechanisms are not completely understood, the potential of metals to generate reactive oxygen species (ROS) and thus to alter cellular reduction–oxidation (redox) states is considered the most important mechanism involved in metal-induced carcinogenicity [145]. Recent research suggests that chronic exposure to ROS causes oxidative stress by disrupting the balance between the levels of ROS produced and the potential of cellular antioxidant systems to remove them. Prolonged and persistent oxidative stress causes changes in cellular redox homeostasis and leads to abnormal activation of redox-sensitive signaling molecules. Oxidative stress also damages biomacromolecules, such as DNA, proteins, and lipids, and eventually induces a variety of chronic and degenerative diseases including cancer, cardiovascular disorders, diabetes, rheumatoid arthritis, Alzheimer's and Parkinson's disease [17].

Accumulating evidence provides a correlation between metal-induced oxidative stress and increased cancer risk (Figure 5). Due to the increasing utilization of toxic metals in industry and medicine as well as their inefficient recycling, environmental accumulation of carcinogenic metals may result in subsequent increases in cancer incidence [106, 146]. This makes understanding the relationships among metals, oxidative stress, and carcinogenicity of great interest. Such knowledge could improve risk assessment and the design of anticancer therapeutics [17].

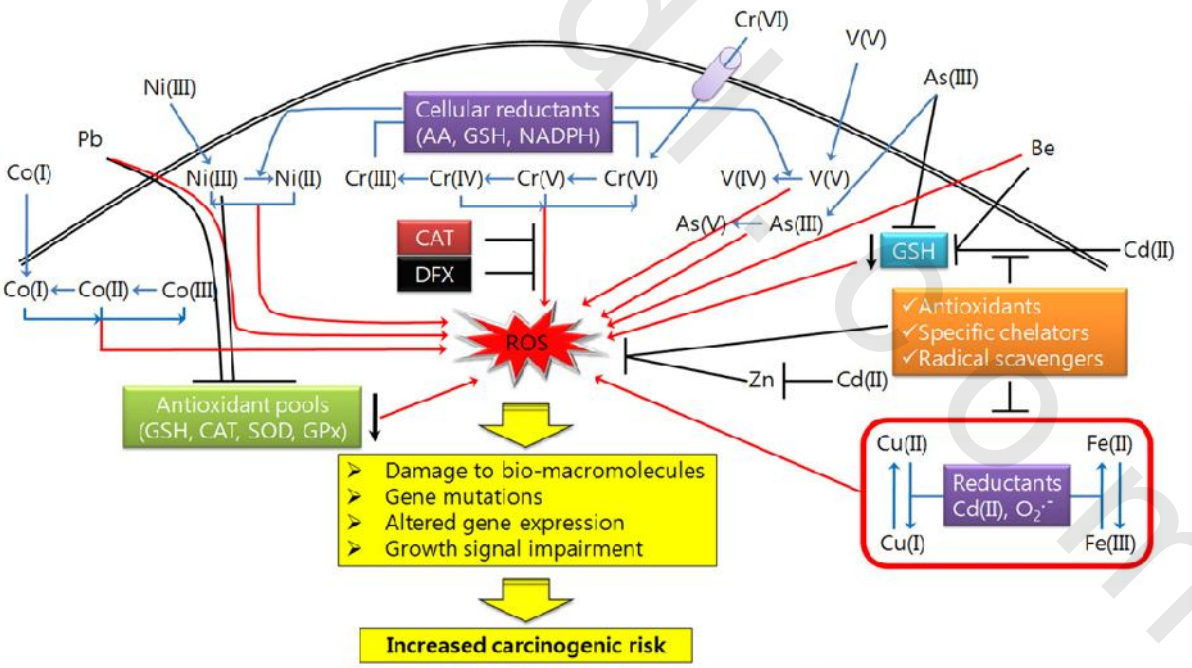

Figure 5: General scheme of metal-induced reactive oxygen species (ROS) formation and increased carcinogenic risk [17].

Common mechanisms of metal-mediated ROS generation

Metal ions produce intracellular ROS in a direct and indirect manner, where the Fenton-type reaction is one of the most well-known mechanisms. During this reaction, a transition metal ion reacts with H_2O_2 to generate the highly toxic $^{\bullet}OH$ and an oxidized metal ion [17].

$$metal^{n+} + H_2O_2 \rightarrow metal^{n+1} + {}^{\bullet}OH + OH^- \quad (1)$$

Many metals, such as Fe (II), Cu, Cr (III), (V) and (IV), Co (II), Ni (II), and V (IV), can generate free radicals via the Fenton-type reaction, although their abilities to generate free radicals differ [17]. While the efficiencies of Co (II) and Ni (II) to generate $^{\bullet}OH$ are very low due to their high redox potentials, Fe (II) produces the toxic radical more readily [147]. Neither the significance of the Fenton-type reaction under physiological conditions nor *in vivo* mechanisms by which free Fe or Cu ions mediate the generation of $^{\bullet}OH$ via the Fenton-like reaction are completely understood. Another key mechanism in metal-induced ROS generation is the Haber–Weiss reaction. In this reaction, $O_2^{\bullet -}$ mediates $^{\bullet}OH$ generation from H_2O_2 and also participates in the reduction of Fe (III) leading to the Fenton reaction [17].

$$metal^{n+1} + O_2^{\bullet -} \rightarrow metal^{n+} + O_2 \quad (2)$$

$$metal^{n+} + H_2O_2 \rightarrow metal^{n+1} + {}^{\bullet}OH + OH^- \quad (3)$$

$$O_2^{\bullet -} + H_2O_2 \xrightarrow{metal^{n+}/metal^{n+1}} O_2 + {}^{\bullet}OH + OH^- \quad (4)$$

The Haber–Weiss reaction can involve metals, such as Cr (III), (IV), (V), and (VI), V (IV), and Co (I) and Co (II). The Haber–Weiss-type mechanism of $^{\bullet}OH$ generation is likely to be more predominant *in vivo* than the Fenton-type reaction, based on ROS production in the immune function of macrophages during phagocytosis [17]. It is commonly accepted that the step to convert $O_2^{\bullet -}$ to H_2O_2 is too slow, unless a suitable metal ion is present as a catalyst for the Haber–Weiss reaction. *In vivo*, intracellular free iron is present in very low quantities under normal physiological conditions. However, the release of excess free iron from iron-containing molecules can occur in response to stress, which stimulates *in vivo* ROS production [148].

Many studies have focused on metal-induced toxicity and carcinogenicity, emphasizing their role in the generation of reactive oxygen and nitrogen species in biological systems, and the significance of this therein. Metal-mediated formation of free radicals may cause various modifications to DNA bases, enhanced lipid peroxidation, and changes in calcium and sulphydryl homeostasis [3, 149].

5. **Oxidative damage to biomolecules**

5.1. **Lipid peroxidation**

It is known that metal-induced generation of oxygen radicals results in the attack of not only DNA in the cell nucleus, but also other cellular components involving polyunsaturated fatty acid residues of phospholipids, which are extremely sensitive to oxidation. Whilst the ability of the hydroxyl radical (generated via Fenton chemistry) to initiate lipid peroxidation is unquestionable, it is necessary to also consider the diffusion-limited reactivity of the hydroxyl radical toward sugars, nucleotides, or proteins. The mechanism, proposed more than two decades ago, involves the formation of a Fe (II): Fe (III) complex (or a Fe (II)–O2–Fe(III) species). The maximal rates of lipid peroxidation are observed when the ratio of Fe (II): Fe (III) is 1:1. It has been also demonstrated that ADP–Fe(II) promoted the peroxidation of phospholipid liposomes, but only after a lag phase. Catalase, superoxide dismutase and hydroxyl radical scavengers did not extend the lag phase or inhibit the subsequent rate of lipid peroxidation [(149)].

Several experimental models of iron overload *in vivo*, confirmed increased polyunsaturated fatty acids (PUFA) oxidation of hepatic mitochondria, as well as lysosomal fragility. It has been observed that, following oral intake of carbonyl iron in rats, mitochondrial lipid peroxidation occurred. Experiments also showed that this was accompanied by substantial decrements in mitochondrial metabolism. These observations suggest that mitochondrial PUFA are a preferential target for iron-driven peroxidation. The deleterious process of the peroxidation of lipids is also very important in arteriosclerosis, cancer and inflammation [(149)].

The overall process of lipid peroxidation consists of three stages: initiation, propagation and termination and is presented in (Figure 6) [(149, 150)]. Once formed, peroxyl radicals (ROO•) can be rearranged via a cyclisation reaction to endoperoxides (precursors of malondialdehyde) with the final product of the peroxidation process being malondialdehyde (MDA) (Figure 6). The major aldehyde product of lipid peroxidation other than malondialdehyde is 4-hydroxy-2-nonenal (HNE) (Figure 6). Malondialdehyde is mutagenic in bacterial and mammalian cells and carcinogenic in rats. Hydroxynonenal is weakly mutagenic but appears to be the major toxic product of lipid peroxidation. In addition, HNE has powerful effects on signal transduction pathways, which in turn have a major effect on the phenotypic characteristics of cells. Peroxidation of lipids is an autocatalytic process which is terminated for example by the recombination of radicals ($R^{\bullet} + R^{\bullet} \rightarrow$ non-radical product) or depletion of the substrate [(149)].

Malondialdehyde can react with DNA bases G, A and C to form adducts M_1G, M_1A and M_1C, respectively (Figure 6) [(149, 150)]. M_1G adducts were found to range in tissue at levels ranging from below the limit of detection to as high as 1.2 adducts per 10^6 nucleosides (which corresponds approximately 6000 adducts per cell). M_1G has also been detected in human breast tissue by ^{32}P-postlabeling as well as in rodent tissues. Site-specific experiments confirmed that M_1G is mutagenic in *E. coli*, inducing transversions to T and transitions to A. The mutation frequencies are comparable with those reported for 8-oxo-G in similar systems. M_1G is repaired by both bacterial and mammalian nucleotide excision repair pathways and is also repaired in *E. coli* by mismatch repair. Studies employing NMR spectroscopy indicate that M_1G undergoes rapid and quantitative ring opening to form N^2-oxopropenyl-G when it is

present in duplex DNA; however, not when it is present in single stranded DNA. While the reactive functionality of M_1G is present in the major groove, the reactive functionality of N^2-oxo-propenyl-dG is present in the minor groove of DNA. The interconversion of M_1G and N^2-oxo-propenal-dG within the DNA may lead to the formation of DNA–DNA interstrand crosslinks or DNA–protein crosslinks [149].

Etheno adducts

There are also other exocyclic DNA adducts that arise from lipid peroxidation. For example etheno -dA, -dC and -dG have been detected by both ^{32}P-post-labeling and GC–MS. While the precise pathway of their formation in DNA is unknown, adducts can readily be generated in vitro by exposure of DNA to a peroxidising lipid. The biological activity of etheno adducts involves transitions to A (induced by N^2, 3-etheno-dG) and transversions to T in *E. coli* (induced by 1, N^2-etheno-dG). It has been demonstrated that etheno-dA and -dC are strongly genotoxic but weakly mutagenic when introduced on single-stranded vectors in *E. coli*. Etheno-dA induces predominantly transitions to G whereas etheno-dC induces transversions to A and transitions to T. Studies dealing with the repair of etheno adducts have shown that etheno-dA is removed by the action of 3-methyladenine glycosylase and its mammalian homolog alkyladenine glycosylase (AAG). In addition to efficient removal by glycosylases, other repair pathways should also be considered [149].

Propano adducts

It has been demonstrated that hydroxyl propanodeoxyguanosines (HO-PdGs) are present in human DNA [149]. These adducts are most probably derived from the reaction of DNA with acrolein and crotonaldehyde generated by a lipid peroxidation process. Acrolein and crotonaldehyde are mutagenic in bacteria and mammalian cells. However, the mutagenic potency of HO-PdGs has not been evaluated by site-specific approaches, due to the instability of these adducts, which renders their incorporation into oligonucleotides unviable. Therefore, a novel post-oligomerization strategy for the synthesis of oligonucleotides containing the acrolein-derived HO-PdG was reported, which should make it possible to construct the requisite adducted vectors. Experiments with unsubstituted adduct PdG revealed that this induces base pair substitution mutations in *E. coli* with high efficiency. Little is known about the repair of HO-PdGs. There may be a possibility that PdG or HO-PdGs are substrates for base excision repair enzymes [149].

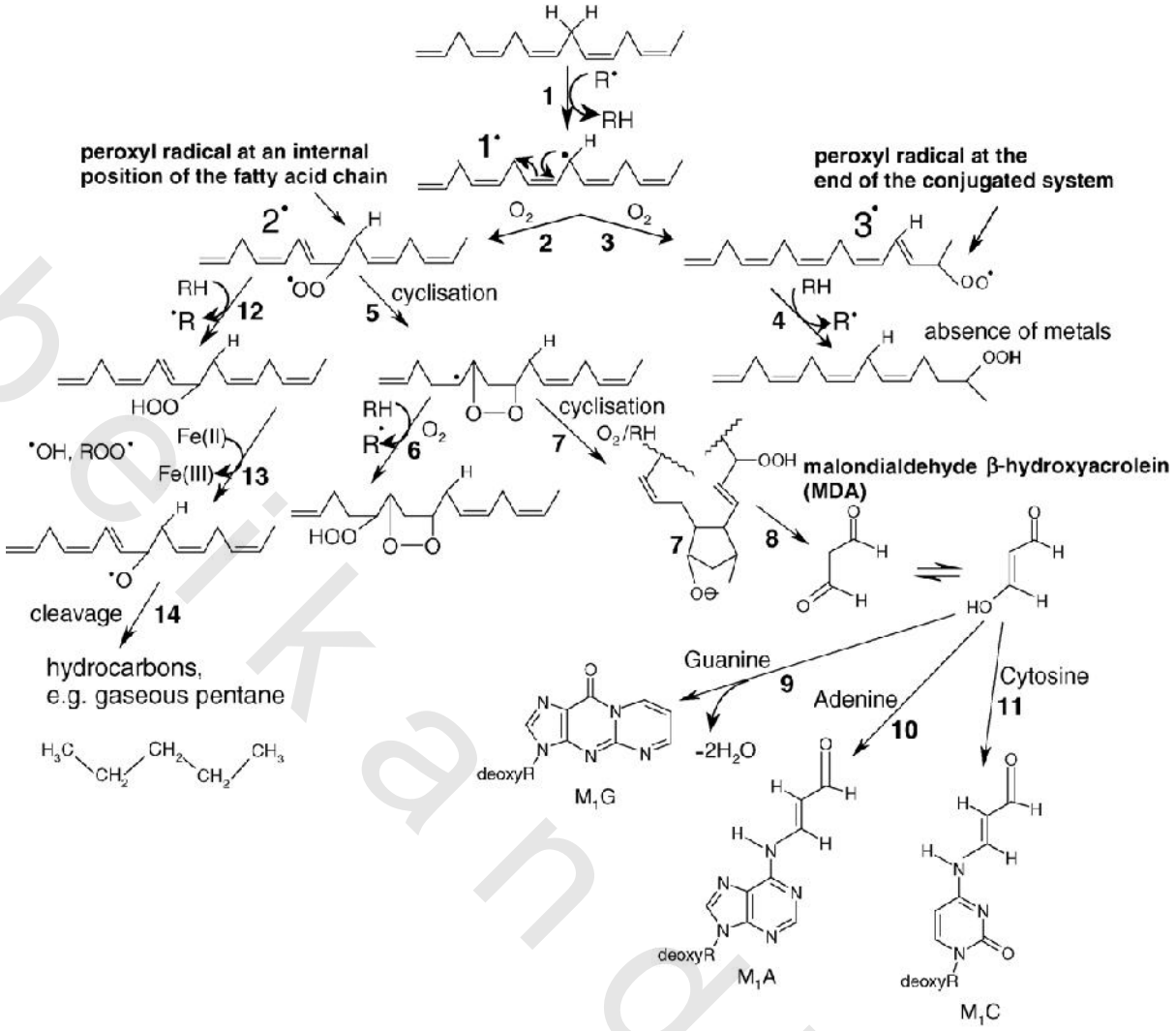

Figure 6. Various pathways of lipid peroxidation. The methylene groups of polyunsaturated fatty acids are highly susceptible to oxidation and their hydrogen atoms, after the interaction with radical R$^{\bullet}$, are removed to form carbon-centred radicals 1$^{\bullet}$ (reaction (1)). Carbon-centred radicals react with molecular dioxygen to form peroxyl radicals (reactions (2) and (3)). If the peroxyl radical is located at one of the ends of the double bond (3$^{\bullet}$) it is reduced to a hydroperoxide which is relatively stable in the absence of metals (reaction (4)). A peroxyl radical located in the internal position of the fatty acid (2$^{\bullet}$) can react by cyclisation to produce a cyclic peroxide adjacent to a carbon-centred radical (reaction (5)). This can then either be reduced to form a hydroperoxide (reaction (6)) or through reaction (7) it can undergo a second cyclisation to form bicyclic peroxide which after coupling to dioxygen and reduction yields a molecule structurally analogous to the endoperoxide. Compound 7 is an intermediate product for the production of malondialdehyde (reaction (8)). Malondialdehyde can react with DNA bases G, A, and C to form adducts M_1G, M_1A and M_1C (reactions (9)–(11)). Peroxyl radicals located in the internal position of the fatty acid (2$^{\bullet}$) can, besides cyclisation reactions, (reaction (5)) also abstract hydrogen from the neighbouring fatty acid molecule, creating thus lipid hydroperoxides (reaction (12)). They can further react with redox metals (e.g. iron) to produce reactive alkoxyl radicals (RO$^{\bullet}$) (reaction (13)) which, after cleavage (reaction (14)) may form, e.g. gaseous pentane; a good marker of lipid peroxidation [149].

5.2. **Proteins**

Mechanisms involved in the oxidation of proteins by ROS were elucidated by studies in which amino acids, simple peptides and proteins were exposed to ionizing radiations under conditions where hydroxyl radicals or a mixture of hydroxyl/superoxide radicals are formed [151]. The results of these studies demonstrated that reactions with hydroxyl radicals lead to abstraction of a hydrogen atom from the protein polypeptide backbone to form a carbon-centred radical, which under aerobic conditions reacts readily with dioxygen to form peroxyl radicals [149]. The peroxyl radicals are then converted to the alkyl peroxides by reactions with the protonated form of superoxide ($HO_2^{\bullet}$). In the absence of ionizing radiation the same reactions can be initiated by hydroxyl radicals produced under in vivo conditions by the Fenton reaction. Thus in the absence of radiation, proteins are resistant to damage by H_2O_2 and by other simple oxidants unless transition metals are present. Metal-catalyzed damage to proteins involves oxidative scission, loss of histidine residues, bityrosine crosslinks, the introduction of carbonyl groups, and the formation of protein-centred alkyl, $R^{\bullet}$, alkoxyl, $RO^{\bullet}$, and alkylperoxyl, $ROO^{\bullet}$ radicals [149]. The alkoxyl radical derivatives of proteins are capable of undergoing peptide bond cleavage. Peptide bond cleavage can also occur by hydroxyl radical-initiated attack of the glutamic acid and proline residues of proteins to form a mixture of various products. Protein damage is likely to be repairable and is a known non-lethal event for a cell, however, evidence has been reported that two mitochondrial proteins-aconitase and adenine nucleotide-translocase may be important targets of long-term oxidative damage [151].

The side chains of all amino acid residues of proteins are susceptible to oxidation by ionizing radiation and by the action of ROS/RNS [151]. Since proline, histidine, arginine, lysine, and cysteine residues in proteins are highly sensitive to oxidation by redox metals, redox metal (iron, copper)-mediated oxidation of a protein may be a site-specific process. It is believed that the iron (II) binds both to high- and low-affinity metal binding sites on the protein, most probably involving the above-mentioned amino acids. The Fe (II)–protein complex reacts with H_2O_2 via the Fenton reaction to yield an active oxygen species, e.g. $^{\bullet}OH$, ferryl ion, at the site. While it has been proposed by many authors that the hydroxyl radical represents the major species responsible for the oxidation of proteins, clear experimental evidence is still missing. A study by Welch et al. (2001) [152] demonstrated the site-specific modification of ferritin by iron which involved the oxidation of cysteine, tyrosine, and also some other residues. Whilst the hydroxyl radical scavenger HEPES protected the protein against oxidation, catalase did not, confirming the site specific oxidation of ferritin. The oxidation of myoglobin by H_2O_2 yields ferrylmyoglobin, which contains two oxidizing equivalents: the ferryl complex and an amino acid radical [149].

Using Electron paramagnetic resonance (EPR) spectroscopy Giulivi and Cadenas (1998) [153] showed that the spectra of the amino acid radicals consisted of a composite of three signals attributable to a peroxyl radical, a tyrosyl radical and radicals in an aromatic amino acid-containing peptide. The aromatic amino acid radical was observed to be relatively long lived and in close proximity to the heme iron. Hence, it has been proposed that this is the first site of the protein radical. Reduction of the ferryl complex by tyrosine (Tyr) is described by the reaction:

$$Fe\ (IV)=O + Tyr\ OH + H^{+} \rightarrow Fe(III) + H_2O + Tyr\ O^{\bullet}$$

and alternatively by other amino acids leads to the subsequent formation of other amino acid radicals via an electron-transfer process that occurs throughout the protein. This view suggests that the protein radical(s) is highly delocalized within the globin moiety in a dynamic process which encompasses electron tunneling through the backbone chain, or H-bonds, leading to the formation of secondary radicals [149].

The amino acid residue side chains that are most vulnerable to attack by various ROS and RNS lead to the formation of the following products: arginine → glutamic semialdehyde; glutamate → 4-hydroxy-glutamate; histidine → 2-oxo-histidine; tyrosine → 3,4-dihydroxy phenylalanine, Tyr–tyr cross-linked proteins, 3-nitro-tyrosine; Valine → 3, 4-hydroxy valine; cysteine→cys–S–S–cys, cys–S–S–R disulphied; proline → glutamic semialdehyde, 2-pyrrolidone-4-hydroxy-proline; methionine→ methionine sulphone and sulphoxide [151].

In view of the fact that protein carbonyl groups are generated by many different mechanisms and a number of highly sensitive methods have been developed for the assay of protein carbonyl groups, the concentration of protein carbonyl groups is a good measure of ROS mediated protein oxidation [149].

Nitric oxide reacts rapidly with superoxide radical to from the highly toxic peroxynitrite anion $ONOO^-$ which is able to nitrosate the cysteine sulphydryl groups of proteins, to nitrate tyrosine and tryptophan residues of proteins and to oxidize methionine residues to methionine sulphoxide. However, the process of modification of proteins by peroxynitrite is strongly inhibited by physiological concentration of CO_2 since peroxinitrite is known to react rapidly with CO_2 to form the $ONOOCO_2^-$:

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^-$$

The nitration of tyrosine residues, which is irreversible process, may prevent the phosphorylation or adenylylation of tyrosine residues of regulatory proteins.

When superoxide is depleted from the biological environment via a dismutation reaction, $NO^{\bullet}$ interacts directly with the biological substances (for example with the hem iron of guanylate cyclase) to form a complex:

$$Fe(II)\{\text{guanylate cyclase}\} + NO^{\bullet} \rightarrow Fe(II)\{\text{guanylate cyclase}\}-NO^{\bullet}$$

Protein oxidation by ROS is associated with the formation of many different kinds of inter- and intra-protein cross-linkages, including those formed, *(i)* by addition of lysine amino groups to the carbonyl group of an oxidized protein; *(ii)* by interaction of two carbon-centred radicals obtained by the hydroxyl radical-driven abstraction of hydrogens from the polypeptide backbone; *(iii)* by the oxidation of sulphydryl groups of cysteine residues to form –S–S– crosslinks; *(iv)* the oxidation of tyrosine residues to form –tyr–tyr– cross-links [149].

Cysteine and methionine residues of proteins are particularly susceptible to oxidation by ROS [223]. However, oxidation of the sulphur amino acids is reversible. The oxidized products of cysteine, intra-molecular (P1–S–S–P1) and inter-molecular (P1–S–S–P2) protein cross-

linked derivatives can be repaired by disulphide exchange reactions catalyzed by thiol transferases.

The oxidation of methionine (Met) residues of proteins leads to the formation of a mixture of the S– and R– isomers of methionine sulphoxide, Met–(S)–SO and Met–(R)–SO:

$$\text{Met} + \text{ROS} \rightarrow \text{Met–(S)–SO} + \text{Met–(R)–SO} + \text{products}$$

Because almost all forms of ROS are able to oxidize methionine residues of proteins to methionine sulphoxide, it was proposed that the cyclic oxidation and reduction of methionine residues of proteins serves an important antioxidant function to protect cells from oxidative damage [(149)].

Oxidation of proteins is associated with a number of age-related diseases and ageing [(154,155)]. The concept of the role of protein oxidation in ageing is supported by animal studies showing that the process of ageing is often associated with the accumulation of oxidized forms of proteins. The accumulation of oxidized proteins in living systems may be: *(i)* due to an increase in the steady state level of ROS/RNS and/or to a decrease in the antioxidant capacity of an organisms; *(ii)* a decrease in the ability to degrade oxidized proteins due to either a decrease in the protease concentrations and/or to an increase in the levels of protease inhibitors [(149)].

5.3. **Oxidative nuclear and mitochondrial DNA Damage**

The hydroxyl radical is known to react with all components of the DNA molecule: damaging both the purine and pyrimidine bases and also the deoxyribose backbone [(156)]. Permanent modification of genetic material resulting from these "oxidative damage" incidents represents the first step involved in mutagenesis, carcinogenesis and ageing. In fact, it is well established, in various cancer tissues free radical-mediated DNA damage has occurred. To date, more than 100 products have been identified from the oxidation of DNA. ROS-induced DNA damage involves single- or double stranded DNA breaks, purine, pyrimidine, or deoxyribose modifications, and DNA cross-links. DNA damage can result either in arrest or induction of transcription, induction of signal transduction pathways, replication errors and genomic instability, all of which are associated with carcinogenesis [(157, 158)].

The hydroxyl radical is able to add to double bonds of DNA bases at a second-order rate constant in the range of $(3–10) \times 10^9\ M^{-1}\ s^{-1}$ and it abstracts an H-atom from the methyl group of thymine and each of the five carbon atoms of 2' deoxyribose at a rate constant of approximately $2 \times 10^9\ M^{-1}\ s^{-1}$ [(230)]. While OH-adduct radicals of DNA bases are generated via an addition reaction, the allylic radical derived from thymine and carbon-centred sugar radicals arise by abstraction reactions [(156)]. Further reactions of base and sugar radicals generate a variety of modified bases and sugars, base-free sites, strand breaks and DNA–protein cross-links [(149)].

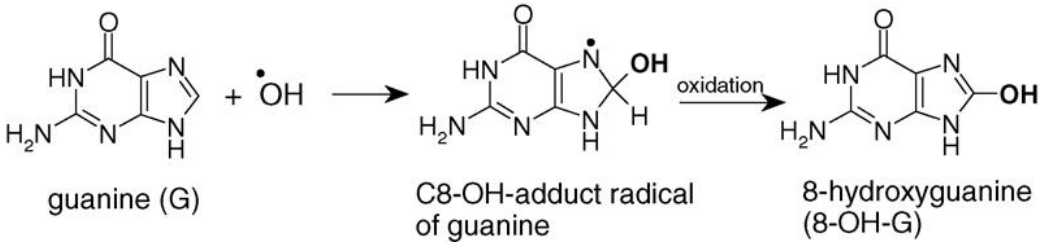

Figure 7. Reaction of guanine with hydroxyl radical [149].

An example illustrating the mechanisms of 8-hydroxyguanine (8-OH-G) formation is given in (Figure 7). The presence of 8-OH-G in human urine was first reported by Ames and co-workers. This oxidized DNA product is important because it is both relatively easily formed and is mutagenic and carcinogenic. It is a good biomarker of oxidative stress of an organism and a potential biomarker of carcinogenesis. We note that 8-hydroxyguanine undergoes keto-enol tautomerism and therefore 8-OH-G is often called 8-oxoguanine or 8-oxo-G, however, 8-oxo-G and 8-OH-G are equivalent [149].

This base modification occurs in approximately one in 10^5 guanidine residues in a normal human cell. Ionizing radiation, a carcinogenic and exogenous source of ROS, induced both urinary and leukocyte biomarkers of oxidative DNA damage. Tobacco smoking, another carcinogenic source of ROS, increases the oxidative DNA damage rate by 35–50%, as estimated from the urinary excretion of 8-oxo-G and the level of 8-oxo-G in leukocytes by 20–50% [149].

Measurements by Kasai et al. (2001) [159] demonstrated that factors such as hard physical labour, day–night shift work, smoking and low meat intake significantly increased the 8-oxo-G level, while moderate physical exercise, such as sports reduced its level. These findings were comparable with previous data obtained from studies on rats and suggest that our lifestyle may significantly affect the level of oxidative damage we sustain [149].

In addition to ROS, reactive nitrogen species (RNS), such as peroxynitrites and nitrogen oxides, have also been implicated in DNA damage [160]. Upon reaction with guanine, peroxynitrite has been shown to form 8-nitroguanine. Due to its structure, this adduct has the potential to induce G: C→T: A transversions. While the stability of this lesion in DNA is low, in RNA, however, this nitrogen adduct is stable. The potential connection between 8-nitroguanine and the process of carcinogenesis is unknown [149].

In addition to the extensive studies devoted to the role of oxidative nuclear DNA damage in neoplasma, there exists evidence about the involvement of the mitochondrial oxidative DNA damage in the carcinogenesis process [161]. Mutations and altered expression in mitochondrial genes encoding for complexes I, III, IV and V, and in the hypervariable regions of mitochondrial DNA, have been identified in various human cancers. The following points account for the fact that mitochondrial DNA is more susceptible to oxidation than nuclear DNA [161]: *(i)* under physiological conditions, the mitochondria convert ~5% of oxygen consumed into superoxide anion and subsequently hydrogen peroxide; *(ii)* mitochondrial DNA repair capacity is limited, since they lack entirely the feature of nucleotide excision repair; *(iii)* mitochondrial DNA is not protected by histones [161].

Hydrogen peroxide and other reactive oxygen species have been implicated in the activation of nuclear genes that are involved in mitochondrial biogenesis, transcription, and replication of the mitochondrial genome. Although the region of tumor cells that possess mutated mitochondrial DNA and the extent to which mitochondrial DNA alterations participate in the cancer process have not been satisfactorily established, a significant amount of information supporting the involvement of the mitochondria in carcinogenesis exists [(162)]. This connection supports the fact that fragments of mitochondrial DNA have been found to be inserted into nuclear DNA, suggesting a possible mechanism for activation of oncogenes [(149)]. As observed with oxidative genomic DNA modification, oxidative damage and the induction of mutation in mitochondrial DNA may participate at multiple stages of the process of carcinogenesis, involving mitochondria-derived ROS, induction of mutations in mitochondrial genes, and possibly the insertion of mitochondrial genes into nuclear DNA [(162)]. As described above, oxygen radicals may induce a number of DNA base alterations that can lead to mutagenesis. However, there are specific and general repair mechanisms that can repair DNA base modifications [(163, 164)]. Of interest is the fact, that the efficiency of repair mechanisms may be enhanced following exposure to reactive oxygen species because expression of many DNA repair enzymes is up regulated following oxidative stress [(149)].

Since in nuclear DNA, ~90 % of oxidized bases are repaired by single nucleotide repair mechanisms and the remaining 10 % by long-patch base excision repair, the single nucleotide base excision repair is the primary pathway for repair of 8-OH-G. The first evidence of a repair mechanism for the 8-OH-G lesion was observed in irradiated mouse liver, where levels of this lesion were found to decrease with time. A repair enzyme was partially purified from *E. coli* and was later found to be identical to the cloned DNA repair enzyme, formamidopyrimidine–DNA glycosylase FPG protein, previously isolated from *E. coli*. While significant knowledge of the DNA repair mechanisms in nuclear DNA exists, much less is known about the repair systems in the mitochondria. However, compared with nuclear DNA repair mechanisms, DNA repair capacity in the mitochondrion appears to be rather low. The impaired repair capacity may lead to mitochondrial dysfunction and the onset of degenerative diseases [(149)].

6. **Oxidative stress, cell signaling and cancer**

Cells communicate with each other and respond to extracellular stimuli through biological mechanisms called cell signaling or signal transduction [(165)]. Signal transduction is a process enabling information to be transmitted from the outside of a cell to various functional elements inside the cell. Signal transduction is triggered by extracellular signals such as hormones, growth factors, cytokines and neurotransmitters [(166)]. Signals sent to the transcription machinery responsible for expression of certain genes are normally transmitted to the cell nucleus by a class of proteins called transcription factors. By binding to specific DNA sequences, these factors regulate the activity of RNA polymerase II. These signal transduction processes can induce various biological activities, such as muscle contraction, gene expression, cell growth and nerve transmission [(167)].

While ROS are predominantly implicated in causing cell damage, they also play a major physiological role in several aspects of intracellular signaling and regulation. It has been

clearly demonstrated that ROS interfere with the expression of a number of genes and signal transduction pathways [168]. Because ROS are oxidants by nature, they influence the redox status and may, according to their concentration, cause either a positive response (cell proliferation) or a negative cell response (growth arrest or cell death). While high concentrations of ROS cause cell death or even necrosis, the effects of ROS on cell proliferation occurred exclusively at low or transient concentrations of radicals. Low concentrations of superoxide radical and hydrogen peroxide in fact stimulate proliferation and enhanced survival in a wide variety of cell types. ROS can thus play a very important physiological role as secondary messengers. Other examples include regulation of the cytosolic calcium concentration, regulation of protein phosphorylation, and activation of certain transcription factors such as NF-κB and the AP-1 family factors [169].

ROS and metal ions primarily inhibit phosphorserine/threonine-, phosphotyrosine- and phospholipid phosphatases, most probably by interacting with sulphydryl groups on their cystein residues, which are oxidized to form either intramolecular or intermolecular disulphide bonds [165, 168]. These structural changes alter protein conformation which leads to the up regulation of several signaling cascades, most importantly growth factor kinase-, src/Abl kinase-, MAPK- and PI3- kinase-dependent signaling pathways. These signaling cascades lead to the activation of several redox-regulated transcription factors (AP-1, NF-κB, p53, HIF-1, NFAT). Figure 14 summarizes ROS-induced signaling pathways [149].

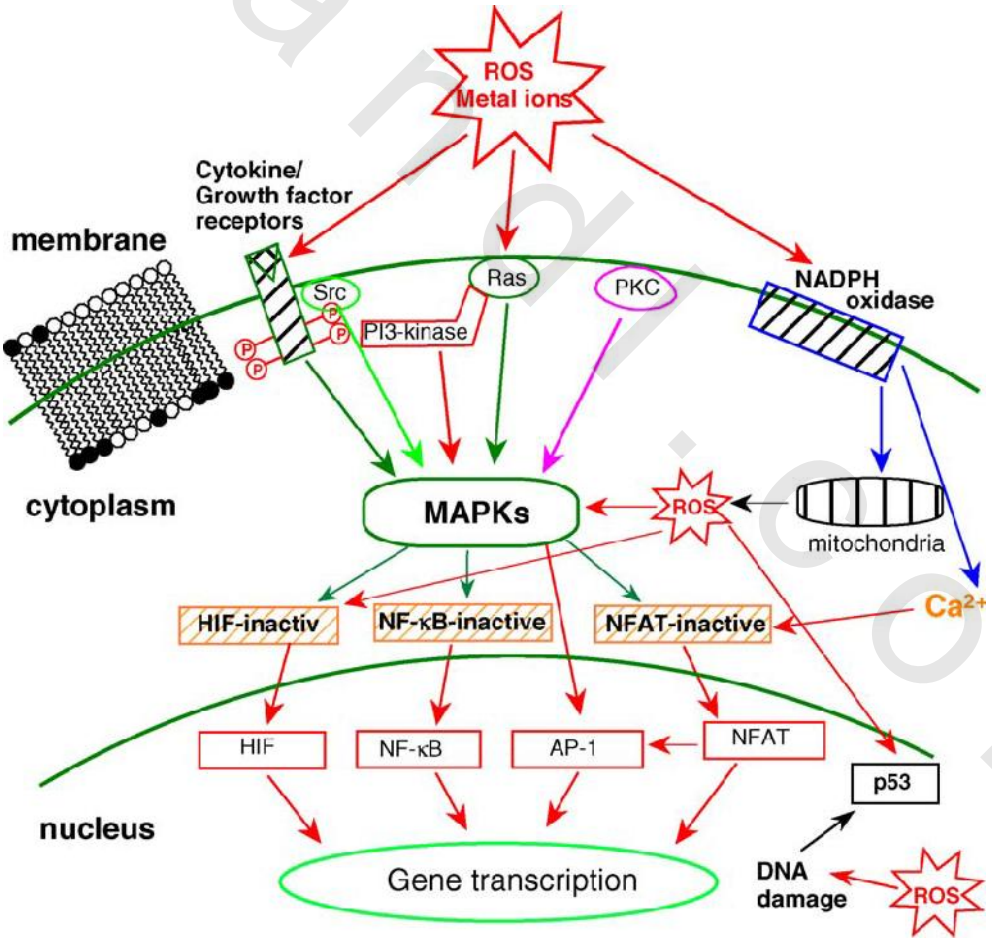

Figure 8. ROS and metal ions-induced signaling pathways [149].

6.1. **Cytokines and growth factor signaling**

A variety of cytokines and growth factors that bind to receptors of different classes have been reported to generate ROS in nonphagocytic cells. Growth factor receptors are tyrosine kinases (RTKs) that play a key role in the transmission of information from outside the cell into the cytoplasm and the nucleus. The information is transmitted via the activation of mitogen-activated protein kinases (MAPKs) signaling pathways [170-172].

ROS production as a result of activated growth factor receptor signaling includes epidermal growth factor (EGF) receptor [149], platelet-derived growth factor (PDGF) receptor [173] and vascular endothelial growth factor (VEGF) [149]. Further examples involve cytokine receptors (TNF-α, and IFN-γ) or interleukin receptors (IL-1β). Cytokines receptors fall into a large and heterogenous group of receptors that lack intrinsic kinase activity and are most directly linked to ion channels or G proteins. Cytokines such as TNF-α, IL-1 and interferon (IFN-γ) were among those first reported to generate ROS in nonphagocytic cells. It is generally accepted that ROS generated by these ligand/receptor-initiated pathways can function as true second messengers and mediate important cellular functions such as proliferation and programmed cell death [149].

Abnormalities in growth factor receptor functioning are closely associated with the development of many cancers [174]. Several growth factor receptors (EGF, PDGF and VEGF) are affected by carcinogenic metals such as nickel, arsenic, cobalt and beryllium [175]. The EGF receptor is associated with cell proliferation in normal cells. Nickel has been found to increase expression of the EGF receptors and overexpression of the EGF receptor has been observed in lung and urinary cancers [176]. Exogenous oxidative stress appears to stimulated secretion of heparin-binding EGF. VEGF is involved in proliferation and angiogenesis and also is induced by carcinogenic metals (Co, Ni and As) and hypoxia. Arsenic-induced VEGF expression appears to be associated with p38 [177]. Activation of both EGF and VEGF results in increases in cellular Ca (II). A similar effect was observed in a various cell types following treatment with Ni (II), Cd (II) and Be (II) compounds. The VEGF is probably most strongly activated by hydrogen peroxide. The PDGF is found in endothelial cells, fibroblasts and mesenchymal cells; the overexpression of PDGF has been found in lung and prostate cancers [149].

6.2 **Non-receptor tyrosine kinases**

In addition to receptor tyrosine kinases, several non-receptor protein kinases (PTKs) belonging to the *Src* family (*Src* kinases) and Janus kinase (JAK) are also activated by ROS [178]. For example hydrogen peroxide and superoxide radical induce tyrosine phosphorylation of several PTKs in different cell types, including fibroblasts, T and B lymphocytes, macrophages and myeloid cells. It is noteworthy that *Src* has been activated by As (III) and organic Cr (III) compounds and by UV radiation as well as by various ROS [175]. Over expressed *Src* has been found in colorectal, breast, pancreas, bladder and head/neck cancers. Activated *Src* binds to cell membranes by myristilation and initiates MAPK, NF-κB and PI3K signaling pathways (Figure 8) [149].

6.3. **Ras**

Ras gene products are membrane-bound G proteins whose main function is to regulate cell growth and oppose apoptotic effects. Ras is activated by UV radiation, ROS, metals and mitogenic stimuli. Ras genes were found to be mutated in 30% of lung, skin, liver, bladder and colon cancers [149]. As, Ni, Fe and Be are linked with ras mutations (in two ras genes, H- and K-ras) and cancer [179]. H-ras mutations were observed in rats following dimethylarsinic acid administration in drinking water. Ni (II) compounds induced K-ras mutations and kidney tumors in rats. Mutations to ras caused by metals have not been reported in human cancers [149].

6.4. **Protein tyrosine phosphatases**

Protein tyrosine phosphatases (PTPs) are probably the best-characterized direct targets of ROS. Reversible inactivation of PTPs by ROS plays an important role in the redox control and cell signaling. It has been shown that inhibition of PTPs by ROS may directly trigger PTKs. The effects of ROS occur through targeting the cysteine-containing residues of the active sites of tyrosine phosphatases [180]. Cysteine residues are most susceptible to oxidative damage by hydrogen peroxide and other oxidants, producing sulphenic acid intermediates, which can further react with thiols to form catalytically inactive PTP disulphides. Superoxide radical was also shown to regulate the activity of PTPs very efficiently, in particular PTP-1B via cysteine residues [149].

6.5. **Serine/threonine kinases**

All receptor serine/threonine kinases described in mammalian cells are members of TGF-β superfamily. TGF-β1 is the prototype of this large family of polypeptide growth factors. Unlike RTKs-linked growth factors, TGF-β1 typically inhibits the growth of most target cells. The TGF- β1 has been shown to stimulate ROS production in a variety of cells [181]. TGF-β1 has also been shown to suppress the expression of antioxidant enzymes in some cells. TGF-β1 inhibited the expression of Mn-SOD, Cu, Zn-SOD and catalase in rat hepatocytes [149].

Akt is a serine/threonine kinase, recruited to the cell membrane by PI3k and activated by phosphorylation. The end result of Akt activation is stimulation of growth pathways and inhibition of apoptotic pathways. Conversely, inhibition of akt may result in apoptosis. VEGF activation by ROS in mouse muscle cells occurs via the PI3K/Akt pathway [149].

Calcium has been well recognized as a signaling factor involved in the regulation of a wide range of cellular processes involving cell proliferation, cell differentiation and apoptosis [149]. Experiments revealed that ROS induce release of calcium from intracellular stores, resulting in the activation of kinases, such as protein kinases C (PKCs) a member of serine/threonine kinases. In addition, ROS and metals can directly activate some serine/threonine phosphorylation processes. Among serine/threonine kinases, PKC is subjected to a rather complicated cellular redox regulation. PKC contains several cysteine rich regions both in the zinc finger of the regulatory domain and in the catalytic site which can be modified by various oxidants [182]. Both regulatory and catalytic domains of the PKC are susceptible to H_2O_2-induced oxidative modification; in fact treatment of different cell types using e.g. H_2O_2 leads to stimulation of its activity. One of the possible mechanisms of the

PKC activation is tyrosine phosphorylation and conversion to the Ca^{2+}/phospholipid-independent form. It appears certain that oxidant-induced PKC activation plays a critical role in cancer proliferation and clearly this has important functional consequences on downstream signaling pathways; i.e. activation of MAPKs, defined transcription factors and proto-oncogenes [(182)].

The group of proteins termed mitogen-activated protein kinases (MAPKs) relay signals generated by exogenous and endogenous stimuli to intracellular space via phosphorylation of proteins. During this process of intracellular communication, MAPKs interact with upstream mediators, involving growth factor receptors, G proteins, tyrosine kinases and downstream mediators, such as nuclear transcription factors [(149)].

A number of studies reported that the serine/threonine kinases of the MAPK family can be regulated by oxidants. There are four known MAPK families (MAPKs): extracellular-regulated (ERKs), c-jun-NH_2-terminal kinase (JNKs), p38 MAPK and the big MAPK-1 (BMAPK-1), of which serine/threonine kinases are important in the process of carcinogenesis including cell proliferation, differentiation and apoptosis [(183)]. Deregulation of MAPK function has been reported for skin, breast and cervical cancers in humans. Products of NOX1 activity, superoxide and hydrogen peroxide can activate the MAPK cascade at the level of MEK and ERK1/2. The experimental studies on the up regulation of MAPKs by H_2O_2 treatment have shown that the activation of each signaling pathway is type- and stimulus - specific. For example, it has been reported that endogenous H_2O_2 production by the respiratory burst induces ERK but not p38 kinase activity [(184)]. Conversely, exogenous H_2O_2 activates p38 kinase, but not ERK in rat alvedor macrophages. The ERK pathway has most commonly been associated with the regulation of cell proliferation. The balance between ERK and JNK activation is a key factor for cell survival since both a decrease in ERK and an increase in JNK is required for the induction of apoptosis [(149)].

Carcinogenic metals have been shown to activate MAPKs [(185)]. Arsenic (III) and chromium (VI) activates ERK1and ERK2, JNK and p38 in the human cells. Beryllium difluoride activates ERK1, JNK and p38 in human breast cancer cells. BMAPK-1 is not activated directly by metal ions; however, indirect activation by hydrogen peroxide has been well documented [(149)].

6.6. **Nuclear transcription factors**

Probably the most significant effect of metals and ROS on signaling pathways has been observed in the mitogen-activated protein kinase (MAPK) pathways. This involves activation of nuclear transcription factors. These factors control the expression of protective genes that repair damaged DNA, power the immune system, arrest the proliferation of damaged cells, and induce apoptosis. The nuclear transcription factor NF-κB, is involved in inflammatory responses and AP-1 is important for cell growth and differentiation. p53 is a gene whose disruption is associated with more than half of all human cancers. The p53 protein guards a cell-cycle checkpoint, and inactivation of p53 allows uncontrolled cell division. The nuclear factor of activated T cells (NFAT) regulates cytokine formation, muscle growth and differentiation, angiogenesis and adipogenesis. HIF-1 regulates the expression of many cancer-related genes including VEGF, enolase, heme oxygenase 1 and lactate dehydrogenase A [(149)].

NF-κB (Nuclear factor- kappa B)

NF-κB is a DNA binding protein that regulates several genes involved in cell transformation, proliferation, and angiogenesis [186]. NF-κB activation has been linked to the carcinogenesis process because of its role in differentiation, inflammation, and cell growth. Carcinogens and tumor promoters involving toxic metals, UV radiation, phorbol esters, asbestos, alcohol and benzo(a)pyrene are among the external stimuli that activate NF-κB [187].

AP-1 (Activator protein 1)

AP-1 proteins have also been found to participate in oncogenic transformation through interaction with activated oncogenes such as H-ras [149].

p53

The nuclear factor plays a key role in protecting a cell from tumourigenesis [188]. Many studies have been devoted to mutations in p53 gene caused by direct action of reactive oxygen species (ROS) or by carcinogenic metals [149].

At the hepatocyte level, hepatocarcinogenesis occurs in distinctly defined stages: ‘‘initiation’’ which is negatively regulated by the rate of hepatocyte apoptosis, ‘‘promotion’’ which is characterized by a selective increase in cell proliferation and decrease in apoptosis of preneoplastic hepatocytes, and ‘‘progression’’ where both cell proliferation and apoptosis appear to be increased [189, 190]. Such an imbalance of the proliferation-apoptosis process may result from the loss of coordinated response to growth factors and cytokines among which the insulin-like growth factors (IGF-I and -II) stand as suitable candidates [191]. Indeed, the IGFs are synthesized and secreted in extracellular fluids by fetal as well as adult hepatocytes. The interaction of IGF-I and -II with type-I IGF receptor (IGF-IR) plays a pivotal role in the proliferation of a variety of cell types, in the control of cell cycle progression in G1, in the regulation of the early phases of tumourigenicity [192], in the maintenance of the tumourigenic phenotype [193], and in the prevention of apoptosis [192-194].

7. **Insulin-like growth factor 2 (IGF-2)**

Mature IGF-2 is a 67 amino acid (7.5 kDa) peptide produced mainly by liver, but it is also secreted by most tissues where it can act in an autocrine or paracrine manner. There is considerable evidence that IGF-2 regulates cell growth, differentiation and metabolism. It is particularly important in promoting fetal growth, being highly expressed during embryogenesis. The effects of the IGFs overlap. They are both potent mitogens, their relative potency depending on the cell type [195].

The IGF-2 gene (30 kb) is located next to the insulin gene on 11p15.5. IGF-2 is initially synthesized as prepro-IGF-2 (20.1 kDa, 180 AAs) consisting of A–E domains and a 24-residue signal peptide (Figure 9). Post-translational processing begins with cleavage of the signal peptide to yield pro-IGF-2 (1–156). This is followed by O-linked glycosylation of the

89-residue E-domain that may promote further processing [195]. Pro-IGF-2 then undergoes sequential proteolysis to mature IGF-2 (1–67) that lacks the E-domain. Prohormone convertase 4 (PC4) is the protease thought to cleave the E-domain. Incomplete processing of pro-IGF-2 results in various peptides (10–18 kDa) containing all or part of the E-domain, known collectively 'big' IGF-2. These are secreted into the circulation, normally accounting for 10–20% of total IGF-2. The glycosylation on big IGF-2 may promote ternary complex formation in serum. Big IGF-2 also forms binary complexes with IGFBP2, IGFBP3 and IGFBP5 [196,197].

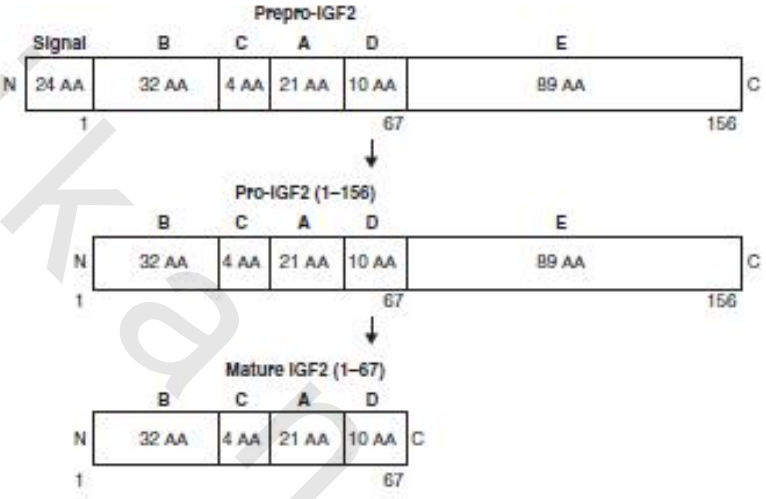

Figure 9. Structure and processing of prepro-IGF-2. The IGF-2 gene (30 kb) is located on 11p15.5 comprising nine exons. Exons 7, 8 and 9 are encoding. Translation generates prepro-IGF-2 (180 AAs, 20.1 kDa) consisting of five domains (A, B, C, D and E) and a 24-residue N-terminal signal peptide. Proteolysis of the signal peptide by signal peptidase yields pro-IGF-2 (1–156). The C-terminal E domain is glycosylated promoting further processing. Sequential proteolysis removes the E-domain giving mature IGF-2 (1–67) that is secreted. Incompletely processed pro-IGF-2 peptides (10–18 kDa) containing all or part of the E-domain are also secreted making up 10–20% of total circulating IGF-2. These peptides are called 'big' IGF-2 [195].

Quantitatively IGF-2 is the predominant circulating IGF present in adults at a concentration of ~700 ng/ml, three times that of IGF-1. Serum IGF-2 is low in neonates, climbing in early childhood and then remaining at similar concentrations throughout life, although it may decrease slightly in healthy elderly subjects [198]. Concentrations are similar in both genders. Free IGF-2 circulates at picomolar concentrations, similar to insulin. The portion of IGF-2 bound to IGFBPs has a relatively long half-life (10–16 h) compared with that of free IGF-2 (a few minutes) [195].

7.1. **IGF-2 signaling**

IGF-2 signals via three receptor complexes namely the IGF1 receptor (IGF1R), insulin receptor isoform A (IR-A) and the IGF1R–IR-A hybrid receptor (Figure 10). IGF1R binds both IGFs with comparable and high affinity [(199)]. It is thought to mediate most of the biological effects of IGF-2. IR-A is an alternatively spliced IR isoform that lacks exon 11 (11) encoding 12 amino acid residues at the C-terminus of the ligand-binding a subunit. This enables it to bind IGF-2 with an affinity 15% of that for insulin, much higher than its affinity for IGF-1 [(200)]. While insulin binding stimulates glucose uptake, IGF-2 binding is mitogenic [(201)]. Although IR-A is widely expressed throughout life, its physiological role in adults is unclear [(195)].

In common with insulin and IGF-1, binding of IGF-2 to the IGF1R activates a receptor tyrosine kinase (RTK) associated with the β-subunit leading to an intracellular response [(202-204)]. Autophosphorylation of the β-subunit by the RTK recruits insulin receptor substrates (IRS) 1–4. Phosphatidylinositol 3-kinase (PI3-K) then binds to IRS1 via its regulatory subunit and is activated, in turn activating Akt (protein kinase B). This has a number of intracellular effects, which ultimately promote cell survival and mitogenesis. First, it inhibits apoptosis by inactivating BAD (BCL-2 antagonist of cell death). It also phosphorylates tuberous sclerosis complex (TSC1/2) leading to activation of mammalian target of rapamycin (mTOR) and subsequent ribosomal protein synthesis that is required for mitogenesis. Akt also has the metabolic action of leading to GLUT4 translocation, which promotes cellular glucose uptake [(195)].

By recruiting other adaptor proteins to the receptor complex, ligand activation of IGF1R also leads to activation of the MAPK pathway that transmits the proliferative signals generated at the cell surface to the nucleus. It causes the change in expression of proteins required for cellular proliferation. Phosphorylation of IRS proteins recruits the adaptors Shc and growth factor receptor-bound protein 2 (Grb2), which along with son-of-sevenless form a complex activating the GTP binding protein Ras. There is further phosphorylation and activation of Raf-1 and the kinases (MEK1/2 and ERK1/2) that leads to the activation of transcription factors involved in cell proliferation [(195)].

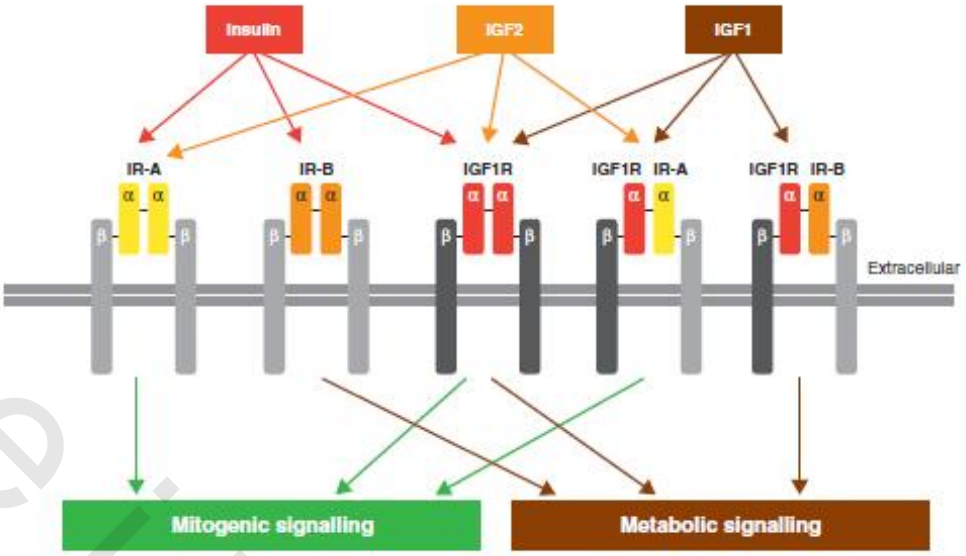

Figure 10. Ligands and receptors of the insulin-like growth factor (IGF) system. The ligands of the IGF system signal by binding to three different receptor complexes, namely the insulin receptor (isoforms A and B), the IGF1 receptor (IGF1R) and hybrids of the IGF1R and insulin receptor. The receptor complexes are a2b2 tetramers. Ligand binding to the extracellular a-subunit results in activation of a receptor tyrosine kinase located in the transmembrane b-subunit. This activates the intracellular signaling pathway leading to a biological response, which can be mitogenic or metabolic in nature. Activation of IR-A and IGF1R initiate mitogenic signaling whereas activation of IR-B causes metabolic responses. Ligand binding to the hybrid receptors can activate either type of signaling depending on the I-R isoform in the complex [204].

7.2. **Physiological regulation of IGF-2**

Genetic factors play a significant role in the regulation of IGF-2. The proportion of its variance attributable to genetic factors is 66%, compared with 38% for IGF-1. Transcription is regulated by genomic imprinting, an epigenetic mechanism that restricts expression to the paternal allele in most tissues. Imprinting is achieved by methylation of the differentially methylated region (DMR) on the maternal allele [195]. It prevents excessive expression of IGF-2, which could lead to proliferation and tumors. IGF-2 is transcribed from four promoters (P1–P4) in a tissue-specific manner. During embryogenesis, transcription occurs from P2–P4 resulting in monoallelic expression. In adults, there is also expression from P1 in liver which is biallelic accounting for the high circulating IGF-2 concentrations in adults [195].

Cellular responsiveness to IGF-2 is influenced by changes in receptor expression. Increased IR-A expression during embryogenesis [201] and increased IR-A: IR-B ratio during dedifferentiation [205] promote its action. IGF2 also binds to the widely expressed IGF2R, a 250 kDa monomeric, cell surface protein. It is thought to promote endocytosis and lysosomal

degradation of IGF-2, thereby antagonizing its action and acting as a tumor suppressor [206]. IGF2R also binds lysosomal enzymes intracellularly, transporting them from the Golgi to lysozomes. The IGF2R gene, like IGF2, is imprinted but expressed from the maternal allele. This reciprocal imprinting may regulate the relative abundance of the two proteins. A soluble form of IGF2R cleaved from the cell surface binds IGF-2 in serum and is thought to reduce IGF-2 bioactivity in vivo [195, 207]. Because the IGFs promote growth, it is logical that they are both nutritionally regulated, their concentrations indicating the availability or otherwise of substrate from the diet. Down-regulation of IGF-2 during starvation may protect the individual from hypoglycemia, which would occur if its concentration did not decrease in parallel with ternary complexes. Specific nutrients also influence IGF-2, notably down-regulation by vitamin C and vitamin D [208].

8. **Defense Mechanisms**

The toxicity of heavy metals may be attributable to the binding of metals to sulfydryl groups in proteins such as GSH, resulting in an inhibition of activity or interference with structure, or the displacing of an essential metal element leading to deficiency effects [209]. Additionally, persistent exposure to heavy metals may induce the generation of free radicals and reactive oxygen species, probably leading to oxidative stress. Repair of stress-damaged proteins and chelation of metals involving heat shock proteins (Hsps) and metallothionein (MT) are thus recognized as potential mechanisms, in part, of metal detoxification. Although mechanisms by which heavy metals interact have not been clearly elucidated, a number of biomolecules, including GSH, MT, and Hsps have been predominantly recognized as major interactive mediators when evaluating interactions based on metal mixture exposure [210].

8.1. **Glutathione**

Glutathione (GSH) plays a crucial role in the development of heavy metal mixture toxicity. GSH levels (measured as non-protein thiols) in the kidney appear to vary with time and are associated with an interactive effect of the metal mixture at low doses [211]. Due to its non-enzyme antioxidant status, the regulation of GSH upon interaction with a heavy metal mixture is attributable to neutralization of oxidative radicals. GSH might also sacrifice its sulfhydryl group to protect enzymes or other active proteins exposed to a metal mixture [210].

8.2. **Heat shock proteins**

Heat shock proteins (Hsps), a family of universal stress proteins localized in several cellular organelle systems, are commonly altered under a metal mixture-induced insult. Hsp induction patterns have been recognized as potential biomarkers of early cellular responses to exposure as well as prominent mediators of toxicity in target organelle systems. Their induction profile may delineate specific cellular responses to a stressor. Once Hsps are

induced, they provide protective effects to the organism or target cell population. Both *in vitro* and in vivo low dose studies indicate that Hsps appear to be induced in a metal/metalloid-, dose-, and time-specific manner [212, 213]. Exploitation of these proteins enables an evaluation of the magnitude of the stressor and the responsibility of the organism as well as a risk assessment of metal mixtures even at a low dose. By combining other toxicity endpoints, investigations of Hsp profiles might be applicable as either an endpoint or a mediator for evaluating metal mixture toxicity [210].

8.3. **Metallothionein**

Metallothionien (MT), a metal-specific stress protein with various isoforms in many tissues, is pivotal for regulating the interaction of a heavy metal mixture [210, 211]. Metallothionein is a transition metal-binding protein that is found in all eukaryotes and in some prokaryotes. Mammalian MTs constitute a superfamily of non-enzymatic polypeptides (61–68 amino acids), which are characterized by low molecular weight (6–7 kDa), distinctive amino acid composition (high cysteine content) and a high content of sulphur and metals (metal thiolate clusters) [19]. *In vivo*, the metal-binding involves mainly Zn(II), Cu(I), Cd(II) and Hg(II), while in vitro additional and diverse metals such as Ag(I), Au(I), Bi(III), Co(II), Fe(II), Pb(II), Pt(II) and Tc(IV) may be bound to apothionein (the metal-free form) . However, under physiological conditions mammalian MTs mostly contain Zn [212]. In mammals, distinct metallothionein isoforms designated MT-I through MT-IV have been found [213]. MT-I and MT-II are present in all organs, whereas MT-III is expressed mainly in brain and MT-IV is most abundant in certain stratified tissues [19].

In mammals, MT-I+II consist of 61 and 62 amino acids, respectively, which are devoid of aromatic amino acids, while one-third of the residues are cysteines (in total 20) that form metal thiolate clusters. In the polypeptide chain, cysteines are arranged in series of motifs: Cys-X-Cys, Cys-X-Cys-Cys, Cys-X-X-Cys (X is a non-Cys residue), which are absolutely conserved across species [214]. The cysteine sulfhydryl groups bind and coordinate 7 mol of divalent metal ions [i.e. Zn(II) or Cd(II)] per mol MT-I+II, while the molar ratio for Cu(I) and Ag(I) is 12 [19].

8.3.1. **Biosynthesis of metallothionein**

Transcriptional regulation

MT-I+II are regulated in a coordinate manner and are rapidly increased by various pathological conditions [215, 216]. Administration of essential or toxic metals like Zn, Cu, Cd, Hg increase MT-I+II biosynthesis by inducing their transcription, for which several *cis*-acting DNA elements, or metal response elements (MREs) in the promoter region are binding sites for trans-acting transcription factors [212, 214]. MT-I+II gene transcription is initiated when metals occupy the MRE-binding transcription factor-1 (MTF-1), which is a multiple Zn finger protein; the only known mediator of the metal responsiveness of MT-I+II [214].

ROS also increase the MT-I+II transcriptional response, as shown by exposure to free radicals like superoxide anions and hydroxyl radicals, which rapidly increase MT-I mRNA levels in a dose-dependent manner [217]. It is well known that metallothionein synthesis occurs in response to oxidative stress in the cytosol and nucleus. It has been also found that oxidative stress in mitochondria induces the synthesis of metallothionein, which may contribute to regulation of mitochondrial ROS production [19]. In addition, MT-I+II are also increased by glucocorticoids like corticosterone and dexamethasone, which signal transcription through glucocorticoid response elements (GREs) present in the gene regulatory region; catecholamines (norepinephrine, isoproterenol) can also activate MT-I+II gene transcription [212, 215].

Proteolysis of Metallothionein

MT-I+II are to some degree regulated by intracellular protein degradation, which takes place in both lysosomal and non-lysosomal compartments [217]. Turnover rates of cytosolic apothioneins versus lysosomal metal-bound MT-I+II proteins are quite different, in that lysosomal MT-I+II proteolysis occurs more readily than in the cytosol, although bound metals stabilize MT-I+II proteins and prevent their lysosomal proteolysis. In the cytoplasm, the 26S proteasome complex degrades apothionein, which due to the lack of metals has a shorter half-life than MTs [19].

Localization of metallothionein

Inside cells, MT-I+II are distributed in cytoplasm and sub-cellular organelles like lysosomes and mitochondria. Depending on the phase of the cell cycle, differentiation or in the event of toxicity, MT-I+II are rapidly translocated to the nucleus, as seen during early S-phase and in oxidative stress [217, 218]. Due to their small size, MT-I+II can diffuse through nuclear pore complexes, although nuclear trafficking relies on specific cytosolic partner proteins and the appearance of nuclear binding proteins, which in the presence of ROS enhance the nuclear localization of MT-I+II [219]. In addition, perinuclear localization of MT mRNA may contribute to the nuclear import of MT-I+II proteins [19].

By immunolocalization assay it was observed the presence of metallothionein in the cytoplasm and in the nuclei of fibroblast cells treated with trace elements such as Zn, Cu and Fe and co-treated with the same metals. The significance of its presence in nucleus of the cells treated with heavy metal may be to protect the cell from DNA damage and apoptosis as well as regulating gene expression during certain stages of the cell cycle. The nuclear localization of metallothionein also could be important in providing a pool of intracellular Zn, which can be released and donated to other metalloproteins and transcription factors [220].

8.3.2. **Function of metallothionein**

Metallothionein is implicated in a diversity of intracellular functions, but the only consensus among researchers thus far concerns its role in the detoxification of heavy metals, which is due mostly to its high affinity for these metals [19]. In accordance with the chemical nature of abundant disulfide bonds, the following biological roles of metallothionein are

proposed: *i)* detoxification of metals including non- essential and excess essential metals, storage of essential metals; *ii)* sequestration of ROS and nitrogen species (RNS) and electrophiles [221]; *iii*) intracellular trafficking of Zn [222]. Furthermore, the role of metallothionein in apoptosis has been aggressively investigated and the vast majority of studies show that metallothionein plays a protective role with respect to apoptosis [223, 224]. However, many possibilities are likely, since MT-I+II are indeed multipurpose proteins involved in a broad range of functions, which also include immune defense responses, protein–protein and protein–nucleotide interactions, regulation of Zn fingers and Zn-containing transcription factors, mitochondrial respiration, thermogenesis, body energy metabolism, angiogenesis, cell cycle progression, cell survival and differentiation [225]. Some of these MT-I+II actions may have therapeutic relevance in a range of acute and chronic disorders, in which inflammation and oxidative stress play a central pathophysiologic role [300]. Accordingly, MT-I+II may signal through diverse molecular pathways [226].

Role of metallothionein in the toxicity of heavy metals

The role of metallothionein in detoxification has been particularly well studied and was originally examined using an MT-inducing agent. MT transgenic cell lines and mice have been used to demonstrate that metallothionein provides protection against the toxicity of heavy metal such as Cd and Hg. Protection against metal toxicity has been attributed primarily to MT-I and -II, although MT-III is thought to play a role in Zn homeostasis in neurons. The protective role of metallothionein against Cd toxicity is well established in mammals based on several models [19].

Metallothionein is protective against the lethality not only of Cd, but also other metals such as Zn, Cu, Fe, Pb, Hg and As. The possible mechanisms by which metallothionein may protect against metal toxicity include: *i)* reduction of metal uptake into cells; *ii)* sequestration of metal within cells; and *iii)* enhanced metal export out of cells [227]. Since metallothionein contains high levels of sulphur, the mutual affinity of sulphur and transition metals makes the binding of these metals to metallothionein thermodynamically stable [228]. Intracellular Zn and Cu are normally bound to metallothionein. Zn is known to be the most effective metallothionein inducer. Park et al. (2001) [227] have demonstrated that following repeated injection of Zn and Cu, wild-type mice were more tolerant to Zn- and Cu-induced lethality than MT-null mice. Jiang et al. (2002) [229] demonstrated that Zn-induced metallothionein was able to bind intracellular Cu, quench the redox cycling activity of Cu, inhibit Cu-dependent oxidative stress in membrane phospholipids, and prevent Cu dependent apoptosis in HL-60 cells. However, the Cu-bound metallothionein is redox inactive in the absence of oxidative stress [19]. Furthermore, in the $MT^{+/+}$ cells, it has been observed that in cells treated with metals the apoptotic and necrotic index was lower with respect to MT null cells. This was associated with the presence of metallothionein, which plays a protection role against both metal toxicity and the harmful effects of free radicals generated by the Fenton reaction [25].

Metallothionein as reservoir of Zn

Mobilization of Zn from metallothionein by an oxidative reaction may either constitute a general pathway by which Zn is distributed in the cell or be restricted to conditions of oxidative stress in which Zn is needed in antioxidant defense systems [19].

Release of Zn from metallothionein; there are two major concerns regarding Zn transfer from metallothionein to other proteins. The first is the energetic feasibility of Zn transfer from metallothionein to other proteins, and the second is the specificity of Zn transfer [(228)].

As suggested above, the cluster structure of Zn–MT provides a chemical basis by which the cysteine ligand can induce oxidoreductive properties. This structure allows for thermodynamic stability of Zn in metallothionein, while permitting Zn to retain kinetic liability. The sulphur ligand confers redox activity on the Zn–MT complex, and can be oxidized and reduced with the concomitant release and binding of Zn in an oxidoreductive environment. The release of Zn makes Zn available for the functional demand of other molecules [(228)]. Therefore, Zn–MT could provide a pool of intracellular Zn, which can be released and donated to other metalloproteins and transcription factors [(19)].

Zn release from metallothionein is modulated by both GSH and glutathione disulfide (GSSG). GSH inhibits Zn release in the absence of GSSG, indicating that metallothionein is stabilized at relatively high cellular GSH concentrations. The presence of GSSG results in Zn release. The rate of Zn release depends linearly on the amount of GSSG: the more oxidative the redox state becomes, the more efficiently Zn is released from metallothionein [(228)]. Moreover, cellular disulfides other than GSSG also react with metallothionein to release Zn with high efficiency [(19)].

Zn transfer from metallothionein*;* normal cellular activity requires Zn mobilization and transfer from one location to another or from one Zn-binding site to another [(228)]. In cell-free systems, studies have shown that Zn transfers from metallothionein to carbonic anhydrase [(230)], alkaline phosphatase and bovine carboxypeptidase. Zn exchange between metallothionein and Zn finger transcription factors serves as a mechanism for the regulation of gene expression through activation or inhibition of DNA binding by estrogen receptors [(231)]. In addition, a direct interaction between metallothionein and apo-Zn-binding peptides during the process of Zn transfer has been demonstrated in a cell-free system [(19)].

Metallothionein as an antioxidant

An antioxidant role for mammalian metallothionein is well documented. The induction of metallothionein under radical generating circumstances has led to the speculation that MT might be involved in free-radical-scavenging activity [(232)]. For instance, metallothionein is reported to mitigate the cardiotoxicity of doxorubicin both *in vivo* and *in vitro* mainly due to its free-radical scavenging properties. Further, acute alcohol hepatotoxicity and hepatic oxidative stress are significantly inhibited in MT-transgenic mice. However, in these transgenic mice, a role for Zn independently of metallothionein has also been demonstrated in protecting from alcoholic liver injury [(233)]. Cells isolated from mice deficient in metallothionein by targeted disruption of MT-I and -II genes were found to be more sensitive to the toxic effects of oxidants. These cells do not have altered levels of GSH, CAT or GPx. In vitro experiments clearly show that metallothionein can scavenge superoxide anions or hydroxyl radicals, phenoxyl radicals and nitric oxide [(19)].

Metallothionein might function as an expendable target for oxidants due to its highly enriched cysteine residue structure. These cysteines, while involved in thiolate clusters, are quite labile and freely exchange native metals with electrophiles [(19)]. Structurally, the 20

cysteines of metallothionein are very close, which seemingly would favor intramolecular disulfide bond formation [228]. A number of *in vitro* studies have shown that metallothionein can form dimmers through disulfide bond formation under oxidative conditions, although intramolecular oxidation of the cysteine thiolates and mixed disulfide formation has also been demonstrated [231]. Because oxidative condition is believed to release Zn and other metals from metallothionein, by Sagher et al. (2006) [234] it has been postulated a reaction in which cells, under oxidative stress, mobilize Zn from Zn–MT for use for the hundreds of Zn-containing proteins. The loss of the Zn from metallothionein as a result of oxidation would yield oxidized thionein (T(o)). T(o) can be reduced to T by the Trx system [235]. Thioredoxin (Trx) may be only one of the possible cellular reducing systems that can reduce T(o). It is known that oxidized GSH can oxidize MT and cause the release of Zn from Zn–MT and that reduced GSH can reduce T(o), which can bind Zn. Of interest were the findings that certain selenium compounds, such as selenocystamine, can accelerate these reactions [236]. Alternatively, metallothionein may function as an antioxidant indirectly by affecting two important metals, Zn and Cu, which may facilitate antioxidant enzymes such as superoxide dismutase [19].

8.4. **Antioxidant defense systems**

The cells contain antioxidant molecules to defend against excess of ROS produced by accumulation of Cu or Fe. In cells, a limited amount of ROS is produced in normal metabolic processes in the cytoplasm and peroxisomes, whereas the bulk of these substances are generated as a side product during oxidative phosphorylation in mitochondria [237]. In sub toxic concentrations, these products may act as second messengers in intracellular signal transduction pathways [19].

Nevertheless, the cell contains antioxidant molecules to defend against excess production of ROS. A good antioxidant should: (1) specifically sequench free radicals; (2) chelate redox metals; (3) interact with other antioxidants within the "antioxidant network"; (4) have a positive effect on gene expression; (5) be readily absorbed; (6) work in both the aqueous and/or membrane domains [4]. The harmful effects of ROS are balanced by the antioxidant action of antioxidant enzymes and non-enzymatic antioxidants [19].

The most efficient enzymatic antioxidants involve superoxide dismutase, catalase and glutathione peroxidase. Non-enzymatic antioxidants involve Vitamin C, Vitamin E, carotenoids, thiol antioxidants (glutathione, thioredoxin and lipoic acid), natural flavonoids, a hormonal product of the pineal gland, melatonin and other compounds. Some antioxidants act in a hydrophilic environment, others in a hydrophobic environment, and some act in both environments of the cell [149].

Certain antioxidants are able to regenerate other antioxidants and thus restore their original function. This process is called an "antioxidant network". The redox cycles of vitamins E and C form such an antioxidant network. The capacity to regenerate one antioxidant by another is driven by the redox potentials of the [Red/Ox] couple [149].

8.4.1. **Enzymatic antioxidants**

Superoxide dismutase (SOD)

One of the most effective intracellular enzymatic antioxidants is superoxide dismutase (SOD) (EC1.15.1.1). Superoxide dismutase is the antioxidant enzyme that catalyzes the dismutation of $O_2^{\bullet-}$ to O_2 and to the less-reactive species H_2O_2. Superoxide dismutase exists in several isoforms, differing in the nature of the active metal centre and amino acid constituency, as well as their number of subunits, cofactors and other features. In humans there are three forms of SOD: cytosolic Cu, Zn-SOD, mitochondrial Mn-SOD, and extracellular SOD (EC-SOD). SOD destroys $O_2^{\bullet-}$ with remarkably high reaction rates, by successive oxidation and reduction of the transition metal ion at the active site in a "Ping-Pong" type mechanism [149].

Catalase

Catalase (EC 1.11.1.6) is an enzyme present in the cells of plants, animals and aerobic bacteria. Catalase is located in a cell organelle called the peroxisome. The enzyme very efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen. Catalase has one of the highest turnover rates for all enzymes: one molecule of catalase can convert ~6 million molecules of hydrogen peroxide to water and oxygen each minute:

$$2H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$

The significantly decreased capacity of a variety of tumors for detoxifying hydrogen peroxide is linked to a decreased level of catalase [149].

Glutathione peroxidase

There are two forms of the enzyme glutathione peroxidase, one of which is selenium-independent (glutathione-*S*-transferase, GST, EC 2.5.1.18) while the other is selenium-dependent (glutathione peroxidase EC 1.11.1.19). These two enzymes differ in the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanisms. Glutathione metabolism is one of the most essential of antioxidative defense mechanisms [149].

Humans have four different Se-dependent glutathione peroxidases. All glutathione peroxidases enzymes are known to add two electrons to reduce peroxides by forming selenoles (Se-OH). The antioxidant properties of these selenoenzymes allow them to eliminate peroxides as potential substrates for the Fenton reaction. Glutathione peroxidase acts in conjunction with the tripeptide glutathione (GSH), which is present in cells in high (micromolar) concentrations [223]. The substrate for the catalytic reaction of GPx is H_2O_2, or organic peroxide ROOH. Glutathione peroxidase decomposes peroxides to water (or alcohol) while simultaneously oxidizing GSH:

$$2GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

$$2GSH + ROOH \xrightarrow{GPx} GSSG + ROH + H_2O$$

Significantly, glutathione peroxidase competes with catalase for H_2O_2 as a substrate and is the major source of protection against low levels of oxidative stress [149].

8.4.2. **Non-enzymatic antioxidants**

In the cytosol high micromolar levels of the antioxidants vitamin C, β-keto acids such as pyruvate and purine species such as urate can act as ROS scavengers. Similarly, the membrane bound antioxidants vitamin E and lipoic acid can serve a protective function in a more hydrophobic environment. Of especial importance from the view point of thiol-dependent signaling are the cellular tripeptide GSH (γ-glutamylcysteinylglycine) [238], Trx1 and Trx2 system and Metallothionein [19].

Glutathione (γ-glutamylcysteinylglycine)

Glutathione (GSH) is a tripeptide present in the cell at millimolar concentrations and functions as a redox buffer to maintain the overall cellular redox state. Upon oxidation, it forms glutathione disulfide (GSSG), which can be reduced by GR using NADPH as an electron source. If oxidized glutathione GSSG is accumulated inside cells, the ratio of GSH/GSSG is a good measure of oxidative stress [19].

The major protective roles of GSH against oxidative stress are: (1) GSH is a cofactor of several detoxifying enzymes against oxidative stress, e.g. GPx, glutathione-S-transferase and others; (2) GSH participates in amino acid transport through the plasma membrane; (3) GSH scavenges hydroxyl radical and singlet oxygen directly, detoxifying H_2O_2 and lipid peroxides by the catalytic action of GPx; (4) GSH is able to regenerate the most important antioxidants, vitamins C and E, back to their active forms; (5) GSH can reduce the tocopherol radical of vitamin E directly or indirectly via reduction of semidehydroascorbate to ascorbate [239].

The maintenance of normal GSH status is essential for cellular protection, as inhibition of GSH synthesis abolishes this protection. This proposal is based on observations that GSH depletion increases metal toxicity in rats, mice, and cultured cells, in an *in vitro* study on the effects of Cu ions on the free-radical scavenging properties of GSH, showed that besides preventing the occurrence of a redox-active Cu, a Cu–GSH complex could also act by itself to scavenge free-radical species. GSH sequesters redox-active Cu ions, thus preventing them from catalyzing free-radical generation. The formation of Cu–GSH complexes could be

regarded as an additional mechanism in the protection of cells against excessive Cu exposure [19].

Thioredoxins system (Trx1 and Trx2)

In addition to GSH, the cell also possesses two proteins (Trx1 and Trx2) that can reverse disulfide formation. Thioredoxins (Trx) are small redox-active proteins, 12 kD oxidoreductase with two cysteines residues, that complement the GSH system in protection against oxidative stress. Oxidation of Trx1 or Trx2 releases the apoptosis signal regulating kinase 1 (ASK1) and allows for the initiation of apoptosis [19].

Hansen et al. (2006) [240] have observed that some metals such as arsenic (As), cadmium (Cd) and mercury (Hg) can oxidize Trx1 and Trx2, but had little effect on cellular GSH/GSSG. In contrast, other metals (Cu, Fe and Ni) oxidized GSH/GSSG but did not oxidize Trx1 or Trx2. These findings show that differences in metal-induced toxicity are associated with differential effects on the major thiol antioxidant systems [240].