STUDY OF MALONDIALDEHYDE AND GLUTATHIONE PEROXIDASE IN BLOOD AND SALIVA OF ORAL CANCER PATIENTS

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BRANCH – VI

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DECLARATION BY THE CANDIDATE

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Your dissertational study titled "STUDY OF MALONDIALDEHYDE AND GLUTATHIONE PEROXIDASE IN BLOOD AND SALIVA OF ORAL CANCER PATIENTS" presented before the ethical committee on 7th Jan. 2014 has been discussed by the committee members and has been approved.

You are requested to adhere to the ICMR guidelines on Biomedical Research and follow good clinical practice. You are requested to inform the progress of work from time to time and submit a final report on the completion of study.

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CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

- OSCC - Oral Squamous Cell Carcinoma
- ROS - Reactive Oxygen Species
- RNS - Reactive Nitrogen Species
- AOPP - Advanced Oxidation Protein Products
- MDA - Malondialdehyde
- 4-HNE - 4 hydroxy-2-nonenal
- SOD - Superoxide Dismutase
- CAT - Catalase
- GPx - Glutathione peroxidase
- GST - Glutathione-S-transferase
- GSH - Reduced glutathione
- GR - Glutathione reductase
- NO - Nitric oxide
- TAA - Total antioxidant activity
- TAC - Total antioxidant capacity
- TBARS - Thiobarbituric acid reactive substances
- WDSCC - Well differentiated squamous cell carcinoma
- MDSCC - Moderately differentiated squamous cell carcinoma
- PDSCC - Poorly differentiated squamous cell carcinoma
- OSMF - Oral submucous fibrosis
- OLP - Oral lichen planus
- 8-OHdG - 8- hydroxy-2-deoxyguanosine

Oral cancer is one of the most common malignancy in India and is the major form of cancer worldwide. It contributes to about 30-40% of all cancers. In India, the age standardized incidence rate of oral cancer patients is about 12.6 per 100,000 population and prevalence is also very high. It is 4 times higher than in other countries. The important etiologic agents of oral cancer in India are tobacco, chewing with betel quid or tobacco smoking and alcohol consumption. Oral squamous cell carcinoma (OSCC) makes upto 90 % of all oral cancers. Even though various theories have been established, free radicals play a very important role in the carcinogenesis (**Ganesan and Kumar, 2014)**.

Free radicals are released from various biochemical reactions taking place in our body and also from respiratory chain as a result of occasional leakage. It can be an atom or molecule with one or more unpaired electron. Despite their short half life, they show a high reactivity and damaging activity towards micromolecules like proteins and lipids. These free radicals can be oxygen derived and are called reactive oxygen species (ROS) (**Ganesan and Kumar, 2014).**

Oxidative stress is an imbalance between the production of ROS and the cell's oxidant capacity. This may initiate or promote carcinogenesis in the cell by mutagenesis, cytotoxicity and changes in gene expression. Hence, free radicals are believed to play an important role in the disease progression. Free radicals can produce lipid peroxidation in membrane, oxidative modification of proteins and DNA damage, which indirectly induces cell death, mutation and carcinogenesis **(Gurudath et al., 2012)**.

During lipid peroxidation, free radicals attract the hydrogen atom from the polyunsaturated fatty acid (PUFA) of the plasma membrane and produces peroxide,

which are themselves unstable and more reactive thereby resulting in loss of membrane functions. Lipid peroxidation and continuous degradation produce Malondialdehyde (MDA) and 4 hydroxy-2-nonenal (4-HNE).

The most widely used method of estimating free radical activity and lipid peroxidation is to determine the concentration of MDA. It is a marker for measuring oxidative stress **(Mahadevan and Velavan et al., 2012).**

MDA, a highly reactive three carbon dialdehyde, CH_2 $(CHO)_2$ readily combines with several functional groups on molecules including proteins, lipoproteins, and DNA. MDA-modified proteins may show altered physico-chemical behavior and antigenicity. It has been known to be mutagenic in bacterial and mammalian cells and carcinogenic in rats **(Choudhari et al., 2014)**.

Numerous compounds and enzymes work to overcome the damage caused by ROS and to protect cellular components from oxidative damage. Antioxidants acts as the first line of defense against free radical damage. They are essential for maintaining optimum health and well‑being. Superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) are the three major enzymatic antioxidants, responsible for scavenging free radicals and nascent oxygen. These enzymes catalyze decomposition of ROS **(Gurudath et al., 2012).**

GPx contains selenium in its active center and has a high degree of affinity for hydrogen peroxide (H_2O_2) compared with CAT. GPx, a selenium dependent enzyme, removes both H_2O_2 and lipid peroxide by catalyzing a redox reaction using glutathione. Therefore H_2O_2 mediated intracellular DNA damage is prevented, which is thought to be a prerequisite for carcinogenesis. GPx inactivation has been reported to be caused by oxidative damage to the cell membrane. SOD metabolizes free

radicals and dismutates superoxide anions (O_2^-) to H_2O_2. This protects the cells against O_2^- mediated lipid peroxidation. CAT neutralizes the toxicity by acting on H_2O_2 and decomposing it. It has been reported that superoxide radicals inhibit CAT activity and H_2O_2 suppresses SOD activity in the cell **(Beevi et al., 2004).**

Redox modulation is observed by distinctive changes in the activities of these enzymes in oxidative stress. Therefore an overall balance between ROS production and removal may be more important in various cancers including OSCC **(Gurudath et al., 2012).**

The most routinely used diagnostic procedure in the laboratory involves the analysis of the cellular and chemical constituents of blood. Other biologic fluids are also utilized for the diagnosis of various diseases, out of which saliva offers some distinctive advantages **(Mahadevan and Velavan, 2012).** Saliva based diagnostics are non-invasive, non-infectious and cost-effective screening tools. It is a potential substitute to blood and serum based diagnostic technologies **(Kaur et al., 2016).** The exchange between plasma and saliva takes place in the salivary ducts, which are separated from the systemic circulation by a thin layer of epithelial cells **(Lee and Wong, 2009)**. Saliva acts as a diagnostic fluid for many oral and systemic diseases like diabetes, cancer, cardiovascular, metabolic and neurological diseases **(Mahadevan and Velavan, 2012).** Numerous studies confirm that saliva can be useful for the early detection, diagnosis, prognosis, and prediction of oral precancer and cancer **(Kaur et al., 2016).**

Oxidative stress biomarkers have been quantified in plasma, whole blood, urine, respired gases, muscle and other skeletal tissues. One possible biofluid that has the potential to be utilized for measuring a variety of antioxidants and stress related

biomarkers but still under investigation is saliva. Saliva is an attractive biospecimen due to its ease of collection and adequate amount is produced by the human body for examination **(Evans and Omaye, 2017).** Saliva is one of the most complex, versatile and important body fluid. It reflects a large range of physiological needs and information. Hence, known as the "mirror of the body" **(Wang and Gao et al., 2014).**

In India, salivary analysis is not given much importance and there are only limited studies on analysis of salivary oxidative stress markers in cancer. As saliva is in proximity to oral neoplasms and premalignant lesions, it could be an ideal tool for screening, diagnosis, and management of oral cancer (**Shivashankara and Kavya, 2011).**

Studies have proved that there was an increase in serum and salivary levels of MDA in oral cancer patients compared to normal healthy individuals. Only limited studies exist to compare the levels of MDA in OSCC patients of both saliva and blood simultaneously. Decreased GPx levels in blood were found in most of the studies of oral cancer patients but a few studies have also shown increased levels. There is scarcity of literature for the studies on salivary levels of GPx in oral cancer patients. Moreover, no study has been done to assess the blood and salivary levels of GPx simultaneouly in blood and saliva in OSCC. Therefore, the present study has made an attempt to evaluate the levels of MDA and GPx in both blood and saliva of OSCC patients and to compare with that of the healthy controls and to establish the diagnostic efficacy of saliva in evaluating salivary levels of MDA and GPx in OSCC patients.

AIM

To estimate the levels of MDA and GPx in both blood and saliva of OSCC patients.

OBJECTIVES OF THE STUDY

- To determine the levels of MDA in both blood and saliva of OSCC patients and to compare it with that of healthy controls
- To determine the levels of GPx in both blood and saliva of OSCC patients and to compare it with that of healthy controls
- To correlate MDA and GPx in blood and saliva of OSCC patients.
- To determine the sensitivity and specificity of these markers in blood and saliva of OSCC.

ORAL CANCER

Cancer is the major cause of morbidity and mortality all over the world. It is one of the main causes of death in all countries with its relative position varying with age and sex. The sixth most common cancer in the world is oral and oropharyngeal carcinomas (**Shenoi et al., 2012)**. In India, Oral cancer is among the top three types of cancers. The incidence of oral cancer is highest in India, South and Southeast Asian countries. In India, oral squamous cell carcinoma (OSCC) constitutes about 90-95% of all the oral cancers. The lifetime risk for mortality due to cancer in India for both males and females is estimated to be 61% **(Varshitha, 2015).**

A number of etiological factors has been attributed to the high incidence of oral cancer in India. The common causes for oral cancer are tobacco consumption habit among the patients either as smokeless tobacco or smoking, alcohol consumption. The other causes for oral cancer are positive family history of oral cancer, viral infections like HPV, poor oral hygiene **(Varshitha, 2015).**

However, not all the people who follow these habits develop OSCC. There may be a few genetic characteristics specific to an individual or certain other environmental factors which may either offer protection against OSCC, or may predispose to or even promote OSCC **(Feller, 2012).**

According to World Health Organization (WHO), in developing countries, in males, carcinoma of oral cavity is the sixth commonest cancer after lung, prostate, colorectal, stomach and bladder cancer, while in females, it is the tenth commonest site of cancer after breast, colorectal, lung, stomach, uterus, cervix, ovary, bladder and liver **(Mehrotra and Yadav, 2006).**

The international agency for research on cancer (IARC) confirmed that smoking of various forms of tobacco is carcinogenic in humans. There is increased

exposure to carcinogenic agents such as tobacco-specific nitrosamines released from tobacco chewing with betel quid and to nitrosamines derived from areca nut alkaloids. Furthermore, reactive oxygen species (ROS) involved in multistage carcinogenesis, are also generated in considerable amounts in the oral cavity during chewing/smoking **(Mehrotra and Yadav, 2006).**

OXIDATIVE STRESS AND REACTIVE OXYGEN SPECIES

The oxygen supply is absolutely essential for the existence of higher organisms. Paul Bert (1878) showed that oxygen in high concentrations could damage brain, lungs and other organs **(Choudhari et al., 2014).** In 1954, Gerschman and colleagues for the first time proposed that damaging effects of oxygen could be attributed to formation of oxygen free radical **(Gerschman et al., 1954).**

A free radical is defined as a molecule or a molecular species that contains one or more unpaired electrons and is capable of independent existence**.** ROS include both free radicals as well as non-radical derivatives of oxygen **(Sathyanarayana and Chakrapani, 2006)**

The relation between free radicals and disease can be explained by the concept of 'oxidative stress'. Sies defined oxidative stress as an imbalance between oxidants and antioxidants in favour of oxidants, potentially leading to damage. Products of biological damage are referred as biomarkers of oxidative stress (**Choudhari et al., 2014)**.

Oxygen is required in many metabolic reactions, particularly for the release of energy. During these processes, molecular oxygen is completely reduced and converted to water. However if the reduction of oxygen is incomplete, a series of reactive radicals are formed, as shown below (figure 1):

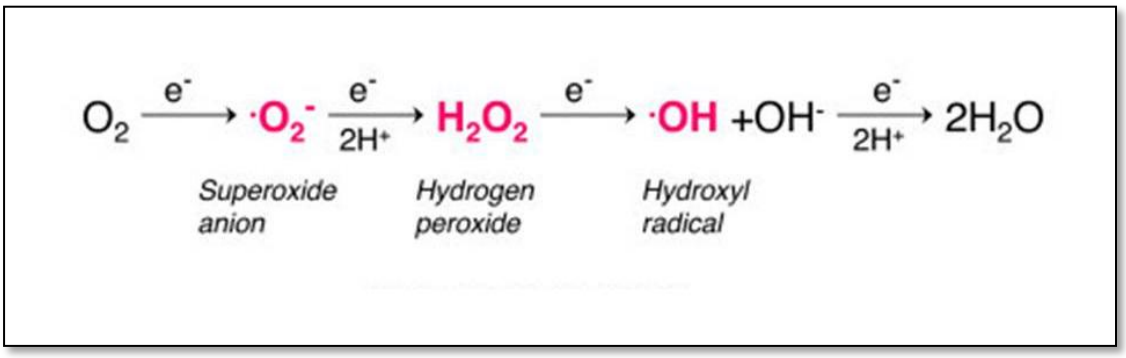


Figure 1: Reactive radicals (in pink) formed from oxygen (Source: Google)

Besides superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl (OH^-) radical, the other free radicals and ROS of biological importance include singlet oxygen($^1O_2$), hydroperoxy radical (HOO^-), lipid peroxide radical (ROO^-), nitric oxide (NO^-) and peroxynitrite ($ONOO^-$). The common characteristic features of free radicals are

- ✓ Highly reactive
- ✓ Very short half life
- ✓ Generate new radicals by chain reaction
- ✓ Damages biomolecules, cells and tissues.

FUNCTION OF ROS IN CELLS

Numerous beneficial functions are performed by ROS in our body such as phagocytosis, apoptosis, detoxification reactions, executioner of precancerous cells and infections. It is involved in signaling pathways to maintain cellular homeostasis in the body. Also regulates many metabolic and cellular processes including proliferation, migration, gene expression, immunity and wound healing. Biochemical reactions are involved in the synthesis of prostaglandins, hydroxylation

of proline and lysine, oxidation of xanthine and other oxidative processes **(Noori, 2012).**

SOURCES OF ROS

Free radical reacts with the nearest stable molecule, snatching its electron to gain stability. The attacked molecule loses its electron and becomes a free radical. The chain reaction cascade resulted in disruption of a living cell.

ROS can be produced from endogenous and exogenous substances. The endogenous sources are mitochondria, cytochrome P450 metabolism, peroxisomes, and inflammatory cell activation, NADPH oxidases (NOX), cyclooxygenases, lipooxygenases, xanthine oxidases. Mitochondria and NADPH oxidases are two major contributors of endogenous ROS in cancer.

Exogenous sources are environmental agents such as non-genotoxic carcinogens, various xenobiotics, ultrasound and microwave radiation. They are necessary for normal cellular functions but when in excess they can cause cellular damage and can lead to cancer **(Choudhari et al., 2014).**

MECHANISM OF ACTION OF ROS IN CANCER

Cancer development is characterised by progressive action of multiple events taking place in a single cell. It can be described by three stages: initiation, promotion and progression. Involvement of ROS was found in all these stages. Depending upon the type of radical involved and its reactivity, the effect of oxidative stress varies at different stage of carcinogenesis.

Initiation: When a normal cell sustains a DNA mutation and when proceeded by a round of DNA synthesis, fixation of the mutation occurs, resulting in an initiated

cell. This initiation by ROS is supported by presence of oxidative DNA modifications in cancer tissue**.**

Promotion: In this stage, clonal expansion of initiated cells takes place by induction of cell proliferation and/or inhibition of apoptosis. This stage shows a strong involvement of oxidative stress. ROS can induce the expansion of mutated cell clones by modulating the genes linked with proliferation or cell death temporarily and by regulating activity of certain transcription factors such as nuclear factor kappa B (NFƙB), nuclear factor erythroid 2-related factor (Nrf2), hypoxia inducible fators (HIF) and p53 associated with the control of cell growth and oncogenesis. This results in NFƙB activation, followed by induction of genes, which encodes the proteins responsible for inhibiting apoptosis. Pro-survival functions occurs when ROS acted at signal-transduction level. Oxidative stress can activate extracellular signal regulated kinase (ERK/MEK) and phosphoinositide 3-kinase (PI3K/AKT) pathways resulting in inactivation of proapoptotic proteins and upregulation of antiapoptotic genes. Lower level of oxidative stress at this stage can stimulate cell division and thus promotes growth of tumor. ROS production during this stage is the main mechanism of ROS-related tumour promotion.

Progression: Generation of large amounts of ROS may cause mutation and inhibit anti proteinases, upregulate matrix metalloproteinases and injure local tissues. In fully developed cancer, increased levels of oxidatively modified DNA bases may be responsible for the genetic instability and tumor metastasis. ROS is reported to be a pivotal factor for triggering angiogenic response, which is essential in tumor metastasis **(Choudhari et al., 2014).**

ROS formed through various events and pathways, react with and damage cellular components and contribute to neoplastic transformation **(Choudhari et al., 2014).** Simplified flowchart depicting the entire sequence of events: (figure 2).

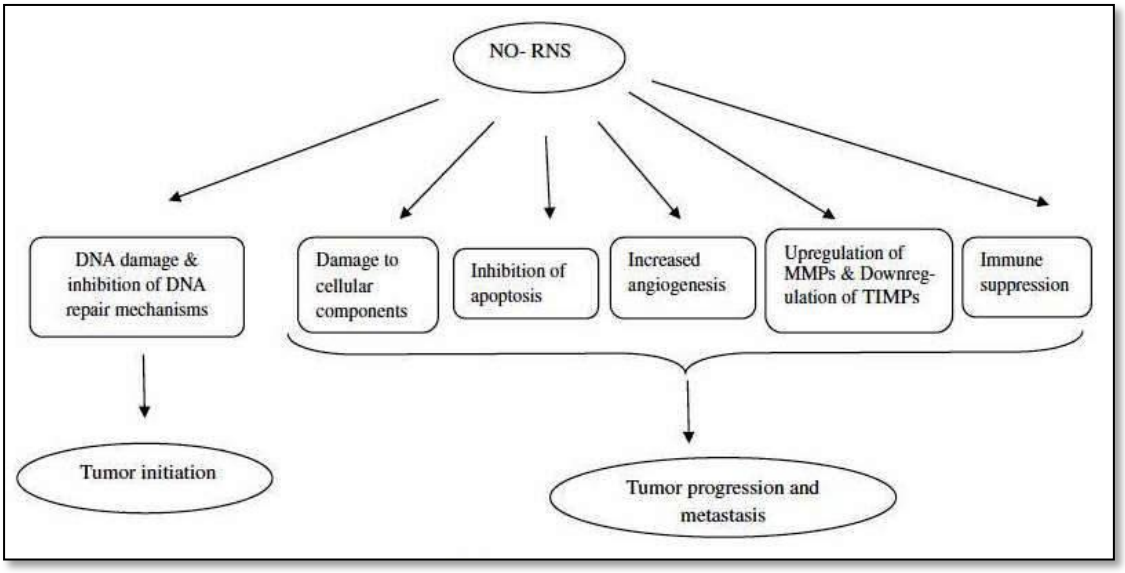


Figure 2: Mechanism of action of ROS in cancer. (Source: Choudhari et al., 2014)

ROS MEDIATED DAMAGE TO BIOMOLECULES AND ITS ROLE IN CARCINOGENESIS

Oxidative nuclear and mitochondrial DNA damage:

DNA, a highly sensitive element to ROS attacks causes permanent modification of genetic material. This is the first step involved in mutagenesis and carcinogenesis.

Following are the effects of DNA damage caused by ROS/RNS:

- ✓ Cause structural alterations in DNA and can produce gross chromosomal alterations
- ✓ Affects cytoplasmic and nuclear signal transduction pathways.
- ✓ Modulates activity of proteins and genes that respond to stress

- ✓ Regulates genes that are related to cell proliferation, differentiation and apoptosis.
- ✓ Not only they cause damage when exposed to H_2O_2 / other oxidants but also suppress DNA repair. This results in elevated DNA lesions and an increased risk of disease.
- ✓ Participates in carcinogenesis via activation of proto oncogenes and inactivation of tumor suppressor genes

Lack of histone proteins and its close proximity to the respiratory chain makes mitochondrial DNA (mtDNA) more at risk for ROS-induced oxidative damage. mtDNA is an important contributor to carcinogenesis as its repair is less complete than chromosomal DNA repair **(Choudhari et al., 2014)**.

Oxidative damage to proteins:

Proteins, major initial cell targets of ROS is characterized by loss of histidine residues, oxidative scission, introduction of carbonyl groups, and formation of protein-cantered alkyl, R^-, alkoxyl, RO^-, and alkylperoxyl, ROO^-, radicals. Protein oxidation is connected with formation of inter and intra-protein cross linkages resulting in fragmentation, cross-linking and aggregation of proteins. Amino acid residue side chains and DNA repair enzymes are very susceptible to attack by ROS and RNS, which results in increased frequency of mutations. Protein oxidation results in earlier formation of protein carbonyls in biological systems. Advanced oxidative protein products (AOPP) produced by different oxidation patterns, results in either NO or H_2O_2 production. This may result in a series of reactions with potential damage to cellular micromolecules **(Choudhari et al., 2014).**

Oxidative damage to lipids:

Methylene group between two double bonds of polyunsaturated fatty acid (PUFA) in cell membranes are more sensitive to ROS damage (figure 3).

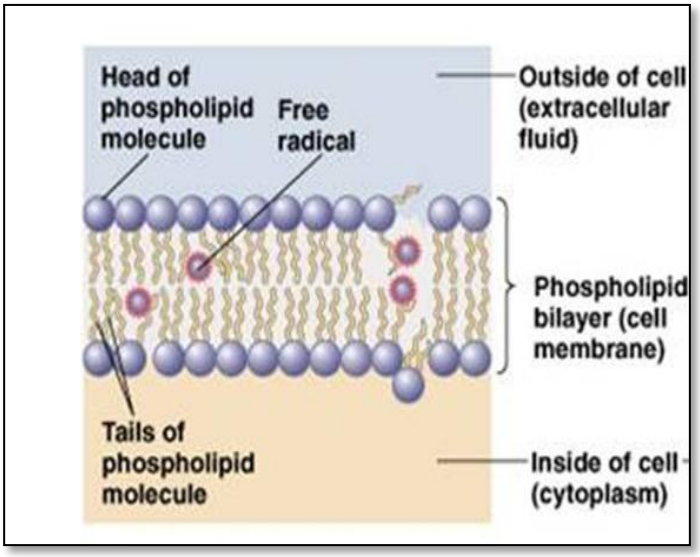


Figure 3: Cell membrane damage by free radicals (Source: Google).

Free radical-induced peroxidation of membrane lipids occurs in 3 stages- initiation, propagation and termination **(Sathyanarayana and Chakrapani, 2006)**

Initiation phase:

This step is characterized by the removal of hydrogen atom (H) from polyunsaturated fatty acids (LH) caused by hydroxyl radical to make water and a fatty acid radical.

$$LH + OH^{\cdot} \longrightarrow L^{\cdot} + H_2O$$

Propagation phase:

The fatty acid radical, an unstable molecule, thereby reacts readily with molecular oxygen, producing peroxy radical ($LOO^{\cdot}$). The radical formed is also an

unstable species that reacts with another PUFA (LH) to form lipid hydroperoxide (LOOH).

$$L^{\cdot} + O_2 \longrightarrow LOO^{\cdot}$$

$$LOO^{\cdot} + LH \longrightarrow LOOH + L^{\cdot}$$

The hydroperoxides are capable of further stimulating lipid peroxidation as they can form alkoxy ($LO^{\cdot}$) and peroxyl ($LOO^{\cdot}$) radicals.

$$2LOOH \longrightarrow LO^{\cdot} + LO_2^{\cdot} + H_2O$$

$$LOOH \longrightarrow LO^{\cdot} + LOO^{\cdot} + \text{aldehydes}$$

Termination phase:

Lipid peroxidation proceeds as a chain reaction until all the available PUFA gets oxidized and results in the formation of reactive aldehydes like malondialdehyde (MDA) and 4 hydroxy-2-nonenal (4-HNE). They have high reactivity with proteins and DNA. On binding to DNA, they become potentially mutagenic **(Sathyanarayana and Chakrapani, 2006).**

MALONDIALDEHYDE AS A MARKER FOR LIPID PEROXIDATION

Malondialdehyde (MDA) is an end-product formed during oxidative stress. It is one of the several products formed during the degradation of phospholipids in cell membrane. Arachidonic acid (AA), released due to the action of phospholipase-A2 is attacked subsequently by ROS (hydroxyl radical OH•) from mitochondria through a non-enzymatic reaction and lipid endoperoxide is formed **(Lorente, 2013).** This lipid endoperoxide, ruptures spontaneously and MDA is formed in the intracellular space (figure 4). MDA is released into extracellular space and finally into the blood.

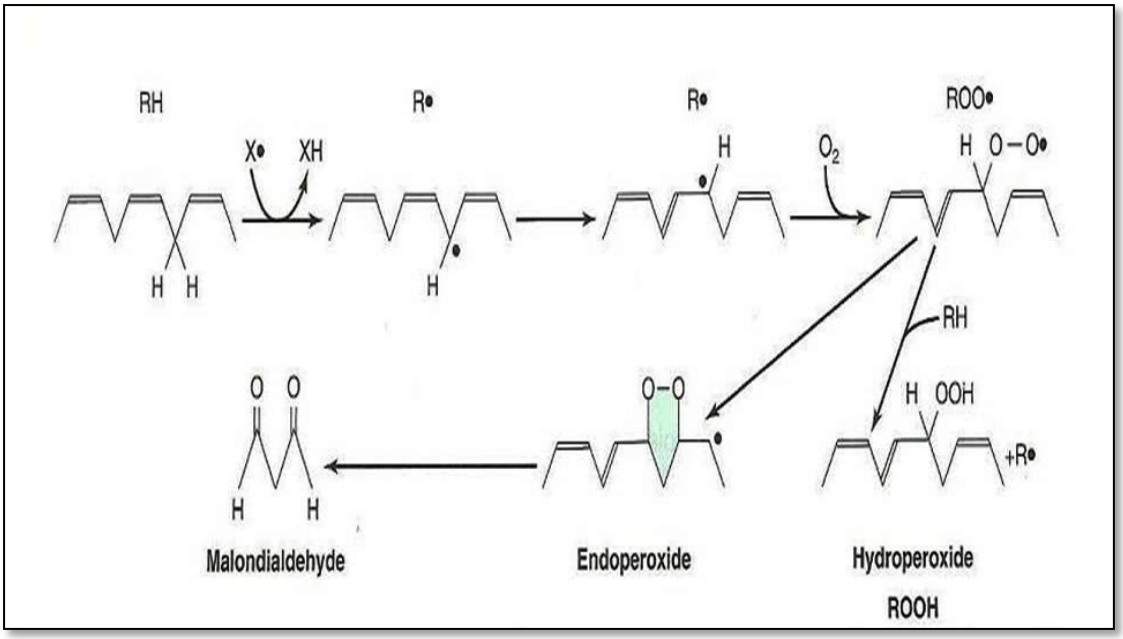


Figure 4: Lipid peroxidation. **(**Source: Sathyanarayana and Chakrapani, 2006)

MDA, a highly reactive three carbon dialdehyde, ($CHO=CH_2=CHO$) readily combines with several functional groups on proteins, lipoproteins, and DNA. MDA-modified proteins may show altered physico-chemical behavior and antigenicity and has been found to be mutagenic in bacterial and mammalian cells and carcinogenic in rats.

Cellular damage due to free radicals can be assessed by measuring the levels of these lipid peroxides. Studies have reported increased levels of lipid peroxidation products such as lipid hydroperoxides, 4-HNE and MDA in oral cancer and precancer patients. This could be due to increased free radicals, which suggests that there may be a relationship between free radical activity and malignancy. Along with ROS, RNS is also known to play role in carcinogenesis **(Choudhari et al., 2014)**.

OXIDATIVE STRESS IN ORAL CANCER AND PRECANCER

Usage of tobacco (smoking and smokeless) and excessive consumption of alcohol are amongst the major risk factors for oral cancer. ROS has been implicated in

oral cancer development in tobacco chewers and smokers as a result of which oxidative stress is increased and antioxidant defences are compromised in oral cancer patients **(Choudhari et al., 2014)**.

Free radicals generated by the use of tobacco cause continuous endogenous damage to cellular DNA and accumulation of such damage plays an important role in oral carcinogenesis. The formation and stabilization of free radicals are affected by the heat generated while smoking as well as the change in pH of body fluids due to tobacco consumption. Moreover, free radicals in the saliva of tobacco users, produced during auto-oxidation of areca nut-polyphenols are pivotal in initiation and promotion of oral cancer. This establishes the role of ROS in oral cancer in tobacco users.

Areca ingredients induce ROS and DNA adducts formation. Alkaline conditions in betel nut chewing are favourable for free radicals formation.

Alcohol, regardless of the form taken, increases the risk of oral cancer as free radicals are produced in excessive amounts. Cytochrome P450 2E1 (CYP2E1) oxidizes ethanol to acetaldehyde, which is again oxidised to acetate. Chronic ethanol ingestion can instigate single nucleotide polymorphism of CYP2E1. Increased CYP2E1 activity leads to increased generation of ROS. This in turn leads to lipid peroxidation and formation of 4HNE, which binds to DNA to form mutagenic adducts.

All the above mentioned factors causes oxidant/ antioxidant imbalance which elevates oxidative stress. This is accompanied by increased lipid peroxidation, oxidative DNA damage, damage to macro and micro-molecules of cells and disturbances of antioxidant defense which can induce malignant process **(Choudhari et al., 2014)**.

ANTIOXIDANT ENZYME SYSTEM

To mitigate the harmful effects of free radicals, the aerobic cells have developed antioxidant defense mechanisms. A biological antioxidant may be defined as a substance (present in low concentration compared to an oxidizable substrate) that significantly delays or inhibits oxidation of a substrate. Antioxidants may be considered as the scavengers of free radicals **(Sathyanarayana and Chakrapani, 2006).**

A good antioxidant should: (i) specifically quench free radicals (ii) chelate redox metals (iii) interact with (regenerate) other antioxidants within the "antioxidant network" (iv) have a positive effect on gene expression (v) be readily absorbed (vi) have a concentration in tissues and biofluids at a physiologically relevant level (vii) work in both the aqueous and/or membrane domains **(Valko et al., 2006)**.

There are different ways of classifying antioxidants **(Sathyanarayana and Chakrapani, 2006):**

I. Antioxidants in relation to lipid peroxidation

   A) Preventive antioxidants - blocks the initial production of free radicals. Eg: Catalase, Glutathione peroxidase.

   B) Chain breaking antioxidants – inhibits the propagative phase of lipid peroxidation. Eg: Superoxide dismutase, Vitamin E, Uric acid.

II. Antioxidants according to their location

   A) Plasma antioxidants

   Eg: β-carotene, ascorbic acid, bilirubin, uric acid, ceruloplasmin, transferrin.

   B) Cell membrane antioxidants. Eg: α-tocopherol.

C) Intracellular antioxidants

Eg: Superoxide dismutase, Catalase, Glutathione peroxidase.

III. Antioxidants according to their nature & action

A) Enzymatic antioxidants

Eg: Superoxide dismutase, Catalase, Glutathione peroxidase, Glutathione reductase

B) Non-enzymatic antioxidants

a) Nutrient antioxidants

Eg: α-tocopherol, carotenoids (β- carotene), ascorbic acid, selenium.

b) Metabolic antioxidants

Eg: Glutathione, ceruloplasmin, albumin, bilirubin, transferrin, ferritin, uric acid

The most efficient enzymatic antioxidants involve Superoxide dismutase (SOD), Catalase (CAT) and Glutathione peroxidase (GPx). The major reactions of these enzymes are outlined below:

SUPEROXIDE DISMUTASE

SOD is the first line of defense to protect cells from the negative effects of superoxide (O_2^-) as it converts O_2^- to hydrogen peroxide and O_2. Its catalytic function was discovered by Joe Mc Cord and Irwin Fridovich in 1968. SOD is grouped as Cu/Zn SOD, Mn SOD and Fe SOD, depending on the metal ion content. Its activity is observed extracellularly as well as intracellularly in mitochondria and cytosolic compartment. SOD activity varies and the highest levels are seen in liver, adrenal gland, kidney and spleen **(Patekar et al., 2013).**

Three different enzyme isoforms of SOD were identified in mammals - SOD1, SOD2 and SOD3. SOD1 contains Ra and Zn in its catalytic centre and was found in cytoplasm, nuclear compartments and in lysosomes. SOD2, a homotetramer, presents Mn as cofactor. It has been localized in mitochondria and has a role to play in the promotion of cellular differentiation and tumorigenesis. It provides protection of pulmonary toxicity induced by hyperoxia. SOD3, which is also a homotetramer, was localized in extracellular fluids like human plasma, lymphatic fluid and cerebrospinal fluid. **(Iannitti et al, 2012)**

CATALASE

CAT is a major primary antioxidant defense component catalyzes the decomposition of hydrogen peroxide, produced by SOD, into water and oxygen. Chemically, it is a tetramer composed of four polypeptide chains containing four porphyrin heme groups. High amounts of CAT have been localised in peroxisomes, microsomes and cytosol of hepatocytes. Kidney and red blood cells have also known to show its increased activity. **(Patekar et al, 2013).**

GLUTATHIONE PEROXIDASE

In 1957, Gordon C. Mills discovered an enzyme, glutathione peroxidase (GPx), which protected hemoglobin from oxidative breakdown by hydrogen peroxide. It is one of the most essential of antioxidative defence mechanisms (**Sachdeo and Mody, 2011).**

They are classified as selenium dependent (GPx) and selenium independent (Glutathione-S-transferase, GST) and are found to be present intracellularly in the cytosol and mitochondrial matrix **(Patekar et al, 2013).** They vary from each other by

the number of subunits, the bonding nature of the selenium at the active centre and their catalytic mechanisms. Humans have four different Se-dependent glutathione peroxidases. All GPx 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.

GPx has a high degree of affinity for H_2O_2 than CAT. It reduces and breaks down not only H_2O_2 but also lipid peroxide (LOOH) by catalyzing a redox reaction with reduced glutathione (GSH), which serves as an electron donor. During the GPx-catalyzed reaction, GSH is converted to its oxidized disulfide form (GSSG), which has a decreased ability to reduce peroxide. GSH can be regenerated from GSSG by glutathione reductase (GR), using reduced nicotinamide adenine dinucleotide phosphate (NADPH). During the reaction, NADPH is oxidized to $NADP^+$ and NADPH is regenerated through the pentose phosphate pathway. Therefore, GPx-dependent redox cycle functions as a cellular antioxidant mechanism (figure 5) **(Higuchi, 2014)**

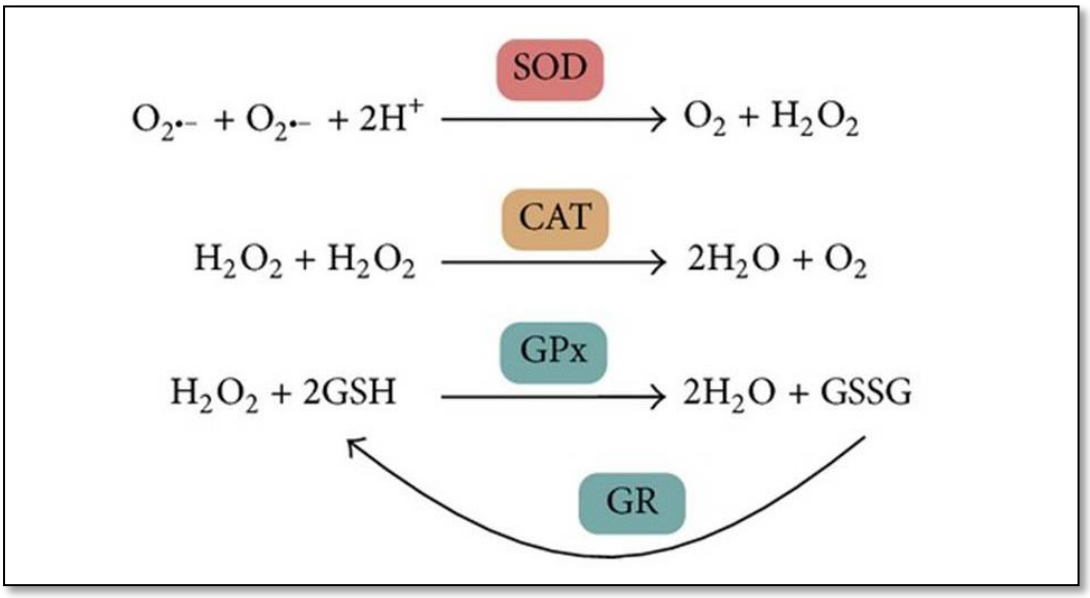


Figure 5: The antioxidant enzyme system (G-SH-reduced glutathione; GS-SG-oxidized glutathione) (Source: Google)

The aberrant expression of this enzyme is induced by oxidative stress and has been associated with a variety of pathologies such as hepatitis, HIV, skin, kidney, breast and intestine cancer **(Iannitti et al., 2012).**

OXIDANTS AS BIOMARKERS IN ORAL CANCER AND PRECANCER

As ROS have a short half-life (seconds), its detection is often difficult. Specific ROS can be assessed in tissue at cellular level. Another way of measuring these biomarkers is by quantification of the oxidative damage of biomolecules in saliva, blood or urine. Stable, specific, or nonspecific derivatives such as lipid peroxidation products, amino acid oxidation products and peptide oxidation products can be measured by changes in their fluorescence, colour, or luminescence. The most commonly studied marker of lipid peroxidation is MDA. Easy technique and low cost of evaluation can make them versatile and useful prognostic tool for identification of oral cancer patients with high risk for recurrence and oral precancer patients with high risk for oral cancer **(Choudhari et al., 2014)**.

ANTIOXIDANTS AND CARCINOGENESIS

Increased amounts of free radicals are formed than normal under pathological conditions. To combat, antioxidant defense mechanisms act at different levels to minimise their harmful effects. Reduced antioxidant activity associated with high levels of oxidative stress have been reported in different cancers of head and neck. Various studies in blood and tissues of oral precancer and cancer, have shown lowered antioxidants or antioxidant capacity, which might be due to

- ✓ Increased utilization of antioxidants to scavenge ROS or RNS
- ✓ Inadequate production of antioxidant enzymes

- ✓ Poor antioxidant defence system in cancerous environment
- ✓ Increased destruction of antioxidants by reactive oxygen metabolites

SALIVA AS A DIAGNOSTIC FLUID

Plasma, whole blood, urine, respired gases, muscle, and other skeletal tissues have been used to measure oxidative stress markers. Saliva is one such biospecimen still under investigation, as it has the potential to be utilized for measuring antioxidants and oxidative stress related biomarkers **(Evans and Omaye, 2017).**

Saliva is secreted from three major salivary glands and numerous minor salivary glands. The whole saliva is a complex mixture of fluids including gingival cevicular fluid, oral, nasal, and mucosal transudate **(Humphrey and Williamson, 2001)**. Oral bacteria and their metabolites, desquamated epithelial and blood cells, food debris and various chemical products are also present in the saliva. In healthy adults, saliva production is estimated to be about 0.75 - 1.5 L per day and has a pH of about 6.2 - 7.4. Biochemically, saliva is an aqueous solution (more than 99% is water) containing numerous organic and inorganic molecules. It may reflect the current physiological condition of the body. Therefore, it is called as "the mirror of health of the organism" **(Farnaud et al., 2010; Yoshizawa et al., 2013**).

The exchange between plasma and saliva takes place by active transport, diffusion across the cell membrane by passive diffusion directed by the concentration gradient in the salivary ducts, which are separated from the circulation system by a thin layer of epithelial cells (figure 6) **(Lee and Wong, 2009).** Saliva can potentially be used for long-term monitoring of oral diseases.

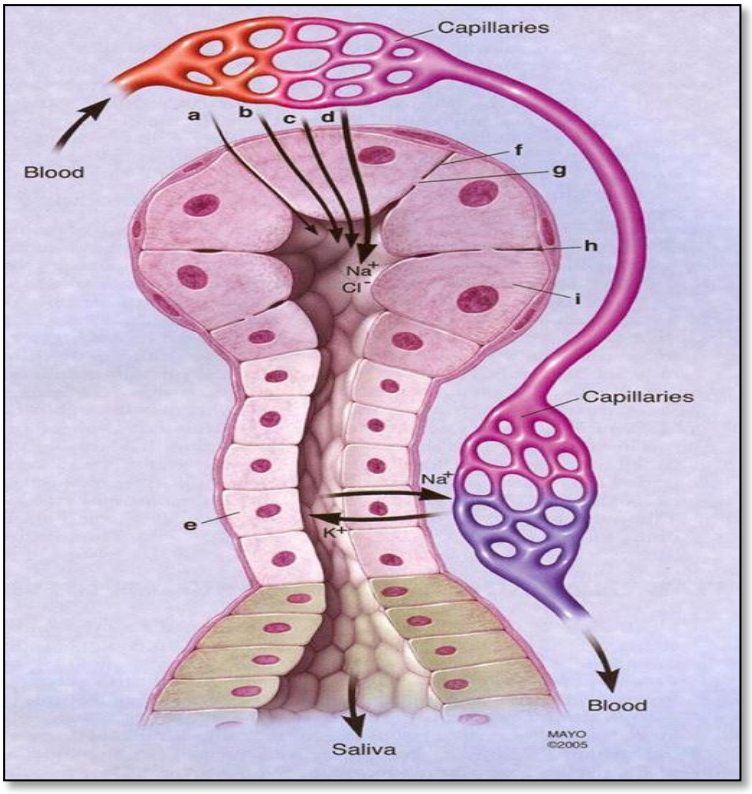


Figure 6: Mechanism of molecular transport from serum into salivary gland ducts. A. Active transport. B. Passive diffusion. C. Simple filtration. D. Acinar cells actively pump sodium ions (Na+) into the duct. E. Duct cells pump Na+ ions back into blood. F. Cell membrane. G. Pore on the cell membrane. H. Intracellular space. I. Acinar cell. [Source: Lee and Wong, 2009]

Similar to plasma and tissues, ROS and RNS in saliva play an important role in redox-dependent signaling and are necessary for physiological functions. Antioxidant enzymes such as SOD, CAT, and GPx are present in saliva **(Battino et al., 2002)**. Their function is to protect oral cavity against the ill effects of ROS/RNS. Saliva is intended to be the first line of defense against free radicals **(Tothova et al., 2015).**

Apart from periodontitis and dental caries, there are so many oral and systemic disorders that were studied in relation to salivary markers of oxidative stress. Oral

precancerous lesions such as lichen planus and leukoplakia and patients with OSCC were found to be associated with higher MDA **(Metgud and Bajaj, 2014)**.

Some of the properties of human saliva that had attracted clinicians or researchers to use this non-invasive fluid are:

- ✓ Non-invasive
- ✓ Simple collection protocols
- ✓ Non-infectious sample
- ✓ Easily disposal
- ✓ Easily transportable
- ✓ Cost effective
- ✓ Not subject to cultural and religious "taboos"
- ✓ Safe and effective
- ✓ Higher patient compliance (**Khurshid and Zohaib et al., 2016)**

STUDIES ANALYSING OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN SYSTEMIC DISORDERS

Schiavon and Guidi et al., 1994 measured plasma GPx in various stages of different renal diseases and compared it with the following indices of kidney function: serum creatinine, creatinine clearance, and urinary excretion of α_1-microglobulin, $ß_2$-microglobulin, albumin and N-acetyl-ß-D-glucosaminidase. GPx appeared significantly reduced in most of the renal diseases and showed a significant correlation with most of the renal function indices. Authors have suggested that measurement of GPx can be used as an adjunctive index for the assessment of kidney alterations.

Bakan and Taysi et al., 2003 reported lower levels of serum GPx, SOD, GSH and higher NO and MDA in chronic lymphocytic leukemia patients compared with the control group. However, there was no statistically significant difference in the parameters on the basis of stages in these patients. Authors concluded that significant changes in antioxidant defense system leads to enhanced action of oxygen radical, resulting in lipid peroxidation.

Arana and Cutando et al., 2006 assessed oxidative stress by measuring GPx, GR, GSH and glutathione disulfide (GSSG) in saliva of diabetic patients and heroin addicts. GPx and GR levels were significantly higher in diabetics and lower in drug addicts than controls. Both group of patients had significantly lower levels of GSH and higher GSSG than controls. Authors have suggested that saliva may be suitable for determining the prognosis and evolution of these diseases and its oral manifestations.

Hassan and Keen et al., 2013 reported significantly higher plasma levels of MDA, NO and SOD and significantly lower levels of GSH and GPx in lichen planus of skin patients than in controls. Authors concluded that an increased lipid peroxidation and an imbalance in the antioxidant defense mechanism may play a role in its pathogenesis.

Ayala and Munoz et al., 2014 found MDA to be significantly modified in alzheimer's disease, cancer, diabetes, liver disease, parkinson's disease.

Khalil Arjmandi and Moslemi et al., 2016 reported a significant increase in MDA and a significant decrease in GPx, SOD, TAA and Se levels in the serum of breast cancer patients in one day before and after the end of radiation therapy. The level of the CAT enzyme had no significant changes. The results showed some

changes in the status of TAA, SOD and GPx which are associated with age, body mass index and clinical stage of the disease.

Ramadan and Hemida et al., 2017 have observed decreased preoperative serum level of SOD and GPx antioxidants and increased level of MDA in epithelial ovarian cancer. These findings were associated with advanced tumor stage. Their study confirmed the role of oxidative stress in development of epithelial ovarian cancer.

STUDIES ANALYSING OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN ORAL DISORDERS OTHER THAN PRECANCER/CANCER

Cimen et al., 2003 and **Arikan et al., 2009** found increased MDA level and decreased anti-oxidant enzymes (SOD, GPx, and CAT) in recurrent aphthous stomatitis patients.

Sarode and Shelar et al., 2012 evaluated salivary MDA in healthy adults, matched for gender and age, with and without dental caries. MDA values were increasing with higher oral hygiene index but were not statistically significant. Authors have concluded that there is an association between presence of dental caries and salivary MDA levels.

Miricescu and Totan et al., 2013 in a study of chronic periodontitis showed a significantly higher levels of salivary MDA & lower levels of salivary GPx when compared to control group.

Kurku and Kacmaz et al., 2015 investigated oxidative stress in saliva of smokers along with their serum and found that the total oxidant stress, oxidative stress index, MDA and NO levels were found to be higher in the serum samples, and the levels of total sulfhydryl groups were lower in smokers compared with the controls.

Among the smokers, salivary MDA levels were higher before and after smoking, GPx levels were lower than the controls and salivary NO levels after smoking were higher than both those of the control group and the levels before smoking. They have concluded that the saliva samples can also be useful in showing oxidant-antioxidant balance in smokers.

STUDIES ANALYSING OXIDATIVE STRESS AND/OR ANTIOXIDANT STATUS IN BLOOD OF PRECANCEROUS AND OSCC PATIENTS

Beevi et al., 2004 analyzed the blood levels of lipid peroxidation products (MDA and lipid hydroperoxide), enzymatic (SOD, CAT, GPx) and non enzymatic (GSH, Vitamin E & Vitamin C) antioxidants and nitric oxide products[nitrite (NO_2^-), nitrate (NO_3^-) and total nitrite (TNO_2^-)] of 15 OSCC patients with clinical stage III/IV with age and sex matched healthy subjects. Nitric oxide products were significantly elevated, whereas enzymatic and non-enzymatic antioxidants were significantly lowered in OSCC patients. They have concluded that oxidative stress is increased and antioxidant defenses were compromised in OSCC. A weak antioxidant defense system makes the mucosal cells more vulnerable to the genotoxic effect of ROS. This creates an intracellular environment more favorable for DNA damage and disease progression.

The findings of the studies conducted by **Manoharan et al., 2005, Sharma et al., 2009, Srivastava et al., 2012, Shilpashree et al., 2013** correlated with the findings of the study done by **Beevi et al., 2004.** They have proved that TBARS level gradually increased whereas antioxidants level gradually reduced with increasing stages of oral cancer patients.

Sabitha and Shyamaladevi et al., 1999 analyzed SOD, catalase, GPx, GR, GST, glucose-6-phosphate dehydrogenase (G6PDH) and MDA levels from the blood samples of stage III oral cancer patients before initiating radiotherapy and after the sixth week of radiotherapy. MDA showed a significant increase and antioxidant enzymes showed a significant decrease in untreated and irradiated oral cancer patients when compared with normal subjects, representing the lack of antioxidant defense. Radiation induces lipid peroxidation by inactivating the antioxidant enzymes, thereby rendering the system inefficient in management of the free radical attack. Thus, the degree of radiation affects the extent of the depression of the antioxidant enzyme activities and increases lipid peroxidation. Similar findings with regard to GPx were reported by **Sachdeo and Mody, 2011.**

Thomas and Sethupathy, 2015 evaluated the oxidative stress (TBARS) and antioxidant levels (SOD, catalase, GPx and GSH) levels in blood samples of healthy subjects, 20 oral precancer & 20 OSCC patients. Increased TBARS and decreased antioxidant levels were found in oral cancer patients. They also found that MDA levels in WDSCC was greater as compared to MDSCC and PDSCC, but this difference was statistically not significant. No correlation exists in lipid peroxidation between degrees of differentiation of malignant oral lesions. As the disease progresses from precancerous to cancerous state, levels of antioxidants declined further. Hence the authors have concluded that these antioxidant markers would be suitable for predicting the prognosis of oral cancer.

The findings of the studies regarding GPx and/or MDA, conducted by **Chole et al**., **2010, Gurudath et al., 2012, Rai et al., 2015, Misra et al., 2016, Nyamati et al., 2016** also correlated with the findings of the study done by **Thomas and Sethupathy, 2015.**

Bagul and Ganjre et al., 2013 reported significantly increased levels of SOD and GPx in OSCC patients compared to controls. The cause behind this may be increased oxidative stress and higher levels of circulating free radicals in patients with OSCC. The body's defense mechanisms would play an important role in the form of antioxidants and try to minimize the damage, adapting itself to the above stressful situation. Thus the increased activity of antioxidant enzyme may be a compensatory regulation in response to oxidative stress.

Khan and Malik et al., 2017 assessed various biochemical, inflammatory and antioxidative parameters in sera of 50 OSCC patients versus 20 healthy controls. Percent (%) fold increase of MDA, AGEs, AOPPs, IL-1, TNF-α. MMP-2, MMP-9 and MMP-11 were found to be 64.85, 65.52, 68.28, 37.72, 15.97, 9.62, 42.12, 15.42 and 30.35, respectively in OSCC as compared to controls. Authors found a significant decrease of GSH, SOD, CAT, Vitamin-A, Vitamin-E, and GR i.e. 73.59, 72.34, 77.57, 26.49, 17.24 and 57.67%, respectively except GPx which was 75.57 times increased in OSCC patients.

Evaluation of oxidant–antioxidant status in blood and tumor tissue samples in OSCC patients in comparison with the healthy controls by **Gokul et al., 2010, Huo et al., 2014** showed similar findings. MDA and NO were significantly elevated in the blood and tissue samples of OSCC patients. In tissues, SOD and CAT were significantly reduced while in erythrocytes, reduced CAT and raised SOD was seen. They concluded that this oxidant–antioxidant imbalance may be considered as one of the factors responsible for pathogenesis of cancer. Findings of **Korde et al., 2011** with regard to MDA were similar to the above mentioned studies.

STUDIES ANALYSING OXIDATIVE STRESS AND/OR ANTIOXIDANT STATUS IN SALIVA OF PRECANCEROUS AND OSCC PATIENTS

Rai et al., 2006 analyzed the salivary samples of lipid peroxidation product (MDA) in 25 cases of leukoplakia, 47 cases of OSMF, 21 cases of candidiasis, 67 cases of dental caries, 62 cases of oral cancer and 50 healthy subjects. They have found significantly elevated levels of salivary MDA in periodontitis, leukoplakia, OSMF and cancer as compared to controls. These findings indicate a role of free radicals in its pathogenesis.

Similar findings were reported by **Kaur et al., 2016,** who analyzed oxidative DNA and lipid damage using salivary 8-OHdG, MDA, and vitamins C and E in OLP, oral leukoplakia, OSMF, OSCC and controls. Significantly higher levels of salivary 8-OHdG and MDA and lower levels of vitamins C and E were found in OSCC and precancer patients compared to healthy controls. Authors concluded that instead of individual biomarker approach, a combination of 8-OHdG, MDA, vitamin C, and vitamin E had a high specificity and sensitivity for the diagnosis of oral pre-cancer and OSCC.

Shivashankara and Kavya, 2011 also found that changes correlated with progression of cancer as evident by more pronounced changes in WDSCC, compared to MDSCC and premalignant lesions. Oxidative stress is involved in etiopathology of oral cancer, as evident from elevated MDA and decreased glutathione. Authors concluded that the salivary parameters could be of use in diagnosis and prognosis of oral cancer.

Vlkova et al., 2012 compared salivary markers of oxidative and carbonyl stress such as TBARS, AOPP, advanced glycation end products (AGEs) and TAC in 16 patients with oral premalignant lesions (leukoplakia, lichen planus, erythroplakia)

and age-matched healthy controls. Significantly higher salivary TBARS and AGEs and lower TAC and expression of SOD were found in patients than in controls. No differences were found in AOPP. Markers of lipoperoxidation and carbonyl stress were increased in patients with oral premalignant lesions. Authors concluded that decreased antioxidant status potentially due to decreased expression of antioxidant enzymes might be responsible for these findings.

Similar study conducted by **Agha-Hosseini et al., 2012** in the salivary MDA, TAC & 8-OHdG of OLP, OSCC and controls revealed no significant differences in TAC and MDA levels between OLP and control, and also between OLP and OSCC patients. MDA and 8-OHdG were significantly higher but TAC was lower in OSCC patients than control.

Shetty et al., 2014 evaluated salivary MDA in 65 healthy controls, 115 potentially malignant disorders (PMD) and 50 OSCC patients. A consistent elevation in the levels of salivary MDA was observed in controls with tobacco related habits, PMD and in OSCC. The authors have concluded that salivary MDA analysis can be used as an efficient, noninvasive tool for the early diagnosis of PMD and OSCC for planning comprehensive treatment protocol.

Shankarram et al., 2015 assessed oxidative stress status by measuring TAC, GPx, SOD, 8-OHdG and MDA in saliva of periodontitis, oral cancer and healthy controls. Highly significant elevation of all oxidative stress marker levels except for that of SOD over healthy group. Oral cancer showed increased levels.

STUDIES ANALYSING OXIDATIVE STRESS AND/OR ANTIOXIDANT STATUS IN BOTH BLOOD AND SALIVA OF PRECANCEROUS AND OSCC PATIENTS

Ergun et al., 2011 assessed the oxidative stress (MDA) and antioxidant profile (TAA) in 21 recently diagnosed OLP and healthy controls using serum and salivary samples. Lower serum TAA and higher salivary MDA was seen in OLP. A significant correlation was found between serum and saliva TAA in patients with OLP and in the control group. Significant correlation was also found between serum and saliva MDA values in control group. A significant inverse correlation was found between salivary MDA and TAA values in the control group.

Ganesan and Kumar, 2014 assessed the varying levels of lipid peroxides (MDA) in saliva, serum and tissue in 10 oral pre cancer and 20 OSCC and also various forms of tobacco usage with sex as an added parameter. Significantly elevated levels of MDA were seen in saliva, serum and tissue in OSCC followed by oral leukoplakia when compared to controls. The results clearly indicate the increase in lipid peroxidation in oral pre cancer and oral cancer with no significant difference between gender groups. Authors correlated the increase in salivary concentration of MDA with increase in serum. These findings reinforce the concept that saliva is available, easy to obtain and relatively risk free diagnostic biofluid for assessing biomarkers.

Metgud and Bajaj, 2014 also found enhanced MDA levels & decreased GSH levels in saliva and serum of oral leukoplakia and OSCC patients as compared to controls, indicating that tumor processes cause an imbalance of oxidant-antioxidant status in cell structures.

Rasool et al., 2014 estimated lipid peroxidation and antioxidant status in OSCC patients & compared the sensitivity and specificity of circulating biomarkers (MDA, Sialic acid, CAT, SOD, GSH and Neuraminidase) with β-2 microglobulin (β-2MG) at different thresholds in blood and saliva using receiver operating characteristics (ROC) curve design. MDA and Sialic acid were significantly increased in plasma of OSCC patients as compared to controls whereas antioxidant level was significantly decreased. ROC analysis indicated that MDA in saliva is a better diagnostic tool as compared to MDA in blood and β-2MG in blood is better diagnostic marker as compared to β-2MG level in saliva.

SOURCE OF DATA

Data was collected from patients visiting the cancer centres in Trichy, Thanjavur and Coimbatore. Informed consent (Annexure I) was obtained from all the patients before collection of samples. The study was performed after obtaining approval from the institutional ethical committee.

METHOD OF COLLECTION OF DATA

The sample for the present study comprised a total of 50 patients of both sexes with an age distribution between 38 and 75 years and were divided into two groups of 25 patients each.

Study group: Patients who were histopathologically diagnosed with OSCC (n = 25)

Control group: Normal healthy individuals with clinically normal oral mucosa (n = 25)

INCLUSION CRITERIA

Histopathologically diagnosed new cases of OSCC were included in the study group.

EXCLUSION CRITERIA

- Patients undergoing treatment such as chemotherapy and radiotherapy.
- Patients with any other systemic disorders.
- Patients under antioxidants.

METHODOLOGY

After obtaining an informed consent (Annexure-I), patients from both the groups were subjected to thorough clinical examination. Each patient's complete medical, dental history and clinical photographs were recorded. Following procedures were done in each patient of both the groups.

1. Collection of blood
2. Collection of saliva

MATERIALS REQUIRED

- Disposable syringe
- Heparinised tubes for collecting blood
- Clot activator tubes
- Plain test tubes
- Sterile container for saliva
- Cooling centrifuge
- Micropipettes with plastic disposable pipette tips
- Water bath
- Eppendorf tubes
- Cuvettes
- Ransel glutathione peroxidase enzyme kit
- Reagents for malondialdehyde estimation
- Distilled water
- Beaker
- Stirrer
- Measuring cylinder

- Test tube stand
- Spectrophotometer
- Auto analyzer

SAMPLE COLLECTION – BLOOD

- ✓ Under aseptic precautions, 5ml of venous blood was collected from antecubital vein of all the subjects using sterile disposable syringe.
- ✓ Haemolysed and lipemic samples were avoided.

PROCESSING OF BLOOD SAMPLES

- ✓ 2 ml of whole blood was transferred to a heparinized tube and the rest 3ml was transferred to a clot activator tube.
- ✓ Serum separated by centrifugation (3000 rpm for 15 min) of clot activator tubes was stored at -80^0C until analysis, to estimate MDA by thiobarbituric acid method by using spectrophotometer.
- ✓ For GPx estimation, dilute 1 volume of the hemoglobin reagent with 4 volumes of redistilled water.
- ✓ Dilute 0.05 ml heparinized whole blood with 1 ml diluting agent (R3) provided in the antioxidant enzyme kit; incubate for 5 minutes and add 1 ml of Hemoglobin reagent.
- ✓ Samples were then centrifuged in a cooling centrifuge and the supernatant was stored at -80^0C until analysis of GPx by Ransel antioxidant enzyme kit provided by RANDOX Laboratories Ltd (Antrim, United Kingdom) and samples were processed on Rx Monza automated analyzer.

SAMPLE COLLECTION - SALIVA

5ml of unstimulated salivary sample were collected from each subject between 8-11 am to avoid circadian variations.

Patients were given detailed information about the collection protocol:

- ✓ Refrain from eating or drinking atleast 90 minutes prior to salivary collection.
- ✓ Rinse mouth with distilled water prior to collection of sample.
- ✓ To sit in a comfortable position with eyes open and head tilted slightly forward.
- ✓ Avoid swallowing and oral movements during collection
- ✓ To pool the saliva in the floor of the mouth and to drain passively for 10 minutes over the lower lip into a sterile plastic container. This was done until 5 ml of saliva was obtained.

PROCESSING OF SALIVA SAMPLES

Saliva samples were immediately centrifuged (1000 g, 10 minutes) at 4°C to remove cell debris. The resulting supernatants were immediately transferred to 2 separate aliquots:

1st group of aliquots were used for estimating MDA.

2nd group of aliquots were used for estimating GPx.

The resulting supernatants were immediately deep-frozen at –80°C and stored for later analysis. GPx was assayed using the Ransel antioxidant enzyme kit provided by RANDOX Laboratories Ltd (Antrim, United Kingdom) and samples were processed on Rx Monza automated analyzer. MDA was estimated using thiobarbituric acid method by using spectrophotometer.

ESTIMATION OF GLUTATHIONE PEROXIDASE

This method is based on that of Paglia and Valentine, 1967. GPx catalyses the oxidation of GSH by Cumene Hydroperoxide. In the presence of GR and NADPH the oxidised Glutathione (GSSG) is immediately converted to the reduced form with a concomitant oxidation of NADPH to $NADP^+$. The decrease in absorbance at 340 nm is measured.

Reaction principle:

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

$$GSSG + NADPH + H+ \xrightarrow{GR} NADP^+ + 2GSH$$

Reagent components and concentration:

Contents		Concentration
R 1a.	**Reagent** Glutathione	4 mmol/l
	Glutathione reductase	≥ 0.5 U/l
	NADPH	0.34 mmol/l
R 1b.	**Buffer** Phosphate buffer	0.05mol/l; pH 7.2
	EDTA	4.3 mmol/l
R2	**Cumene hydroperoxide**	0.18 mmol/l
R3	**Diluting agent**	

Reagents preparation:

- One vial of reagent R 1a was reconstituted with 6.5 ml of buffer R 1b.
- 10 microlitre R2 was diluted with 10 ml of saline and mixed thoroughly by shaking vigorously.

- The contents of one vial of diluting agent R3 was reconstituted with 200 ml of redistilled water.

Procedure:

- ✓ Select GPx in the Run Test screen and carry out a water blank as instructed.
- ✓ Pipette into a test tube:

Sample	10 µl
Reagent R1	500 µl
Cumene R2	20 µl

Mix and aspirate into the analyser. The values were expressed in U/L.

ESTIMATION OF MALONDIALDEHYDE

MDA can be measured as Thiobarbituric Acid Reactive Substances (TBARS). First used in 1978, the measure of TBARS is still a commonly used and convenient method of determining the relative lipid peroxide content of biological samples sets. Lipids are both most likely to form peroxides and the most reactive in the TBARS assay.

Principle:

Free MDA is typically quite low, requiring release of MDA by acid treatment of proteins present in the biological samples and breakdown of peroxides by heat and acid to produce a MDA-TBA adduct (figure 6) that absorbs light at 530-540 nm. The intensity of the color at 535 nm corresponds to the level of lipid peroxidation in the

sample. Removal of protein by precipitation eliminates potentially interfering amino acids that may react with Thiobarbituric acid (TBA).

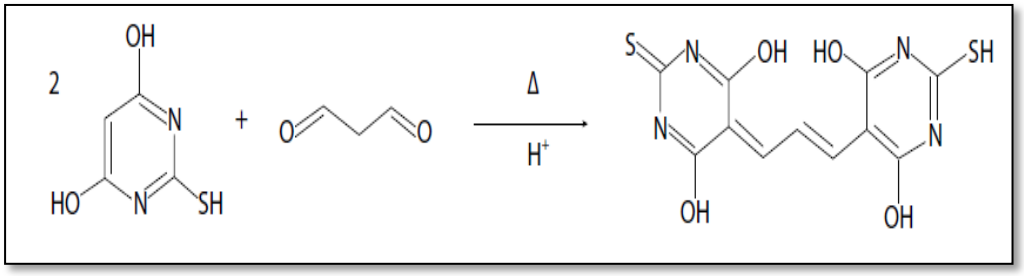


Figure 7: Reaction between MDA and TBA to form the MDA-TBA adduct (Source: Google).

Reagents:

- N/12 sulphuric acid (0.083N) -------> 0.23 ml of 36 N sulphuric acid / 100 ml of distilled water.
- 10% / 10g of Trichloroacetic acid (TCA) in 100 ml of distilled water.
- Thiobarbituric acid (TBA)

  A mixture of equal volume of 0.67% of TBA aqueous (670 mg/50 ml of distilled water).
- Butanol (n-butanol) used for extraction and blank.

Procedure:

- ✓ Around 0.25 ml serum was mixed thoroughly with 0.25 ml of 10 % TCA and mixture was allowed to stand for 5 minutes at room temperature.
- ✓ This was further centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded.
- ✓ The precipitate collected was washed twice with dilute sulfuric acid.
- ✓ About 1 ml of N/12 dilute sulfuric acid was added to the tube containing precipitate and was mixed well with 0.15 ml of TCA reagent.

- ✓ This was further centrifuged at 3000 rpm for 10 minutes and the supernatant was discarded.
- ✓ Mix with 2 ml of distilled water and 1 ml of TBA reagent (TBAR).
- ✓ Reaction mixture was heated in boiling water bath at 95^{o}C for 1 hour and allowed to cool with tap water.
- ✓ Then 3.5 ml of n-butyl alcohol was added and centrifuged at 3000 rpm for 15 minutes.
- ✓ Butanol layer was taken from tube and absorbance of butanol layer was measured at 530 nm by spectrophotometer against blank using distilled water.
- ✓ Same procedure was followed for MDA analysis in saliva.

Calculation:

Serum/salivary lipid peroxides in terms of MDA = $\frac{A \times 3.5 \times 1000 \times 10^3}{1.56 \times 10^5 \times 1000 \times .25}$

= $89.74 \times A$ μmol/l, where A = absorbance.

STATISTICAL METHODS

All the parameters were tabulated for statistical significance using Statistical Package for Social Science (SPSS) software. The differences in the levels of MDA and GPx in OSCC and control group were statistically analyzed using T test, followed by Pearson correlation coefficient test to assess the association between the parameters in blood and saliva and Receivers operating characteristic (ROC) curve analysis to determine sensitivity and specificity.

ARMAMENTARIUM

Figure 8: Materials used for sample collection and analysis.

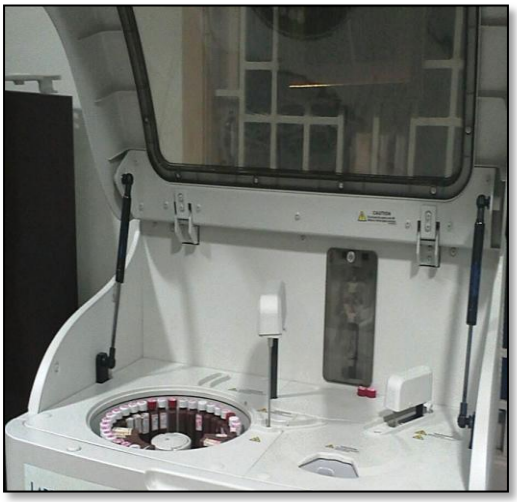


Figure 9: Autoanalyzer

Figure 10: UV visible spectrophotometer

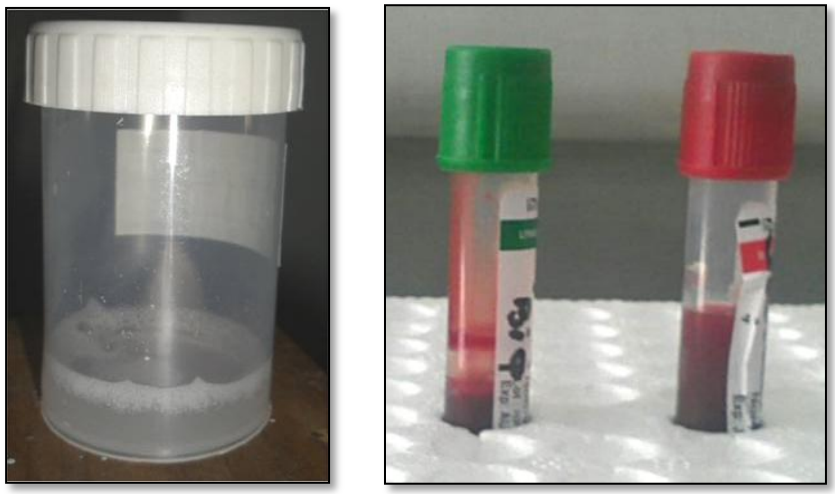


Figure 11: Saliva and blood samples

Figure 12: Placement of tubes for centrifuging.

Figure 13: Photograph showing Glutathione peroxidase enzyme estimation kit.

Figure 14: Placement of reagents and samples for Glutathione peroxidase estimation in analyzer.

Figure 15: Photograph showing reagents used for Malondialdehyde estimation.

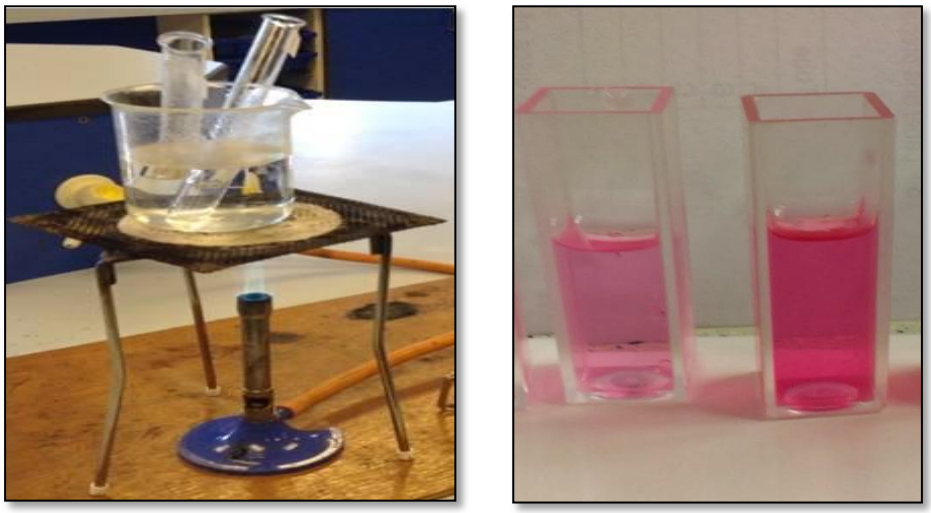


Figure 16: Mixture was heated in a boiling water bath and a pink coloured complex was formed.

Table - 1

DESCRIPTIVE STATISTICS FOR MDA LEVELS IN BLOOD OF BOTH STUDY AND CONTROL GROUPS

GROUP	N	MEAN (µmol/L)	STANDARD DEVIATION	MINIMUM (µmol/L)	MAXIMUM (µmol/L)
CASES	25	13.3292	1.58981	11.03	15.97
CONTROLS	25	3.2896	0.91864	2.30	4.74

µmol/L = micromoles / litre

The mean value of MDA levels in blood of OSCC patients and in control group were 13.3292 µmol/L and 3.2896 µmol/L respectively.

Table - 2

COMPARISON OF MDA LEVELS IN BLOOD OF STUDY GROUP WITH CONTROL GROUP

Comparison groups	't' value	P value
Controls and Cases	-27.339	0.000

T test was used for the comparison of mean blood levels of MDA between the control and the study groups and the difference was found to be highly significant ($p < 0.01$).

Table -3

DESCRIPTIVE STATISTICS FOR MDA LEVELS IN SALIVA BOTH STUDY AND CONTROL GROUPS

GROUP	N	MEAN (µmol/L)	STANDARD DEVIATION	MINIMUM (µmol/L)	MAXIMUM (µmol/L)
CASES	25	9.2892	0.76631	8.27	10.67
CONTROLS	25	2.7244	0.95610	1.55	4.53

µmol/L = micromoles / litre

The mean value of MDA levels in saliva of OSCC patients and in control group were 9. 2892 µmol/L and 2.7244 µmol/L respectively.

Table - 4

COMPARISON OF MDA LEVELS IN SALIVA OF STUDY GROUP WITH CONTROL GROUP

Comparison groups	't' value	P value
Controls and Cases	-26.789	0.000

T test was used for the comparison of mean salivary levels of MDA between the control and the study groups and the difference was found to be highly significant ($p < 0.01$).

Table - 5

DESCRIPTIVE STATISTICS FOR GPX LEVELS IN BLOOD OF BOTH STUDY AND CONTROL GROUPS

GROUP	N	MEAN (U/L)	STANDARD DEVIATION	MINIMUM (U/L)	MAXIMUM (U/L)
CASES	25	121.2660	21.28547	92	152
CONTROLS	25	219.2948	28.88628	171.53	260

U/L = units per litre

The mean value of GPx levels in blood of OSCC patients and in control group were 121. 2660 U/L and 219.2948 U/L respectively.

Table - 6

COMPARISON OF GPX LEVELS IN BLOOD OF STUDY GROUP WITH CONTROL GROUP

Comparison groups	't' value	P value
Controls and Cases	13.660	0.000

T test was used for the comparison of mean blood levels of GPx between the control and the study groups and the difference was found to be highly significant ($p < 0.01$).

Table -7

DESCRIPTIVE STATISTICS FOR GPX LEVELS IN SALIVA BOTH STUDY AND CONTROL GROUPS

GROUP	N	MEAN (U/L)	STANDARD DEVIATION	MINIMUM (U/L)	MAXIMUM (U/L)
CASES	25	74.0104	19.76245	47.07	102.90
CONTROLS	25	140.0960	17.57459	116.40	170

U/L = units per litre

The mean value of GPx levels in saliva of OSCC patients and in control group were 74.0104 U/L and 140. 0960 U/L respectively.

Table - 8

COMPARISON OF GPX LEVELS IN SALIVA OF STUDY GROUP WITH CONTROL GROUP

Comparison groups	't' value	P value
Controls and Cases	12.494	0 .000

T test was used for the comparison of mean salivary levels of GPx between the control and the study groups and the difference was found to be highly significant ($p < 0.01$).

Table 9

CORRELATION OF GPX AND MDA IN BLOOD AND SALIVA OF OSCC GROUP

PARAMETERS	PEARSON'S CORRELATION Value (r)	p value
Blood and salivary GPx	0.757	0.000
Blood and salivary MDA	0.940	0.000
Blood GPx and MDA	-0.840	0.000
Salivary GPx and MDA	-0.845	0.000

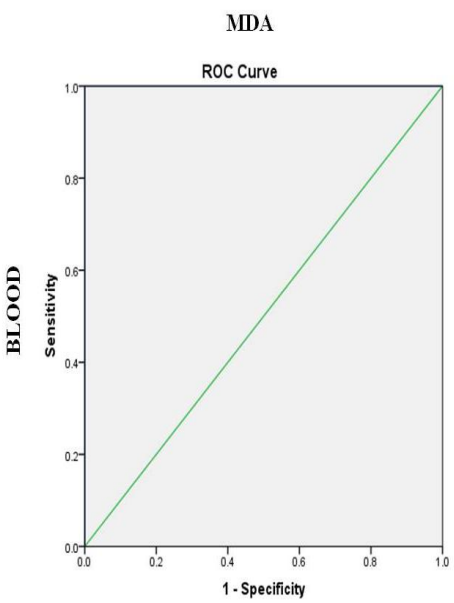
Pearson's correlation coefficient test was done to correlate MDA and GPx in blood and saliva of OSCC subjects.

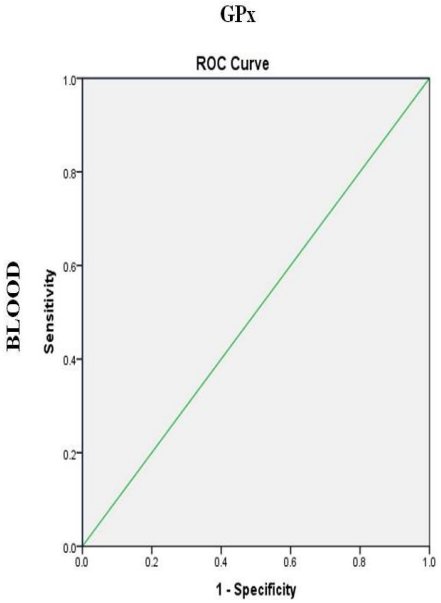
- ✓ Blood and salivary GPx levels showed a strong positive correlation and p value was found to be statistically highly significant ($p < 0.01$).
- ✓ Blood and salivary MDA levels showed a strong positive correlation and p value was found to be statistically highly significant ($p < 0.01$).
- ✓ GPx and MDA levels in blood showed a strong negative correlation and p value was found to be statistically highly significant ($p < 0.01$).
- ✓ GPx and MDA levels in saliva showed a strong negative correlation and p value was found to be statistically highly significant ($p < 0.01$).

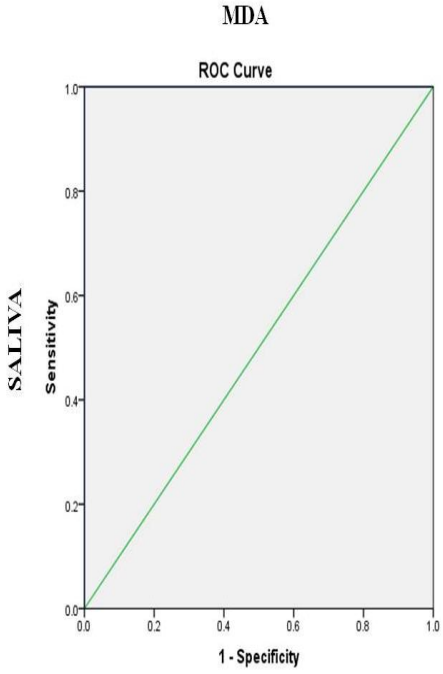
Table-10

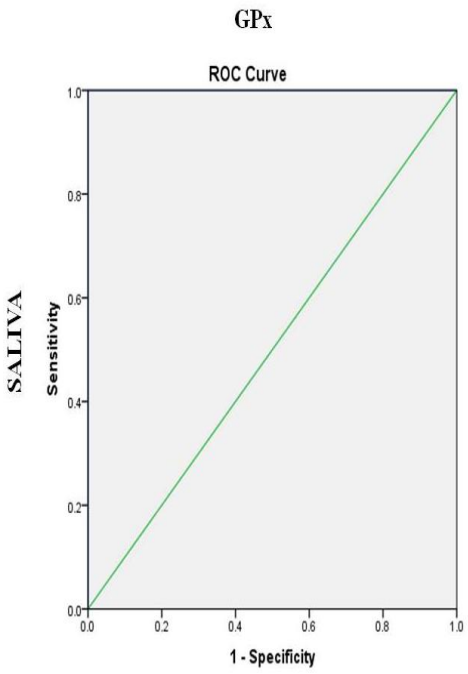
SENSITIVITY AND SPECIFICITY OF MDA AND GPx IN BLOOD AND SALIVA OF THE CONTROL AND PATIENTS OF OSCC

Receivers operating characteristic (ROC) curves were constructed at different thresholds to evaluate discriminatory efficacy of the parameters between patients and controls in blood and saliva separately.









The area under curve (AUC) computed in saliva and blood for GPx and MDA was 1.00, statistically significant ($P < 0.01$).The optimum threshold values of MDA level in blood and saliva obtained were ≥ 7.88 μmol/L and ≥ 6.40 μmol/L respectively. The optimum threshold values of GPx level in blood and saliva obtained were ≤ 161.76 U/L and ≤ 109.65 U/L respectively. The sensitivity of MDA and GPx obtained was 100 % in saliva and in blood. The specificity record of MDA and GPx levels obtained were 100% in saliva and in blood.

Cancer is an event occurring at the genetic level and DNA damage is the final step resulting in carcinogenesis. Viruses, chemicals, irradiation and the genetic makeup of the individual are the multiple factors that play a role in carcinogenesis. The two important agents of DNA damage are ROS and RNS (**Beevi et al, 2004)**. However, they are effectively neutralized by highly powerful cellular antioxidant enzymes. Oxidative stress results when the balance is lost between ROS production and antioxidant defense, leading to oxidative damage of the cellular macromolecules (**Srivastava et al, 2012).**

Disruption of this delicate oxidant/antioxidant balance in the body seems to play a causative role in carcinogenesis. With increasing evidences, the role of oxidative stress is found in several human pathological conditions such as gastrointestinal ulcerogenesis, rheumatoid arthritis, ischemic heart disease, several autoimmune disorders, metabolic disorders, neurodegenerative disease and cancer. ROS and RNS were found to be involved in all the three stages of multistep carcinogenesis and can result in DNA damage, activate procarcinogens, initiate lipid peroxidation, inactivate enzyme systems and alter the cellular antioxidant defense system (**Srivastava et al, 2012)**.

Malondialdehyde (MDA) is a major reactive aldehyde resulting from the peroxidation of biological membrane PUFA. MDA is mutagenic, genotoxic agent and potential carcinogen in mammalian system, which readily reacts with deoxy nucleosides to produce adducts causing DNA damage. So, lipid peroxidation has gained importance because of its involvement in various diseases including cancer (**Metgud and Bajaj, 2014)**

Glutathione peroxidase (GPx) is one of the important enzymatic antioxidant involved in the protection of cells against peroxidation. It catalyses the degradation of H_2O_2 and O_2. Most of its activity was found in blood within the erythrocytes and only 1 to 2% in plasma **(Sachdeo and Mody, 2011)**.

Involvement of oxidative stress have been reported in oral conditions like lichen planus, recurrent aphthous ulcer and periodontitis. Recent studies have also shown the association between oxidative stress with potentially malignant disorders like OSMF and leukoplakia. Oral cancer has been a major concern worldwide, as it accounts for the sixth most common malignancy in the world (**Srivastava et al, 2012).**

The present study was carried out in 25 histopathologically diagnosed patients of OSCC who have not undergone any treatment and 25 normal healthy individuals. Saliva and blood samples were collected from all the subjects of both the groups. The samples were centrifuged and analysed for the following parameters:

- Malondialdehyde
- Glutathione peroxidase

MDA level was evaluated by thiobarbituric acid method using spectrophotometry and GPx was evaluated using Ransel antioxidant enzyme kit.

In the present study, all individuals in the study group were in the age range of 38-75 years. Gender distribution was 18(72%) males and 7(28%) females. Most commonly affected site was buccal mucosa (10/25cases) followed by tongue (6/25 cases), palate (2/25), retromolar trigone (2/25) and 1 case each in angle of the mouth (1/25), labial mucosa (1/25), lower lip and buccal mucosa (1/25), lower lip and angle of the mouth (1/25), buccal mucosa and angle of the mouth (1/25). 14(56%) cases

were affected with MDSCC, 9(36%) with WDSCC and 2(8%) with PDSCC. The results were discussed under the following headings:

- MDA levels in blood of OSCC subjects
- MDA levels in saliva of OSCC subjects
- GPx levels in blood of OSCC subjects
- GPx levels in saliva of OSCC subjects
- MDA levels in both blood and saliva of OSCC subjects
- GPx levels in both blood and saliva of OSCC subjects
- Correlation of blood and salivary MDA and GPx levels in OSCC subjects.
- Sensitivity and specificity of MDA and GPx levels in blood and saliva of OSCC subjects

I. MDA LEVELS IN BLOOD OF OSCC SUBJECTS

In the present study, the mean MDA levels in blood of healthy controls and OSCC patients were 3.2896 μmol/L and 13.3292 μmol/L respectively. This difference between the control group and study group was found to be statistically highly significant ($p<0.01$).

Our findings correlated with several studies that have found significantly increased levels of MDA in oral cancer groups with differing clinical stages **(Beevi et al., 2004, Manoharan et al., 2005, Sharma et al., 2009, Gokul et al., 2010, Srivasatva et al., 2012, Shilpashree et al., 2013, Huo et al., 2014). Sabitha and Shyamaladevi, 1999** observed a significant increase ($p < 0.01$) in MDA levels before

initiating radiotherapy and a highly significant increase ($p < 0.001$) after radiotherapy in OSCC subjects.

Our findings were in agreement with that of studies by **Chole et al., 2010, Korde et al., 2011, Thomas and Sethupathy, 2015, Rai et al., 2015, Misra et al., 2016** and **Nyamati et al., 2016** in healthy controls, oral precancer and OSCC patients. They observed a statistically significant increase in serum MDA levels in oral precancer and oral cancer patients compared to control group. **Chole et al., 2010** concluded that oxidative stress is more intense in oral cancer and precancer and suggested antioxidant therapy as an adjunct in the treatment of oral precancer and cancer.

Khan and Malik et al., 2017 found highly significant MDA levels ($p<0.05$) and 64.85 percent fold increase of MDA in OSCC patients compared to controls.

The increase in MDA levels in blood as shown in this study might be due to the decomposition products of PUFA, a major component in the cell membranes of erythrocytes and other cells. PUFA is considered highly susceptible to oxidative attack (**Srivasatva et al., 2012**). Erythrocytes are exposed continuously to oxidative stress. Although the reducing capacity of the normal erythrocytes is greater than its oxidising potential, lack of antioxidant defense leads to an increase in membrane lipid peroxidation (**Sachdeo and Mody, 2011).** Oxidative attack results in alterations in respect to membrane's fluidity and permeability with consequent leakage into the plasma. Thus, large volumes of MDA may be due to the leakage into the plasma or inadequate clearance of free radicals by the cellular antioxidants (**Srivasatva et al., 2012**). Owing to its high cytotoxic properties, MDA modulate cell growth by activating signal transduction pathways, therefore acting as tumor promoters and co-carcinogenic agents (**Metgud and Bajaj, 2014).**

II. MDA LEVELS IN SALIVA OF OSCC SUBJECTS

In the present study, mean MDA levels in saliva of healthy controls and OSCC patients were 2.7244 μmol/L and 9. 2892 μmol/L respectively. This difference between the control group and study group was found to be highly significant ($p<0.01$).

Our findings of elevated MDA levels in saliva of OSCC patients were in accordance with **Rai et al., 2006**, **Shivashankara & Kavya, 2011, Agha-Hosseini et al , 2012, Shetty et al., 2014** and **Kaur et al., 2015** in their study of comparison between healthy individuals, oral precancer and OSCC.

Warnakulasuriya et al., 2008 in a study on Japanese patients with oral leukoplakia observed positive staining of MDA adducts in the dysplastic epithelial cells. Therefore these tissue-bound MDA adducts might be the source of the salivary MDA **(Vlkova et al, 2012).**

III. GPx LEVELS IN BLOOD OF OSCC SUBJECTS

In the present study, mean GPx levels in blood of healthy controls and OSCC patients were 219.2948 U/L and 121.2660 U/L respectively. This decrease from normal to OSCC was highly significant ($p<0.01$).

Our findings were in agreement with several studies that have found significantly decreased levels of GPx in oral cancer groups with differing clinical stages **(Beevi et al., 2004, Manoharan et al., 2005, Sharma et al., 2009, Srivastava et al., 2012 and Shilpashree et al., 2013). Sabitha and Shyamaladevi, 1999, Sachdeo and Mody**, **2011** observed a significant decrease ($p < 0.01$) in GPx levels before initiating radiotherapy and a highly significant decrease ($p < 0.001$) after radiotherapy in OSCC subjects.

Gurudath et al., 2012, Thomas and Sethupathy et al., 2015 and **Nyamati et al., 2016** found the mean GPx level gradually decreased from healthy individuals to potentially malignant and OSCC patients and the result was statistically highly significant ($P < 0.001$).

Decrease in GPx levels might occur as the consequence of overwhelming free radicals by the elevated levels of lipid peroxides. Low levels of GPx suggested that most cancer cell types couldn't detoxify hydrogen peroxide (**Gurudath et al., 2012)**. Decrease in antioxidant enzymes may also be due to increased scavenging by lipid peroxides as well as sequestration by tumor cells to meet the demands of a growing tumor **(Shilpashree et al., 2013).**

Insufficient power of a depleted antioxidant defense system for a prolonged time might also result in enhanced lipid peroxidation. According to Blunt and Fridovich, GPx may be inactivated by superoxide anions during oxidative stress conditions and toxic ligands such as MDA could partially inhibit GPx inactivity. Disturbed antioxidant enzymes status might also be due to the deprivation of trace elements such as Copper, Manganese, Zinc and Selenium. With reduced GPx activity, detoxification of H_2O_2 to H_20 remains incomplete (**Sharma et al., 2009)**. The reduced level of selenium in the serum might also be a reason for the decrease in GPx activity, as its activity partially depends upon the selenium concentration. According to Etlemble et al, 1979 the efficiency of this enzyme might be impaired due to the circulating inhibitor of this enzyme produced by the tumor itself (**Sachdeo and Mody**, **2011)**.

Bagul and Ganjre et al., 2013 found statistically significant ($p<0.05$) increase in the levels of GPx in OSCC patients as compared with control subjects. Our finding was not in accordance with that of **Bagul and Ganjre et al., 2013,** who suggested that

the increased GPx levels might be due to the high oxidative stress and lipid peroxidation in the initial stages of OSCC and increased level of free radicals in their body. As a compensatory mechanism body tries to increase the levels of antioxidants to counteract carcinogenesis. **Khan and Malik et al., 2017** found greatly increased levels of GPx (75.57 times) in OSCC patients compared with control group. Our finding was not in accordance with that of **Khan and Malik et al., 2017**, who proposed that in the presence of SOD and CAT deficiency in OSCC patients, GPx comes into action so as to combat oxidative stress and scavenge hydrogen peroxide to less toxic molecules. In addition, enhanced activity of GPx converts the available GSH to oxidized form leaving behind insufficient active GSH levels.

IV. GPx LEVELS IN SALIVA OF OSCC SUBJECTS

In the present study, the mean levels of GPx in saliva of OSCC patients and in control group were 74.0104 U/L and 140.0960 U/L respectively.This decrease from normal to OSCC was highly significant ($p<0.01$).

Using the keywords: Glutathione peroxidase, saliva, oral cancer, Oral squamous cell carcinoma in Google and PubMed search, only one study was available that estimated the levels of Glutathione peroxidase in saliva of periodontitis, oral cancer patients and healthy controls using ELISA kits (**Shankarram et al., 2015)**.

Shankarram et al., 2015 in the study of periodontitis, oral cancer and healthy controls assessed GPx, TAC, SOD, 8-OHdG and MDA by ELISA kits and found highly significant elevation of all oxidative stress marker levels in saliva except for that of SOD over healthy group. Our finding of decreased salivary GPx levels in

OSCC was in disagreement with that of **Shankarram et al., 2015.** This difference in finding might be due to:

- Inclusion/exclusion criteria has not been mentioned. In our study, only histopathologically diagnosed new cases of OSCC were included. Patients already under chemotherapy/radiotherapy, any other systemic disorders and patients under antioxidants were excluded.
- Different methodology
- Number of patients with different histopathological grades of OSCC

V. MDA LEVELS IN BOTH BLOOD AND SALIVA OF OSCC SUBJECTS

Several studies have taken serum samples as a tool for measuring oxidative stress and antioxidant status, and only a few studies have taken saliva samples. Only limited studies exist to compare the levels of MDA in OSCC patients of both saliva and serum simultaneously (**Ganesan and Kumar, 2014, Metgud and Bajaj, 2014** and **Rasool et al., 2014).**

Studies by **Ganesan and Kumar, 2014, Metgud and Bajaj, 2014** and **Rasool et al., 2014** reported significantly elevated levels of MDA ($p<0.001$) in saliva and serum of OSCC patients when compared to control group.

The increased MDA levels (in saliva and serum) were not only due to tobacco consumption but also as a result of the magnitude of oxidative stress. This is in favour of the hypothesis that altered ROS metabolism in cancer cells leads to production of large amounts of ROS as compared to non-neoplastic cells and the suppression of the antioxidant system that mediate the defense mechanisms in the body (**Metgud and Bajaj, 2014)**.

VI. GPx LEVELS IN BOTH BLOOD AND SALIVA OF OSCC SUBJECTS

Using the keywords: Glutathione peroxidase, saliva, blood, serum, plasma, oral squamous cell carcinoma, oral cancer in Google and PubMed search, yielded no results. As no other published study was available to compare the levels of GPx in OSCC patients of both saliva and serum simultaneously, the statistical significance of our findings could not be compared.

VII. CORRELATION OF BLOOD AND SALIVARY MDA AND GPx LEVELS IN OSCC SUBJECTS

In the present study, correlation between blood and salivary GPx levels was done and the results showed highly significant strong positive correlation. None of the study had correlated between blood and salivary GPx in OSCC. Hence the significance of our results cannot be compared or correlated.

A highly significant strong positive correlation between serum and salivary MDA levels was found in the present study. **Ganesan and Kumar, 2014** observed an increase in salivary concentration of MDA with increase in serum. But statistical tools were not used to justify the observation.

The positive correlation seen between the blood and salivary levels of GPx and MDA might be due to passage of molecules from the blood into the saliva by passing through the spaces between cells by transcellular (passive intracellular diffusion and active transport) or paracellular routes (extracellular ultrafiltration) **(Lee and Wong, 2009).** Hence the increase in MDA levels and decrease in GPx levels in circulation was also reflected in saliva.

In the present study, blood levels of MDA and GPx showed a highly significant strong negative correlation. This finding was consistent with the study of **Sharma et al., 2009.** A moderate negative correlation was observed by **Srivastava et al., 2012** but it was not statistically significant. Our findings were not in line with **Khan and Malik et al., 2017,** who observed a weak positive correlation between GPx and MDA.

Similarly, salivary levels of MDA and GPx also showed a highly significant strong negative correlation. None of the study had correlated salivary GPx and MDA in OSCC, so significance of our results cannot be compared or correlated.

The compensatory mechanism to counterbalance the effects of ROS results in reduced levels or activities of antioxidants and this could be the possible explanation for the negative correlation between MDA and GPx levels.

VIII. SENSITIVITY AND SPECIFICITY OF MDA AND GPx IN BLOOD AND SALIVA OF OSCC

In order to check whether MDA and GPx levels in blood and saliva could discriminate between OSCC and healthy controls, a complete statistical Receiver Operating Characteristic (ROC) curve analysis was performed. The area under curve (AUC) computed in saliva and blood for GPx and MDA was 1.00, statistically significant ($P < 0.01$).The optimum threshold values of MDA level in blood and saliva obtained were ≥ 7.88 μmol/L and ≥ 6.40 μmol/L respectively. The optimum threshold values of GPx level in blood and saliva obtained were ≤ 161.76 U/L and ≤ 109.65 U/L respectively. The sensitivity of MDA and GPx obtained was 100 % in saliva and in blood. The specificity record of MDA and GPx levels obtained were 100% in saliva

and in blood. Hence, it meant that MDA and GPx levels in saliva and blood were equally reliable diagnostic tests.

Rasool et al., 2014 observed a high sensitivity (86.67 %) in saliva and low sensitivity (73.33%) in blood. The specificity was found to be 100% in saliva and 90% in blood and hence MDA in saliva was considered as a better diagnostic test than MDA in blood. Findings with regard to MDA were partly in agreement to that of **Rasool et al, 2014.** None of the study had assessed the sensitivity and specificity for blood and salivary GPx in OSCC, so significance of our results cannot be compared and needs further research to conclude our findings.

There were certain limitations in the present study- smaller sample size**,** cases and controls were not age/sex matched and other antioxidant enzymes were not estimated. A major issue was the analytical method. As the results seem to vary extremely between laboratories, correct reporting on the used methods in the articles is more important for the reproducibility and comparability of results.

Although the present study showed significant results, the scope for further research remains open, as there is a paucity of similar literature for comparisons at present. The results should be viewed as a pilot for extending studies with larger sample sizes, with various clinical stages and histopathological grades of OSCC for more accurate results. Only then the markers can be used for prediction of malignancy, for early detection and prevention of cancer and preventive measures in a clinical setting.

The present study was conducted in 25 newly diagnosed histopathologically confirmed cases of OSCC and 25 healthy controls. Blood and saliva samples were collected from all the subjects in study and control group and analysed for MDA and GPx. The values were tabulated and the results were analyzed using T test, Pearson correlation coefficient test and ROC analysis.

The analysis showed the following results:

- ✓ A highly significant difference was observed between the mean blood levels of MDA in healthy controls and OSCC patients and were found to be raised in OSCC with $p < 0.01$.
- ✓ A highly significant difference was observed between the mean salivary levels of MDA in healthy controls and OSCC patients and were found to be raised in OSCC with $p < 0.01$.
- ✓ A highly significant difference was observed between the mean blood levels of GPx in healthy controls and OSCC and were found to be decreased in the OSCC with $p< 0.01$.
- ✓ A highly significant difference was observed between the mean salivary levels of salivary GPx in healthy controls and OSCC, and were found to be decreased in the OSCC with $p< 0.01$
- ✓ A highly significant strong positive correlation was observed between blood and salivary GPx levels in OSCC subjects.
- ✓ A highly significant strong positive correlation was observed between blood and salivary MDA levels in OSCC subjects.
- ✓ A highly significant strong negative correlation was observed between GPx and MDA levels in blood in OSCC subjects.

- ✓ A highly significant strong negative correlation was observed between GPx and MDA levels in saliva in OSCC subjects.
- ✓ The sensitivity and specificity of MDA and GPx were found to be 100 %, both in blood and saliva.

Our findings of increased MDA and decreased GPx levels with a strong negative correlation proved that the antioxidants are depleted during the course of neutralizing ROS in OSCC patients. Thus, our findings reemphasized the role of ROS in oral carcinogenesis. Additionally, significant positive correlation between serum and salivary levels with high sensitivity and specificity highlights saliva as a valid, convenient and an equally reliable diagnostic biofluid as blood for measuring biomarkers of antioxidants and oxidative stress. MDA and GPx can be considered as potential biomarkers for assessing oxidative stress and antioxidant status in OSCC.

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ANNEXURE 1

INFORMED CONSENT FORM

STUDY OF MALONDIALDEHYDE AND GLUTATHIONE PEROXIDASE IN BLOOD AND SALIVA OF ORAL CANCER PATIENTS.

Name: Age/Sex: Date:

I, _____ aged _____ have been informed about my in the study:

1. I agree to give my personal details like name, age, sex, address, previous dental history and the details required for the study to the best of my knowledge.
2. I will cooperate with the dentist for my intra oral examination or extra oral examination.
3. I permit the dentist to take saliva and blood samples required for the study.
4. If am unable to participate into study for reasons unknown. I can withdraw from the study.

In my full consciousness and presence of mind, after understanding all the procedure in my own language, I am willing and give my consent to participate in this study.

Signature / Thumb impression

ஆராய்ச்சி ஒப்புதல் கடிதம்

வாய் புற்று நோயினால் பாதிக்கப்பட்ட நோயாளிகளின் உமிழ் நீர் மற்றும் இரத்தத்தில் மெலோன்டை ஆல்டிஹைடு மற்றும் குளூட்டோதையோன் பாராகஸிடேஸ் அளவினை ஆராய்ந்து அறிதல்.

பெயர்.....
வயது.....
பாலினம்.....

என் பெயர், வயது, பாலினம், நான் மேற்கொள்ளும் சிகிச்சை பற்றிய முழு விவரங்களையும் கொடுக்க நான் முழு மனதுடன் ஒப்புக்கொள்கிறேன்.என்னுடைய வாயின் முன் பகுதி அல்லது வெளிப்பகுதியை மருத்துவர் பரிசோதனை செய்ய ஒத்துழைக்கிறேன்.மேற்கண்ட ஆராய்ச்சிக்காக என் உமிழ் நீர் மற்றும் இரத்தம் எடுக்க அனுமதி அளிக்கிறேன்.
ஆராய்ச்சி சம்பந்தப்பட்ட விபரங்களை முழமையாக புரிந்து கொண்ட பிறகு என் முழு மனதுடன் இந்த ஆராய்ச்சியில் பங்கு கொள்ள சம்மதிக்கிறேன்.

இப்படிக்கு

இடம்:
தேதி:

ANNEXURE 2

A. GLUTATHIONE PEROXIDASE (GPx) AND MALONDIALDEHYDE (MDA) LEVELS IN BOTH BLOOD AND SALIVA OF 25 OSCC PATIENTS

S.NO	GPx (U/L)		MDA (µmol/L)	
	BLOOD	SALIVA	BLOOD	SALIVA
1.	99.23	55.23	12.43	9.09
2.	152.00	49.85	11.56	8.33
3.	106.42	52.05	13.64	9.09
4.	121.62	64.09	11.05	9.27
5.	146.12	58.14	11.03	8.71
6.	150.09	101.6	14.65	10.02
7.	143.80	102.9	15.24	8.29
8.	136.55	99.73	14.55	8.67
9.	99.00	100	12.24	8.27
10.	125.24	87.45	13.32	10.19
11.	106.76	102	14.44	9.88
12.	108.07	66.54	15.55	9.54
13.	92.03	78.37	13.36	8.94
14.	150.24	68.55	12.45	9.22
15.	149.31	48.63	14.53	10.54
16.	127.65	62.01	12.44	8.41
17.	107.33	101.2	11.21	9.31
18.	99.03	73.44	12.11	9.55
19.	92.00	56.33	11.34	8.54
20.	139.19	81.24	15.97	10.22
21.	121.24	69.55	11.66	10.58
22.	109.36	84.02	14.11	9.01
23.	148.04	47.07	15.34	8.34
24.	96.10	49.71	13.56	10.67
25.	105.23	90.56	15.45	9.55
Mean	121.2660	74.0104	13.3292	9.2892

B.GLUTATHIONE PEROXIDASE (GPx) AND MALONDIALDEHYDE (MDA) LEVELS IN BOTH BLOOD AND SALIVA OF 25 CONTROLS

S.NO	GPx (U/L)		MDA (µmol/L)	
	BLOOD	SALIVA	BLOOD	SALIVA
1.	199.34	155.7	3.43	2.12
2.	250.30	150	3.09	1.83
3.	198.22	120.6	2.45	2.2
4.	171.53	128.2	2.37	4.53
5.	260.00	142.3	4.47	1.55
6.	240.11	130.1	2.41	2.54
7.	242.21	117.6	2.3	3.71
8.	189.45	168.3	2.32	3.24
9.	193.61	116.8	4.56	1.77
10.	249.53	163.9	3.3	4.05
11.	259.01	131.3	4.22	2.33
12.	232.51	134.1	2.37	2.61
13.	196.45	146.3	2.81	1.78
14.	201.23	118.2	2.35	2.06
15.	210.44	128.8	3.03	4.09
16.	253.66	146.2	4.55	3.76
17.	199.72	165.6	3.38	2.33
18.	186.27	119.4	3.71	3.57
19.	181.35	170	2.34	2.44
20.	212.34	140.5	2.76	4.15
21.	197.12	155	3.56	1.57
22.	202.66	161.3	4.74	3.83
23.	257.21	134.6	2.32	2.55
24.	243.45	141.2	4.71	1.67
25.	254.65	116.4	4.69	1.83
Mean	219.2948	140.0960	3.2896	2.7244