ASSOCIATION OF MCP-1 AND sCD40- L IN GINGIVAL CREVICULAR FLUID AND SALIVA OF CHRONIC PERIODONTITIS SUBJECTS WITH CARDIOVASCULAR DISEASE'S (CVD'S) – A CASE CONTROL STUDY.

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PAKISTAN

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A thesis submitted in fulfillment of the requirements for the award of the degree of Master of Philosophy (Physiology)

DEPARTMENT OF PHYSIOLOGY

BAHRIA UNIVERSITY HEALTH SCIENCES CAMPUS KARACHI

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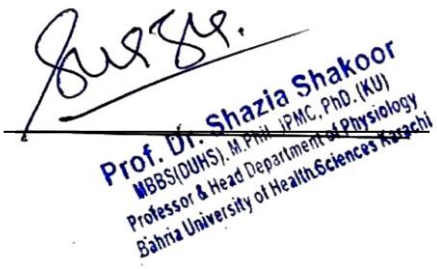
Principal Supervisor's Signature: 

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TO MY BELOVED FAMILY

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ABSTRACT

Periodontal disease refers to pathologic inflammatory response affecting the supporting structures including alveolar bone, gingival connective tissue surrounding the teeth. Characteristically it is chronic in nature, preceded by oral biofilm formation accompanied with the deposition of bacterial plaque and calculus. Periodontitis is the sixth leading cause of human chronic degenerative disease that increases the risk for CVD's due to the strong correlation between the subgingival microbiota and the pathogens present in the vascular lesions. Periodontitis if left untreated, results in alveolar bone loss and exfoliation of the involved teeth. Traditional periodontal diagnostic methods included assessment of clinical parameters and radiographs. Though efficient, these conventional techniques are inherently limited in that only a historical perspective, not current appraisal, of disease status can be determined. Advances in the use of oral fluids as possible biological samples for objective measures of current disease state, treatment monitoring, and prognostic indicators have boosted saliva and other oral-based fluids to the forefront of technology. Oral fluids contain locally and systemically derived mediators of periodontal disease, including microbial, host-response, and bone-specific resorptive markers. Amogst them most important biomarkers in oral fluids are MCP-1 and sCD40 L that represent inflammatory mediators, collagen degradation and bone turnover-related molecules that have emerged as possible measures of periodontal disease activity. So this study has aimed to determine the association between elevated levels of MCP-1 and sCD40 L in saliva and Gingival Crevicular Fluid, of severely affected Chronic Periodontitis patients with well established progression and acute precipitation of Cardio vascular disease. A total of 84 subject's 42 cases and 42 controls between the age of 35- 68 with severe periodontitis along with horizontal alveolar bone loss (grade 4) having cardiac disease were selected as cases and individuals with periodontitis without cardiac diseases were opted as controls in the study. After informed consent detailed demographics, cardiovascular, medical and family history of the patients, grading of alveolar bone loss, periodontal and gingival status, probing depth, gingival index, plaque index, clinical attachment level was carefully recorded on the evaluation form. GCF and Salivary samples were extruded and the sCD40 L and MCP -1 levels were quantified in a solid phase, sand-which technique using Elisa kit. This study identified a correlation of the bio molecular

markers sCD40-L and MCP -1 that constitute an important pathway leading to cardiovascular loads in chronic periodontal patients. The significant positive results were observed that suggests that these two inflammatory mediators, soluble CD40 ligand (sCD40 L) and monocyte chemoattractant protein-1 (MCP-1) has been established in progression and acute precipitation of CVD's and this pathway is considered as one of the mechanisms that may lead to increasing severity of periodontal disease and its systemic effects. Timely detection and diagnosis of disease significantly affect the clinical management of periodontal patients by offering earlier, less invasive, and more cost-effective treatment therapies. This study will also educate the patients to look for a collaborative effort of health practitioner and dentists for early monitoring and intervention along with appropriate oral hygiene care that will potentially reduce systemic cardiac illness in chronically affected periodontal patients.

Key words: Periodontitis, sCD40 L, MCP -1, Elisa, GCF.

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AA	Amino Acid
AAP	American Academey of Periodontology
ACVD	Advanced Cardiovascular Disease
Anti CL	Anti Cardiolipins
AP	Accute Periodontitis
APC	Antigen Presenting Cell
CAD	Coronary Artery Disases
CAL	Clinical Attachment Loss
CAMs	Cell Adhesion Molecules
CBVD	Cerebrovascular Disease
CCL2	Chemokine Ligand 2
CDC	Centre of Disease Control
CEJ	Cemento Enamel Junction
COPD	Chronic Obstructive Pulmonary Disease
COX1	Cytochrome c Oxidase 1
COX2	Cytochrome c Oxidase 2
CP	Chronic Periodontitis
CRP	C-Reactive Proteins
CVD's	Cardio Vascular Diseases
CVS	Cardio Vascular System
DC	Dendritic Cells
DM	Diabetes Mellitus
GBD	Global Burden of Diseases
GCF	Gingival Crevicular Fluid
HS	High Sensitivity
HSPs	Heat Shock Proteins
HTN	Hypertension
IE	Infective Endocarditis
IFNγ	Interferon Gamma

LIST OF ABBREVIATIONS

IgG	Immunoglobulin G
IL	Inter Leukeins
JE	Junctional Epitheluim
LDL	Low Density Lipoprotein
LPs	Lipopolysaccharides
MCP-1	Monocyte Chemoattractant Protein-1
MI	Myocardial Infarction
MMP	Matrix Metallo Proteinases
mRNA	Messenger Ribonucelic Acid
mSBI	Mean Sulcular Bleeding Index
NCD	Non Communicable Disease
NFKb	Nuclear Factor Kappa beta
NO	Nitrous Oxide
PAD	Peripheral Artery Disease
PAMPs	Pathogen Associated Molecular Patterns
PD	Periodontitis
PGE_2	Prostaglandin E_2
RBL	Radiographic Bone Loss
RPD	Removable Partial Denture
sCD40L	Soluble Cluster of Differentiation 40 Ligand
SMC	Smooth Muscle Cells
TGF-β	Transforming growth factor beta
TM	Transmembrane
TNF	Tumer Necrosis Factor
t-PA	Tissue Plasminogen Activator
VCAM-1	Vascular Cell Adhesion Molecule-1
WHO	World Health Organization
YLD	Years Lost to Disability

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CHAPTER 1

INTRODUCTION

1.1 BACK GROUND:

Periodontitis is an inflammatory condition that affects the supporting tooth structure called periodontium. A Tooth constitute two major parts one is the Clinical or Dental crown that is exposed to the oral cavity and the other part is the one that is attached just beneath the crown known as root. Both the crown and root meets at a junction called as cement-enamel junction (CEJ). Dental pulp cavity present in the centre is supplied by blood vessels, lymph vessels and sensory nerve tissue. (Ghannam, Bordoni et al., 2019). Periodontium is a unique and a diverse structure comprising of gingiva, alveolar bone, cementum and periodontal ligament. Periodontal ligaments have the ability to withstand sheer and occlusal stresses due to the thick fibrous tissue that alleviates the forces applied on maxilla and mandible. A calcified covering over the root surface known as cementum comprises of extensions of periodontal ligaments known as Sharpey's fibre. Thus a periodontal apparatus consists of alveolar bone supporting the tooth structure, covered externally by gingiva (De Jong, Bakker, Everts et al., 2017). Just below gingival sulcus is the junctional epithelium that controls the bacterial migration and has a unique characteristic to cause periodontal infection which is an irreversible loss of supporting tooth structure by osteoclast activation and osteoblast inhibition. (Kassebaum, N.J.Bernabé, E.et al., 2014).

The hard tissue of a tooth represents enamel, dentine and cementum where as the pulp cavity is the soft tissue of the tooth. Dental crown is covered by the hardest tissue known as enamel beneath which lies the dentine surrounding the pulp cavity and root canals (Dorozhkin et al., 2016). The organic content constitutes primarily collagen and non- collagen protein and the inorganic content comprises hydroxyl apatite crystals (Nanci ., 2017).

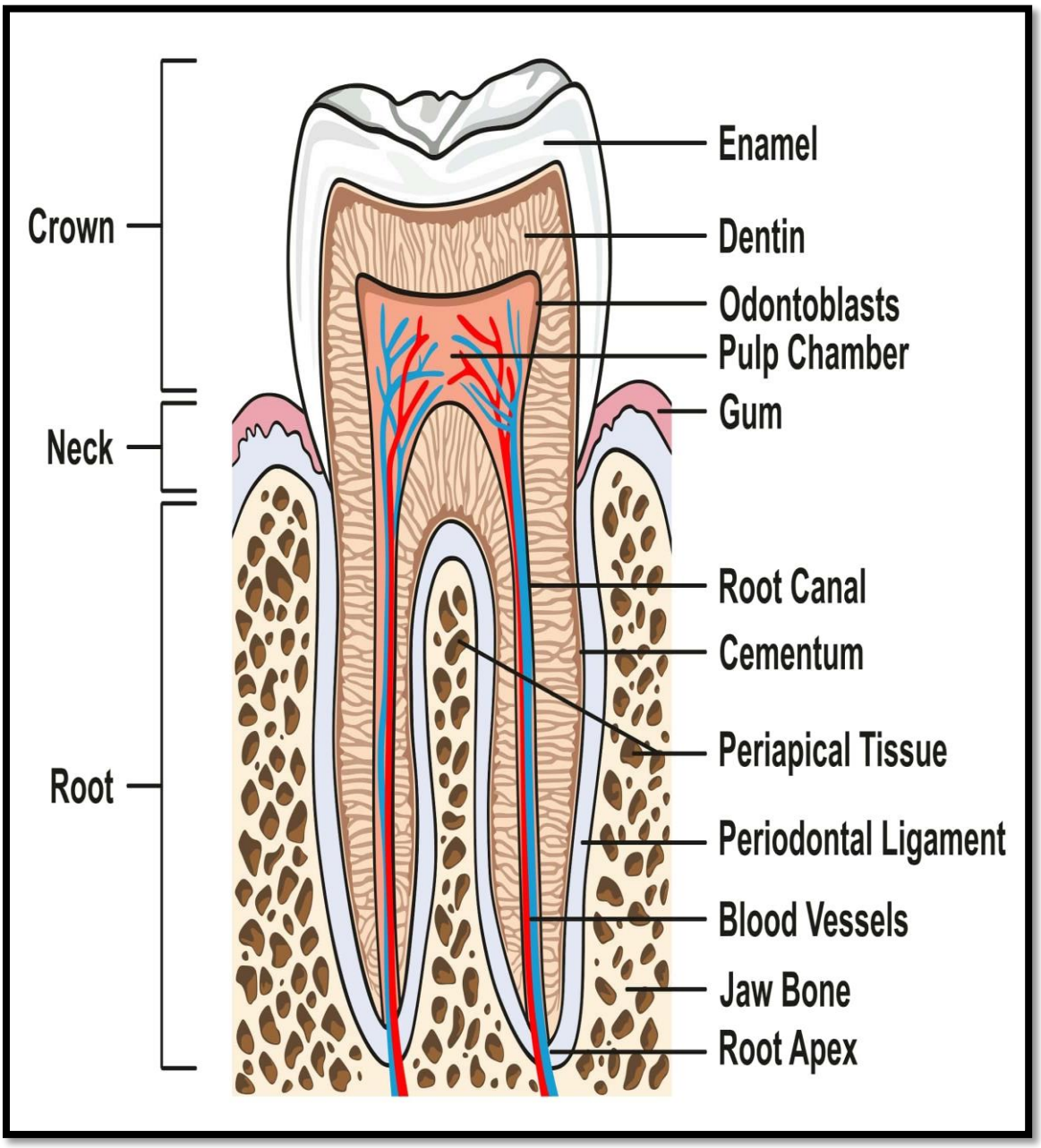


Figure 1.1(a) : Structure of a Tooth (DoroZhkin et al , 2016)

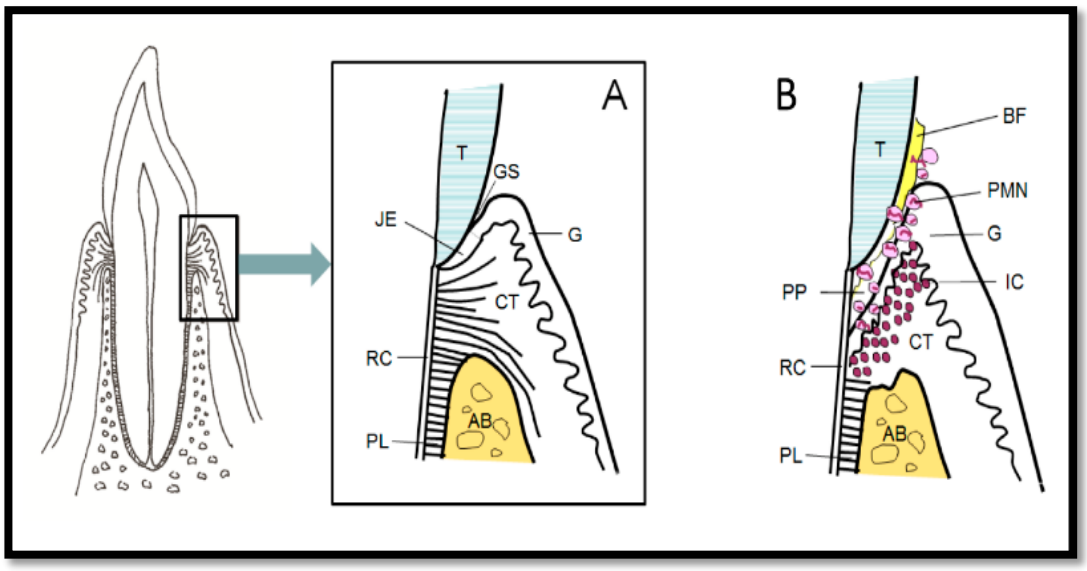


Figure 1.1 (b): The anatomical structure of the periodontium in health (A) and in periodontitis (B). Abbreviations: Alveolar bone (AB), bacterial biofilm (BF), connective tissue (CT), gingiva (G), gingival sulcus (GS), inflammatory cells (IC), junctional epithelium (JE), polymorphonuclear neutrophils (PMN), periodontal ligament (PL), periodontal pocket (PP), root cementum (RC), and tooth (T).

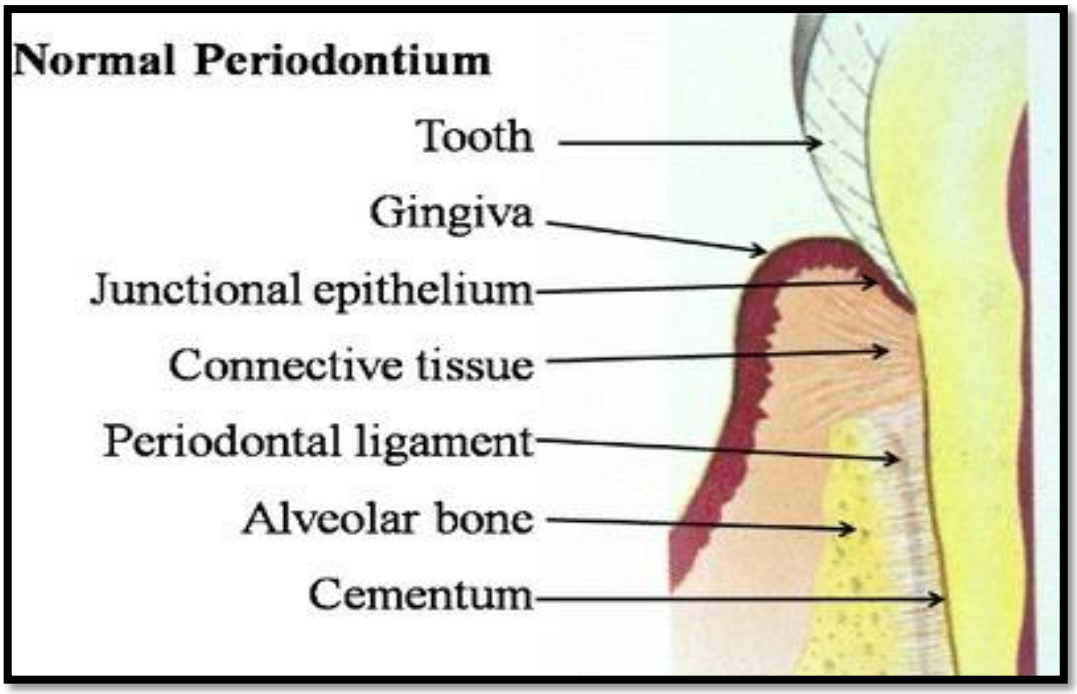


Figure 1.1 (c): Components of periodontium (Farran, 2020, dental town .com)

Periodontitis is seen in both adults and children and is broadly referred to as chronic or aggressive periodontal disease presenting as a vital characteristic of non uniform bone loss and formation of periodontal pockets.(Sanz, M.et al., 2022). A non Communicable Disease (NCD) , sixth most common leading cause of human chronic degenerative disease with a high prevalence worldwide is 30 -35 % (Al Jehani et al., 2014) . As per National Health and Nutrition mild to moderate form of periodontitis in adult population is recorded to be 45- 50% (Kassebaum, N.J; Bernabé, E.et al., 2014). Prevalence rate recorded in Eygpt is 27.7% for mild, 25.2% for moderate and 45% for severe forms of periodontitis. Nepal has recorded to have 47.6%, Australia 23% and Saudi Arabia to have 40% of the population to have mild to moderate forms. Periodontal problems in Pakistan are reported to be 31 % according to W.H.O (Bokhari, Suhail, Malik et al., 2106). Severe or chronic forms (referred to as Chronic Periodontitis) constitute 11.24 % globally in third and fourth decade people. (Kinane DF et al., 2012). In Britain adult aged between 32 – 43 years is prevalence for severe forms of periodontitis is recorded to be 42 % and70 % in the age group of 55- 65 years. (Al Qahtani, Joesph, Deepthi et al., 2017)

Chronic Periodontitis (CP) is a multi factorial slowly progressing inflammatory condition that involves the abnormal destruction of gingival tissues, periodontal ligaments and alveolar bone that comprises of the vital supporting elements of the teeth eventually resulting in complete tooth loss and collapsed bite function in an individual. (Attstrom R et al., 2015). Acute periodontitis is characterized by pain, discomfort and inflammation of periodontal structures causing infection and destruction of tissues. (Herrera, Retamal-Vades, Alonso et al., 2018). Advanced stage results in tooth loss due to imbalance between host resistance and oral micro flora that severely affects connective tissue and the alveolar bone. (Jensen, A et al., 2018). Predisposing factors include anatomical abnormalities, supra and sub gingival calculus restorative overhangs, smoking, stress diabetes mellitus and various systemic diseases that progresses the inflammatory condition. (Knight, E.T et al., 2016).

Chronic periodontitis is initiated by various etiological factors including systemic, environmental, microbial and local. Dental plaque, calculus deposited over the tooth and inadequate oral hygiene along with tobacco smoking that aggravate periodontitis (Donovan, Marzola, Murphy et al., 2017). Accumulation of Dental plaque which is a sticky, colorless oral mirobial film and lack of immune responses leads to gingival inflammation causing irreversible loss of periodontal apparatus. Bacteria form a matrix of extracellular polymeric substances which incorporate within the biofilm, containing streptococcus mutans that adheres to the surface of tooth as well as oral mucosa. Dental plaque accumulates due to improper tooth brushing strategies', poor oral hygiene and no dental visits or follow-ups. (Yu, Zhao, Mei, et al., 2017). Calculus is a calcified layer which forms when dental plaque is not removed by regular tooth brushing. The harsh surface and permeable structure of analytics give a perfect substrate to bacterial colonization and fill in as a supply for lethal bacteria antigen. Bacteria of calculus like Porphyromonas gingivalis localized in periodontal inflammation persist if left untreated (Lai and Walters, et al., 2016)

Potential risk factors of periodontitis include systemic diseases such as obesity, osteoporosis, cardiovascular diseases, pregnancy and hormone imbalances. Socio economic conditions, age and genetic factors are the environmental factors affecting the periondtium (Madiba, Bhayat et al., 2018). Approximately over 400 microbes are present in oral flora, out of which 100 microbial species are present in sub gingival flora but only a small number of them are associated in the progression of the disease. Organisms involved in chronic periodontitis are Actinomycetemcomitans, Bacteroidesforsythu, prevotellaintermedia, Porphyromonasgingivals, Pepto-streptococcus, Fusobacterium nucleatum, GramNegative anaerobic rods and Spirochetes all form a biolfilm (Al- Jehani, et al., 2017). Bacterial LPS antigen invades and a host response occurs by the formation of inflammatory mediators that leads to tissue destruction by collagen breakdown and action of enzymes like proteinases, prostaglandins and pro inflammatory cytokines (Chen, Deng et al., 2018). Smoking is one the risk factors affecting the periodontal apparatus. In smokers a greater prevalence of attachment loss, recession and severe destructive periodontal disease is observed. Refractory periodontal diseases in association with the frequency and duration of smoking

owing to bone loss have been evidenced by clinical and epidemiological studies (Attia, Mohamed, Alharthi et al., 2017).

Epidemiological and pathological associations between periodontal and systemic diseases have still been debated and investigated and according to global burden of diseases (G. B. D. Risk Factors Collaborators, 2016) an approximate of 41 million(71%) deaths occurring globally are due to high prevalence of non communicable diseases (NCDs) with in line association from sedentary life style , unhealthy pattern of diet , poor oral hygiene care provoking systemic Cardio vascular risk that compromises the health care economy, equating to two-thirds of all health costs in the society (Gonzalez-Quesada C et al., 2013).

GLICKMAN'S CLASSIFICATION OF HORIZONTAL ALVEOLAR BONE LOSS (Grade 4) refers to progressive periodontal infection caused by the bacteria, exposing the furcation areas involving the roots of teeth and alveolar bone ultimately resulting in teeth to loosen and fall. Oral cavity becomes a dangerous source of contamination and infection spreads through blood to internal organs. (Barbara Buffoli et al., 2019). Due to inflammation, the surrounding structures are infiltrated by activated lymphocytes, macrophage and neutrophils, releasing cytokines and causing the activation of acute phase reactants like CRP and fibrinogen (Van Dyke TE et al., 2013).

One of the first proposed classifications is the one by Glickman, This classification system probably is the most widely used and it describes the main characteristics of furcation lesions as four grades:

Grade I: involvement: it is the incipient or early lesion. The pocket is supra-bony, involving the soft tissue; there is slight bone loss in the furcation area. Radiographic change is not usual, as bone changes are minimal.

Grade II: involvement: the bone is destroyed on one or more aspects of the furcation, but a portion of the alveolar bone and periodontal ligament remain intact, thus allowing only partial penetration of the probe into the furcation area. The radiograph may or may not reveal the grade II furcation involvement.

Grade III: involvement: the inter-radicular bone is completely absent, but the facial and/or lingual orifices of the furcation are occluded by gingival tissue. Therefore, the furcation opening cannot be seen clinically, but it is essentially a through and through tunnel. If the radiograph of the mandibular molars is taken with a proper angle and the roots are divergent, these lesions will appear on the radiograph as a radiolucent area between the roots. The maxillary molars present a diagnostic difficulty owing to roots overlapping each other.
Grade IV: involvement: the inter-radicular bone underneath the roof of furcation is completely destroyed. The gingival tissue is also receded apically so that the furcation opening is clinically visible. The radiographic image is essentially the same as in grade III lesions.

In 1975 Hamp, Nyman and Lindhe, proposed a classification system referring to the horizontal attachment loss and based on three degrees:
Degree I: horizontal attachment loss < 3 mm of the total width of the furcation area.
Degree II: horizontal attachment loss > 3 mm but not encompassing the total width of the furcation area.
Degree III: “through and through” destruction of the periodontal tissue in the furcation area.

Later on, a sub-classification referring to the vertical bone loss from the furcation fornix was introduced to complement the horizontal classification (I–III):
Subclass A: vertical bone loss 3 mm or less.
Subclass B: vertical bone loss from 4 to 6 mm.
Subclass C: bone loss from the fornix of 7 mm or more.

Clinical features of periodontitis include redness of gums, swelling, bleeding, pocketing, attachment loss and bad odor and taste. Severe pain may occur due to abscesses causing tooth loss. It may present without the pain sensation due to altered periodontal nociception, Pain sensation is determined by bacterial virulence, host responses and suppression of inflammatory responses. (Gaurilcikaite, E. et al., 2017).

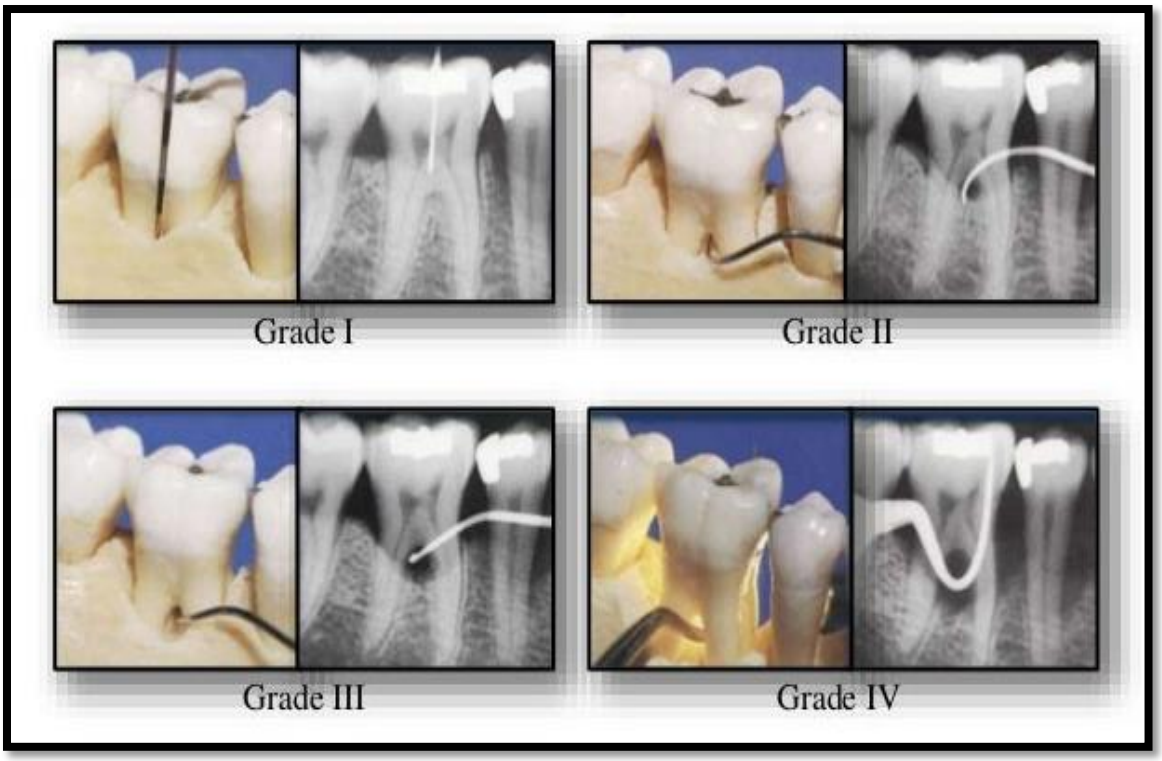


Figure 1.1 (d): Glick mans classification of alveolar bone loss

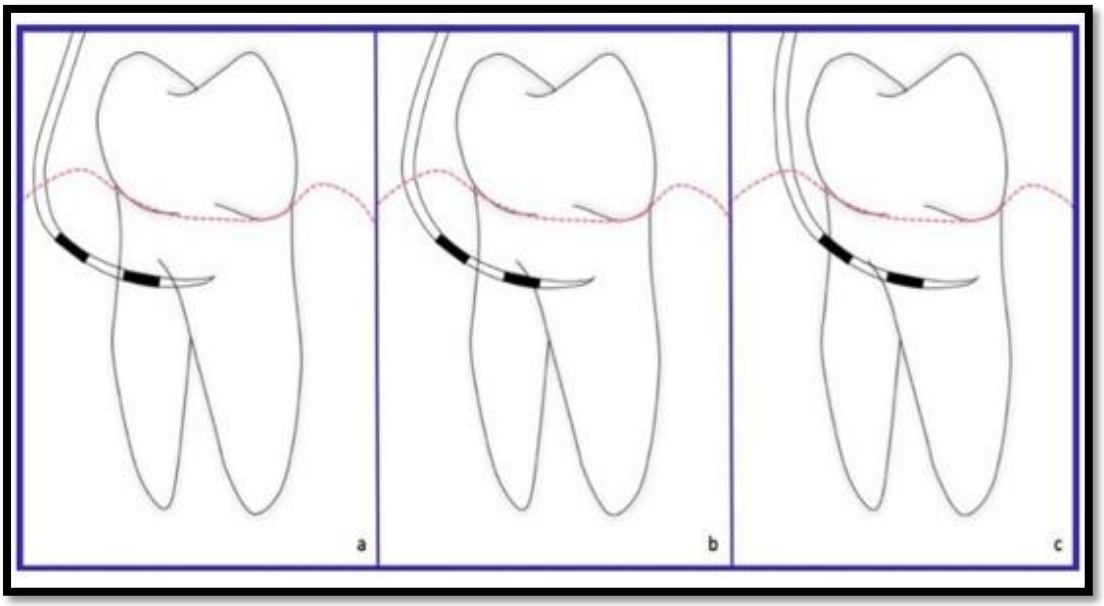


Figure 1.1(e): Schematic representation. Non-exposed furcation lesion (NE): (a) Class I: Incipient lesion. Horizontal attachment loss of 2 mm or less; (b) Class II: Horizontal attachment loss of 3 mm or more; (c) Class III: Total horizontal attachment loss (through and through).

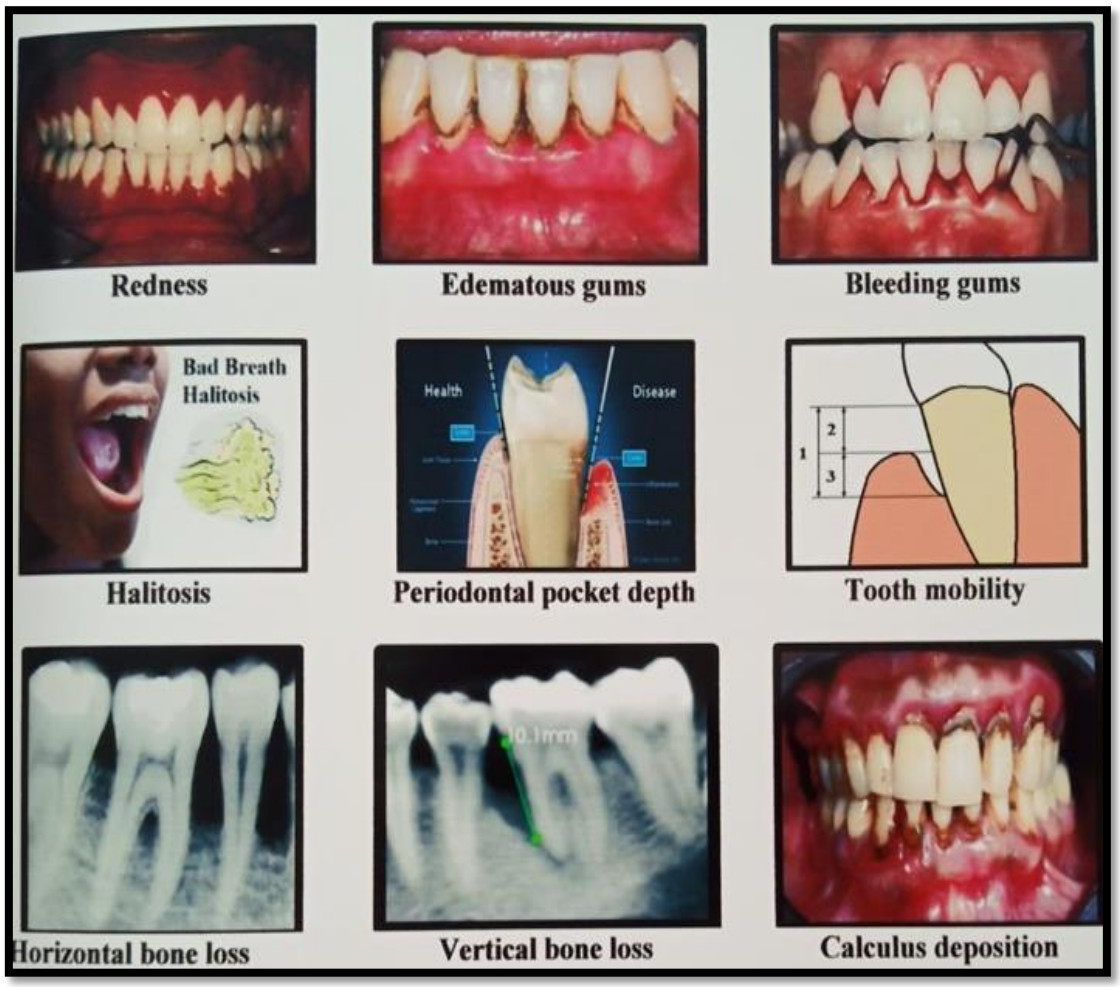


Figure 1.1 (f): Clinical features of Periodontitis

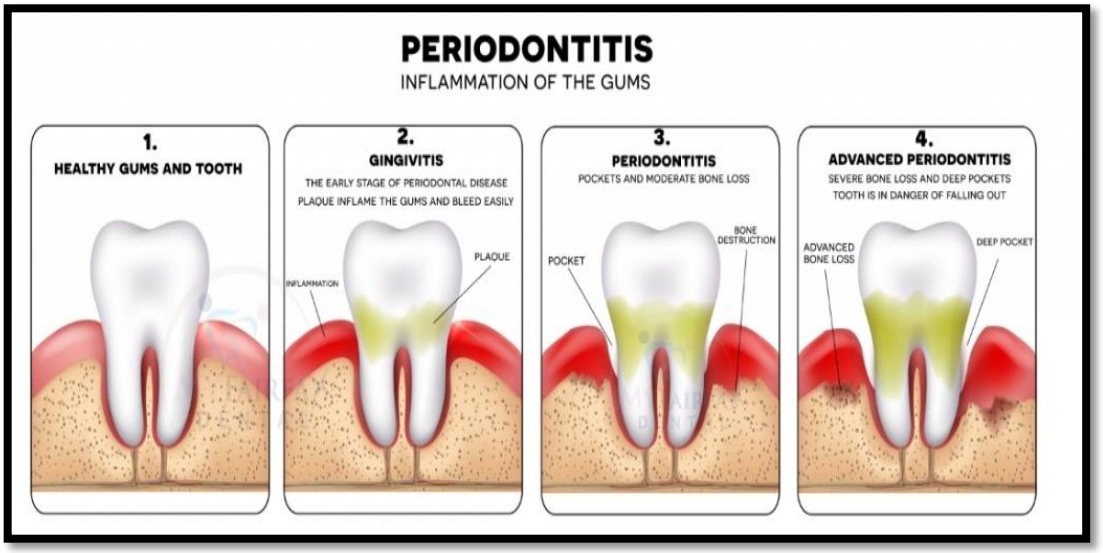


Figure 1.1 (g): Periodontitis: inflammation of gums (myfairfaxdental. Com)

Periodontal disease can be diagnosed mainly on clinical and radiographic examination which ascertains severity and prognosis of the disease. Clinical parameters include probing depth, clinical attachment level, bleeding and radiographic analysis of alveolar bone (Mittal, S, Komiyama, M, et al., 2022). Family and medical history with specific periodontal markers such as salivary leukocytes, lactoferrin and hemoglobin aids in appropriate diagnosis.The known etiological factors should be removed and the patients should be advised of the various risk factors and their association with the systemic diseases. (Tonetti, M.S, Sanz, M, et al., 2019)

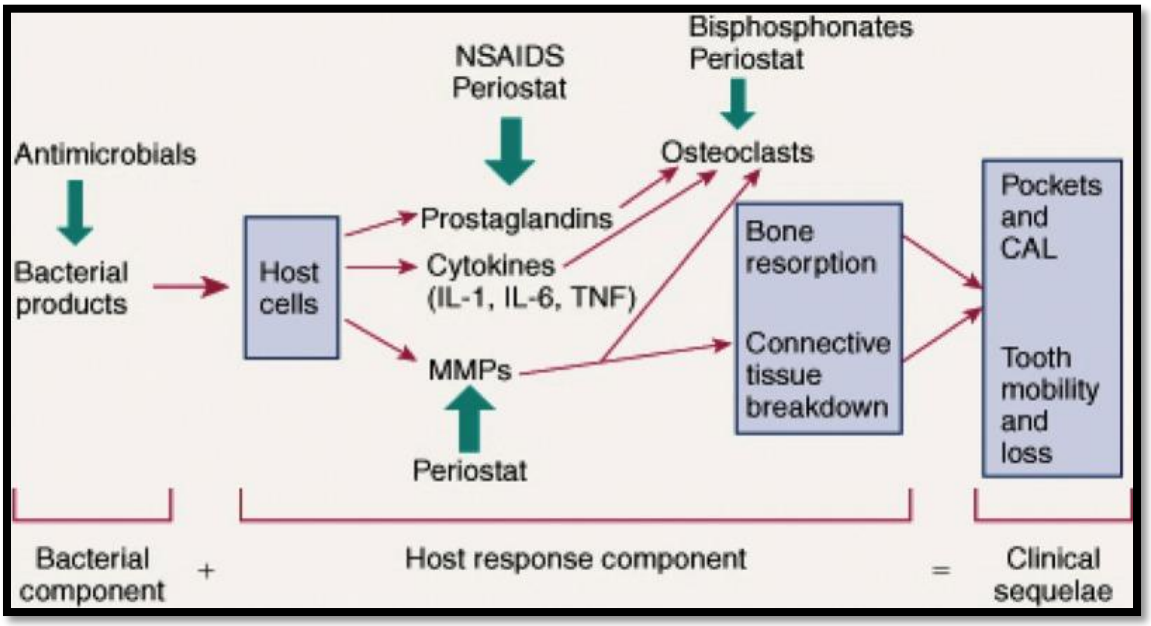
Table 1.1 (a): American academy of periodontotlogy 2019 guidelines of periodontitis

	Slight (Mild)	Moderate	Severe (Advanced)
Probing depths	>3 & <5 mm	≥5 & <7 mm	≥7 mm
Bleeding on probing	Yes	Yes	Yes
Radiographic bone loss	Up to 15% of root length or ≥2 mm & ≤3 mm	16% to 30% or >3 mm & ≤5 mm	>30% or >5 mm
Clinical attachment loss[1]	1 to 2 mm	3 to 4 mm	≥5 mm

In chronic periodontitis destruction of supporting tooth structure occurs due to imbalance between host's inflammatory response and sub gingival microbiota resulting in colonization of anaerobic bacteria. Alveolar bone resorption in periodontitits is due to increased osteoclastic activity which impairs the bone remodeling process. Bone resroption in periodintitis that causes severe inflammatory changes is due to Lipopolysaccharide released from gram negative bacteria that affects the bone mass (Gilowski,Ł;Wiench, R; et al., 2014). Junctional epithelium forms a seal between gingiva and root surface and provides protection against the exposure of oral microbe. Inflammatory changes causes' apical migration of junctional epithelium to the root surface causing destruction of collagen fibers leading to pocket formation. Pocketing disrupts the balance between tissue regeneration and removal as it suppresses the activity of osteoblast,

keratinocytes and fibroblasts while the osteoclasts, neutrophils and macrophages are stimulated to enhance the degradation process (Henderson, B. et al., 2018). The most important pathological characteristic is alveolar bone resorption caused by osteoclasts that regulates a cascade of inflammatory cytokines and enzymes. Enzymes such as matrix metal proteinases (MMP) 1, 8, 9 and 13 degrades type 1 collagen by assisting osteoclast activation and migration (Belibasakis, G.N. et al., 2012)

Damage to periodontal tissue is caused by a molecular mechanism of dysregulation between anti-inflammatory and pro-inflammatory cytokines that maintains the homeostasis by regulating cell signaling and immune responses. Lipopolysaccharides released from gram negative bacteria during inflammatory reactions consistently raise the level of cytokines that results in aggressive alveolar bone loss (Ben-Sasson, S.Z; et al 2009). Inflammatory cytokines IL -1, 6, 7 and TNF plays a very important role in the periodontal pathogenesis. Immune responses of host are primarily affected by IL-1 as it promotes the activation of Th1 and Th2cells and determines severity of the diseases (Gilowski, Ł; Wiench, R; et al., 2014). Nuclear factor Kappa are a group of complex elucidating protein known as inhibitor IkB found in abundance in periodontal infection (Ambili, Janam, Babu et al.,2017). Another important pro inflammatory cytokines responsible for the pathogenesis of periodontitits is tumor necrosis factor alpha found in saliva and GCF of healthy as well as inflamed periodontal tissue that causes systemic inflammation so it is considered as an important biomarker and a diagnostic tool for screening and managing periodontal diseases (Eivazi, Falahi, Eivazi et al., 2017). Prostaglandins (PGE2) produced by COX-1 and COX-2 are arachidonic acid derivative responsible for inflammation. In periodontium PGE-2 is produced by fibroblasts and macrophages which enhance osteoclastic activity resulting in bone resorption and gingival inflammation (Deo and Bongate, et al., 2015).



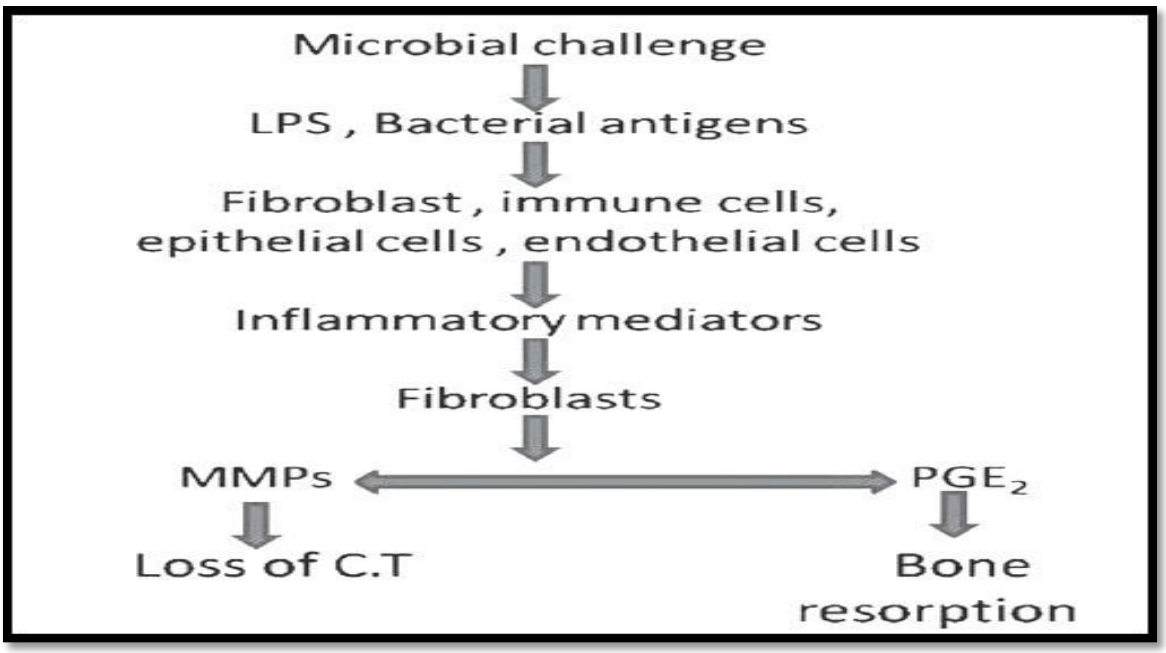


Figure 1.1 (h): Host response to Prostaglandins enhancing Osteoclastic activity and CT breakdown resulting in clinical sequelae

Periodontitis can also cause various systemic diseases that affect CVS, cerebral Respiratory and endocrine system. Endotoxins such as gram negative anaerobic bacteria directly or indirectly cause systemic illness (Colombo, A.P.V.; et al 2016).

Multiple risk factors are involved in the development of chronic periodontitis, the most crucial factor is the presence of microorganisms along with host related risk factors including heavy deposits of calculus and plaque, various modifications of oral hygiene , age, gender and inherited/biological characteristics ,socio economic conditions, chronic degenerative diseases like diabetes , sedentary life style causing obesity , smoking , human immuno deficiency virus, all interact and render an increasing risk of development of chronic non communicable periodontitis. (Kinane DF et al., 2012). Approximately 45% of deaths in Europe results from cardiovascular disease (CVD) of which dyslipidemia, hypertension, stroke and ischemic myocardial disease are the leading cause of these CVD related deaths (Dietrich T et al ., 2013).

There is a crucial link between periodintitits and heart diseases like stroke and arthrosclerosis which functions as silent killers by infecting the blood vessels. Periodontal pathogens found in atherosclerotic plaque like P.gingivalis, P. intermedia, actinomycetemcomitans, and T. forsythia travels from the oral cavity into the remote locations (Velsko, I.M.; Chukkapalli, S.S.; et al 2014). P.gingivalis found in atherosclerotic plaque and circulating leukocytes in periodontal infections alters the microbiota of gut leading to endotoxemia, increased epithelial permeability and endothelial dysfunction (Hajishengallis, G. et al., 2015).

In atherosclerosis a pro inflammatory environment is promoted by continuous microbial invasion in the vessel wall that potentiates the endothelial lesions and the cytokines enhancing the vascular inflammation are interleukins and tumor necrosis factor (Preshaw et al., 2020). There is a close association between peripheral artery disease (PAD) and chronic periodontitis as the affected vascular epithelium produces arterial stenosis and a reduced blood flow that concomitantly produces enhanced systemic inflammation. This bacteremia leads to lipo polysaccharide (LPS) production activating circulating monocytes (Aoyama et al., 2017).

Chronic periodontitis is also linked to neuro inflammation such as Parkinson’s disease. Patients with brain injury presents with poor oral hygiene and impaired

periodontal conditions resulting in the release of inflammatory cytokines into the blood stream and damaging the blood brain barrier. Lipo polysaccharides produced from periodontal pathaogen such as T. dentolica and P. gingivalis destructs the brain tissue worsening the neurological conditions. (Bui et al., 2019)

Oral cavity is a known reservoir for respiratory infection such as COPD, asthama and pneumonia as the pathogens of oral cavity leaks through the saliva and causes respiratory tract infection called Lemierre's syndrome (Akkaoui etal 2020).

Hormonal changes during pregnancy leads to increased vascular permeability in gingival tissues , patients with advanced periodontitis and hormonal change eases the diffusion of pathogenic microorganisms that leak into the fluid and can infiltrate placenta potentially affecting the fetal tissues , rupturing the fetal membrane and causing premature delivery or miscarriages (Ye et al., 2020).

GCF (gingival crevicular fluid) located in periodontal pocket determines microenvironment of periodontium. It is an important effective periodontal diagnostic marker containing TGF – b present in epithelial, connective tissue and hematopoietic cells. TGF – b elicits pro inflammatory and anti inflammatory properties which causes cancer progression (Astuti, L.A.; Hatta, M.; et al., 2018). Oral periodontal diseases and caries has contributed to the most years lost to disability (YLD) in age-standardized prevalence rates from 354 diseases and injuries across 195 countries as reported by G.B. D. Disease Injury & Incidence & Prevalence Collaborators, 2018. It has been estimated that cardiovascular mortality in worldwide populations is independently associated with severe periodontitis (Ide M et al., 2013) due to biologically plausible proposed mechanism of oxidative stress elevation of CRP C reactive protein and bacteraemia by translocation of circulating oral micro flora that induces systemic inflammatory response either directly or indirectly that results in the development of atherothrombogenesis (Borgnakke WS et al., 2103).

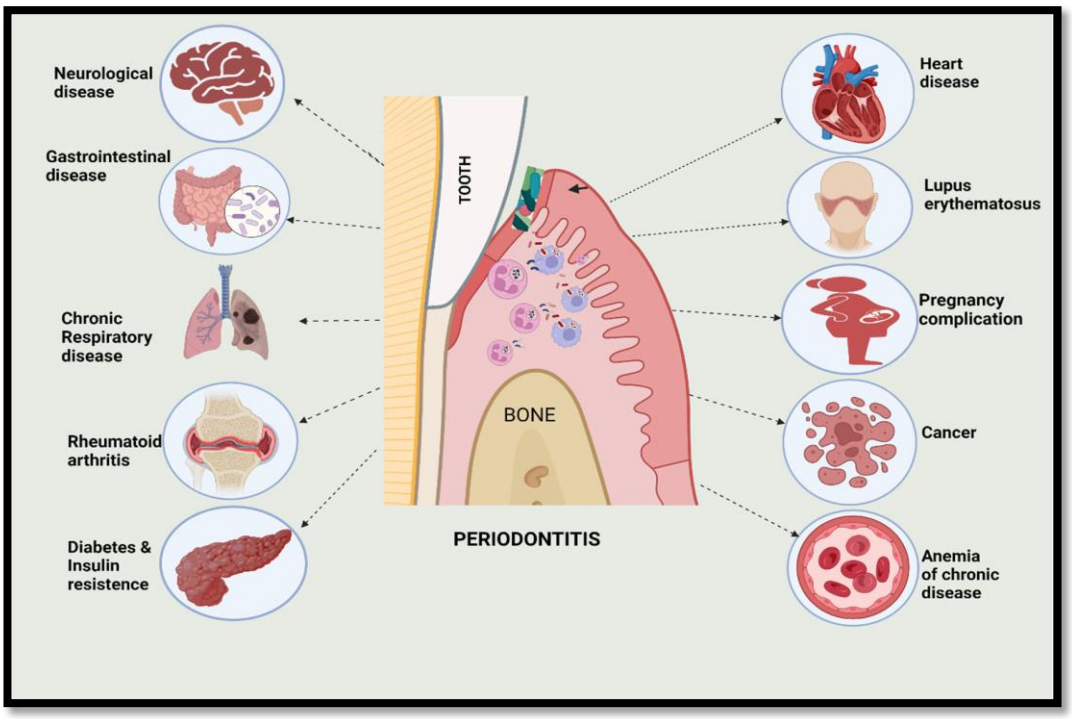


Figure 1.1 (i): Representation of diverse systemic diseases and their relationship with periodontitis. This figure was created with Biorender.com (accessed on 9 September 2022).

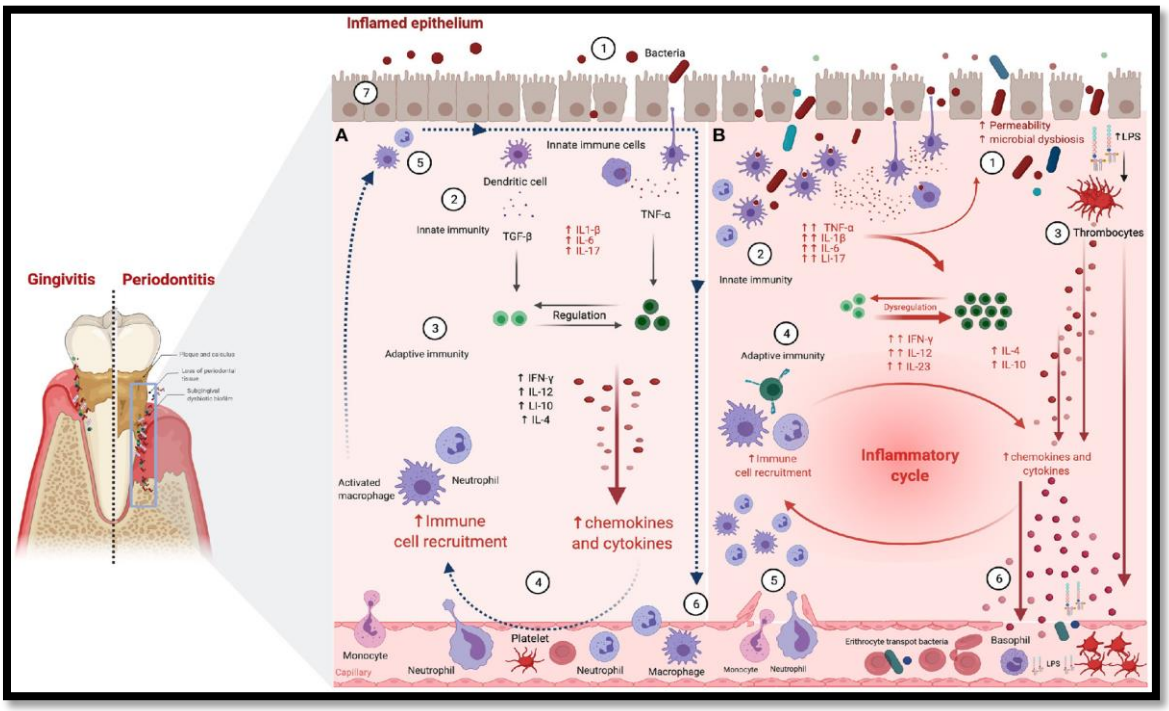


Figure 1.1 (j): Periodontitis associated immune and inflammatory process. A) Representing resolved inflammation and infection clearance. B) Representing unresolved periodontal inflammation and infection remaining.

Periodontitis is activated by the intracellular signaling pathways stimulation of which leads to the production of bio film containing numerous oral microbial organisms causing low grade chronic inflammation (Kinane DF et al., 2012). Inflamed epithelium forms ulcerated pockets that allows virulence factor and bacteria to disseminate through these pocketed epithelium, to enter into the blood stream causing measurable effects on the physiology of vascular system perpetuating CVD's (Linden GJ et al ., 2013).There is an association of numerous biomarkers with the increased risk of development of CVD (Schenkein HA et al., 2013) of which the most critical mediators is monocyte chemoattractant protein-1 (MCP-1), a chemokine predominantly involved in the atheromatous plaque formation by migration and amplified responses of monocyte resulting in thrombogenic events (Gonzalez-Quesada C et al., 2013). MCP-1 produced by cells of periodontal tissues has a very important role in producing cardiac disease by recruiting immune cells such as macrophages and monocytes at the site of infection causing tissue destruction. The levels of MCP-1 are higher in patients having severe periodontal infections than healthy individuals (Huang, Y., Li, S., et al 2020).

MCP-1 aChemokine (CC-motif) ligand 2 (CCL2), is from family of CC chemokines. It has a vital role in the process of inflammation, where it attracts or enhances the expression of other inflammatory factors/cells. It leads to the advancement of many disorders by this main mechanism of migration and infiltration of inflammatory cells like monocytes/macrophages and other cytokines at the site of inflammation. MCP-1 has been inculpated in the pathogenesis of numerous disease conditions primarily cardiovascular diseases. All chemokine receptors are seven transmembrane G-protein coupled receptors belonging to rhodopsin or serpentine receptor family. The primary sources of monocyte chemoattractant protein-1 are epithelial cells, endothelial cells, smooth muscle cells, monocytes/ macrophages, fibroblasts, astrocytes and microglial cells which are regulated by several other cytokines and factors (C.M. Sena et al., 2013). They direct the migration and infiltration of monocytes, microglia, memory T lymphocytes at the place of injury and infection in various disorders. The

endothelial function is essential for the homeostasis of the body and its dysfunction is correlated with several pathophysiological conditions, including atherosclerosis and hypertension. Endothelial cells control cellular adhesion, thromboresistance, proliferation of smooth muscle cells, and inflammation of the vessel wall by generating a broad variety of factors. The disrupted bioavailability of NO is the characteristic of endothelial dysfunction (C.M. Sena et al., 2013). Vascular endothelium injury contributes to the inflammation by secretion of cytokines and chemokines (MCP-1) and to the expression of adhesion molecules by endothelium damage. MCP-1 activation of CCR2 has been observed to be responsible for vascular endothelium renewal after injury, angiogenesis, and collateral formation (I.K. Kwaifa., et al 2020).The MCP-1 is responsible for the migration of monocytes to the sub-endothelium and attracting leucocytes to the site of injury, thus facilitating atherogenic and thromboembolic potential. The monocytes get matured as macrophages, releasing cytokines, and combine with the accumulated oxidized LDL to form foam cells. The smooth muscle cells also proliferate and migrate to the sub-endothelial space due to the effect of proinflammatory cytokines and growth factors interaction. There is formation of fatty streak which eventually leads to atherosclerotic plaque. The MCP-1 level in human atherosclerosis has been correlated with plaque vulnerability (D.P. Ramji et al., 2015).

Significant raised levels of s CD40 L is found in localized ulcerated periodontal tissue that accelerates widespread inflammatory systemic loads. Higher circulating levels of sCD40 L or recombinant ligands for CD40 receptors on monocytes promotes leukocytes there by activation increases leukocyte binding to platelets and endothelium (Lievens D et al ,.2012). Significant raised levels of both of these markers are found in localized ulcerated periodontal tissues and serum thereby accelerating widespread inflammatory systemic loads of CVD’s in an individual (Lievens D et al ,. 2012). The MCP-1 up regulation stimulates the release of cytokines and a Soluble modulator CD40 ligand (sCD40L) which is a co stimulator molecule for helper T cells, activation of it causes expression of cell adhesion molecules and plethora of inflammatory biomolecules .(Giannini S et al., 2012).

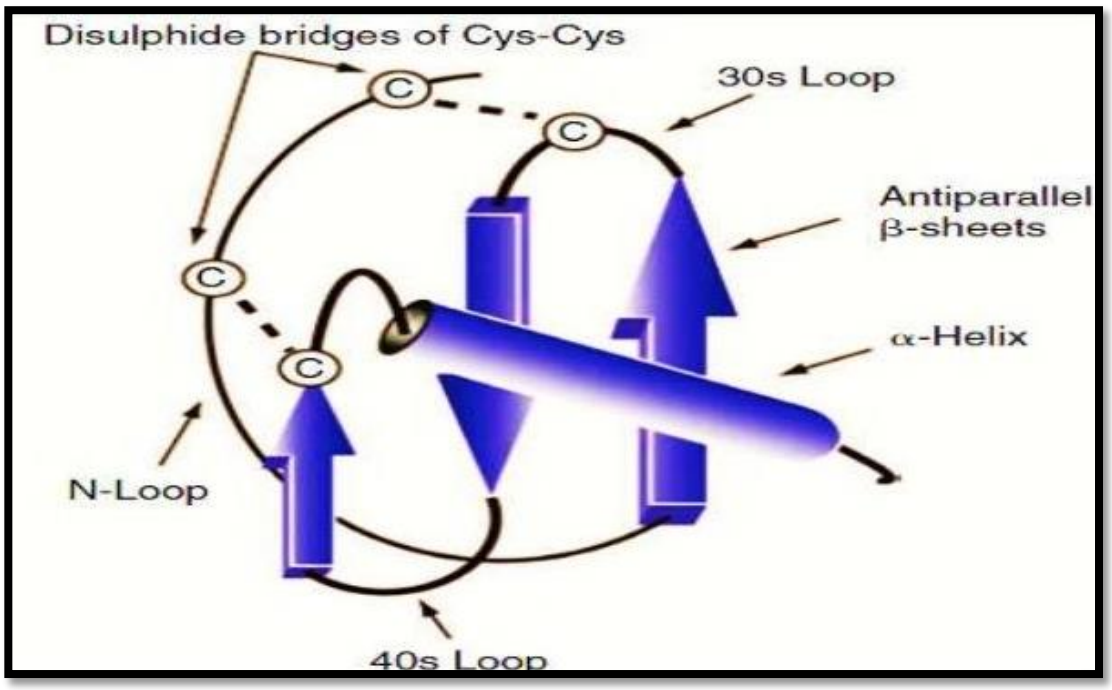


Figure 1.1 (k): Structure of MCP-1, Satish L.,Deshmane et al Journal of Interferon &Cytokine Research, Volume 29, Number 6, 2009, 315 9/26/2015

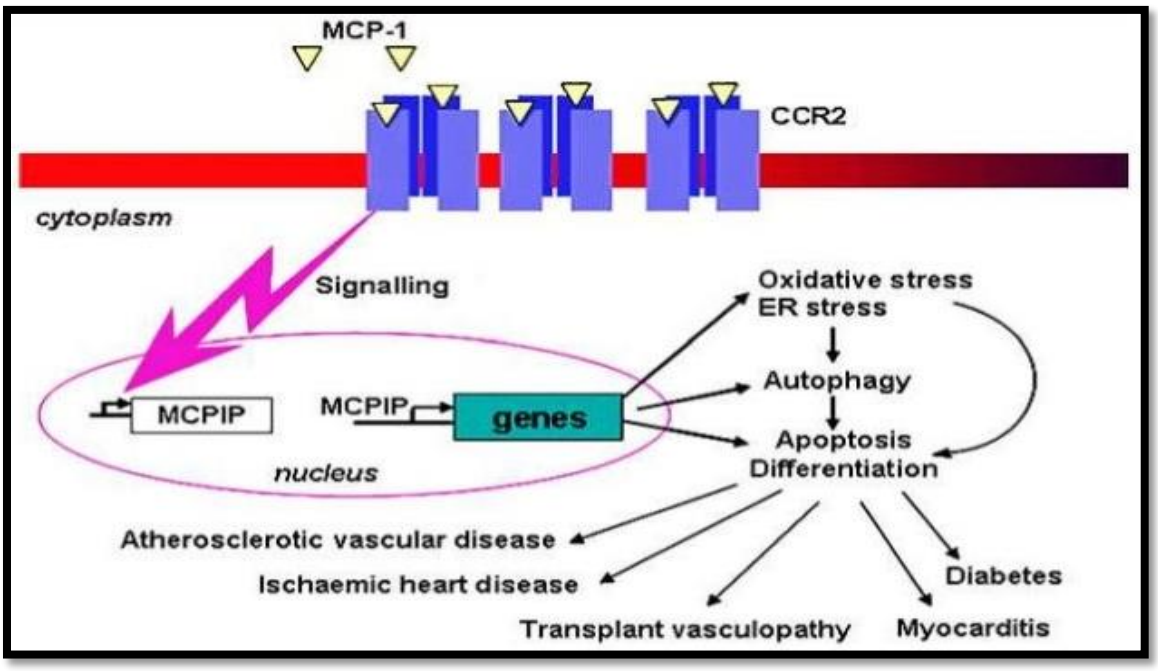


Figure 1.1 (l): Role of MCP – 1 in cardiovascular diseases Jianli niu and pappachan e, Kolattujudy clinic science (2009) 117, 95-109 9/26/2015

CD40L is a 33 kDa type II transmembrane protein belonging to the Tumor Necrosis Factor (TNF) superfamily. The CD40L gene (CD40LG) encodes a 261 amino acid (AA) protein with a 22 AA cytoplasmic domain, a 24 AA transmembrane (TM) domain, and a 215 AA extracellular domain. (Van Kooten, C., et al 2000). CD40L is highly expressed by panoply of hematopoietic and non-hematopoietic cells. CD40L can be further expressed or over expressed by activated cells, the most characteristic are activated and/or differentiated T cells .Like other members of the TNF family, active CD40L at the cell surface or in its soluble form is composed of homotrimers. This multimeric conformation of CD40L is of crucial importance for effective interaction with CD40 and the subsequent intracellular signaling. Moreover, the soluble forms of CD40L retain their ability to form trimers, which bind CD40 and deliver biological signals. Membrane bound CD40L can be cleaved at methionine 113 of the extracellular domain and shed as a soluble form (Elgueta, R.; et al., 2009). The principal isoform (isoform 1) is encoded by 5 exons. The second CD40L isoform (isoform 2) is poorly described. It is a truncated 240 AA protein lacking exon 4 in the CD40LG (extracellular domain), and the functional consequence of this is unknown. Membrane bound CD40L is expressed on B cells and dendritic cells (DCs). It is not expressed on non-activated T cells and platelets, but is weakly expressed on non-activated macrophages, neutrophils and endothelial cells. It is highly expressed on activated T cells and platelets from which it can be cleaved as a soluble form, but it is not cleaved from B cells, DCs and macrophages. There is no up-regulation in neutrophils and endothelial cells, regardless of whether they are activated (Lievens, D; et al., 2009).

The CD40-CD40L system is a pathway which is associated with both prothrombotic and proinflammatory effects. CD40 and its ligand were first discovered on the surface of activated T cells, but its presence on B cells, antigen-presenting cells, mast cells, and finally platelets, is evident. The soluble form of CD40L (sCD40L) is derived mainly from activated platelets and contributes to the pathophysiology of atherosclerosis and atherothrombosis. Indeed, sCD40L has autocrine, paracrine, and endocrine activities, and it enhances platelet activation, aggregation, and platelet-leucocyte conjugation that may lead to atherothrombosis (Chen.Y et al., 2006).

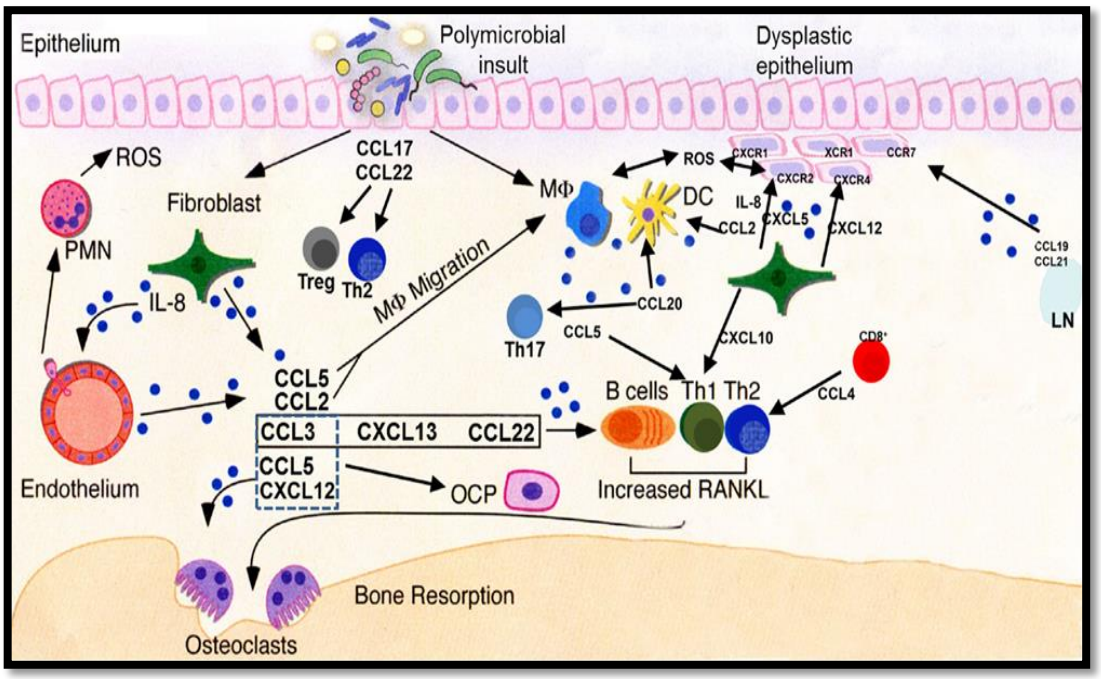


Figure 1.1 (m): Chemokines function in periodontitis and oral carcinogenesis Front. Immunol, 05 May 2015 Sec. Molecular Innate Immunity Volume 6 – 2015 https://doi.org/10.3389/fimmu.2015.00214

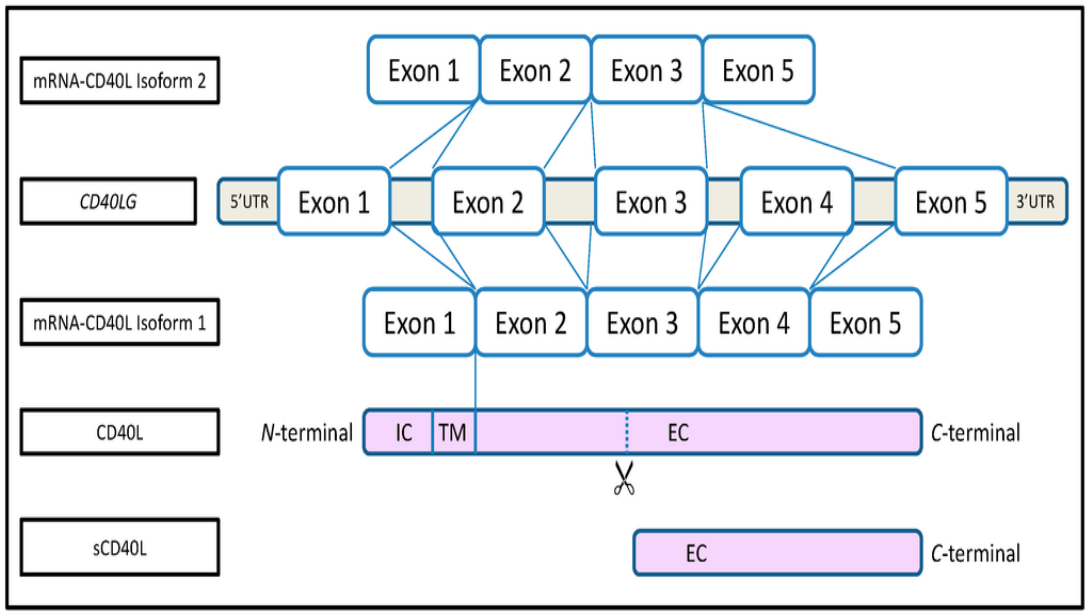


Figure 1.1 (n): Sheme of the CD40 ligand gene structure and its different isoforms.Intracellular domain (IC), transmembrane domain (TM), extracellular domain (EC).

CD40 ligand is abundantly present on platelets and activated T cells, existing as soluble and membrane-bounded forms. Binding of CD40L to CD40 receptors is expressed constitutively on B cells, platelets , endothelial cells, macrophages and smooth vascular cells that stimulates chemokines and pro inflammatory cytokine, matrix metalloproteinases (MMP) and CAMS resulting in activation of tissue factor producing thrombotic activity along with pro-coagulant effects, on the endothelium causing different forms of coronary heart diseases such as atherosclerosis, angina and acute coronary syndromes (Davì G et al ., 2012). The systemic challenge of bacterial toxins derived from the periodontal lesions represents an important link between periodontitis, monocytic inflammatory response, and metabolic dysregulation in certain disease states.

CD40L found in platelet cytoplasm and is being docked in the platelet α-granules. Despite being non-nucleated cells devoid of DNA apart from mitochondrial DNA platelets can retro transcribe RNA using a spliceosome that lead to detectable RNA messages for cytokines, CD40L is also produced de novo by activated platelets. (Doescher, A.; et al 2014) Recently, some RNA-seq studies did not find CD40L mRNA in platelets. This result suggests that a preformed protein is synthesized by megakaryocytes and stored in α-granules before platelet fragmentation. After stimulation by different agonists, platelets undergo a degranulation process via a well characterized mechanism, and either export the α-granule molecules to the membrane in a fixed form or secrete them as a soluble form. Granules fuse with the platelet membrane and display their fixed CD40L on the surface. This process occurs within seconds to minutes after stimulation (Henn, V.; et al 2001). CD40L is thus expressed on the platelet surface only after activation, and this molecule is identical in terms of structure and physiological function to membrane bound CD40L expressed in activated T-lymphocytes and other cells. It can notably generate signals for the recruitment and extravasation of leukocytes. It induces, through the engagement of CD40, the secretion of chemokines and the expression of adhesion receptors in endothelial cells (Henn, V; et al 2001). It provides a powerful link between platelets and the immune system: CD40L expressed on activated platelets induces dendritic cell maturation, B-cell iso-type switching, and augments CD8+ T-cell responses in both in vitro and in vivo model. Platelets do not maintain CD40L on their surface for long. It is cleaved and released in a

soluble form and may also be carried on the surface of microparticle-derived platelets. Platelets are the major source of sCD40L in the circulation. The normal range of sCD40L in the serum of a healthy adult is estimated to be 0.79 to 4.7 ng/mL, by means of immunoassay techniques (Chaturvedi.R,et al., 2014).

Exposure to periodontal infections has been postulated to perpetuate various inflammatory events; one of the primary being those involved in coronary artery disease (CAD). Low-grade bacteremia and endotoxemia in the periodontitis-affected patients appear to cause systemic effects on the vascular physiology. (Paquette DW, et al., 2017)

One of the important biomarker for periodontitis is miRNA- 1226-5p detected in body fluids is present in saliva, GCF and serum of patients with periodontal pathology. The identification of miRNA biomarker helps in prognosis and clinical diagnosis of periodontitis. It also facilitates alveolar bone lineage and new periodontal fibroblast development in regulating the homeostasis and integrity of periodontal tissues. It also contributes to osteoclast formation, morphogenesis of bone, neutrophil adherence and migration from blood capillaries into inflamed tissues (Olsen, I.; Singhrao, S.K.et al., 2017).

Immunological cells like lympocyte, neutrophil and macrophage maintains a constant interaction with the bacteria of periodontium by transmigration to gingival sulcus through junctional epithelium and releases alpha defensins which activates keratinocytes (Gursoy, U.K.; et al., 2013). The keartinocytes induces adaptative immune responses by stimulating complement system, opsonizing and killing the invading bacteria of the periodontium. Keratinocytes also activate PAMPS (pathogen associated molecular patterns) such as toll like receptors 1-9 (TLRs 1-9), cathelicidins, chemokines, cytokines which enhances periodontal immune responses (Elmanfi, S. et al., 2018). Dentritic cells act as antigen presenting cells (APC’s) producing a strong phagocytic activity enhancing a maturation process that involves their migration to lymph nodes that promotes CD4 T cells , B cells and Helper T cells activation. Uncontrolled up regulation of helper T cells induces osteoclast genesis that causes alveolar bone resorption (Song, L.et al., 2018).

Intensive research reveals that bacteria associated with chronic periodontitis results in various systemic diseases by raising the levels of systemic and local antibody response. High serum IgG antibody response to pathogens of periodontium serves as a major risk factor to cardiovascular events (Palm, F.et al., 2014).Cardiovascular disease (CVD) contributes to high rate of mortality due to dyslipidemia, stroke and ischemic infarction so this study will aims to identify the association of severe periodontitis with the induction and precipitation of systemic inflammatory responses due to translocation of circulating oral micro flora.

Secondly, the biologically plausible proposed mechanism of oxidative stress result in an elevated levels of MCP-1 and sCD40 L in saliva and GCF, which directly or indirectly results in the development of atherothrombogenesis.

Treatment of peridontitis is categorized as conservative, conventional and surgical. Regular brushing, flossing, avoiding alcoholism and smoking along with dietary modification are all included in conservative measurement (Lertpimonchai, Rattanasiri, Vallibhakara et al., 2017). Conventional therapy includes scaling and root planning which is the mechanical debriment of pathogenic bacteria from the tooth surface (Yap and Pulikkotil. et al., 2019). Surgical treatment includes gingevectomy, bone grafting and flap raising procedures such as Widman flap surgery and laser (Deas Moritz, Sagun Jr et al., 2016). However early awareness and intervention and incorporation of effective method to prevent severe periodontal condition through scaling and root planning will demonstrates significant reduction in bacteremia along with co-morbid of systemic diseases , shedding the burden of mortality and morbidity in various populations. Also periodontal therapy aims at eliminating the periodontal pathogens that raises the levels of these biomarkers, thereby minimizing the immune-mediated deleterious effects (Schenkein HA et al., 2013).

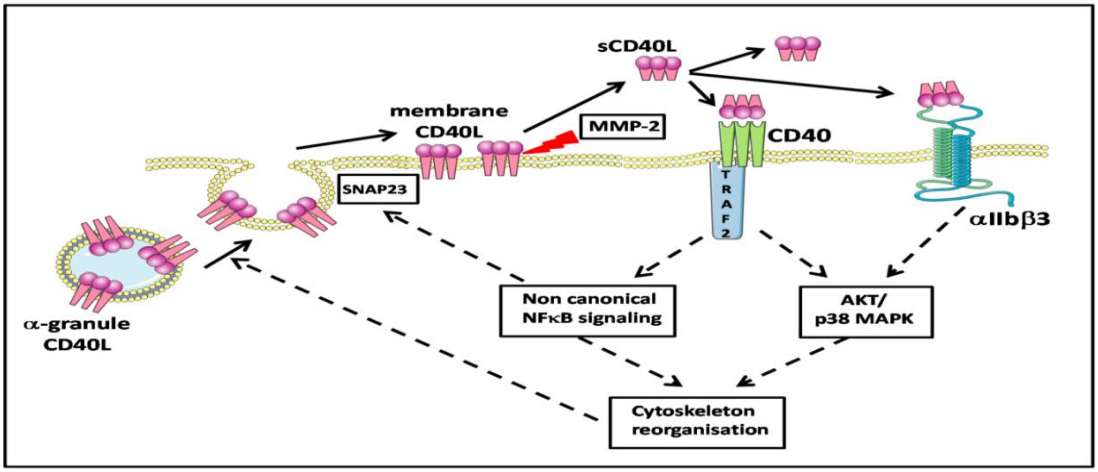


Figure 1.1 (o): Schematic overview of the regulation of platelet CD40L and the role of sCD40L in signaling after binding to platelet CD40 and αIIbβ3 inducing an auto-amplification loop. Synaptosomal-associated protein 23 (SNAP23), mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB), protein kinase B (AKT) matrix metalloproteinase-2 (MMP-2), TNF receptor associated factor 2 (TRAF2

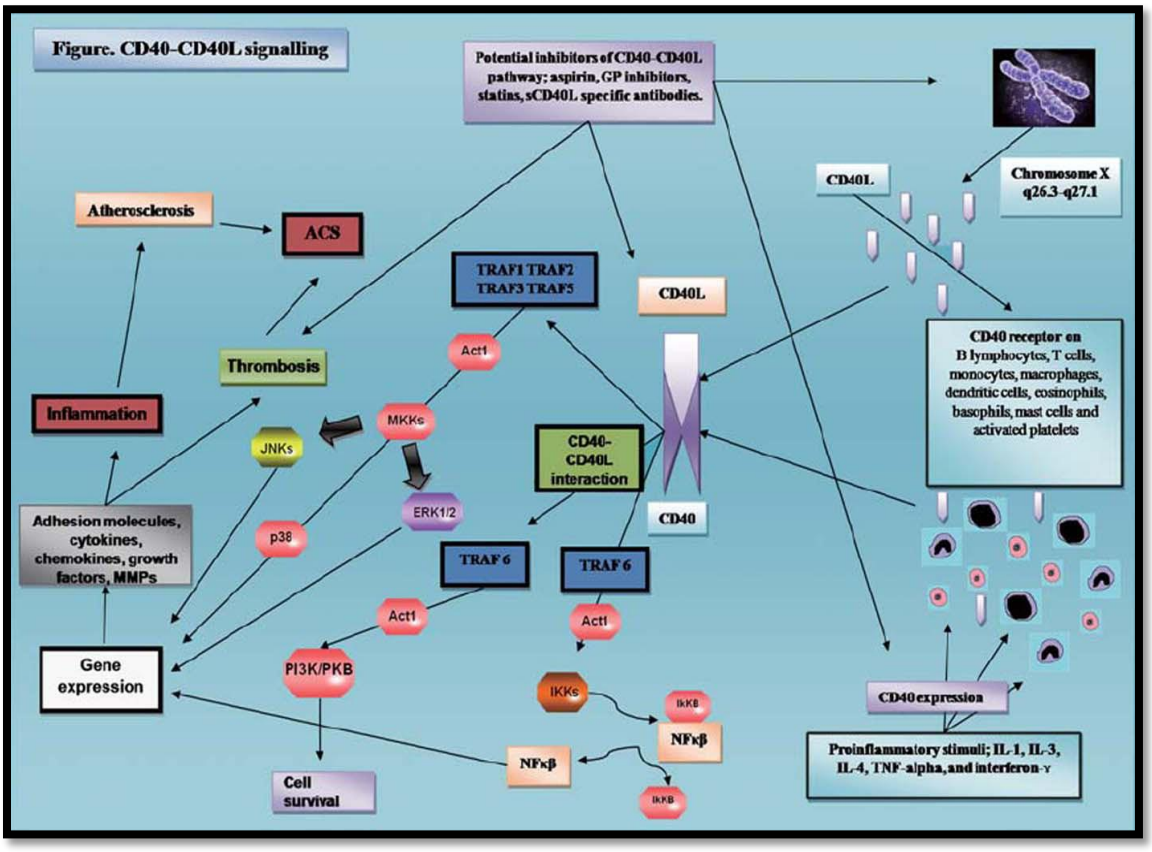


Figure 1.1 (p): Proinflammatory stimuli increasing expression of both CD40 and CD40L. Once CD40 is expressed on cell surface it interacts with its ligand sCD40L, and intracellular pathways conduct the signal.

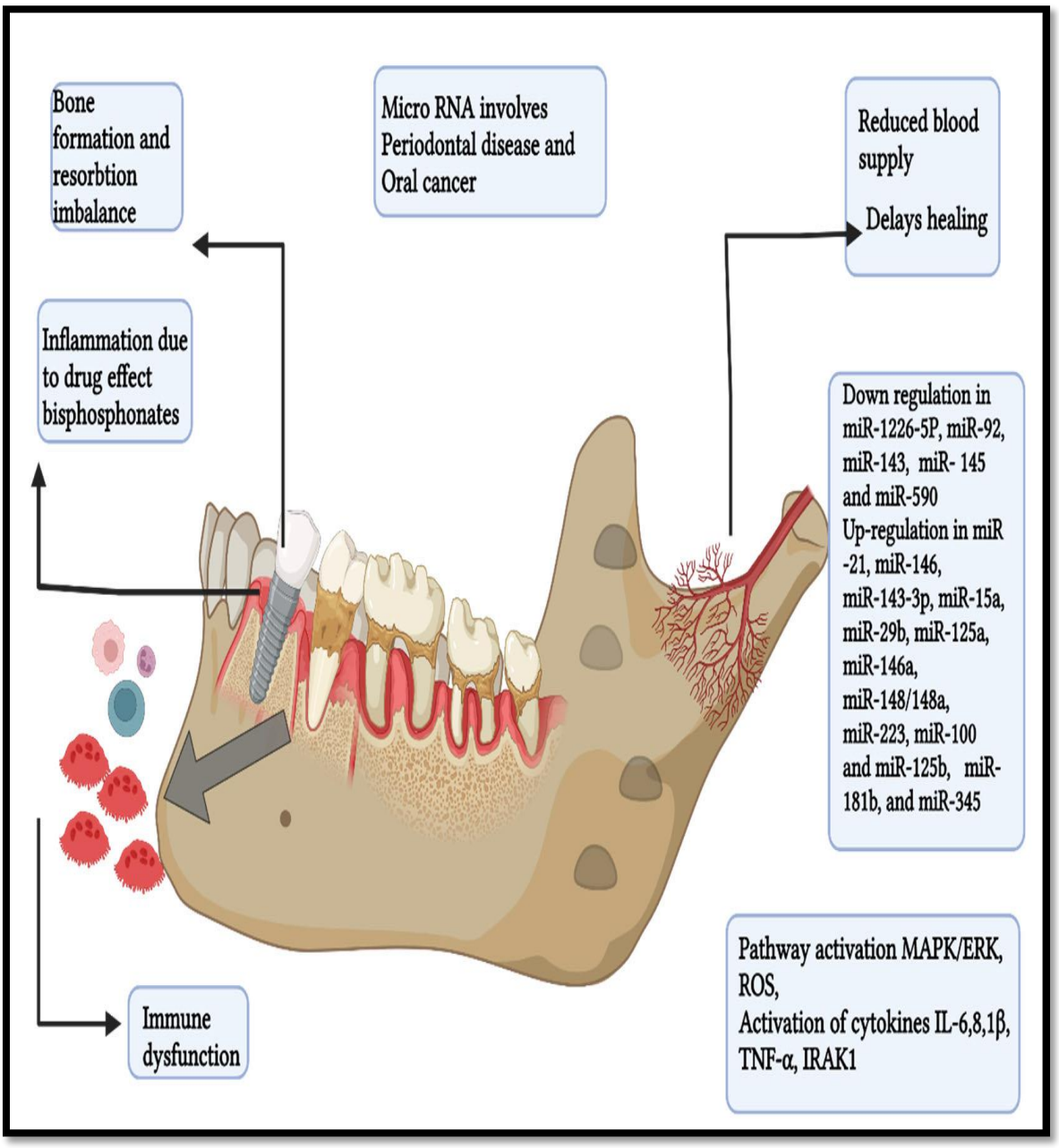


Figure 1.1 (q): Schematic diagram showing events in the oral cavity. This figure was created with Biorender.com (accessed on 9 September 2022).

The primary goal of periodontal therapy is to reduce the infectious and inflammatory challenge and to stop the progressing tissue destruction. Removal of pathogenic biofilms and suppression of inflammation can discontinue the periodontal tissue degradation; however, only limited regain of lost tissues occurs, depending on the form of tissue defects, systemic health status, and age (Reynolds, M.A.; Kao, R.T.; et al., 2016). In advanced cases, the active anti-infective treatment phase is often combined with surgery to eliminate residual pockets—with the aim of improving the ecology at periodontal sites—or sometimes with adjunctive systemic antimicrobials to reduce pathogen burden. In smokers, however, the treatment outcome is compromised, which makes smoking cessation an essential part of their periodontal therapy (Ryder, M.I.; Couch, E.T.; et al., 2018). Although anti- infective treatment reduces total bacterial counts, proportions of periodontal pathogens, as well as the number of sites colonized with pathogens, many of the species return with time. Therefore, daily oral hygiene of the patient and continuing professional supportive periodontal therapy are necessary to maintain the outcome and strengthen the long-term success of the treatment. Moreover, patients with advanced disease and masticatory dysfunction and bite collapse due to severe tooth loss have an obvious need for complex rehabilitation of the bite function as well as esthetic treatment. After treatment, however, periodontitis patients with prosthodontic reconstructions have still an increased risk for tooth loss, and many patient-related factors such as age, socioeconomic status, non-compliance, and diabetes are associated with abutment tooth loss (Müller, S.; Eickholz, P.; Reitmeir, P.; et al 2013). Since untreated periodontitis increases systemic low-grade inflammation, another treatment goal is to improve this condition. Although intensive mechanical periodontal treatment of patients with severely damaged periodontal tissues can cause an acute systemic inflammatory response and impair endothelial function, this occurs only transiently and, after six months, a significantly improved endothelial function is reached. Furthermore, periodontal treatment has been shown to reduce atherosclerotic biomarkers (e.g., IL-6, TNF) of individuals with cardiovascular disease or diabetes as well as to improve the glycemic status (Hba1c levels) of diabetic patients (Wang, X.; Han, X.; Guo, X.; et al ., 2014).

In conclusion periodontal disease is multifactorial and the imbalance between tissue loss and gain can occur due to various reasons, including aggressive infection, uncontrolled chronic inflammation, weakened healing, or all of the above simultaneously. Thus, successful disease management requires an understanding of different elements of the disease at the individual level and the design of personalized treatment modalities, including immunotherapies and modulators of inflammation (Alvarez, C.; Rojas, C.; Rojas, L.; Ca_erata, et al., 2018).

Figure 1.1 (r): Oral hygiene techniques (conservative method) Staro ftexas dental assisting school, brushing and flossing (2020) Conventional therapy

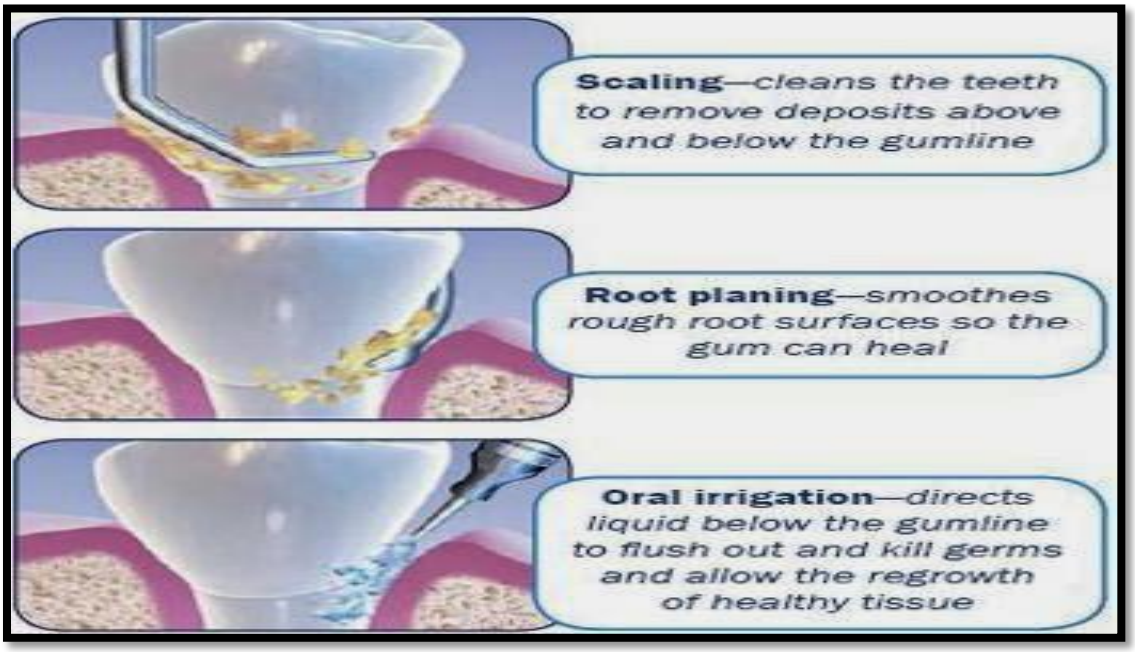


Figure 1.1 (s): Star of texas dental assisting school. Scaling-root-planing (2020)

1.2 RESEARCH GAP/ RATIONALE OF STUDY:

1.2.1 Theoretical gap:

Theoretically various postulation has stated that periodontal disease is interlinked with CVD risk but the causation and its association with each other that is, one results in causation of other has also not yet been updated. Uncertainty regarding the chronic periodontal disease, that preceeds to tooth decay and CVD risk due to increased plethora of inflammatory bio molecule MCP-1 and sCD40 L in gingival crevicular fluid, saliva, GCF and serum is the common cascador linking the two conditions has remained un-bridged. A correlative association has not yet been explored in a periodontal disease setting, so through this study, we have explored the existence of significant correlative association between the levels of MCP-1 and sCD40 L in saliva and GCF, in severely affected chronic periodontitis patients resulting in well established precipitation and acute progression of CVD's.

1.2.2 CONTEXTUAL GAP / ANALYSIS: N/A

1.2.3 METHODOLOGICAL GAP / ANALYSIS:

This study aims to bridge the wide gap between general medicine and dentistry by clarifying that chronic periodontitis is associated with the high CVD‘s risk due to these biomarkers (MCP-1 and sCD40 L) leading to worsening of life quality. However, incorporation of effective method like oral hygiene care and periodontal treatment, scaling and root planning will demonstrate a reduction in bacteremia along with co-morbid of systemic diseases thus shedding the burden of mortality and morbidity in various populations. Also periodontal therapy aims at eliminating the periodontal pathogens which are responsible for raising the levels of these biomarkers, thereby minimizing the immune-mediated deleterious effects.

1.3 PROBLEM STATEMENT:

Approximately 45% of mortality in worldwide population results from cardiovascular disease (CVD) due to dyslipidemia, stroke and ischemic. This study has aimed to identify that severe periodontitis is associated with the induction and precipitation of systemic inflammatory responses due to translocation of circulating oral micro flora. Secondly, the biologically plausible proposed mechanism of oxidative stress results in an elevated levels of MCP-1 and sCD40 L in saliva and GCF, which directly or indirectly results in the development of atherothrombogenesis.

1.4 RESEARCH QUESTION / HYPOTHESIS OF THE STUDY:

Null hypothesis: There is no association between elevated levels of MCP-1 and sCD40 L in saliva and GCF of a chronic periodontitis patients and the well established precipitation of acute Cardio Vascular Diseases.

Alternate hypothesis: There is an association between elevated levels of MCP-1 and sCD40-L in saliva and GCF of a chronic periodontitis patients and the well established precipitation of acute Cardio Vascular Diseases.

1.5 OBJECTIVE:

To determine the association between elevated levels of MCP-1 and sCD40-L in saliva and Gingival Crevicular fluid, of severely affected chronic periodontitis patients with well established progression and acute precipitation of Cardio vascular disease.

1.6 SIGNIFICANCE OF THE STUDY:

This study promotes individual awareness of maintaining appropriate oral hygiene and effective periodontal treatment along with CVD risk assessment in chronic periodontitis patients that promotes the reduction of bacteremial episodes and systemic levels of CRP , E selectins , IL-6 along with bio molecular markers, MCP-1 and sCD40 L of non communicable disease and co-morbid of systemic diseases that produces thrombotic activity along with pro-coagulant effects, on the endothelium causing coronary heart diseases such as atherosclerosis, angina and acute coronary syndromes lowering the burden of mortality and morbidity in various populations. It enables the practitioners including dentist and a cardiologist to promote the awareness of oral and general health maintenance in the population by increasing the number of periodontal assessment, coordinating referrals and comprehending the links between these interlinked disorders amongst the population. Mostly periodontal disease is under treated as it presents without pain, so it enables the dentists to carefully evaluate the history for the presence CVD's and MetS in their patients, which can be rectified in an appropriate manner like lifestyle modifications, induction of physical exercise, consumption of high-fiber-whole grain diet, smoking and alcohol cessation.

CHAPTER 2

LITERATURE REVIEW

Periodontitis is a chronic inflammatory response involving the supporting structure of the teeth including alveolar bone, gingival and periodontal connective tissue surrounding the teeth. Characteristically it is chronic in nature, preceded in response to the formation of oral bio film accompanied with the deposition of bacterial plaque.(Lang NP et al ., 2015). It commences with inflammation of gingiva by a group of bacteria adhering to tooth surfaces referred to as plaque induced gingivitis. This condition is present in its acute state but if left untreated progresses to chronic condition causing irreversible loss of gingival clinical attachment, periodontal ligaments resorption of alveolar bone; pocketing and eventual tooth loss (Kinane, Stathopoulou and Papapanou, 2017).Gingivitis and periodontitis are the two primary types of periodontal diseases. Gingivitis is a reversible condition causing by acute gingival inflammation treated with maintenance of good oral hygienic care and without subsequent loss of periodontal tissue (Lang NP et al., 2015). Gingivitis if untreated can lead to progressive periodontal destruction over the period of time. In contrary, periodontitis is induced by chronic inflammation that extends beyond the gingiva, causing irreversible connective tissue breakdown and attachment loss of structural root, and alveolar resorption in a susceptible individual (Dye BA et al., 2012).

Geographical variation plays a very important role in the prevalence of periodontitis as reported by W.H.O that 20-50% of the cases accounting for chronic periodontitis are attributed to differences in behavioral, socio-economical, systemic conditions and patterns of oral hygiene. Lack of oral hygiene awareness, low socio economic status and inappropriately expensive dental health care system enables the habitants of developed countries to become more prone in acquiring a compromised well established periodontal conditions as compared to the developed countries (Al Qahtani et al., 2017).

Glickman's classification of horizontal alveolar bone loss (Grade 4) refers to progressive periodontal infection caused by the bacteria, exposing the furcation areas involving the roots of teeth and alveolar bone ultimately resulting in teeth to loosen and fall (Barbara Buffoli et al., 2019). This loss of supporting structure causes ulcerated pocket formation occurs due to apical migration of the gingival epithelium.

Currently, the proposed classifications are based on the extension of the defect and the degree of horizontal/vertical attachment loss. Glickman (table 2.1) in 1953 developed a classification system in order to describe the extension and main characteristics of the furcation defect (Grade I–IV). Hamp, Nyman and Lindhe and Tarnow and Fletcher proposed to measure the horizontal/vertical attachment loss, respectively. Moreover, other classifications have been proposed in an attempt to describe the anatomy of the furcation more completely, describing the number of remaining bony walls (Heins, P.J et al 1968) the morphology of the existing bone (Easley, J.R et al 1969) and the relationship between root trunk and vertical/horizontal attachment loss (Hou, G.L. et al 1998). Most of the classifications of furcation lesions are unable to convey all the relevant information related to the marginal tissue position and its relationship with the furcation involvement (clinically exposed/non-exposed furcation). This information could be important for diagnosis, prognosis and treatment planning as well as for the communication between clinicians and researchers.

Table 2.1: Classification system; extension and main characteristics of furcation defect.

Glickman, I.	Grade I: Incipient lesion. Suprabony pocket and slight bone loss in the furcation area. Grade II: Loss of interradicular bone and pocket formation but a portion of the alveolar bone and periodontal ligament remain intact. Grade III: Through-and-through lesion. Grade IV: Through-and-through lesion with gingival recession, leading to a clearly visible furcation area.
Hamp, S.E.	Degree I: Horizontal attachment loss < 3 mm. Degree II: Horizontal attachment loss > 3mm not encompassing the total width of the furcation area. Degree III: Horizontal through-and-through destruction of the periodontal tissue in the furcation area.
Rosemberg, M.M.	**Horizontal** Degree I: Probing < 4 mm. Degree II: Probing > 4 mm. Degree III: Two or three furcations classified as degree II are found. **Vertical** Shallow: Slight lateral extension of an interradicular defect, from the center of the trifurcation in a horizontal direction. Deep: Internal furcation involvement but not penetrating the adjacent furcation.

Periodontitis if untreated proceed to progressive periodontium destruction, resulting in mobility of tooth, loss of tooth and compromised function of mastication (Eke PI et al., 2012). Human oral cavity comprises of approximately 700 different bacterial strains, predominating in various habitats particularly in periodontal sulcus and teeth. Streptococcus oralis, Streptococcus sanguis (S. sanguis) Actinomyces naeslundii and Actinomyces odontolyticus typically colonize the supragingival areas of the tooth. (Wei L, Thornton-Evans GO et al ., 2012). Sub gingival sulcus is predominantly colonized by Veillonella parvula, Actinomyces naeslundii, Streptococcus oralis, Streptococcus sanguis, Actinomyces odontolyticus, (Wei L, Thornton-Evans GO et al., 2012). Periodontal pocket formation due to chronic inflammation is associated with a concomitant colonization of supra gingival

anaerobic microbiota comprising of microaerophilic gram-negative bacilli, anaerobs and oral bacterial microbial as such as P. gingivalis (Eke PI et al., 2015).

The prevalence and incidence of periodontitis has been the most challenging task in epidemiology, so to characterize there are different diagnostic approach, criteria and methods (Wei L, Thornton-Evans GO et al., 2012). World Health Organization (WHO) in 1982 presented a more reliable and reproducible data to assess periodontitis in suspected individual referred to as "Community periodontal index of treatment needs" (Eke PI et al., 2015).

Given Proposed case definition of peridontitis by American Academy of Periodontology (AAP) and Centers for Disease Control and Prevention (CDC) are as follow:

1. No periodontitis" is graded as "No symptoms of mild, moderate, or severe periodontitis.
2. Mild periodontitis" is graded as "≥2 interproximal sites with clinical attachment loss (CAL)≥3 mm, and ≥2 interproximal sites with periodontal probing depth (PPD)≥4 mm (not on same tooth) or one site with PPD≥5 mm.
3. Moderate periodontitis" is graded as "≥2 interproximal sites with CAL≥4 mm (not on same tooth), or ≥2 interproximal sites with PPD≥5 mm (not on same tooth)."
4. Severe periodontitis" is graded as "≥2 interproximal sites with CAL≥6 mm (not on same tooth) and ≥1 interproximal site with PPD≥5 mm" (Eke PI et al ., 2012) .

According to American academy of Periodontology 1999 guidelines /American dental association classification of chronic periodontitis is based on site and severity of the disease. On the basis of site it is categorized as generalized and localized. Extent and severity of periodontal diseases is based on the degree of damage produced and identification of specific factors that attributes in the case management of the chronic condition. Clinical attachment loss (CAL) is determined in the initial stages; if it is not evident then radiographic bone loss (RBL) is assessed. Revised guidelines of 1999 of American Dental Association stated that severity of chronic periodontitis is characterized as stage 1 (mild) with probing depth of <4mm along with 1-2mm CAL and coronal third of radiographic bone

loss(< 15%), stage 2 (moderate) with probing depth of <5 mm with 3-4mmCAL and coronal third of radiographic bone loss (15-33%) , stage 3 (severe) with probing depth of > 6mm , CAL of > 5mm , radiographic bone loss of middle 3rd of the root or beyond associated with vertical bone loss , furcation involvement and tooth loss . (perio.org/ 2017 wwdc; the good practioner's guide to periodontology, 2016; Attia et al., 2017)

United States (US) adults have high prevalence rate of peridontitis , contributing to a very high risk of chronic infection, that constitutes an important health risk concern of public health (Wei L, Thornton-Evans GO et al ., 2012). A recent survey by National Health and Nutrition Examination Survey (NHANES), showed 46% of the US adult population aging 30 years or above suffered from periodontitis, with 8.9% severely affected by chronic periodontitis "(Eke PI et al ., 2015).

From 2012 to 2013, US population reported periodontitis in 44.7% (SE: ± 2.4%) of the adults aged ≥30 years" (Eke PI et al., 2015) .From 2013 o 2014 as reported by NHANES the periodontal estimation was consistent with the 47.2% (SE: ±2.1%) So combined period from 2012 – 2014 the prevalence of periodontitis was 45.9%, (141 million adults aged ≥30 years) (Eke PI et al., 2015).

Prevalence of chronic periodontitis in population of adults aged 35 to 40 years is reported to be 42%, while in Germany according to WHO one out of five are the sufferers of the chronic form of the disease. 10.7% of Portugal population has chronic periodontitis (Machado et al., 2019).

Asian countries have higher prevalence rate of chronic periodontitits among which India is documented to have 85% (Akhilesh et al., 2016). 96% recorded in Pakistan by a National oral health survey, 18% population have periodontal problems out of which 31% has periodontitis (Bokhari, Suhail, malik et al., 2015).

Prevalence in Arabia was categorized on the basis of periodontal pocket depth, 52% mild pocket depth and 32% deep pocketing. A study conducted among the Saudian population revealed that 6 mm of clinical attachment loss and 6mm of pocketing in one out of 10 participants (Elabdeen, Mustafa, Hasturk et al., 2015).

Periodontium, a complex structure comprising of cementum, alveolar bone, gingival and periodontal ligament all of this makes up periodontal apparatus. The major function of periodontium is to keep the tooth supported on the socket, maintain its integrity and serves as a barrier from the mastication forces and oral micro flora (Katancik et al., 2016).

Healthy gingival has stippled appearance and is coral pink in color that encircles the tooth resisting the food friction by tightly adhering to the alveolar bone. Inflamed gingiva has edematous appearance, red in color that bleeds excessively due to accumulation of bacterial plaque (Chen, Ahmad, Li et al 2015). Clinically chronic periodontitis exhibits changes such as bleeding on probing, attachment loss, pocket formation, halitosis, redness, edema, furcation involvement, horizontal and vertical bone loss ultimately leading to tooth loss (Meseli et al., 2017).

Mineralized connective tissue bearing the tooth is alveolar bone. Vascularized, fibrous connective tissue structure that attaches to the cementum and is called periodontal ligament. Cementum is the calcified layer over the tooth root that adheres to the alveolar bone. It has the ability to withstand sheer occlusal stresses and ability to repair and maintain potential integrity of tooth root (Jiang et al., 2016).

Various factors predisposes to the etiology in progression and initiation of periodontitis including environmental, microbial, local and systemic factors (Natto, Ahmad, Alsharif et al 2018). Calculus and plaque are the local factor that causes inflammation to gingiva resulting in irreversible loss of periodontal apparatus.

Plaque is a sticky matrix of extracellular polymeric material formed by the bacteria called as microbial film. This along with pathogens of gingival blood stream that contains inflammatory mediators and pathogens are the major risk factors for systemic diseases (Colombo and Tanner, 2019).

Smoking is a well-recognized risk factor for PD and ACVD. Among toxic products generated during smoking, nicotine is one of the most harmful. This substance is

responsible for vasoconstriction that compromises delivery of nutrients to the periodontium. In addition, nicotine significantly suppresses cellular/humoral immune responses and causes neutrophil dysfunction. Similarly, toxic products produced during burning of tobacco induce the atherogenic mechanism by increasing oxidation of LDL, causing chronic inflammation in the intima layer and subsequent endothelial dysfunction. Smoking increase platelet aggregation, blood viscosity and shifts the pro- and antithrombotic balance toward increased coagulation (Li H et al., 2019).

Dental plaque becomes calcified due to improper tooth brushing and flossing leading to the formation of sub and supra gingival calculus that varies according to the degree of mineralization. Lingual and facial aspects of mandibular and maxillary teeth respectively are the sites of deposition of calculus (Balaji, Niazi, Dhanasekaran, et al 2019). Pathogens such as Porphyromonas gingvalis, L .acidophilus, and Treponema Denticola, Aggregatibacter Actinomycetecomitans and Staphylococcus aureus are the major pathogens initiating and progressing periodontal infection (Han, Wang and Ge et al 2017). Chronic periodontitits is caused by the pathogens such as Porphyromonas gingivalis, Treponema denticola and Tannerella forsythia. Tobacco smoking and chewing causing premature tooth loss is the most important factor responsible for poor oral and periodontal health status of an individual. Another important factor is the removable partial denture (RPD) that causes gingival inflammation , tooth mobility, alveolar bones loss , deep periodontal pocket and heavy plaque deposition(Qureshi, Hamid, Khan et al 2018) .

Host immune responses are initiated by bacterial Lipo-polysaccharides that produce inflammatory mediators such as MMP, prostaglandins and cytokines that enhances collagen breakdown. (Franco, Patricia, Timo et al., 2017). Several systemic conditions have a strong associative relationship with chronic periodontitis like cardiovascular diseases (CVD’s), diabetes, hypertension and obesity (Siddiqi, Zafar and Sharma et al., 2019).

CVD' are closely related to periodontitis due to the reason that systemic inflammation progresses in moderate to chronic forms and while treating them causes a significant reduction in the levels of inflammatory mediators . The bacterial pathogens may enter the bloodstream through the necrotic / damaged tissue which further invades the cardiovascular system (Bui.Almeida-da- Silva, Huynh et al., 2019).

Obesity compromises the periodontal therapy by delaying the healing process. Studies have demonstrated that adipokines such as leptin, Visafatin and adiponectin produced by periodontal cells are regulated by pathogenic microbes of periodontium (Arboleda, Vargas, Losada et al., 2019).

First line of immune defense against the pathogenic Lipo polysaccharide antigen is produced by leukocytes. Monocytes secrete cytokines such as IL- 1 and TNF that causes bone resorption. Collagen destruction, pocket formation and gingival attachment loss is caused by (matrix metallo proteinases) MMP (Bosshardt et al., 2017). Transcription mediator initiating periodontal inflammation representing cytokine expression at molecular level is known as Nuclear Factor kappa B (NFKB). This molecular cytokine in turn enhances the production of inflammatory mediators such as MMP and prostaglandins. Osteoclastic cell activation leading to bone resorption by RANK activation is produced by IL-1 NFKB, TNF (Pan, Wang and Chen. 2019).

Studies have revealed that macrophages secrete a large amount of TNF alpha that is particularly isolated from GCF (Gingival crevicular fluid) and serum of patients with chronic periodontitis (Kany, Vollrath and Relja, 2019). This TNF alpha in turn stimulates the production of prostaglandins E2 and collagenase that potentiates bone resorption (Martineez-Anguilar, Carrillo, Sauri-Esquivel et al., 2019).

WHO in 2012 has estimated approximately 17.5 million deaths (31%) of the total in which the attributable major cause of mortality is Atherosclerotic cardiovascular disease (ASCVD), peripheral arterial disease (PAD).

Cerebro Vascular disease (CBVD) and Myocardial Infarction (MI) as the most common types pathogenesis of all shares a common implicative factor that is inflammation (World Health Organization.Global status report on non-communicable diseases 2014 [Updated 2014].

Atherosclerosis is a chronic inflammatory condition contributing to CBVD/stroke and CVD, pathogenesis involves accumulation of fatty plaques composed of calcium cholesterol esters , cholesterol along with fibrous matrix complex (these cholesterol- fat rich streaky plaques contains numerous fibroblast and immune cells) which are deposited within the intima of the vessel walls leading to narrowing and thickening of the arterial lumen causing a decreased blood flow and blood clot formation called thrombus rupture of which cause it to travel distally and occlude the arteries causing stroke and myocardial infarction (Libby P et al ., 2019).

Atherosclerosis individuals have elevated concentration of serum inflammatory biomarkers, such as C-reactive protein (CRP) (Shah SH et al., 2013) cell adhesion molecules, various inflammatory cytokines and fibrinogen (Blake GJ et al., 2012). In atherosclerosis in addition to the accumulation of cholesterol debris deposited on the vascular wall , pathogenesis of the disease is further aggravated by immune mediators such as TNF- alpha , IL-1 and IL-6 which are released by foam cells or leukocytes, (Cardoso et al., 2018).

A pro-inflammatory environment is produced by persistent microbial inflammation. Infection can drive autoimmunity to vascular cells, thus initiating the atherosclerotic process (Hamilton et al., 2017). Chronic Periodontitits due to its sustained inflammatory nature correlates with an increased risk for cardiovascular disease. Bacteremia and systemic inflammation not only lead to the aggravation of endothelial lesions, but also potentiates inflammatory processes in the vascular wall (Leira et al., 2019). Vascular inflammation is, in turn, modulated by

proinflammatory cytokines such as TNF, IL-1, and IL-6, both in chronic periodontitis and in CVDs. A decrease in systemic inflammatory biomarkers secondary to periodontal treatment leads to beneficial features contributing to the reduction of cardiovascular risk (Preshaw et al., 2020). However, even if periodontal interventions result in a reduction of systemic inflammation and endothelial dysfunction in the short term, there is no significant evidence that these treatments actually prevent atherosclerotic vascular disease or are able to modify its outcomes (Cardoso et al., 2018).

Sustained periodontal inflammatory states will alter the count of circulating neutrophils due to increased bone marrow output or mobilization of the marginal granulocyte pool (Cecoro et al., 2020). Uptake of advanced glycation end products (AGEs) during PD is reported to drive pro-inflammatory signals that, in turn, trigger redox-sensitive transcription factors deemed responsible for hyper-permeability of the endothelial cell, VCAM-1 (vascular cell adhesion molecule 1) activation, chemotaxis and the release of TNF, IL-1, IL-6 into the bloodstream (Furutama et al., 2020). These circulating inflammatory mediators are able then to modify endothelial function, leading to impaired vasodilation and causing significant alterations in the vascular structure (Cecoro et al., 2020). TNF-_ and IL-6 in particular, are associated with diminishing nitric oxide production and endothelial dysfunction, both known precursor factors for atherosclerosis and CVDs. The relationship between Periodontitis and CVDs has been traced back to factors such as transfer of periodontal bacteria to atheromatous plaques, change of lipid metabolism (Ferguson et al., 2020), endothelial dysfunction, shared genetic risk factors, as well as pro-inflammatory cytokine (especially IL-6 and TNF-_) spillover in the bloodstream coming from periodontal tissues. Severe Periodontitis increases the risks for acute myocardial infarction and stroke and it is known that periodontal treatment significantly reduces their incidence (Bui et al., 2019; Cecoro et al., 2020). It has been reported that chronic inflammation in PD has a significant impact on the long-term clinical outcomes in patients with atrial fibrillation (Imamura et al., 2013).

Chemokines are a family of small, signaling proteins having small molecular weight of 8–14 kDa secreted by the cells of immune system. These are chemotactic cytokines responsible for the regulation of movement of other cells in response to a chemical stimulus (chemotaxis) thus emanating their name as chemokines. The family of chemokines is characterized into four families with two main subgroups (CXC and CC) and two small subgroups (CX3C and C) (A. Zlotnik, O.et al., 2000). This classification is broadly based on the presence of the cysteine amino acids nearby N-terminus. Monocyte chemoattractant protein-1(MCP-1)/CC chemokine ligand-2 (CCL2) belongs to the CC family which have cysteines adjoined closely to N-terminus. The cysteine residues are linked by disulfidebridge between the first and third cysteine and between the second and fourth cysteine (E. Van Coillie et al., 1999). The other MCPs found in humans other than MCP-1 are MCP-2(CCL8), MCP-3(CCL7) and MCP-4(CCL13) sharing about ~ 60% homology. (J. Lin et al, 2014) .CCL2 is the first purified and best characterized human CC chemokine, identified because of its monocyte chemotactic property in-vitro in human cell lines. The location of MCP-1 gene in chromosome is at 17q11.2-q21.1 with three exons and two introns. The MCP-1 precursor molecule consists of hydrophobic amino terminal signal peptide of 23 amino acids. The mature protein contains 76 amino acids after the cleavage of signal protein, forming monomeric polypeptide. MCP- 1 values in the range of 350-450pg/ml. (S.L. Deshmane et al., 2009).

The endothelial function is essential for the homeostasis of the body and its dysfunction is correlated with several patho-physiological conditions, including atherosclerosis, hypertension and diabetes. Endothelial cells control cellular adhesion, thrombo resistance, proliferation of smooth muscle cells, and inflammation of the vessel wall by generating a broad variety of factors. The disrupted bioavailability of NO is the characteristic of endothelial dysfunction (C.M. Sena et al., 2013). Vascular endothelium injury contributes to the inflammation by secretion of cytokines and chemokines (MCP-1) and to the expression of adhesion molecules by endothelium damage. MCP-1 activation of CCR2 is responsible for vascular endothelium renewal after injury, angiogenesis, and collateral formation (I.K. Kwaifa., et al 2020). The MCP-1 is responsible for

the migration of monocytes to the sub-endothelium and attracting leucocytes to the site of injury, thus facilitating atherogenic and thromboembolic potential. The monocytes get matured as macrophages, releasing cytokines, and combine with the accumulated oxidized LDL to form foam cells. The smooth muscle cells also proliferate and migrate to the sub-endothelial space due to the effect of proinflammatory cytokines and growth factors interaction. There is formation of fatty streak which eventually leads to atherosclerotic plaque. The MCP-1 level in human atherosclerosis has been correlated with plaque vulnerability (D.P. Ramji et al., 2015).

Approximately 10 – 15 % of a generalized inflammatory response is evoked by the presence of chronic periodontitis which increases the risk for CVD a major systemic disease states. A strong association exists between oral infection and myocardial diseases as clearly stated by epidemiological studies due to the strong correlative association between the subgingival microbiota and the pathogens present in the vascular lesions (Mahendra J et al ., 2014).

Most of the studies concluded that the levels of mediators like CRP found in individuals, with both CVD and periodontitis were additive relative to levels found in patients with either condition. Individuals suffering from periodontitis on average presented with 14–15% greater risk of developing CVD from prospective trials while the odds increased to more than 100% when analyzing case-control studies compared to healthy individuals (Packard RR et al., 2018).

There is a Strong association between periodontitis and atherosclerosis initiated by inflammatory mechanisms by the bacteria of periodontal lesions like Porphyromonas gingivalis which is an invasive periodontal bacterium along with its byproducts invade the systemic circulation (Takeuchi H et al., 2012). These bacteria exacerbate the expression of pro-inflammatory cytokines causing up-regulation of endothelial cell activation, promoting activated leukocytes infiltration resulting in the propagation of the atherosclerotic plaques (Packard RR et al., 2018). The mainstay of pathogenesis is the higher levels of GCF of periodontits patients having MCP-1 and sCCD40L mediator molecules that involves bio-molecules movement

in the inflammatory and immune cascade levels that initiates and propagates an atherosclerotic state increasing the systemic inflammatory load.

A potent monocyte activator sCD40 L ligation on human monocytes induces phenotypic changes that cause T-cell activation by the monocytes enhancing the inflammatory response by releasing CC-chemokine, MCP-1 from mononuclear cells. MCP-1 induces the infiltration of the activated leukocytes into the atherosclerotic lesion, which directly activate smooth muscle cells, macrophages, and T cells inside the vessel wall (Stumpf C et al., 2016).

CD40 ligand (CD40L) is a transmembrane glycoprotein structurally related to TNF-α which was originally seen on activated CD4+ T cells but recently found in large numbers of platelets (Phipps RP., 2000). CD40 ligand exists in two forms, membrane bound and soluble (sCD40L), which further bind to CD40 receptor, that is constitutively expressed on B cells, macrophages, endothelial cells, and vascular smooth muscle cells resulting in production of cytokines, connective tissue-degrading enzymes, and up regulation of inflammatory responses. The normal value of sCD40 L ranges between 406.83-547.36 pg/ml (Schonbeck U, et al., 2003). It also promotes thrombotic activity by enhancing the expression of tissue factor in platelets as well as via direct effect on the pro-coagulant activity of the endothelium (Zhou L., et al., 1998). The systemic challenge of bacterial toxins derived from the periodontal lesions represents an important link between periodontitis, monocytic inflammatory response, and metabolic dysregulation in certain disease states. Exposure to periodontal infections has been postulated to perpetuate various inflammatory events; one of the primary being those involved in coronary artery disease (CAD). Low-grade bacteremia and endotoxemia in the periodontitis-affected patients appear to cause systemic effects on the vascular physiology (Paquette DW, et al., 2017). Gingival crevicular fluid (GCF) is an inflammatory exudate that can be collected at the gingival margin or within the gingival crevice, the analysis of which provides for an accurate quantitative biochemical assessment of the local cellular metabolism that reflects the patient's periodontal health status (Champagne CM, et al., 2003) .

Dissemination of bacterial dental plaque in the circulation can results in platelet activation leading to increased expression of both surface and soluble CD40 L that interacts with CD40 receptor on macrophages, endothelial and fibroblasts to promoting progressive atherosclerosis and its destabilization (Tonetti MS et al., 2014). So there is a positive correlative association between sCD40 L and MCP-1 levels in GCF levels of chronic periodontal patents as one of the important pathways leading to the propagation of cardiovascular events in patients with periodontal disease (Tonetti MS et al ., 2014).

In chronic periodontitis, natural immune mechanisms comes into interplay which activates numerous pro inflammatory reactants like CRP , fibrinogen and PAI-1 propagating systemic inflammation They cause repair and regeneration of a various tissues, neutralization of virulent pathogens and complement factors activation. Various Meta-analytical reports have significantly reported that inflammation response plays a positive role for an association between periodontal disease and CVD (Loos BG, 2015).

Infective endocarditis (IE) is an infection of the endocardial surface of the heart, impacting one or more heart valves, caused by bacteremia originating from the oral cavity, gut, skin, or mucous membranes. The transient bacteremia caused by these pathogens activates an inflammatory cascade similar to that which is associated between periodontitis and CAD. A low-grade bacteremia from an oral source with good oral hygiene, such as tooth brushing, yet most healthy individuals do not develop IE. Historically, it was felt that IE was a threat only to those with rheumatic or congenital heart disease, with studies suggesting that turbulent flow across valves in these conditions results in endothelial damage, making them more susceptible to IE. However, while the incidence of rheumatic heart disease has decreased over time, cases of infective endocarditis have continued to increase and almost half of IE patients have structurally normal hearts prior to diagnosis. The exact means by which certain individuals are susceptible to IE is yet to be elucidated, but it appears that a complex network of immunologic and thrombotic factors is at play that ultimately provides bacteria with an environment in which to thrive and counteract an effective immune response (Liesenborghs L, et al ., 2020). Despite efforts

towards earlier diagnosis and surgical intervention, there is significant morbidity and mortality associated with IE. Approximately 80% of IE is caused by staphylococci or streptococci. Viridans group streptococci, most commonly found in the oral cavity was found in 16% of patients in one large cohort (Murdoch DR, et al., 2009). As early as the 1950s, the American Heart Association published guidelines for antibiotic prophylaxis in patients with rheumatic or congenital heart disease, due to studies showing antibiotics decreased bacteremia after dental extraction. A 2005 study of patterns of IE in congenital heart disease patients cited dental procedures and infections as the leading "cause" of IE and emphasized on the use of antibiotics for lower risk patients (Di Filippo S, et al., 2006).

Atherosclerotic disease is a focal thickening of vascular intima residing between the endothelial lining and smooth muscle cell (SMC) layers of blood vessels in response to an immune response (Ross R. et al 1999). Endothelial dysfunction is the earliest change in atherosclerotic formation. The primary etiological factor of atherosclerosis is not known. However, other risk factors significantly contribute to the development and progression of this pathology, such as aberrant profile of plasma cholesterol, smoking, hypertension, DM, and increased levels of inflammatory mediators including CRP and cytokines (Libby P.2002). Atherosclerosis starts with accumulation of low density lipoprotein (LDL) within the intima layer where it is oxidized. This in turn activates increased expression in nearby endothelial cells of cell surface proteins such as ICAM-1, vascular cell adhesion molecule-1 (VCAM-1), and selectins (Libby P. 2002). Adhesion of circulating inflammatory cells (monocytes, lymphocytes) to these adhesion molecules is increased by their diapedesis into the inflamed intima site. The initial development of the atherosclerotic lesion occurs through differentiation of monocytes to macrophages that scavenge on LDL, thus forming foam cells and subsequently fatty streaks (Libby P.2002). Later, a T-leukocyte induced-cell-mediated immune response with increased level of inflammatory cytokines such as INF-γ, TNF-α, and IL-1β further accelerate atherogenesis (Jawień J. et al., 2008). T-cell-associated mediators stimulate migration and mitosis to form a fibrous pseudo-capsule around the lesion. Macrophages loaded with lipids undergo apoptosis leading to formation of a necrotic core underneath the fibrous capsule,

which renders it susceptible to rupture, thus leading to formation of fatal thrombosis (Ross R. et al 1999).

Cumulative evidence from literature over the last decades has supported the role of PD as an independent risk factor for ACVD (Sanz M, et al., 2020). The presence of certain periodontal pathogens, Gram-negative anaerobes in particular, in subgingival biofilm has been associated with increased risk of myocardial infacrtion MI. The hallmark of periodontitis is elevation in the levels of Gram-negative bacteria that are characterized by their ability to trigger an intense immune response via their mechanism of pathogenicity, such as lipopolysaccharide (LPS) (Cekici A, et al., 2014). Moreover, some of these bacterial species possess the potential to invade deeper tissues, reaching the circulation and inducing a systemic immune response away from their original habitat (Velsko IM, et al., 2014). In vivo and in vitro studies have suggested that periodontal bacteria associated with chronic inflammation may compromise the epithelial-barrier function by epithelial-mesenchymal transition (Abdul kareem AA et al., 2018). Epithelial-mesenchymal transition comprises cellular events starting with loss of polarity, cytoskeletal, and adhesion proteins, ending with loss of epithelial-phenotype and acquisition of mesenchymal-like characteristics. This results in loss of epithelial sheet coherence and formation of micro ulceration thus, facilitating the penetration of motile periodontal pathogens/virulence factors to the underlying connective tissue and exposed blood vessels. On the other hand, periodontal bacteria can invade host cells as part of their defensive strategy to evade host immune responses. This intracellular localization provides not only protection from the body's defensive mechanisms but also a shelter from action of antimicrobials. Periodontopathogens such as P. gingivalis residing within the cells either stay dormant or multiply by modulating cellular machinery (Hertzén E, et al., 2010). Once multiplied, P. gingivalis leaves the epithelial cells via the endocytic recycling pathway to infect other cells or gain access to the circulatory system. The trafficking of P. gingivalis into endothelial cells is positively influenced by bacterial load, and certain virulent proteins such as gingipains, fimbriae, and hemagglutinin A .Further, invasion of gingival epithelial and endothelial cells by P. gingivalis could be synergized by Fusobacterium nucleatum and T. forsythia (Inagaki S., et al 2006).

Two mechanisms have been proposed to explain how PD influences ACVD. First, a direct mechanism by which periodontal pathogens invades endothelial cells. This notion is supported by polymerease chain reaction assays for atherosclerotic plaques (Ford PJ et al., 2006). Analysis of cardiovascular specimens containing thrombus tissues demonstrated that Streptococcus mutans was the most prevalent bacteria (Nakano K, et al., 2009). Atherosclerotic lesions formed in coronary arteries also exhibited the presence of other bacteria such as P. gingivalis, Prevotella intermedia and T. forsythia. It is not clear how the presence of bacteria intracellularly influences atherosclerosis but some pathogens, e.g., P. gingivalis, could trigger foam cell formation or their persistence within the cells, and thereby provoke a state of secondary inflammation that leads to endothelial dysfunction.Increased systemic levels of inflammatory cytokines due to PD is the second suggested mechanism (indirect pathway). PD stimulates a systemic inflammatory response which results in chronically elevated levels of different cytokines, also related to atherosclerotic vascular disease, such as IL-1β, IL-6, IL-8, TNF-α and monocyte chemoattractant protein-1(MCP-1). Some can enhance rapid hepatic synthesis and secretion of intravascular plasma proteins such as CRP protein and fibrinogen (Caúla AL, et al., 2014). Additionally, bacterial products such as LPS could enter the circulation and induce a potent immune response.These aforementioned factors could initiate atherosclerosis by their action on endothelial cells, modulating lipid metabolism, and increasing oxidative stress (Ford PJ et al., 2006).

CRP, TNF, t-PA , LDL-C , fibrinogen, hepatoglobin , IL-8 and PAF are the biomarkers of endothelial dysfunction and dyslipidemia which are not only the risk factors of CVD but are also an important augmented inflammatory marker of peridontital diseases (Buhlin K et al., 2012). An association between periodontitis and modest systemic inflammatory response has been concluded by the possible spilling over of these mediators from the periodontal lesions into the systemic circulation which affects the tissues and organs located distally from the oral cavity like liver that may be affected leading to induction of an acute-phase response influencing various body system, consequently developing atheromas (Teles R et al., 2012).

Bacteria in sub gingival plaque called P .gingivalis induce periodontal inflammatory destruction and atheromatous lesions. (Tomás I et al., 2012) Human atherosclerotic plaque has high concentration of Chlamydia pneumoniae (C. pneumoniae) (Kebschull M et al., 2013). Apoptosis, foam cell production and endothelial dysfunction in the intima of coronary artery is caused by P. gingivalis. Rupture of atherosclerotic plaques as well as periodontal destruction is caused by MMP (Behle JH et al., 2016). Chronic periodontal patients have increased levels of IL-6 that stimulates hepatic cells to produce CRP, pro-coagulant mediators (Loos BG, 2015). CRP is synthesized in liver which induces systemic inflammation. Elevated levels of CRP (≥2.1 mg/l) are an important marker for atherosclerosis, stroke and MI has are closely associated with increased levels of CRP (>2.1 mg/l) (Behle JH et al., 2016).

PAI-1, fibrinogen, (vWF), as well as markers of platelet activation are thrombotic markers, implicated in association between CVD and periodontitis. Higher fibrinogen plasma levels causes higher blood viscosity, blood volume and shear stress, which enhances endothelial cell activation , platelet activation subsequently atheroma formation (Rizzo M et al., 2012).

Heat-shock proteins (HSPs) produced under stress (inflammation) has a protective effect on human cells, including endothelial cells, and can further interact with both the adaptive and innate immune responses (Rizzo M et al., 2012). Bacteria such as P gingivalis in inflammation expresses antigens which resembles human HSPs, stimulating the production of autoreactive T-cells and antibodies to human cells causing atherosclerosis (Rizzo M et al., 2012).

Also elevated levels of HSPs are found in periodontitis patients (Rizzo M et al., 2012) and therefore cross-reactivity exist betweeen human endothelial cell-HSPs and periodontal bacterial-HSPs concluding that inflammation in atherosclerosis may be promoted by HSP-induced immune responses (Ford P et al 2012). Anti-cardiolipin (anti-CL) and anti-oxidized low-density lipoprotein (anti-oxLDL) antibodies increases CVD and also found in the GCF of periodontitis patient (Ford P et al 2012).

Endothelial dysfunction is the earliest stage of atherosclerosis and a possible link between PD and CVD's. Several studies have linked periodontitis to endothelial dysfunction and this relationship is sustained by several shared biomarkers of periodontitis, CVD's and endothelial dysfunction (Gupta S et al 2020). Upon initiation of periodontitis, expression of inflammatory cytokines markedly increases together with alteration in the lipid profile which could contribute to the development and aggravation of thrombogenesis and thromboembolic events. PD is significantly associated with up regulation of biomarkers responsible for endothelial dysfunction and dyslipidemia such as CRP, tissue plasminogen activator (t-PA),and LDL-cholesterol (C), TNF-a . Additionally, periodontitisis associated with higher levels of other inflammatory serum biomarkers including von Willebrand factor (vWF), fibrinogen, and endothelial progenitors'cells. CVD is associated with more severe periodontitis and this was marked by higher serum level of high sensitivity (hs)-CRP (Gupta S et al 2020). Elevated level of hs-CRP due to periodontitis exerts stress additional to the previously existing inflammatory activity of atherosclerotic lesion; consequently, increasing the risk of CVD (Deepa D, et al., 2016).

Pharmacological and non pharmacological treatments are aimed for treating periodontitis. Conservative, conventional and surgical approaches are included in non pharmacological means. Conservative method encompasses regular tooth brushing, flossing, cessation from smoking and alcoholism and intake of healthy diet such as leafy vegetables and citrus fruit, supplements and yogurt (Woelber, Bremer, Vach et al., 2017).

Scaling and root planning (SRP) are included in conventional therapy which over the years were considered as gold standard for plaque and calculus elimination (Tanwar et al., 2016). Scaling was previously performed by curettes but now ultrasonic scalers have nowbeen used and have gained much attention for rendering the root surface free from calculus. Root planning is performed by curettes to render the cementum free from infection and also to smoothen out the root surface making it less vulnerable for microbial deposits (Yan, Zhan, Wang et al., 2020).
Non accessible areas like furcation areas , indented grooves and deep pockets where the pathogens harbor sub-gingivally cannot alone be treated by conventional

mechanical procedures of debriment and cleaning of pathogenic micro organism but instead a combination of chemical agents and antimicrobial are used to suppress the microbials growth and progression (Takeshi Kikuchi., et al 2015). Surgical techniques like flap debriment, gingevectomy, modified Widman flap and laser procedure are the techniques employed apart from conservative and conventional methods (Deas et al., 2016). Treatment by drugs such as antimicrobials reduces the bacterial load and pocket inflammation. Patients who respond poorly to conventional therapy, those with acute periodontal infections leading to systemic manifestations and medically compromised patients are prescribed with antibiotics. Doxycycline is the drug of choice to treat periodontal diseases (Barca et al., 2015).

A substantial body of evidence supports the relationship between PD and CVD's. Although many studies have reported that periodontal therapy significantly increases surrogate markers of CVD's within a short time, followed by improvement in systemic inflammation and endothelial function (Boyapati R et al ., 2020) invasive dental procedures including periodontal treatment have also been associated with decreasing the risk of cardiac conditions (Nordendahl E et al ., 2018). Dental practitioners have to be aware of the association between these two diseases. Patients with severe periodontitis should be advised to see a physician to check for signs of CVD's. Patients should be informed that PD is associated with increased risk of cardiovascular complications and therefore their periodontal condition requires treatment. Furthermore, subjects with CVD's have to adhere to proper oral hygiene measures and regular check-ups with a dental practitioner (Herrera D et al., 2020). Thus, cardiologists should notify patients with atherosclerosis about the importance of good oral and dental health. Patients should be advised of the need to have regular home and professional dental care. Furthermore, the physician should recommend referral to a dentist or peridontist for oral and periodontal examination, assessment and treatment when necessary Cooperation between the dentist and the cardiologist is of paramount importance for patients on anticoagulant/anti platelet medication prior to any oral or periodontal surgeries to avoid any complications such as excessive bleeding and ischemic events (Herrera D et al., 2020).

OPERATIONAL DEFINITIONS

Periodontium:

Periodontal apparatus supporting the tooth structure in a socket of mandibular and maxillary arches. It comprises of cementum, alveolar bone, gingiva and periodontal ligament (Lang NP et al., 2015).

Periodontal disease:

Pathologic chronic inflammatory responses affecting the supporting structure of the teeth including alveolar bone, gingival and connective tissue (periodontal tissues) is surrounding the teeth (Lang NP et al ., 2015).

Chronic Periodontitis:

A response of inflammation resulting in the formation of oral biofilm accompanied with the deposition of bacterial plaque in the periodontal tissues (Lang NP et al., 2015).

Glickman's Classification (grade 4) of horizontal alveolar bone loss:

A progressive periodontal infection caused by the bacteria, exposing the furcation areas involving the roots of teeth and alveolar bone ultimately resulting in teeth to loosen and fall (Barbara Buffoli et al., 2019).

Severe Perodontitis:

Ulcerated pocket formation at the apical migration of the gingival epithelium causing progressive periodontium destruction, mobility and loss of tooth and overall compromised function of mastication (Dye BA et al., 2012) graded as "≥2 interproximal sites with CAL≥6 mm (not on same tooth) and ≥1 interproximal site with PPD≥5 mm" (Eke PI et al ., 2012) .

Mild Periodontitis:

Graded as≥2 interproximal sites with clinical attachment loss (CAL)≥3 mm, and ≥2 interproximal sites with periodontal probing depth (PPD)≥4 mm (not on same tooth) or one site with PPD≥5 mm (Eke PI et al ., 2012).

Moderate Periodontitis:

Graded as ≥2 interproximal sites with CAL≥4 mm (not on same tooth), or ≥2 interproximal sites with PPD≥5 mm (not on same tooth) (Eke PI et al., 2012).

Non Communicable Periodontitis:

A condition affecting peridontium in the presence of microorganisms along with host related risk factors including heavy deposits of calculus and plaque, various modifications of oral hygiene, age, gender and inherited/biological characteristics ,socio economic conditions, chronic degenerative diseases like diabetes , sedentary life style causing obesity , smoking and human immuno deficiency virus (Wei L, Thornton-Evans GO et al ., 2012).

Microbial Peridontitis:

Ulcerated pocket formation due to chronic inflammation caused by anaerobic subgingival microbiota composed of microaerophilic gram-negative bacilli, anaerobs and oral bacterial microbilas such as P. gingivalis (Eke PI et al ., 2012).

Gingival Crevicular Fluid (GCF):

It is a fluid that is derived from gingival plexus of the blood vessels in the gingival sulcus that represents as an inflammatory exudate in the pathological state while in healthy individual it is considered as an altered tissue transudate (Zohaib khurshid et al., 2017).

Tumor necrotic factor alpha (TNF alpha):

A significant pro- inflammatory cytokine produced immune mediators by the specific response of the cells such as neutrophils, adipocytes, keratinocytes, macrophages and fibroblasts (Victor M. Mart-nez-Aguilar., et al 2019).

Plaque index:

An index which is used to measure accumulated deposits within the gingival pocket. Around marginal gingiva of tooth that is calculated by assessing tooth's all four sites and is visible with a naked eye (Silness and Loe Plaque Index., Marwa et al 2017).

Plaque Index = (2+1+1+2) /4= 1.5

Mean Sulcuar Bleeding (mSBI):

An index used to measure edema of sulcular region, bleeding on probing and gingival color. The criteria for scoring as described by Muhlemann and Son are as follows:

Score 0= No bleeding on probing and healthy marginal and papillary gingival.

Score 1= Bleeding occurs on probing and gingiva is healthy.

Score 2= No swelling / edema, bleeding occurs on probing and change in gingival color.

Score 3= Slight swelling/edema, change in gingival color and bleeding on probing.

Score 4= Prominent edema, change in gingival color and bleeding on probing.

Score 5= Marked edema, change in gingival color and bleeding occurs on probing.

Mean value is calculated by: Scores for these units are added and then divided by 4 (all four sites of a single tooth) (Pradeep AR et al., 2017).

Vertical bone loss:

Vertical pattern of resorption/loss of inter-dental alveolar bone is known as Vertica bone loss. Vertical defect occurs when the distance from alveolar bone crest to the base is greater than or equal to 3mm (Gupta et al , 2019).

Clinical Attachment Loss (CAL):

Measured in millimeters, it represents the level of receded ginigva from the reference point, calculated by adding the gingival marginal level from Cemento-enamel junction to the probing depth. Categorized as:

Mild: 1-2 mm (CAL)

Moderate: 3-4 mm (CAL)

Severe: greater than 5 (CAL)

(American Dental Association 1999 guidelines, Attia et al., 2017)

CHAPTER 3

METHODOLOGY

3.1 STUDY DESIGN:

Case Control study

3.2 SUBJECTS:

84 adult individuals , males and female subjects between the age of 35-68 having poor oral hygiene condition, loss of periodontal attachment and horizontal bone loss (grade 4) with cardiac disease were selected as cases and individuals with periodontitis without cardiac condition were selected as controls.

3.3 PLACE OF SAMPLE COLLECTION / SETTING:

Out-patient department of Oral Diagnositics, Periodontology and Oral Maxillofacial Surgery Department (OMFS) of Bahria University Dental College, BUDC- BUHS Karachi. (Figure 3.1)

3.4 INCLUSION CRITERIA:

1. Cardiac patients who had severe chronic periodontitis with horizontal alveolar bone loss (grade 4) and at least 20 natural teeth residing in the oral cavity excluding third molars.
2. Controls were opted who had chronic periodontitis but were without cardiac disease.
3. Severe Gingivitis with bleeding gums and severe tooth loss excluding the third molars with cardiac disease were also opted as cases.

3.5 EXCLUSION CRITERIA:

1. Patients with history of severe allergies, pregnancy, lactation, or any invasive treatment within past 6 months.
2. History of clinically evident chronic inflammatory condition.
3. Patients who underwent invasive scaling procedures in the last 3 months or who had consumed antibiotic within a month were not included in the study.

3.6 DURATION OF THE STUDY:

1. **Individual study period**: - 90 days (3 month) and approximately 1 hour on each patient.
2. **Total study period:** - 6 months after approval of synopsis from Ethical review committee (ERC) and Faculty review committee (FRC).

3.7 SAMPLE SIZE ESTIMATION:

Sample size was calculated using the method for "unmatched Case-Control study**"** on www.openepi.com **(Open Epi Version 3).** Hypothetical proportion of control with exposure was found to be 40% and hypothetical proportion of cases with exposure is 11.24% with two sided confidence interval (1 – alpha) is 95%. Least extreme Odds Ratio to be detected was 0.19%. The required sample for cases and controls was 42 and 42 respectively. Total sample size was 84.

SAMPLE SIZE FOR UNMATCHED CASE-CONTROL STUDY

For:

Two-sided confidence level(1-alpha)	95
Power(% chance of detecting)	80
Ratio of Controls to Cases	1
Hypothetical proportion of controls with exposure	40
Hypothetical proportion of cases with exposure:	11.24
Least extreme Odds Ratio to be detected:	0.19

	Kelsey	**Fleiss**	**Fleiss with CC**
Sample Size – Cases	37	35	42
Sample Size – Controls	37	35	42
Total sample size:	74	70	84

References

Kelsey et al., Methods in Observational Epidemiology 2nd Edition, Table 12-15

Fleiss, Statistical Methods for Rates and Proportions, formulas 3.18 &3.19

CC = continuity correction

Results are rounded up to the nearest integer.

Results from Open Epi, Version 3, open source calculator--SSCC

3.8 SAMPLING TECHNIQUE:

Convenience Sampling Method (Non – probability Sampling).

3.9 HUMAN SUBJECTS AND CONSENT FORM:

Total number of patients enrolled in the study was 84 (42 cases and 42 controls). Written informed consent was taken from each patient prior to enrollment in the study (Annexure- C).

3.10 MATERIALS:

1. **DRUGS: N/A**
2. **CHEMICALS: N/A**
3. **PROFORMA/ QUESTIONNAIRE:** Subject evaluation proforma/ questionnaire (Annexure –D)
4. **ANY OTHERS:**

(A) EQUIPMENT:

1. ORTHOPANTOMOGRAM X-RAY (OPG):

The orthopantomogram also known as orthopantomograph, pantomogram or an OPG is a 2D panoramic single image radiograph of the mandible, maxilla and teeth. The OPG machine employed in the oral radiology department at Bahria University Dental College was the DURR Dental, Vista Pano with make #DG-07C11T2, Turkey. (Figure 3.2a)

2. CALIRATED PERIOODONTAL PROBE :

The University of North Carolina probe (UNC15) with calibrations minimum of 1mm marking was employed to measure the periodontal pocket depth and clinical attachment loss. It provides measurement limit up to 15mm. (Figure 3.2b)

3. SPECTROPHOTOMETER:

Analysis of baseline samples was performed according to manufacturer's protocol.

The Spectrophoptmeter Thermo Fisher Scientific (Model 51119700DP) was employed using sandwich technique. This sandwich kit is for the accurate quantitative detection of Human monocyte chemotactic protein1;monocyte chemotactic and activating factor also known as MCP-1 in biological fluid sample such as serum , saliva, GCF, plasma or tissue homogenates. (Figure 3.2 c)

(B) KIT USED :

MCP-1 and sCD40 L levels in GCF and saliva obtained from the study participants were measured using a commercially available solid phase sandwich enzyme-linked immunosorbent assay, in Bahria University's (BUMDC) MDRL laboratory using ELISA kits (Cat # E0124 Hu. and Cat#E0251Hu, Bioassay Technology Laboratory , 1008 Junjiang Inter. Bldg.228 Ningguo Rd, Yangpu dsit , Shangai , Co., LTD, China) as according to manufacturer's instructions. (Figure 3.3)

(C) OTHERS:

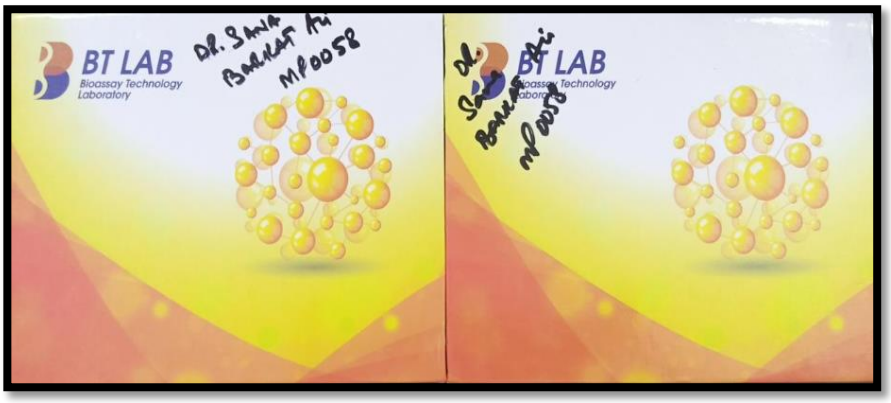
- A curette (Figure 3.4 a)
- 1-5ul color coded Hirschmann's microcapillary pipettes (Figure 3.4b)
- 0.5 ml Eppendorfs(Figure 3.4c)

Figure 3.1: Out-patient department of Oral Diagnosis, Oral Maxillofacial Surgery Department (OMFS) and MDRL of Bahria University Dental College, BUDC- BUHS Karachi.

Figure: 3.2(a) Orthopantomogram, Oral Radiology Department, Bahria Dental College.

Figure:3.2 (b) – Calibrated Probe UNC 15 (Mellissa Boyd et al, 2016)

Figure 3.2 (c) – Thermoscientific Spectrophotometer



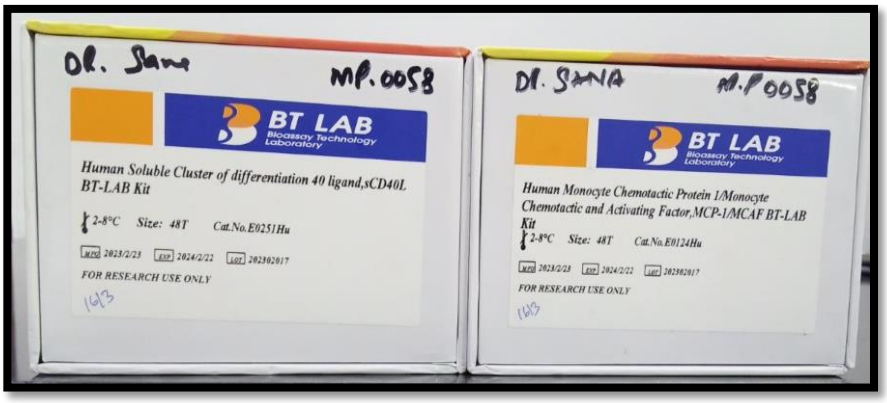


Figure 3.3: Human MCP-1 and s CD 40 L Elisa Kit

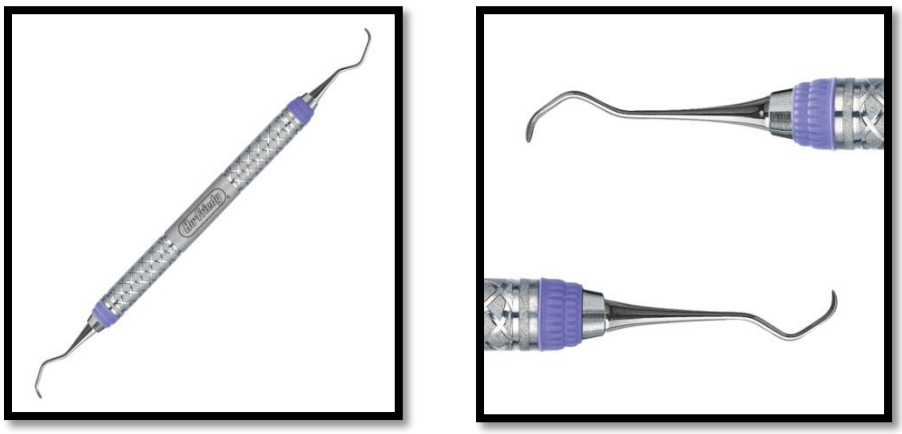


Figure 3.4 (a) : A Curette

Figure 3.4(b): 1-5ul color coded Hirschmann's microcapillary pipettes

Figure 3.4 (c): Eppendorf tube

3.11 PARAMETERS OF THE STUDY:

(A) CLINICAL PARAMETERS:

1. Probing depth (PD) (Attia et al ., 2017)

- The periodontal probe is placed in sulcus in six different locations around each affected tooth, that is, mesiobuccal, straight buccal, distobuccal, distolingual, straight lingual and mesiolingual.
- The distance from the base of the pocket to the gingival margin is measured in millimeters using the calibrations on the periodontal probe UNC15.
- The periodontal pocket probing depth was determined in both cases and control groups.

2. Clinical attachment loss (CAL) (Hafez Diab et al.,2017)

- The periodontal probe was placed parallel to the long axis of affected tooth until the resistance was felt and the measurement was recorded in millimeters.
- The periodontal probe was placed at six different locations around each affected tooth that is mesiobuccal, straight buccal, distobuccal, distolingual straight lingual and mesiolingual.
- Cemento enamel junction was identified to be the reference point to determine the clinical attachment level of the gingiva.
- The periodontal pocket depth was determined and was added to the distance from the cement enamel junction to the gingival margin.
- The clinical attachment loss is calculated by adding the gingival marginal level to the probing depth, which demonstrates the recorded level of gingiva from the reference point.

- Clinical attachment loss was determined by means of UNC -15 calibrated periodontal probe in both the cases and control groups.

3. A plaque index (PI). (MarwaMadi et al ., 2017)

- The plaque accumulation per tooth was assessed and scoed at four site namely mesial, dital, lingual and buccal/labial using Silness – Loe Index.
- The average mean score was calculated by adding the plaque index score at four sites and then divided by 4. the grading is done as follows :

  0= no paque

  1= thin plaque layer only at gingival margin detectable by scraping with probe.

  2= moderate plaque layer along the gingival margin, free interdetnal spaces but visible to the naked eye.

  3= abundant plaque accumulation at the gingival margins and interdetnal spaces.
- Plaque index was determined in both the cases and control groups.

4. A gingival index (GI).

- This index was first introduced by Loe and Silness in 1963 designed to assess the severity and quality of gingival inflammation in an individual or population.
- Gingival inflammation was assessed on the basis of color, consistency and bleeding on probing.
- Gingival index scores was taken each site on a 0-3 scale with 0 being normal and 3 being severe inflammation characterized by edema, redness, swelling and spontaneous bleeding.
- Gingival index was determined in both the cases and control groups.

➢ **Healthy patients were classified by:**

1. GI < 1
2. PD < 3 mm
3. CAL = 0.

- **Severe Periodontitis patients were classified and graded as:**

1. "≥2 interproximal sites with CAL≥6 mm (not on same tooth) and
2. ≥1 interproximal site with PPD≥5 mm.

B. RADIOGRAPHIC PARAMETERS:

1. Vertical Bone Loss (VBL) Gupta et al ., 2019)

- Vertical bone loss of the affected tooth was determined using orthopantomogram(OPG) by first identifying the landmark
- The vertical distance in millimeters was measured from the crest of the alveolar bone till the base of defect.
- A tooth was considered to have a vertical defect when the distance from the crest of alveolar bone to the base of defect was > or equal to 3mm.
- The radiographic alveolar bone was measured by computer added software program namely Durr Vista soft version 2.0.1
- Vertical bone loss was assessed in both case and control groups.

2. **Horizontal Alveolar Bone loss:-** As classified by Glickman and was assessed in both cases and control group.

Table 3.1: Glickman classification of horizontal Alveolar bone loss

Glickman	Grade I:	Incipient lesion. Suprabony pocket and slight bone loss in the furcation area.
	Grade II:	Loss of interradicular bone and pocket formation but a portion of the alveolar bone and periodontal ligament remain intact.
	Grade III:	Through-and-through lesion.
	Grade IV:	Through-and-through lesion with gingival recession, leading to a clearly visible furcation area.

C. BIOCHEMICAL PARAMETERS:-

1. **Biochemical markers:** MCP-1 and sCd40L was assessed in gingival crevicular fluid (GCF) using Human monocyte chemotactic protein 1and Human soluble Cluster of Differential 40 ligand Elisa kit.

   (a) Gingival Crevicular fluid sample collection for MCP-1and sCd40L:

   1. Clinical evaluation to ascertain the periodontal attachment using UNC 15 probe (Hu-freidy®) was done to achieve a reliability index score 0.8 using Dahlberg's formula.
   2. Supra gingival cleaning of plaque and debris with a curette was done during sample collection to prevent salivary contamination.
   3. 1-5ul color coded Hirschmann's microcapillary pipettes was gently placed , extra-crevicularly at the marginal gingival without penetrating into the gingival crevices and a standardized volume of 2 µl of Gingival Crevicular Fluid was collected from the subject from the most severely afflicted site of periodontitis.(Figure 3.5 a)
   4. GCF and salivary collection was done on the individual patient in a single day to control spontaneous bleeding (if occurs) from chronically infected patients and avoid contamination at the collection sites.
   5. The samples collected were carefully dispensed into airtight and sterilized, 0.5 ml Eppendorfs bearing 398 µl of sample diluent, and a blast of air was passed through the micro capillary pipettes to enable complete removal of contamination. (Figure 3.5 b)
   6. All the samples were kept in cryobox and stored at −80°C until analyzed. (Figure 3.5 c)
   7. Thawing of the samples and analysis was done only once for the assay (using a solid phase sandwich enzyme-linked immunosorbent assay)
   8. Concentration of MCP-1 and sCd40L in the samples was determined by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, E0124Hu and E0251Hu, China) in accordance with manufacturer’s instruction.
   9. Results were analyzed statistically.

Figure 3.5 (a): 2 µl of sample was collected from the subject

Figure 3.5 (b): Collected samples dispensed into airtight and sterilized, 0.5 ml Eppendorfs.

Figure 3.5 (c): Samples kept in cryobox and stored at −80°C.

(b) Analysis of gingival crevicular fluid levels of MCP-1 by enzyme-linked immunosorbent assay:

MCP-1 level in GCF were analyzed using commercially available enzyme-linked immunosorbent assay kit as per the manufacturer's instruction (Bioassay Technology Laboratory).

1. Prior to use, the kit, buffer solution and samples were thawed at room temperature for 30 minutes and the sample was mixed in the vortex mixer. (Figure 3.6 a)
2. The standards were reconstituted to 120ul of the standard (1920ng/L) with 120ul of standard diluent to generate a 960ng/L standard stock solution.
3. Allow the standard to sit for 15 minutes with gentle agitation to make dilutions.
4. Serial dilution of standard stock solution (960ng/L) 1:2 with standard diluent to produce 480ng/L, 240ng/L, 120ng/ L and 60ng/L solutions were prepared. Standard diluent serves as the zero standards (0ng/L).
5. The assay was performed at room temperature.
6. Add 50ul standard to standard wells.(Figure 3.6 b)
7. Add 40 ul sample to sample wells and then add 10ul anti- MCP-1 antibody to sample wells, and then add 50ul streptavidin –HRP to sample wells and standard wells. (Figure 3.6 c).
8. Mix well. Cover the plate with sealer. Incubate in spectrophotometer for 60 minutes at 37^0 C. (Figure 3.6 d)
9. Remove the sealer and wash the plates 5times with wash buffer. Soak wells with 300ul wash buffer for 30seconds to 1 minute for each wash .for automated washing, aspirate all wells and wash 5 times with wash buffer. Blot the plate onto the paper towels or other absorbent material.
10. Add 50 ul substrate solutions A to each well and then add 50ul substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37^0 C in the dark. (Figure 3.6 e)
11. Add 50ul of Stop Solution to each well, the blue color will change to yellow immediately. (Figure 3.6 f)

12. Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450nm within 10 minutes after adding the stop solution.
13. Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations were performed with computer-based curve fitting software that can be determined by regression analysis. (Figure 3.6 g).

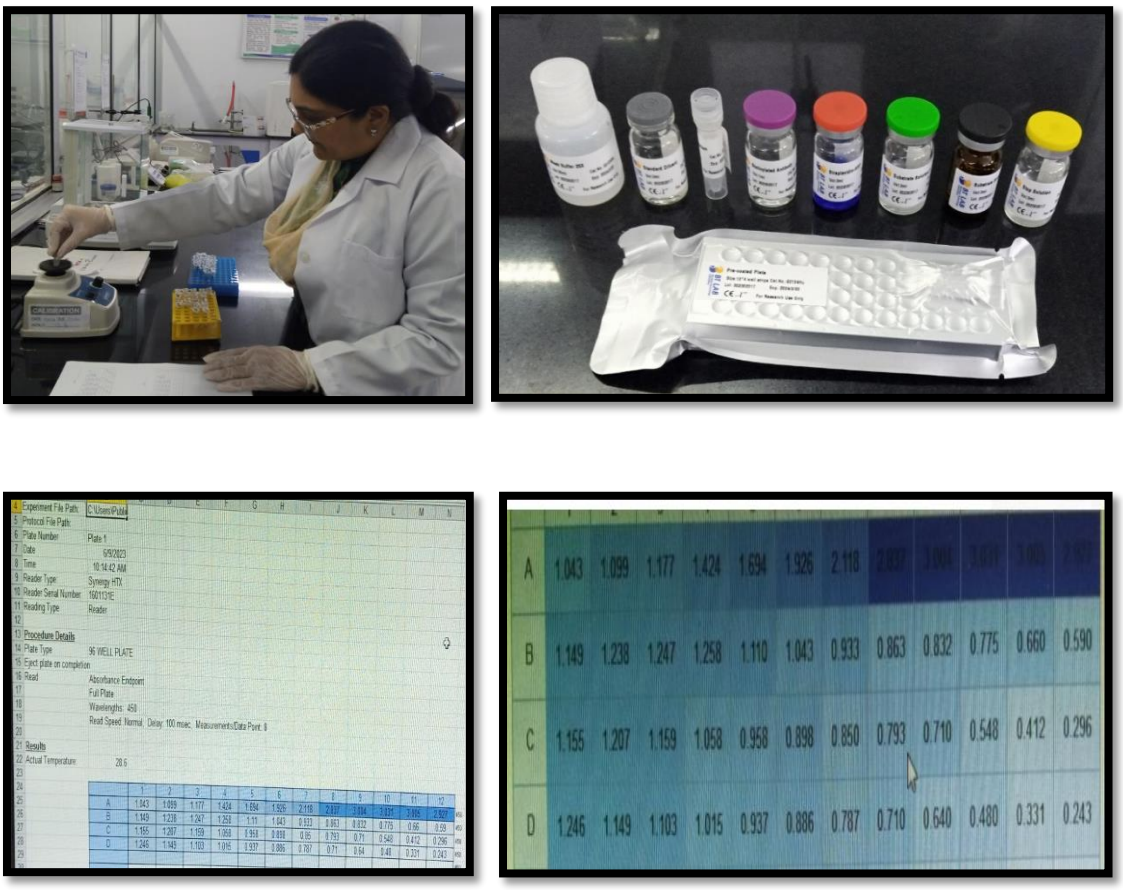


Figure 3.6 (a): MCP-1; Thawing of Buffer solution at room temperature for 30 minutes, sample mixing done in the vortex mixer and pre reading taken.

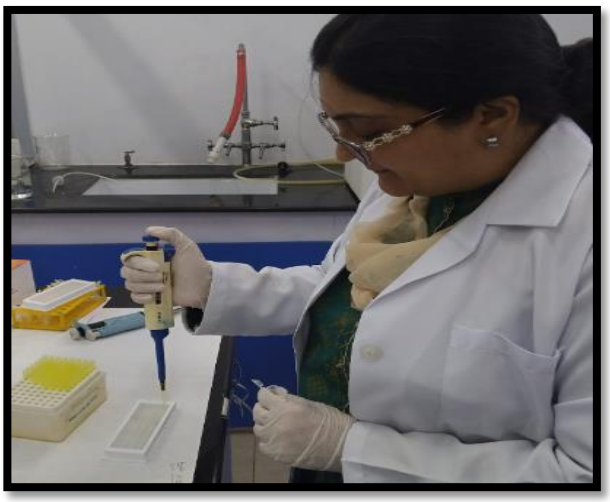


Figure 3.6 (b): MCP-1; Adding 50ul standard to standard wells

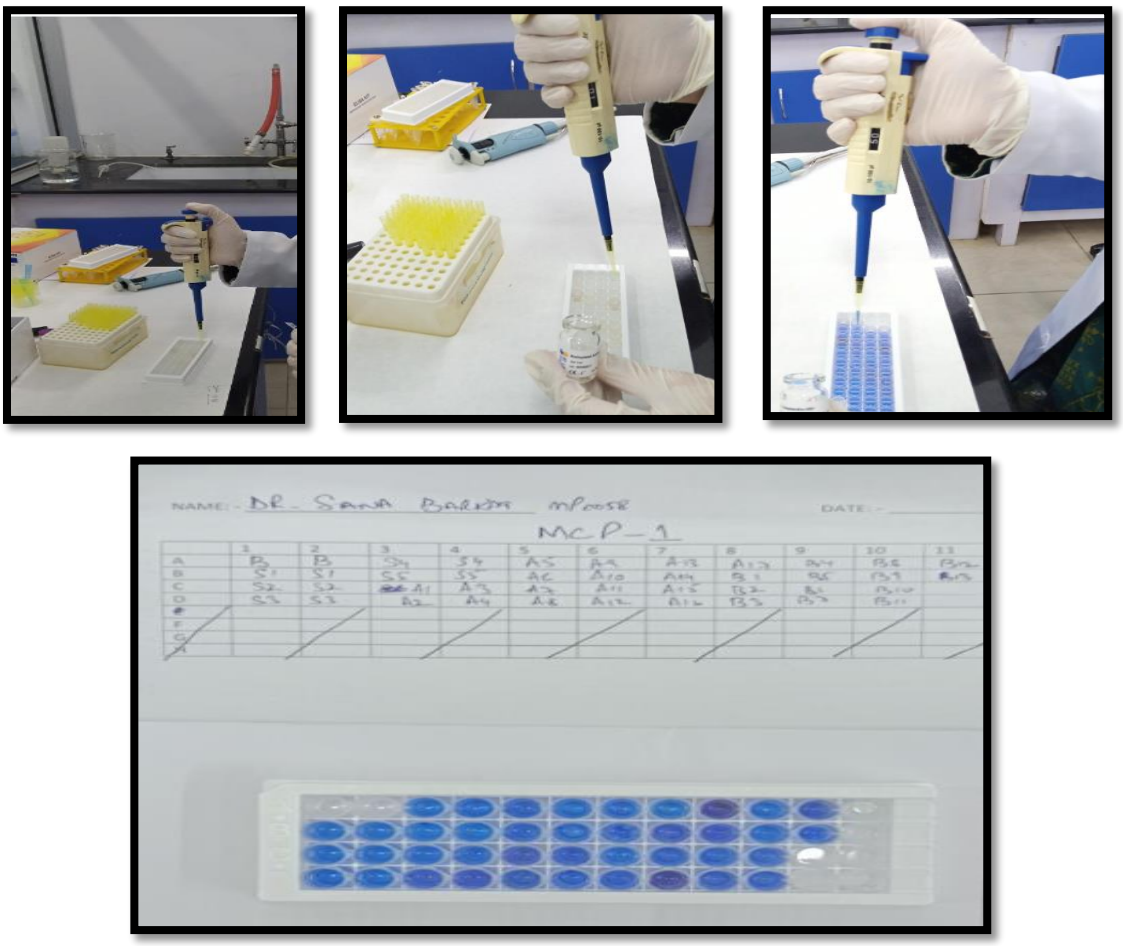


Figure 3.6 (c): MCP-1; Adding 40 ul sample to sample wells , adding 10ul anti-MCP-1 antibody to sample wells, then add 50ul streptavidin –HRP to sample wells and standard wells.

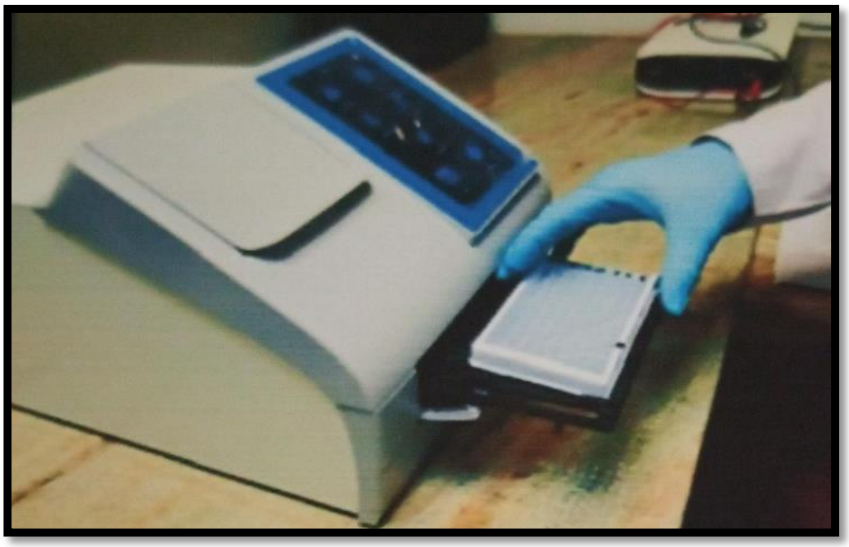


Figure 3.6 (d): MCP-1;Sixty minutes of sample incubation of done in Spectrophotometer at 37^0 C.

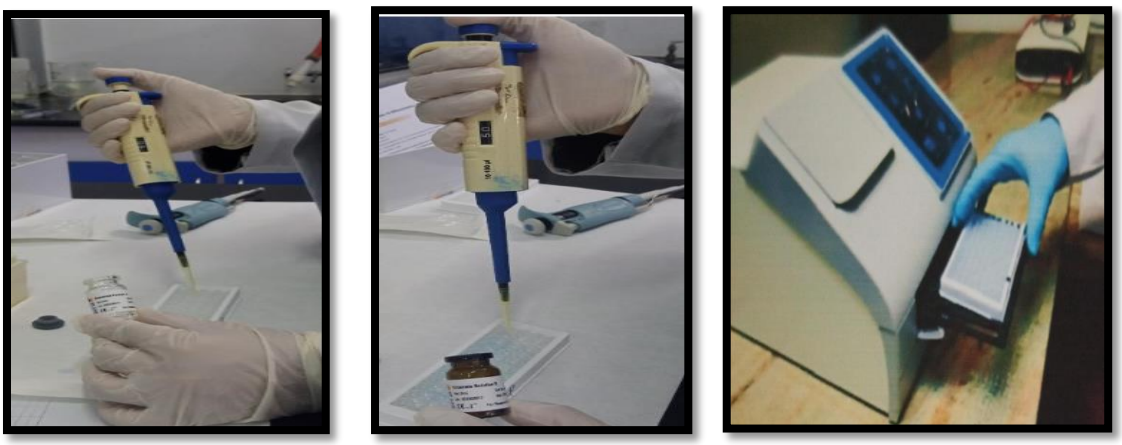


Figure 3.6 (e): MCP-1; Adding 50 ul substrate solution A to each well and then add 50ul substrate solution B to each well. Incubated for 10 minutes at 37^0 C in Spctrophotometer.

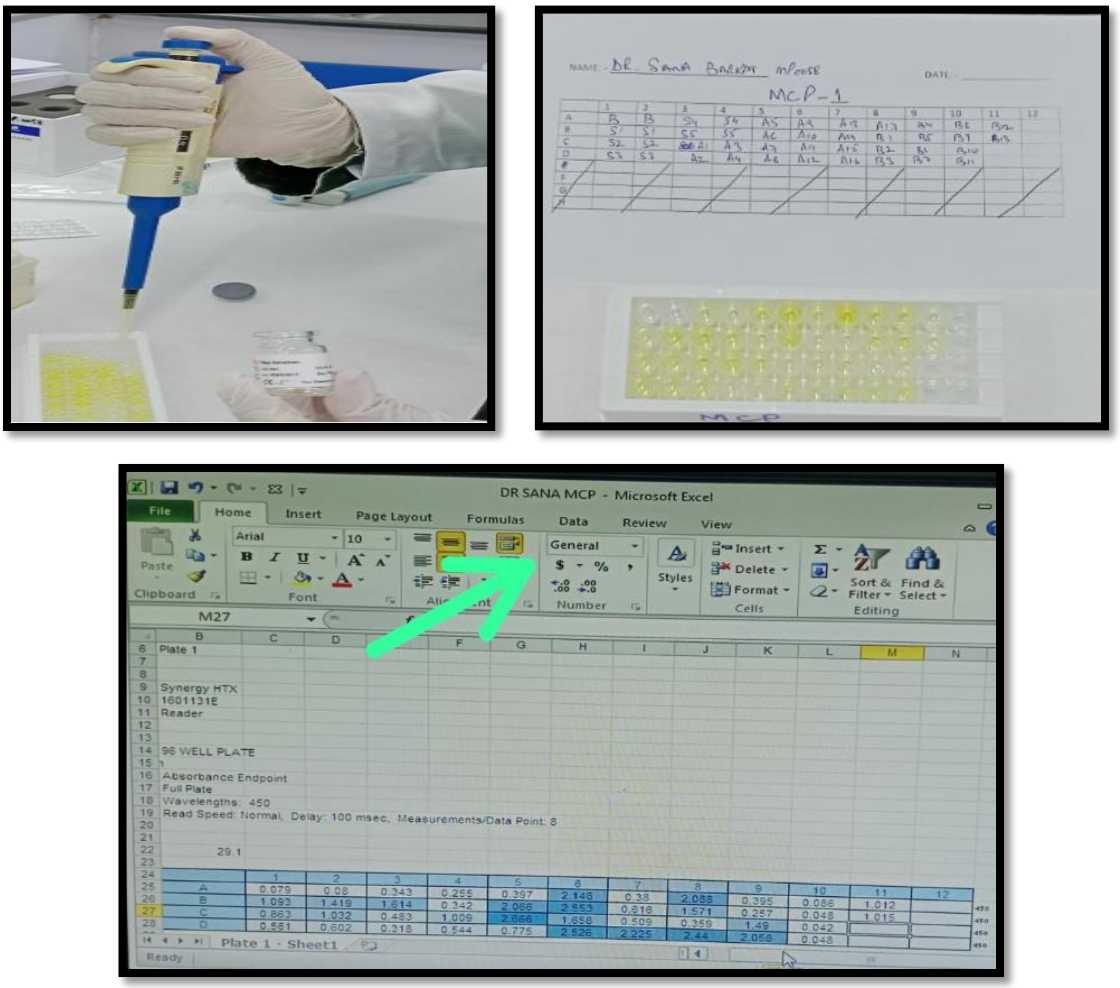


Figure 3.6 (f): MCP-1; Adding 50ul of Stop Solution to each well, the blue color changed to yellow immediately. Post readings taken.

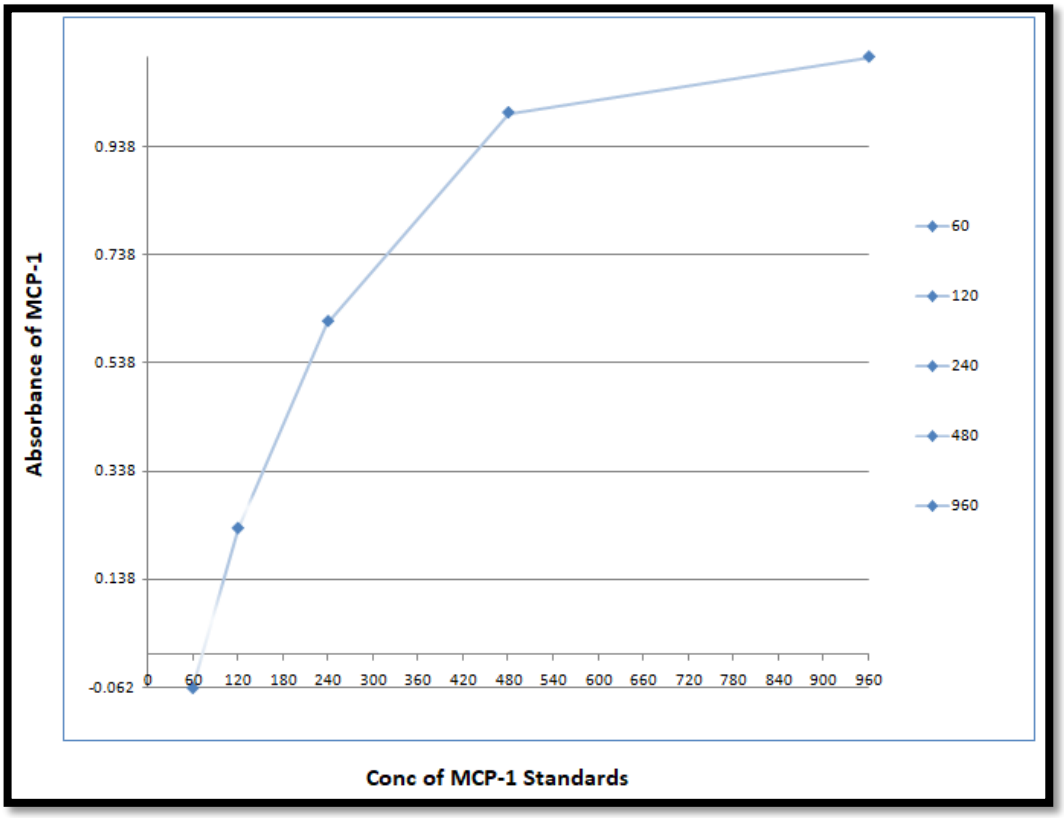


Figure 3.6 (g): MCP-1: Standard curve plotted by regression analysis.

(c) Analysis of gingival crevicular fluid levels of sCD40L by enzyme-linked immunosorbent assay:

sCD40L level in GCF were analyzed using commercially available enzyme-linked immunosorbent assay kit as per the manufacturer's instruction (Bioassay Technology Laboratory).

1. Prior to use, the kit, buffer solution and samples were thawed at room temperature for 30 minutes.(Figure 3.7 a)
2. The standards were reconstituted to 120ul of the standard (32ng/L) with 120ul of standard diluent to generate a 16ng/L standard stock solution.
3. Allow the standard to sit for 15minutes with gentle agitation t make dilutions.
4. Serial dilution of standard stock solution (16ng/L) 1:2 with standard diluent to produce 800ng/L, 400ng/L, 300ng/L, 200ng/L and 100ng/L solutions were prepared. Standard diluent serves as the zero standards (0ng/L).
5. The assay was performed at room temperature.
6. Add 50ul standard to standard wells. (Figure 3.7 b)
7. Add 40 ul sample to sample wells and then add 10ul anti- MCP-1 antibody to sample wells, and then add 50ul streptavidin –HRP to sample wells and standard wells. (Figure 3.7c).
8. Mix well. Cover the plate with sealer. Incubate in spectrophotometer for 60 minutes at 37^0 C. (Figure 3.7 d)
9. Remove the sealer and wash the plates 5times with wash buffer. Soak wells with 300ul wash buffer for 30seconds to 1 minute for each wash. For automated washing, aspirate all wells and wash 5 times with wash buffer. Blot the plate onto the paper towels or other absorbent material.
10. Add 50 ul substrate solutions A to each well and then add 50ul substrate solution B to each well. Incubate plate covered with a new sealer for 10 minutes at 37^0 C in the dark. (Figure 3.7 e)
11. Add 50ul of Stop Solution to each well, the blue color will change to yellow immediately. (Figure 3.7 f)

12. Determine the optical density (OD value) of each well immediately using a micro plate reader set to 450nm within 10 minutes after adding the stop solution.
13. Construct a standard curve by plotting the average OD for each standard on the vertical (Y) axis against the concentration on the horizontal (X) axis and draw a best fit curve through the points on the graph. These calculations were performed with computer-based curve fitting software that can be determined by regression analysis. (Figure 3.7 g).

Figure 3.7 (a): Scd40L; Thawing of Buffer solution at room temperature for 30 minutes, sample mixing done in the vortex mixer and pre reading taken.

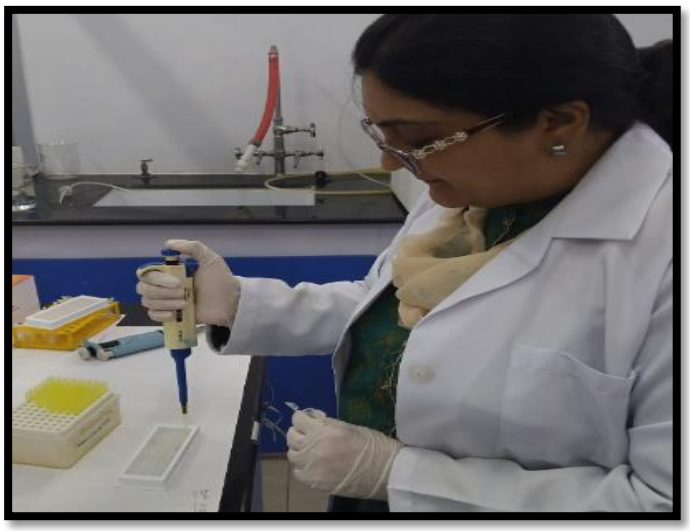


Figure 3.7 (b): sCD40L; Adding 50ul standard to standard wells

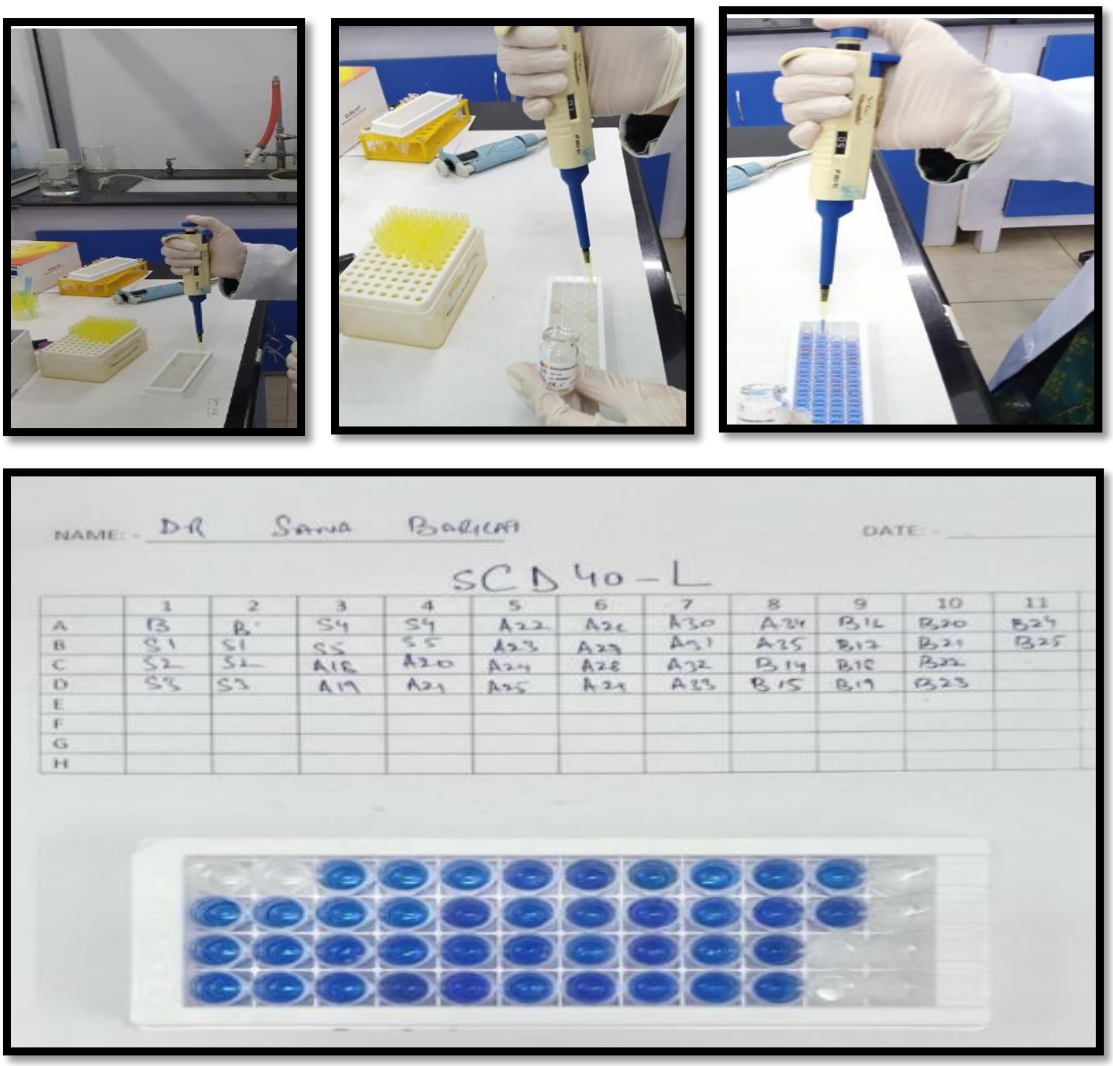


Figure 3.7 (c): sCD40L; Adding 40 ul sample to sample wells, adding 10ul anti-MCP-1 antibody to sample wells, then add 50ul streptavidin –HRP to sample wells and standard wells.

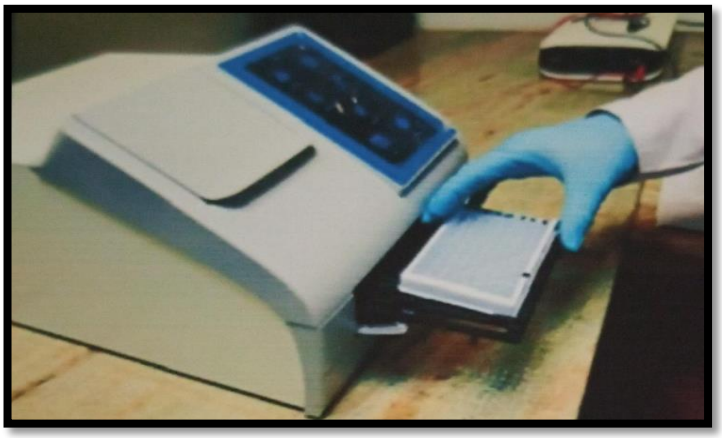


Figure 3.7(d): sCD40L; Sixty minutes of sample incubation of done in Spectrophotometer at 37^0 C.

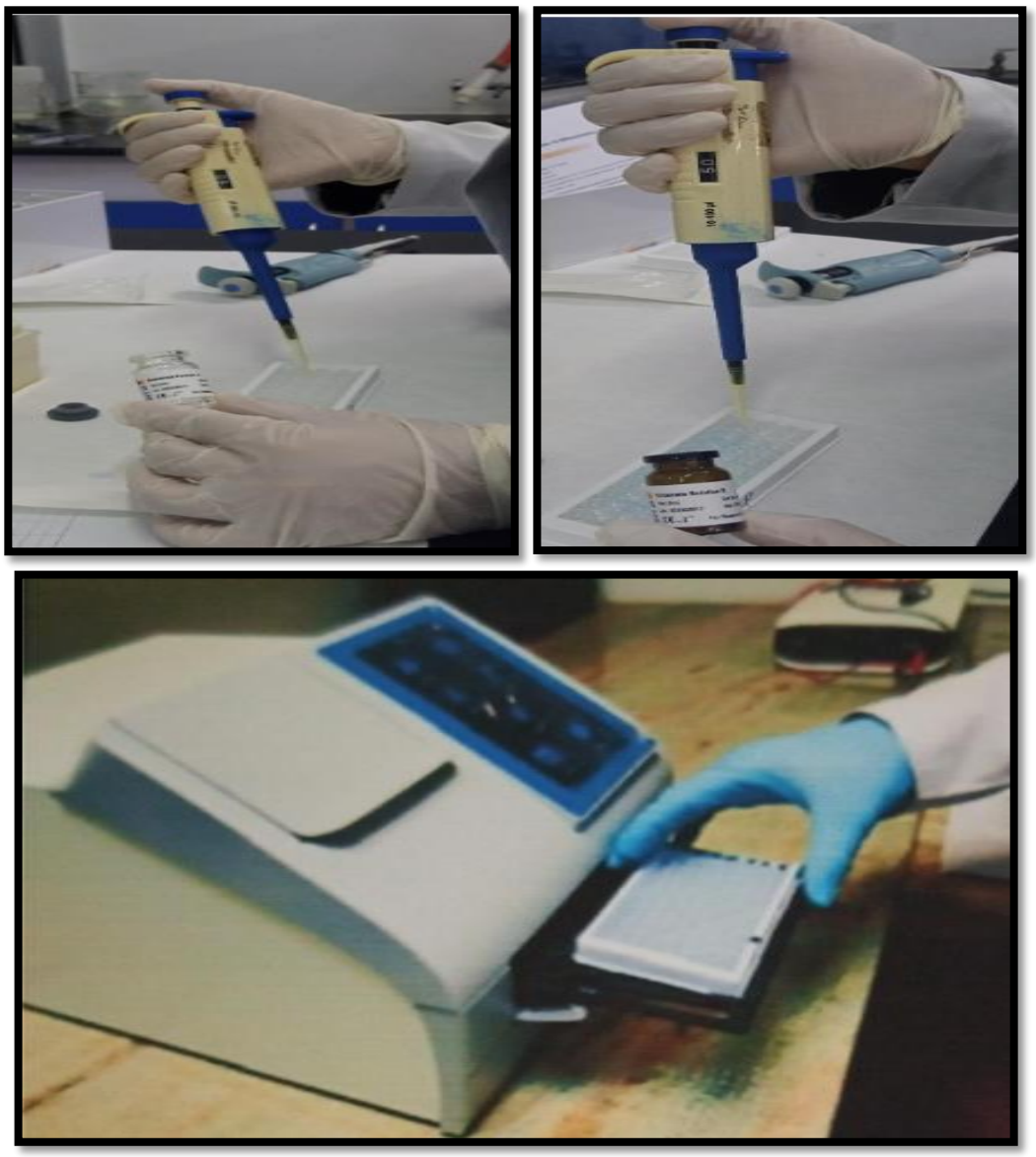


Figure 3.7 (e): sCD40L; Adding 50ul substrate solution A to each well and then add 50ul substrate solution B to each well. Incubated 10minutes at 37^0C in Spctrophotometer.

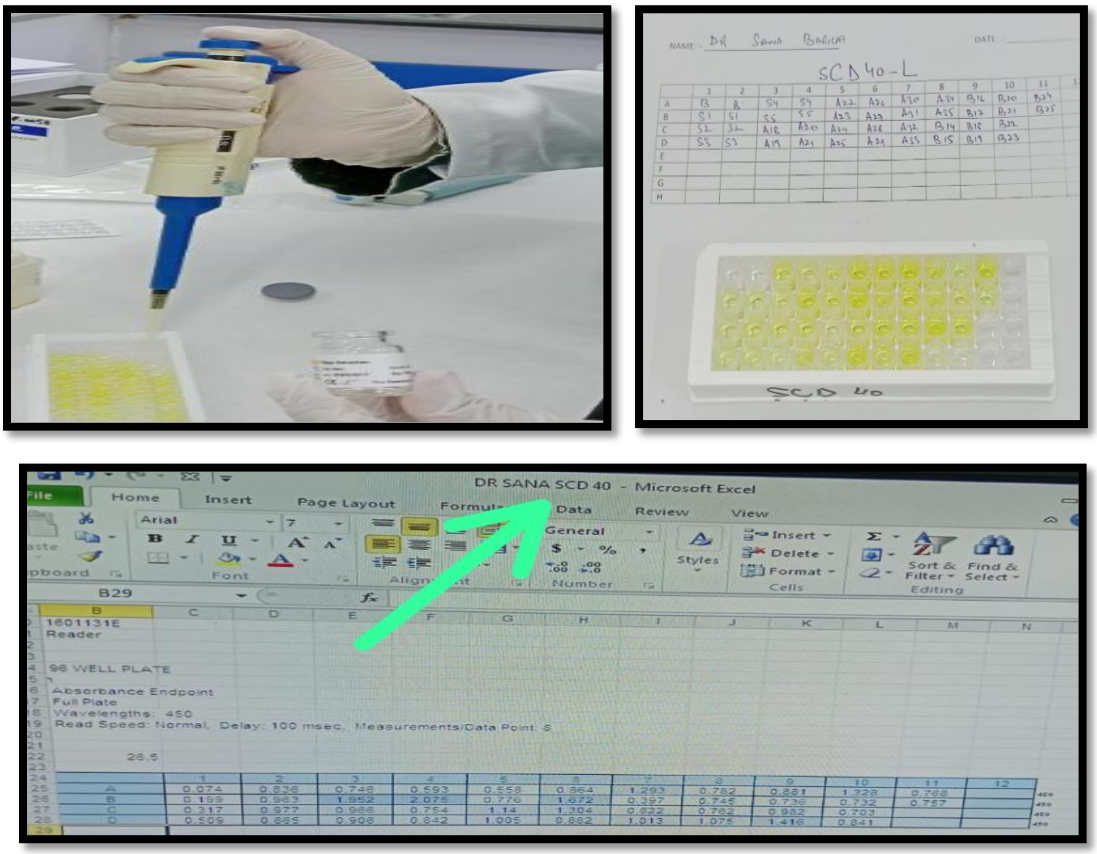


Figure 3.7 (f): sCD40L; Adding 50ul of Stop Solution to each well, the blue color changed to yellow immediately. Post-reading taken.

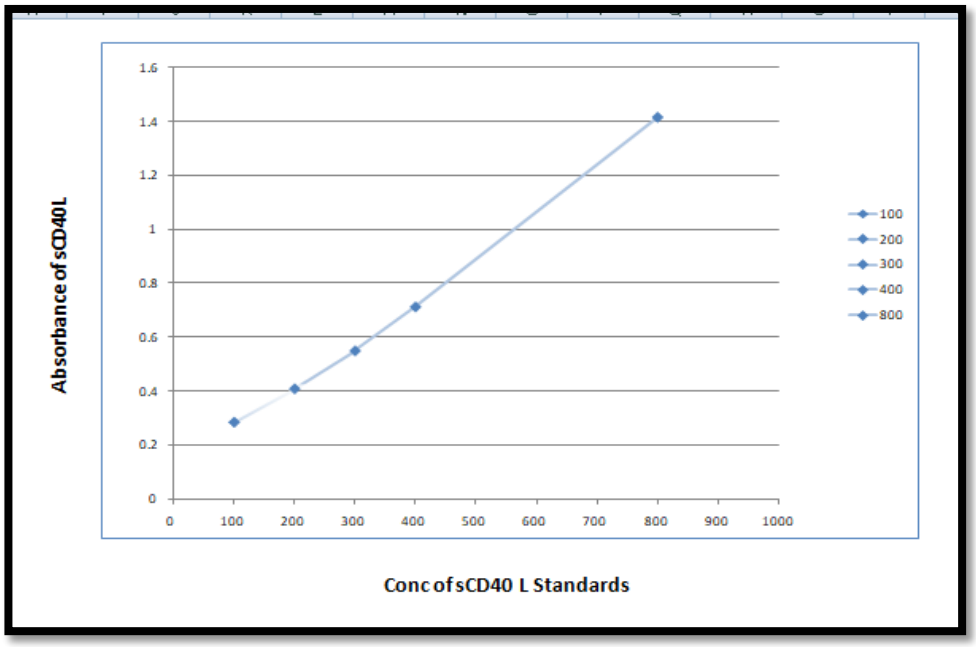


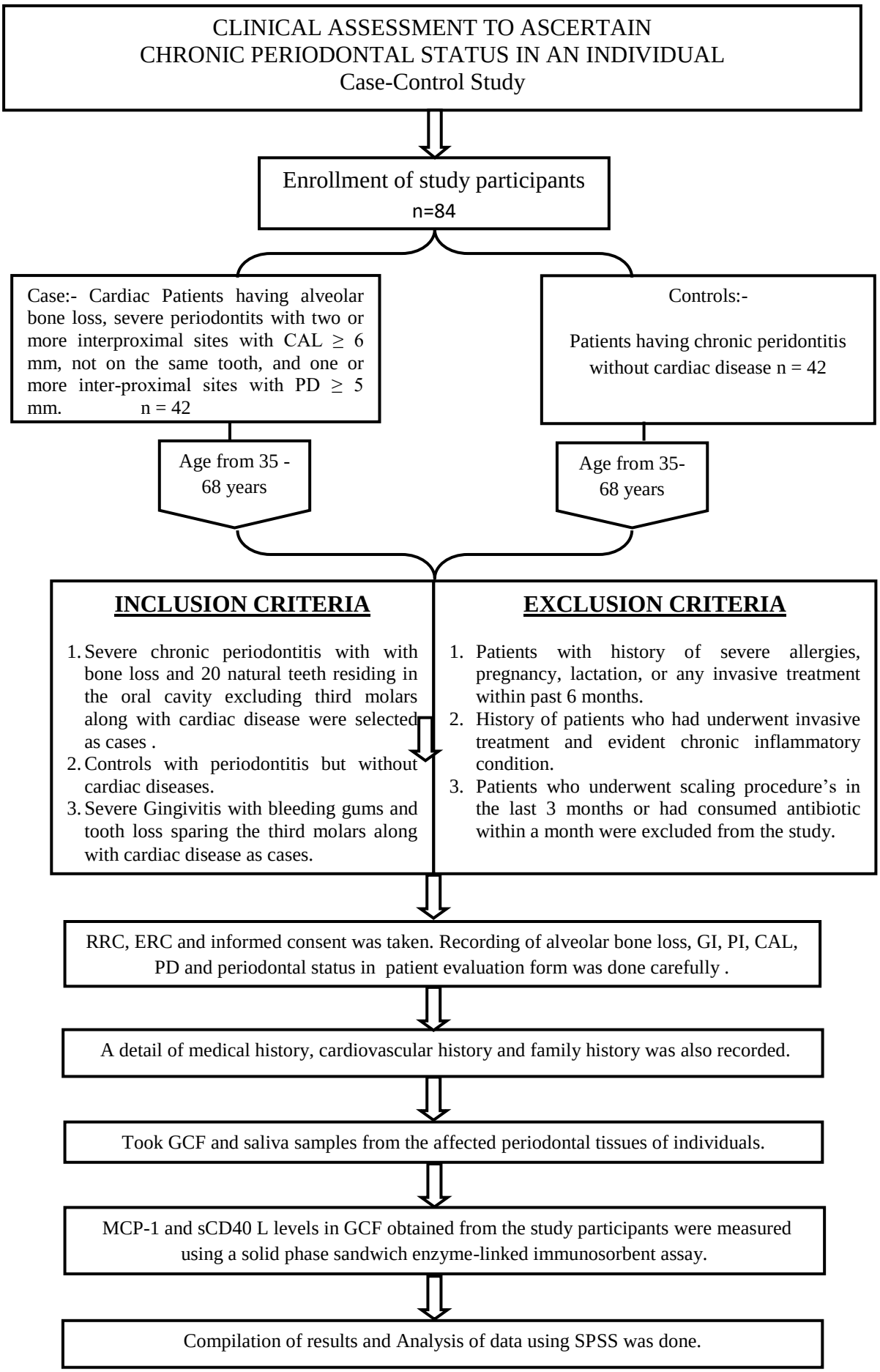
Figure 3.7 (g): sCD40L: Standard curve plotted by regression analysis.

3.12 PROTOCOL/ PROCEDURE OF THE STUDY :

This case control study was conducted after obtaining RRC and ERC of Bahria University Health science (BUHS).Written informed consent form was taken from all study participants. Patients suffering from severe/chronic periodontitis with cardiac diseases were taken as cases and patients with moderate to severe periodontitis without cardiac diseases were taken as controls as per guidelines of American Academy of Periodontology. (Heiko Z et al., 2015). The patients were recruited from Oral Diagnostic, Periodontotlogy and Oral Maxillofacial Department (OMFS) of Bahria University Health Sciences (BUHS-BUDC). Demographic details, family and medical/systemic disease history were recorded with specific emphasis on cardio vascular diseases. Through the Subject evaluation form the dental health status, gingival and periodontal conditions, probing depth, gingival index, plaque index, clinical attachment levels and horizontal bone loss was carefully recorded. Grade (1, 2,3,& 4) horizontal bone loss was observed on an OPG (Orthopantogram). Clinical evaluation to ascertain the periodontal attachment using UNC 15 probe (Hu-freidy®) was done to achieve a reliability index score 0.8 using Dahlberg's formula.Supra gingival cleaning of plaque and debris with a curette was done during sample collection to prevent salivary contamination. 1-5ul color coded Hirschmann's microcapillary pipettes was gently placed , extra-crevicularly at the marginal gingival without penetrating into the gingival crevices and a standardized volume of 2 µl of Gingival Crevicular Fluid was collected from the subject from the most severely afflicted site of periodontitis.GCF and salivary collection was done on the individual patient in a single day to control spontaneous bleeding (if occurs) from chronically infected patients and avoid contamination at the collection sites. The samples collected were carefully dispensed into airtight and sterilized, 0.5 ml Eppendorfs bearing 398 µl of sample diluent, and a blast of air was passed through the micro capillary pipettes to enable complete removal of contamination. All the samples were stored at −80°C until analyzed. Thawing of the samples and analysis was done only once for the assay (using a solid phase sandwich enzyme-linked immunosorbent assay).

Concentration of MCP-1 and sCd40L in the samples was determined by a commercially available enzyme-linked immunosorbent assay (ELIS A) kit (Bioassay Technology Laboratory, E0124Hu and E0251Hu, China) in accordance with manufacturer's instruction. Results were analyzed statistically.

3.13 FLOW CHART / ALGORITHM OF STUDY:



3.14 STATISTICAL ANALYSIS:

Data is analyzed using IBM-SPSS version 23.0; Counts with percentages are reported on baseline characteristics, cardiovascular history, Medical alerts, life style issues, brushing habits, past treatment history, habits, periodontal status of cases and outcomes on biomarkers for cases and control samples. Association of the factors with studied group is tested using Pearson Chi Square test. Independent sample t-test is used to compare the mean of bio markers MCP1 and SCD40L between cases and control samples. Binary logistic regression analysis is used to estimate the odds ratio with 95% confidence interval for studied risk factors using univariate and multivariate analysis. P-values less than 0.05 were considered statistically significant. Pie diagram and bar charts are also used to give graphical presentation of study outcomes.

CHAPTER 4

RESULTS

4. 1 BASELINE CHARACTERISTICS OF STUDIED SAMPLES

In the present study there were one hundred samples that were divided between two study groups. Among control samples there were 26% between age 31 – 40 years, 36% were between 41 – 50 years old, 28% were between 51 – 60 years old, 10% were >60 years old, 78% were male gender, 22% were female gender, 12% were businessman, 26% were on daily wages, 16% were housewife, 12% were retired, 26% were on service and 8% were unemployed, among cases there were 24% between age 31 – 40 years, 34% were between 41 – 50 years old, 32% were between 51 – 60 years old, 10% were >60 years old, 74% were male gender, 26% were female gender, 16% were businessman, 12% were on daily wages, 26% were housewife, 12% were retired, 26% were on service and 8% were unemployed. Pearson Chi Square test did not give any significant association of these baseline characteristics between cases and control samples ($p>0.05$).

4.2: ASSOCIATION OF CARDIOVASCULAR HISTORY WITH STUDIED GROUPS

Table- 4.2 reports the association of cardiovascular history with studied groups, in control group none of the sample was reported for known heart disease, in cases 6% were reported for chest pain or exertion, 40% were reported for coronary artery disease, 66% were reported for high blood pressure, 2% were reported for Endocarditic, 20% were reported for Hyper cholestremia, 4% were reported for Previous heart attack and 28% were reported for Valvular heart disease. Pearson Chi Square test did give a significant association of Coronary artery disease, High blood pressure, Hyper cholestremia and Valvular heart disease with cases and control samples ($p<0.05$)

Table 4.1: Baseline Characteristics of Studied Samples (n=100)

Characteristics		Group				p-value
		Controls (n=50)		Cases (n=50)		
		N	***%***	***N***	***%***	
Age Group	31 - 40 years	13	26	12	24	0.97
	41 - 50 years	18	36	17	34	
	51 - 60 years	14	28	16	32	
	>60 years	5	10	5	10	
Gender	Male	39	78	37	74	0.64
	Female	11	22	13	26	
Occupation	Businessman	6	12	6	12	0.99
	Daily wages	13	26	13	26	
	Housewife	8	16	8	16	
	Retired	6	12	6	12	
	Service	13	26	13	26	
	Unemployed	4	8	4	8	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.2: Association of Cardiovascular history with studied groups

Known Heart Disease		Group				p-value
		Controls (n=50)		Cases (n=50)		
		N	***%***	***n***	***%***	
Chest pain or exertion	Yes	-	-	3	6	0.07
	No	50	100	47	94	
Coronary artery disease	Yes	-	-	20	40	<0.01*
	No	50	100	30	60	
High blood pressure	Yes	-	-	33	66	<0.01*
	No	50	100	17	34	
Endocarditic	Yes	-	-	1	2	0.31
	No	50	100	49	98	
Hyper cholestremia	Yes	-	-	10	20	<0.01*
	No	50	100	40	80	
Previous heart attack	Yes	-	-	2	4	0.15
	No	50	100	48	96	
Valvular heart disease	Yes	-	-	14	28	<0.01*
	No	50	100	36	72	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

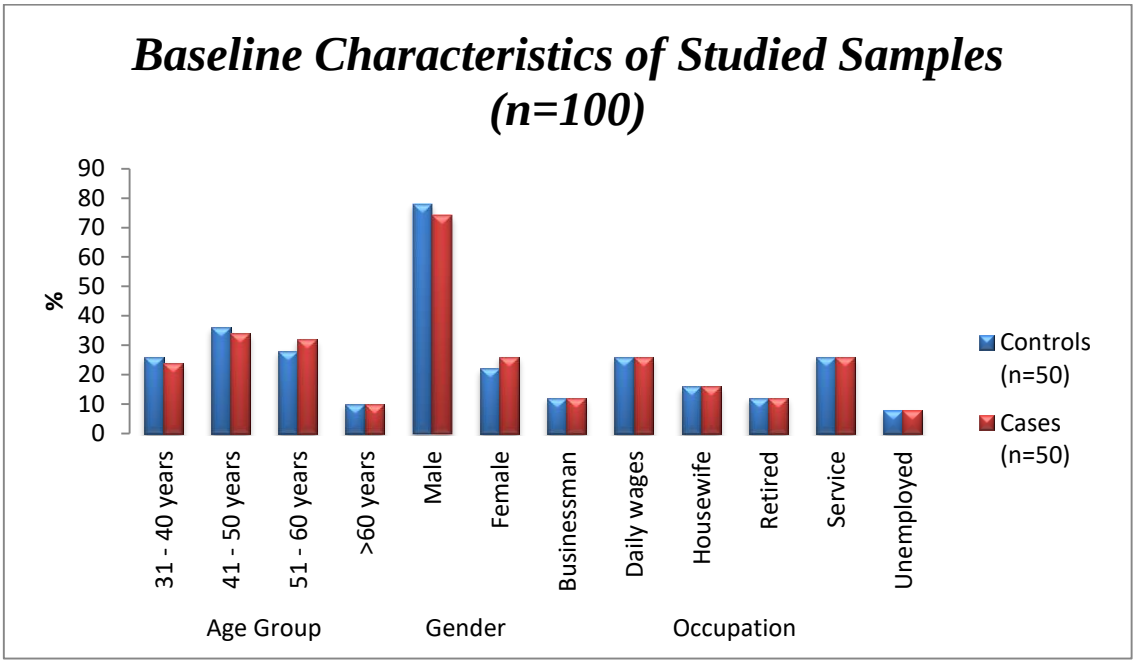


Figure 4.1 (a): Showing the descriptive baseline characteristics between cases and control samples. In controls majority were age 41 – 50 years old, male gender and on daily wages.

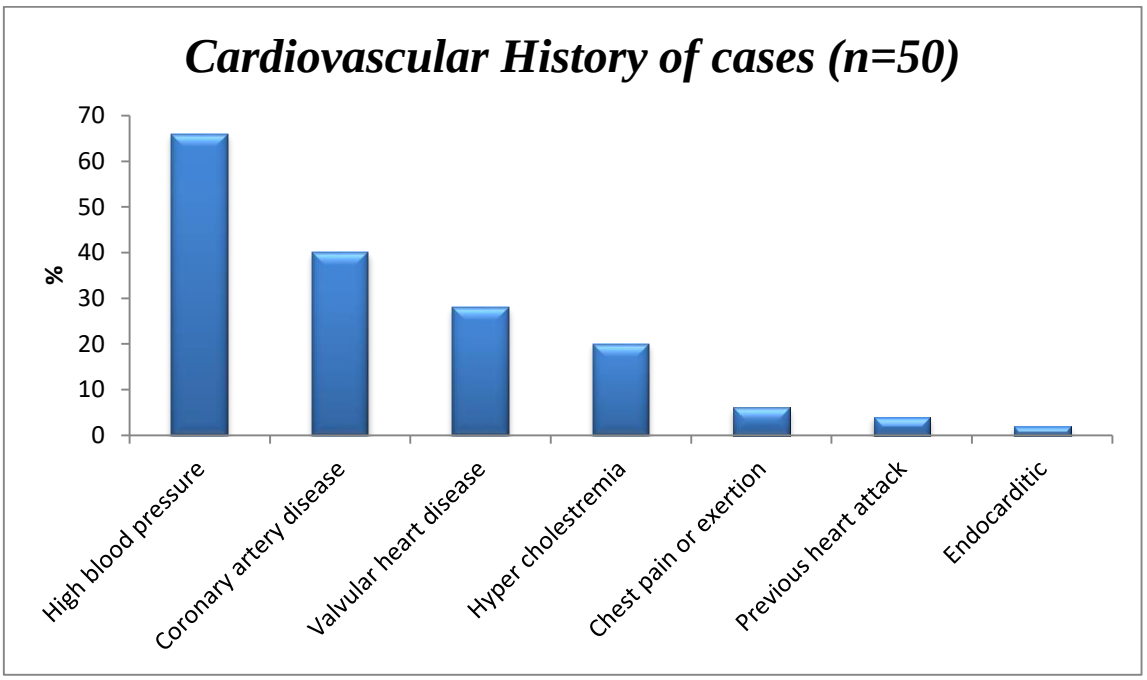


Figure 4.2 (a): Showing cardiovascular history of cases, most were reported high blood pressure, least was endocarditis.

4.3: ASSOCIATION OF MEDICAL ALERTS WITH STUDIED GROUPS

Table- 4.3 reports the association of medical alerts with studied groups, in control group samples none was reported for hypertensive, 30% were reported for diabetes, none were reported for high cholesterol, 8% were reported for Peptic ulcer disease, 6% were reported for back pain, 4% reported for back pain, 4% were reported for insomnia, 14% were reported for Asthama,6% were reported for Athritis, none was reported for stroke, 6% were reported for depression, 2% were reported for sciatica, 6% were reported for glaucoma,8% were reported for allergy, 4% were reported for jaundice and 4% were reported for some other medical alerts, whereas among cases 80% were reported for hypertensive, 42% were reported for diabetes, 46% were reported for high cholesterol, 12% were reported for peptic ulcer disease, 4% were reported for back pain, 4% reported for back pain, 4% were reported for insomnia, 6% were reported for Asthama,6% were reported for Athritis, none was reported for stroke, none was reported for depression, 2% were reported for sciatica, none was reported for glaucoma, none was reported for allergy, 4% were reported for jaundice and 4% were reported for some other medical alerts. The association of hypertensive, high cholesterol, and allergy with cases and control samples was statistically significant using Pearson Chi Square test ($p<0.05$).

4.4: ASSOCIATION OF LIFE STYLE ISSUES WITH STUDIED GROUPS

Table-4 reports the association of life style with studied groups, among control samples none was reported to ever had raised cholesterol, 42% do exercise once in a week, 30% do exercise twice in a week, 20% do exercise thrice in a week, 20% said they don’t do exercise, 32% were reported for smoking, in cases 56% were reported to ever had raised cholesterol, 38% do exercise once in a week, 22% do exercise twice in a week, 4% do exercise thrice in a week, 36% said they don’t do exercise, 48% were reported for smoking. The association of raised cholesterol was found statistically significant ($p<0.05$) using Pearson Chi Square test.

Table 4.3: Association of Medical Alerts with Studied groups

Systematic Disease		Group				p-value
		Controls (n=50)		Cases (n=50)		
		N	***%***	***n***	***%***	
Hypertensive	No	50	100	10	20	<0.01*
	Yes	-	-	40	80	
Diabetes	No	35	70	29	58	0.21
	Yes	15	30	21	42	
High cholesterol	No	50	100	27	54	<0.01*
	Yes	-	-	23	46	
Peptic ulcer disease	No	46	92	44	88	0.50
	Yes	4	8	6	12	
Back pain	No	47	94	48	96	0.64
	Yes	3	6	2	4	
Insomnia	No	48	96	48	96	0.99
	Yes	2	4	2	4	
Asthama	No	43	86	47	94	0.18
	Yes	7	14	3	6	
Athritis	No	47	94	47	94	0.99
	Yes	3	6	3	6	
Stroke	No	50	100	50	100	N.A
	Yes	-	-	-	-	
Depression	No	47	94	50	100	0.07
	Yes	3	6	-	-	
Sciatica	No	49	98	49	98	0.99
	Yes	1	2	1	2	
Glaucoma	No	47	94	50	100	0.07
	Yes	3	6	-	-	
Allergy	No	46	92	50	100	0.13
	Yes	4	8	-	-	
Jaundice	No	48	96	50	100	0.15
	Yes	2	4	-	-	
Others	No	48	96	48	96	0.99
	Yes	2	4	2	4	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.4: Association of life style issues with studied groups

Life Style Issues		Group				p-value
		Controls (n=50)		Cases (n=50)		
		N	*%*	*n*	*%*	
Have you ever had raised cholesterol?	Yes	-	-	28	56	<0.01*
	No	50	100	22	44	
How many times per day do you exercise? (Brisk Walking)	Once	21	42	19	38	0.30
	Twice	15	30	11	22	
	Thrice	4	8	2	4	
	None	10	20	18	36	
Do you smoke?	Yes	16	32	34	68	<0.01*
	No	34	68	26	52	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

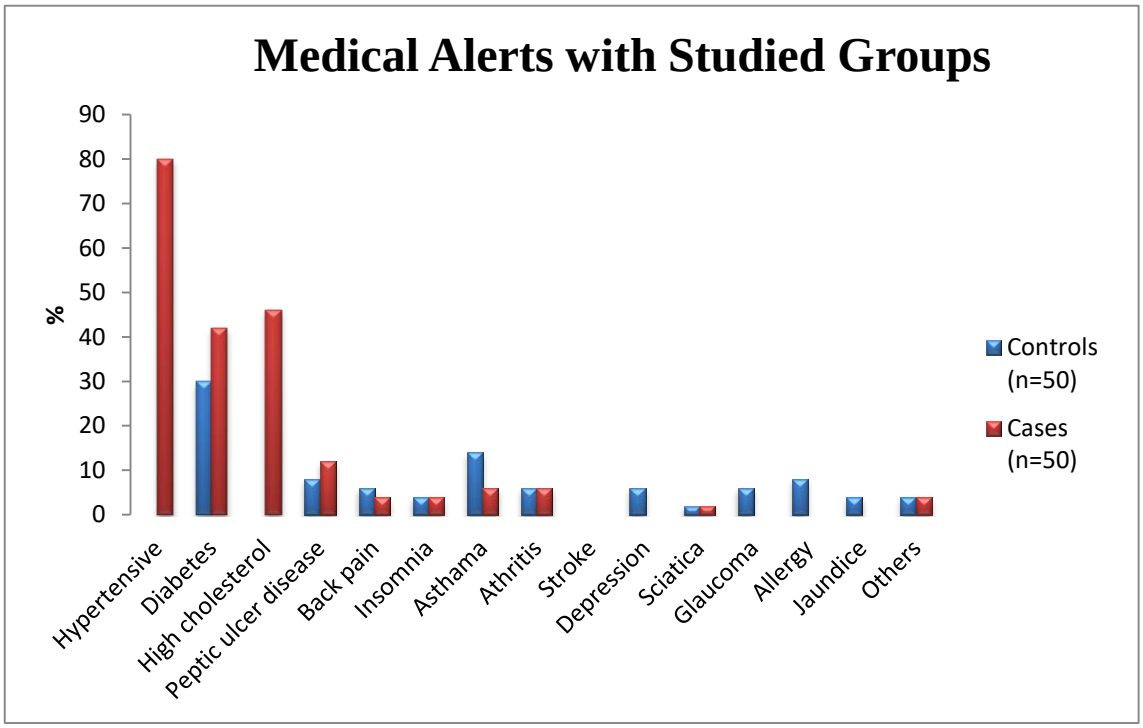


Figure 4.3 (a): Showing descriptive medical alerts in cases and control samples, in cases majority were found hypertensive, in control majority were diabetes.

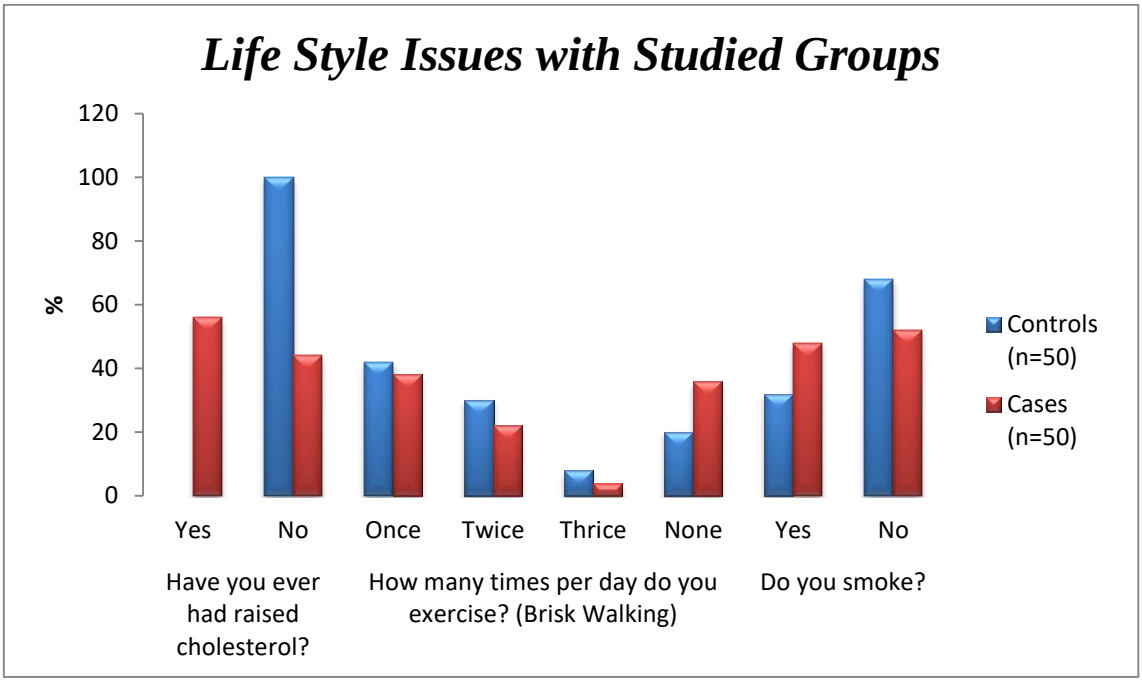


Figure 4.4 (a): Showing life style issues of cases and controls, in cases all were reported for high cholesterol, in control none was found with this complain / issue.

4.5: ASSOCIATION OF BRUSHING HABITS WITH STUDIED GROUPS

Table 4.5 reports the association of brushing habits with studied groups, in control group there were 82% samples found to clean their teeth using brush, 2% uses brush and finger, 12% uses brush and miswak, 2% uses finger only and 2% uses miswak only, there were 22% samples reported BD frequency, 8% reported occasionally, 58% reported OD frequency, and 12% reported TDS frequency, morning time was reported by 58% samples, 8% done in night, 22% done in morning and night 12% done in morning, evening and night. In cases there were 90% samples found to clean their teeth using brush, 4% uses brush and finger, and 6% uses miswak only, there were 22% samples reported BD frequency, 10% reported occasionally, 64% reported OD frequency, and 4% reported TDS frequency, morning time was reported by 66% samples, 8% done in night, 26% done in morning and night none was done in morning, evening and night. Pearson Chi Square test didn't give any significant association of brushing habits with studied groups ($p>0.05$).

4.6: ASSOCIATION OF PAST TREATMENT HISTORY WITH STUDIED GROUPS

Table 4.6 reports the association of past treatment history with studied groups, in control samples 42% were reported for extraction, 52% were reported for scaling and 54% were reported for filling in the past, in cases 70% were reported for extraction, 74% were reported for scaling and 38% were reported for filling. The association of extraction and scaling with studied groups was considered statistically significant using Pearson chi square test ($p<0.05$).

4.7: ASSOCIATION OF HABITS WITH STUDIED GROUPS

Table 4.7 reports the association of habits with studied groups, in control group 64% reported for Pan, 90% reported for chalia, 22% were reported for tobacco use, 16% were reported for niswar, 40% were reported for smoking, and 40% were reported for guttka use. In cases 70% reported for Pan, 90% reported for chalia, 26% were reported for tobacco use, 12% were reported for niswar, 48% were reported for smoking, and 30% were reported for guttka use. None of the habit did give significant association between cases and control samples using Pearson Chi Square test ($p>0.05$).

Table 4.5: Association of brushing habits with studied groups

Variables		*Group*				*p-value*
		Controls (n=50)		*Cases (n=50)*		
		N	*%*	*n*	*%*	
Type	Brush	41	82.0	45	90.0	0.07
	Brush, finger	1	2.0	2	4.0	
	Brush, Miswak	6	12.0	0	0.0	
	Finger	1	2.0	0	0.0	
	Miswak	1	2.0	3	6.0	
Frequency	BD	11	22.0	11	22.0	0.52
	Occasionally	4	8.0	5	10.0	
	OD	29	58.0	32	64.0	
	TDS	6	12.0	2	4.0	
Time	Morning	29	58.0	33	66.0	0.09
	Night	4	8.0	4	8.0	
	Morning and Night	11	22.0	13	26.0	
	Morning, Evening and Night	6	12.0	0	0.0	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.6: Association of past treatment history with studied groups

Variables		*Group*				*p-value*
		Controls (n=50)		*Cases (n=50)*		
		n	*%*	*n*	*%*	
Extraction	Yes	21	42	35	70	<0.01*
	No	29	58	15	30	
Scaling	Yes	26	52	37	74	0.02*
	No	24	48	13	26	
Filling	Yes	27	54	19	38	0.10
	No	23	46	31	62	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.7: Association of habits with studied groups

Habits		***Group***				***p-value***
		Controls (n=50)		***Cases (n=50)***		
		n	***%***	***n***	***%***	
Pan	No	18	36	15	30	0.52
	Yes	32	64	35	70	
Chalia	No	5	10	5	10	0.99
	Yes	45	90	45	90	
Tobacco	No	39	78	37	74	0.64
	Yes	11	22	13	26	
Niswar	No	42	84	44	88	0.56
	Yes	8	16	6	12	
Smoking	No	30	60	26	52	0.42
	Yes	20	40	24	48	
Guttka	No	30	60	35	70	0.29
	Yes	20	40	15	30	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

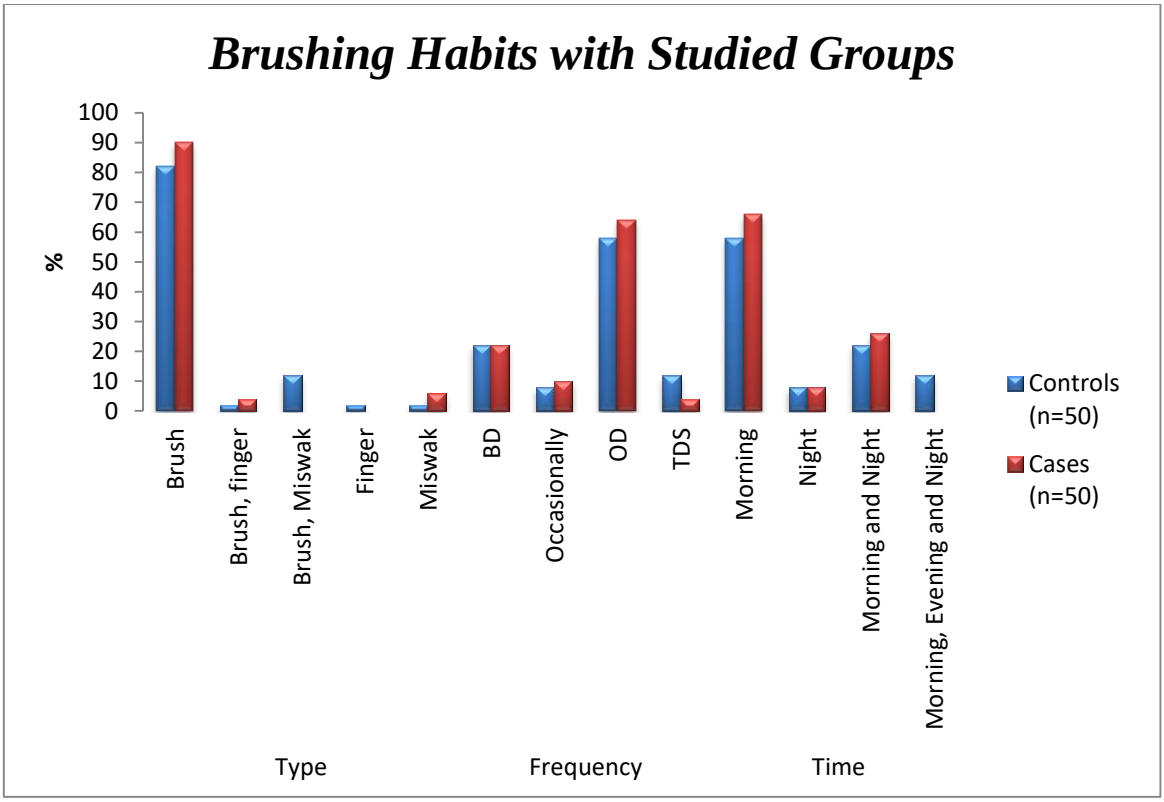


Figure 4.5 (a): Showing the brushing habits with studied groups, majority of cases uses brush, on OD frequency, in morning time only.

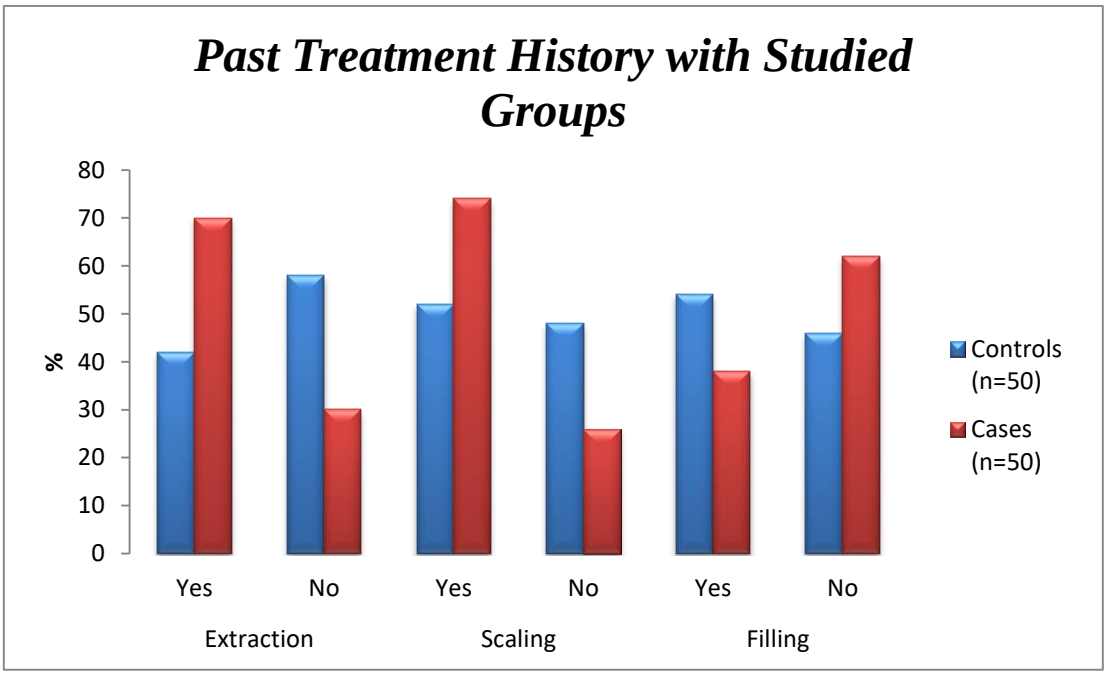


Figure 4.6 (a): Showing the past treatment history with studied groups, in cases majority were reported for extraction and scaling.

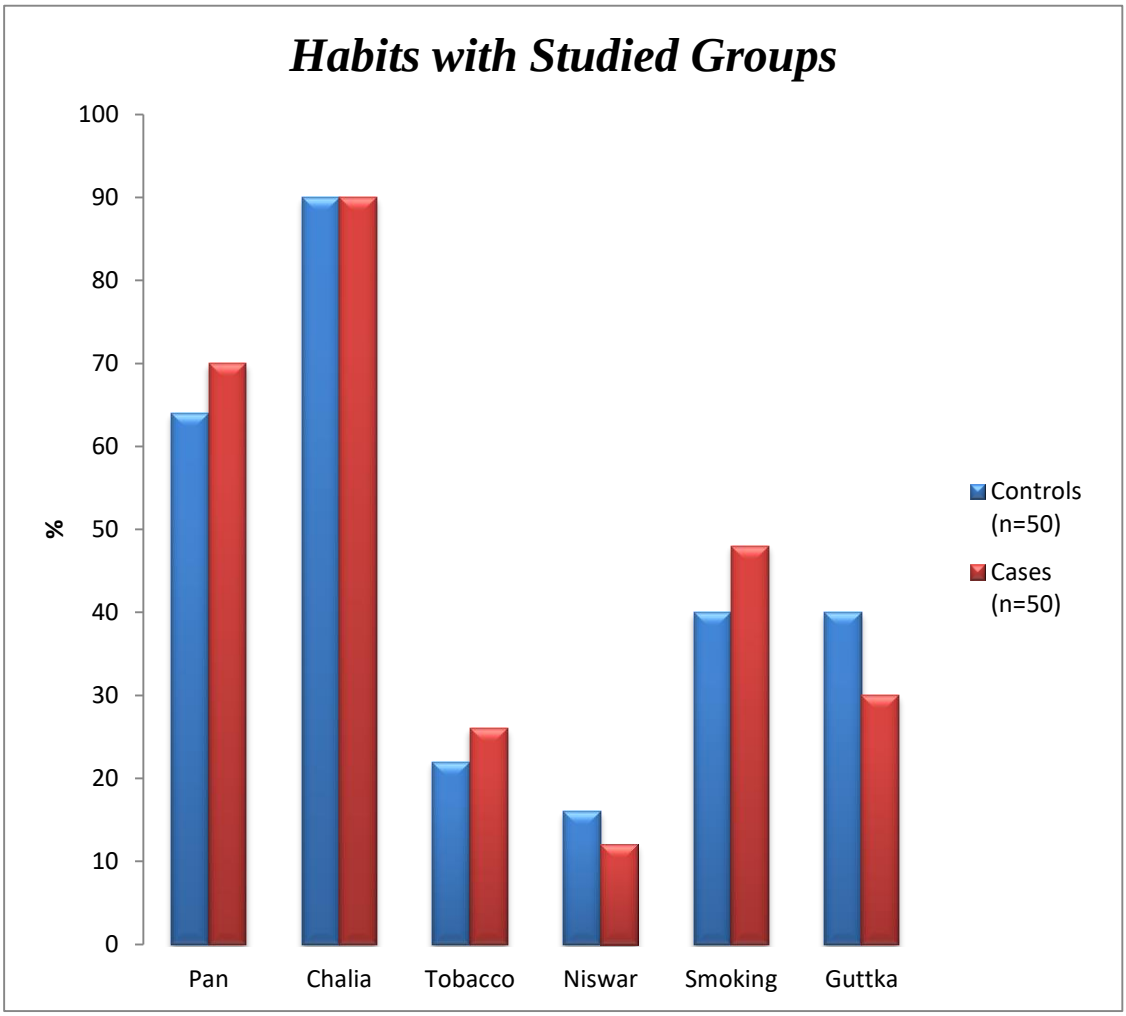


Figure 4.7 (a): Showing the habits of studied groups, in cases most were found with habit of chalia , pan , and smoking.

4.8: ASSESSMENT OF PERIODONTAL STATUS IN CASES

Table 4.8 reports the assessment of periodontal status in cases, there were 4% found with moderate and 96% were found with severe periodontitis status, 24% cases were grade-3 and 76% cases were found grade-4 horizontal alveolar bone loss. The chi square test give significant differences in the proportions ($p<0.01$).

4.9: ASSOCIATION OF PERIODONTITIS WITH AGE AND GENDER

Table- 4.9 reports the association of periodontitis with age group and gender of cases, there were 50% cases of moderate periodontitis with age 41 – 50 years old and male gender, among severe periodontitis cases 33.3% were aged 41 – 50 years old and 75% were male gender, however the association of periodontitis status with age group and gender was statistically insignificant ($p>0.05$).

4.10: ASSOCIATION OF GRADES WITH AGE AND GENDER

Table-4.10 reports the association of grades with age group and gender of cases, there were 50% cases of grade-3 with age 41 – 50 years old and 75% were male gender, among grade-4 cases cases 28% were aged 41 – 50 years old and 73.7% were male gender, however the association of grades with age group and gender was statistically insignificant ($p>0.05$).

4.11: MEAN COMPARISON OF MCP-1 AND sCD40L WITH STUDIED GROUPS

Table-4.11 reports the mean comparison of biomarkers between cases and control group samples, results showed in control group mean MCP1 was 286.5 (SD=±54.3), and mean SCD40L was 409.2 (SD=±122.4), whereas in cases the mean MCP1 was 464.4 (SD=±175.2) and mean SCD40L was 487.3 (SD=±144.8). Independent sample t-test did give a significant mean difference for the MCP1 and SCD40L between cases and control group samples ($p<0.05$).

Table 4.8: Assessment of periodontal status in cases

Variables		***Cases (n=50)***		***p-value***
		N	***%***	
Periodontitis	Moderate	2	4.0	<0.01*
	Severe	48	96.0	
Grade: Horizontal alveolar bone loss	Grade 3	12	24.0	<0.01*
	Grade 4	38	76.0	
*p<0.05 was considered statistically significant using sign test				

Table 4.9: Association of periodontitis with age and gender

Variables		***PERIODONTITIS***				***p-value***
		Moderate (n=2)		***Severe (n=48)***		
		n	***%***	***n***	***%***	
Age Group	31 - 40 years	0	0.0	12	25.0	0.19
	41 - 50 years	1	50.0	16	33.3	
	51 - 60 years	0	0.0	16	33.3	
	>60 years	1	50.0	4	8.3	
Gender	Male	1	50.0	36	75.0	0.43
	Female	1	50.0	12	25.0	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.10: Association of grades with age and gender

Variables		***Grade***				***p-value***
		Grade-3 (n=12)		***Grade-4 (n=38)***		
		n	***%***	***n***	***%***	
Age Group	31 - 40 years	3	25.0	9	23.7	0.49
	41 - 50 years	6	50.0	11	28.9	
	51 - 60 years	2	16.7	14	36.8	
	>60 years	1	8.3	4	10.5	
Gender	Male	9	75.0	28	73.7	0.92
	Female	3	25.0	10	26.3	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.11: Mean Comparison of MCP-1 and sCD40L with Studied Groups

Biomarkers	***Groups***				***p-value***
	Controls (n=50)		***Cases (n=50)***		
	Mean	***SD***	***Mean***	***SD***	
MCP-1	286.5	54.3	464.4	175.2	<0.01*
sCD40L	409.2	122.4	487.3	144.8	<0.01*
*p<0.05 was considered statistically significant using Independent sample t-test					

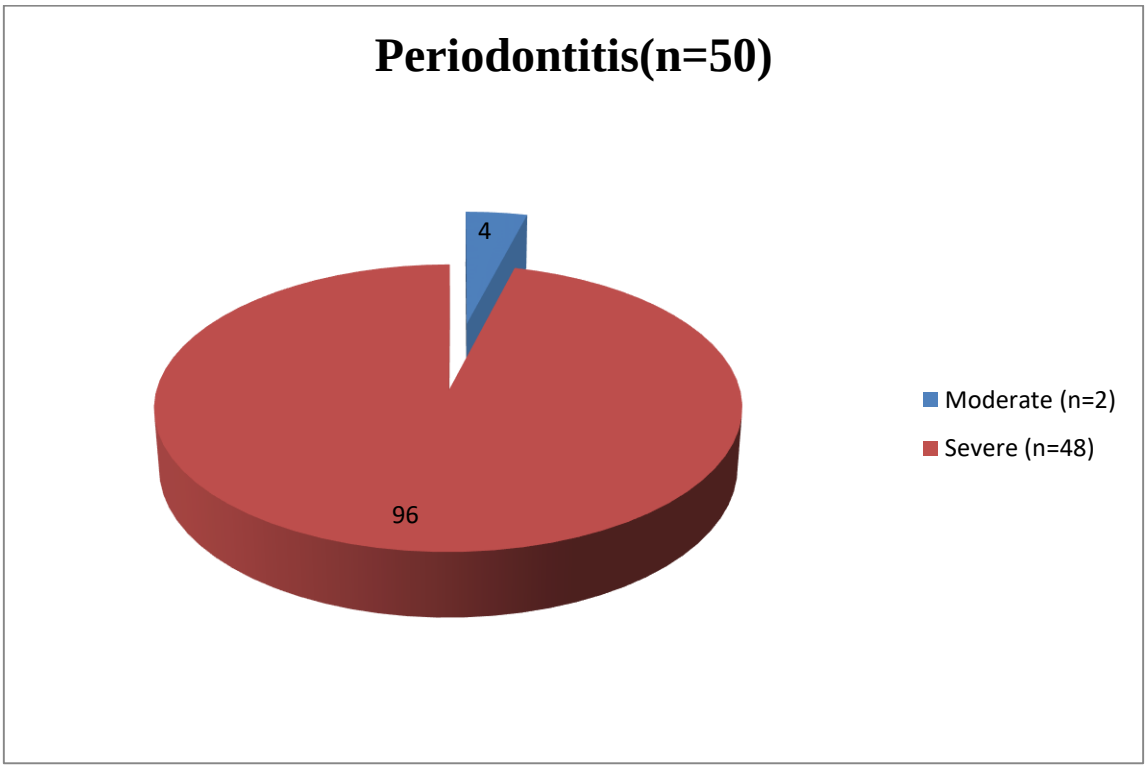


Figure 4.8 (a): Showing in cases most were severe periodontitis cases.

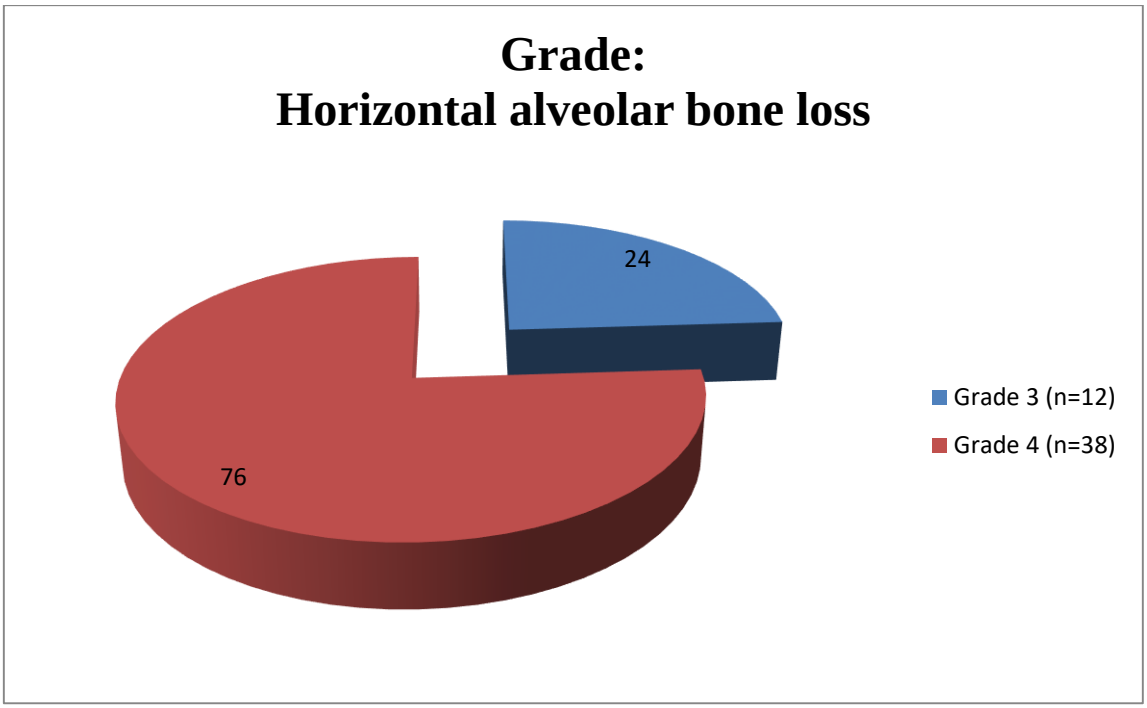


Figure 4.9 (a): Showing in cases most were grade-4 horizontal alveolar bone loss.

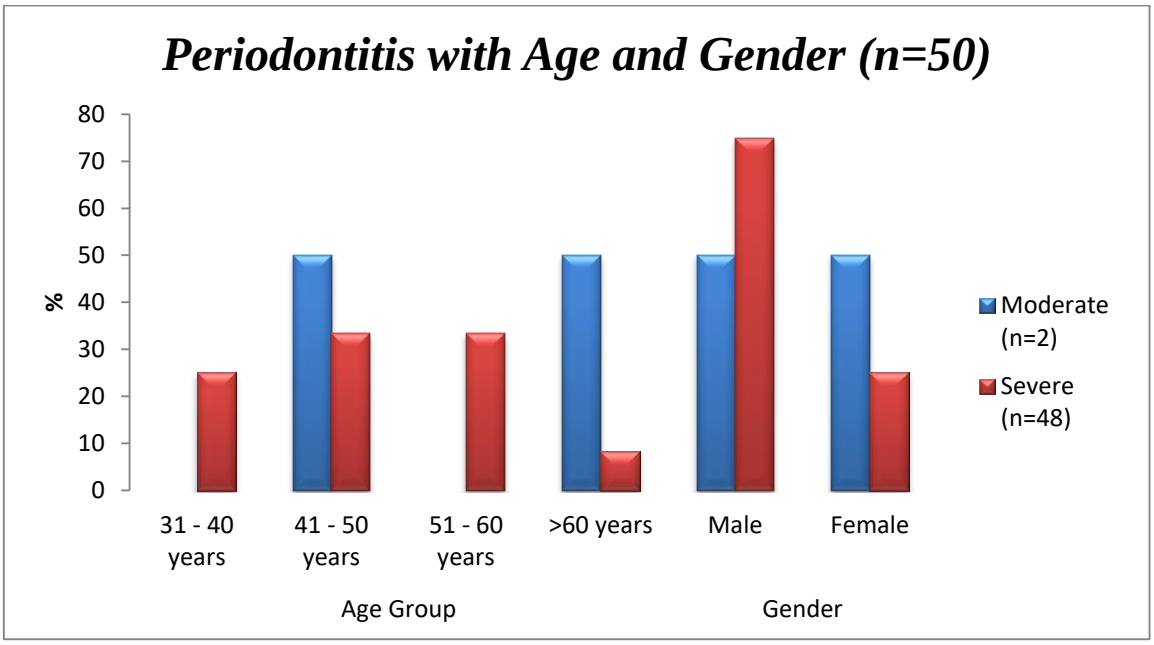


Figure 4.10 (a): Showing periodontitis with age and gender, majority were age 41 – 50 years with severe status, and male gender.

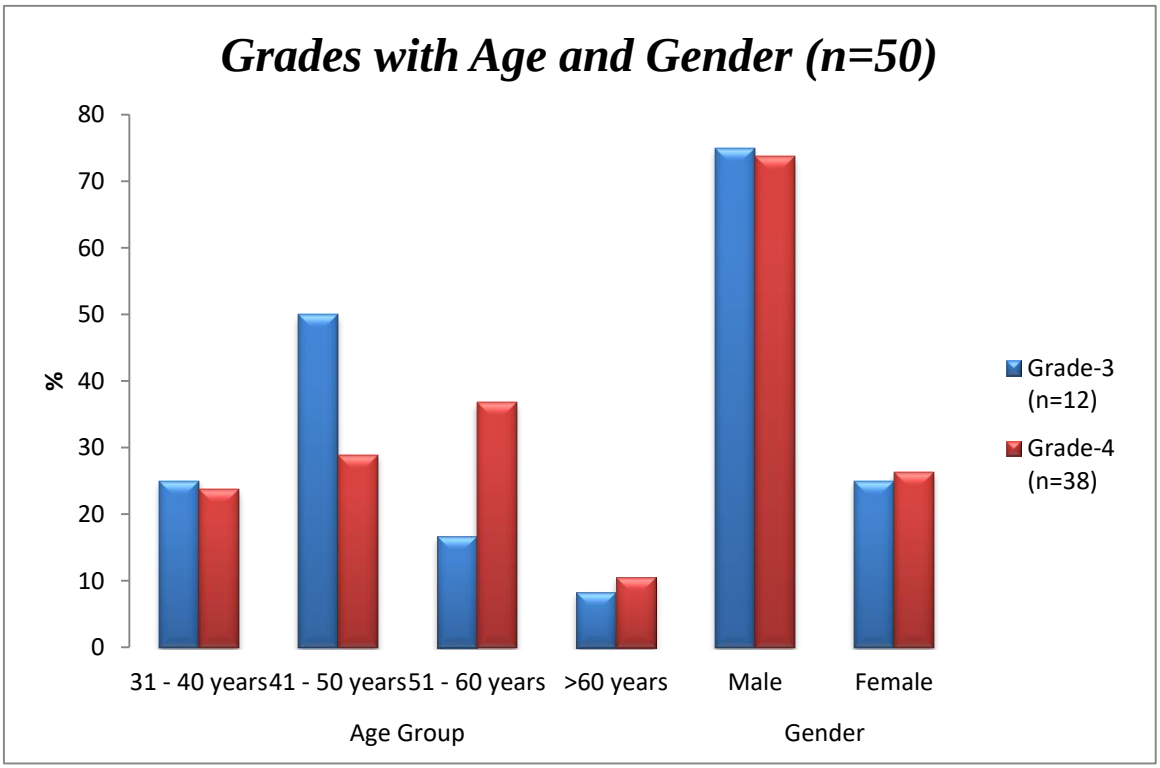


Figure 4.11 (a): Showing the grades with age and gender, majority cases of grade-4 were found with age 51 – 60 years old, and male gender.

4.12: ASSOCIATION OF MCP-1 AND sCD40L WITH STUDIED GROUPS

Table-4.12 reports the association of biomarkers with studied groups, in control group there were 12% raised MCP1 and 14% were raised SCD40L whereas in cases 64% were raised MCP1 and 52% were raised SCD40L. The association was considered statistically significant using Pearson Chi Square test ($p<0.01$).

4.13: ESTIMATION OF ODDS RATIO USING BINARY LOGISTIC REGRESSION MODEL

Table-4.13 reports the odds ratio with 95% confidences interval for cases using binary logistic regression analysis. In univariate model it was observed that samples with male gender, [OR=0.80 C.I (0.31-2.01)], done filling in past [OR=0.52 C.I (0.23-1.15)] were found less likely to be found with cases, and samples who done extraction in past [OR=3.22 C.I (1.41-7.35)], done scaling in past [OR=2.62 C.I (1.13-6.09), raised MCP1 [OR=1.01 C.I (1.00-1.01)] and raised SCD40L [OR=1.0 C.I (1.00-1.00)] were found more likely to be found with cases. Odd for extraction, scaling, MCP1 and SCD40L were found statistically significant ($p<0.01$). In multivariate model association of gender, filling with cases was negative and association of extraction, MCP1 and SCD40L with cases was found positive, the odds for extraction [OR=9.23 C.I (2.17-39.2)], scaling [OR=5.60 C.I (1.36-23.0)], MCP1 [OR=1.01 C.I (1.00-1.01)] and SCD40L [OR=1.0 C.I (1.00-1.00)] were statistically significant ($p<0.05$).

Table 4.12: Association of MCP1 and sCD40L with Studied Groups

Biomarkers		***Groups***				***p-value***
		Controls (n=50)		***Cases (n=50)***		
		n	***%***	***n***	***%***	
MCP1	Raised	6	12	32	64	<0.01*
	Not Raised	44	88	18	36	
SCD40L	Raised	7	14	26	52	<0.01*
	Not Raised	43	86	24	48	
*p<0.05 was considered statistically significant using Pearson Chi Square test						

Table 4.13: Estimation of Odds ratio using Binary logistic Regression Model

Parameters	***Univariate OR (95% C.I)***	***Multivariate OR (95% C.I)***
Age (years)	1.0 (0.95-1.05)	1.0 (0.93-1.08)
Male	0.80 (0.31-2.01)	0.49 (0.13-1.78)
Extraction	3.22* (1.41-7.35)	9.23* (2.17-39.2)
Scaling	2.62* (1.13-6.09)	5.60* (1.36-23.0)
Filling	0.52 (0.23-1.15)	0.61 (0.19-1.97)
MCP1	1.01* (1.00-1.01)	1.01* (1.00-1.01)
SCD40L	1.0* (1.00-1.00)	1.0* (1.00-1.00)
Dependent variable : Group **odds ratio with p<0.05 was considered statistically significant*		

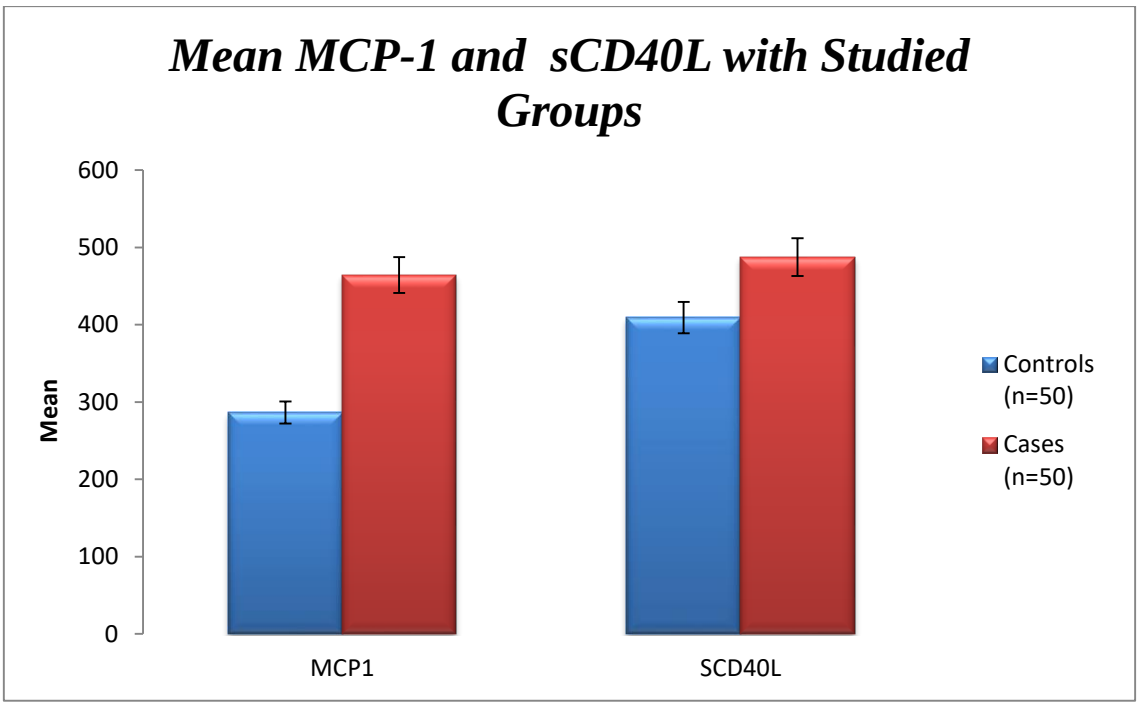


Figure 4.12 (a): Showing in cases mean MCP1 and SCD40L were found higher than control group.

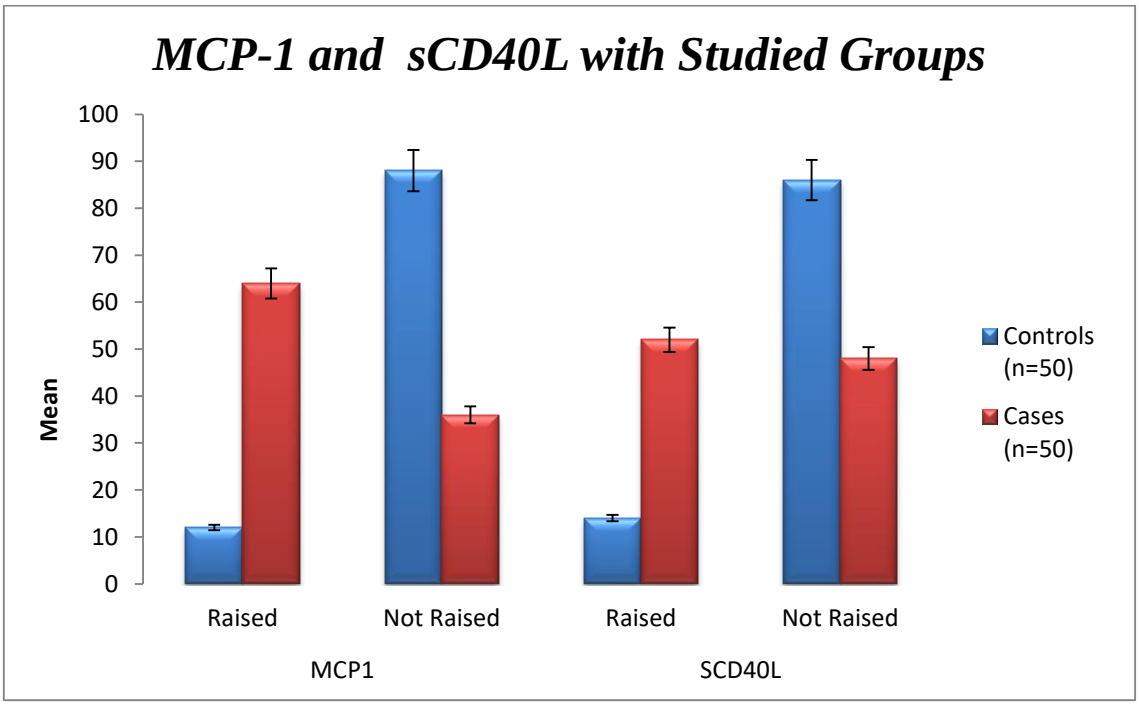


Figure 4.12 (b): Showing in cases mostly MCP-1 levels were raised as compared to sCD40L.

CHAPTER 5

DISCUSSION AND CONCLUSION

5.1 SEQUENCE OF DISCUSSION EXPERIMIENT / HYPOTHEIS WISE:

A total of 100 patients (50 cases and 50controls) completed the study. Patients were enrolled in the study as per American Academy of periodontology guidelines diagnosed with severe chronic periodontitis.Chronic inflammatory periodontal diseases are among the most common human infections with 10–15% of the population afflicted with advanced forms of the disease. (Leishman SJ et al 2010). In the present study control samples were 26% between age 31 – 40 years, 36% were between 41 – 50 years old, 28% were between 51 – 60 years old, 10% were >60 years old, 78% were male gender, 22% were female gender, among cases there were 24% between age 31 – 40 years, 34% were between 41 – 50 years old, 32% were between 51 – 60 years old, 10% were >60 years old, 74% were male gender, 26% were female gender. The male gender in both the cases and control were more as compared to females which might be due to the reason that during enrollment oral hygiene and periodontal status in males could not meet the appropriate standards of healthy periodontium. This is in coherence with a study by Schulze and colleagues where the periodontal status was unsatisfactory in male participants (Schulze et al., 2016). However, non significant results were found between the genders among the two groups in the present study. One of the most common risk factor in the initiation of periodontitis is inadequate oral hygiene. Attaining optimal oral hygiene has been regarded as strongly associated to the prevention of gingivitis that may later on preceed to periodontitis and bone loss in the susceptible host (Lertpmonchai et al., 2017). A pronounced relationship has been observed between poor oral hygiene and increased accumulation of dental plaque, high prevalance and increased severity of periodontal disease (Bhardwaj et al., 2014). The accumulation of dental plaque and calculus is the consequence of inappropriate tooth brushing technique, infrequent brushing of teeth, type of cleaning aids and failure to

carry out irreugualr dental visits. In the present study patients were inquired of brushing frequency by means of a proforma (Annexure-D), categorized as once a day, twice thrice and occassionally. Majority of the participants brushed once a day (58%) followed by others who brushed twice (22%) and occasionally (8%). Ideally cleaning and brushing of teeth is advised twice a day (Fotedar et al., 2016) but majority of the patients in the current study with chronic perioodntitis in both the groups carried out brushing once a day. Hence the present fact could not coincide with a study demonstrating the significance of tooth brushing, twice daily along with proper tooth brushing techniques and dental flossing, undoubtedly is effective in preventing periodontitis and other oral diseases (Zimmermann et al., 2015).

In the present study other risk factors included were smoking, tobacco chewing, betel nut (chalia), gutka and naswar. In the control group 64% reported for Pan, 90% reported for chalia, 22% were reported for tobacco use, 16% were reported for niswar, 40% were reported for smoking, and 40% were reported for guttka use. In cases 70% reported for Pan, 90% reported for chalia, 26% were reported for tobacco use, 12% were reported for niswar, 48% were reported for smoking, and 30% were reported for guttka use both males and females. The male participants of the present study indulged in smoking presented with changes in the clinical parameters that coincides with a study on smokers presenting with increased periodontal pocket depths and clinical attachment loss (Bergstrom, 2014). Though smoking appears to be one of the significant risk factors in the development and progression of periodontal disease but present study supported the fact that non smokers also presents with similar plaque accumulation, pocket depths and clinical atrachment loss coincinding with the study (Jacob, 2017).

However, there are non significant results in between the groups. Studies have revelaed that tobacco smoke induces alterations to the 3-OH fatty acids present in lipid A in a manner that is consistent with a microflora of reduced inflammatory potential. Smokers have significant reductions in the 3-OH fatty acids associated with the consensus (high potency) enteric LPS structure as compared with non-smokers with chronic periodontitis. Thus, smoking is associated with specific structural alterations to the lipid-A-derived 3-OH fatty acid profile in saliva that are consistent with an oral microflora of reduced inflammatory potential. (Buduneli N., et al 2011).

In the present study association of cardiovascular history with studied groups revealed that in control group none of the sample were reported for known heart disease, in cases 6% were reported for chest pain or exertion, 40% were reported for coronary

artery disease, 66% were reported for high blood pressure, 2% were reported for Endocarditic, 20% were reported for Hypercholestremia, 4% were reported for previous heart attack and 28% were reported for Valvular heart disease.The presence of periodontal infection further evokes a generalized inflammatory response thereby posing a risk for numerous systemic disease states, one of them being a higher risk of CVD. A clear evidence of an epidemiological association between oral infections and CVD is demonstrated via the studies that coincide with the present study showing a significant correlation between the subgingival microbiota and the pathogens detected in the vascular lesions. (Mahendra J., et al 2014) Links between periodontitis and atherosclerosis have been predicted based on inflammatory mechanisms initiated by bacteria associated with periodontal lesions. Invasive periodontopathic bacteria like Porphyromonas gingivalis and their byproducts reach systemic circulation. (Takeuchi H., et al 2012). The adaptive immune response to the presence of these bacteria exacerbates the expression of pro-inflammatory cells which in turn up-regulate the endothelial cell activation, promote infiltration of activated leukocytes, and influence the propagation of the atherosclerotic lesions (Packard RR .,et al 2018). The present study is in coherence with Takeuchi H & Packard RR which gave a significant association of Coronary artery disease, High blood pressure, hypercholestremia and Valvular heart disease with cases. Mean comparison of biomarkers between cases and control group samples in the present study, showed that in control group mean MCP1 was 286.5 (SD=±54.3), and mean SCD40L was 409.2 (SD=±122.4), whereas in cases the mean MCP1 was 464.4 (SD=±175.2) and mean SCD40L was 487.3 (SD=±144.8). This give a significant mean difference for the MCP1 and SCD40L between cases and control group sample so the presnt study reveals that the mainstay of the pathogenesis is the involvement of bio-molecules in the inflammatory and immune cascade such as MCP-1 and sCD40 L, two such mediator molecules that not only initiates and propagate an atherosclerotic state but also be involved in the progression of periodontal disease. The increase in concentrations of both these mediators has been independently documented both locally in GCF, as well as systemically in patients with severe periodontitis in some healthy controls and mostly in cases as supported by (Pradeep AR.,et al 2009). The findings of the present study substantiated the probable pathogenic role played by the two markers in causing severe periodontal disease and increasing the systemic inflammatory load that coincides with the study carried out by (Gupta M, et al 2103). Kiener et al. in their study documented sCD40 L to be a potent monocytes activator.

Ligation of CD40 on human monocytes induces phenotypic changes that would influence T-cell activation by the monocytes and also enhance or prolong their inflammatory response. (Kiener PA., et al 1995) Aukrust et al. has further specifically proved in their study that sCD40 L induces dose-dependent and specific release of MCP-1 from peripheral blood mononuclear cells (Aukrust P., et al 1999).

A study by Giannini et al. supporting the present study in their in vivo model documented that level of MCP-1 and soluble vascular cell adhesion molecule-1 significantly correlates with the levels of CD40 L expressed by platelets (Giannini S., et al 2012). The explanation proposed is that increased sCD40 interacts with its receptor on monocytes and enhances the release of CC-chemokine, MCP-1 from mononuclear cells. MCP-1 further promotes the infiltration of these activated leukocytes into the atherosclerotic lesion, and these in turn may directly activate smooth muscle cells, macrophages, and T cells inside the vessel wall, promoting atherogenesis (Stumpf C et al 2016) also concluded that platelet CD40 L plays a pivotal role in atherosclerosis by increasing platelet-leukocyte and leukocyte endothelium interactions, and increase in MCP-1 through CD40 ligand seems to be a crucial phenomenon in acerbating atherosclerosis. The present study has also aimed at highlighting the existence of this correlation between the levels of MCP-1 and sCD40L in GCF of patients with severe chronic periodontitis. Dissemination of dental plaque bacteria in the circulation can induce platelet activation leading to increased expression of both surface and soluble CD40 L, which can interact with CD40 receptor on endothelial cells, macrophages, and fibroblasts to promote a wide array of functions causing progressive atherosclerosis and its destabilization (Imamura T., et al 2016). A positive correlation was observed between sCD40 L and MCP-1 levels in the present study which suggests that this phenomenon is one of the pathways that leads to the propagation of cardiovascular events in patients with periodontal disease.

The present study reveals that when assessment of periodontal status in cases were carried out, there were 4% with moderate and 96% were with severe periodontitis status, 24% cases were grade-3 and 76% cases were found grade-4 horizontal alveolar bone loss. This corroborates the notion of Papapanou and Wennström that angular periodontal bony lesions demonstrate a higher risk of progressive chronic periodontitis than periodontal sites with horizontal bone morphology (Papapanou PN., et al 2008). However, the current study differs from a retrospective case-control studies which reported no statistically significant differences in progressive angular and horizontal

bone loss in periodontitis patients (Greenstein B., et al 2009). The reason for bone resorption in chronic periodontitis is that the gingival crevicular fluid (GCF) contains a complex array of protein components that not only irrigate the gingival sulcus but also are released into the oral cavity (Ebersole J ., et al 2013). GCF is derived from gingival capillary beds (serum components) and from both resident and emigrating inflammatory cells. This fluid contains an array of innate, inflammatory, and adaptive immune molecules and cells whose role is to contribute to the interaction of host and bacteria in this ecological niche. Studies demonstrate that GCF contains mediators that can stimulate bone resorption. The primary factor responsible is IL-1α, with IL-1β and PGE2. This exudate is an accessible source of extracellular matrix derived biologic markers of periodontal bone resorption (Gupta G., et al 2013).

The present study also reveals the association of life style with studied groups, among control samples none was reported to have had raised cholesterol, in cases 56% were reported tor have raised cholesterol , this corborates with the study carried out by

(Feng L.,et al 2020) that age is linked with worsening lipid profiles resulting in periodontal infections . The absorption, synthesis, and metabolism of lipoproteins cholesterol change with age through complex mechanisms Old age is a predisposing factor for disruption of lipid levels, so the elderly are more likely to have CVD (Pollock RD., et al 2015).

A study by (sanz, M. et al. 2018) proposed that untreated diabetes mellitus, leads to metabolic disorders caused by hyperglycemia. Poor glycemic control in patients with diabetes has been shown to raise the level of systemic inflammation markers, e.g. interleukin-1ß, in the gingival crevicular fluid of a periodontal pocket, which is associated with the onset and severity of periodontal disease. Gram-negative bacteria in the periodontal pockets elevate serum inflammatory markers such as C - reactive protein which can induce hyperinflammatory immune cells and promote the release of proinflammatory cytokines leading to insulin resistance. The current study is contradictory and shows non significant results as the cases were selected as having chronic periodontitis and cardiac diseseases however diabetes was considered as a confounding factor.

5.2 IMPLICATION OF THE STUDY:

5.2.1 THEORETICAL IMPLICATIONS:

This study contributes to the fact that pro inflammatory cytokines can recruit leukocytes, lymphocytes, and macrophages to produce a local inflammatory response, resulting in smooth muscle cell proliferation and extracellular matrix deposition. This can lead to accelerated atherosclerosis and, in some cases, instability in the fibrous cap leading to plaque rupture and CVD's.Through this study , it is hypothesized that periodontitis can trigger an inflammatory cascade in the oral cavity, in moderate to severe cases, stimulating a systemic cascade as a result of transient bacteremia. To support this hypothesis testing of 50 case samples of chronic periodontitis and CVD's was carried out that showed approximately 78% of the specimens were positive for at least one periodontal pathogen, suggesting a potential role for these pathogens in atherosclerosis. Thus relationship between PD and atherosclerosis (CVD's) through this study confirms that oral pathogens: "(1) disseminate from the oral cavity and reach the systemic vascular tissue, (2) can be found in the affected tissues, (3) live within the affected site, (4) invade affected cell types in vitro, (5) induce atherosclerosis in humans.

5.2.2 PRACTICAL IMPLICATIONS:

Infective endocarditis (IE) a form of CVD, is an infection of the endocardial surface of the heart, most commonly impacting one or more heart valves, that is thought to be caused by bacteremia originating from the oral cavity, gut, skin, or mucous membranes. The transient bacteremia caused by these pathogens activates an inflammatory cascade of immunologic and thrombotic resulting in significant morbidity and mortality associated with IE. IE caused by Gram-positive cocci, such as Staphylococcus aureus, its treatment requires prolonged antibiotic therapy, and half of patients with IE undergo valvular surgery. Therefore, the risk of IE caused by bacteremia of periodontal infection may result in elevated Valvular Heart Disease.This study have implictaed the indications for reduced antibiotic therapy for the prevention of IE and has highlighted the clinical significance of mantainance of oral hygiene as poor oral health has a profound effect on general health, and it is thereofore important to examine periodontal

infections along with rigorous evaluation of oral conditions. The findings of the current studyhas substantiated the probable pathogenic role played by the two markers MCP-1 and sCd40 L in causing severe periodontal disease and increasing the systemic inflammatory load.The present study showed a significant positive association and an increase in the quantity of sCD40 L corresponded with an increase in levels of MCP-1 in both GCF and serum, and so far this is the first study documenting such interrelationship between the two markers in patients with periodontal disease.

5.2.3 POLICY IMPLICATIONS:

This research documented that improvement in oral health is important in preventing progression of various systemic illnesses. Thus, treatment, as well as prevention of periodontal disease, should not be considered in isolation, but as an integral part of the maintenance of general health of the patients. For this, a collaborative effort is needed between medical and dental practitioners with the former becoming more aware of clinical presentations of periodontal disease in their patients and their early referral to dentists, thus, minimizing chances of any systemic effects. Similarly,dentists should not only render treatment for periodontal disease, but also look for any confounding risk factors forperiodontitis and CVD (overweight, hypertension, diabetes) and help in their management along with their medical counterparts.

5.3 LIMITATIONS AND STRENGTH OF THE STUDY:

A) Limitations:

1. This study focuses mainly on the association of peridontitis with CVD’s but confounding factors like alcoholism and diabetes affecting and having an association with periodontal disease was not assessed.
2. Single centric study conducted in BUHSCK – Bahria Unversity Dental College.
3. Short duration of study of 3 months to fulfil the requirement of M.Phil degree.
4. Radiological investigations without CBCT technique due to budget constraints.
5. Gender specific relationship with the disease was not assessed as the condition of chronic peritonitis affects equally in both males and females.

B) Strength:

1. Patient having CVD's will be advised to maintain a good oral hygiene so as to prevent the aggravation of systemic cardiac illness and that in severe condition of peridontitis it will have a negative impact on Cardivascular health.
2. The physician will also liaise with the dental surgeon while treating chronic peridontitis patients who are on anticoagulant therapy so as to avoid excessive bleeding or ischemic events.
3. By having the early awareness, the patient will consider periodontal treatment as the integral part health status maintenance which will increase the early referrals to dentists to avoid the worst systemic outcome.

5.4 FUTURE RESEARCH DIRECTIONS/ RECOMMENDATIONS:

Open avenues for future studies in CVD patients with chronic periodontitis are:

1. To evaluate the patients with periodontitis for higher risk for cardiovascular diseases, such as myocardial infarction or stroke, and actively manage all their cardiovascular risk factors such as smoking, exercise, excess weight, blood pressure, lipid and glucose management, with sufficient periodontal therapy and maintenance.
2. To evaluate the CVD patients with periodontitis who are at higher risk for subsequent CVD complications, and advice them to regularly adhere to the recommended dental therapeutic, maintenance and preventive regimes.
3. To educate the patients to consults his/her physician if any of these risk factors are not appropriately controlled. Oral health education should be provided to all patients with periodontitis and a tailored oral hygiene regime, including twice - daily brushing, interdental cleaning and, in some cases, the use of adjunctive chemical plaque control, may be appropriate.
4. To evaluate full - mouth probing and bleeding scores in subjects presenting with a diagnosis of CVD via thorough oral examination that embeds a comprehensive periodontal evaluation.
5. To manage people with CVD, if periodontitis is diagnosed, as soon as their cardiovascular status permits, irrespective of the level of CVD or specific medication, nonsurgical periodontal therapy should be provided, preferably in 30 - to 45 - min sessions, in order to minimize a spike of acute systemic inflammation
6. To highlight the role of local haemostatic agents (such as oxidized cellulose, absorbable gelatin sponges, sutures, tranexamic acid mouthwashes, compressive gauze soaked in tranexamic acid) used and dental clinicians should consider the confounding effect of local anaesthetic with vasoconstrictors.
7. To premedicate the patients with a risk of endocarditis with antibiotics following current guidelines (European or the American guidelines).

8. To encourage the people with cardiovascular disease who have extensive tooth loss to pursue dental rehabilitation to restore adequate mastication for proper nutrition.
9. To educate people with CVD's that gum disease is a chronic condition, which may aggravate therefore requires lifelong attention and professional care, encouraging them the need to clean the teeth and gum carefully at home.
10. To inquire the patients with CVD about the signs and symptoms of periodontitis, including bleeding gums during brushing oreating, loose teeth, spacing or spreading/drifting of the teeth, oral malodor and/or abscesses of the gums or gingival suppuration.
11. Liaison of physician with the dental surgeon over periodontitis management in CVD patients on anticoagulant/antiplatelet therapy prior to the oral intervention and/or periodontal surgery, so as to avoid excess bleeding or the risk of ischaemic events.
12. Redoing previous work connecting periodontitis and metabolic/cardiovascular health with measurement of indicators of the presence and severity of mental illness.
13. Studying periodontal disease in pregnancy and the effect of treating it on metabolic factors (e.g., diabetes).
14. Investigating inflammatory markers for predicting severity and timing of possible interventions, and monitoring of these patients.
15. Identifying interventions that would have the greatest overall impact across domains, and the groups of individuals, who would benefit most from periodontal disease prevention and treatment.
16. Exploring genetic, epigenetic and environmental factors in large population studies that put individuals at shared risk for periodontal disease, mental illness, metabolic and cardiovascular disease.

5.5 CONCLUSION:

In periodontitis, inflammation is the key component for the spread of microorganisms. The inflammatory cytokines play an important role as mediators of inflammation and are involved in different pathways. There is an interaction between the microorganism present and the host's immune response. In periodontitis, there is a feedforward loop between host and microbiota variables that is responsible for the onset and persistence of systemic diseases like CVD's. This study comprehensively summarizes as to how periodontitis goes from a seemingly mild, localized infectious disease, to a potentially life-threatening, chronic generalized hyper-inflammation condition. A strong, positive and significant correalation of salivary biomarkers MCP-1 and sCD40L in chronic periodontitis patients with CVD's was observed. In conclusion, higher circulating levels of MCP-1 and sCd40-L are associated with higher long-term cardiovascular mortality in individuals with chronic periodontitis and this study supported a key role of these cytokines in patients with cardiovascular disease. Higher severity of periodontal disease and poor oral hygiene leads to greater manifestation of systemic inflammation in patients with CVD's. The ultimate goal of the study is to continue opening a dialogue on the many facets of periodontal disease that may establish early diagnosis and treatment of many complex systemic conditions under an integrative framework.

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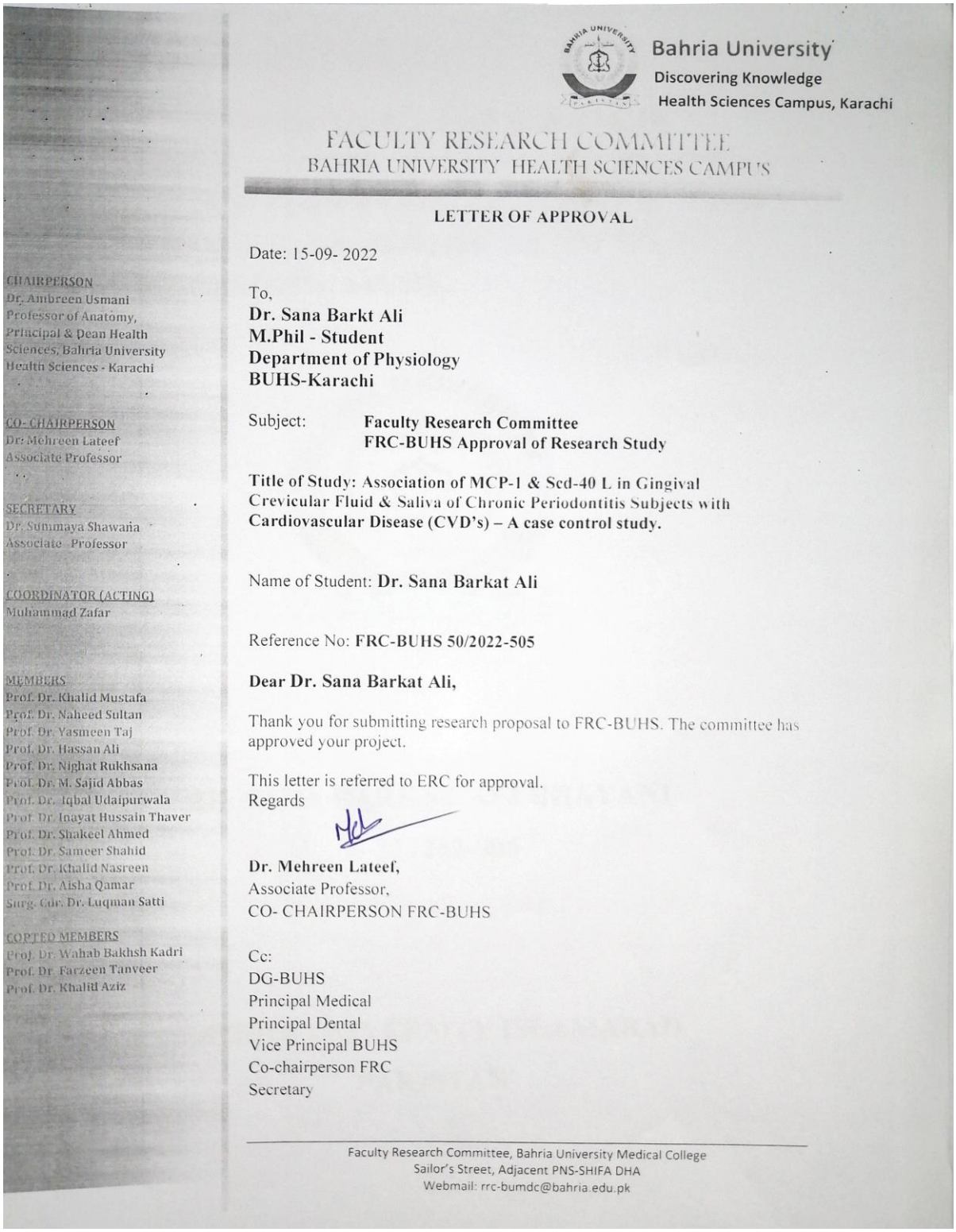
ANNEXURE A

FRC APPROVAL LETTER



Bahria University
Discovering Knowledge
Health Sciences Campus, Karachi

FACULTY RESEARCH COMMITTEE
BAHRIA UNIVERSITY HEALTH SCIENCES CAMPUS

LETTER OF APPROVAL

Date: 15-09- 2022

To,
Dr. Sana Barkt Ali
M.Phil - Student
Department of Physiology
BUHS-Karachi

Subject: **Faculty Research Committee**
FRC-BUHS Approval of Research Study

Title of Study: Association of MCP-1 & Scd-40 L in Gingival Crevicular Fluid & Saliva of Chronic Periodontitis Subjects with Cardiovascular Disease (CVD's) – A case control study.

Name of Student: **Dr. Sana Barkat Ali**

Reference No: **FRC-BUHS 50/2022-505**

Dear Dr. Sana Barkat Ali,

Thank you for submitting research proposal to FRC-BUHS. The committee has approved your project.

This letter is referred to ERC for approval.
Regards

Dr. Mehreen Lateef,
Associate Professor,
CO- CHAIRPERSON FRC-BUHS

Cc:
DG-BUHS
Principal Medical
Principal Dental
Vice Principal BUHS
Co-chairperson FRC
Secretary

CHAIRPERSON
Dr. Ambreen Usmani
Professor of Anatomy,
Principal & Dean Health Sciences, Bahria University Health Sciences - Karachi

CO- CHAIRPERSON
Dr. Mehreen Lateef
Associate Professor

SECRETARY
Dr. Summaya Shawana
Associate Professor

COORDINATOR (ACTING)
Muhammad Zafar

MEMBERS
Prof. Dr. Khalid Mustafa
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Prof. Dr. Yasmeen Taj
Prof. Dr. Hassan Ali
Prof. Dr. Nighat Rukhsana
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Prof. Dr. Iqbal Udaipurwala
Prof. Dr. Inayat Hussain Thaver
Prof. Dr. Shakeel Ahmed
Prof. Dr. Sameer Shahid
Prof. Dr. Khalid Nasreen
Prof. Dr. Aisha Qamar
Surg. Cdr. Dr. Luqman Satti

COPTED MEMBERS
Prof. Dr. Wahab Bakhsh Kadri
Prof. Dr. Farzeen Tanveer
Prof. Dr. Khalil Aziz

Faculty Research Committee, Bahria University Medical College
Sailor's Street, Adjacent PNS-SHIFA DHA
Webmail: rrc-bumdc@bahria.edu.pk

ANNEXURE B

ERC APPROVAL LETTER

Bahria University
Discovering Knowledge
Health Sciences Campus Karachi

ETHICAL REVIEW COMMITTEE
LETTER OF APPROVAL

Date: 18-Oct-22

Reference:
FRC-BUHS 50/2022-505

PATRON
Prof. Ambreen Usmani
Principal & Dean
Health Sciences(BU)

CHAIRPERSON
Dr. Quratulain Javaid

SECRETARY
Dr. Ambreen Surti

MEMBERS
Prof M Alamgir
Prof Anis Jafarey
Prof Aisha Qamar
Ms Nighat Huda
Surg Cdre Amir Ejaz
Prof Reza H Syed
Ms Shabina Arif
Mr M Amir Sultan
Prof Dr Rafat Murad
Ms NajmusSahar Ilyas

Dr. Sana Barkat Ali
MPhil Student
Department of Physiology
BUHS-Karachi

Subject: Institutional approval of research study

Title of Study: Association of MCP-1 & sCD-40 L in gingival crevicular fluid & saliva of chronic periodontitis subjects with cardiovascular disease (CVD's) – A case control study.

Principal Investigator: Dr. Sana Barkat Ali

Reference No: ERC 63/2022

Dear Dr. Sana Barkat Ali,

Thank you for submitting the above mentioned study proposal. ERC Bahria University Health Sciences Campus has reviewed this project in the meeting held on 17-September-2022 and gives approval. Kindly notify us when the research is complete.

Regards,

18/10/2022
DR. AMBREEN SURTI
Secretary, ERC
BUHS

18/10/2022
DR. QURATULAIN JAVAID
Chairperson, ERC
BUHS

Cc:
DG-BUHS
Principal BUHS

BUHS Karachi, DHA Phase – II Adjacent PNS SHIFA Karachi
Office No. +92-21-99332688 Ext: 1026 |Tel: +92-21-35319491-9 | Web: www.bahria.edu.pk/bumdc/

ANNEXURE C

SUBJECT CONSENT FORM (ENGLISH VERSION)

WRITTEN INFORMED CONSENT OF THE PARTICPANT

You have fully understood that you are being asked to participate in this research voluntarily for the purpose of obtaining a standardized volume of 2 µl of gingival crevicular fluid (GCF) and saliva from the most severely afflicted site of periodontitis which aims to diagnose the cause of precipitation of systemic effects of acute cardio vascular diseases.

You have clearly understood that you will be asked questions regarding the evaluation of your general health and clinical evaluation will be done to ascertain the periodontal attachments via probing. You have also been explained in detail the nature and significance of participating in the project and you have understood the provided explanation.

You have been explained that radiological investigations (OPG) advised to you are to evaluate your dental health status and to monitor your disease process. For this purpose you fully agree to give the OPG to the researcher. You also agree to give all relevant information needed, in full and to the best of your knowledge to the researcher.

RISKS: You have been well informed that there will be no risk to you while obtaining the GCF or salivary samples from the chronically infected periodontal site of infection and if any bleeding occurs during the procedure will be properly maintained.

COMPENSATION: It is clarified to you that no incentive, reward, monetary, financial assistance or reimbursement will be provided to you for participating in the study whereas you do have the right to withdraw from the study at any time.

CONFIDENTIALITY: You have been told that findings of your disease or condition and your data will be kept strictly confidential and will be used only for the benefit of community, publications and paper presentations.You are also well aware of the fact that any information that you give regarding your health will also be kept thoroughly confidential.

CONTACT: You have been told that you can contact Dr. Sana Barkat Ali at 021-35319496 extension # 1067 at Bahria University Health Sciences during office hours (8:30 am – 4:00 pm) if you have any queries regarding your participation.

I hereby confirm that I have read and understood whatever has been stated above and based on the same I voluntarily consent to participate in the study.

Name of Participant: _____S/o, D/o, W/o_____

Signature of Participant: _____

Name of Researcher: _____

Signature of Researcher: _____

Date: _____

SUBJECT CONSENT FORM (URDU VERSION)

شرکت کنندہ کی تحریری باخبر رضامندی

آپ مکمل طور پر سمجھتے ہیں کہ آپ سے اس تحقیق میں رضاکارانہ طور پر حصہ لینے کے لیے کہا جا رہا ہے تا کہ اس کی شدید ترین متاثرہسے جی سی ایف یا تھوک کے نمونےکا معیاری حجم حاصل کیا جا سکے۔ پیریڈونٹائٹس جس کا مقصد وجہ کی تشخیص کرنا ہے اور یہ شدید قلبی عروقی امراض کے نظامی اثرات کو روکے گا ۔

آپ اچھی طرح سے سمجھتے ہیں کہ آپ کی عمومی صحت کی تشخیص کے حوالے سے سوالات پوچھے جائیں گے اور طبی جانچ پڑتال کے ذریعے پیریڈونٹل منسلکات کا پتہ لگانے کے لیے کی جائے گی۔ آپ کو پروجیکٹ میں حصہ لینے کی نوعیت اور اہمیت کے بارے میں بھی تفصیل سے بتایا گیا ہے اور آپ فراہم کردہ وضاحت کو سمجھتے ہیں۔

اس مقصد کے لیے آپ محقق کو OPGکی تفصیلی رپورٹ دینے سے مکمل اتفاق کرتے ہیں۔ آپ تمام متعلقہ معلومات کو مکمل طور پر اور آپنی بہترین معلومات میں بھی اتفاق کرتے ہیں۔

خطرات:

آپ کو اچھی طرح سے مطلع کیا گیا ہے کہ انفیکشن کے دائمی طور پر متاثرہ پیریڈونٹل سائٹ سے جی سی ایف یا تھوک کے نمونے حاصل کرنے کے دوران آپ کو کوئی خطرہ نہیں ہوگا اور اگر طریقہ کار کے دوران کوئی خون بہہ رہا ہے تو اسے اچھی طرح سے برقرار رکھا جائے گا۔

معاوضہ:

آپ کواضح کیا جاتا ہے کہ آپ کو مطالعہ میں حصہ لینے کے لیے کوئی ترغیب، انعام، مالی، مالی امداد یا معاوضہ فراہم نہیں کیا جائے گا جبکہ آپ کو کسی بھی وقت مطالعہ سے دستبردار ہونے کا حق حاصل ہے۔

رازداری:

آپ کو بتایا گیا ہے کہ آپ کی بیماری یا حالت کے نتائج اور آپ کے ڈیٹا کو سختی سے خفیہ رکھا جائے گا اور صرف کمیونٹی، اشاعتوں اور کاغذی پیشکشوں کے فائدے کے لیے استعمال کیا جائے گا۔ آپ اس حقیقت سے بھی بخوبی واقف ہیں کہ آپ اپنی صحت کے حوالے سے جو بھی معلومات دیں گے اسے بھی مکمل طور پر خفیہ رکھا جائے گا۔

رابطہ:

آپ کو بتایا گیا ہے کہ بحریہ یونیورسٹی ہیلتھ سائنسز میں ڈاکٹر ثناء برکت علی سے 021 35319496. ایکسٹینشن# 1067 پر دفتری اوقات میں (8:30 بجے تا شام 4:00 بجے) رابطہ کر سکتے ہیں اگر آپ کو اپنی شرکت سے متعلق کوئی سوال ہو۔

میں یہاں اس بات کی تصدیق کرتا ہوں کہ میں نے جو کچھ اوپر بیان کیا گیا ہے اسے پڑھ اور سمجھ لیا ہے اور اسی کی بنیاد پر میں مطالعہ میں حصہ لینے کے لیے رضاکارانہ طور پر رضامندی دیتا ہوں

حصہ لینے والے کا نام: _____

S/o, D/o, W/o _____

شرکت کنندہ کے دستخط: _____

محقق کا نام: _____

محقق کے دستخط: _____

تاریخ: _____

ANNEXURE D

SUBJECT EVALUATION FORM/ QUESTIONNAIRE:

QUESTIONNAIRE

A. PERSONAL INFORMATION:

Date:				
Age:				
Gender:	Male		Female	
Ethnicity				
Occupation:				
Email:				
Phone number				

B. CARDIOVASCULAR HISTORY:

DO YOU HAVE KNOWN HEART DISEASE ○ **YES** ○ **NO**

○ High blood pressure(if yes please specify)	○ Chest pain or exertion	○ Enlarge heart	○ Coronary artery disease
○ Previous heart attack Date:		○ Idiopathic cardiomyopathy	○ Hypertrophic cardiomyopathy
○ Valvular heart disease Date diagnosed:		○ Congestive heart failure	○ Pericarditis
○ Endocarditis	○ Congenital heart disease Type:	○ Previous heart surgery Type:	○ Tachycardia mediated cardiomyopathy

C. MEDICAL ALERT (SYSTEMIC DISEASE):

	YES	NO	DETAILS
CNS	☐	☐	_____
CVS	☐	☐	_____
RESPIRATORY	☐	☐	_____
ENDOCRINE	☐	☐	_____
JOUNDICE	☐	☐	_____
GIT	☐	☐	_____
OTHER	☐	☐	_____

PREVIOUS HOSPITALIZATION /

SURGERY / GENERAL / ANESTHESIA: _____

CURRENT MEDICATION: _____

ALLERGIES: _____

D. FAMILY HISTORY:

Is there a family history of any of the following (Grandparents, parents, brothers, sisters)?

Heart Disease	
High Blood Pressure(if yes please specify)	
Stroke	
High Cholesterol	
Diabetes	
Any cancers	
Mental Health Problems	
Glaucoma	
Blood clots	
Asthma	
Arthritis	
Any other hereditary conditions	

E. LIFESTYLE ISSUES:

1) Have you ever had raised cholesterol?
2) How many times per week do you exercise?
3) How much brisk walking do you do per day?
4) Do you smoke?
5) If yes, estimated number per day?
6) If infrequent smoker, number per week?

F. BRUSHING HABITS:

FREQUENCY: OD BD TDS OCCASIONALY TIME: M E N

TYPE: BRUSH MISWAK FINGER MANJAN OTHERS:_____

G. PAST DENTAL TREATMENT HISTORY:

EXTRACTION Y N SCALING Y N FILLING Y N

H. HABITS:

PAN ☐ CHALIA ☐ TOBACCO ☐ NISWAR ☐

GUTTKA ☐ MANPURI ☐ SMOKING ☐

SUBJECT EVALUATION FORM:

A. ASSESSMENT OF PERIODONTAL STATUS:

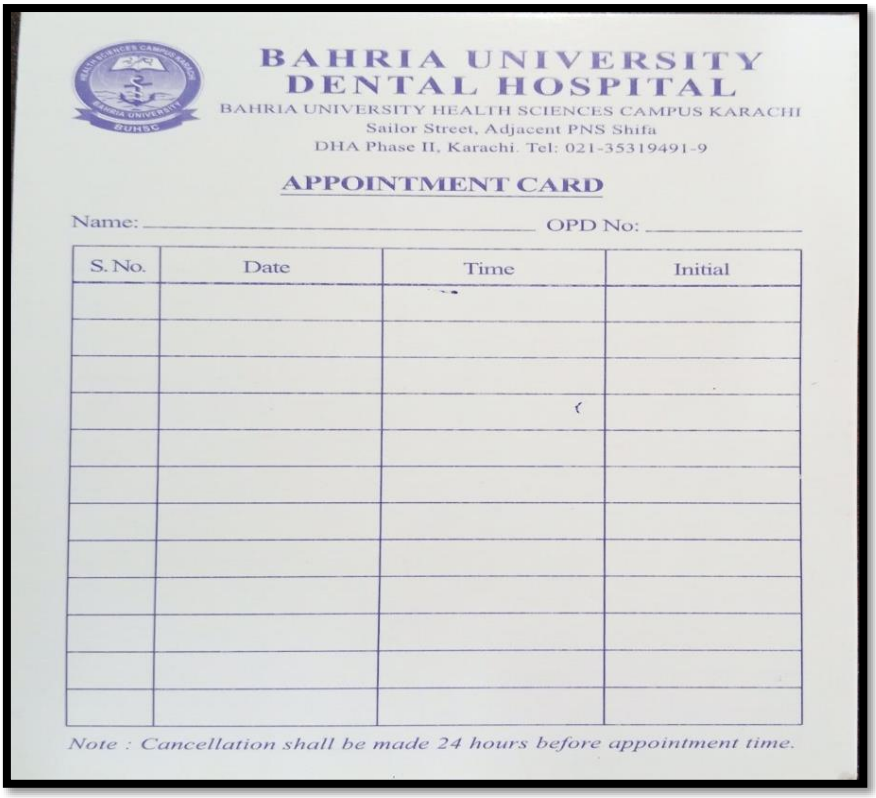
HEALTHY PATIENTS	**YES** ☐	**NO** ☐
a) GI < 1	b) PD < 3 mm	c) CAL = 0.
PERIODONTITIS :-	**YES** ☐	**NO** ☐
a) **Mild:-** ☐ Graded as≥2 interproximal sites with clinical attachment loss (CAL)≥3 mm, and ≥2 interproximal sites with periodontal probing depth (PPD)≥4 mm (not on same tooth) or one site with PPD≥5 mm	b) **Moderate :-** ☐ Graded as ≥2 interproximal sites with CAL≥4 mm (not on same tooth), or ≥2 interproximal sites with PPD≥5 mm (not on same tooth).	c) **Severe:-** ☐ With two or more inter proximal sites with CAL ≥ 6 mm not on same tooth . One or more inter-proximal sites with PD ≥ 5 mm.

B. EVALUATION OF HORIZONTAL ALVEOLAR BONE LOSS:

Horizontal Alveolar Bone loss:-			
Grade 1	Incipient bone loss	YES	NO
Grade 2	Partial bone loss	YES	NO
Grade 3	Total bone loss through and through	YES	NO
Grade 4	Bone loss similar to grade 3 along with gingival recession exposing the furcation areas	YES	NO

ANNEXURE E

HOSPITAL CARD:



BAHRIA UNIVERSITY DENTAL HOSPITAL

BAHRIA UNIVERSITY HEALTH SCIENCES CAMPUS KARACHI

Sailor Street, Adjacent PNS Shifa

DHA Phase II, Karachi. Tel: 021-35319491-9

APPOINTMENT CARD

Name: ______________________ OPD No: ____________

S. No.	Date	Time	Initial

Note : Cancellation shall be made 24 hours before appointment time.

S. No.	Date	Time	Initial

ANNEXURE F

TURNITIN PLAGIARISM REPORT

ORIGINALITY REPORT

7%	6%	5%	0%
SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

PRIMARY SOURCES

1	www.ijdr.in Internet Source	3%
2	pubannotation.org Internet Source	1%
3	Vesile Elif Toy, Tamer Ataoglu, Abubekir Eltas, Husniye Gul Otlu, Aysun Bay Karabulut. "Obesity as a Modifying Factor of Periodontal Therapy Outcomes: Local and Systemic Adipocytokines and Oxidative Stress Markers", Research Square Platform LLC, 2022 Publication	1%
4	heart.bmj.com Internet Source	1%
5	Hina Makkar, Mark A. Reynolds, Abhishek Wadhawan, Aline Dagdag, Anwar T. Merchant, Teodor T. Postolache. "Periodontal, metabolic, and cardiovascular disease: Exploring the role of inflammation and mental health", Pteridines, 2018 Publication	<1%