ESTIMATION OF SALIVARY LEVELS OF HEAT SHOCK PROTEINS 60 IN CARDIOVASCULAR DISEASED PATIENTS WITH CHRONIC PERIODONTITIS

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CERTIFICATE

This is to certify that this dissertation titled "**ESTIMATION OF SALIVARY LEVELS OF HEAT SHOCK PROTEINS 60 IN CARDIOVASCULAR DISEASED PATIENTS WITH CHRONIC PERIODONTITIS** " is a bonafide record of work done by **Dr. A. ARCHANA MEENAKSHI** under my guidance during the study period of 2009-2012.

This dissertation is submitted to **THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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Abstract

Background :

The association between periodontal disease and cardiovascular disease have been reported in various earlier studies, but the pathological mechanism behind the relation is still inconclusive. Recently phylogenetically conserved nature of Heat shock proteins 60 (Hsps60) has led to the proposition that they may provide a link between Periodontal disease and Cardiovascular disease.

Saliva is a non invasive diagnostic medium that has been used to assess several inflammatory disorders including Periodontal disease This study aims at assessing the levels of Heat shock protein 60 (Hsp60) in systemic health and cardiovascular diseased groups with Chronic Periodontitis.

Materials and methods:

Salivary samples were collected from 49 subjects and they were divided into two groups, Group A-23 Subjects in systemic health; Group B-26 patients with Cardiovascular disease (with chronic periodontitis). Hsp60 levels in both the groups were estimated by sandwich Enzyme Linked Immuno Sorbent Assay (ELISA). Statistical analysis was performed using the student ‘T ‘test and Pearson’s correlation.

Results:

There was no significant difference in the salivary Hsp60 levels between systemically healthy and cardiovascular diseased patients with periodontitis ($p = 0.3244$).

Conclusion

The preliminary results indicate that Salivary Hsp60 levels may not be a potential marker for CVD but a larger sample size would be necessary to confirm the hypothesis .

Key words: Hsp60, Periodontitis, Atherosclerosis , ELISA

CONTENTS

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LIST OF ABBREVIATIONS

CVD	- Cardio Vascular Disease
CHD	- Coronary Heart Disease
CRP	- C Reactive Protein
ELISA	- Enzyme Linked Immuno Sorbent Assay
GCF	- Gingival Crevicular Fluid
GroEL	- Growth of Escherichia Coli Large
Grps	- Glucose regulated proteins
HDL	- High Density Lipid
HSP	- Heat Shock Protein
HumHSP	- Human Heat Shock Protein
ICAM	- InterCellular Adhesion Molecule
IFN	- Interferon
Ig	- Immunoglobulin
IL	- Interleukin
IMT	- Intima Media Thickness
LDL	- Low Density Lipid

LPS	- Lipopolysaccharide
MMP	- Matrix Metalloproteinase
MycHSP	- Mycobacterial Heat Shock Protein
NK	- Natural Killer
PAMPs	- Pathogen Associated Molecular Patterns
PBMC	- Peripheral Blood Mononuclear Cells
PBS	- Phosphate Buffered Saline
PPD	- Periodontal Probing Depth
RA	- Rheumatoid Arthritis
TCR	- T Cell Receptor
TGF	- Transforming Growth Factor
TLR	- Toll Like Receptor
TNF	- Tumor Necrosis Factor
VCAM	- Vascular Cell Adhesion Molecule
WBC	- White Blood Cell

INTRODUCTION

Periodontitis, a chronic tissue-destructive inflammatory disease is predominantly induced by specific Gram-negative bacteria colonizing the gingival crevice. Continuous stimulation of exogenous antigens from red complex bacteria such as, *Porphyromonas gingivalis (Pg), Treponema denticola (Td) ,and Tanerella forsythia (Tf)* has been traditionally thought to be important determinants of disease pathogenesis.[131] In recent years, the role of endogenous antigens has gained considerable attention.

Heat Shock Proteins are expressed in all eukaryotic and prokaryotic cells including Gram - positive and Gram-negative bacteria. They are the most highly conserved group of proteins in phylogeny with respect to biochemical function, mode of regulation and structure.[33,34]

Heat Shock Proteins have a direct role both in the adaptive and the innate immune responses. Hsps are capable of eliciting innate immune responses in a variety of target cells including monocytes, macrophages, dendritic cells and endothelial cells in a peptide-independent manner.[74] Hsp generated immune response could potentially thus contribute to pathogenesis of periodontal inflammation. Further more, Molecular mimicry between bacterial Hsp60 of *P.gingivalis* and human fibroblast may allow microorganisms to evade the host defenses and thereby contribute to the etiopathogenesis of periodontitis.

There has been an increasing interest in the impact of oral health on atherosclerosis and subsequent cardiovascular disease (CVD).[55] Several epidemiological studies suggest that periodontal disease may be a risk factor for CHD.[60] This association has however been difficult to prove, due to common etiologic factors with CHD like smoking, low socio economic status and unfavourable health care practice of the individual. The causal link between Periodontitis and CVD is therefore yet to be established. On the other hand several lines of evidence link *P.gingivalis* with CVD.[56]

Choi et al 2001[19] isolated *P. gingivalis* heat shock protein-specific T-cells in atherosclerotic plaque from subjects with severe atherosclerosis. Hsp60 has therefore been postulated to one be potential mechanism through with periodontal disease which mediates its systemic effects.

Saliva is a diagnostic medium that has been widely used as a marker for periodontal disease in recent times.[43] The presence of several inflammatory markers and host derived products makes it a potential candidate for assessment of several systemic diseases.[136] Point-of-care saliva diagnostic technologies are being developed for oral diseases such as oral cancer, CVD, Peripheral Artrial Disease, Cerebrovascular Disease.[54]

Heat Shock Proteins 70 (Hsp70) has been recently identified in human whole saliva and is thought to positively correlated with CVD.[16,36] Similarly Salivary Hsp60 levels may be used as a potential marker for Cardiovascular disease considering the number of reports that have suggested a close

association between *Porphyromonas gingivalis* and Cardiovascular disease .We have previously reported that there is a significant increase in salivary Hsp60 levels in periodontal disease when compared to health. There is however, as yet scant evidence that correlates salivary Hsp60 levels with periodontal disease and Cardiovascular Disease.

AIMS AND OBJECTIVES

The aim of Present Study is

1. To estimate the levels of salivary heat shock protein (Hsp60) in systemically healthy patients with Chronic Periodontitis and
2. To compare these levels with that of Cardiovascular Disease Patients with Chronic Periodontitis

REVIEW OF LITERATURE

Periodontitis is a chronic inflammatory disease characterized by mononuclear cell infiltration into the gingival tissues leading to connective tissue destruction and alveolar bone resorption. **Seymour et al** [127] stated that although periodontal bacteria are the causative agents in periodontitis, subsequent progression and disease severity are thought to be determined by the host immune responses.

The pathogenic species present in sub gingival biofilm release an array of virulence factors that can evade antibacterial host defence mechanisms and then cause damage to host tissue via immune or inflammatory interactions, which typically consist of neutrophils, monocytes/ macrophages, T cells and B cells. **Hirsch et al**[57] proposed that Collagen type 1, a major component of the periodontium, has been considered to be one of the target antigens of this autoimmune response due to the fact that high titers of anticollagen type I antibody are found in the sera, and that collagen type I-specific T-cell clones can be identified in the inflamed gingival tissues, of periodontitis patients

Major periodontopathic bacteria such as Porphyromonas gingivalis, Actinobacillus actinomycetem comitans, Fusobacterium nucleatum, Prevotella intermedia, Bacteroides forsythus, and Campylobacter rectus are reported to produce Hsps homologous to Escherichia coli GroEL. The frequency of seropositivity antibodies to P. gingivalis GroEL were significantly higher in periodontitis patients than in periodontally healthy control subjects. The presence of complexes of bacteria residing in dental plaque of which the red

complex (*Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola*) are thought to be closely associated with pathogenesis of periodontal disease, identified by **Socransky SS. & Haffajee AD**[131]

These red complex bacteria are involved in the production of many virulence factors, such as capsule, Lipopolysaccharide, fimbriae, GroEL heat shock protein, outer membranous protein of gram negative bacteria. These antigens are believed to result in activation of host immune and inflammatory responses, which involves the generation of cytokines, recruitment of inflammatory cells, and the activation of osteoclasts.

Role of Self Antigens In Periodontal Disease:

In recent years it has been recognized that the immune response to self antigens may contribute to the disease process. High titres of anti collagen type I antibody have been identified in sera and collagen type specific T cell clones can be identified in infected gingival tissues in periodontitis. Exogenous antigens may themselves help to elicit responses from host self antigens in the following way. An exogenous antigen may present with structural similarities with certain host antigens; thus, any antibody produced against this antigen (which mimics the self – antigens) bind to the host antigens and amplify the human response.[56] The most striking form of molecular mimicry is observed in Group B-hemolytic streptococci, which stores antigens in humans, and is responsible for the cardiac manifestations of Rheumatic fever

Heat Shock Proteins:

Since the first report on the heat-induced appearance of chromosomal puffings in salivary gland tissue of Drosophila busckii in 1962, a new research domain has been intensively explored. This research resulted in the discovery of a large number of related proteins and their physiological role in many prokaryotic and eukaryotic organisms, tissues, and individual cells and at the level of subcellular structures. These proteins were originally called "heat shock proteins" because they were discovered in salivary glands and other tissues of Drosophila melanogaster recovering from a so-called transient sublethal heat shock, during which body temperature was increased; 5°C above normal body core temperature . Such a mild heat shock elicited a heat shock response, characterized by the synthesis of new heat shock proteins normally almost absent in tissues of adult animals and by an increased synthesis of constitutively present or cognate heat shock proteins. The increased resistance toward stressful events has been described in a great variety of organisms, organs, and tissues, including the heart as followed by a transient increased tolerance to high, normally lethal temperatures.[110]

GroEL – GroES complex

Hsp60 is a 60 kilodalton oligomer composed of monomers that form a complex arranged as two stacked heptameric rings. GroEL has the form of a double heptameric ring, with a large central cavity in which the substrate protein is bound via hydrophobic interactions. The 2 rings stack back to back.

Each subunit of HSP60 has three domains; the apical domain, the equatorial domain, and the intermediate domain.[15] Two non contiguous segments at the N and C termini of the molecule form the equatorial domain (sub domains). The equatorial domain contains the binding site for ATP and for the other heptameric ring.

Likewise, intermediate domain consists of 2 segments. The intermediate domain binds the equatorial domain and the apical domain together. The intermediate domain induces a conformational change when ATP is bound allowing for an alternation between the hydrophilic and hydrophobic substrate binding sites. In its inactive state, the protein is in a hydrophobic state. When activated by ATP, the intermediate domain undergoes a conformational change that exposes the hydrophilic region.[34] This insures fidelity in protein binding. Apical domain consists of a single sequence connecting intermediate domains

The co chaperonin GroES also exists as a heptamer. It can bind to either GroEL ring to form a cap on the central cavity. In the absence of GroES, the 2 rings have identical 3D structure symmetric with respect to inter ring interface. The individual domains of the subunits have been proposed to engage in specific roles during chaperonin action. The apical domain recognizes the folding intermediates to be sequestered in the central cavity, as well as the flexible loops at the binding interface of GroES. Their interior cavity surface is lined with hydrophobic residues in the polypeptide acceptor state, which can bind non native polypeptides or help unfold misfolded

intermediates that expose hydrophobic surface regions, in the protein folding and protein release status, on the other hand, the interior cavity lining becomes hydrophilic.[148]

Nomenclature and Families of Heat Shock Proteins:

The applied nomenclature is primarily derived from the trigger leading to the synthesis of these proteins. Because heat shock was the first discovered trigger of the heat shock response leading to enhanced transcription of certain genes, the related products of this transcriptional activity have been called heat shock proteins.[80] On the basis of the adopted nomenclature throughout this survey heat shock genes will conventionally be designated as hspgenes,while the related proteins are called Hsps. **Craig et al in 2003**[22] proposed that proteins the synthesis of which was increased upon glucose starvation are called glucose-regulated proteins (Grps). For the majority of these genes and proteins the name is associated with a molecular mass indication like for instance dHsp27, Hsp60 Hsp70 respectively by an indication of the compartment in which they reside, like mitochondrial mt-Hsp75.

Furthermore, distinction has been made between the proteins almost absent under nonstressed conditions but synthesized immediately after cellular stress, and the proteins that are constitutively synthesized in the tissue.[22] The first are called inducible proteins, while the second class is known as cognate proteins, like for instance Hsc70.

Classification of various Hsps in families is based on their related function and size, which can vary from 10 to 170 kDa. The Hsp70 family can

stand for a typical example. The proteins of this family range in weight between 70 and 78 kDa. All Hsp70 family members bind ATP. Constitutive or cognate members are Hsc70, Hsp75 and Grp75, while the inducible member is Hsp72, commonly called Hsp70. In the spectrum of Hsps, the so-called Grps form a special group. As mentioned earlier, the synthesis of these proteins increases when extracellular glucose concentrations are low. Other triggers, however, can also lead to enhanced Grp synthesis, like depletion of intracellular calcium stores or inhibition of protein glycosylation. Grps reside in various Hsp families and comprise Grp58, Grp78, Grp94, and Grp170. They are all localized in the endoplasmic reticulum.[4]

Functions of Heat Shock Proteins :

Function of Chaperones and Chaperonins Under Nonstressed conditions:

Most likely, the primary physiological function of Hsps is to fulfill chaperoning activity. Molecular chaperones have been defined as a nonrelated class of proteins that mediate the correct folding of other proteins, but do not take part in the final assembly of new Structures.[34] Although every newly synthesized protein contains within its amino acid sequence the necessary information for ultimate correct folding, this process can be hampered by several factors. For instance, during their synthesis, incomplete amino acid sequences may already associate with other unfinished parts of peptide chains or with totally completed peptides. For correct association and/or folding the amino acid chains need to be entirely synthesized and therefore have to be

kept in the unfolded monomer state. Second, proteins that need to be translocated to other cellular compartments should also be kept in an unfolded or semi-folded state to pass intracellular membranes. Such physical state can be achieved through binding to chaperone proteins .[33]

Chaperonins consist of a class of Hsps that assist in correct protein assembly at a later stage than the chaperones. This process occurs when completed protein chains are released from the ribosomes or are transported to such cell organelles as mitochondria. In eukaryotic cells, a distinction is made between two groups of chaperonins. Group I consists of Hsp60 and Hsp10, both residing in the mitochondria. Group II comprises the TCP1 T-complex polypeptide (TCP1) subfamily, the members of which can be found in the cytosol. TCP1 is, among others, involved in the folding of actin and tubulin and thus indispensible for proper functioning of the cytoskeleton.[48]

Function of Chaperones and Chaperonins Upon Stress:

Compared with the nonstressed situation, much less is known about the various Hsps that exert their chaperone function during and after stress. This lack of insight is in part due to the fact that under these circumstances general protein synthesis is completely disturbed and new Hsps appear in various cellular compartments[78]. The stress-mediated translocation of both Hsc70 and Hsp70 into the cellular nucleus, in particular to the nucleolus. Upon heat shock, translocation occurs within 60 min and terminates, 3 hour later, the time at which the highest content of these proteins is reached. It is well known that heat shock negatively affects the organization of several functional

structures in the cell. Striking alterations occur in the cytoskeleton, more specifically in intermediate filaments. The microtubular network is not affected, but intermediate filaments aggregate to form a tight perinuclear network. This phenomenon, however, is reversible since normal distribution is gradually reappearing after the stress is relieved.[79]

Heat Shock Protein 60 and Periodontal Disease:

Heat Shock proteins are grouped in families according to their molecular mass, and the human and bacterial cognates are very similar, sharing more than 50% sequence homology at the amino acid level. In response to stress stimuli, including high temperature, mechanical stress, infection, surgical stress, and oxidant and cytokine stimulation, cells produced high levels of heat shock proteins to protect themselves against these unfavourable conditions. They participate in vital physiological process in the cell such as folding, assembly, translocation of polypeptides across membranes and play a role in protein repair after cell damage[110] .

The immune system has a bias toward recognition of microbial antigens for protecting the host from infection at birth. Much data suggest that an important initial line of defense in this regard involves autologous heat shock proteins, especially highly conserved Hsp60s[7] . Infection is stressful to the infectious agent, as well as to the host, and it seems obvious that bacteria require increased production of molecular chaperones to survive the infectious process. Given the high degree of amino acid sequence homology between Hsps of different species, the presence of antibodies against Hsps may, on the

one hand, be helpful to protect the host from recurring infection; on the other hand, presence of such antibodies may contribute to autoimmunity through cross reactivity between Hsps and tissue-specific proteins containing similar epitope motives. [60]

Hsp60 is a mitochondrial chaperonin that function as a chaperonin to assist in folding linear amino acid chains into their respective three-dimensional structure. Hsp60 has been shown to be released from specific cells like peripheral blood mononuclear cells (PBMCS) when there are Lipopolysaccharides (LPS) or GroEL present. This suggests that the cell has different receptors and responses to human and bacterial Hsp60.[50] In addition, it has been shown that Hsp60 has the capability of activating monocytes, macrophages and dendritic cells and also of inducing secretion of a wide range of cytokines

M.D.A. Petit et al 1999[107] investigated the proliferative responses of PBMCs of patients with periodontitis (n = 10) and controls with gingivitis (n=12) to recombinant mycobacterial Hsp60 (MycHsp60) and Hsp70 (MycHsp70), as well as recombinant human Hsp60 (HumHsp60) and Hsp70 (HumHsp70) and also the proliferative responses to Candida albicans and purified protein derivatives of Mycobacterium (PPD). Mean responses to HumHsp60, MycHsp60, and HumHsp70, MycHsp70 were significantly lower for patients compared with controls. The level of IFN-γ in the supernatants of the cells stimulated with Hsp's was lower in the patients compared with controls. This concurs with the current hypothesis that periodontitis patients

have a depressed Th1 response. He found that with an increasing estimated subgingival bacterial load, periodontitis patients mount a decreasing immune response to Hsps, and the poor reactivity to Hsps may be a susceptibility factor for destructive periodontal disease and may need to be considered in the pathogenesis of this condition

K. Ueki (2002)[142] demonstrated that serum antibodies to both HumHsp60 and Porphyromonas gingivalis GroEL were elevated in periodontitis patients compared with healthy subjects. The stimulatory effect of human and bacterial Hsp60 on the production of tumour necrosis factor-α (TNF-α) was examined in phorbol myristate acetate stimulated THP-1 cells(Human monocytic cell line). The activity of Hsp60 was inhibited by anti-CD14 and anti-Toll-like receptor 4 (TLR4) antibodies, suggesting that both CD14 and TLR4 mediate Hsp60 signalling. Immunohistochemical analysis demonstrated that Hsp60 is abundantly expressed in periodontitis lesions[96]. Therefore, it is postulated that periodontopathic bacteria stimulate the cells in the periodontium to up-regulate the expression of Hsp60, which in turn may stimulate macrophage and possibly other cells to produce proinflammatory cytokines. These mechanisms may be involved in the chronicity and tissue destruction of periodontal disease.

Argueta JG et al (2006)[9] investigated whether the Toll-like receptor (TLR) family plays a functional role as a Porphyromonas gingivalis GroEL receptor. Human macrophage-like THP-1 cells (Human monocytic cell line) were used and the nuclear factor-κB (NF-κB) activity of cells stimulated with

a recombinant Porphyromonas gingivalis GroEL was measured with a luciferase assay. Flow cytometry analysis was used to determine the binding to THP-1 cells of fluorescein isothiocyanate (FITC)-labeled GroEL. He observed by luciferase assay that the purified recombinant GroEL was able to stimulate NF-κB transcriptional activity in THP-1 cells. Flow cytometry analysis showed that the FITC-labeled GroEL bound to THP-1 cells in a dose-dependent fashion. He concluded that Porphyromonas gingivalis GroEL induces its intracellular signaling cascade in Th1 cells via TLR2 or TLR4 and via a combination of both receptors.[102]

Fukui M et al (2006)[40] stated that Salivary IgA to GroEL may have a protective role by reducing the inflammatory response induced by GroEL derived from periodontopathogenic bacteria.Hsp60 was shown to induce a secretion of proinflammatory cytokines in professional antigen presenting cells and to enhance the activation of T cells in primary stimulation . Hsp60 is expressed on the surface of different eukaryotic cell lines increases the activation of T cells in primary stimulation.[29] Although Heat Shock Proteins are typically regarded as being intracellular they can be expressed on the surface of mononuclear cells and Hsp60 has been identified in serum of healthy individuals.

Lundqvist C et al (1994)[81] found the expression of Hsp60 to be higher in gingival epithelial cells of inflamed tissue samples from periodontitis patients compared with samples from periodontally healthy individuals. **Ando et al (1995)**[6] demonstrated that the serum from a

periodontitis patient, contained antibodies that reacted with A actinomycetemcomitants, F.nucleatum, and P.nigerscens Hsp60 and Hsp70, suggesting that these proteins may be involved in the pathogenesis of periodontitis.

Tabeta K et al (2000)[134] reported that the gingival tissue extracts from healthy or periodontitis patients contain antibodies to the GroEL protein of porphyromonas gingivalis. They also demonstrated that diseased periodontal tissue reacted more strongly to porphyromonas gingivalis GroEL and human Hsp60 compared with controls. The authors showed that peripodontitis patients had a higher antibody titer against porphyromonas gingivalis GroEL than healthy subjects.

Immune responses to bacterial HSP may generate cross reacting immunity to self Hsp and precipitate damaging inflammatory responses. Endogenous rather than bacterial Hsp are more likely to be involved in signalling innate responses in periodontal disease.[103] Human but not periodontopathic bacterial Hsp60 can induce TNF-α production in macrophages and this activity is mediated at least in part by CD14, & TLR 4, both of which are known to be LPS receptors. Bacterial homologue of Hum Hsp60,[118] *Porphyromonas gingivalis* GroEL and *Aggregatibacter actinomycetemcomitants* GroEL did not show TNF α inducing activity. Proinflammatory cytokine production induced by autologous Hsp60 could be another pathway leading to periodontal tissue destruction[123].

Epitope Mapping of Heat Shock Proteins 60 (GroEL) From Porphyromonas gingivalis In Periodontitis:

Heat shock proteins (Hsp) are highly conserved through evolution and are structurally similar in all living organisms, so that immune responses against hsp can cross-react and produce anti-self reactivity[101] . Immune responses to the cross-reactive determinants in bacterial hsp are thought to be generated early in life and the immunologic memory may function as a `common barrier' against infections. On the other hand, cross-reactive determinants in human Hsp may function as a trigger for autoimmune reactions[9].

Periodontal disease is a chronic infectious disease; with Porphyromonas gingivalis being one of the most frequently implicated pathogens. Compared with other pathogens, periodontal bacteria persist for many years or decades in periodontal pockets and thus present a long-term challenge, including bacterial Hsp, to the host immune system. Continuous exposure to Hsp from periodontal bacteria may cause cross-reactions with human Hsp and that the cross-reactions may modify destructive forms of periodontal tissue.[6]

Among Hsp families, Hsp60 (GroEL) homologs are major heat shock protein antigens in various bacterial infections. They are antigenically cross-reactive and serologically detectable in a wide range of Gram-negative bacteria as to be considered key molecules for auto-immune reactions. Pg GroEL showed sequence similarity with human Hsp60 and r-GroEL was a

highly antigenic protein which was frequently recognized by sera from patients with periodontitis.[22] PgGroEL may have cross-reactive determinants with human Hsp60. Therefore, mapping of B-cell epitopes on PgGroEL is important to investigate the potential of Pg GroEL as a trigger for auto-immune reactions production in periodontitis patients for a better understanding of auto-immunity in the pathogenicity of periodontal disease.[99]

Elevated Humoral Immune Response to Heat Shock Protein 60 (Hsp60) Family in Periodontitis Patients:

Heat shock proteins, particularly the Hsp60 family of proteins, are thought to play important roles in the causal relationship between microbial infections and autoimmunity because of conservation of the amino acid sequence during evolution and their strong immunogenicity.

Recently, representative periodontopathic bacteria such as P. gingivalis, Bacteroides forsythus and A. actinomycetemcomitans were shown to express Hsp60 family proteins which are homologous with E.coli GroEL. Serum antibody to the periodontopathic bacteria-derived Hsp60 was frequently detected in periodontitis patients. Chronic inflammatory periodontal disease is characterized by connective tissue destruction and alveolar bone resorption. Although periodontopathic bacteria are the primary aetiological agents, the ultimate determinant of disease progression and clinical outcome is the host's immune response. It has been reported that GroEL-like protein belonging to the Hsp60 family can be expressed by periodontopathic bacteria such as Porphyromonas gingivalis and

Actinobacillus actinomycetemcomitans. Further, antibodies against P.gingivalis GroEL are present in serum from periodontitis patients. **Ando et al**[6]. demonstrated that Hsp60 is expressed in periodontitis tissues using an antihuman Hsp60 antibody which cross-reacted with bacterial GroEL.

Therefore, it can be hypothesized that the immune system could be triggered by bacterial antigens, GroEL for example, which share a high degree of homology with self Hsp60 proteins, resulting in an aberrant immune response and chronicity of inflammation. In addition, recent epidemiological reports suggested that periodontal diseases are associated with increased risk factors for coronary heart disease. The immune response to Hsp60 of P.gingivalis Clamydia pneumoniae has been implicated in the pathogenesis of atherosclerosis, it is possible to assume that the antibodies against Hsp60 derived from periodontopathic bacteria have similar effects on the process of vascular endothelial injury.

Accumulation of Human Heat Shock Protein 60-Reactive T Cells in the Gingival Tissues of Periodontitis Patients:

Periodontitis is a chronic inflammatory disease characterized by mononuclear cell infiltration into the gingival tissues, leading to connective tissue destruction and alveolar bone resorption. Although periodontal bacteria are the causative agents in periodontitis, subsequent progression and disease severity are thought to be determined by the host immune responses. Collagen type 1, a major component of the periodontium, has been considered to be one of the target antigens of this autoimmune response due to the fact that high

titres of anticollagen type I antibody are found in the sera, and that collagen type I-specific T-cell clones can be identified in the inflamed gingival tissues, of periodontitis patients. Despite being highly homologous between prokaryotic and eukaryotic cells, Hsp60s are strongly immunogenic, and immune responses to microbial Hsp60s are speculated to initiate chronic inflammatory diseases in which autoimmune responses to human Hsp 60 may be central to pathogenesis. **Anderton et al[5].**

Major periodontopathic bacteria such as P. gingivalis Aggregatibacter actinomycetemcomitans, Fusobacterium nucleatum, Prevotella intermedia, Bacteroides forsythus , and Campylobacter rectus are reported to produce Hsp's homologous to Escherichia coli GroEL. The frequency of seropositivity and titres of antibodies to human Hsp60 and P. gingivalis GroEL were significantly higher in periodontitis patients than in periodontally healthy control subjects. Furthermore, affinity-purified serum antibodies to human Hsp60 and P. gingivalis GroEL cross-reacted with P. gingivalis GroEL and human Hsp60, respectively. These results suggest that an immune response based on the molecular mimicry between P.gingivalis GroEL and human Hsp60 may play a role in periodontitis

K Tabeta (2000)[134] examined the presence of antibodies to the 60-kD human and Porphyromonas gingivalis GroEL Hsp60 in the sera and inflamed gingival tissues of periodontitis patients. Western blot analysis clearly demonstrated that the number of periodontitis patients showing a positive response to Porphyromonas gingivalis GroEL and it was higher than the

number of periodontally healthy subjects. For HumHsp60, a higher frequency of seropositivity was found in the periodontitis patients than in the healthy subjects.In addition, the periodontitis patients demonstrated stronger reactivity compared with the healthy subjects. Quantitative analysis of serum antibodies by ELISA also demonstrated that the levels of antibodies in the sera of patients were significantly higher than those of control subjects. He suggested that molecular mimicry between GroEL of the periodontopathic bacterium Porphyromonas gingivalis and autologous HumHsp60 may play some role in immune mechanisms in periodontitis.

KYamazaki,(2004)[156] examined the proliferative response of peripheral blood mononuclear cells (PBMC), as well as the cytokine profile and T-cell clonality, for periodontitis patients and controls following stimulation with recombinant HumHsp60 and Porphyromonas gingivalis GroEL. The nucleotide sequences within complementarity-determining region 3 of the T-cell receptor (TCR) chain were compared between Hsp60-reactive peripheral blood T cells and periodontitis lesion-infiltrating T cells. Periodontitis patients demonstrated significantly higher proliferative responses of PBMC to HumHsp60, but not to Porphyromonas gingivalis GroEL, than control subjects. The response was inhibited by anti-major histocompatibility complex class II antibodies[46]. Analysis of the nucleotide sequences of the TCR demonstrated that HumHsp60 - reactive T-cell clones and periodontitis lesion-infiltrating T cells have the same receptors, suggesting that Hsp60-reactive T cells accumulate in periodontitis lesions. Analysis of the cytokine profile

demonstrated that Hsp60-reactive PBMC produced significant levels of gamma interferon in periodontitis patients, whereas Porphyromonas gingivalis GroEL did not induce any skewing toward a type1 or type 2 cytokine profile. He suggested that periodontitis patients have HumHsp60 -reactive T cells with a type 1 cytokine profile in their peripheral blood T-cell pools.

Ford P et al (2005)[39] examined the nature of the inflammatory infiltrate and the presence of HumHsp60 and GroEL in 31 carotid endarterectomy specimens. HumHsp60 expression was evident on endothelial cells and cells with the appearance of smooth muscle cells and lymphocytes

T. Honda (2006)[58] compared the gene expression profile of inflammatory mediators including proinflammatory cytokines and other inflammatory molecules, and anti-inflammatory cytokines by using quantitative real-time polymerase chain reaction in gingivitis and periodontitis lesions. Interleukin (IL)-1β, interferon (IFN)-γ and RANKL, Transforming growth factor (TGF)-β1 tended to be higher in periodontitis, whereas tumour necrosis factor (TNF)-α and IL-12 , P 40, IL-10 and IL-4 showed no difference. Heat-shock protein 60 (Hsp60) expression was up-regulated significantly in periodontitis. He concluded that autoimmune response to Hsp 60 may exert in periodontitis lesion, and suggest that perhaps subtle differences in the balance of cytokines may result in different disease expression.

Jarjour WN et al (1991)[64]**,** who suggested that the difference in the levels of anti-HSP antibodies seen in sera of patients with various rheumatoid

and other inflammatory diseases compared to normal controls, could merely reflect disease-associated polyclonal B cell activation. Subsequent investigations have suggested that such antibodies or specific T cells against HSP were associated with immune and autoimmune diseases.

The Link between Periodontal Disease and Systemic Disease

There is increasing evidence that chronic infections are associated with cardiovascular diseases (CVDs). These infections include Helicobacter pylori, Chlamydia pneumoniae, cytomegalovirus, and, more recently, periodontopathic bacteria such as P.gingivalis.

Common susceptibility involves a genetically determined phenotype, which leads to a greater risk of both atherosclerosis and infection. In atherogenesis, inflammation plays a continuous role from endothelial cell expression of adhesion molecules to the development of the fatty streak, established plaque, and finally plaque rupture. Exposures to infections like periodontal disease have been postulated to perpetuate inflammatory events in atherogenesis In this hypothesis, in the presence of periodontal pathogens, a susceptible person develops periodontal disease. This same person would also be susceptible to atherosclerosis.

The term periodontal disease is used to describe a group of conditions that cause inflammation and destruction of the attachment apparatus of the teeth (i.e., gingiva, periodontal ligament, root cementum, and alveolar bone). Periodontal disease is caused by bacteria found in dental plaque, and about 10 species have been identified as putative pathogens in periodontal disease,

mainly gram-negative rods. Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Bacteroides forsythus are the gram-negative bacteria most commonly associated with periodontitis.

Periodontitis lesions exhibit gingival inflammation as well as destruction of the periodontal ligament and alveolar bone. This leads to bone loss and apical migration of the junctional epithelium, resulting in the formation of periodontal pockets. **Page et al**[102] proposed that periodontitis may affect the host's susceptibility to systemic disease in three ways: by risk factors, by subgingival biofilms acting as reservoirs of gram-negative bacteria, and through the periodontium acting as a reservoir of inflammatory mediators.

Factors that place individuals at high risk for periodontitis may also place them at high risk for systemic diseases such as cardiovascular disease. Among the environmental risk factors and indicators shared by periodontitis and systemic diseases, such as cardiovascular disease, are tobacco smoking, stress, aging, race or ethnicity, and male gender .There is increasing evidence that chronic infections are associated with cardiovascular diseases (CVDs). These infections include Helicobacter pylori, Chlamydia pneumoniae, cytomegalovirus, and, more recently, periodontopathic bacteria such as Porphyromonas gingivalis.

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Pathophysiology of Inflammation in Atherosclerosis

Inflammatory processes have become an integral part of the pathophysiology of atherosclerosis and are presumed to be involved from the initiation to the progression and final stages of infarction. Normal endothelium does not allow for the attachment of leukocytes. When initial damage of the endothelium occurs, either by infection or by an atherogenic diet, the endothelial cells express adhesion molecules that allow leukocytes to bind to them. These adhesion molecules are called 'vascular cell adhesion molecules' (VCAM) and 'intercellular adhesion molecules' (ICAM). Selectins and integrins also support leukocyte attachment (**Libby *et al.*, 2002**).[77]

Once this attachment is established, the atheroma accumulates more lipids and promotes the production of various chemokines and growth factors that stimulate the recruitment of monocytes and macrophages. These chemokines also promote the migration of smooth-muscle cells. These muscle cells respond to the inflammatory stimuli by secreting specific enzymes (metalloproteinases) that are able to degrade elastin and collagen. Further, these metalloproteinases may disintegrate the fibrous capsule holding the cholesterol plaque together, and cause plaque rupture. Plaque rupture greatly increasesthe risk of myocardial infarction and stroke.

During the past two decades, there has been an increasing interest in the role of chronic infections as risk factors for atherosclerosis (**Ross, 1999**).[121] In a recent meta-analysis, the odds ratio of chronic infection for early atherogenesis was 3.0 (**Kiechl *et al.*, 2001**).[71] Intervention with

roxithromycin for the treatment of *Chlamydia pneumoniae* among unstable angina patients (ischemic syndromes) decreased cardiac events.[48]

However, a more recent study with the randomized controlled trial design, and with an acceptable follow-up time period, failed to show an effect of antibiotic treatment on the prevention of cardiac events.[97] This relationship between chronic inflammation and atherogenesis has been recently expanded to include other pro-inflammatory processes related to a hyperactive immune response[13] or autoimmune reaction to microbial or other metabolic stimuli. For example, systemic lupus erythematosus patients have been found to be at a higher risk for developing cardiovascular disease.[96] These generalized hyperinflammatory states may be characterized by elevated CRP concentrations (**Libbyet al 2000**)[77].title to atherosclerosis.

Oral Infections and Coronary Heart Disease

Coronary heart disease (CHD) is the most important clinical manifestation of atherosclerosis. **Mattila and colleagues**[86] were the first to show a statistical association between dental infections and advanced coronary atherosclerosis. This original finding has been further investigated in many clinical and experimental studies. Some study designs are better suited for the establishment of causal inferences between dental infections and CHD.

Systemic Inflammation:

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The second hypothesis is that of systemic inflammation and increased circulating Cytokines and inflammatory mediators. In this hypothesis, inflammation leads to an increase in the levels of circulating cytokines, which in turn damage the vascular endothelium and ultimately result in

atherosclerosis. The circulating cytokines of interest include C-reactive protein (CRP), Interleukin-1, Interleukin-6 (IL-6), tumour necrosis factor alpha (TNF-a), and prostaglandin. The highest relative risk for myocardial infarction was found to be the levels of CRP together with the ratio of total cholesterol to high-density lipid.[2]

CRP is a powerful marker of vascular risk and there is some evidence for a direct role in vascular dysfunction and atherogenesis. It is produced by the liver and is stimulated by TNF-a and IL-6, leading to a decrease in nitric oxide availability and an increase in angiotensin 1 receptors. It binds to low density lipids, increasing their uptake by macrophages and hence an increase in foam cell formation. For these reasons, CRP has been postulated as a major mechanism for atherosclerosis.[30]

Among other possible risk factors, evidence linking chronic infection and inflammation to cardiovascular disease has been accumulating. It is clear that periodontal disease is capable of predisposing individuals to cardiovascular disease, given the abundance of gram-negative species involved, the readily detectable levels of proinflammatory cytokines, the heavy immune and inflammatory infiltrates involved, the association of high peripheral fibrinogen, and the white blood cell (WBC) counts. There are several proposed mechanisms by which periodontal disease may trigger pathways leading to cardiovascular disease through direct and indirect effects of oral bacteria. First, evidence indicates that oral bacteria such as Streptococcus sanguis and Porphyromonas gingivalis induce platelet

aggregation, which leads to thrombus formation. These organisms have a collagen-like molecule, the platelet aggregation-associated protein, on their surface. Possibly, antibodies reactive to periodontal organisms localize in the heart and trigger complement activation, a series of events leading to sensitized T cells and heart disease.

Furthermore, one or more periodontal pathogens have been found in 42% of the atheromas studied in patients with severe periodontal disease P**ockley et al 1999**[110] studied proteolytic enzymes referred to as gingipains R, which are released in large quantities from P. gingivalis. After entering the circulation, gingipains R can activate factor X, prothrombin, and protein C, promoting a thrombotic tendency through the ultimate release of thrombin, subsequent platelet aggregation, conversion of fibrinogen to fibrin, and intravascular clot formation[49]. An exaggerated host response to a given microbial or LPS challenge, is reflected in the release of high levels of proinflammatory mediators such as PGE2, TNF-a, and IL-1b. These mediators have been related to interindividual differences in the T-cell repertoire and the secretory capacity of monocytic cells.[15] Typically, peripheral blood monocytes from these individuals with the hyper inflammatory monocyte phenotype secrete 3- to 10-fold-greater amounts of these mediators in response to LPS than those from normal monocyte phenotype individuals.[66]

LPS from periodontal organisms being transferred to the serum as a result of bacteremias or bacterial invasion may have a direct effect on endothelia so that atherosclerosis is promoted. LPS may also elicit recruitment

of inflammatory cells into major blood vessels and stimulate proliferation of vascular smooth muscle, vascular fatty degeneration, intravascular coagulation, and blood platelet function. These changes are the result of the action of various biologic mediators, such as PGs, ILs, and TNF-a on vascular endothelium and smooth muscle. Fibrinogen and WBC count increases noted in periodontitis patients may be a secondary effect of the above mechanisms or a constitutive feature of those at risk for both cardiovascular disease and periodontitis.[38]

Periodontitis as an infection may stimulate the liver to produce C-reactive protein (CRP) (a marker of inflammation), which in turn will form deposits on injured blood vessels. CRP binds to cells that are damaged and fixes complement, which activates phagocytes, including neutrophils.[30] These cells release nitric oxide, thereby contributing to atheroma formation CRP is a powerful marker of vascular risk and there is some evidence for a direct role in vascular dysfunction and atherogenesis. It is produced by the liver and is stimulated by TNF-a and IL-6, leading to a decrease in nitric oxide availability and an increase in angiotensin 1 receptors. It binds to low density lipids, increasing their uptake by macrophages and hence an increase in foam cell formation. For these reasons, CRP has been postulated as a major mechanism for atherosclerosis**. Ebersole et al.**[32] found that patients with adult periodontitis have higher levels of CRP and haptoglobin than subjects without periodontitis. Both CRP and haptoglobin levels decline significantly after periodontal therapy.

Infection:

The third hypothesis is direct infection of the blood vessels by bacteria. In this hypothesis, the bacterial pathogens get into the bloodstream, and subsequently invade the endothelium leading to endothelial dysfunction, inflammation, and atherosclerosis. A number of studies have shown bacteria in the arteries, but a study by **Ford and colleagues**[39] used real-time polymerase chain reaction to show P gingivalis in 100% of the arteries. Fusobacterium nucleatum was found in approximately 80% of the arteries, Tannerella forsythia was found in just under 50%, and C pneumoniae was found in just under 30%. H pylori and Haemophilus influenzae were both found in approximately 4% of the arteries. Oral organisms can and do invade blood vessel walls, but it is unclear that whether they cause atherosclerosis or simply invade an already damaged artery.[71]

Heat Shock Proteins in Cardiovascular System:

To date, acute and chronic ischemic heart disease is one of the major causes of death among people in the Western world, despite numerous exogenous pharmacological protective measures like calcium antagonists, coronary vasodilators, and blocking agents of the angiotensin converting enzyme and b-adrenoreceptors.

Upregulation of the synthesis of Hsps is one such phenomenon leading to improved tolerance to ischemia in experimental models. In both the vascular and cardiac compartment, heat shock proteins are present and can be

induced by specific stressors. The type of proteins expressed in the vascular compartment is somewhat different from that expressed in the heart.

In nonstressed, adult mice such Hsps as Hsp27, Hsc70, Hsp70, and Hsp84 are constitutively expressed in a variety of tissues, including the heart. In the heart of this species these four Hsps are clearly present, but at low levels compared with other tissues.

In unstressed rats the heart contains relatively high a Beta crystalline levels, whereas intermediate levels are found for Hsp27. In both rat and human heart, Hsp27 can be found in endothelial cells, smooth muscle cells, and cardiomyocytes, whereas a B-crystallin is only present in cardiomyocytes.

In heat- shocked rats the Hsp70 content is significantly lower in heart and brain than in colon, liver, kidney, and spleen. In these tissues, stress-induced synthesis of new Hsps occurs very rapidly. Within minutes Hsp70 Mrna transcripts are present, whereas protein accumulation reaches its maximum at 12 h after stress induction.

In the later time domain, the cardiac Hsp70 content slowly decreases but remains detectable up to 12 h after the initial stimulus. Immunohistologically, Hsp70 was found to be present in the nucleus of cardiomyocytes, in fibroblasts, and in endothelial cells in the coronary vessel wall within 3 h after stress induction. In the heart, Hsp27 concentrations double during the first 16 h after heat shock, whereas aB-crystallin increases by 20%.

In the heart, the Hsp70 synthesis seems to be developmentally regulated in both left and right ventricles. In the fetal heart very low Hsp70 levels are found. These levels, however, increase upon development and peak after the first 2 wk after birth. In contrast, the Hsc70 protein contents remain unchanged during left ventricular development, whereas they decrease with age in the right ventricle.

B-Crystallin plays an exceptional role in normal cardiac development, activating genetic programs responsible for cardiac morphogenesis. As early as 8.5 days postconception, aB-crystallin can be detected in the mouse heart and is uniformly distributed in atria and ventricles. In the endothelial cushion, pulmonary trun aorta, and endothelium, however, the protein seems to be absent.

Upon exposure to environmental stress, all cell types in the blood vessel wall respond with the synthesis of Hsps. Aside from heat, vascular Hsp synthesis is induced by such triggers as circulating hormones, reactive oxygen species (ROS). Nitric oxide (NO) is probably involved in heat shock-mediated Hsp70 synthesis in blood vessels, because the NO synthase (NOS) inhibitor Nvnitro- L-arginine (L-NNA) also inhibits Hsp70-gene transcription.

Hsps and Atherosclerosis:

In atherosclerotic plaques of human blood vessels several members of the Hsp families have been detected. The pathophysiological significance of the pertinent presence of Hsps in those plaques is still unclear. The most plausible explanation is that they reflect the stressful condition of the cells

within the developing plaques. Cell proliferation, inflammation, and chronic ischemia all are events taking part in the multifactorial process of atherosclerosis. The current knowledge about Hsps in atherosclerotic blood vessels has been reviewed by **Xu and Wick**[153] .

Berberian et al[16] Were the first to describe the presence of high concentrations of the major inducible Hsp70 protein in the center of atherosclerotic plaques in human blood vessels. Interestingly, high concentrations of this protein are colonized with infiltrating macrophages and are particularly localized at the border of necrotic zones in the vessel wall. Aside from Hsp70, increased concentrations of Hsp60 and Hsp90 and Hsp27 have been found in these pathological tissues. A linear relationship between the concentration of Hsp60 and the number of infiltrating T lymphocytes exists. **Xu et al**.[154] also found coexpression of Hsp60 and the intercellular adhesion molecule- 1 (ICAM-1), vascular cell adhesion molecule, and E-selectin in endothelial cells exposed to cytokines and low-density lipoproteins, whereas others found coexpression of Hsp60 and ICAM-1 after exposure to endotoxin.

In a great number of patients with coronary heart disease, high plasma concentrations of anti-Hsp60 antibodies have been documented .One of the etiological factors of atherosclerosis, i.e., oxidized low-density lipoproteins (Ox-LDL; a cytotoxic lipoprotein), induces Hsp70 synthesis in cultured human endothelial cells[44] . Because smooth muscle cells are among the cell types

involved in the formation of atheroma, it is obvious to address the question whether Ox- LDL also induce Hsp70 synthesis in these cells..

However, compared with cultured human endothelial cells from umbilical veins, the degree of Hsp70 synthesis is significantly lower. Interestingly, there are indications that smooth muscle cell necrosis as a consequence of circulating toxins is inhibited by treatment of exogenously administered Hsp70.[41] Protection seems to be related to the binding of Hsp70 to the external leaflet of the sarcolemma and not to its internalization in the cell. Smooth muscle cell proliferation following scrape wound injury is also inhibited by a preceding heat shock leading to the expression of Hsps in these cells.

Another component of atherogenesis is the shear stress acting on the vascular endothelial cell. Large variations in shear stress, which is in fact the tangential component of the hemodynamic force, activate atherogenesis-related genes in the endothelium at lesion-prone sites.[70] Among others, multiple protein kinases leading to protein phosphorylation are activated. The small Hsp27, which is expressed in endothelial cells of the vascular wall, is phosphorylated, among others, upon changes in shear stress, whereas its expression level remains unchanged.[29]

The characteristics of the tissue samples obtained during cardiac surgery, the hemodynamic circumstances, and serum hormone levels at the moment of tissue preservation should be documented in great detail, since it has been shown in experimental animals that sudden changes in these

parameters can affect the synthesis of Hsps. Increased hemodynamic loading through aorta banding and injection of catecholamines, vasopressin, or angiotensin II were found to stimulate Hsp synthesis[78]

Cross-Reactivity / Molecular Mimicry:

The fourth hypothesis is that of crossreactivity or molecular mimicry. In this hypothesis, the periodontal bacteria induce a local immune response, which subsequently cross-reacts with self-antigens expressed on the vascular epithelium. This in turn leads to vascular inflammation and atherosclerosis.[90] Recently, there has been increasing awareness that immune responses are central to atherogenesis, and a mechanism by which infection may initiate and facilitate the progression of atherosclerosis can be explained in terms of the immune response to bacterial heat shock genes and heat shock proteins (Hsps).[17]

All cells express Hsps on exposure to various forms of stress, including temperature, context, human heat shock protein 60 (hHsp60) shows a remarkable similarity with a very large number of autoantigens. In terms of atherosclerosis, factors such as bacterial lipopolysaccharide, cytokines, and mechanical stress may induce the expression of host protective hHsp60 on endothelial cells. Because of the homologous nature of Hsps among species, cross-reactivity of antibodies to bacterial Hsp (termed GroEL) with hHsp60 on endothelial cells may subsequently result in endothelial dysfunction and the development of atherosclerosis.[148]

The presence of risk factors such as high blood cholesterol would enhance the expression of hHsp60 and adhesion molecules by endothelial cells and result in progression from early fatty streak lesions to severe and irreversible atherosclerotic alterations. Anti-Hsp60/65 were shown to be cross-reactive with those of other bacteria and were able to lyse stressed but not unstressed endothelial cells.[87]

GroEL proteins, belonging to the Hsp60 family, have been reported to be major antigens in several pathogenic bacteria. An Escherichia coli GroEL homologue has been identified in the periodontopathic bacteria P gingivalis, Fnucleatum, and Actinobacillus actinomycetemcomitans, which was immunogenic and was recognized by serum antibodies in patients with periodontal disease. [59]

GroEL antigens share a high degree of homology with hHsp60 proteins and antibody to hHsp60 cross-reacts with this periodontopathic bacterial GroEL. Patients with periodontal disease were shown to have a higher positive response to P gingivalis GroEL than healthy controls and cross-reactivity between anti-P gingivalis GroEL antibodies in the serum of these periodontitis patients with hHsp60 and between antibodies to hHsp60 with Pgingivalis GroEL has been demonstrated.

Tabeta and colleagues[135] in their study were able to show that there are higher levels of antibodies Hsp60, P gingivalis, and GroEL in patients with artherosclerosis compared with periodontitis and controls. They were also able to show that there was a high correlation between the anti-GroEL and anti-

Hsp60 antibodies. The cross-reactivity observed by a number of GroEL-specific T-cell lines to hHsp60 and hHsp60-specific lines to GroEL, again suggesting molecular mimicry of GroEL and hHsp60. **Tabeta and colleagues**[134] in a study were able to show that there are higher levels of antibodies HSP60, P gingivalis, and GroEL in patients with artherosclerosis compared with periodontitis and controls . GroEL-, hHsp60- and P gingivalis-specific T-cell lines from peripheral blood and from human atherosclerotic plaques were identified. The artery T-cell lines specific for GroEL, hHsp60, and P gingivalis demonstrate a predominant Th2 phenotype in the CD4 subset and a Tc predominance in the CD8 subset with a high proportion of CD8 cells.

HSP 60 and Host Immune Response :

Wassner A et al[146] described the cloning and characterization of CD4 and CD8 T lymphocytes isolated from inflamed gingival tissue obtained from four patients with chronic periodontitis..Clones were raised with phytohemagglutinin and interleukin – 2 and tested for proliferation in response to whole cell antigens of P.gingivalis, Prevotella intermedia Aggregatibacter actinomycetemcomitants, Human collegen type 1 , and two bacterial heat shock proteins using flow cytometry analysis . Most clones were reactive with P.intermedia, it seems that the immune response is not strictly directed against this particular microorganism, as clones reactive with one of the other bacteria were also obtained from two patients.[81] He proposed that collagen specific CD4 Th2 – like T cells contribute to the chronicity of Periodontiits but rather their modes of activation might be controlled by Th0-like T cells specific for

periodontitis associated bacteria suggesting that autoimmune component might be involved in the pathogenesis of periodontitis . Pro inflammatory cytokine production induced by autologous HSP 60 could be another pathway leading to periodontal tissue destruction.[60]

The Heat shock proteins are conserved through evolution and are structurally similar in all living organisms so that immune responses against Hsp can cross-react and produce anti-self reactivity. Immune responses to the cross-reactive determinants in bacterial Hsp are thought to be generated early in life and the immunologic memory may function as a "common barrier" against infections. Cross reactive determinants in human Hsp may function as a trigger for autoimmune reactions.[21]

Hsp cytokine effects are mediated through via the CD14/ Toll-like receptor complex signal transduction pathways leading to the activation of nuclear factor κ B (NF-κB) and mitogen – activated protein kinases (MAPKs) i.e., ERKs (p42 and p44 extracellular signal reductase kinases), JNK (c-Jun NH2- terminal Kinase) , and p38 kinase . The CD14 and TLR receptor complexes are Pattern Recognition Receptors involved in the innate immunity for the pathogen recognition and host defense. [32]

The outcome of an immune response is determined by the particular subsets of leukocytes that are recruited and subsequently activated within the lesion. A Th1 cytokine response has shown to dominate the lesion of atherosclerosis with production of gamma interferon (INF α), Tumor necrosis factor α (TNFα) and interleukin -2. IFN α deficient mice have been shown to

develop lesions of reduced size, lipid accumulation and cellularity but with increased fibrosis and collagen content, resulting in a more stable plaque.[47]

The Th-1 inhibitory cytokine IL-10 is expressed in human atherosclerotic lesions in association with reduced inflammation. The Th2 cytokine IL-4 is uncommon in atherosclerotic lesions. Monocyte Chemoattractant Protein 1 (MCP1) exhibits potent chemotactic activity for monocytes and has been demonstrated in human atherosclerotic plaques. IFN inducible protein 10 (IP 10) specifically chemo attractants activated T cells. IFN-α induces the expression of IP-10, which in turn, upregulates IFNαproduction. Thus contributes to the lesion. Neovascularization and healing are inhibited by IP-10 and this may increase the severity and instability of the plaque.[89] Influence of periodontal therapy on intima–media thickness of the arterial wall.

The intima–media thickness of the arterial wall is a parameter of atherosclerosis. The carotid intima– media thickness is highly correlated with coronary artery disease and cerebral disease. In a pilot study, no significant difference in brachial artery intima–media thickness was noted before and 3 months after non-surgical periodontal treatment that included systemic antimicrobial therapy, a reduction in the carotid intima–media thickness was observed after periodontal treatment. In a longitudinal study Non-surgical debridement was performed and completed within 4 weeks. Echo-Doppler cardiography of the carotid artery was evaluated before and 1, 6 and 12 months after the periodontal treatment.[15] The results showed that the carotid

intima–media thickness was significantly reduced at 6 and 12 months after treatment, and the decrease in the carotid intima–media thickness was detected at multiple sites along the carotid axis: at the carotid bifurcation and at 1 and 2 cm from the bifurcation. This indicates a beneficial effect of periodontal treatment on the carotid intima–media thickness.[59]

Methods of Hsp60 Detection

The increasing interest in Hsp as markers of exposure to environmental stress or diseases requires a generally applicable method for Hsp determination.[66] There are many Classical methods that evaluate Hsp at the protein level

Enzyme-linked immunosorbent assay

It is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. In ELISA an unknown amount of antigen is affixed to a surface, and then a specific antibody is washed over the surface so that it can bind to the antigen. This antibody is linked to an enzyme, and in the final step a substance is added that the enzyme can convert to some detectable signal[73].

Performing an ELISA involves at least one antibody with specificity for a particular antigen. The sample with an unknown amount of antigen is immobilized on a solid support (usually a polystyrene microtiter plate) either non-specifically (via adsorption to the surface) or specifically (via capture by another antibody specific to the same antigen, in a "sandwich" ELISA).

After the antigen is immobilized the detection antibody is added, forming a complex with the antigen. The detection antibody can be covalently linked to an enzyme, or can itself be detected by a secondary antibody which is linked to an enzyme through bio conjugation. Between each step the plate is typically washed with a mild detergent solution to remove any proteins or antibodies that are not specifically bound. After the final wash step the plate is developed by adding an enzymatic substrate to produce a visible signal, which indicates the quantity of antigen in the sample.[85] Older ELISAs utilize chromogenic substrates, though newer assays employ fluorogenic substrates enabling much higher sensitivity. ELISA is used to detect serum antibodies to periodontal pathogens and has been used in recent studies to quantify specific pathogens.

Handley HH et al (1995)[50] has examined by ELISA the serum IgG antibody levels to McyHsp65 and HumHsp60, as well as to the Escherichia coli Hsp60, GroEL, in patients with Rheumatoid arthritis, Systemic lupus erythematosus (SLE), Reiter's syndrome, active tuberculosis, and normal controls.

Tabeta K et al (2001)[134] demonstrated the presence of antibodies to the Hsp60 of Actinobacillus actinomycetemcomitans in the sera of periodontitis patients and periodontally healthy control subjects by enzyme-linked immunosorbent assay using a recombinant A. actinomycetemcomitans GroEL as an antigen.

Watanabe S etal (2006)[147] established an ELISA system to measure anti-Hsp60 IgG and IgA antibody titre in sera of Rheumatoid arthritis patients **Goretzk Li et al (2006)**[44] stated that immunoassays for the quantification of the HSP biological markers help to define more precisely the significance of heat shock proteins in cancer. **Shamaei-Tousi A et al (2007)**[128] measured plasma levels of Hsp10, Hsp60 by immunoassay and related to other plasma measures of inflammation.

Western Blot / Immunoblotting

Many authors have demonstrated the expression of human and bacterial Hsp60 by western blot It is a powerful method for detecting a particular protein in a complex mixture which combines the superior resolving power of gel electrophoresis, the specificity of antibodies, and the sensitivity of enzyme assays. This multistep procedure is commonly used to separate proteins and then identify a specific protein of interest. Two different antibodies are used in this method, one specific for the desired protein and the other linked to a reporter enzyme. It is a technique for the analysis of proteins within the cell. Proteins are electrophoresed through a SDS polyacrylamide gel so as to separate the molecules according to size, and are then transferred to a membrane and hybridized with an antibody against the specific protein of interest.

Heat shock proteins in saliva:-

Molecular chaperones were considered to be intracellular, but there is increasing evidence demonstrating their cytoprotective and immune modulator properties outside the cell, exerting cytokine-like effects and influencing immune recognition. There is scant evidence regarding Hsp60 in saliva. However the major extracellular chaperone Hsp70 was found in saliva, indicating a possible effect of Hsp70 on mucosal surfaces. Hsp70 has been found to be present in human blood sera. Cpn10 and Cpn60 are present in pancreatic juice, but Hsp70 is not. These observations raise the possibility that molecular chaperones may be present in other secretory fluids, such as human saliva.

Fabian TK et al (2007)[35] summarized the immune-modulatory role of the 70-kDa stress protein family, with special attention on the potential impact of salivary Hsp70 on oral defense mechanisms. Three major facets of Hsp70-induced immune activation are : 1) the appearance of Hsp70 on the surface of certain tumor cells or virally infected cells, leading to their phagocytosis and subsequent lysis; 2) the role of extracellular uncomplexed Hsp70 as a danger signal, leading to the secretion of proinflammatory cytokines from antigen-presenting cells and T lymphocytes and of nitric oxide from macrophages as well as to complement activation; 3) receptor-mediated uptake of peptide-loaded Hsp70 to antigen-presenting cells and cross-presentation of the Hsp70-peptide complex as an antigen to cytotoxic T cells and natural killer lymphocytes. The immune-activating effect of salivary Hsp70 may also be

highly important in oral defense, especially in areas where molecular and cellular participants of the immune response appear on the surface of the oral cavity (i.e. several lesions of the mucosa and the periodontal tissues).[58]

Fabian TK (2003)[36] Human whole saliva was collected from six participants under resting conditions and secretory stimulation. The samples were precleared by centrifugation and sterile filtered. Salivary volume, protein concentration and amylase activity were determined. For detection of Hsp70 saliva proteins were separated on a 12.5% SDS polyacrylamide gel. Semi-dry Western blot analysis was used with a primary antibody against the inducible form of Hsp70. There was a significant decrease of Hsp70 and a non-significant decrease of total protein concentration during stimulation, whereas the activity of salivary amylase increased significantly. Stimulation significantly increased the Hsp70, total protein and amylase outputs as well as the amylase/protein ratio, and decreased the HSP 70/amylase and Hsp70/protein ratios. He concluded that Hsp70 is secreted to saliva, but unlike amylase is not transported by the exocytotic secretory mechanisms of acinar cells. Passive transport mechanisms of Hsp70 from blood serum or from salivary gland cells may be major routes of salivary Hsp70 secretion.

Fejerdy L (2004)[37] investigated whether repeated, short-term heat and mechanical stimulation of the salivary glands can specifically modify the salivary Hsp70 concentration in the human whole saliva. Both kind of stimulation increased the secretory rate significantly, during stimulation, but it decreased to control level in resting phases. Hsp70 concentration increased

after the first stimulation in the case of mechanical stress and after the second stimulation in the case of heat stimulation. In contrast, a significant confluent increase of total protein concentration and amylase activity occurred after the first stimulation in the case of heat stimulation and after the second stimulation in the case of mechanical stress.

MATERIALS AND METHODS

Study Population

A study design of 49 patients were included into the study and were divided into two groups

Group A- 23 patients who are systemically healthy with Chronic periodontitis who attended the out patient Department of periodontology, Ragas Dental College and Hospitals, Chennai,

Group B- 26 patients of Cardiovascular disease with chronic Periodontitis patients

Cardiovascular Diseased Patients --- Patients who had multiple coronary vessel block and who were scheduled for coronary artery bypass surgery in Madras Medical Mission Hospital, Mogappair, Chennai were enrolled in the study.

Informed consent was obtained from all the patients. The patients were informed that this research work was in no way directly related to the therapy or cure of the disease. The study was undertaken following approval from the institutional review board.

Patients of all age group were included in the study

Exclusion criteria

- Patients who have undergone scaling or extraction 3 moths prior to sample collection
- Patients who were immunocompromised

Clinical Evaluation:

Periodontal Parameters

Clinical evaluation was done using mouth mirror and William's periodontal probe. The probing depth, clinical attachment loss, bleeding on probing was evaluated.

Cardiovascular Parameters

Blood sugar level – Fasting , Post prandial , Random ; Total serum cholesterol , LDL , HDL , VLDL , Triglycerides , ESR, Total Count , differential count , HB%, Carotid IMT were evaluated

Sample Collection:

Saliva collection:

Salivary collection is done according to the technique by **Navazesh et al (2001).**[94] 5ml of unstimulated saliva is collected by spitting method. The patients were advised to refrain from intake of any food or beverage (water exempted) one hour before the test session. The subjects were advised to rinse his or her mouth several times with distilled water and then to relax for five minutes. The patient is asked to lean the head forward over the container with

the mouth slightly open and allow the saliva to drain into the container with the eyes open. The time lasted for saliva collection is five minutes, the saliva is collected in a sterile disposable plastic container and the samples were centrifuged and stored at -20^{o} C and used for further analysis.

Armamentarium

1. centrifuge Tubes
2. Micropipettes
3. Micropipette tips
4. Pasteur pipette
5. Laboratory Centrifuge
6. Refrigerator
7. Autoclavable containers for saliva collection
8. Ice pack (for transfer)

Reagents Required:

1. Hsp60 ELISA KIT(ASSAY DESIGNS)

Enzyme linked immunoassay for HSP 60:-

saliva samples were diluted in sample Dilutent(1:50)

- Six polypropylene tube each with one of the following standard values was prepared.100mg/ml,50mg/ml.,25mg/ml,12.5mg/ml,6.25mg/ml, 3.125mg/ml.
- To the 96 pre coated micro litre wells, 100 micro ml of prepared Hsp 60 standard (Tube #1 to Tube#2), sample and zero standard (0mg/ml) was added and incubated at room temperature for 60 mins.

- Liquid from all the wells were aspirated and washed with 300microliter of 1x wash buffer for 6 times using automatic washer.
- After the 6 wash, 100 micro litre of previously diluted Anti Hsp 60(Goat polyclonal antibody) was added to each well and incubated at room temperature for 60 mins .the plates were washed with wash buffer as described previously.
- 100 micro litre of previously diluted HRP (Horse radish peroxidise) conjugate was added to each well excepting the blank, and incubated at room temperature for 30 mins .plates were washed with wash buffer for 6 times.
- 100 micro litre of TMB (Tetramethylbenzidine) substrate was added to every well colour developed was visible within 1minute of addition to the wells.
- Finally 100 micro litre of stop solution 2 was added to the wells.
- Microplate reader was set according to the manufacturers instruction at a wavelength of 450 mm and absorbance was measured.

Statistical analysis:-

The salivary levels of Hsp60 in Group A and Group B were compared by calculating the mean and standard deviation for each group. Student T test was used for statistical analysis and P value was calculated. Correlation coefficient of salivary Hsp60 in cardiovascular disease i.e., Group B with IMT values, mean probing depth and lipid profile of the same group of patients were analysed using Pearson's correlation.

RESULTS

The present study assessed the levels of salivary Hsp60 levels in Chronic Periodontitis in two groups of patients. Group A comprised of patients in Systemic health while group B comprised of patients with Cardiovascular Disease. Sandwich Elisa Method was used to assess the salivary Hsp60 levels.

49 saliva samples were collected from the 2 groups. Group A and Group B (23 Periodontitis with Systemic health and 26 Periodontitis with cardiovascular disease). The saliva samples were centrifuged to detect Hsp60 between the two groups

Hsp60 Saliva

The mean salivary level of HSP 60 in Systemic Health Patients with Chronic periodontitis was 30.15ng/dl whereas the mean salivary level of Hsp60 in Cardiovascular Disease patients with Chronic Periodontitis was 34.29ng/dl**. (Table 1**). There was no statistically significant difference between the two groups (p= 0.324).

Correlation coefficient for Salivary HSP 60 levels with Chronic Periodontits with Cardiovascular Disease

There was a weakly positive (0.098, 0.196) but statistically insignificant (p= 0.168) correlation between salivary Hsp60 levels and probing depth values in both Group A and Group B patients. However there was no statistically significant correlation between the, salivary Hsp60 values and either IMT values or the Lipid profile in patients with CVD (0.35, 0.368) (**Table 2).**

Table No: 1 HSP 60 levels in saliva – Comparison between Chronic periodontitis patients who are systemically healthy and who are with Cardiovascular disease

Group		Mean	Standard deviation	P value
Saliva	Chronic periodontitis with systemic health	26.04	14.36	0.32441
	Chronic periodontitis with Cardiovascular disease	34.30	14.82	

Table No: 2 Pearson's correlation for Salivary levels of Hsp 60 with IMT values, Lipid Profile , Probing depth of CVD patients and Systemically healthy patients

Comparison groups		Correlation Coefficient (r)	P Value
SALIVARY HSP 60 LEVELS	IMT values	0.079	0.350
	Lipid Profile	-0.184	0.368
	Probing depth of CVD Patients	0.098	0.316
	Probing depth of systemically healthy group	0.196	0.168

DISCUSSION

Periodontal disease involves an inflammatory process that develops in the gingiva in response to bacterial antigens in the subgingival biofilm. The pathogenic species present in sub gingival biofilm cause damage to host tissue via immune or inflammatory interactions, which typically consist of neutrophils, monocytes/ macrophages, T cells and B cells [93,100]

The immune response is typically activated through exposure to bacterial antigens with specific Pathogen associated molecular patterns (PAMPs) such as LPS, fimbriae, & Outer membranous protein, etc. In addition to these PAMPs, self antigens have also been reported to be involved in host inflammatory immune response. Hsp60 is a mitochondrial chaperonin that functions in the folding linear amino acid chains into their respective three-dimensional structure.[34, 148] Hsp60 has been shown to be released from specific cells like peripheral blood mononuclear cells (PBMCS) when there are Lipopolysaccharides (LPS) or GroEL present. Hsp60 was shown to induce a secretion of proinflammatory cytokines in professional antigen presenting cells and to enhance the activation of T cells in primary stimulation.[146] Hsp60 has thus been evaluated as a potential marker for periodontal disease activity. We have previously reported that salivary Hsp60 levels are significantly increased in periodontal disease when compared to health, but the sensitivity and specificity of this marker could not be determined.[66] We have extended this study to include CVD patients as a speculative role for these proteins has been proposed in the etiopathogenesis of Atherosclerosis .

Saliva was collected by the method, reported by **Navazesh et al (2008)**[94] Care was taken to ensure that, saliva was collected between 9-11 AM to avoid variation due to circadian periodicity that has been shown to affect salivary analytes. The ELISA method was used to evaluate Hsp60 in preference to immunoblotting as it is versatile, accurate, reproducible, and economical. Previous studies done by **Tabeta K et al (2001),**[135] **Watanabe S et al (2003)**[147] **and Handley HH et al (1996)**[50] provide evidence that supports the use of this method.

In this study, patients were divided only into 2 groups. Group A systemic healthy group with Chronic Periodontitis and Group B Cardiovascular diseased group with Chronic Periodontitis. As discussed previously a periodontal health group was not included in this study because we have reported previously that there is significant increase in Salivary Hsp60 levels in Periodontal disease when compared to health.

In this study, we estimated that the mean salivary level of Hsp60 in group A was 30.15 ng/dl whereas the mean salivary level of Hsp60 in group B was 34.29 ng/dl. Although Salivary Hsp60 values in Group B Patients was increased when compared with Group A patients there was no statistically significant difference between these two groups.

The Hsp60 levels were then correlated with the probing depth of both Group A and Group B patients for the evaluation of the results with the severity of the disease. The results showed a positive correlation (0.098 & 0.196) but there was no statistically significant difference between the two. These results suggest

that there is no direct linear correlation between the severity of periodontal disease and salivary Hsp60 levels.

The Hsp60 levels are upregulated in the early stages of periodontitis probably as a response to increase in the P.gingivalis levels in tissue.[134] Previous evidence suggests that early immunomodulatory events may have cascading effect on the tissue destructive process in periodontal disease.[22] The evidence for the involvement of Heat shock proteins in the pathogenesis of atherosclerosis has arisen from a number of studies.[15,20]

The heat shock protein expression in the vasculature serves to protect it against the effects of physiological and pathological insults such as mechanical stress, shear stress, hypercholesterolemia, free radicals, cytokines, and infections. The intensity of heat shock protein expression has been evaluated to positively correlate, with the severity of atherosclerosis. The endothelial cell cytotoxicity and the anti-Hsp65/60 antibodies in individuals with atherosclerosis provide evidence for the role of these proteins as the disease pathogenesis.

Human atherosclerotic plaques express a spectrum of inflammatory cytokines, including interferon-g, interleukin-1a and -1b, and tumour necrosis factor-α, all of which have been shown to increase heat shock protein expression in a range of cell types. Heat shock proteins may also be expressed by lipid-laden "foam" cells in the atherosclerotic plaque, in that in vitro exposure to oxidized LDL induces Hsp60 expression by monocytic cell lines and Hsp70 expression by human endothelial cells[23,74].

Several studies reported that they were able to show that there are higher levels of antibodies Hsp60, *P gingivalis*, and GroEL in patients with artherosclerosis compared with periodontitis and controls.[135,84]

The results of the study indicate that there was a positive (0.079) but not significant correlation between the Hsp60 values and IMT values observed in group B patients. However there was a negative (-0.186) but statistically not significant correlation between the Hsp60 values and the lipid profile of group B patients.

Previous reports have suggested that salivary markers such as inflammatory cytokines may strongly correlate to the systemic inflammatory status.[136] Although Hsp 60 levels has been postulated to predict its role in the pathogenesis of Atherosclerosis , there is no direct evidence for its role in hyperlipidemia. Therefore its lack of association of Hsp60 levels and HDL/LDL ratio is not surprising. On the other hand, Hsp60 has been associated with the atheromatous plaque which in turn has been intimately associated with IMT. It is therefore a little surprising that salivary Hsp60 levels showed no correlation with IMT.

However the present results must be viewed in the light of the fact that Salivary Hsp60 levels may not truly indicate their circulatory levels .Indeed there is very little conclusive evidence about an absolute correlation between any of the salivary constituents and their circulatory levels. The possibility of Hsp60 being a common link between Periodontal Disease and Cardiovascular Disease cannot be

ruled out from the results of this study. This study only indicates that salivary Hsp60 may not be an ideal marker of disease activity.

The results must also be interrupted with care because of the several limitations of the study which include,

1. A Small sample size
2. Circulatory levels of Hsp60 were not assessed.
3. Hs60 levels were not assessed in relation to severity of CVD so a role for developing pathogenesis could not be ascertained.

SUMMARY AND CONCLUSION

The aim of the present study was to estimate the salivary levels of Hsp60 in systemically healthy subjects and cardiovascular patients with chronic periodontitis and then they were correlated with probing depth, the IMT values and lipid profile.

49 patients were included in the study and divided into Group A and Group B (Group A - 23-Patients who are systemically healthy with chronic periodontitis) who attended the outpatient Department of periodontology, Ragas Dental College and Hospitals, Chennai and (Group B -26 Patients of Cardiovascular Disease with chronic periodontitis) who had multiple coronary vessel block and who were scheduled for coronary artery bypass surgery in Madras Medical Mission Hospital, Mogappair Chennai were enrolled in the study.

5ml of unstimulated saliva was collected prior to phase I therapy both in periodontitis patients and cardiovascular patients before treatment and processed for ELISA Analysis. Statistical analysis was done using student 'T' test and Pearson correlation.

From the Results we conclude that

There is no statistically significant upregulation of salivary Hsp60 levels in chronic periodontitis patients with cardiovascular disease when compared to that of systemically healthy subjects. However future studies with larger sample size should be conducted to add more credibility for the findings of the present study.

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RAGAS DENTAL COLLEGE

DEPARTMENT OF PERIODONTICS AND IMPLANTOLOGY

ESTIMATION OF SALIVARY LEVELS OF HEAT SHOCK PROTEIN 60 IN CARDIOVASCULAR PATIENTS

PROFORMA

CASE NO:

NAME: AGE: SEX: M/F.

ADDRESS:

MEDICAL HISTORY:

DIABETES: YES/NO If yes for how many years:

HYPERTENSION: YES/NO If yes for how many years:

PAST DENTAL HISTORY:

PERSONAL HISTORY:

SMOKING: YES/NO/FORMER SMOKER.

CIGARETTE/BEDI/OTHER. 1(<10 per day)
2(10-20 per day)
3(>20 per day)

TOBACCO CHEWING: YES/ NO.

ALCOHOL INTAKE: YES / NO.

BRUSHING: TWICE/ ONCE.

FINGER/ BRUSH/PASTE.

DIET: VEG/ NON VEG.

FAMILY HISTORY OF CAD:

BODY MASS INDEX:

BLOOD INVESTIGATIONS:

BLOOD SUGAR: FF PP RBS TOTAL SERUM CHOLESTEROL:
ESR: LDL:
TOTAL COUNT:
HB:
DIFFERENTIAL COUNT:

ORAL EXAMINATION:

POCKET DEPTH:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

CLINICAL ATTACHMENT LOSS:

18	17	16	15	14	13	12	11	21	22	23	24	25	26	27	28
48	47	46	45	44	43	42	41	31	32	33	34	35	36	37	38

SIMPLIFIED ORAL HYGEINE INDEX:

16	11	26	31	36	46

DEBRIS SCORE:

CALCULUS

SPECIMENS OBTAINED:

CONSENT FORM

I _____ aged about_____ years on my own volition hereby give my consent to be part of the clinical study conducted by Dr.A.Archana Meenakshi Department of periodontics, Ragas Dental college,Chennai.

I have been explained in detail in the language known to me about the procedure to be carried out and I give my consent to take my salivary samples as it may be applicable for experimental procedures.

PATIENT SIGNATURE:

NAME : DATE:

DOCTOR SIGNATURE: