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Is Neutrophil Gelatinase-Associated Lipocalin a valid marker of periodontal disease?

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Abstract
Aim of work*: The current study tested Neutrophil gelatinase-associated lipocalin (NGAL) as a reliable inflammatory marker of periodontal disease.*
Subjects and Methods*: Samples were taken from gingival crevicular fluid from 60 systemically healthy individuals. Fifty patients with stage II grade A periodontitis (study group), and ten were periodontally and clinically healthy subjects (control group).Whole mouth periodontal parameters [plaque index (PI), gingival index (GI), clinical attachment loss (CAL) and probing depth (PD)] were recorded.*
Results*:Neutrophil gelatinase associated lipocalin GCF levels were significantly higher in periodontitis patients thanperiodontal healthy subjects.*
Conclusion*: Estimation of GCF NGAL levels showed significantly higher levels in periodontitis patients than periodontal healthy individuals, we suggest that NGAL could be considered a potential inflammatory biomarker of periodontal disease.*
Keywords: *NGAL, Lipocalin2, periodontitis.*

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I .Introduction

Periodontitis is a microbe driven chronic inflammatory disease. It is primarily characterized by an imbalance between the commensal microbiota and the host response. However,the interaction of the oral microbiome and the host response is complex.The complexity arises from the wide range of bacterial taxa resident within the subgingival microbiota,and also from the intricacy of the immune and inflammatory responses of the host operative within the gingival tissues, and whether these responses are protective or destructive influences.[1]That activate the host's immune response, causing destruction of the tooth supporting tissues,leading to tooth loss.[2]

One of the most important causes of periodontal disease is the dental plaque that contains many groups of the bacterial community,this bacteria are the most abundant component with about 500 species estimated to exist in subgingival plaque, these many types of bacteria have been classified according to their ability and strength to cause damage withinSocransky complexes. Socransky and his colleagues have investigated the periodontal microflora in health and disease. These complexes have been color coded as purple, yellow, green, orange and red.[3]

The main pathogens of periodontal disease are Gram-negative bacteria, including *Porphyromonas gingivalis* (*P.g*), *Prevotella intermedia* (*P.i*), *Treponema denticola* (*T.d*), *Tannerella forsythia* (*T.f*) and *Aggregatibacter actinomycetemcomitans* (*Aa*). These bacteria cause an imbalance in the oral flora of periodontitis susceptible hosts, which leads to excessive infiltration of immune cells.[4]

After several of days of accumulation of dental plaque, filamentous and rod forms of bacteria appear with a mild leukocytic immune response.At 5-10 days, neutrophils enter the tissues resulting in the intensification of gingivitis with edema and spontaneous bleeding, increased probing pocket depths.As the immune response intensifies, macrophages and neutrophils degranulate releasing toxic compounds, causing apoptosis and eventually necrosis. The pocket lining ulcerates, and the bacteria invade the inner soft tissues, eventually reaching the vascular bed and disseminating throughout the body.[5]

Then, monocytes activated by the bacterial lipopolysaccharide (LPS) secrete inflammatory mediators such as prostaglandin E2 (PGE2), thromboxane B2, interleukin-1β (IL-1β), IL-6, and tumor necrosis factor alpha (TNF-α). Macrophages and T-helper cells secrete receptor activator of nuclear factor kappa B ligand

(RANKL), leading to an increased ratio of RANKL to osteoprotegerin (OPG) and increased binding of RANKL to RANK on macrophage precursor cells. This promotes differentiation and activation of the precursor cells into osteoclasts, which begin to resorb the adjacent alveolar bone.[6]

Matrix metalloproteinases (MMPs) are activated and break down proteins in the connective tissue surrounding the lesion. In part, the ratio of these MMPs to tissue inhibitors of matrix metalloproteinases (TIMPs) determines the relative speed and magnitude of tissue destruction.In inflamed periodontal tissue the balance between MMPs and their inhibitors TIMPs is disrupted. As a result of this disruption, pathological alterations in the types and quantities of MMPs are present. This leads to excessive breakdown of the extracellular matrix, basement membrane, and alveolar bone.[7]

Diagnosis of periodontitis is usually performed by clinical assessment including clinical attachment level (CAL), probing depth (PD), and radiographic findings. This process needs to be repeated at regular intervals in order to determine the patient's disease status. These clinical parameters give good indicators for determining disease status; but, they only provide information about past periodontal tissue destruction and do not elucidate current disease activity nor predict future activity due to low sensitivity and positive predictive value.[8]

A biomarker is an objective measure that has been evaluated and confirmed either as an indicator of physiologic health, or a pharmacologic response to a therapeutic intervention.[9] These biomarkers can be found in many biologic fluids. Biomarkers in serum can potentially provide information at the patient level; while, that in gingival crevicular fluid (GCF) can potentially provide information at the site level. However, saliva contains both local and systemically derived markers and provides information at the patient level.[8]

Gingival crevicular fluid is an inflammatory exudate whose constituents are derived from host cells and microorganism related processes and whose composition reflects the ongoing events in the periodontal tissues, Therefore, extensive research on GCF components has been performed, to identify GCF parameters that could help in periodontal disease diagnosis, estimation of disease activity and progression, proper treatment planning, and monitoring of treatment outcomes.[10]

Neutrophil gelatinase associated lipocalin (NGAL), also known as lipocalin 2, mostly expressed by neutrophils and oral epithelial cells, participates in multiple physiological and pathophysiological processes, including hematopoietic cell apoptosis, inflammation modulation, and metabolic disease pathogenesis.Interleukin-1β (IL-1β),lipopolysaccharide (LPS)and tumor necrosis factor-α (TNF-α) are the strongest inducers of NGAL.[11][12]

NGAL is reported to play an important role in mediating innate immunity response to bacterial infections by sequestrating iron-loaded siderophores.[13] It is also a neutrophils chemoattractant, promoting their maturation, adhesion and extravasation, and their phagocyte capacity, besides, it activates regulatory T cells.[14, 15]

However, limited studies were available in the literature regarding distinct role of GCF NGAL in periodontal diseases immunopathogenesis. This raises the question about the validity of NGAL as an inflammatory marker in periodontitis and the ability of using NGAL ELISA to differentiate between periodontal health and disease.

II .Subjects and Methods

Patient selection:

The present study was carried out on 60 subjects of both genders (30 males, 30 females), aged between 30 to 60 years old. Fifty patients of them were diagnosed with stage II grade A periodontitis according to the classification of the American Academy of periodontology 2017(study group). Tenwere periodontally and clinically healthy subjects (control group). They were selected from department of Oral Medicine and Periodontology Clinic, Faculty of Dentistry, Mansoura University. The study protocol was reviewed and approved by the ethical committee of the Faculty of Dentistry, Mansoura University under the number (A1010720). Patients were asked to sign an informed consent after explaining the steps, method and benefits.

Inclusion and Exclusion criteria:

The inclusion criteria were stage II grade A periodontitis patient aged 30 -60 years, with clinical attachment loss (CAL) measuring 3-4 mm, with probing depth >4mm, no history of antibiotic or periodontal therapy in the last 3 months. While the exclusion criteria were systemic diseases / conditions that could influence the progression of periodontitis or the treatment response (e.g. Diabetes Mellitus, smoking and pregnancy).[16, 17]In addition, Those who suffer from obesity and kidney failure [18, 19], and those with unacceptable oral hygiene level during re-evaluation of phase I therapy, teeth with gingival recession, and endodontic involvement, were also excluded.

Study design

The current study classified into two groups; study group: 50 patients with periodontitis stage II grade A with free systemic disease and control group: 10 periodontal healthy subjects.

Material and Methods

GCF samples were collected from the selected pockets using sterile paper points.The paper points were inserted into the periodontal pocket (gingival crevice) until light resistance is felt and kept on-hold for 30s. Any paper point contaminated with blood were excluded and discarded. The paper points were placed into sterile Eppendorf tubes containing 100µl of Phosphate Buffer Saline PH 7.4 and stored immediately at -80°C for further analysis.

GCF samples were collected and recorded for all patients and healthy subjects.Whole mouth periodontal parameters [plaque index (PI), gingival index (GI), clinical attachment loss (CAL) and probing depth (PD)] were recorded. NGAL evaluations were done by Enzyme Linked Immunosorbent Assay (ELISA), supplied by Bioassay Technology Laboratory (China) Cat. No. E1719Hu.

Principle of the test

The plate has been pre-coated with Human NGAL antibody. NGAL present in the sample is added and binds to antibodies coated on the wells. Biotinylated Human NGAL antibody is then added and binds to NGAL in the sample. Then, Streptavidin-HRP is added and binds to the Biotinylated NGAL antibody. After incubation, unbound Streptavidin-HRP is washed away during a washing step. Substrate solution is then added and color develops in proportion to the amount of Human NGAL. The reaction is terminated by addition of acidic stop solution and absorbance (OD) is measured at 450 nm.The results were automatically calculated using BioRad ELISA system (USA).A standard curve was plotted relating the OD to the concentration of standards. The NGAL concentration in each sample was interpolated from this standard curve. Figure (1)

III. Results

RegardingNGALvaluesin GCF levels,there were statistically significant differences between the study and control groups, with markedly higher values in the study group. Where mean ± SD of NGAL of patients group was 243.3±36.77 and mean ± SD of NGAL of control group was 187.3±14.8. Table (1)

Regarding plaque index; there were statistically significant differences between patients group and control group with (Median, range) of **PI** were 2.4 (1.9 – 2.9), 0.75 (0.3 – 1) for groups (patients and control), respectively.Regarding gingival index; there were statistically significant differences between patients group and control group with (Median, range) of **GI** were 1.87 (1.55 – 2.4), 0 (0 – 0.9) for groups (patients and control), respectively. Table (2)

Regarding probing depth; there were statistically significant differences between patients group and control group with (Median, range) of **PD** were 3.28 (2.7 – 4.1), 1.5 (1 – 1.5) for groups (patients and control), respectively.Regarding clinical attachment loss; there were statistically significant differences between patients group and control group with (Median, range) of **CAL** were 2.8 (2.2 – 3.2), 0 for groups (patients and control), respectively. Table (2)No significant correlation was noticed between GCF NGAL level and the clinical parameters among our studied population. Table (3)

IV. Discussion

Periodontal diseases are characterized by a destruction of collagen fibers and other extracellular matrix constituents in periodontal tissues. A possible mechanism for the degradation of periodontal extracellular matrix is the independent and/or cooperative action of both human and bacterial proteinases.Most likely, periodontal tissue destruction is mediated to a significant extent by the host-cell-derived MMPs.[20]

However, during theperiodontitis pathogenesis,a variety of cytokines, chemokines, arachidonic acid metabolites, and inflammatory mediators including proteolytic enzymes are released as a result of the activation of the host's adaptive and innate immunity by the microbiological agents. These released molecules were blamed for a percentage of the periodontitis associated soft tissues degradation and bone resorption.Being an exudate that reflects the events in the periodontium, GCF may be used to detect the levels of certain biologically active substances.[21]

In the current study, baseline GCF NGAL levels were tested among both periodontitis group and periodontal healthy group. However, its level in periodontitis group was statistically significantly higher compared tothat of healthy group(<0.001)**.**

In consistent with this result, **Tan et al.** demonstrated elevated levels of serum and salivary lipocalin-2 in periodontitis patient that was correlated with disease severity.[22] In this respect**, Westerlund et al**. reported higher lipocalin-2 activity in GCF of periodontitis patients, compared to periodontal healthy controls, and demonstrated immunohistochemically that extravasated PMNs were its major source. Supporting this issue,

lipocalin-2 deficiency may decrease neutrophil infiltration, myeloperoxidase activity, and expression of TNF-α and IL-1β.[12]

Our results were in accordance with**Ceylan et al.** study.In their study, they tested the percentage of GCF NGALand semaphorin3A among patients with stage III grade C periodontitis, patients with gingivitis and healthy people before and after treatment. **Ceylan et al**. confirmed that the percentage of NGAL of GCF in periodontitis patients is much higher compared to healthy controls, and that NGALshowed strong positive correlations with TNF-α and positive correlations with Semaphorin3A. Finally, **Ceylan et al**confirmed that NGAL and TNF-α levels in GCF are correlated with clinical parameters and could prove useful as non-invasive screening tools for periodontitis.[11]

In another study conducted by **Pradeep et al**in 2016 on the ratio of lipocalin 2 in tears and GCF in obese patients and patients with periodontitis. It was discovered that, patients with periodontitis had higher levels of lipocalin 2 than individuals without the condition. Additionally, much higher NGAL levels was found among those who are obese and have periodontitis. However, **Pradeep et al** confirmed that Lipocalin2 may be an important inflammatory marker which may help link obesity and chronic periodontitis.[18]

Since our study is a preliminary study, we recommend further studies with a larger population, evaluation of NGAL after treatment and a longer follow-up period to assess the relationship between NGAL and other inflammatory cytokines and to test the role of NGAL in the inflammatory process.

V. Conclusions

Estimation of GCF NGAL level showed significantly higher levels in periodontitis patients than periodontal healthy individuals, wesuggest that NGAL could be considered a potential inflammatory biomarker of periodontal disease.

Ethics approval

The study methodology was authorized by the Mansoura Faculty of Dentistry's Ethics Committee under the number A10010720.

Author's contributions

J.M.Y: designed the study, controlled all study procedures, analyzed the clinical data, wrote and edited the manuscript. A.A.B: performed the biochemical assessment and analyzed the data.M.F.K: obtained the biological samples and clinical data. Each author contributed to the final document of the manuscript and discussed the findings.

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Table (1): NGAL levels in patient and control groups.

Group	Mean	SD	95% CI		P value
			Lower Bound	Upper Bound	
Patients	243.3	36.77	232.85	253.75	**< 0.001**
Control	187.3	14.8	176.71	197.89	

SD, standard deviation, **CI**;Confidence Interval,**P**; p value, **T, T** test is used,

Table (2): Clinical assessment of patient and control groups

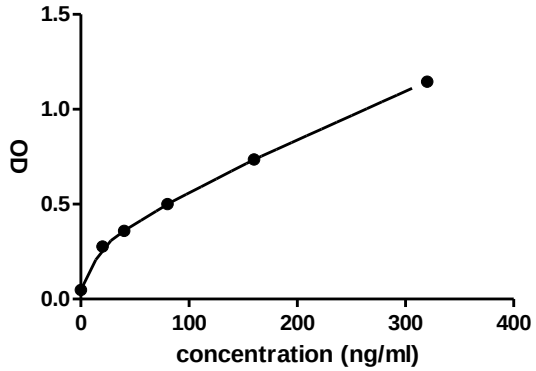
	Patients	Control	P value
plaque index (Median, range)	2.4 (1.9 – 2.9)	0.75 (0.3 – 1)	**< 0.001**
Gingival index (Median, range)	1.87 (1.55 – 2.4)	0 (0 – 0.9)	**< 0.001**
periodontal probing depth (Median, range)	3.28 (2.7 – 4.1)	1.5 (1 – 1.5)	**< 0.001**
clinical attachment loss (Median, range)	2.8 (2.2 – 3.2)	0	**< 0.001**

Mann-Whitney U test was used

Table (3): Correlation ofGCF NGAL concentrations with clinical parameters in patient and control groups

		plaque index	Gingival index	periodontal probing depth	clinical attachment loss
Patients	R	0.133	0.063	0.201	0.042
	P value	0.359	0.663	0.162	0.775
Control	R	-0.059	0	0.409	-
	P value	0.871	1	0.241	-

Spearman correlation is used



NGAL STANDARD CURVE

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