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***Research Article***

# COMPARING EXPRESSION OF NOD-LIKE RECEPTOR FAMILY, PYRIN DOMAIN CONTAINING 3 (NLRP3) IN GENERALIZED AGGRESSIVE PERIODONTITIS (G-AGP) AND HEALTHY GINGIVA

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## ABSTRACT
Periodontal diseases are poly microbial infections resulting in bone destruction and tooth loss. Host's response to these infections includes both mechanisms of innate immune responses and acquired immune responses. Bacteria and especially gram-negative bacteria play an important role in prevalence of periodontal diseases. Also, it is determined that some periodontopathic bacteria make inflammatory cells to express NLRP3 which is one of the most important parts of inflammasome. According to this, the current research tries to survey the expression of NLRP3 in generalized aggressive periodontitis. Gingiva tissue samples were collected from 20 people having clinically healthy gingiva and 25 people having G-AgP (total of 45 samples). After RNA extraction and cDNA preparation from each sample the amount of expression of NLRP3 was surveyed by the Real-time PCR technique. Conducting this research it was determined that expression of NLRP3 in tissues with G-AgP is significantly higher than the expression of NLRP3 in healthy gingiva ($P \approx 0.034$); but regarding its correlation with high tissue destruction cases (CAL and/or PD≥7mm) no significant statistical correlation was observed. Based on the current research findings it is concluded that in G-AgP the increased expression and production of NLRP3 is observed which results in inducing release of inflammatory cytokines and aggravating inflammation in the periodontal tissues.

***Keywords:*** *NLRP3, Aggressive Periodontitis, Poly Microbial Infections, Healthy Gingiva*

## INTRODUCTION
One of the concerns of periodontists is the periodontal diseases and inflammatory changes resulted from supporting tissues of the teeth such as gingival recession, platelet formation, joints and bone loss (Jin and Lee, 2008). Bacteria and especially gram-negative bacteria play an important role in prevalence of periodontal diseases. Periodontopathic bacteria stimulate cells constituting the periodontal tissues and make them express different types of inflammatory mediators (Wang and Ohura, 2002); however, the severity of periodontal diseases is related to the balance of amount of microorganisms and the host's immune responses (Tohme *et al.*, 1999).

Xue *et al.*, (2015) compared the expression of NLRP3, NLRP1 and AIM2 in patients suffering from periodontitis. To this aim, 65 samples of gingiva tissues were collected and divided into 3 groups: Patients with chronic periodontitis, patients with G-AGp and healthy people. Real-time PCR was used for determining the expression of mRNA related to NLRP3, NLRP1 and AIM2. Also immunohistochemistry technique was used for determining the propagation of NLRP3, NLRP1 and AIM2 in gingival epithelium and gingival connective tissue. They observed that expression of NLRP3 in chronic and aggressive periodontitis samples was higher than the healthy gingiva tissue. NLRP1 was hardly observed in healthy gingiva tissue; whereas, expression of AIM2 in chronic periodontitis was higher than other groups. They concluded that expression of NLRP3, NLRP1 and AIM2 is different between patients suffering from chronic and aggressive periodontitis (Xue *et al.*, 2015).

Zhao *et al.*, (2014) surveyed the correlation between NLRP3 and cell line MG-63 apoptosis infected by *Aggregatibacter actinomycetemcomitans* (A.a). To this aim, firstly they infected the Osteoblastic MG-63 cell line with A.a and they found out that A.a results in significant increase of cell line MG-63 apoptosis. Also, they found out that following the infection of MG63 cells with A.a the expression of NLRP3 and ASC significantly increases and this is also accompanied by increase of IL-1β and IL-18. They knock

downed the expression of NLRP3 by Specific small RNA (SiRNA) and weakened the apoptosis of MG63 cells infected with A.a.; These researchers concluded that A.a results in stimulating apoptosis of human osteoblasts and at least a part of this is conducted through NLRP3 (Zhao *et al.,* 2014).

In a research conducted by Park *et al.,* (2013), they surveyed the secretion of IL-1β during infection with *P. gingivalis*. *P. gingivalis* is one of the periodontopathogens. Since IL-1β is one of the inflammatory cytokines regulating the innate immune system and its excessive discharge results in gingival tissue loss, the signal induced by P.g in differentiated THPI cells was surveyed. After conducting real time PCR the inflammasome compounds in gingival tissue reached a normal level and it was concluded that P. gingivalis results in discharge of IL-1β through activating Casp1 and ultimately results in cell death. Also, cell death and discharge of IL-Iβ depend on activation of path of NLRP3 and AIM2. Activation of NLRP3 is caused by release of ATP and lysosomal injury (Park *et al.,* 2013).

Beli Basaki *et al.,* (2013) surveyed the effect of *P.gingivalis* as a part of biofilm of gingival tissue on the expression of inflammasome of NLRP3 and AIM2 (inflammasome independent from NLR and IL-IB) in gingival fibroblasts. To this aim, 10 types of gingival biofilms and 10 types except for Porphyromonas gingivalis were used for contact with cells for 6 hours. Taqman Real time PCR technique was used for analyzing the expression of gene of different combinations of inflammasome and IL-1β. As a conclusion, in 10 types of gingival biofilms, the expression of NLRP3 had a reduction but AIM2 had no change. They expressed that this reduction of innate immune response of human could be in favor of staying alive and resistance of biofilm types in gingival tissue (Belibasakis *et al.,* 2013).

Hung *et al.,* (2009) surveyed the role of NLRP3 protein (Cryo-pyrin) and adapter protein having second CARD (ASC) in detecting molecules resulting in host's immune response against Porphyromonas gingivalis in periodontal diseases; because *P.gingivalis* is one of the common separated types of periodontal diseases and periodontal disease results in periodontal loss and NLRP3 and ASC result in cell death through activating inflammasome and inflammatory response. To this aim, type A7436 P.gingivalis cell culture in monocyte and macrophage cells and THPI (Knock out control cell of NLRP3 and ASC) for inducing cell death and measuring it through viability test and Real time PCR technique and ELISA for surveying IL-1β caused by activating NLRP3 inflammasome and measuring cell death through PI exclusion assay and ASC western analysis. Cell death measuring with the increased PI color showed that induced cell death by *P.gingivalis* in THPI is depended on NLRP3 and ASC and independent from IL-1 and morphological changes along with necrosis due to loss of membrane integrity and release of cell contents and cell death by accumulation of ASC and it is depended to ASC and NLRP3 (Huang *et al.,* 2009).

## MATERIALS AND METHODS

The current research is an analytical-correlational survey. Data collection tools include interview with patients, examining patients, observing radiographic stereotypes and observation results and filling out the information forms. Individuals participating in this study were all Iranians above 18 and non-smokers and they had not used anti-inflammatory drug before the surgery and they had visited the department of periodontics, faculty of dentistry and clinics related to faculty of dentistry, Shahid Beheshti University of Medical Sciences and Health Services. All individuals have deliberately filled out the consent form of participating in the study.

The sampling method was non-random and samples were gathered from the available individuals. Number of selected samples was estimated regarding the prevalence of the diseases and by the use of Cochran formula and at most 20 samples were estimated for each group. However, for patient group (aggressive periodontitis), 25 samples were gathered due to possibility of lack of cooperation.

Rest (Qiagen-Germany) was used for conducting statistical analysis of expression of gene and SPSS 16.0 software was used for surveying other variables. Firstly, the statistical descriptive indicators (mean, SD, maximum and minimum) were calculated. For comparing the expression of Moesin and TLR4 in three studied groups, in case of subordination of data from normal distribution, the one-way ANOVA test is used and otherwise, the Rest software is used. The acceptable error rate in this research is less than 0.05.

***Collecting and Preserving the Gingival Tissue Sample***

Gingival tissue samples were collected following the surgery and they were immediately put into Cryo tubes containing RNA Later (RNA Later is a RNA preservative that could preserve RNA for 24 hours at 37°C, for a week at room temperature, for a month at 4°C and for a long time at -20°C. This material is a product of QIAGen and it is allocated to preserving special tissue. In case we want to preserve tissue at -70°C the tissue must be separated from RNA Later. In this research, samples were put into RNA Later inside a fridge (4°C) for 24 hours until the RNA Later fully penetrated the tissue and then they were transferred to -20°C and until collecting all samples they were remained at -20°C. The ratio of tissue to RNA Later must be 1ml of the solution per each 100mg of the tissue. However, the tissue must be fully immersed in the solution and not outside the solution.

*Homogenizing or Leveling out the Tissue*

By homogenizing or leveling out the tissue it means that at the end of this stage, the tissue is completely destroyed and lost its mass form and turns into a solution.

AT this stage the samples frozen at -20 are brought out and by the use of Eppendorf sampler (made in Gernmany) and Nuclasefree sampler tip made by Boeko in Germany the RNA Later of the tissue is discharged and the tissue becomes RNA Later free.

Then, by the use of ROCHE kit (High pure RNA Isolation kit) with product number: 12033674001, the tissue homogenization and Total RNA extraction stages were conducted and all stages were conducted based on kit protocol.

RNA was extracted from the gingival tissue samples and its concentration and purity were measured by the use of a bio-photometer device. RNA concentration was around 40-50µg/ml and OD 260/280 around 1.6-1.9 indicating desirable quality of RNA. For surveying the quality, the extracted RNA was electrophoresis on 2% agarose gel which is shown in figure 1.

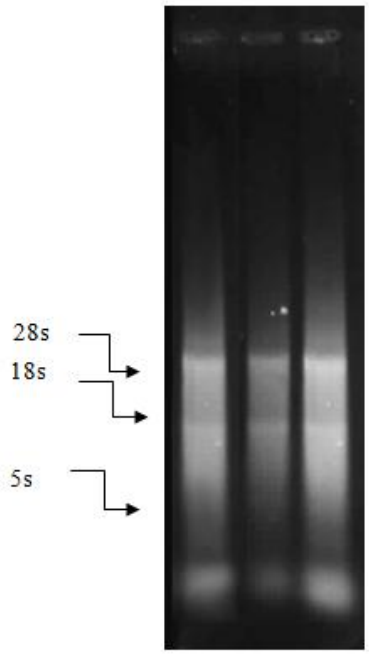



**Figure 1: Bands Related to 3 Extracted RNA Samples on 2% Agarose Gel, 28s, 18s and 5s Bands Related to rRNA Indicating the Desirability of Extracted RNA**

*Determining the Quality of cDNA*

cDNA is gathered from all samples and PCR reaction was conducted for determining the quality of cDNA for GAPDH (as an internal control and reference gene). PCR reaction indicates the appropriate synthesis

of cDNA. Figure 2 shows electrophoresis of 5 PCR reactions related to GAPDH gene on 2% agarose gel along with DNA ladder.

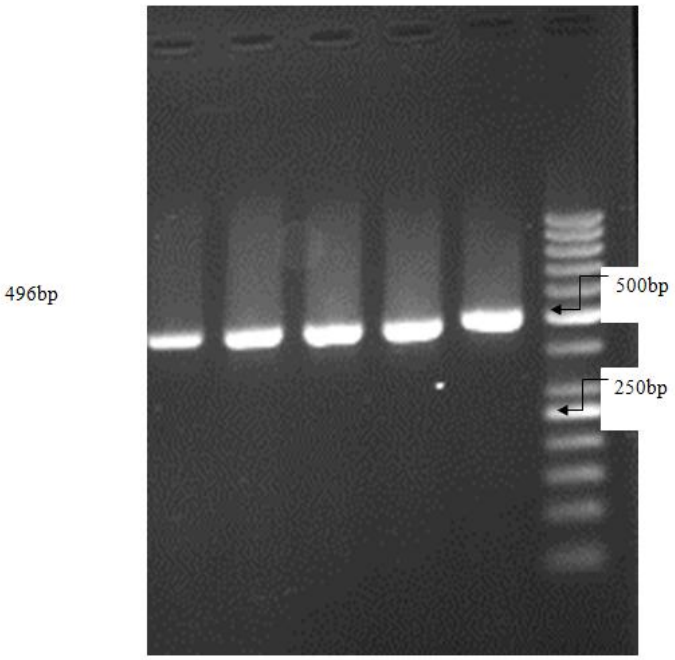



**Figure 2: Electrophoresis of PCR Products Related to GAPDH Gene on 2% Agarose Gel of PCR Product is 496bp which is Shown by Arrows and they are Electrophoresis alongside the 50bp Size Marker**

*Validation of Real-Time PCR Tests*
*Melt Curve Analysis*
The reaction was conducted on all samples. Melt curve results of this reaction shows that the whole PCR product was related to the specific amplification of the area and there was no non-specific product. Results are shown in figure 3.

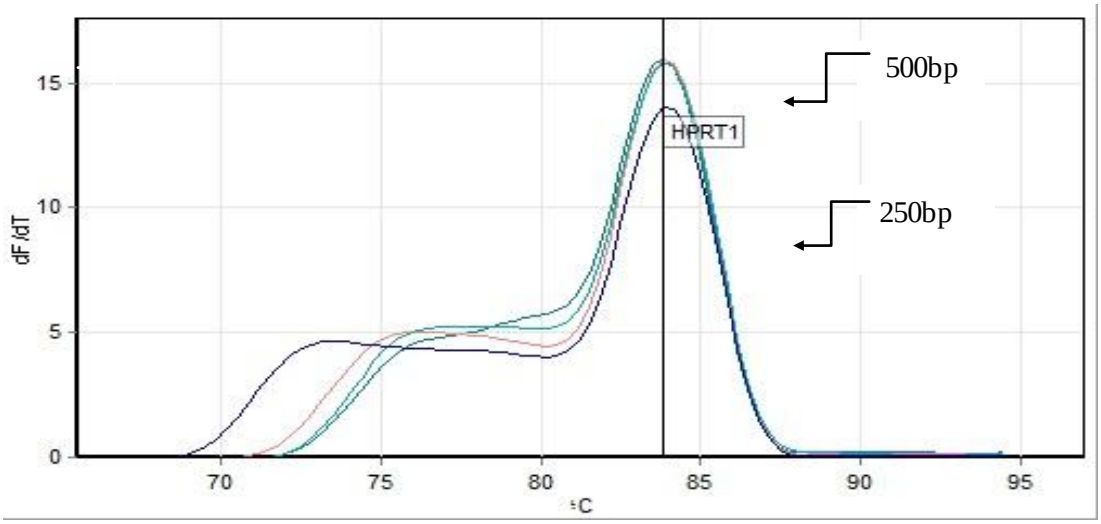



**Figure 3: Melt Curve of PCR Product of HPRT Gene: Melting Point of PCR Product Resulted from Amplification of the Area is around 84°C**

The reaction was conducted on all samples. The melt curve results of this reaction show that the whole PCR product was related to specific amplification of the area and there was no non-specific product. Results are shown in figure 4.

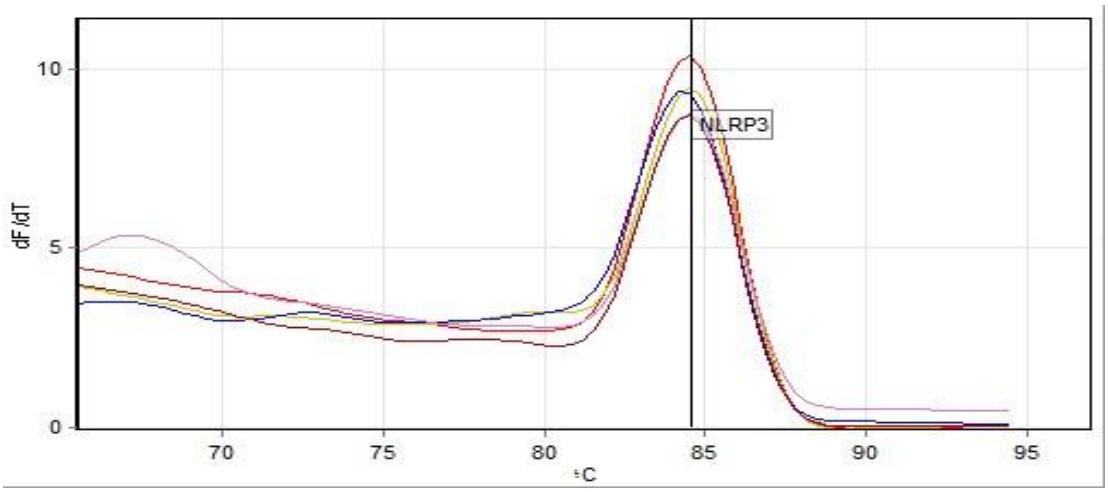
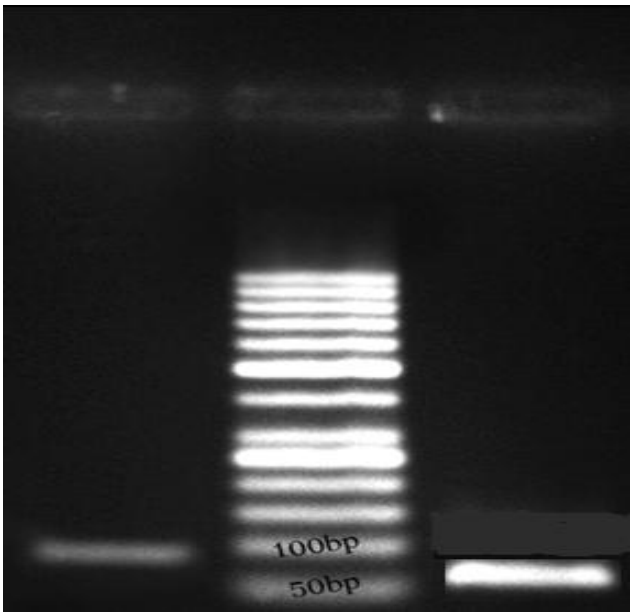



**Figure 4: Melt Curve of PCR Product of NLRP3 Gene, Melting Point of the PCR Product Resulted from Amplification of the Area is around 84.5°C**

*Determining the Specificity of Real Time PCR Reaction Products*

Real time RT-PCR reaction products were electrophoresis on 2% agarose gel in order to confirm this reaction and the specificity of the product.




Ladder

(50-1000bp)

**Figure 5: Electrophoresis of Real Time PCR Reaction Products on 2% Agarose Gel for HPRT1 and NLRP3 Genes, Products alongside 50bp Size Marker; Name of each Band is Mentioned with its Size in the Following Figure**

*Calculating Efficiency of Real Time PCR Reactions for HPRT1 and NLRP3 Genes*

In order to calculate the efficiency, different dilutions of cDNA were prepared and then the reaction of real time PCR was conducted on them so that their efficiency would be achieved.

The achieved efficiency for the HPRT1 was 1.19 and R and $R^2$ amounts were close to 1 indicating the linear reaction.

The achieved efficiency for NLRP3 gene was 1.09 and R and $R^2$ amounts were close to 1 indicating the linear reaction.

*Comparing the Aggressive Periodontitis Patients Group and Healthy Periodontal Group*

In this part the data of aggressive periodontitis and healthy group was compared. Results are shown in table 1 and diagram 1.

**Table 1: Results Achieved from Comparing the Aggressive Periodontitis Group and Healthy Group**

| Gene | Type | Reaction Efficiency | Expression | Std. Error | 95% C.I. | P(H1) | Result |
|---|---|---|---|---|---|---|---|
| HPRT | REF | 0.7731 | 1.000 | | | | |
| NLRP3 | TRG | 0.7118 | 2.860 | 0.319 - 25.446 | 0.037 - 274.775 | 0.034 | UP |

As above table shows, in terms of expression of NLRP3 gene, compared to healthy cases, the aggressive periodontitis has a significant increase (P<0.05).

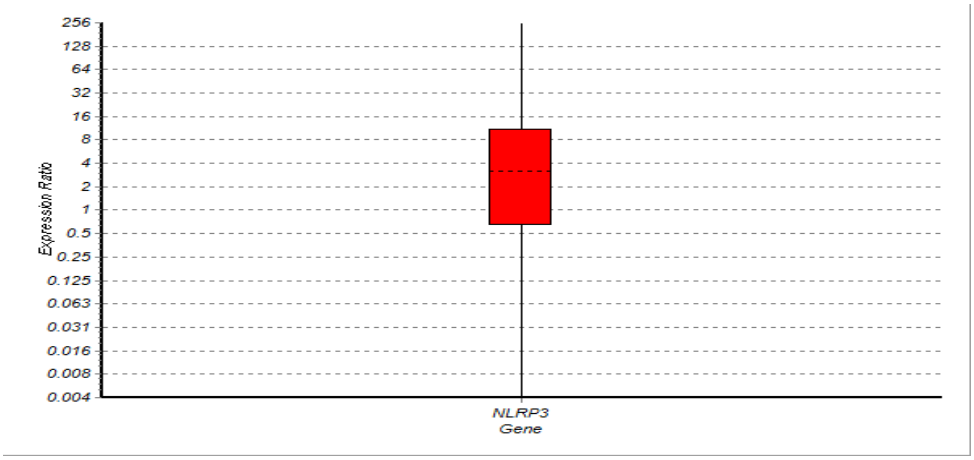



**Diagram 1: Results Achieved from Comparing the Expression of NLRP3 Gene between Aggressive Periodontitis Group and Healthy Group (it must be Noted that Healthy Group is Considered as the Base) As the Diagram Shows, Expression of NLRP3 in Aggressive Periodontitis Group is about Three Times the Healthy Group**

Comparing the aggressive periodontitis cases with CAL above or equal to 7 millimeters and cases with CAL below 7 millimeters:

In this part, aggressive periodontitis group data (CAL≥7mm) are compared with CAL<7mm cases. Results are shown in table 2 and diagram 2.

**Table 2: Results Achieved from Comparing Aggressive Periodontitis (CAL≥7mm) and CAL<7mm**

| Gene | Type | Reaction Efficiency | Expression | Std. Error | 95% C.I. | P(H1) | Result |
|---|---|---|---|---|---|---|---|
| HPRT | REF | 0.7416 | 1.000 | | | | |
| NLRP3 | TRG | 0.6984 | 0.833 | 0.039 - 7.547 | 0.006 - 52.829 | 0.830 | |

As table 2 shows, in terms of expression of NLRP3 gene, there is no difference observed between aggressive periodontitis with CAL≥7mm and CAL<7mm (P>0.05).

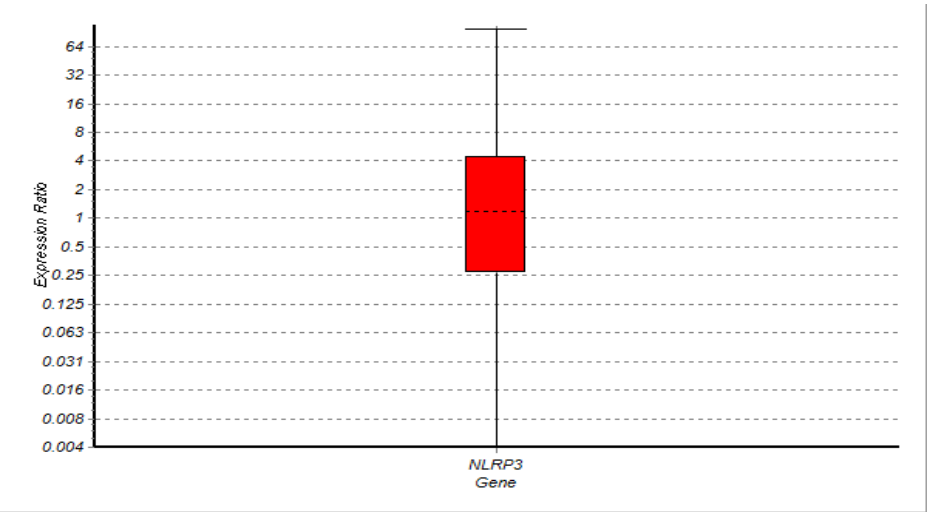



**Diagram 2: Results Achieved from Comparing the Expression of NLRP3 Gene between Aggressive Periodontitis Group (CAL≥7mm) and CAL<7mm (it must be Noted that Group having CAL<7mm is Considered as the Base)- as the above Mentioned Diagram Shows, Expression of NLRP3 in Cases with CAL Equal or above 7mm is Nearly One Time of the Cases with CAL below 7mm which is Insignificant**

*Comparing Cases of Aggressive Periodontitis with PD above or Equal to 7mm with Cases having PD below 7mm:*

In this part, data of aggressive periodontitis group with PD≥7mm and cases with PD<7mm are compared. Results are shown in table 3 and diagram 3.

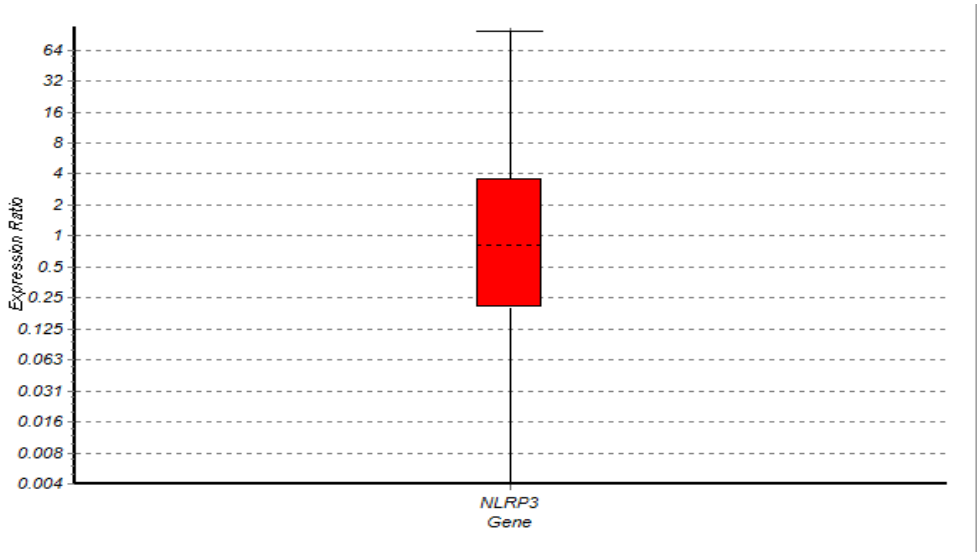



**Diagram 3: Results Achieved from Comparing the Expression of NLRP3 Gene between Aggressive Periodontitis Group with PD≥7mm and PD<7mm (it must be Noted that Group having PD<7mm is Considered as the Base); as the above Mentioned Diagram Shows, Expression of NLRP3 in Cases with PD Equal or above 7mm is Nearly 0.7 Times of Cases with PD below 7mm which is Insignificant**

**Table 3: Results Achieved from Comparing Aggressive Periodontitis with PD≥7mm and PD<7mm**

| Gene | Type | Reaction Efficiency | Expression | Std. Error | 95% C.I. | P(H1) | Result |
|---|---|---|---|---|---|---|---|
| HPRT | REF | 0.7416 | 1.000 | | | | |
| NLRP3 | TRG | 0.6984 | 0.706 | 0.065 - 6.703 | 0.006 - 50.231 | 0.652 | |

As table 3 shows, in terms of expression of NLRP3 gene, there is no difference shown between aggressive periodontitis with PD≥7mm and PD<7mm (P>0.05).
It must be mentioned that in cases with aggressive periodontitis, CAL≤5mm and PD≤5mm were not available; thus, the expression of genes was studied in cases of CAL>7mm and PD>7mm in patient group.
It must be noted that in tables 4 and 5 the descriptive statistical indicators of research main variables in two healthy and aggressive periodontitis group are respectively brought.

**Table 4: Statistical Descriptive Indicators of the Healthy Group**

| Statistical Indicator / Variables | Mean | SD | Minimum | Maximum | No |
|---|---|---|---|---|---|
| Age (year) | 24.5 | 2.65 | 18 | 54 | 20 |
| CAL (millimeter) | 2.89 | 0.93 | 1.2 | 2.8 | 20 |
| PD (millimeter) | 2.57 | 0.67 | 1.2 | 2.75 | 20 |

**Table 5: Statistical Descriptive Indicators if Aggressive Periodontitis Group**

| Statistical Indicator / Variables | Mean | SD | Minimum | Maximum | No |
|---|---|---|---|---|---|
| Age (year) | 24.5 | 2.41 | 12 | 56 | 25 |
| CAL (millimeter) | 7.07 | 1.23 | 4.4 | 9.4 | 25 |
| PD (millimeter) | 6.54 | 0.29 | 3.5 | 9.3 | 25 |
| Expression of STAT1 | 2.860 | 0.319 | 0.899 | 3.683 | 25 |

## RESULTS AND DISCUSSION

Generally, conducting the current research it was determined that expression of NLRP3 gene which is the main molecule in formation of inflammasome, in aggressive periodontitis cases is significantly higher than the healthy cases; but regarding its correlation with high tissue loss (CAL and or PD equal or above 7mm, no statistically significant correlation was observed).
Xue *et al.*, (2015) pointed out that expression of NLRP3 in chronic periodontitis cases and aggressive periodontitis cases is higher than the healthy gingival tissue.
As it is observable, there is a tremendous similarity between the above mentioned research result and our research findings; and we also encountered the significant expression of NLRP3 (2.86 times increase) in aggressive periodontitis samples compared to the healthy samples. Huang *et al.*, (2015) reported that in patients having chronic periodontitis and Diabetes mellitus type 2, hyperglycemic could result in intensifying the gingivitis through activating the NLRP3 path which results in more tissue loss.
Despite the difference in study design, it could be claimed that there is a kind of similarity between the results, because we also find out that in inflammatory conditions of periodontal tissues (aggressive periodontitis), in higher expression of NLRP3 it is observed that aggressive periodontitis has higher speed and intensity in destroying tissue and maybe a part of this could be related to the activation of NLRP3;

which by formation of inflammasome provides the base for production of inflammatory cytokines of IL-1 family that have an important role in destroying soft and hard tissues; and release of IL-1 family cytokines (such as IL-1 and IL-18) results in increased expression of NLRP3. Thus, tissue inflammation is a self-amplification effect on expression or activation of NLRP3 (Bauernfeind *et al.*, 2009).

Zhao *et al.*, (2014) claimed that Aggregatibacter actinomycetemcomitans result in stimulating apoptosis of human osteoblasts and at least a part of this effect happens through NLRP3.

Park *et al.*, (2013) concluded that *Porphyromonas gingivalis activates Caspae1 and results in secretion of IL-1β* and ultimately results in cell death and occurrence of cell death and secretion of IL-1β depends on activation of NLRP3 path.

Huang *et al.*, (2009) reported that cell death induced by Porphyromonas gingivalis in THP1 cells is depended on NLRP3.

Although, the research design in above mentioned studies is different from our research, but, since *Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans* are considered as the most important periodontopathic bacteria for the aggressive periodontitis, it could be claimed that there is a kind of similarity between the achieved results; because for infiltration of inflammatory cells into the inflamed gingival tissue, there is a need for spacing; a part of which happens through programmed cell death (apoptosis as the most important type) so that the adequate space is provided for establishment of inflammatory cells in inflamed position. Thus, along with intensifying the inflammation, NLRP3 may induce apoptosis of the basic tissue cells for establishment of inflammatory cells in periodontal tissues and this may result in more tissue loss. Also, following the destruction of tissue cells, the power of tissue restoration decreases as well.

***Conclusion***

In general, from the findings achieved from this research it is concluded that in generalized aggressive periodontitis, an increased expression and production of NLRP3 is observed which induces the release of inflammatory cytokines and results in intensifying the inflammation in periodontal tissues. On the other hand, NLRP3 could induce apoptosis of periodontal tissue cells and provide the base for more tissue loss.

**REFERENCES**

**Bauernfeind FG, Horvath G, Stutz A, Alnemri ES, MacDonald K, Speert D, Fernandes-Alnemri T, Wu J, Monks BG, Fitzgerald KA *et al.*, (2009).** Cutting edge: NF-κB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. *The Journal of Immunology* **183** 787–791.

**Belibasakis GN, Guggenheim B and Bostanci N (2013).** Down-regulation of NLRP3 inflammasome in gingival fibroblasts by subgingival biofilms: involvement of Porphyromonas gingivalis. *Innate Immunity* **19**(1) 3-9.

**Huang MT, Taxman DJ, Holley-Guthrie EA, Moore CB, Willingham SB, Madden V, Parsons RK, Featherstone GL, Arnold RR, O'Connor BP and Ting JP (2009).** Critical role of apoptotic speck protein containing a caspase recruitment domain (ASC) and NLRP3 in causing necrosis and ASC speck formation induced by Porphyromonas gingivalis in human cells. *The Journal of Immunology* **182**(4) 2395-404.

**Huang X, Yang X, Ni J, Xie B, Liu Y, Xuan D and Zhang J (2015).** Hyperglucose contributes to periodontitis: involvement of the NLRP3 pathway by engaging the innate immunity of oral gingival epithelium. *Journal of Periodontology* **86**(2) 327-35.

**Jin MS and Lee JO (2008).** Structures of the toll-like receptor family and its ligand complexes. *Immunity* **29**(2) 182-91.

**Park E, Na HS, Song YR, Shin SY, Kim YM and Chung J (2013).** Activation of NLRP3 and AIM2 inflammasomes by Porphyromonas gingivalis infection. *Infection and Immunity* **82**(1) 112-23.

**Tohme ZN, Amar S and Van Dyke TE (1999).** Moesin functions as a lipopolysaccharide receptor on human monocytes. *Infection and Immunity* **67**(7) 3215-20.

**Wang PL and Ohura K (2002).** Porphyromonas gingivalis lipopolysaccharide signaling in gingival fibroblasts-CD14 and Toll-like receptors. *Critical Reviews in Oral Biology and Medicine* **13**(2) 132-42.
**Xue F, Shu R and Xie Y (2015).** The expression of NLRP3, NLRP1 and AIM2 in the gingival tissue of periodontitis patients: RT-PCR study and immunohistochemistry. *Archives of Oral Biology* **60**(6) 948-58.
**Zhao P, Liu J, Pan C and Pan Y (2014).** NLRP3 inflammasome is required for apoptosis of Aggregatibacter actinomycetemcomitans-infected human osteoblastic MG63 cells. *Acta Histochemica* **116**(7) 1119-24.