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Case Report

Achondroplasia and Periodontal Disease - Molecular Testing Methods Case Report

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2. Key words

Achondroplasia; Periodontal disease; Immunophenotyping; LAMP

1. Abstract

Achondroplasia is an autosomal dominant genetic disease with its molecular mutation in gene encoding in type 3 of the receptor for fibroblast growth factor 3 (FGFR3). Although available literature describes limited correlation between achondroplasia and periodontal disease, a detailed immunphenotyping and molecular testing (LAMP, QPCR, PCR) has been performed at our facility showing a constant presence of Aggregatibacter actinomycetemcomitans, a culprit responsible for the advancement of periodontal disease. Although immunophenotyping, did not exhibit any major abnormalities in the number or differentiation of B and T lymphocytes, the percentage values of terminally differentiated and effector memory cells were decreased along with a reduction of neutrophil killing capacity. It is therefore recommended to monitor the progression of the periodontal disease at strict time intervals through an advanced molecular testing assays such as LAMP, in order to determine whether the implemented treatment regimen is correctly designed and progression of the periodontal disease is properly controlled.

3. Introduction

Achondroplasia is an autosomal dominant genetic disease characterized by a fully penetrant form of dwarfism linked to the endochondral bone formation disturbance. The molecular base of this abnormality is a mutation in the gene encoding of type 3 of the receptor for fibroblast growth factor 3 (FGFR3) [1, 2].

The mutation of the FGR3 gene may also lead to an ineffective final protein product or an excessive amount of this protein, negatively affecting the production of periodontal fibroblasts, as well as its quality, leading to an accelerated tooth loss [3]. According to Su et al., 2010, the up-regulation of FGFR3 plays an instrumental role in bone homeostasis resulting in reduced bone mass. They provided evidence, activation of Erk1/2 by FGFR3 caused diminished bone matrix mineralization of bone marrow stromal cells (BMSCs) from wild-type mice as compared to Fgfr3G369C/+ mutants. Additionally, an increased activity of p38 protein yielded a decreased replication and increased osteogenic differentiation of Fgfr3G369C/+ BMSCs. This data points to the plausible cause of periodontal attachment loss in individuals with ACH [4, 6].

Another tenable cause of periodontal destruction could be due to a misbalance of an immune system regulation, leading to an osteoclastic alveolar bone loss, through an up-regulation of pro-inflammatory cytokines such as IL-1β, IL-17E (IL-25) and IL-17 [5].

The production of nuclear factor κ B -Ligand (RANKL) by the T and B cells and activation of B cells in the peripheral lymphatic system can give rise of auto-antibodies to components of the periodontal apparatus such as collagen, fibronectin and laminin causing irreversible damage of the periodontal support [4, 6].

A limited number of associations between achondroplasia and periodontal apparatus destruction has been reported. Periodontitis is an inflammatory state causing tooth-supporting apparatus breakdown leading to partial or total tooth loss [7, 8].

Although there are known, systemic conditions (diabetes, acquired immunodeficiency syndrome, stress, obesity), smoking, medications (immunosuppressants, anticoagulants), malnutrition, occlusal trauma, local factors and genetic factors responsible for an advancement of periodontal disease, dysbiotic microbiota, are thought to be the main reason for the initiation and progression of periodontitis. The subgroups of bacteria described by Socransky exhibit different levels of pathogenicity and cause rapid colonization in the sub-gingival environment, radically influencing the balance of the healthy periodontal status [9]. We focused our attention on Porphyromonas gingivalis, Tannerella forsythia, Treponema denticola

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and Aggregatibacter actinomycetemcomitans due to their abilities to cause the progression of periodontal disease and their incidence throughout the course of the disease. The key to success for these species is their dexterity to form close relationships within the community. The interaction of different species and their maturation within the biofilm leads to an increase in the number of Gram-negative and anaerobic bacteria, resulting in the progression of inflammation of the periodontal apparatus [10].

Although the diagnosis of periodontal disease is highly dependent on radiographic analysis and clinical parameters, such as pocket probing depth, clinical attachment loss, bleeding on probing, plaque index, mobility, furcation involvement, they fail to provide a vast amount of information. These methods do not portray the current state of the disease nor provide information about the potential risk for disease advancement [11, 12].

Even though technological advancement has allowed for the development of a variety of techniques for microbe detection, including microbiological assays, enzyme detection, immunoassays as well as polymerase chain reaction (PCR), none of them can precisely quantify the amount dysbiotic pathogenic DNA. The process of sub-gingival periodontopathic bacteria detection can be significantly shortened by the qualitative PCR and Loop-Mediated amplification (LAMP) technique. Moreover, these methods allow for an extremely sensitive and simple way of detection [13, 14].

Our case report presents an achondroplasia patient who is exhibiting a pattern typical of periodontitis in generalized, unstable stage II, grade C. The sub-gingival microbiological status of an individual has been determined by three molecular diagnostic methods. For this purpose, we used PCR, qPCR as well as LAMP, an isothermal technique utilized for the detection of microbial DNA. Additionally, a detailed analysis of the patient was carried out to define and identify whether there are discrepancies within the immunological system [15].

4. Case Report

A 25-year-old female patient diagnosed with a form of achondroplasia had reported to the Department of Restorative Dentistry and Endodontics at Poznan Medical Sciences. Her primary complaint was a progressively increasing mobility of an upper and lower dentition within a seven-year time span. She had reported no pain, although an increasing amount of bleeding on brushing has been noted. General anamnesis revealed no familial history of achondroplasia nor periodontitis. The patient had a history of vitamin D deficiency rickets when she was one year old. Previous dental history revealed a normal rate of deciduous teeth eruption and shedding. No history of advanced periodontal surgery other than a closed flap debridement was executed.

4.1. General Examination

The patient exhibited a short stature (135cm). Large, broadened forehead dimensions, short, mid-facial features, bow legs, short upper and lower extremities.

4.2. Intraoral Examination

An intra-oral examination revealed a pronounced protrusion of an upper anterior segment. Periodontal charting was carried out at initial and recall visit revealing a generalized aggressive periodontal state. (Figure 2, 3, 4) Upper and lower dentition was previously splinted and exhibited marked mobility.

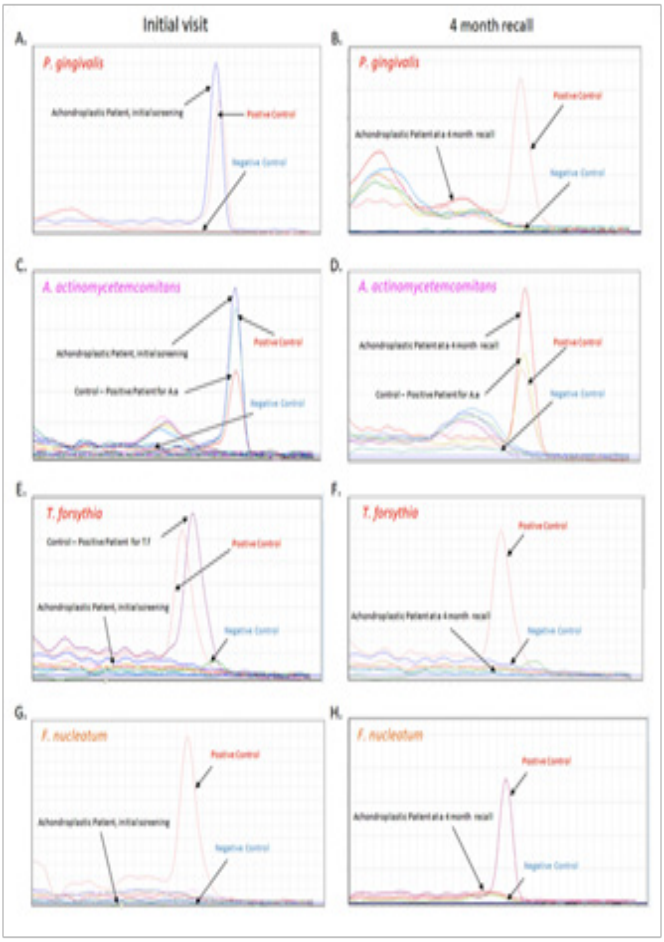


Figure 1: Melting Curve analysis of bacteria utilizing LAMP. (A) Testing for presence of P.gingivalis at the initial visit - a positive result. Melting curve analysis has been obtained and fluorescence value of 2.2 has been detected. (B) Testing for presence of P.gingivalis at a 4 month recall - a negative result. Melting curve analysis has been obtained and fluorescence value of 0.2 has been detected. A positive and negative controls were also provided. (C) Melting Curve analysis of A.a. utilizing LAMP, initial screening of an achondroplastic patient. Testing for presence of A.a at the initial visit, utilizing LAMP, revealed a positive result. Melting curve analysis has been obtained and fluorescence value of 1.1 has been detected. A positive control expressed a value of 0.9 , whereas a negative control was below a detectable limit . (D) Melting Curve analysis of A.a. utilizing LAMP, a 4-month recall, of an achondroplastic patient. A significant increase of fluorescence has been noticed, comparing to the initial screening. (E) Melting Curve analysis of T. forsythia utilizing LAMP, initial screening Testing for presence of T. forsythia at the initial visit, utilizing LAMP, revealed a negative result. Melting curve analysis has been obtained and fluorescence value of 0.1 has been detected. A positive and negative controls were also provided, along with another patient, tested positive for T.forsythia (F) Melting Curve analysis of T forsythia utilizing LAMP, a 4-month recall of an achondroplastic patient. Sample analysis revealed did not detect presence of T.forsythia. Fluorescent peak of positive control expressed a value of 0.64. (G) Melting Curve analysis of F. nucleatum, utilizing LAMP, initial screening Testing for presence of F.nucleatum at the initial visit, utilizing LAMP, revealed a negative result. Melting curve analysis has been obtained and fluorescence value of 0.03 has been detected. A positive and negative controls were also provided. (H) Melting Curve analysis of F.nucleatum utilizing LAMP, a 4-month recall of an achondroplastic patient. Sample analysis revealed did not detect presence of F.nucleatum. Fluorescent peak of positive control expressed a value of 1.8.

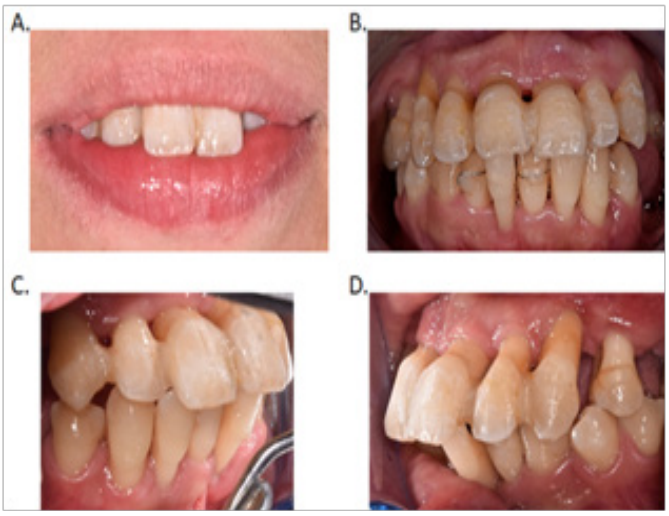
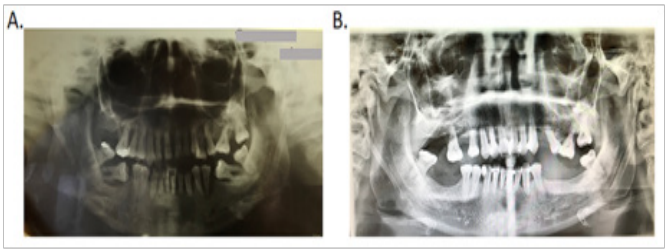
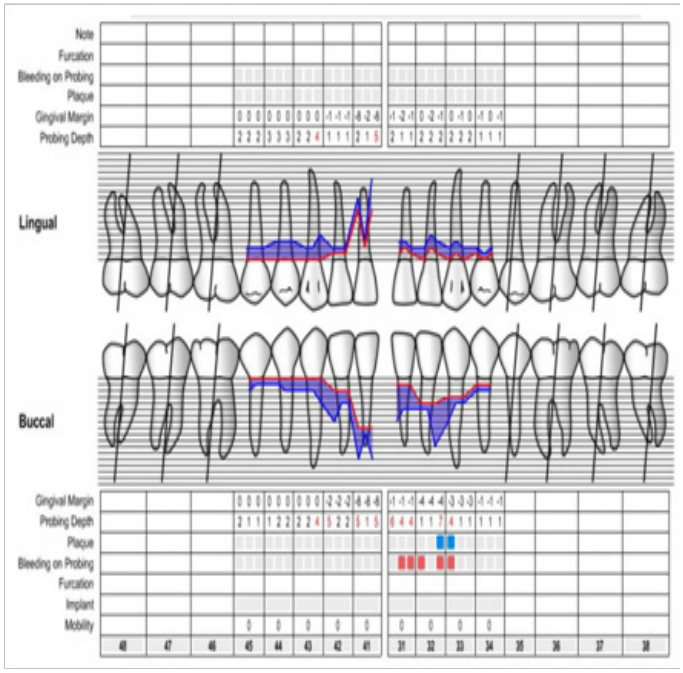


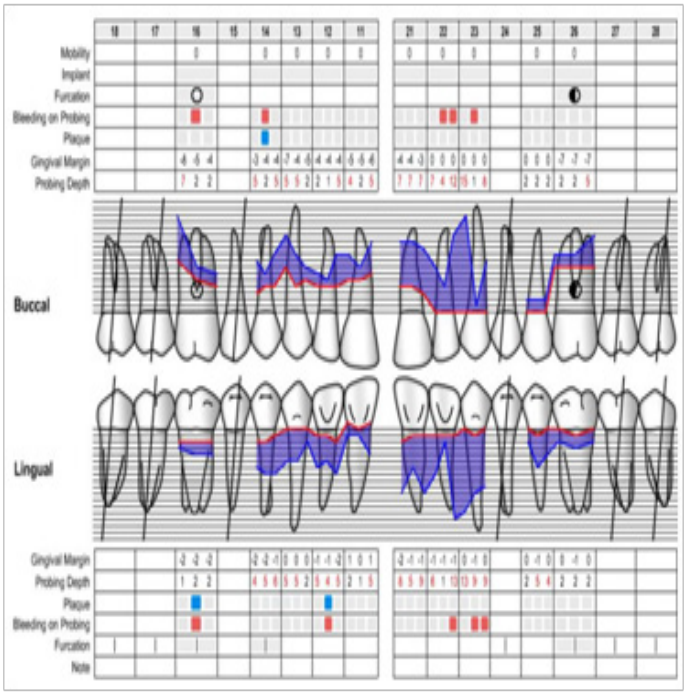
Figure 2: (A) Frontal view, showing central and lateral incisors at rest position (B) frontal view. showing the extent of clinical attachment loss along with multiple recessions (C) Rirgt lateral view. This figure shows the extent of labial central incisor flaring (D Left lateral view. showing the extent of clinical attachment loss along with multiple recessions along with severe malocclusion.



Periodontal chart, initial visit

Figure 3: Patients OPG (A) at the very first visit at the Department of Restorative Dentistry and En-dodontics at Poznan Medical Sciences ; (B) A recall visit, at the Department of Restorative Dentis-try and Endodontics at Poznan Medical Sciences showing partial loss of of the dentition, although, a more stable clinical scenario has been identified clinically with an decreased bleeding on probing (BOP) score and attachment loss reduction as well as an improved hygiene shown by the a reduction of the plaque score.





Periodontal chart, reevaluation

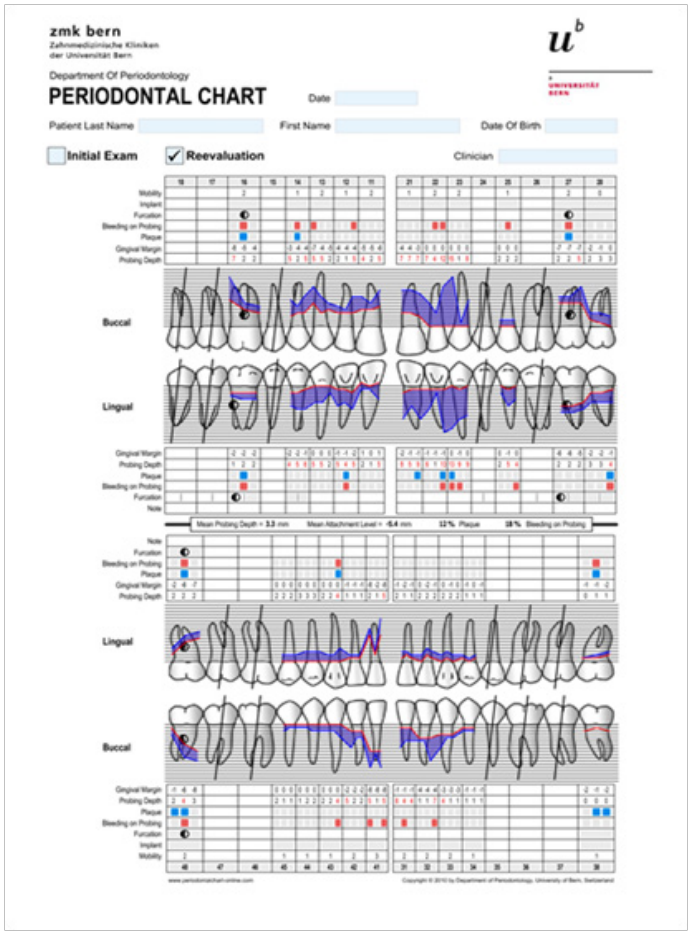


Figure 4: (A) An initial examination, revealed a Mean probing of 3.5mm, a clinical at-tachment loss of 5.8mm . A plaque score of 23% and Bleeding on probing was at 31%; (B) A revaluation visit, showed a mean probing of 3.3mm, a clinical attachment loss of 5.4 mm. A plaque score was at 12% and bleeding on probing (BOP) at 18%

4.3. Morphology with the Smear

Morphological and smear values of the peripheral blood white blood cells (WBC), red blood cells (RBC) and platelets (PLT) showed no abnormalities. Both percentage and the absolute value of lymphocytes, monocytes, neutrophils, basophils and eosinophils were within the norm.

4.4. Immunophenotype Investigations

Data received from the immunophenotypic assessment did not show any symptoms of pathological deficiency in the number of lymphocytes or differentiation of B and T lymphocytes, which could be related to immunodeficiency. However, some B and T cell subpopulations were located out from normal ranges. In the patient's peripheral blood lymphocytes, the percentage and absolute values of basic B, T CD4+, T CD8+ and NK cell subpopulations were located within the age norm. The CD4:CD8 ratio was correct. However, the percentage value of activated T-cells CD3+/HLA-DR+ was above the norm. In the distribution of B lymphocyte subpopulations, the absolute value of switched-memory cells was at the lower end of the age norm, but their percentage value was located within the age norm. In the norm also was the value of non-switched memory cells. An increased percentage of transitional cells and the values of immature-activated cells was observed. Also, the absolute value of transitional cells was at the upper end of the age norm. Within the T-helper cells subpopulations, the values of naive cells, terminally differentiated cells, central memory and effector memory cells were located in the age norm. In the distribution of cytotoxic T lymphocyte subpopulations, the percentage values of terminally differentiated cells and effector memory cells were decreased, and their absolute values were located at the lower end of the age norm.

4.5. Immune cells' functional analysis

To assess the functional activity of phagocytic cells, monocytes and neutrophils, the Phagotest™ and Phagoburst™ (BD Biosciences) were performed (Table 2). The Phagotest™ allows to investigate the engagement of monocytes and neutrophils in phagocytic mechanism and estimate the efficacy of this activity both in a qualitative and quantitative manner. Otherwise, the Phagoburst™ serve for the indication of the ability of monocytes and neutrophils for intracellular killing of phagocytosed microbes. This feature is measured based on the indication of the ability to produce reactive oxygen species (ROS) by phagocytes and the number of released substances [16].

Tests were performed with use the flow cytometry according to the manufacturer's instructions. It was found that the phagocytic activity of studied cells was normal compared to the controls and was in norms to the reference values. However, the production of oxygen radicals by neutrophils under the influence of the mitogen phorbol myristate acetate (PMA) was impaired. Although ROS secretion under the influence of Escherichia coli was within normal limits, weakened functional reserve of the examined cells was observed (Table 1, 2).

Table 1: Immunophenotypic analysis

B cells					**T cells**				
subpopulations	%	ramng %	absolute values [cells/ mm^3]	range[cells/ mm3]	subpopulations	%	ramng %	absolute values [cells/ mm^3]	range[cells/ mm3]
Naïve	79.8	48.4-79.7	173	60-470	Naïve T helper	47.9	16-100	350	100-2300
Memory	20.2	17.5-46.5	44	30-170	Terminally differentiated T helper	1.9	0-7	14	0-100
Non-memory	79.8	53.5-86.7	173	70-480	Effector memory T helper	9.6	23-Jan	70	10-200
Switched memory	9.6	8.3-27.8	21	20-90	Central memory T helper	40.6	18-95	297	180-1100
Non-switched memory	10.7	7.0-23.8	23	Oct-80	Naïve T cytotoxic	36.1	6-100	185	20-1000
Double negative	– 0.1	2.2-10.0	– 0	20-Oct	Terminally differentiated T cytotoxic	– 5	Jul-53	26	30-300
Transitional	8.3	0.9-5.7	18	0-30	Effector memory T cytotoxic	– 9.8	14-96	50	40-600
Immature	12.2	3.2-19.6	27	Oct-40	Central memory T cytotoxic	4.7	20-Jan	24	0-100
Activated	10.3	1.6-10.0	22	20-Oct	Recent thymic emigrants	46.6	7-100	341	50-2400
Plasmablasts	– 0.1	0.4-2.4	0	0-10	T regulatory cells	4.2	10-Feb	31	30-200

Table 2: Results of functional tests

Phagotest™					**Phagoburst™**				
substrate	%	range%	mean fluorescent intensity [MFI]	range[MFI]	substrate	%	range %	mean fluorescent intensity [MFI]	range [MFI]
E. coli	98.9	91.4-98.7	15441	14294-23952	spontaneous	0.6	0.0-1.1	1903	1484-2304
					E. coli	94.9	89.2-97.1	8003	5095-15094
					fMLP	8.0	1.2-3.4	– 1665	1845-3098
					PMA	– 97.3	98.8-100	– 20119	24966-57039

4.6. Measurement of Immunoglobulin Concentration

The effectiveness of the acquired immune response was assessed by measuring the concentration of all immunoglobulin classes: IgM, IgA and IgG. The concentration of four IgG immunoglobulin subclasses was also measured: IgG1, IgG2, IgG3 and IgG4. Antibody concentration was assessed using the Minineph™ test (Binding Site). The measurement was made by the nephelometric method. In assessing the concentration of IgM, IgA and IgG, there were no deviations from the age norm. However, when testing the concentration of IgG subclasses, an increase in IgG2 concentration was found, while the concentration of remaining subclasses remained in the normal range.

4.7. Cause-related Therapy

The patient has received multiple hygiene instructions along with a cause-related therapy in order to eliminate supra-gingival deposits. The ultrasonic scaling has been performed. Next, the collection of sub-gingival pathogens was carried out with sterile endodontic paper points (ISO 35) which were placed within pockets greater than 4 mm for 10 seconds. Collected specimens were subjected for the

detection of periodontal pathogens using LAMP, qPCR and PCR techniques. The patient was also informed about the necessity of a coronally directed roll tooth brushing technique to increase the efficacy of cleaning and reduce soft tissue trauma. Root planing was conducted to reduce pockets greater than 4 mm in depth.

5. Results and Discussion

There are various causes of periodontal disease. Whether it is genetically based or caused by periodontal pathogens, the final outcome leads to an accelerated, premature tooth loss. A misbalance of an immune system regulation may lead to the disturbance of periodontal homeostasis due to lack of regulatory T cells (Tregs). Their suppressive nature may in turn inhibit the production of IL-10, and TGF-β and significantly contribute to the development of periodontitis. Our data shows values of naive and central memory cells, recent thymic emigrants (RTE cells) and regulatory T cells (Tregs) were in the normal range. The expression of CD11a, CD11b, CD11c and CD18 adhesive molecules on the lymphocytes and neutrophils was correct.

Moreover, an inflammatory state leading to periodontal apparatus deterioration can also be caused by a prolonged accumulation of sub-gingival plaque. According to our data, although the mean periodontal probing value was 3.5 mm, there were multiple sites with a probing depth ≥ 5 mm. Similarly, the results obtained by Ebersole et al.; Gunsolley et al showed an association of an increasing depth of periodontal pockets with the rising titers of antibodies, both local or systemic, making them strong indicators of periodontal disease advancement and its recurrence [17, 18].

Innate immune mechanisms, covering also polymorphonuclear neutrophils (PMN) play an instrumental role in the maintenance of periodontal balance. A disruption in quality and quantity of PMNs in periodontal tissue and crevicular gingival fluid may provoke frank dysbiosis, consequently leading to a permanent clinical attachment level loss of dentition [19, 20]. The functional evaluation of phagocytic cells performed in the described case has shown a reduced ability of PMN to the mitogen-activated secretion of reactive oxygen species, an increased FMLP value (8.0 %)(1.2-3.4) and a decreased PMA value of 97.3 (98.8-100) (Table 1). This data coincides with the results obtained by Nibali et al.,.Their data analysis of subjects revealed that patients with AgP and genetic factors exhibited a higher oxidative burst in response to E. coli ($P = 0.002$) [21]. Since neutrophils are in the first line of the non-specific defense against microorganisms, their deficiency or their inability to release oxygen free radicals of a given cell population may be may be the cause of recurrent and difficult-to-treat uncontrollable growth of periodontopathogens.

According to data obtained by Žilinskas and associates ,the stimulated along with non-stimulated PMNs in patients exhibiting periodontal disease showed significantly more superoxide anions ($p<0.01$), than those of healthy individuals [22]. This coincides with data acquired by Matthews et al., 2007 [23, 24].

Our data however, has shown an impaired production of oxygen radicals by neutrophils under the influence of the mitogen phorbol myristate acetate (PMA). This data is comparable to the data of other authors who studied the oxidative function of the venous blood activated PMN in diabetes mellitus type 1, via luminol-dependent CL.Their results also show significantly decreased ($p<0.01$) levels of superoxide generation [25].

The observed immunodeficiency within the peripheral blood neutrophil population may be particularly dangerous for neutrophils present in gingival pockets [26].

Antibodies play a key role in a well-functioning immune system. They serve as opsonins, mediate antibody-dependent cytotoxicity (ADCC), and also limit the infectivity of microorganisms. IgG1 and IgG2 antibody subclasses are attributed to a strong anti-inflammatory effect. The reduced IgG1 concentration is most often the result of reduced immune activity. Quantitative IgG2 disorders are associated with susceptibility to bacterial and viral disorders. Low levels of IgG2 and IgG3 are with susceptibility to upper respiratory tract infections. Simultaneous determination of IgG2 and IgG4 is performed during the diagnosis of immunological deficiencies [27]. In our study, we observed an elevated level of concentration of the IgG2 subclass. Similarly, results obtained by Ardila et al, indicate, periodontal patients had detectable titers both IgG1 and IgG2. High IgG1 and IgG2 antibody levels against A. actinomycetemcomitans occurred in the majority of tested individuals.

Such a scenario is most likely associated with an increased interaction of periopathogenic bacteria. In this case, the reduced non-specific response can be compensated by increasing the number of specific response exponents. However, it is uncertain whether this model is functional enough.

There is a clear interrelationship between three factors, when combined, can lead to destructive phenomena. These are respectively: genetic defect of the FGFR3 gene loci, presence of a red complex pathogens along with A. actinomycetemcomitans, and restricted immunological activity of the host. The genetic disorder causes ineffective final protein product or an excessive amount of this protein negatively influencing periodontal fibroblast production or its quality. This, in turn, could promote persistent inflammatory reaction within a periodontal apparatus leading to unfavorable interactions of bacterial virulence factors with hosts fibroblasts in which pro-inflammatory cytokines can induce osteoclastic bone resorption and disturb the functionality of immune cells [2, 3, 20, 21, 26].

A disturbance within FGFR3 gene loci found in achondroplastic patients and their deleterious effect on periodontal support clearly stresses the need for a regular periodontal status examination. Although clinical parameters of periodontal disease are of enor-

mous value, they present several limitations, therefore, pathoger screening at strict times intervals through molecular diagnostic techniques such as LAMP could be of an enormous value. Figure 1 presents diagnostic data obtained for P.gingivalis, A. actinomy cetemcomitans, T.forsythia as well as F. nucleatum at initial and a 4 month interval. This isothermal method offers an expedient highly sensitive and cost-effective approach to microbiologica investigation. It is essential, such testing is carried out at strictly determined time intervals to monitor pathogen levels to guide pe riodontally affected individuals and reduce chances of disease ad vancement [28].

6. Conclusion

Although no clear association between periodontal disease and ac hondroplasia has been established, due to a limited sample size clinical and radiographic data clearly shows signs of progressing periodontal disease. These symptoms have been correlated with the results obtained from three diagnostic molecular assays. PCR qPCR, and LAMP results indicate a constant presence of Aggrega tibacter actinomycetemcomitans (Figure1) a culprit known for it role in the advancement of periodontal disease. (only LAMP re sults shown) The reduction of neutrophil killing capacity observed in our study may be an essential factor associated with immunode ficiency. Also, although immunophenotyping, has not detected ab normalities in the number or differentiation of B and T lymphocy tes, the percentage values of terminally differentiated and effecto memory cells were decreased. Their absolute values were located at the lower end of the age norm, indicating a possible erroneou immunological response from the host leading to the progressio of the periodontal disease

7. Conflict of interests

The authors declare no conflict of interests.

8. Acknowledgement

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