T.R.N.C

NEAR EAST UNIVERSITY

INSTITUTE OF HEALTH SCIENCES.

TONSIL ANTIMICROBIAL PEPTIDE EXPRESSION LEVELS IN PFAPA (PERIODIC FEVER , APHTHOUS STOMATITIS, PHARYNGITIS, AND CERVICAL ADENITIS) SYNDROME PATIENTS.

BY:-

MARTHA EMMANUEL AGADA

ID NO:- 20156703

A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES, NEAR EAST UNIVERSITY, LEFKOSA. NORTHERN CYPRUS, IN PARTIAL FULFILLMENT OF THE REQUIREMENT FOR THE AWARD OF MASTERS DEGREE IN MEDICAL AND CLINICAL MICROBIOLOGY, NEAR EAST UNIVERSITY, NICOSIA. NORTHERN CYPRUS.

NICOSIA

2018

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ADVISOR:-

ASSIST. PROF. DR. UMUT GAZI.

NICOSIA

2018.

DECLARATION

I hereby declare that the work in this thesis entitled "TONSIL ANTIMICROBIAL PEPTIDE EXPRESSION LEVELS IN PFAPA (PERIODIC FEVER WITH APHTHOUS STOMATITIS, PHARYNGITIS, AND CERVICAL ADENITIS) SYNDROME PATIENTS" is the product of my own research efforts undertaken under the supervision of ASSIST. PROF. DR. UMUT GAZI. This thesis has not in any way been presented previously for another degree or diploma in any University elsewhere. More so, all information provided in this document was obtained and presented in accordance with the academic ethical rules and conduct governing its approval. However, materials and results obtained from other sources have been acknowledged, cited and referenced appropriately.

Name, Surname:

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APPROVAL/CERTIFICATION PAGE

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This study has been accepted by the thesis committee in Medical Microbiology and Clinical Microbiology program as Master Thesis.

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According to the relevant article of the Near East University Postgraduate Study-Education and Examination Regulation, this thesis has been approved by the above mentioned members of the thesis committee and the decision of the board of Directors of the Institute.

> Prof. Dr. K. Husnu Can Baser. Director Of The Institute Of Health Sciences.

DEDICATION

THIS THESIS IS DEDICATED TO:-

GOD ALMIGHTY, MY DEAREST PARENT (MR AND LATE MRS JOSEPH KOGI), MY LOVING HUSBAND (MR EMMANUEL AGADA OTACHE) AND MY BELOVED DAUGHTER (MISS EMMANUELLA-ENE-NOMTEI-DIAMOND AGADA OTACHE).

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MI KPEIK NOM'A.

AN'YA OWOICHO.

NAGODE UBANGIJI.

ABSTRACT

Martha E. A. Tonsil AMP expression levels in PFAPA Patients. Pathology Laboratory of the Near East University Hospital. Graduate School of the Institute of Health Sciences, M.Sc. Thesis in Medical and Clinical Microbiology Department program. Nicosia, 2017.

BACKGROUND: The periodic fever, apthous somatitis, pharyngitis and cervical adenitis (PFAPA) is a disease which was named according to its clinical criteria that includes fever flares accompanied by pharyngitis, adenitis, and/or aphthous stomatitis, asymptomatic intervals between the flares, and onset before 5 years of age. Even though being probably the most common cause of recurrent fever in Western European children, its cause is not yet clear. However, it is thought to be an auto-inflamamtory disease (AID) as the fever attacks are responsive to cortiosteroid, and is not associated with any infectious or autoimmune cause.

AIM: Since another auto-inflammatory disorder called Crohn disease is associated with abnormal antimicrobial production, the same mechanism was hypothesized to be responsible for PFAPA syndrome. For this purpose, the aim of this study is to monitor the AMP levels expressed by PFAPA tonsils.

MATERIALS AND METHODS: Palatine tonsil tissue samples were isolated from seven PFAPA syndrome and six recurrent tonsillitis patients due to group A β -hemolytic streptococci infection (GA β HS). The expression levels of AMPs were compared by immunohistochemistry at the Near East University Hospital Pathology Laboratory. The AMPs focuses were Human β -defensin 1-2 (H β D1-2), LL-37, Rnase7, LEAP-1-2 which were previously shown to be expressed in human tonsils by immunohistochemistry.

RESULTS: The IHC result showed stains seen around and on the germinal center and lymphoid follicle of the tonsil tissue sections. A low expression level of H β D-1, and strong expression levels of H β D-2, LL-37, RNase7, and LEAP-1 were observed in both groups. The expression levels did not change between the two groups except for that for LEAP-1which was significantly higher in GA β HS group. No positive cell was stained with Rabbit IgG isotype control, and LEAP-2 expression was not detected in both group samples as well as in positive control used.

CONCLUSION: Our results suggest that the PFAPA patients did not differ in the expression of AMP in germinal centres and follicles, from GA β HS patients except from LEAP-1 expression. Since LEAP-1 was previously shown to have similar antimicrobial spectrum activity as H β D-1, future studies are recommended to compare the microbiota composition that may further enlighten the epidemiology of the syndrome.

ÖZET

GİRİŞ: PFAPA (periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis) hastalığı ateş, faranjit, adenit ve/veya herhangi bir enfeksiyon yokluğunda aftoz somatiteyi içeren diyagnostik kriterlerine göre isimlendirilmiş bir hastalıktır. Sendromun sebebi tam olarak hâlâ daha tanımlanamamış olması ile birlikte Batı Avrupa çocuk populasyonunda gözlemlenen en yaygın yüksek ateş sebebidir. Yüksek sıcaklığın tek doz kortikosteroide cevap vermesi ateşin enflamasyon kaynaklı olduğunu ve herhangi bir enfeksiyonun yokluğunda oluşması sendromun bir otoinflammatuvar hastalık olabileceğini ima eder.

AMAÇ: Crohn hastalığı olarak da bilinen başka bir oto-enflamatuar bozukluk anormal antimikrobiyal üretimle ilişkili olduğundan, aynı mekanizmanın PFAPA sendromundan sorumlu olduğu varsayılmıştır. Bu amaçla, bu çalışmanın amacı PFAPA bademcikleri tarafından ifade edilen AMP düzeylerini karşılaştırmaktır.

GEREÇ VE YÖNTEM: Palatin bademcik doku örnekleri yedi PFAPA sendromundan ve altı grup A β-hemolitik streptokok enfeksiyonu ile ilişkilendirilmiş tekrarlayan tonsillit hastasından (GAβHS) izole edildi. Yakın Doğu Üniversitesi Hastanesi Patoloji Laboratuvarı'nda AMP'lerin ekspresyon seviyeleri immünhistokimya ile karşılaştırıldı. Odaklanılan AMP'ler daha önce insan bademciklerinde immünohistokimyasal olarak eksprese edildiği gösterilen Human β-defensin 1-2 (HβD1-2), LL-37, Rnase7, LEAP-1-2 idi.

BULGULAR: IHC sonuçları, söz konusu AMP'lerin bademcik doku kesitlerinin germinal merkez çevresinde ve lenfoid dokularında ekpres edilebildiklerini gösterdi. Her iki grup numunesinde, düşük seviyede HβD-1 ve yüksek seviyede HβD-2, LL-37, RNaz7 ve LEAP-1'in ekspresyonu gözlemlendi. GAβHSG grubunda, PFAPA grubuna kıyasla, daha yüksek seviyede ekspres edilen LEAP-1'in dışında diğer AMP'lerin ekspresyon seviyeleri her iki grup arasında herhangi bir değişiklik göstermedi. Negatif kontrol olarak kullanılan tavşan IgG izotip kontrol negatif boyama sonucu vermiştir. Bunun yanında LEAP-2 ekspresyonu ne grup numunelerinde ne de pozitif kontrol olarak kullanılan dokuda saptanabilmiştir.

SONUÇ: Sonuçlarımız, PFAPA hastalarının, GAβHS'li hastalardan, LEAP-1 ekspresyonu haricinde tonsil germinal merkez ve follikül AMP ekspresyonu seviyelerinde herhangi bir

farklılık göstermediğini rapor etmiştir. LEAP-1'in HβD-1 ile benzer antimikrobiyal spektrum aktivitesine sahip olduğu daha önce gösterildiğinden, sendromun epidemiyolojisini daha da aydınlatılması amacı ile gelecek çalışmaların mikrobiyom yapısına odaklanması önerilmektedir.

TABLE OF CONTENT

1. TITLE PAGE	i
2. DECLARATION	ii
3. CERTIFICATION/APPROVAL SHEET	iii
4. DEDICATION	iv
5. ACKNOWLEDGEMENT	v
6. ABSTRACT	viii
7. OZET	х
8. TABLE OF CONTENT	xii
9. LIST OF TABLES	xiv
9. LIST OF FIGURES	XV
10. LIST OF ABBREVIATIONS AND SYMBOLS	xvi
CHAPTER 1.	
1.0 INTRODUCTION	1
1.1 AIMS AND OBJECTIVES OF THE RESEARCH/STUDY	3
CHAPTER 2.	
2.0. LITERATURE REVIEW	5
2.1. PFAPA SYNDROME, THE SIGNS AND SYMPTOMS	5
2.1.1. PFAPA SYNDROME	5
2.1.2. KEY PRINCIPLES OF AUTOINFLAMMATORY PATHOMECHANISMS	6
2.1.3.1. THE MAJOR SIGNS AND SYMPTOMS OF PFAPA	8

2.1.3.2. CLINICAL CHARACTERISTICS OF OF PFAPA SYNDROME PATIENTS	10
2.2. GENERAL DIAGNOSIS OF PFAPA SYNDROME	11
2.2.1. THOMAS ET AL. DIAGNOSTIC CRITERIA FOR PFAPA SYNDROME	12
2.2.1.1. CLASSIC THOMAS CRITERIA	13
2.2.1.2. INCOMPLETE THOMAS CRITERIA	13
2.3. TREATMENT OF PFAPA	13
2.3.1. CORTICOSTEROIDS	13
2.3.2.1. TONSILLECTOMY	14
2.3.2.2. EFFECTS OF TONSILLECTOMY	15
2.4. THE ROLE OF IMMUNE SYSTEM IN TONSILS AND THEIR EPITHELIUM	16
2.5. ANTIMICROBIAL PEPTIDES	20
2.5.1. DEFINATION OF ANTIMICROBIAL PEPTIDES (AMPs)	20
2.5.2. TYPES OF ANTIMICROBIAL PEPTIDES	20
2.5.3. CHARACTERISTICS OF AMPs	20
2.5.4. ANTIMICROBIAL PEPTIDES MECHANISM OF ACTION	27
2.5.5. EXPRESSION LEVELS OF THE AMPS IN TONSILS OF SOME DISE CONDITIONS	ASED 30
CHAPTER 3.	
3.0. MATERIALS AND METHODS	32
3.1.0. STUDY POPULATION	32
3.2. SAMPLE PREPARATION	32

3.3. IMMUNOHISTOCHEMISTRY	33			
3.4. IMMUNOHISTOCHEMISTRY SEMI-QUANTITATION METHOD FOR SCOR (POSITIVE CELL DISTRIBUTION) AND STAINING INTENSITY OF THE CELLS (%	ING			
	37			
3.5. STATISTICAL ANALYSIS	37			
CHAPTER 4.				
4.0. RESULTS	39			
4.1. CHARACTERISTICS OF STUDY GROUP	39			
4.2. AMPs EXPRESSION PATTERN LEVELS OF PFAPA GROUP AND GABHS GROUP				
IN TONSIL TISSUE SECTION SAMPLE	39			
CHAPTER 5.				
5.0. DISCUSSION	43			
CHAPTER 6.				
6.0. CONCLUSSION AND RECOMMENDATION	47			
6.1. CONCLUSSION	47			
6.2. RECOMMENDATION	47			
REFERENCES	49			
APPENDIX	60			
LIST OF TABLES.				

1. CLINICAL CHARACTERISTICS OF SOME AIDs.

2. THOMAS ET AL. DIAGNOSTIC CRITERIA FOR PFAPA SYNDROME.

3. PFAPA SYNDROME LITERATURE SUMMARY ON EFFECT OF TREATMENT FOR RECURRENT TONSILLITIS.

4. TONSILS AND THEIR EPITHELIUM'S DESCRIPTION.

5. ORAL AND AIRWAYS EPITHELIUM ANTIMICROBIAL GENES EXPRESSION SUMMARY.

LIST OF FIGURES.

1. A STRATIFIED DISPLAY OF PERIODIC FEVER FREQUENCY OF THE AUTOINFLAMMATORY DISEASE SYNDROMES.

2. THE KEY PRINCIPLE INVOLED IN INNATE IMMUNE SYSTEM RECOGNITION BY PATTERN RECOGNITION RECEPTOR (PRR).

3. SCHEMATIC PRESENTATION OF A TYPICAL PFAPA SYNDROME PATIENT'S CLINICAL FEATURES.

4. TONSILLECTOMY.

5. TONSILS AND THROAT'S ANATOMICAL POSITIONS IN THE BODY.

6. ANTIMICROBIAL PEPTIDE'S DIRECT KILLING MECHANISM OF ACTION.

7. THE PROPOSED MECHANISM OF ACTION FOR CATIONIC PEPTIDES.

8. AN OVERVIEW OF ANTIMICROBIAL'S WIDE-RANGE OF CELLULAR INTERRACTIONS.

9. IMMUNOHISTOCHEMISTRY SCHEMATIC BASIC STEPS.

10. IMMUNOHISTOCHEMISTRY STAINED SLIDES OF AMPS EXPRESSION PATTERN LEVELS OF BOTH GROUPS IN TONSIL TISSUE SECTION SAMPLE.

11. HISTOGRAM REPRESENTATION FOR THE MEAN AND STANDARD ERROR OF MEAN (SEM) AMPs SEMI-QUANTITATION EXPRESSION SCORES IN GA β HS GROUPS AND PFAPA GROUP.

LIST OF ABBREVIATIONS AND SYMBOLS.

- PFAPA Periodic Fever with Aphthous Stomatitis, Pharyngitis, and Cervical Adenitis. AIDS - Autoinflammatory Diseases.
- FAPA Fever, Aphthous Stomatitis, pharyngitis, and Cervical Adenitis.
- CRP C-Reactive Protein.
- ESR Erythrocyte Sedimentation Rate.
- AMPs Antimicrobial Cationic Peptide.
- $H\beta Ds$ Human- β -defensins.
- LL-37 cathelicidin.
- RNase7 Ribonuclase7.
- LEAP Liver Expressed Antimicrobial Peptide.
- CAPS Cryopryin-Associated Periodic Syndromes.
- FCAS Familial Cold Autoinflammatory Syndrome.
- MWS Muckle-Wells Syndrome.
- NOMID Neonatal-Onset Multisystem Inflammatory Disease.
- HPF Hereditary Periodic Fever syndromes.
- FMF Familial Mediterranean Fever.
- TRAPS Tumour Necrosis Factor (TNF)-Associated Periodic Syndrome.
- HIDS Hyper-IgD Syndrome (Mevalonate Kinase Deficiencies).
- DAMP Danger-Associated Molecular Pattern;
- DIRA Deficiency of IL-1Ra;

DITRA, Deficiency of IL- 36Ra;

- ER Endoplasmic Reticulum;
- IL=1- interlukin 1
- IL-1 receptor; IL-1Ra IL-1 Receptor Antagonist (Protein).
- MKD Mevalonate Kinase Deficiency.
- PAMP Pathogen-Associated Molecular Pattern.
- PRR Pattern Recognition Receptor.
- MWMDO Merriam-Webster Dictionary Medical Online.
- WBC White Blood Count.
- AA Autoinflammatory Alliance.
- IL-1 β inhibitor- Anakinra.
- IBD Inflammatory Bowel diseases.
- kDa Kilodallons.
- (IGF-1)- Insulin-like growth factor 1(sulfation factor).
- TGF- α Tumor Growth Factor-Alpha.
- hCAP Human Cationic Antimicrobial Peptide.
- $P2 \times 7$ -SFK-Akt-CREB/ATF1- signaling pathway activated by LL-37 in keratinocytes.
- mRNA Messenger RNA.
- HGPC Human Gingival Pithelial Cells.
- TLR Toll-Like Receptor.
- RT-PCR Real-Time-polymerised Chain Reaction.

- ECP Eosinophil Cationic Peptide.
- LPS Lipopolysaccharides.
- PGN Peptidoglycan.
- SAR Seasonal Allergic Rhinitis.
- HPA Human Protein Atlas.
- FITC Fluorescein Isothiocyanate.
- IHC Immunohistochemistry.
- FFPE Formalin-Fixed Paraffin Embedded.
- IBS- innova bioscience.
- TBS- tris-buffer saline

1.0. INTRODUCTION

The immune defense system protects a child's health status despite constant exposure to immunological antigens. But, when the defense system is compromised, the child becomes endangered and vulnerable to illnesses (Sheppard, 2017). This illness might be autoimmune disease, infectious diseases or even autoinflammatory diseases such as PFAPA (Brown et al., 2010; Padeh et al., 2017; Wekell et al., 2016).

PFAPA, which is thought to be an autoinflammatory disease, is a formulated acronym gotten from the word periodic fever, apthous stomatitis, pharyngitis and cervical adenitis (Wekell et al., 2016; Padeh et al., 2017; Ahmed et al., 2014). PFAPA usually occur at an early onset in children 2<5 years old (Ahmed et al., 2014). It is the most commonly exhibited syndrome of which genetic etiology is still unknown among other autoinflammatory diseases presenting recurrent fever as a common symptom (Wekell et al., 2016). The known genetic known genetic etiology of these autoinflammatory diseases' syndrome originates from deficiency in proteins of the innate immune system (Padeh et al., 2017; Tousseau, 2014; Ahmed et al., 2014). The differential diagnosis of PFAPA includes; recurrent tonsillitis, juvenile idiopathic arthritis, Behçet's disease, cyclic neutropenia, Chrohn's disease, and several other infectious diseases (Berlucchi and Nicolai, 2004).

Raimann successively described these periodic fever syndromes in 1948 in respect to their laboratory and clinical findings. The findings identified an unrelated group of disorders of unknown cause, characterized by short episodes of illness regularly being repeated for several years alternated with healthy periods (Berlucchi et al., 2003; AA, 2016; Berlucchi and Nicolai, 2004). AIDs group are unique and known by how they can cause inflammation without being provoked/triggered in the absence of autoantibodies or autoreactive T cells (Wekell et al., 2016; Padeh et al., 2017). They all exhibit recurrent fever as a common primary symptom together with various other symptoms associated with no contagious primary genetic conditions (Tousseau, 2014). These genetic conditions occur as a result of a malfunction in the protein of the innate immunity and Th1 activation responsive to IL-1 blockade (Wekell et al., 2016; Stojanov et al., 2012). These syndromes can only be considered after malignancy,

allergies, immunodefficiencies, infections, and autoimmune diseases are excluded (Wekell et al., 2016; Stojanov et al., 2012).

Epidemiologically, PFAPA as a periodic fever syndrome was first described in 1987 by Marshall et al. and later referred to as fever, aphthous stomatitis, pharyngitis, and cervical adenitis (FAPA) syndrome by Feder and Bialecki in 1989, and in same year, renamed as PFAPA syndrome by Marshall et al. in order to point out the uniqueness of the clockwork periodicity of fever attacks (Pignataro et al., 2009; Brown et al., 2010; Ahmed et al., 2014; Berlucchi et al., 2003). The incidence has been described in many parts of the world, especially in places like Norway and Sweden, with an annual incidence of at least 3 per 10,000 children under the age of 5 years, with more cases occurring in boys in several PFAPA cohorts (Wekell et al., 2016; Stojanov et al., 2011; Førsvoll et al., 2012). A cumulative incidence of PFAPA was also reported by Tejesvi et al. (2016), estimating that the illness affects about 2 in 10,000 children up to 5 years of age, making PFAPA the most common periodic childhood febrile syndrome among the AIDs. Cheung et al. (2017) reported an incidence of 2.3 in 10,000 European cohort children. It was stated that every pediatrician is likely to encounter at least 1 case of PFAPA in his or her career (Brown et al., 2010). Today, PFAPA diagnosis is not only at a much higher incidence in children under the age of 5 years, but also is discovered in older children and adults, with the first cases reported in 2008 by (Padeh et al., 2017; Wekell et al., 2016).

Genetic etiology of PFAPA is still not yet understood, but the quick response of the fever attacks to corticosteroid caused its suspicion to be an AID (Wekell *et al.*, 2016). Moreso, as a diagnostic criteria, inflammatory markers such as; C-Reactive Protein (CRP), Erythrocyte Sedimentation Rate (ESR) and leukocyte (white blood cells)'s concentration, which are indicative of a prominent, acute inflammatory reaction in periodic fever syndromes, have high elevated level (Simmonds, 2014). But, in PFAPA, these indicators return to normal level, symptoms subside and patients resume daily activities in-between episodes, distinguishing PFAPA syndrome from all other AIDs (Brown et al., 2010).

Recurrent tosillitis is known to aggravate PFAPA febrile episodes, which can effectively be treated with tonsillectomy and an early onset treatment with corticosteroids. A dramatic

response to corticosteroids and total recovery after tonsillectomy were observed as effective recurrent tonsillitis treatment. These two best treatment option for PFAPA syndrome, are aimed at shortening episode's duration, preventing episodes from occurring and also controlling symptoms during the episodes of fever (Nasrin et al., 2012; Phillip, 2017; Batu et al., 2016; Brown et al., 2010; Gentileschi et al., 2017; Vanoni et al., 2016). The palatine tonsils which is part of the lympho-epithelial tissue located at the common openings of the gastrointestinal and respiratory tract, appears to function as the host's first line of defense against exogenous microorganisms, such as fungi, virus and bacteria by manifesting specific antibodies, antimicrobial peptides, B and T-cell activity in response to a variety of antigens (Bogefors et al., 2014; Kaygusuz et al., 2003). These antimicrobial peptides (AMPs) initiate cellular, humoral and innate immunity at systemic and local levels (Ball et al., 2007; Nasrin et al., 2012).

The composition of the host antimicrobial cationic peptide (AMPs) molecules expressed by the tonsils, helps in selectively targeting microbes, thereby splitting and killing them once they bind and predominantly fit in into the microbial membranes they are of these negatively charged microbes (Ball et al., 2007). However, past investigations revealed a downregulation of the expression of these AMPs in different disease manifestations in PFAPA syndrome patients and other diseases such as seasonal allergic rhinitis (SAR) (Eminaga et al., 2001 (Bogefors et al., 2014). Therefore, in this study, it is hypothesized that the previous observation of altered tonsil microbiota in PFAPA cases is associated with a change in the level of AMPs production by tonsil epithelial cells. For this purpose, tonsils samples from PFAPA patients will be used in immunohistochemistry to monitor the expression levels of human- β -defensin 1-2, cathelicidin (LL-37), RNase7 (Ribonuclase7), Hepcidin and Liver expressed antimicrobial peptide-1 (LEAP-2) previously detected in tonsillar tissues of the groups involved in this study (Ball et al., 2007; Bhattacharjee, 2016; Duraiyan et al., 2012; HPA, 2017).

1.1 AIMS AND OBJECTIVES OF THE RESEARCH/STUDY.

A. To achieve screening of antimicrobial peptides expression levels in tonsil samples of PFAPA syndrome patients compared with that of recurrent tonsillitis due to group A beta-

haemolytic streptococci tonsil samples isolated in children using immunohistochemistry method.

B. To provide up-to-date information on the etiology of PFAPA syndrome as an autoinflammatory disease, by summarizing what has been explored and established.

2.0. LITERATURE REVIEW.

2.1. PFAPA SYNDROME, THE SIGNS AND SYMPTOMS

2.1.1. PFAPA SYNDROME

Among the autoinflammatory disease syndromes (AIDs) in childhood, periodic fever, aphthous stomatitis, pharyngitis and cervical adenitis, commonly referred to as "PFAPA" syndrome, is the only one known to be of an unknown genetic defect and also predominates over others in exhibiting recurrent fever condition (Wekell et al., 2016; Padeh et al., 2017). The other AIDs exhibiting same recurrent fever episodes include the cryopyrin-associated periodic syndromes (CAPS) and non-cryopyrin-associated periodic syndromes, but not limited to only these (Figure 1 and Table 1) (Tousseau, 2014).

AIDs posses the ability to cause inflammations in the absence of autoantibodies and autoreactive T cells (Padeh et al., 2017; Stojanov et al., 2011). The known genetical etiology of these syndromes originates from deficiency in proteins of the innate immune system (Padeh et al., 2017; Tousseau, 2014; Ahmed et al., 2014). They have no infectious cause, but all exhibit homogenous symptoms, with recurrent fever as the most dominant symptom (Figure 1 and Table 1) (Tousseau, 2014). Other symptoms which are primary genetic conditions but not contagious, often occur alongside the recurrent fever (Tousseau, 2014).



FIGURE 1: A STRATIFIED DISPLAY OF PERIODIC FEVER FREQUENCY OF THE AUTOINFLAMMATORY DISEASE SYNDROMES (Wurster et al., 2011).

2.1.2. KEY PRINCIPLES OF AUTOINFLAMMATORY PATHOMECHANISMS

Pathomechanism of cellular inflammatory reactions in AIDs can be initiated as a result of intracellular stress or engagement of pattern recognition receptors (PRR), with either pathogen-associated molecular pattern (PAMPs) or damaged-associated molecular pattern (DAMPs) (Umut, 2016; Holzinger et al., 2015). In addition to the above, Mogensen (2009) reported also that PRR recognizes the diverged localization of abnormal, self or unfamiliar molecular complexes during the breakdown of pathogen-specific molecules such as lipopolysaccharide (LLP) (Figure 2). This action induces an innate immune response and also triggers PRR-mediated signaling (Umut, 2016; Mogensen, 2009; Turgut, 2016).

FIGURE 2: THE KEY PRINCINPLE INVOLVED IN INNATE IMMUNE SYSTEM RECOGNITION BY PATTERN RECOGNITION RECEPTOR (PRR).



The principles involved in autoinflammatory pathomechanism (2.1.2.) are driven by four major keys processes:

(1) Defective post-translational processes also known as "protein-misfolding", generates the endoplasmic reticulum stress (ER-stress) and increases the reactive oxygen species (ROS),

often observed in tumor necrosis factor receptor-associated periodic syndrome (TRAPS) or mevalonate kinase deficiency (MKD) (Holzinger et al., 2015).

(2) Another mechanism known to cause autoinflammation is known as the gain-of-function mutations mechanisms, which result in enhancing the expression of proinflammatory genes, also observed in stimulator of interferon genes-associated vasculopathy with onset in infancy (SAVI) (Holzinger et al., 2015; Cheung et al., 2017). Moreover, the gain-of-function of inflammasome protein such as NOD-like receptor pyrin containing 3 (NLRP3) enhances the inflammatory response in cryopyrin-associated auntoinflammatory syndromes (CAPS) (Holzinger et al., 2015).

(3) Another mechanism involved is the unbalanced ratio of cytokine to endogenous antagonist which causes an uncontrolled activity of interlukin often seen in deficiency of IL-1Ra (DIRA) or deficiency of IL-36Ra (DITRA) in keratinocytes (Holzinger et al., 2015).

(4) The abnormal activation of the IL-1 β pathway via the inflammasome is a common mechanism in the pathogenesis of AIDs (Ariyanto, 2014; Cheung et al., 2017).

Cheung *et al.* (2017) hypothesized that, major role in the pathogenesis of PFAPA secondary to a genetic defect that leads to a dysregulation of IL-1 β secretion, might be triggered by IL-1 β as it also occurs in other AIDs. Without any identifiable genetic cause, IL-1 β expression at mRNA and protein level was shown to be elevated during PFAPA febrile attacks (Cheung et al., 2017).

The increase in IL-1 β (which plays a central role in inflammatory response) production is believed to occur as a result of less penetrant and frequent polymorphism Q705K and C10X in NLRP3 and CARD8 genes accordingly, which enhances inflammasome activity in initiating classical autoinflammatory syndromes with penetrant inheritance patterns. In this regard, classical PFAPA is a significant part of CARD8 polymorphism (Cheung et al., 2017). Stojanov *et al.* (2011) reported also the activation of IL-1 β during PFAPA flares.

However, mutations in a variety of genes involved in controlling such inflammasome function can result in autoinflammatory disease in patients. This might be the cause of the genetically unexplained large number of patients with AIDs (Ariyanto, 2014; Cheung et al., 2017). An example of such inflammasome participating gene is Nod-like receptor pyrin 3 (NLRP3) (Table1) as in the case of hereditary inflammatory diseases in CAPS, in which upon activation,

the sensor multiprotein complex NLRP3 inflammasome assembles together with the adaptor protein ASC and procaspase-1 (Ariyanto, 2014; Cheung et al., 2017). This action results to the automatic hydrolysis of proteins to peptides by enzymatic actions leading to the maturation of caspase-1 which occurs as a result of the formation of inflammasome and extracellular release or rather activation of the proinflammatory cytokines IL-1 β and IL-18 seen at the protein level and transcript during PFAPA febrile episodes (Cheung et al., 2017). Moreso, the oligomerization of the inflammasome causes the cleavage and activation of procaspase-1, which in turn promotes the release of functional IL-1 β from their unfunctional precursor, pro-IL-1 β (Cheung et al., 2017).

TABLE 1: CLINICAL CHARACTERISTICS OF SOME AIDs (Allergy/Immunology

 Rare Diseases (2015).

	FMF	TRAPS	HIDS	PFAPA	CAPS
Gene	Gene MEFV		MEVK	Unknown	NLRP3
Age of onset	< 20	< 20	< 1	< 5	< 1
Inheritance	AR	AD	AR	Ś	AD
Ethnicity	Armenian, Turks, Seph Jews, Arabs	Irish Scottish	sh Scottish Dutch French		Any
Fever length	1-3 days	> 5 days	3-7 days	3-4 days	1-3 days
Clinical Features	Rash, serositis	Rash, myalgia, conjunctivitis/ periorbital edema	Cervical LAD, splenomegaly, vomiting, rash, high IgD levels	Cervical LAD, stomatitis	Variable by type
Amyloidosis	Common	Common	Rare	No	Variable
Treatment	Colchicine, IL-1 blockade	Steroids, TNF or IL-1 blockade	NSAIDs, steroids, IL-1 blockade	Steroids, tonsillectomy	IL-1 blockade

2.1.3.1. THE MAJOR SIGNS AND SYMPTOMS OF PFAPA

The overlapping clinical features of the AIDs and the much common PFAPA syndrome in children, makes it difficult to distinguish between them (Ariyanto, 2014). The four major symptoms of PFAPA syndrome are seen in its name (Figure 3) (Vigo and Zulian, 2012; Feder and Salazar, 2009).

PERIODIC FEVER- This is the most dominant of all PFAPA's symptom, patients often look and feel normal in between the fever episodes with an abrupt onset and termination (Wurster et al., 2011). Recurrences of fever that may last from a few days up to over two years are followed by symptom-free intervals of varying duration, occurring as a result of a malignancy, a non infectious inflammatory disorder or a recurrent infection (MMD, 2016). Some diseases with a similar set of symptoms include the Behest syndrome, Crohn's disease and the Still's disease (Berlucchi and Nicolai, 2004).

APHTHOUS STOMATITIS- Its a usual disorder of the oral mucosa manifested by the formation of canker sores on flexible mucous membranes of the mouth (MWMDO, 2017). The canker sore is a small sensitive painful ulcer cavity in the lining of the mouth, generally characterized by a burning, itching, or stinging sensations, which may come before the appearance of any lesion by some hours, and pain that is often out of proportion to the extent of the ulceration. It worsens by physical contact, especially with certain foods and drinks that are acidic (MWMDO, 2017).

PHARYNGITIS- It is defined as an inflammation of the pharynx (also referred to as the back of the throat). It is a common cause of sore throat, typical of respiratory tract infection (MWMDO, 2017).

CERVICAL ADENITIS- It is a condition which often occurs with serious infections of the throat and is distinguished by an enlarged, tender lymph nodes of the neck. Most lymph nodes respond well to oral antibiotic treatment. However, some may need to be opened and drained as a form of treatment. Children associated with fever (MMD, 2009) often experience this abnormal situation.

This syndrome as described by Phillip (2017), includes all clinical features with the episodes of fever starting suddenly and last for 3-7 days; occur routinely every few weeks, usually between 3-6 weeks (Phillip, 2017). The cause of PFAPA is not yet known; hence the need for further research in order to define its origin is of great essence (Feder and Salazar, 2009).

FIGURE 3: SCHEMATIC PRESENTATION OF A TYPICAL PFAPA SYNDROME PATIENT'S CLINICAL FEATURES (Simmons and Daniel, 2016).



2.1.3.2. CLINICAL CHARACTERISTICS OF PFAPA SYNDROME PATIENTS.

PFAPA syndrome should be considered when periodic fever is non-contagious, especially among family members, when the patient is often seen in good health condition in between febrile fever episodes and when there is no history of chronic illness (Feder and Salazar, 2009). PFAPA syndrome fever episode commences in majority of its patients who are <5(between 2-5) years old with a 29.8 days mean interval between those episodes, even though occasionally, an episode might be skipped especially during summer (Feder and Salazar, 2009). Fever episode could last up to 1 year long with a maximum temperature duration ranging between 39.2 °C and 42.1 °C (Feder and Salazar, 2009).

However, in some patients, later onset of the attacks may also occur, with no seasonality or a change in frequency of episodes, while patients may also experience a relapse after long-term reduction of the disease (Tasher et al., 2006). Feder and Salazar, (2009) reported that tonsillar (pharyngeal) erythema characterized by white patches which indicates the presence of rare polymorphous nuclear leukocytes seen on tonsils of few patients, occurs more often than the other symptoms such as aphthous stomatitis (<1 cm or less).

Moreover, children experiencing PFAPA syndrome have other symptoms which include joint pain, rash, abdominal pain, headache, vomiting or diarrhea; while in between episodes the affected children are healthy (Phillip, 2017; Wurster et al., 2011). During attacks, children

experience an enlarged neck gland, and often complain more of pain in the throat and mouth but, they recover completely between the attacks, which eventually stop by late childhood (Wekell et al., 2016).

However, PFAPA syndrome is predictable since most patients outgrow their condition after the age of 10 years. As children age, the time between the episodes will increase and the children continue to grow and develop normally (Tousseau, 2014; Phillips, 2017).

Frequencies of ccurrence are recorded in (Table 3) with a general higher frequency recorded in male than in female (Licameli et al., 2008). Moreso, some studies, such as those on the clinical characteristics of PFAPA patients from Turkey and the United States of America (USA), recorded also a higher occurrence of PFAPA syndrome in male than female with a ratio of 1.8:1 respectively out of 71 patients from Turkey (the largest PFAPA cohort ever), which wasn't looked into in regards to those from United States of America (USA) (Batu et al., 2016). Other related discovered occurrences include that of Stojanov *et al.* (2011) female 8 (38%) and male 13 (62%) out of 21 PFAPA syndrome patients; Tasher *et al.* (2006) female 21 (39%) and male 33 (61%) out of 54 PFAPA syndrome patients; while in contrast to the above mentioned cases, Ahmed *et al.* (2014) reported a lesser frequency of male 17 (47.2%) and female 19 (52.8%) out of 36 PFAPA syndrome patients.

However, in regards to pharyngitis which was mentioned earlier by Feder and Salazar, (2009) as the most common PFAPA syndrome patient's symptom occurring during the episode than the other symptoms, Batu *et al.* (2016) reported a percentage of 33.6%; Tasher *et al.* (2006) 52 (96%); while, Ahmed *et al.* (2014) 25 (69.44%). This is in contrast with a study reported by Stojanov *et al.* (2011), who reported a lower occurrence of 18 (86%). Previous studies has also reported low levels of circulating lymphocytes such as CD4+ and CD8+ during febrile episodes of PFAPA, which could be as a result chemotaxis in germinal centre of the tonsils (Førsvoll et al., 2015).

2.2. GENERAL DIAGNOSIS OF PFAPA SYNDROME

In diagnosing PFAPA syndrome, there is no particular laboratory test, being that the disease is diagnosed based on symptoms and physical examination observed (Philip, 2017; Ariyanto,

2014; Førsvoll and Øymar, 2007; Batu et al., 2016). Some of the diagnostic criteria includes; a detailed case history of the patient, and exclusion of other diseases with similar symptoms such as; autoimmune diseases, infections and immunodeficiency diseases (Brown et al., 2010; Førsvoll and Øymar, 2007).

Though, pharyngitis, aphthous stomatitis and cervical adenitis occurring in the absence of any infection should be the basis for PFAPA syndrome's diagnosis (Brown et al., 2010; AA, 2016). Markers of inflammation-elevated level such as that of c-reactive protein, serum amyloid A, white blood cells, etc, noticed during febrile episodes, gradually reduces by adolescent stage but terminates in others onset of the symptoms (early stage of the syndrome). Though these test are unspecific, patients have to be diagnosed after experiencing three fever flare episodes. Some feverish episodes could last up to five days and reoccur at regular intervals without any sign of infection at this period (Batu et al., 2016; AA, 2016; Phillip, 2017; wurster et al., 2011; Førsvoll and Øymar, 2007).

In addition to the above, Brown *et al.* (2010) and Phillip (2017), reported that, one should consider the quick recovery of patient's febrile episode as a result of the administration of corticosteroids when concluding diagnose on PFAPA syndrome.

S/N	THOMAS CRITERIA.
1	Regular recurring fevers with an early age of onset (<5 years of age)
2	Symptoms in the absence of upper respiratory tract infection with at least one of the
	following clinical signs:
	(a) aphthous stomatitis,
	(b) cervical lymphadenitis and,
	(c) pharyngitis,
3	Excluding cyclic neutropenia,
4	Completely asymptomatic interval between episodes,
5	Normal growth and development.

2.2.1. TABLE 2: THOMAS DIAGNOSTIC CRITERIA FOR PFAPA SYNDROME

The diagnostic criteria below were modified by Thomas *et al.* (1999) ten years after it was proposed by Marshall *et al.* (1989) as reported by Berlucchi and Nicolai, 2004; Lantto et al., 2016; Førsvoll and Øymar, 2007; Ariyanto, 2014. Thomas criteria (Table 2) comprises of two classes as described below:

2.2.1.1. CLASSIC THOMAS CRITERIA: This is a long-term outcome of a patient who met the criteria modified by Thomas *et al.* (1999) and had complete recovery from fever episodes after surgical removal of their tonsils (Lantto et al., 2016).

2.2.1.2. INCOMPLETE THOMAS CRITERIA: This is a long-term outcome of a patient that did not meet those criteria modified by Thomas et al., but had complete recovery from fever episodes after surgical removal of their tonsils (Lantto et al., 2016).

Other AIDs sharing similar recurrent fever symptom with PFAPA syndrome pose a challenge in trying to manage serious complicated cases treatment measures (Tousseau, 2014). In between fever attacks, classic PFAPA patient do not exhibit any symptom or any signs of chronic inflammation. PFAPA syndrome differential diagnosis comprises of other individual symdromes such as juvenile idiopathic arthritis (JIA), cyclic neutropenia (CN), recurrent tonsillitis, familial mediterrannean fever (FMF), hyper-immunoglobulin syndromes (HIDS) and behcet's disease (Feder et al., 1992; Scimeca et al., 1996; Thomas et al., 1999; Padeh et al., 1999; Lee et al., 1999; Dahn et al., 2000; Feder et al., 2000; Scholl *et al.*, 2000) (reviewed in Berlucchi and Nicolai, 2004).

2.3. TREATMENT OF PFAPA

The aim of treating PFAPA syndrome is to shorten episode duration, prevent episodes from occurring and also to control the symptoms during episodes of fever (Phillip, 2017). PFAPA syndrome may have other treatment options, but the most effective treatment options are use of corticosteroids and tonsillectomy (Batu et al., 2016; Vanoni et al., 2016; Tasher et al., 2016).

2.3.1. CORTICOSTEROIDS

Generally, single dosage of prednisone (0.5–2mg/kg) or beta-methasone (0.2 mg/kg), or lower dosages of corticosteroids or its equivalent prednisone administered (mean 0.6 mg/kg/day,

range 0.15–1.5 mg/kg/day) orally at onset of episode, will rapidly resolve the flare and dramatically shorten episode of PFAPA very fast within few hours (Table 3) (Vanoni et al., 2016; Licameli et al., 2008; Tejesv et al., 2016; Phillip, 2017; Batu et al., 2016).

While, cimetidine has poor efficacy and only effective by 30%, colchicine has gastro-intestinal side effects (Phillip, 2017). But when both are used regularly, they may also prevent future episodes in about a third of the children (Phillip, 2017). Regarding with this treatment, there may be a short interval between episodes, and the next episode may occur earlier than expected (Vanoni et al., 2016; Phillip, 2017). Moreso, IL-1 inhibitors (anakinra (ANA)) and pidotimod are both effective with good positive effect in suppressing the disease flares and avoiding recurrences during long-term follow-up with no reported side effects after their administration (Gentileschi et al., 2017).

This was useful in differentiating attacks of PFAPA from other autoinflammatory disease syndromes such as FMF or other hereditary periodic fever syndromes. Medical treatment of PFAPA syndrome does not guarantee a change in the results established being that the syndrome has satisfactory natural history, effective treatment of the episodes induces a rapid reduction of episodes and offers a healthier family lifestyle, as episodes often interfere with quality family lifestyle (Vanoni et al., 2016; Phillip, 2017). Although there is yet to be a satisfactory treatment measure, and a continues and repeated treatment therapy may result to side effects (Stojanov et at., 2011).

2.3.2.1. TONSILLECTOMY

Tonsillitis happens to be the major cause of primary care visit to the hospital, and can be terminated by tonsillectomy as the common choice of treatment procedure (Tasher et al., 2006; Vanoni et al., 2016; Nasrin et al., 2012). Report of previous studies has it that, tonsillectomy can be considered based on the presumption of a chronic tonsillar infection provoking the symptoms. This procedure is reserved for prolonged complicated tonsillar disease and selected patients not responding to other medical treatment options (Vanoni et al., 2016; Licameli et al. 2008; Gentileschi et al., 2017).

Tonsillectomy (Figure 4) is an effective treatment for recurrent tonsillitis patients with regularly recurring fever episodes (Feder and Salazar, 2009) for those who failed to meet the classic Thomas criteria (2.2.1.1), especially those children with a late onset of symptoms (Lantto et al., 2016; Brown et al., 2010; Tasher et al., 2006). A greater number (>80%) of

PFAPA syndrome patients were reported cured after the procedure (Philips, 2017). This treatment method should be looked into especially for patients needing steroids more frequently than once every 3-4 weeks (Philips, 2017).

FIGURE 4: TONSILLECTOMY (DIO. 2018).



This is a surgical procedure carried out in order to treat recurring cases of tonsillitis or a tonsillar abscess (severe infection). Before the operation, the patient is first placed under general anesthesia. The figure above illustrate how surgeons use scalpel to cut away each tonsil from the back of the throat with the help of Forceps which are used for both lending precision to the use of the scalpel and to remove the tonsils once they have been cut (DIO, 2018).

2.3.2.2. EFFECTS OF TONSILLECTOMY

The positive effect of tonsillectomy was manifested by resolving the symptoms of all PFAPA patients and a complete reduction of flare after tonsillectomy (Table 3) (Garavello et al., 2011; Philip, 2017; Licameli et al., 2008).

However, as a result of the anatomically position of the palatine tonsil (as a secondary lymphoid organ), perfectly situated at the entrance of both respiratory and gastrointestinal tract, it plays a greater part of defense in the body's immune system, comprising of humoral, innate and cellular immunity at both systemic and local stages (Ball et al., 2007). Therefore, due to growing knowledge acquired over the years regarding the immunological role played by tonsils, Nasrin *et al.* (2012) pointed out that, a surgical removal of these tonsils will result in poor health status of PFAPA patients that will become less privilege, especially in underdeveloped countries. For this reason, patient who underwent tonsillectomy are thought to

TREATMENT FOR RECURRENT TONSILLITIS (Licameli et al., 2008).								
	Symptom							
Source	Patients, No.	Female to Male Ratio	Fever (Duration, d)	AU, %	Pharyngitis, %	CA, %	ТА	Treatment
Marshall et al ¹	12	5:7	40°C (5)	75	75	67	Not performed	3 Patients had relief with prednisone
Thomas et al ¹²	94	42:52	40°C (3.8)	71	69	82	7 of 11 had complete cessation	Corticosteroid treatment worked remarkably well
Padeh et al ³	28	8:20	(Mean, 4.3)	68	NR	100	100% Cessation (3 surgeries)	23 Patients responded well to prednisone
Abramson et al ¹³	4	4 M	41°C (5)	25	100	75	100% Cessation	TA
Galanakis et al ¹⁴	15	4:11	40°C (4-6)	30	100	80	100% Cessation	Tonsillectomy
Dahn et al ¹⁵	5	1:4	>39°	40	60	60	All "significantly improved"	ТА
Berlucchi et al ¹⁶	5	1:4	39°C-40°C (4-5)	40	100	100	100% Cessation	Tonsillectomy, TA
Parikh et al ¹⁷	2	1:1	(>38.5°C)	50	100	100	None improved	Tonsillectomy, TA
Current study	27	14:13	39°C-40°C (5)	37	55	52	96% Cessation	Tonsillectomy, TA

be associated with weakened levels of humoral and cellular immune responses (Nasrin et al., 2012; Tejesvi et al., 2016).

TABLE 3: - PFAPA SYNDROME LITERATURE SUMMARY ON EFFECT OF

Abbreviations: AU, aphthous ulcers; CA, cervical adenitis; NR, not reported; PFAPA, periodic fever, aphthous ulcers, pharyngitis, and adenitis; TA, tonsillectomy with adenoidectomy.

2.4. THE ROLE OF IMMUNE SYSTEM IN TONSILS AND THEIR EPITHELIUM

Immune system is the biological complex system that comprises of a complex network of both chemicals and cells that act together to provide protection for the body against pathogenic organisms and substances (Gazi, 2016; ImIr, 2016; Ahmed and Hashish, 2000). It is composed of the two main branches: innate and adaptive immune systems (Gazi, 2016; ImIr, 2016; Ahmed and Hashish, 2000). Innate immune system functions basically on providing both chemical and physical barrier against infections, recognition of pathogens invasion by the cells of the tonsil and initiating antimicrobial response (secretions) (Gazi, 2016; ImIr, 2016; Mogensen, 2009). However, the onset of PFAPA's febrile episode, which is associated with an increase in the levels of cytokines, is regarded as a proof of the participation of innate immune response in pathogenesis (Førsvoll et al., 2013). This is ensued by the activation and

reallocation of T cell to inflammation sites, which is an indication of an adaptive immune response as well (ImIr, 2016, Gazi, 2016; Førsvoll et al., 2013).

Polymorphous nuclear cells such as (neutrophils), keratinocytes and endothelial cells can cause chemokine ligan-10 (CXCL10) production, a chemo-attractant that can also be directly produced by an early innate process/mechanism (Gazi, 2016; ImIr, 2016; Førsvoll et al., 2013). This chemo-attractant which may connect innate immune response to an adaptive immune response, attracts T cells to inflammation site in secondary lymphoid organs such as the tonsil, as expressed in different T helper 1(Th-1) type of inflammatory diseases (Gazi, 2016; ImIr, 2016; Førsvoll et al., 2013). CXCL10 influence in innate immune system activation may last long during PFAPA syndrome fever febrile attacks serving as a marker in children clinically (Førsvoll et al., 2013). Likeswise, adaptive response initiation indicated by variations in T cells observed (Førsvoll et al., 2013).

Tonsils as a secondary lymphoid organ include lymphocytes and are involved in trapping of foreign materials coming into the body via the oral route (ImIr, 2016). Furthermore, specific antimicrobial peptides, such as human beta-defensin-1-3 (h β d-1-3), cathelicidin (LL-37), ribonuclease (Rnase7), etc, which serves as the host's natural defence system, are being expressed to provide further protection (Bogefors et al., 2014; Kaygusuz et al., 2003; Ball et al., 2007; Nasrin et al., 2012).

The microbiota, an ecological community of commensal, symbiotic and pathogenic microorganisms, comprises of all forms of organism at all levels, and are all directly involved in developing response in both innate and adaptive immunity for the balance of immune system by an optimal microbiota-host interaction (Lantto et al., 2015). The tonsil epithelium barrier serves as first line of defence against those invading microorganisms (Lantto et al., 2015).

PFAPA syndrome tonsil samples had revealed in past studies a great difference in manifestation of large number of microorganisms from those found in control group, while, pathogenic bacteria were not detected in PFAPA patient sample indicating that it is not an infectious disease (Tejesvi et al., (2016). This was first analysed by Pignatoro *et al.* (2009) using molecular method and achieved a negative result from PFAPA syndrome patient tonsil
tissue sample. Meaning that cytokine production dysregulation in tonsil's inflammasomes could be protected or triggered by tonsillar community differences such as that of different tonsil, oral cavity, etc from those found in healthy control microbiota (Tejesvi et al., (2016). This could be caused by various factors such as genetic individual make-up of the host, environment, level of contact/interaction with microorganisms in mucous membrane, diet or in natural water where *Cynobacteria species (Pseudanabaena catenata)* is in abundance (also dominant in PFAPA cases) and are capable of carrying out photosynthesis (Tejesvi et al., (2016). A fluctuation in microbiota could result to clinical symptoms (Tejesvi et al., (2016).

Туре	Epithelium	Capsule.	Crypts	Location
Adenoids (also	Ciliated	Incompletely	No crypts,	Roof of <u>pharynx</u>
termed	pseudostratified	encapsulated	but small	
"pharyngeal	columnar (respiratory		folds	
tonsils")	epithelium)			
	Ciliated			Roof of <u>pharynx</u>
Tubal tonsils	pseudostratified			
i ubai tonsiis	columnar (respiratory			
	epithelium)			
Palatine tonsils	Non-keratinized	Incompletely	Long,	Sides of <u>oropharynx</u>
	stratified squamous	encapsulated	branched	between palatoglossal
				and palatopharyngeal
				arches
Lingual tonsils	Non-keratinized	Incompletely	Long.	Behind terminal sulcus
	stratified squamous	encapsulated	branched	(tongue).

TABLE 4: TONSILS AND THEIR EPITHELIUM'S DESCRIPTION.

Tonsils positions and sizes differs, in humans (Figure 5), they are positioned in the front (anterior), on the top (superior), at the back (posterior) and at the bottom (inferior) (Blausen_gallery, 2014). They each measure up to 2.5cm in length, 2.0cm in width and 1.2cm in thickness (Blausen_gallery, 2014).

FIGURE 5: TONSILS AND THROAT'S ANATOMICAL POSITIONS IN THE BODY

Hard Palate Nasopharynx Soft Palate Uvula Palatine Tongue Lingual Tonsil Laryngopharynx

(Blausen_gallery, 2014).

Tonsils and Throat

Aside physically acting as a protective shield, the epithelium cells (Table 4) can also secrete biological effectors such as antimicrobial peptides, etc, that are very important in relating homeostatically with microbiota and act to prevent the invasion of immunological pathogens (Mogensen, 2009).

Antimicrobial peptides (AMPs) can be expressed not just when secreted as a mediator, but also when influenced by microbe-associated molecular patterns (MAMPs) which initiate intracellular signaling when engages with pattern recognition receptor (PRR) (Chapter 2.1.2) (Chu and Mazmanian, 2013; Mogensen, 2009). As natural antibiotics of the host's defence system, the AMPs play major functions in microbial community regulation, such that its

downregulation depicts an association with a change in the gut microbiota, leads to chronic inflammatory bowel diseases (IBD) such as chrohn's disease (CD) (Artis, 2016).

2.5. ANTIMICROBIAL PEPTIDES

2.5.1. DEFINATION OF ANTIMICROBIAL PEPTIDE

AMPs are part of the innate immune response, which protects our body against numerous pathogens found in all classes of life, in the oral mucosa and the airway epithelium (Nasrin et al., 2012; Nilsson et al., 1999; Diamond et al., 2008; Zasloff et al., 2002). Unlike most usual antibiotics, AMPs seems to posses the capacity of boosting the host immune system by fighting against foreign materials (Ball et al., 2007; Kwapisz et al., 2009). They posses different structure and could be as small as 1-10 kDa in size, which are capable of exhibiting a wide-range of antimicrobial activity against series of immunological agents (Ball et al., 2007).

2.5.2. TYPES OF ANTIMICROBIAL PEPTIDE (AMPS)

The tonsillar mucosa barrier and epithelial cells lining the mammalian respiratory tracts which serve as first line of defence are known to express antimicrobial factors such as cathelicidins (LL-37) and beta defensins in abundance, thereby having a very important role in the host defence against invading pathogens (palatine tonsil) (Diamond et al., 2000b).

Palatine tonsils also are greatly exposed to pathological microorganism invasion, which can stick to the tonsil and possibly lead to an infection such as those seen in cystic fibrosis (Pácová et al., 2010; Ball et al., 2007; Nilsson et al., 1999; Diamond *et al.*, 2000b). The mechanisms that can be used to prevent such invasion of pathogens are a constant production of these AMPs (Ball et al., 2007). Amongst the AMPs, the human β -defensins-1-2 (H β D-1-2) (Pácová et al., 2010), cathelicidines (LL-37) (Dixon et al., 2012), Ribonuclease7 (RNase7) and the liver expressed antimicrobial peptide-2 (LEAP-2) were shown to be strongly expressed by the palatine tonsils (Ball et al., 2007).

2.5.3. CHARACTERISTICS OF EACH AMPs

Generally, they all possess the capacity of highly positively charged peptides that enable them to defend the host's immune system, thereby functioning as the body's natural antibiotics (Ball

et al., 2007; Nilsson et al., 1999). They are also known to destroy both gram-positive and gram-negative bacteria and alter other cells such as cancer cells (Ball et al., 2007; Nasrin et al., 2012).

DEFENSINS

Defensins are small but highly cationic peptides of about 3-5 kDa in size, which were previously identified in phagocytic cells as a result of their distinct function in innate immune response. α and β -defensing are the two main subgroups classified under defensing (Diamond et al., 2008). Despite of possessing an abundant cationic residue and almost the same secondary physical form, they have different cysteine motifs (Diamond et al., 2008). Both α and β-defensins exhibit a wide range of antimicrobial activity towards all bacteria (Diamond et al., 2008; Lehrer and Ganz, 2002). While, α -defensing possess an acidic pro-region that has to go through another cleavage process before releasing its matured peptide, the β -defensions matures by just a signal peptidase (Beckloff and Diamond, 2008). Past studies reported that, β defensins-1-4 were the first to be discovered, and their expression which is activated by the presence of pathogens, led to the discovery of innate immune response in the airways (Diamond et al., 2008; Kaiser and Diamond, 2000). Pathogens such as Pseudomonas aeruginosa and Escherichia coli lipopolysaccharide presence can induce the expression of βdefensins genes (Kaiser and Diamond, 2000). Pseudomonas aeruginosa intratracheal introduction/instillation in mouse, led to an increase in the level of mouse β -defensin-3 while, β -defensin-2 increased in the tracheal epithelium Bals *et al.* (1999) (reviewed in Diamond et al., 2008), thereby indicating an innate immune in respiratory tract (Diamond et al., 2008).

Epithelial cells and some specific type of cells generating from the myeloid lineage such as the bone marrow or spinal cord still expresses the four types of β -defensins (Diamond et al., 2004; Diamond et al., 2008). These peptides present in respiratory tract in the airways epithelium can also be found in saliva (Laube et al., 2006; Diamond et al., 2001; Dale et al., 2006; Diamond et al., 2008; Ball et al., 2007). This therefore suggests that, they perform a major function in the airways by fighting against bacteria invasion and creating a stable equillibrium with commensal bacteria in the oral tract (Diamond et al., 2008).

While fighting against bacteria that invades the crypt and surface epithelium during acute infection, monocytes, T cells and dendritic cells are led to the infection area to fight the immunological antigens (Schwaab et al., 2009). But when the infection becomes chronic, the numbers of antigen presenting cells, dendritic cells and macrophages reduces even when the expression levels of H β D-1-3 are still same (Schwaab et al., 2009). This in turn leads to failure in immunomodulatory effect and reoccurrence of the infection caused by the biofilm colonies, which are highly resistant to antibiotics (Schwaab et al., 2009). H β D-1-3 may prevent bacteria from causing deep infection to the neck after invasion, but will not be able to stop tonsil abscess from forming (Schwaab et al., 2009).

Numerous immunological antigens of the oral cavity such as *Porphyromonas gingivalis*, *Prevotella melaninogenica*, *Actinobacillus actinomycetemcomitans*, *streptocococcus mutant* are inhabited in the oral cavity that in turn do not initiate any disease because the mouth where the tonsil is located is known to be a major site that ellicit innate immune response, such as the synthesis of various AMPs in other to limit the adhering and invading of pathogens (Ball *et al.* 2007). While in the case of the airways, it is predominantly sterile (Diamond et al., 2008). Tissue biopsy of gingivitis patient has been reported in previous studies to express an increasing level of h β D-2, h β D-3 and mRNA (Diamond et al., 2008; Ball et al., 2007).

H β D-2 expression was demonstrated using an immunohistochemistry technology on tissue samples collected from patients infected with *Candida albicans* (Diamond et al., 2008). Using an *in vitro* study, *Porphyromonas gingivitis* was shown to induce h β D-1 and h β D-2 expression levels in cultured human gingival epithelial cells (HGEC), while, h β D-2 and h β D-3 had increased levels upon stimulation with *Actinobacillus actinomycetemcomitans* and *Fusobacterium nucleatum* (Table 5) (Diamond et al., 2008).

CATHELICIDINES.

Broad variety of tissues such as; mouth, airways, intestine and the skin, including immune cells that are continuously threatened by microorganisms, can express cathelicidins (LL-37) (Pácová et al., 2010); (Bals et al., 1998). Cathelicidins also exhibits strong antimicrobial action in opposition to *Streptococcus mutans* (the main cause of dental caries in the oral cavity), and *Staphylococcus aureus* (a bacterial agent that colonizes the nasal cavity) (Leszczynska et al.,

2013). Whenever throat infection is constantly repeated, expression of cathelicidins is upregulated in the palatine tonsils (Song et al., 2006; Nasrin et al., 2012).

The cathelicidines (LL-37) also functions as an agent for cell movement (chemotaxis) by acting as a chemical stimulus for neutrophils and monocytes (chemotaxis), and might also function in cutaneous wound repair by forming blood vessels and promoting cell growth (Sorensen et al., 2001; Koczulla et al., 2003; Pácová et al., 2010; Diamond et al., 2008). The wound healing process occurs when LL-37 interact with keratinocytes in the course of wound closure, which causes the formation of a barrier against microorganisms (Duplantier and van Hoek, 2013).

TABLE 5: ORAL AND AIRWAYS EPITHELIUM ANTIMICROBIAL GENESEXPRESSION SUMMARY.

ORAL CAVITY			
Peptide	Site of Expression	Regulation	Stimulant
hβD-1	Gingival epithelium	Inducible	Porphyromonas gingivalis
hβD-2	Salivary glands, epithelium	Inducible	Fusobacterium nucleatum, Actinobacillus actinomycetemcomitans, IL- 1b
hβD-3	Keratinocytes	Inducible	Fusobacterium nucleatum, Actinobacillus actinomycetemcomitans
LL-37	Gingival Epithelium Neutrophils	Inducible Constitutive	Vitamin D
AIRWAYS			
Peptide	Site of Expression	Regulation	Stimulant
hβD-1	Ciliated epithelium Plasmacytoid DCs	Constitutive Inducible	- Enveloped viruses

hβD2-	Ciliated epithelium	Inducible	Bacteria, Toll-Like Receptor
			agonists, cytokines
hβD-3	Ciliated epithelium	Inducible	Bacteria, Toll-Like Receptor
			agonists, cytokines
LL-37	Ciliated epithelium	Inducible	Vitamin D
	Neutrophils	Constitutive	-

 β -defensing genes expression in most systems is often a function of the pathogenic microorganisms that invades that system. Table 5 showed cathelicidin's mRNA and its proteins being induced using an *in vitro* method by physiological concentration of vitamin D. These expressions were observed in the airways epithelium (Kaiser and Diamond, 2000; Diamond et al., 2008).

LIVER EXPRESSED ANTIMICROBIAL PEPTIDE (LEAP).

LEAP-1 possess an unusual distinctive expression pattern that enables it to be expressed in many tissue samples, but with a higher expression pattern seen in liver than the other tissues tested, such as the heart, tonsil, brain, etc (Krause et al., 2000). Not only does it exhibit a high level of expression in the liver but also interact effectively with other components of the immune system such as the tonsil, spleen, lymph node, etc, proven that its capacity is more than just inhibiting immunological agents at the epithelium (Krause et al., 2000).

LEAP-2 is known to exhibit a wide range of antimicrobial activities in opposition to Grampositive immunological antigens (Ball et al., 2007). Its tonsillar pattern of expression is distinct with an equal division of transcript between lymphoid tissue and tonsil's epithelium (Ball et al., 2007).

RIBONUCLEASE (RNASE)

The RNase is a superfamily that functions in host immune system in order to prevent infection by pathogenic microorganisms. This family includes the human antimicrobial RNase3 and RNase7, a 14.5 kDa protein. Earlier studies had proven that RNase's mechanisms of actions are a function of how they disrupt the cell membrane (Figure 6 and 7). While RNase7 mechanism of action is established by exhibiting a broad range of antimicrobial activity on pathogens such as protozoans, viruses, bacteria and fungi, RNase3 acts against only helminthes (Torrent et al., 2010; Mojsoska and Jenssen, 2015; Sarawilcox, 2004). They both posses and share a similar structural form, high cationic characteristics and approximately 40% of amino acid identity (Torrent et al., 2010).

Both RNases uses lipid vesicle as a membrane model for their activity such as damaging the lipid bilayer (Torrent et al., 2010). This unique activity is exhibited in a well-defined time (Torrent et al., 2010). Mutational change in their genetic information also revealed a positive and aromatic surface-exposed residue and a surface lysine cluster involved in anti-microbial effects for RNase3 and RNase7, respectively (Torrent et al., 2010).

RNase3 was secreted by activated eosinophils during inflammation, which gave it the initial characteristic name "eosinophil cationic protein (ECP)". This protein, which is related to inflammation, is often released by the degranulation of eosinophils for it to be able to mediate its antibacterial influence, which serves as a marker for some disease diagnosis, such as PFAPA syndrome (Torrent et al., 2010). RNase3 has a high affinity for lipopolysaccharides (LPS) and functions in *Escherichia coli* cell agglutination, which is a major distinctive feature that leads to the death and lyse of *Escherichia coli* cell (Torrent et al., 2010). RNases7 was first identified as a skin antimicrobial peptide due to its major involvement in immune response stimulated by inflammatory agents and bacterial infection at the epithelial tissue level, which includes the gut, genitourinary and respiratory tracts and the skin (Torrent et al., 2010).

RNase3 takes longer incubation period of about 4 hours to cause sufficient damage to microbes, killing about 80% of *Staphylococcus aureus* cells (Torrent et al., 2010). While, within a short incubation period of about 2 hours, *Escherichia coli* and *Staphylococcus aureus* endogenous RNases are released in regards to RNase7 antimicrobial activity (Torrent et al., 2010). This demonstrates RNase7 as an AMP with an extraordinary higher affinity for peptidoglycan leakage and lipopolysaccharide binding capacity than in RNase3 (Torrent et al., 2010).

HEPCIDIN

This is an AMP that was first noticed in urine sample and ultra filtrate (Kwapisz et al., 2009). The word "hep" is carved from hepatocyte where it is synthesized in abundance, while "cidin" signifies its antimicrobial major function (Kwapisz et al., 2009). Several studies suggested the use of hepcidin as a diagnostic and management clinical tool for an array of iron and iron-related test (Kwapisz et al., 2009). Pathomechanism of hepcidin related to several diseases had been confirmed (Kwapisz et al., 2009). Previous investigations reported that using animal prototype suggest its gene encoding (HAMP, 19q13; OMIM 606464) an IL-6-inducible acute phase protein to be a major regulator in absorbing irons, especially during iron metabolism (Roetto et al., 2003; Strnad et al., 2011; kwapisz et al., 2009). It is an important marker in stress inducement (Strnad et al., 2011). HAMP synthesis is carried out by the liver, and expressed significantly first in the liver, to a lesser level in the brain and heart, intestine and very low in tonsils, trachea, lungs, and salivary gland (Kwapisz et al., 2009; Strnad et al., 2011; Osenses, 2017).

However, it plays a major role in the regulation of iron level and posses a weak antimicrobial peptide activity against GAβHS, *Staphylococcus aureus* and *Staphylococcus epidermidis*, strong activity against *Escherichia coli* ML35P, but with not effective on *Pseudomonas auriginosa*. Its AMP activity is also antifungal against *Candida albicans*, *Aspergillus fumigatus* and *Aspergillus niger* (Osenses, 2017; Kwapisz et al., 2009).

Moreover, an invitro study used an animal prototype as mentioned earlier to confirm the major function of hepcidin as a major iron regulator, by decreasing its usage in the intestine, transportation across the placenta and how it is being released from macrophages (Kwapisz et al., 2009). An overloaded iron can control the production of hepcidin in the liver, likewise signals by inflammations, etc (Kwapisz et al., 2009). From the mechanism of its action which is not completely understood, it can also be referred to as an iron-regulatory hormone, which causes low serum level depending on its interactions with ferroportin (a popular iron exporter in mammals) often expressed on liver cells, reticulo-epithelial macrophages surfaces (Kwapisz et al., 2009). This is carried out by hepcidin expression modulation and maintaining iron

modulation and balance (Kwapisz et al., 2009). This iron synthesis by different simulating factors can be divided into negative and positive regulators (Kwapisz et al., 2009).

The positive regulators include; its inflammatory role as a type II acute-phase reactant, which is induced by an inflammatory cytokine IL-6 decreasing the level of serum iron, its absorption and release from macrophages during infection and also its HAMP gene expression regulated by STATS3 and BMP/SMAD pathways (as a signaling molecule), which causes an increase in iron reserve activated by hemochromatosis protein (HFE) (Kwapisz et al., 2009; Osenses, 2017). Its negative regulators includes; hypoxia and anaemia which regulates its expression by regulating erythrocytes production via erythropoietin resulting in a decrease in hepcidin mRNA (Kwapisz et al., 2009; Osenses, 2017).

2.5.4. ANTIMICROBIAL PEPTIDE'S MECHANISM OF ACTION

The host AMP's cationic ability enables them to selectively target microbes, deactivate a portion of the outer membrane, bind and predominantly fit in into the crack they created on the target microbial membranes of these negatively charged microbes. They disrupt the membrane, thereby splitting and killing them directly (Figure 6 and 7) (Mojsoska and Jenssen, 2015; Sarawilcox, 2004; Duplantier and van Hoek, 2013).

FIGURE 6: ANTIMICROBIAL PEPTIDE'S DIRECT KILLING MECHANISM OF ACTION.



(A) A display of membrane disruption by the peptide. (B) A more precise extra/intracellular actions by the molecules (Mojsoska and Jenssen, 2015).

FIGURE 7: THE PROPOSED MECHANISM OF ACTION FOR CATIONIC PEPTIDES (Sarawilcox, 2004).



Generally the cationic antimicrobial peptides first interact with the anions of the outer membrane and displace the magnesium ions present on the surface of the outer membrane. Following this action, they then exert antimicrobial activity by binding tightly to the antigen of interest (anionic membrane lipopolysaccharide (LPS)), neutralize a portion of the membrane and gradually fit into it, change their position across the outer membrane thus; disrupting the membrane (Figure 7) (Sarawilcox, 2004; Duplantier and van Hoek, 2013; Yeaman and Yount, 2003; Hurdle et al., 2011).

FIGURE 8: AN OVERVIEW OF ANTIMICROBIAL'S WIDE-RANGE OF CELLULAR INTERRACTIONS (Duplantier and van Hoek, 2013).



Over 1,480 antimicrobial peptides have been discovered generally, possessing unique characteristics of antiviral, antifungal, anticancer and antibacterial broad-spectrum activities (Wang et al., 2009). Most of them are both amphipathic and cationic and also vary in sizes of about 12-100 amino acids and structure, having both the ability to bind and disrupt microbial membranes, thus; killing them (Figure 6, 7 and 8) (Mojsoska and Jenssen, 2015; Yeaman and Yount, 2003; Hurdle et al., 2011). They also function in chemotaxis of most leukocytes to infectious or inflammatory sites and posses the ability to inhibit both nucleic acid synthesis, cell wall synthesis and protein biosynthesis one's they gained entrance into the bacterial cell

(Duplantier and van Hoek, 2013; Brogden, 2005). A satisfactory biological system interaction is achieved as a result of their cationic design (Zasloff , 2002; Diamond et el., 2008).

2.5.5. EXPRESSION LEVELS OF THE AMPS IN TONSILS OF SOME DISEASED CONDITIONS

The tonsils have the form of a connective tissue-like epithelium (Table 4) that acts as a barrier against invading immunological antigens and serves as a point location where the innate immune system, adaptive immune system and pathogens interact with each other (Karchev, 1988). In this regard, nasal mucosa was screened and the expression of H β Ds-1 and 3 were observed to be downregulated in seasonal allergic rhinitis (SAR) patients using PCR (Bogefors et al., 2013). While, using immunohistocmistry, result revealed an upregulation for H β Ds-1 and 2 in the submucosa and surface epithelium, while, a lesser stain was observed in H β Ds-3 in same SAR patient sample (Bogefors et al., 2012).

LL-37, RNase7, LEAP-1 and 2 expressions by the palatine tonsils in the upper airways were found to be related to inflammatory diseases, which prompt further screening of other AMPs (Bogefors et al., 2012). Below are the expression levels of AMPs in patient tonsils related with some diseased conditions.

A study report from Ball et al. (2007) revealed that the palatine tonsils surface and crypt epithelium from recurrent tonsillitis and control subject express the following antimicrobial cationic peptides; LL-37, HBD-1-3, LEAP-1 and LEAP-2 (Ball et al., 2007). The antimicrobial peptides were confined within fresh tissue section from 5 patients suffering from sleep disorder as control subject and 19 patients of an acute recurrent tonsillitis situation who underwent tonsillectomy and the levels monitored using fluorescent were immunohistochemistry (Ball et al., 2007). However, LL-37 and HBD-1 and 3 was shown to exhibit a downregulated expression level in palatine tonsils of those suffering from acute recurrent tonsillitis in comparison with the healthy control group (Ball et al., 2007). LL-37 peptide synthesis and transcript were observed to be expressed in tonsil epithelium of both groups, but its staining pattern indicated a downregulation at the surface epithelium of recurrent tonsillitis patient (Ball et al., 2007).

Though, a contrast by Song *et al.* (2006) revealed that the downregulation only occurred in control group (Ball et al., 2007). This was thought to be as a result of chronic inflammation of

the tonsil triggering an increased LL-37 expression, while LEAP-2 was upregulated and differ from other AMPs expression pattern which was evident by its multiple transcript expression in both groups (Ball et al., 2007). This can in turn influence the microbiota composition as in the case of Chrohn's disease in which the altered antimicrobial cationic peptide's concentration lead a chronic inflammation of the bowel (Ball et al., 2007).

A group of antimicrobial peptides in the upper airways were studied from tonsil's tissue sections of a recurrent tonsillitis patient and patient suffering from seasonal allergic rhinitis (SAR) (Bogefors et al., 2014). The AMPs were localized using immunohistochemistry staining technique, which revealed a downregulated expression observed in the AMPs, especially Rnase7 and LEAP-2, which was thought to be as a result of T helper 2 cytokines presence dominant in allergic patients (Bogefors et al., 2014). LL-37 showed a low expression too, but anti and pro-inflammatory roles were thought to cause this as LL-37 and H β Ds attracts polymorphous nuclear cells (neutrophils), T cells and monocytes, which in-turn modulate inflammatory process (Bogefors et al., 2014).

Defensins were reported to upregulate inflammatory factors such as interlukin 1 and tumour necrosis factor alpha (TNF- α), but in conjuction with LL-37 and LEAP-2, they cause the downregulation of IL-10 in monocytes as compared to healthy individuals (Bogefors et al., 2014). This proves AMPs roles in both adaptive and innate immune system and susceptibility of allergic patients to respiratory tract infections related to low defense level of the antimicrobial peptides (Bogefors et al., 2014) In contrast to that, study reported by Lauden *et al.* (2011) showed that RNase7 is expressed in tissue biopsies and nasal secretions (Bogefors et al., 2014).

Demir *et al.* (2016) reported also a higher expression level of both LL-37 and H β D-1-3 observed in tonsil tissue sections from 100 children diagnosed with hypertropy (control group) than in 100 recurrent tonsillitis patients (subject group) using immunohistochemistry.

3. MATERIALS AND METHODS.

3.1. STUDY POPULATION

This study was conducted at the Near East University (NEU) Training and Research hospital, an affiliated institution of the Near East University located in Nicosia, Turkish Republic of Northern Cyprus. The project was approved by the Near East University Scientific Research Assessment Ethics Committee (project no: YDU/2016/42-346) for the use of human palatine tonsils. Study included 13 children who were admitted to the hospital within (2012-2016) period, and were divided into two groups: Group 1 included those with periodic fever, apthous stomatitis, pharyngitis and cervical adenitis (PFAPA) syndrome and group 2 included patients with recurrent tonsillitis as a result of Group A beta-haemolytic streptococci (GAβHS). "PFAPA group" comprises of 7 tonsil samples while "GAβHS group" comprises of 6 tonsil samples obtained from participants. After getting all patients' informed consents, patients' database (clinical history, reason for tonsilectomy, vaccination status) was obtained from the hospital information system.

Patients were all from Turkish Republic of Northern Cyprus. The study did not cover any patient record from 2017 (study year) because of the rear existent nature of PFAPA syndrome. The PFAPA group were diagnosed according to the modified diagnostic criteria proposed by Thomas *et al.* (1999) (2.2.1 Table 2), as there is no particular laboratory test but mainly by physical examination (Phillip, 2017; Brown et al., 2010; Førsvoll and Øymar, 2007). Majority of PFAPA patients had late-onset of fever while few of them had an early-onset. Those patient treated with corticosteroids (single dosage of prednisone (0.5–2mg/Kg)) and cured were excluded from the study.

On the other hand, for GA β HS, subjects underwent a general laboratory test at the NEU hospital for their disease/cases to be identified. Children diagnosed with PFAPA syndrome and recurrent tonsillitis due to GA β HS at the hospital had their tonsils surgically removed. The surface epithelium was immediately dissected, instantly fixed in formaldehyde and stored as paraffin embedded tissues at -4^oC in order to preserve its morphology.

3.2. SAMPLE PREPARATIONS

Fixed Formalin Paraffin Embedded (FFPE) samples were obtained by fixing dissected tonsils obtained from tonsillectomy in 10% formaldehyde solution for about 24 hours. This method produces a chemical cross-linking of proteins within the tissue, in order to prevent degradation of the sample and in turn preserves its morphology by terminating all ongoing

processes in the cell and freezing the components in order to keep them as they were at the time they were fixed.

After fixation, the tissues were further processed by being dehydrated and embedded in plastic cassettes using hot paraffin and stored as frozen paraffin blocks at -10° C. This can be preserved for years for further immunohistochemistry procedure. The process is known as paraffin embedding.

3.3. IMMUNOHISTOCHEMISTRY

Antimicrobial expression levels in tonsil samples isolated were compared by immunohistochemistry technique performed at NEU Hospital Pathology Laboratory using the avidin-biotin complex immunoperoxidase (Vectostain^(R) Elite^(R) ABC-HRP Kit; Vector Laboratories, Burlingame, CA) procedure which exploits the high affinity binding between biotin and avidin. Materials used for this study comprises of both apparatus, patient's sample, the primary, secondary and isotype control antibodies used and chemicals, listed in details in (Appendix 4).

This technique was implemented on both the right (labelled 1) and left (labelled 2) Fixed Formalin Paraffin Embedded (FFPE) palatine tonsil tissue block samples. All primary antibodies were applied using same IHC procedure on all tissue samples after the fixation and paraffin embedding. Thereafter, images were obtained by using a Light microscope (AxioCam HRc Zeiss Scope A_1) using an appropriate magnification and filter sets.

The basic technique involved in IHC protocol used in this study consists of the following steps: (1) fixation – for the maintenance of the morphology, (2) antigen retrieval – to increase availability of proteins for detection, (3) blocking – to minimize endogenous peroxidase activity and irritating or poor background signals and (4) antibody labeling and visualization – viewing under a light microscope in other to obtain results. It entails other minor steps too as well. Thus;



FIGURE 9: IMMUNOHISTOCHEMISTRY SCHEMATIC BASIC STEPS. (1110*432-canertm.com).

The FFPE tissue blocks were sectioned using a microtome set into thin sections of $4.0\mu m$, floated out on hot water bath to flatten, and mounted on a poly-L-lysine coated microscope labeled glass slide. This allows the tissue section to stick firmly onto the slides. Tissue sections were further incubated for 1 hour at 70°C to dry up and deparaffinized/dewaxed in 3 equal concentrations of xylene solution for 5 minutes each, then rehydrated in 3 equal concentrations of 96% ethanol for 3 minutes each, thereafter rinsed in distilled water for 5 minutes to remove any remaining alcohol.

The next stage is "Antigen retrieval", a process whereby those methylene bridges (-CH₂) formed by endogenous peroxidase in the tissue during fixation which mask the antigen "formalin-induced antigen cross-linking" are broken down in order to unmask the antigen's epitope for antibody to recognize and bind. This method can be achieved using either a proteolytic digestion method whereby proteolytic enzymes, or trpsin are used to incubate the tissue sections or a Heat Induced Epitope Retrieval (HIER) method (most commonly used method in the laboratory), whereby heat is applied onto the tissue sections for incubation using either a microwave or a pressure cooker containing an acidic buffer (pH 6) or an alkaline buffer (pH9). Some antigens respond better to one buffer or the other, while some antibodies are more active in one method over the other.

For this study, the HIER was applied using a pressure cooker containing a pre-treatment solution (antigen retrieval agent/buffer) also referred to as the target solution (a mixture of 1800ml distilled water+100ml Target Retrieval solution of high pH (50x)+100ml Target Retrieval solution of low pH (50x)). This was used to standardize the HIER by heating up the tissues sections on the slides in a pressure cooker containing the target solution with the lid covered and programme set for about 20 minutes, and thereafter, allowed to cool at room temperature for 20 minutes also. The tissue sections were washed in distilled water for 5 minutes to cool down before washing 3 times for 5 minutes also in Tris-buffered saline (TBS) wash buffer, while changing the solution after each washing. 2 Liters of TBS wash buffer used was formulated at the laboratory by mixing 1900ml of distilled water+100ml of TBS solution (Tris-buffered saline reference standard of pH7 (Sigma-Aldrich laboratory, Turkey)).

After carefully cleaning the slide, hydrophobic pen was used to draw a hydrophobic barrier around the tissue section. This helps to prevent non-specific interactions of the antibodies and to also allow the use of minimal amount of antibody. The pre-treated tissue sections were then blocked with 10 drops of peroxidase blocking solution (("super block" 125ml): Scy Tek laboratories, Logan, Utah, USA), and incubated in a humidity chamber for 10 minutes in order to terminate endogenous peroxidase activities that may cause a high background staining, leading to a false positive stained results, terminates also any endogenous peroxidase enzyme in the tissue in order to prevent it from reacting with the chromogen that will be applied later in the process. This is then washed in TBS wash buffer 3 times for 5 minutes each and rinsed with distilled water for 5 minutes, then wiped away to remove any excess liquid on the slide. Following blocking, tissue sections were incubated with diluted primary antibodies all purchased from Abcam and diluted in antibody diluent, thus: anti-beta defensing-1-ab14425 (Mouse monoclonal to HBD-1 [M11-14b-D10]) 1:500 diluent; anti-beta defensing 2-ab63982 (Rabbit polyclonal to H_βD-2) 1:500 diluent; anti-cathelicidin-ab69484 (Rabbit polyclonal to LL-37) 1:200 diluent; anti-ribonuclease 7-ab205565 (Mouse monoclonal to RNase7 [4C4]) 1:100 diluent; antihepcidin-ab57611 (Mouse monoclonal to hepcidin) 1:100 diluent and anti-LEAP-2ab122294 (Rabbit polyclonal to LEAP-2) 1:200 diluent. However, for the isotype control, anti-Rabbit IgG (polyclonal isotype control antibody)-ab27478 (Rabbit polyclonal to Rabbit IgG) 1:200 diluent was used for the negative control for primary antibody.

However, positive control sample included in this study are: kidney was used for H β D-1, pancrease for H β D-2, spleen for LL-37, skin for RNase7, liver for hepcidin and skeletal muscles for LEAP-2 as instructed on products data sheet.

The samples were allowed to incubate for 1 hour 30 minutes each at room temperature (25°C) in the humidity chamber with the lid covered, then washed in TBS wash buffer 3 times for 5 minutes each. With the slides placed back into the humidity chamber lying flat, the tissue sections were incubated with diluted secondary antibodies specific to the primary antibodies used, which are directly conjugated to a horseradish peroxidase (HRP). This is achieved by adding 10 drops of Sensitek anti-polyvalent biotinylated secondary antibody (Scy Tek laboratories, Logan, Utah, USA) and allowed to incubate for 20 minutes in the same humidified chamber, then, washed in TBS wash buffer 3 times for 5 minute each and placed back into the humidity chamber.

After the wash steps, 10 drops of 3,3'-Diaminobenzidine (DAB) chromogen mixture (1drop of peroxidase substrate solution "DAB substrate chromogen" + 1ml of DAB substrate buffer) (DAB stain kit, Vector Labs, Burlingame, CA) was applied immediately unto the tissue sections on the slides using a pipette, so that the reaction occurs on the slide and incubated for 5 minutes (timing depends on the desired intensity to be achieved) in the moist humidity chamber then rinsed afterwards with distilled water lightly. DAB is a substrate of HRP, and provides a brown colouration, which is insoluble in alcohol and xylene, for the antigen-antibody complex formed for permanent mounting of a tissue sections. In order to achieve a proper visualization of the immunostain in addition to the DAB detection process, 6 drops of Mayer's hematoxylin counterstain was added onto the tissue sections on the slide and incubated for 5 minutes. This stains the cell nuclei blue and provides a contrast to the brown colour of the DAB chromogen for better visualization, thereby, providing a proper definition to the cellular morphology and nuclei visualization of which it is best achieved using hematoxylin. Sections were washed in distilled water 2 times for 5 minutes each afterwards. In order to view a slide that was already stained permanently with a permanent stain such as DAB chromogen, the sections had to be dehydrated again in 3 equal concentrations of 96% ethanol for 10 seconds each and airdried for few minutes then dewaxed in 3 equal concentrations of Xylene solution for 10 seconds each also.

Following counterstaining of the tissue sections, visualization process was initiated. Three drops of Entalian mounting media was applied onto the slide's tissue section, then, covered with a coverslip carefully while avoiding bubble formation. An AxioCam HRc Zeiss Scope A_1 light microscope was used for viewing using an appropriate magnification and filter sets of 200X for all, except for Rabbit IgG, for which 100X magnification was preferred for the examination of protein expression. Results were obtained and recorded using a semi-quantitative method (Chapter 3.4).

3.4. IMMUNOHISTOCHEMISTRY SEMI-QUANTITATION METHOD FOR SCORING (POSITIVE CELL DISTRIBUTION) AND STAINING INTENSITY OF THE CELLS (%)

The staining intensity was scored using a previously reported semi-quantitation published analysis method (Wang CH, Zhao J and Qiao C, 2012). After the immunohistochemistry staining procedure, 10 fields were chosen at random; expression pattern was evaluated in 1000 cells (100cells/field) using a high-powered (200X) microscopic magnification. Individually, stains were roughly evaluated to get their mean value, which was used, for analyzing the results.

The mean value of each investigator's scores was rated as follows; the samples with 0% no positive cell, 1-10% positive cell, 11-50% positive cell, 51-80% and 81-100% positive cell were scored as 0, 1, 2, 3 and 4, respectively. The intensity of the stained cells was rated on a scale of (0-3). Negative, weak, moderate and strong stained samples were rated as 0, 1, 2 and 3 respectively. Final AMPs expression level score (%) was obtained by multiplying the percentage of positive cell distribution (PCD) with the staining intensity (SI). Average of these values obtained for each AMP was used for statistical analysis computation using the SPSS version 18.0.0 installed on windows 10 (Chapter 3.5).

3.5. STATISTICAL ANALYSIS.

IHC staining result (data) obtained from the semi-quantitation analysis result (Table 6) was analyzed for statistical significance. Data did not meet parametric conditions, thus Mann-Whitney U test (non-parametric method was applied). The program IBM SPSS, PASW statistics version 18.0.0 installed on windows 10. Chicago IL, USA: SPSS Inc. IBM Corp. Released 2010 was used to obtain a statistically significant calculated value (P).

Calculated p-value is represented in Appendix 10. Detailed result is documented in Appendix 9-10. This study hypothesized that, (Null hypothesis) H_0 =There is no statistical significant difference between the AMPs expression level in patient group and control group. Any significant association was supposed as p value <0.05

4.0. RESULTS.

4.1. CHARACTERISTIC OF STUDY GROUPS

A total of 13 pathological tonsil samples of children who underwent tonsillectomy due to PFAPA syndrome conditions (PFAPA group) and recurrent tonsillitis due to group A betahaemolytic streptococci infections (GA β HS group) were included in this study and examined/screened using immunohistochemistry staining protocol. The PFAPA group comprises of 7 participants of which 4 were female and 3 were male, while, the GA β HS group is a total of 6 participants with 2 females and 4 males. PFAPA group age ranges between 3-10 years with a mean age of 5.16 years, while that of GA β HS group is 6-13 years with a mean age of 8.35 years. A statistical significant difference was observed between both groups (P=0.022) in regarding age. The median age (4.5) years old in PFAPA patient showed that the patient are much more younger than those observed in GA β HS group, with the median age (8.1) years old. In regards to gender, there was no statistical significant difference (P=0.592) detected between PFAPA group and GA β HS group.

4.2. AMPs EXPRESSION LEVELS OF PFAPA GROUP AND GAβHS GROUP IN TONSIL TISSUE SECTION SAMPLES

From the slides we were able to see that the staining intensity expressed by all AMPs were prominent on and around the germinal center and the lymphoid follicle of the tonsil's tissue morphology only. So the pictures were retrieved from those locations. There was no difference observed for each AMP that was tested being that they all stained the germinal center and lymphoid follicle at the same levels.

H β D-1 expressed low staining intensity with no difference between both groups (P=0.708). A strong staining intensity for AMP expression on the germinal center and lymphoid follicle were observed for H β D-2, LL-37, and RNase7 expression level with no difference between both groups (P=0.725, 0.172 and 0.584) respectively. The highest staining intensity level around the germinal center and lymphoid follicle was observed for Hepcidin, which was significantly higher in GA β HS patient group in contrast with its expression in PFAPA patient

group (P=0.033). There was no positive expression observed for Rabbit IgG (isotype control) for negative control and LEAP-2 in both PFAPA group and GA β HS group (Figure 10). Including the positive control used which was skeletal muscle tissue.

FIGURE 10: IHC STAINED SLIDES OF AMPS EXPRESSION PATTERN LEVELS OF BOTH GROUPS IN TONSIL TISSUE SECTION SAMPLE.





Fig. 10: Tonsils tissue section stained slides obtained from PFAPA syndrome patient (PFAPA group) and recurrent tonsillitis patient due to group A beta-haemolytic streptococci (GA β HS group) were stained with appropriate primary antibodies against AMPs to be monitored as follows: H β D-1 (A), H β D-2 (B), LL-37 (C), RNase7 (D), Hepcidin (E), LEAP-2 (F), Rabbit IgG (Isotype control) as negative control (G) for GA β HS group and H β D-1 (H), H β D-2 (I), LL-37 (J), RNase7 (K), Hepcidin (L) and Rabbit IgG (Isotype control) as negative control (M) for PFAPA group respectively. Appropriate isotype control (Rabbit IgG/mAb) antibodies were used for negative control. An AxioCam HRc Zeiss Scope A₁ microscope was used for viewing with a magnification of 200X for all except Rabbit IgG, which was viewed using 100X.

FIGURE 11: HISTOGRAM REPRESENTATION FOR THE MEAN AND SEM SEMI-QUANTITATION EXPRESSION SCORES IN BOTH GA β HS AND PFAPA GROUP.



Fig. 11: The histogram represents the averaged AMPs expression levels of semi-quantitation (%) plotted using the Mean and Standard Error of Mean (SEM) scores obtained from statistical analysis results of (Appendix 9) for 7 tonsils samples of PFAPA syndrome patient and 6 control group. The arrows represents the SEM while, the Bars represents the mean values obtained, in regards to the histogram. They are respectively represented as follows: H β D-1 (A), H β D-2 (B), LL-37 (C), RNase7 (D) and Hepcidin (E). No histogram representation for Rabbit IgG and Leap-2 as they all showed 0.00 values for both Mean and SEM in both groups.

5.0. DISCUSSION.

PFAPA syndrome is a very rare syndrome generally occurring in about 2/10,000 children (Tejesvi et al., 2016; Cheung et al., 2017). It is thought to be an autoinflammatory disease with re-occurrencing fever episodes. It is the most common syndrome among the autoinflammatory diseases, with no infectious cause, and an unknown etiology which is yet to be discovered (Brown et at., 2010; Ahmed et al., 2014). It is more prevalent around the Eastern Mediterranean such as Turkey and Cyprus (Cheung et al., 2017, Batu et al. 2016) as well as in Western Europe (Batu et al., 2016).

Children treated with corticosteroid and cured were not included in this study. PFAPA syndrome patient, were diagnosed as in other studies in accordance with Marshall *et al.* (1989) diagnostic criteria modified by Thomas *et al.* (1999) while, those who tested positive for group A beta-haemolytic streptococci were also included in this study. But since healthy tonsil samples were not available for this study, a recurrent tonsillitis patient's tonsil sample was used to compare AMP expression with the results observed in this study.

Data were only retrieved from 2012-2016 with an informed consent from the hospital record. Recurrent tonsillitis, a differential diagnosis of this syndrome has been studied previously to aggravate PFAPA syndrome fever episodes (Vanoni et al., 2016). In previous studies, a downregulation was observed in the level of some of the antimicrobial peptides (H β D-1-2, LL-37, RNase7, Hepcidin, LEAP-2) known to be expressed by the tonsil's epithelium (Tejesvi et al., 2016; Bogefors et al., 2014Ball et al., 2017; Ball et al., 2004).

Since, previous observations of altered AMP production produced by epithelial cells was suggested to give rise to disease such as Crohn's disease (Lantto et al., 2015), a total of 13 tonsil samples (age and sex-matched) obtained from 7 PFAPA syndrome patient (PFAPA group) and 6 patient who administred hospital because of recurrent due to group A beta-haemolytic streptococci infection (GA β HS group) were screened using immunohistochemistry staining protocol to monitor the levels of antimicrobial peptides expression. Rabbit IgG, an isotype control antibody was used as a negative control to confirm the specificities of the

primary antibody produced in rabbit. On the other hand, for primary antibodies produced in mouse, untreated samples were used as negative control.

AMPs are known to exhibit broad-spectrum antimicrobial activities against immunological antigens, induce the release of cytokines production such as IL-1 and also bring down IL-10 levels at contact with infections caused by microbes (Gazi, 2016; ImIr, 2016). They function also in chemotaxis especially the β -defensins, etc. This is often associated with their expression levels detected in tissues (Pacova et al., 2010; Bogefors et al., 2014; Diamond and Ryan, 2011). The presence of an antigen can induce expression of AMP, which function as the host's defense peptides (Ahmed et al., 2016; Bogefors et al., 2014).

Weak stain was detected in lymphoid follicle and germinal center of the tonsil tissue section for H β D-1 in PFAPA group and GA β HS group in this study. This correlate with Ball et al. (2007) results, since the expression was reported to be localized to the surface epithelium of palatine tonsils for both GA β HS group and PFAPA group. Considering the fact that tonsillitis samples used in this study, showed similar immunohistochemistry staining pattern of H β D-1 to be lower than GA β HS group (Ball et al., 2007), these findings indicate that PFAPA patients in comparison to healthy subjects are more susceptible to antimicrobial assaults of tonsil epithelium. In regards to H β D-1 expression level, Pacova *et al.* (2010) reported that H β D-1 is highly triggered by the presence of *Staphylococcus aureus* in healthy nasal mucosa than in healthy tonsil with a low expression though, which is true for this study, even though a diseased tonsil tissue sample was used, it still showed a weak expression level.

On the other hand, H β D-2, and LL-37 expression levels seemed to be different than the one reported by Ball *et al.* (2007). In this paper, these AMPs were mainly expressed by the surface epithelial cells, while our study observed a strong signaling in follicular tissue and germinal centres. Same was reported by Demir *et al.* (2016) in which IHC results reported high level H β D-2 expression in tonsilar surface epithelium.

This can either be due to a real situation or an artifact such as different clones of the same antibodies and techniques, which were used for detection of their expression levels. Technique may include wrong titre for the concentrations used during the IHC protocol procedure, as different working concentrations of primary antibodies were tried; ranging from initial recommended 1:200 dilution (1 vol. of antibody + 199 vol. of antibody diluent) to 1:95 (1 vol. of antibody + 94 vol. of antibody diluent) dilution for LEAP-2, specificity of the antibodies were also checked, but same negative results were observed. IHC procedure was also repeated twice for both the anautomated Ventona Benchmark XT machine and manual method each, with no positive result obtained.

In the referred study, for LEAP-1 expression pattern, PCR was used, while for the rest of the screening, IHC procedure was applied, as also reported by Bogefors *et al.* (2014) for LEAP-2 The other reason may be due to age differences between the subjects. While PFAPA group were much younger with median age of 4.5 years, GA β HS group was observed to be of much more older group with a median age of 8.1 years. Statistically, there is significant difference (P=0.022) between both groups. Although, significant changes in antimicrobial peptide generation have not been properly documented, the possible cause cannot be excluded without confirmative studies (Ponnappan and Ponnappan, 2011). There was also no statistical significant difference difference (P=0.592) between both groups in regards to gender.

The expression pattern was the same between the tonsillitis patient in GA β HS group and PFAPA patients in PFAPA group that were included in this study, except for hepcidin expression levels, which could be as a result of the major role it plays in iron regulation and strong antimicrobial activity against GA β HS, which might have induced its expression as observed in other studies (Kwapisz et al., 2009; Osenses, 2017). Statistically, a significant difference (P=0.033) was observed in this study in the expression pattern of hepcidin between both groups. The PFAPA patient had lower level of AMP expression. These antimicrobial peptides were shown to be effective against mainly Gram-positive bacteria, but were also shown to inhibit the growth of *Neisseria cinerea* and *Saccharomyces cerevisiae*, which is a similar mode of activity as H β D-1. In contrast to this, it was not able to suppress the yeast *Rhodotorula rubra* and Gram-negative bacteria *Escherichia coli BL21* and *Pseudomaonas fluorescens* growth (Krause et al., 2000). This suggests that PFAPA patients, due to the lower level of hepcidin expression, may have higher the production of AMPs, the more the protection against immunological antigens such as bacteria (Bogefors et al., 2014). However, for more precise conclusion, more studies are encouraged.

In addition, altered AMP concentration is also known to alter normal flora, thereby giving rise to disease states such as Crohn's disease (Lantto et al., 2015). So, this difference in hepcidin also indicates that normal flora composition may have also been altered. This can further be investigated by

microbiota study in future. Besides these findings, this study is the first to show the RNase7 expression levels in both tonsils from tonsillitis patients and PFAPA patients. High level of expression was observed on lymphoid follicle's region and the germinal center. RNase7 mechanism of action has been studied previously to establish a broad range of antimicrobial activity on pathogens such as protozoans, viruses, bacteria and fungi, with the exception of helminthes (Torrent et al., 2010; Mojsoska and Jenssen, 2015; Sarawilcox, 2004). Endogenous RNase7 on release takes a short time to dis-membrane *Escherichia coli* and *Staphylococcus aureus*.

One of the weaknesses of this study is the lack of control group, which we could not have during the study. This could not be provided by the hospital, since healthy individuals don't often undergo tonsillectomy. So, an inflamed tonsil sample was used for confirmation. If healthy sample was available, there might still be some expressions observed. However, future studies with the inclusion of control samples and higher sample number i.e the availability of a larger cohort (which is the other weakness of this study) will definitely help to draw more precise conclusions. LEAP-2 expression could not be detected in this study's patient samples as well as the positive control. For positive control, skeletal muscle was preferred as suggested by the product's manual. However, for future studies, using different working concentrations will be for sure a great help to set up a protocol in our laboratories. Other positive control included in this study are as follows: kidney (H β D-1), pancreas (H β D-2), spleen (LL-37), skin (RNase7) and liver (hepcidin).

6.0. CONCLUSION AND RECOMMENDATION.

6.1. CONCLUSION

In conclusion, this study identified a novel peptide "RNases7" with a strong expression in both group's tonsil samples. In accordance with this finding, results obtained from IHC staining protocol of both groups suggest that AMPs expression level is expressed in tonsil epithelium, except LEAP-2, of which possible reasons associated with its non expression has been broadly discussed in the discussion part of this manuscript. This should be taken care of in future studies using a better positive control sample and working concentration.

The significant difference (P<0.05) P=0.033 obtained in hepcidin expression level suggest the influence of microbes such as GA β HS, being that it was established in previous studies that the presence of antigen can trigger AMP's expression. This proves that AMPs play major role in defense mechanism by increasing greatly in surface epithelium in other to prevent pathogens invasion. The levels of rabbit IgG and LEAP-2 were not expressed despite several efforts applied as explained in "Discussion". This does not imply that they do not initiate an influence in the tonsil, as previous studies had already proven their expression especially in healthy tonsil sample. Therefore, their non-expression in this study could also be as a result of the unavailability of a healthy tonsil sample of which the hospital could not provide.

Also, this study suggest that the no statistical significant different (P>0.05) observed in the expression level of H β D-1-2, LL-37, RNase7, LEAP-2 showed that PFAPA group have equal chances of expressing their tonsilar AMPs as well as GA β HS group. There is a statistical significant difference in the age of the participants in both groups, while gender recorded a no significant difference was observed in both groups.

From this conclusion it can be suggested that AMPs expression may contribute to the underlying mechanism involved in PFAPA syndrome pathomechanism. The etiological cause of PFAPA could not be detected till date, that was why it was thought to be an AID. So, if the microbiota is altered as a result of a change in AMP expression levels, then this study suggest that that might be PFAPA syndrome cause also, as in Crohn's disease case.

6.1. RECOMMENDATION

The limitation of this study such as the availability of a healthy tonsil sample for the confirmation restrict its strong conclusion on the expression level of some of the AMPs screened, such as LEAP-2 which could not be expressed. RT-PCR can be used for mRNA extraction in the future as well to see if the change in hepcidin is associated with an altered microbiota. Therefore, with this limitations observed, future study can look into such pending issues.

AMPs mechanism of action should be studied in more advanced manner further. Other antibodies should be tried using different tissue samples and relating it to PFAPA syndrome but with similar case so that a wider view is provided. Also, antibodies specificities should be checked with the antigen to see if it will result to an immune complex before embarking on a research.

Also, cohorts should be expanded at random so as to build better awareness of PFAPA syndrome and provide more comprehensive information on the epidemiology and better treatment option in Northern Cyprus and the world at large, as participants comprising of both patient and control group are limited in this study.

Pediatric specialist, pathologist, and laboratory assistant who evaluate PFAPA syndrome incidence and recurrent tonsillitis associated with oral cavities and the upper airways should have more intense training programs such as seminars, workshops, etc, so that they can be more efficient and effective when it comes to differentiating between recurrent tonsillitis due to infection and vice versa, PFAPA syndrome from other AIDs as they both have overlapping symptoms.

APPENDIX 1. ETHICAL APPROVAL FORM FOR THE RESEARCH STUDY.



YAKIN DOGU UNIVERSITESI

BILIMSEL ARASTIRMALAR DEGERLENDIRME ETIK KURULU.

ARASTIRMA PROJESI DEGERLENDIRME RAPORU.

Toplanti Tarihi : 22.12.2016.

Toplanti No : 2016/42.

Proje No : 346.

Yakin Dogu Universitesi Tip Fakultesi ogretim uyelerinden Yard. Doc. Dr. Umut Gazi'nin sorumlu arastirmacisi oldugu, YDU/2016/42-346 proje numarali ve "**PFAPA hastalarinda tonsil AMP ve TLR ekspresyon duzeyleri**" baslikli proje onerisi kurulumuzca degerlendirilmis olup,etik olarak uygun bulunmustur.

1. Prof. Dr. Rustu Onur	(BASKAN)
2. Prof. Dr. Nerin Bahceciler Onder	(UYE)
3. Prof. Dr. Tamer Yilmaz	(UYE)
4. Prof. Dr. Sahan Saygi	(UYE)
5. Prof. Dr. Sanda Cali	(UYE)
6. Prof. Dr. Nedim Cakir	(UYE)
7. Prof. Dr. Kaan Erler	(UYE)
8. Prof. Dr. Cetin Lutfi Baydar	(UYE)
9. Doc. Dr. Umran Dal	(UYE)
10. Doc. Dr. Eyup Yayci	(UYE)
11. Doc. Dr. Nilufer Galip Celik	(UYE)
12. Yrd. Doc. Dr. Emil Mammadov	(UYE)

APPENDIX 2.A. QUESTIONNAIRE IN ENGLISH.

1ST CASE: TONSILLECTOMY DUE TO PFAPA SYNDROME (PFAPA GROUP). Patient name and surname: Gender: Male (M), Female (F). Date of birth: DD....MM....YY..... Hospital ID. No: Laboratory protocol No:

Questions: When was the on-set of fever? How often do the recurrent tonsillitis occur?

Symptoms: (Mark/Tick the symptoms you have). Cervical adenitis Pharyngitis Apthous stomatitis Periodic fever

Laboratory test: Microbiology test for Throat culture: Positive or Negative.

Treatment:		
Steroid:	Yes or No?	Length of treatment
Tonsillectomy:	Yes or No?	Date of Surgery

2ND CASE: RECURRENT TONSILLITIS DUE TO GROUP A BETA-HAEMOLYTIC STREPTOCOCCI (GAβHS GROUP). Patient name and surname: Gender: Male (M), Female (F). Date of birth: DD...MM....YY..... Hospital ID. No: Laboratory protocol No:

Questions: When was the on-set of fever? How often do the recurrent tonsillitis occur?

Symptoms: (Mark/Tick the symptoms you have). Upper respiratory tract infection (snoring). Pharyngitis (sore throat). Periodic fever.

Laboratory test: Microbiology test for Throat culture: Positive or Negative. Hematology test result for white blood cell (WBC) Biochemistry test for C-reactive protein (CRP)

Treatment:

Steroid:	Yes or No?	Length of treatment
Tonsillectomy:	Yes or No?	Date of Surgery

APPENDIX 2.B. QUESTIONNAIRE IN TURKISH.

1ST CASE: RECURRENT TONSILLITIS DUE TO PFAPA SYNDROME (PFAPA HASTA GRUP). Hasta Adi soyadi: Cinsiyet: Erkek (E), Kiz (K). doğum tarihi: YIL.....AY..... Hasta ID. No: Laboratuvar protocol No:

Questions: Ne ates epizodlariin basladigi yas (Ay)? ne sıklıkla recurrent tonsillitis meydana gelia?

Semptomlar: (Isaret/kene semptomlar var). Adenit. Farangit. Aftoz stomatit. Ates

Laboratuvar sonuclari: Mikrobiyoloji sonuc (Bogas kulturu):

Pozitif or Negatif.

Tedavi:	
Kacinci steroidden sonar tonsillektomi?	Evet or Hayir?
Tonsillektomi kacinci ataktan sonar yapildi?	Evet or Hayir?

2ND CASE: RECURRENT TONSILLITIS DUE TO GROUP A BETA-HAEMOLYTIC STREPTOCOCCI (GAβHS GRUP). Hasta Adi soyadi: Cinsiyet: Erkek (E), Kiz (K). doğum tarihi: YIL.....AY..... Hasta ID. No: Laboratuvar protocol No:

Questions: Ne ates epizodlariin basladigi yas (Ay)? ne sıklıkla recurrent tonsillitis meydana gelia?

Semptomlar: (Isaret/kene semptomlar var). Solonum enfeksyon (Horlama/horultu). Farangit. Ates

Laboratuvar sonuclari: Mikrobiyoloji sonuc (Bogas kulturu): Hematoloji sonuc (WBC)..... Biyokimiya sonuc (CRP)

Tedavi:	
Kacinci steroidden sonar tonsillektomi?	Evet or Hayir?
Tonsillektomi kacinci ataktan sonar yapildi?	Evet or Hayir?
APPENDIX 3: BIBLIOGRAPHY.

Immune defense system- is a host **defense system** comprising of many biological structures and processes within an organism that protects against disease.

Immunological antigens- is a molecule capable of inducing an **immune** response on the part of the host organism, though sometimes **antigens** can be part of the host itself. In other words, an **antigen** is any substance that causes an **immune** system to produce **antibodies** against it.

Periodic fever syndromes-are a set of disorders characterized by **recurrent** episodes of systemic and organ-specific inflammation. ... Most autoinflammatory diseases are genetic and present during childhood.

Apthous stomatitis- is a common condition characterized by the repeated formation of benign and non-contagious mouth ulcers (aphthae) in otherwise healthy individuals. The informal term canker sores is also used, mainly in North America, although this may also refer to any mouth ulcer.

Pharyngitis (/fariŋ'dʒʌitis/)- inflammation of the pharynx, causing a sore throat.

Cervical adenitis (Adenopathy)- is an inflammation of a lymph node in the neck.

Juvenile idiopathic arthritis (JRA)- often referred to by doctors today as **juvenile idiopathic arthritis** (**JIA**), is a type of **arthritis** that causes joint inflammation and stiffness for more than six weeks in a child aged 16 or younger. It affects approximately 50,000 children in the United States

Behçet's disease (BD)- is a type of inflammatory **disorder**, which affects multiple parts of the body. The most common symptoms include painful mouth sores, genital sores, inflammation of parts of the eye, and arthritis. ... **Behçet's** is not contagious.

Cyclic neutropenia- is a disorder that causes frequent infections and other health problems in affected individuals. People with this condition have recurrent episodes of **neutropenia** during which there is a shortage (deficiency) of neutrophils.

Chrohn's disease (/'krəonz dɪ,zi:z/)- is a chronic inflammatory disease of the intestines, especially the colon and ileum, associated with ulcers and fistulae.

Differential diagnosis- is the process of weighing the probability of one disease versus that of other diseases possibly accounting for a patient's illness. The **differential diagnosis** of rhinitis (a runny nose) includes allergic rhinitis (hayfever), the abuse of nasal decongestants and, of course, and the common cold.

Autoinflammatory disease- is **defined** as **illness** caused by primary dysfunction of the innate immune system. This new concept includes a broad number of **diseases**, initially focusing on hereditary recurrent fevers such as the prototype FMF, Familial Mediterranean Fever.

Autoantibodies- an antibody produced by an organism in response to a constituent of its own tissues.

Autoimmune diseases- A *disease* in which the body produces antibodies that attack its own tissues, leading to the deterioration and in some cases to the destruction of such tissue. **T cells (T** lymphocyte)- is a type of lymphocyte (a subtype of white blood **cell**) that plays a central role in **cell**-mediated immunity. **T cells** can be distinguished from other lymphocytes, such as B **cells** and natural killer **cells**, by the presence of a **T-cell** receptor on the **cell** surface.

Etiology of disease- cause, origin; specifically : the cause of a disease or abnormal condition.

IL-1 blockade- especially IL-1 β) is a standard therapy for patients with autoimmune diseases or lymphomas. Anakinra.

Th1- The **T** helper cells (T_h cells) are a type of T cell that play an important role in the immune system, particularly in the adaptive immune system. They help the activity of other immune cells by releasing T cell cytokines. These cells help suppress or regulate immune responses. They are essential in B cell antibody class switching, in the activation and growth of cytotoxic T cells, and in maximizing bactericidal activity of phagocytes such as macrophages.

Innate immunity- The *innate immune system* is always general, or nonspecific, *meaning* anything that is identified as foreign or non-self is a target for the *innate immune* response. The *innate immune system* is activated by the presence of antigens and their chemical properties.

Corticosteroid (/ kɔ:tɪkəʊ'stɪərɔɪd/)- any of a group of steroid hormones produced in the adrenal cortex or made synthetically. There are two kinds: glucocorticoids and mineralocorticoids. They have various metabolic functions and some are used to treat inflammation.

C10X – Polymorphism in CARD8 genes associated with inflammatory activities.

Tonsilectomy (/tɒnsɪ'lɛktəmi /)- is the surgical removal of the tonsils, two oval-shaped pads of tissue at the back of the throat — one tonsil on each side. A *tonsillectomy* was once a common procedure to treat infection and inflammation of the tonsils (tonsillitis).

C-reactive protein (**CRP**)- is one of the plasma **proteins** known as acute-phase **proteins**: **proteins** whose plasma concentrations increase (or decrease) by 25% or more during inflammatory disorders. **CRP** can rise as high as 1000-fold with inflammation.

Erythrocyte Sedimentation Rate- is the **rate** at which red blood cells **sediment** in a period of one hour. It is a common hematology test, and is a non-specific measure of inflammation.

Leukocyte concentration- is a heterogeneous group of nucleated cells that can be found in circulation for at least a period of their life. Their normal *concentration* in blood varies between 4000 and 10,000 per microliter.

Syndrome (/sindrəom/)- can be referred to as a group of signs and *symptoms* that occur together and characterize a particular abnormality.

Tonsillar disease (Tonsillitis)- is inflammation of the tonsils, typically of rapid onset. It is a type of pharyngitis. Symptoms may include sore throat, fever, enlargement of the tonsils, trouble swallowing, and large lymph nodes around the neck. Complications include peritonsillar abscess. It is most commonly caused by a viral infection, with about 5% to 40% of cases caused by a bacterial infection. When caused by the bacterium group A streptococcus, it is referred to as strep throat.

Tonsils- is defined as either of a pair of prominent masses of lymphoid tissue that lie one on each side of the throat between two folds of soft tissue that bound the fauces.

Palatine tonsils- is a pair of soft tissue masses located at the rear of the throat (pharynx). Each **tonsil** is composed of tissue similar to lymph nodes, covered by pink mucosa (like on the adjacent mouth lining). Running through the mucosa of each **tonsil** are pits, called crypts.

Lympho-epithelial- consisting of lymphocytes and epithelial cells lymphoepithelial tissues.

Antimicrobial cationic peptide (AMPs)- also called host defense peptides (HDPs) are part of the innate immune response found among all classes of life. These peptides are potent, broad-spectrum antibiotics, which demonstrate potential as novel therapeutic agents. Antimicrobial peptides have been demonstrated to kill Gram-negative and Grampositive bacteria, enveloped viruses, fungi and even transformed or cancerous cells.^[1] Unlike the majority of conventional antibiotics it appears as though antimicrobial peptides may also have the ability to enhance immunity by functioning as immunomodulators.

Downregulation- the process of reducing or suppressing a response to a stimulus; specifically : reduction in a cellular response to a molecule (as insulin) due to a decrease in the number of receptors on the cell surface.

Upregulation- the process of increasing the response to a stimulus; specifically : increase in a cellular response to a molecular stimulus due to increase in the number of receptors on the cell surface.

Immunohistochemistry (IHC)- refers to the process of selectively imaging antigens (e.g. proteins) in cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues.

Microbiota- is an "ecological community of commensal, symbiotic and pathogenic microorganisms" found in and on all multicellular organisms studied to date from plants to animals. A **microbiota** includes bacteria, archaea, protists, fungi and viruses.

Defensin - A family of potent antibiotics made within the body by neutrophils (a type of white blood cell) and macrophages (cells that can engulf foreign particles). The defensins play important roles against invading microbes. They act against bacteria, fungi and viruses by binding to their membranes and increasing membrane permeability. On a chemical level, the defensins are small peptides unusually rich in the amino acid cysteine (Cys). The human defensins are classified into the alpha-defensins and beta-defensins on the basis of their sequence homology and their Cys residues.

Cathelicidin-related antimicrobial peptides are a family of polypeptides found in lysosomes of macrophages and polymorphonuclear leukocytes (PMNs), and keratinocytes. **Cathelicidins** serve a critical role in mammalian innate immune defense against invasive bacterial infection.

Ribonuclase(/,**r**AIbə(σ)'**nju**:**klieiz**/)- (commonly abbreviated RNase) is a type of nuclease that catalyzes the degradation of RNA into smaller components.

Liver expressed antimicrobial peptide- are a family of mammalian liver-expressed antimicrobial peptides (LEAP). E.g. LEAP-1-2. LEAP-2 is a cysteine-rich, and cationic protein.

Cryopryin-Associated Periodic Syndromes- (CAPS) are a group of rare, inherited, autoinflammatory **diseases**. ... Familial Cold Autoinflammatory **Syndrome** (FCAS) Muckle-Wells **Syndrome** (MWS) Neonatal-Onset Multisystem Inflammatory **Disease** (NOMID) (also called Chronic Infantile Neurologic Cutaneous Articular, or CINCA, **Syndrome**)

Familial Cold Autoinflammatory Syndrome- is a condition that **causes** episodes of fever, skin rash, and joint pain after exposure to **cold** temperatures. ... In addition to the skin rash, episodes are characterized by fever, chills, and joint pain, most often affecting the hands, knees, and ankles.

Muckle-Wells Syndrome (MWS)- also known as urticaria-deafness-amyloidosis **syndrome** (UDA), is a rare autosomal dominant **disease** which **causes** sensorineural deafness and recurrent hives, and can lead to amyloidosis. Individuals with MWS often have episodic fever, chills, and joint pain.

Neonatal-Onset Multisystem Inflammatory Disease (NOMID), also known as chronic infantile neurologic cutaneous and articular syndrome (CINCA)- is a rare genetic periodic fever syndrome which causes uncontrolled inflammation in multiple parts of the body starting in the **newborn** period.

Familial Mediterranean Fever(FMF)- is an inflammatory disorder that causes recurrent **fevers** and painful inflammation of your abdomen, lungs and joints.

Tumour Necrosis Factor (TNF)-Associated Periodic Syndrome- (also known as TRAPS,) is a **periodic** fever **syndrome associated** with mutations in a receptor for the molecule **tumor necrosis factor (TNF)** that is inheritable in an autosomal dominant manner.

Mevalonate Kinase Deficiencies(MKD)- also known as **Hyper IgD Syndrome** (HIDS) MKD (HIDS) is an inherited auto inflammatory **disease** that is most often caused by an inherited autosomal recessive gene mutation of the **mevalonate kinase** gene (MVK), from both parents. ... HIDS is caused by the inherited MVK gene mutation. It is a condition characterized by recurrent episodes of fever, which typically begin during infancy.

Pathomechanisms- The mechanism by which a pathological condition occurs.

prognosis- It is a **prediction** about the course of a disease. **Prognosis** comes from the Greek pro- "before" and gnosis "knowledge." It means to know beforehand, but keep in mind that it is only a probable outcome and not a sure thing. In medicine, it is defined as a forecast of the future course of a disease or disorder, based on medical knowledge.

Febrile episodes- A **febrile** seizure, also known as a fever fit or **febrile** convulsion, is a seizure associated with a high body temperature but without any serious underlying health issue. They most commonly occur in children between the ages of 6 months and 5 years

Erythema- (from the Greek erythros, **meaning** red) is redness of the skin or mucous membranes, caused by hyperemia (increased blood flow) in superficial capillaries. It occurs with any skin injury, infection, or inflammation.

Polymorphonuclear leucocytes- A type of immune cell that has granules (small particles) with enzymes that are released during infections, allergic reactions, and asthma. Neutrophils, eosinophils, and basophils are **polymorphonuclear leukocytes**. A **polymorphonuclear leukocyte** is a type of white blood cell.

Neuro-cognitive deficits- is a reduction or **impairment** of cognitive **function** in one of these areas, but particularly when physical changes can be seen to have occurred in the brain, such as after neurological illness, mental illness, drug use, or brain injury.

Vasculitis- is the designation given to a group of uncommon diseases that result in inflammation of the blood vessels. **Symptoms** of **vasculitis** vary greatly and depend upon the organs affected and the severity of the disease. Diagnosis of **vasculitis** can be confirmed by a biopsy of involved tissue or angiography.

Caucasian ethnicity- The **Caucasian** race (also Caucasoid, or Europid) is a grouping of human beings historically regarded as a biological taxon, which, depending on which of the historical race classifications used, have usually included some or all of the ancient and modern populations of Europe, the **Caucasus**, Asia Minor, North Africa.

Inter-quartile range(**IQR**)- is a measure of variability, based on dividing a data set into quartiles. Quartiles divide a rank-ordered data set into four equal parts. The values that divide each part are called the first, second, and third quartiles; and they are denoted by Q1, Q2, and Q3, respectively.

Asymptomatic- it's a term used to refer to a condition or a person producing or showing no symptoms.

Long-term outcome- means, covering or involving a relatively long period of time.

Prednisone- Prednisone is a synthetic corticosteroid **drug** that is particularly effective as an immunosuppressant **drug**. It is used to treat certain inflammatory diseases (such as moderate allergic reactions), some autoimmune diseases, and (at higher doses) some types of cancer, but it has significant adverse effects.

Beta-methasone- Betamethasone is a medication belonging to the family of corticoids (more precisely, it is a glucocorticoid steroid). It has anti-inflammatory properties and is also used in immunosuppressive treatments, as it lowers immune defenses.

Cimetidine- This **medication** is also used to treat certain stomach and throat problems caused by too much acid (e.g., Zollinger-Ellison syndrome, erosive esophagitis) or a backward flow of stomach acid into the esophagus (gastroesophageal reflux disease-GERD).

Colchicine- a poisonous alkaloid $C_{22}H_{25}NO_6$ that inhibits mitosis, is extracted from the corms or seeds of the autumn crocus, and is used in the treatment of gout and acute attacks of gouty arthritis.

IL-1 β inhibitor anakinra- Anakinra is a recombinant version of the *interleukin 1* receptor *antagonist* (IL1-RA). *Anakinra* differs from native human IL-1Ra in that it has the addition of a single methionine residue at its amino terminus Anakinra blocks the biologic activity of naturally occurring *IL-1*, including inflammation.

Cellular immunity- is a protective **immune** process that involves the activation of phagocytes, antigen-sensitized cytotoxic T cells and the release of cytokines and chemokines in response to antigen.

Humoral immunity- is the aspect of **immunity** that is mediated by macromolecules found in extracellular fluids such as secreted antibodies, complement proteins, and certain antimicrobial peptides. **Humoral immunity** is so named because it involves substances found in the humors, or body fluids.

Ciliated pseudostratified columnar (respiratory epithelium)- is found in the linings of the trachea as well as the upper *respiratory* tract. Non-*ciliated pseudostratified columnar epithelia* are located in the membranous part of male vas deferens. **Respiratory epithelium** is a type of **epithelium** found lining the **respiratory** tract, where it serves to moisten and protect the airways. It also functions as a barrier to potential pathogens and foreign particles, preventing infection and tissue injury by the action of mucociliary clearance.

Oropharynx- plural oropharynges $-fa-rin-(,)j\bar{e}z$ also oropharynxes. : the part of the pharynx that is below the soft palate and above the epiglottis and is continuous with the mouth.

Homeostatically- The tendency of the body to seek and maintain a condition of balance or equilibrium within its internal environment, even when faced with external changes. A simple example of homeostasis is the body's ability to maintain an internal temperature around 98.6 degrees Fahrenheit, whatever the temperature outside.

Pathogen-associated molecular patterns (PAMP)- are **molecules associated** with groups of pathogens that are recognized by cells of the innate immune system. These **molecules** can be referred to as small **molecular** motifs conserved within a class of **microbes**. Pattern recognition receptor.

Damage-associated molecular patterns (DAMPs), also known as alarmins- are **molecules** released by stressed cells undergoing necrosis that act as endogenous danger signals to promote and exacerbate the inflammatory response.

Immunomodulator- is a chemical agent (as methotrexate or azathioprine) that modifies the immune response or the functioning of the immune system (as by the stimulation of antibody formation or the inhibition of white blood cell activity).

Kilodallons- is defined as a measure of molecular weight or mass. One hydrogen atom has mass of 1 Da. Proteins and other macromolecule molecular weights are usually measured in kDa or kD (**kilodaltons**) - 1000 Da. **Dalton** is defined as 1/12th the mass of a carbon atom, and an average amino acid has a molecular weight (MW) of approximately 135

Daltons. Since an average protein has 250 amino acids, this means that an average protein has a MW of approximately 34 kDa.

Epithelium- plural **epithelia** $\langle -l\bar{e}-a \rangle$: a membranous cellular tissue that covers a free surface or lines a tube or cavity of an animal body and serves especially to enclose and protect the other parts of the body, to produce secretions and excretions, and to function in assimilation.

Pathology- it is the scientific study of the nature of disease and its causes, processes, development, and consequences. A pathogen is a disease-causing agent, such as **bacteria**, viruses, fungi or parasites

Phagocytes are **cells** that protect the body by ingesting harmful foreign particles, bacteria, and dead or dying **cells**. Their name comes from the Greek phagein, "to eat" or "devour", and "-cyte", the suffix in biology denoting "**cell**", from the Greek kutos, "hollow vessel".

- Neutrophils. Neutrophils are abundant in the blood, quickly enter tissues, and phagocytize pathogens in acute inflammation.
- Macrophages. Macrophages are closely related to monocytes in the blood. ...
- Dendritic Cells. ...
- B Lymphocytes.

Cysteine motifs- A **cystine** knot is a protein structural **motif** containing three disulfide bridges (formed from pairs of **cysteine** residues). The sections of polypeptide that occur between two of them form a loop through which a third disulfide bond passes, forming a rotaxane substructure.

Neutrophils- they are a common type of white blood cell important to fighting off infections — particularly those caused by bacteria. For adults, counts have less than 1,500 *neutrophils* per microliter of blood

Monocytes- a large phagocytic white blood cell with a simple oval nucleus and clear, greyish cytoplasm, whose function is to destroy certain types of viruses and bacteria to protect the body against the development of infection. Elevated *monocytes*, also referred to as "monocytosis"

Chemotasis (from chemo- + taxis)- is the movement of an organism in response to a chemical stimulus. Somatic cells, bacteria, and other single-cell or multicellular organisms direct their movements according to certain chemicals in their environment.

Keratinocyte- is the predominant cell type in the epidermis, the outermost layer of the skin, constituting 90% of the cells found there. Those *keratinocytes* found in the basal layer (stratum basale) of the skin are sometimes referred to as "basal cells" or "basal *keratinocytes*"

Nasopharynx- The area of the upper throat that lies behind the nose. In contrast to the oropharynx, the area of the throat that lies behind the mouth. The word "**nasopharynx**" is a hybrid -- part Latin, part Greek. "Naso-" is a prefix that has to do with the nose. It comes from the Latin "nasus" for the nose (or snout).

periodontitis- chronic inflammation that typically follows untreated gingivitis and that results in progressive destruction of the **periodontal** ligament, formation of pockets around the teeth, and resorption of alveolar bone chiefly in a horizontal direction with loosening or loss of teeth — called also pericementitis.

Secreted proteases- When the protein material is passed to the small intestine, proteins, which are only partially digested in the stomach, are further attacked by **proteolytic enzymes secreted** by the pancreas. These **enzymes** are liberated in the small intestine from inactive precursors **produced** by the acinar cells in the pancreas.

Toll like receptors (*TLR*)- are a family of pattern recognition receptors (PRR) that function as primary sensors of the innate immune system to recognize microbial pathogens. They are single, membrane-spanning, non-catalytic receptors usually expressed on sentinel cells such as macrophages and dendritic cells that recognize structurally conserved molecules derived from microbes.

Cytokines- any of a number of substances, such as interferon, interleukin, and growth factors, which are secreted by certain cells of the immune system and have an effect on other cells.

Disulfide motif- A single *disulfide* bond enclosing a loop of 65–70 residues is a common feature of every domain in L and H chains. The locations of the intrachain *disulfide* bonds is in the V L (variable light) and C L (constant light) domains of a human antibody structure.

Lymphoid tissues- it is a cylinder of loosely organized cells surrounding small arteries. The part of the body's immune system that is important for the immune response and helps protect it from infection and foreign bodies. *Lymphoid tissue* is present throughout the body and includes the *lymph* nodes, spleen, tonsils, adenoids, and other structures.

Eosinophil cationic protein (ECP)- it is also known, as ribonuclease 3 is a basic **protein** located in the **eosinophil** primary matrix. ... ECP is released during degranulation of **eosinophils**. This **protein** is related to inflammation and asthma because in these cases, there are increased levels of ECP in the body.

Lipopolysaccharides (LPS)- it is also known as lipoglycans and endotoxins, are large molecules consisting of a lipid and a polysaccharide composed of O-antigen, outer core and inner core joined by a covalent bond; they are found in the outer membrane of Gramnegative bacteria, and elicit strong immune responses in animals.

Genitourinary tracts- the system of organs comprising those concerned with the production and excretion of urine and those concerned with reproduction — called also **genitourinary** system, urogenital system, urogenital **tract**.

Peptidoglycan- it is also known as murein, is a polymer consisting of sugars (polysaccharides) and amino acids(peptides) that forms a mesh-like layer outside the plasma membrane of most bacteria, forming the cell wall. The sugar component consists of alternating residues of β -(1,4) linked N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM)

Amphipathic(adjective)- (1) Pertains to a **molecule** containing both polar (water-soluble) and nonpolar (not water-soluble) portions in its structure. (2) Of, or relating to, a **molecule** having hydrophobic and hydrophilic regions.

Oropharyngeal's normal flora- The bacteria involved belonged to the patient's **oropharyngeal flora**: S. aureus, Enterobacteriaceae and Pseudomonadaceae. As a result of this study showing the **oropharynx** to be the source of lower airway colonization/infection, a policy for infection prevention has been outlined.

Psoriasin- calcium-binding protein A7 (S100A7), also known as psoriasin, is a protein that in humans is encoded by the *S100A7* gene. S100A7 is a member of the S100 family of proteins containing 2 EF-hand calcium-binding motifs. S100 proteins are localized in the cytoplasm and/or nucleus of a wide range of cells, and involved in the regulation of a number of cellular processes such as cell cycle progression and differentiation. S100 genes include at least 13 members that are located as a cluster on chromosome 1q21. This protein differs from the other S100 proteins of known structure in its lack of calcium binding ability in one EF-hand at the N-terminus.

S100A7 also displays antimicrobial properties. It is secreted by epithelial cells of the skin and is a key antimicrobial protein against Escherichia Coli by disrupting their cell membranes. This is the reason that in countries with poor sanitation, human skin is exposed to E.coli strains from faecal matter but it does not usually result in an infection.

Real-time RT-PCR- A **real-time** polymerase chain reaction (**Real-Time PCR**), also known as quantitative polymerase chain reaction (qPCR), is a laboratory technique of molecular biology based on the polymerase chain reaction (**PCR**). While **RT-PCR** is used to qualitatively detect gene expression through creation of complementary DNA (cDNA) transcripts from RNA, qPCR is used to quantitatively measure the amplification of DNA using fluorescent dyes.

Seasonal allergic rhinitis- is also known as hay fever, is a type of inflammation in the nose which occurs when the immune system overreacts to allergens in the air. Signs and symptoms include a runny or stuffy nose, sneezing, red, itchy, and watery eyes, and swelling around the eyes.

Cytokine- A proinflammatory **cytokine** or more simply an **inflammatory cytokine** is a type of signaling molecule (a **cytokine**) that is excreted from immune cells like helper T cells (Th) and macrophages, and certain other cell types that promote **inflammation**. Interleukins are proteins that regulate immune and inflammatory responses. Interleukins create communication between leukocytes. Lymphokines are **cytokines**that are produced by lymphocytes. Lymphokines send signals out to other cells, such as macrophages and other lymphocytes, telling them to come over and help.

Interlukin-10- (IL-10), also known as human cytokine synthesis inhibitory factor (CSIF), is an anti-inflammatory cytokine. In humans, interleukin 10 is encoded by the IL-10 gene. IL-10 signals through a receptor complex consisting of two IL-10 receptor-1 and two IL-10 receptor-2 proteins. It plays a role in the regulation of immune responses. It is secreted by antigen-presenting cells, promotes the development of immunologic tolerance, and suppresses the production of inflammatory cytokines. The gene for IL-10 is in chromosome region 1q31-q32.

Peroxidase- an enzyme that catalyses the oxidation of a particular substrate by hydrogen peroxide.

Alkaline phosphatase- Membrane bound glycoprotein with hepatic, osseous, renal and placental isoenzymes; See also PLAP-placental alkaline ... Component of an alternative method in **immunohistochemistry.** It is a catalytic enzyme that can be used instead of peroxidases for both direct and indirect **staining** methods.

Immunoperoxidase staining- It is a type of immunostain used in molecular biology, medical research, and clinical diagnostics. In particular, **immunoperoxidase** reactions refer to a sub-class of immunohistochemical or immunocytochemical procedures in which the antibodies are visualized via a peroxidase-catalyzed reaction.

Immunofluorescence- It is a **staining** method in which an antibody or antigen combines selectively with a fluorescent substance, thus labeling the immunogenic substance and indicating its presence.

Paraffin blocks- (in surgical pathology) a method used in preparing a selected portion of tissue for pathological examination. The tissue is fixed, dehydrated, and infiltrated by and embedded in **paraffin**, forming a **block** that is cut with a microtome into slices 8 µm thick.



Microtome- microtome (from the Greek mikros, **meaning** "small", and temnein, **meaning** "to cut") is a tool used to cut extremely thin slices of material, known as sections. Important in science, **microtomes** are used in microscopy, allowing for the preparation of samples for observation under transmitted light or electron radiation.

Reporter molecule- A gene whose phenotypic expression is easy to monitor; used to study promoter activity in different tissues or developmental stages. Recombinant DNA constructs are made in which the **reporter** gene is attached to a promoter region of particular interest and the construct transfected into a cell or organism. In **molecular** biology, a **reporter** gene (often simply **reporter**) is a gene that researchers attach to a regulatory sequence of another gene of interest in bacteria, cell culture, animals or plants.

Covalent/molecular bond- A **covalent bond**, also called a **molecular bond**, is a **chemical bond** that involves the sharing of electron pairs between **atoms**. These electron pairs are known as shared pairs or **bonding** pairs, and the stable balance of attractive and repulsive forces between **atoms**, when they share electrons, is known as **covalent bonding**.

APPENDIX 4: ANTIBODY'S PRODUCT DATA SHEET BY ABCAM.

OVERVIEW.

S/N	ANTIBODY (PRODUCT'S NAME).	DESCRIPTION	Tested Applications Suitable for:	Species reactivity. Reacts/Predi cted to work with:	Immunogen.	Specification.
1	Anti-beta Defensin 1 antibody. [M11-14b-D10] ab14425	Mouse monoclonal. [M11-14b-D10] to beta defensin 1.	ELISA, WB, IHC-P	Human, Chimpanzee, baboon, macaque monkey.	-synthetic peptide. *DHYNCVSSGGQCLYSACPIFTKIQGTCYRGKAKC CK. *Corresponding to amino acids 1-36 of human beta 1 Defensin.	
2	Anti-beta Defensin 2 antibody. ab63982	Rabbit polyclonal to beta Defensin 2.	WB, IHC-P, IHC-FR	Human	-synthetic peptide derived from beta 2 Defensin.	Human 7kDa beta 2 Defensin.
3	Anti-cathelicidin antibody. ab69484	Rabbit polyclonal to cathelicidin.	ICC/IF, IHC-P, WB	Human	A 17 amino acid peptide near the carboxyl terminal of the human cathelicidin. *POSITIVE CONTROL:Human spleen tissue lysate.	Ther is 71% homology between the immunogen and mouse cathelicidin. This antibody has been published (see pubmed 21724991)with mouse samples, but an appropriately sized band in mouse spleen or small intestine was unable to be seen by WB. it may be that certain lots have worked with mouse, but reactivity with mouse after testing was unable to be guaranteed.
4	Anti-Ribonuclease 7 antibody [4c4] ab205565.	Mouse monoclonal [4c4] to Ribonuclease.	IHC-C, WB, ICC/IF	Human	Recombinant full length protein corresponding to human Ribonuclease 7. -Recombinant human Ribonuclease 7.protein produced by CHO-based proprietary suspension cell line. -Data base link:- Q9H1E1POSITIVE CONTROL- CHOEBNAL T85 cell line lysate. U2O3. -cells experiencing Ribonuclease 7: Human skin tissue	
5	Anti-Hepcidin antibody ab57611.	Mouse monoclonal to hepcidin.	WB, IHC-P	human	Recombinant full length protein corresponding to human Hepcicin 99 25-85. -Sequences:- SVFPQQTGQLAELQPQDRAGARASWMPMFQRRR RRDTHFPICIFCCGCCH.	
6	Rabbit IgG, polyclonal-Isotype control. ab27478.	Rabbit polyclonal to Rabbit IgG.	IHC-P, CHIP, IP, ICC/IF			

7	Anti-LEAP 2 antibody.	Rabbit	IHC-P	human	Antigen sequence: - PIPEVSSAKR RPRRMTPFWR	
	ab122294.	polyclonal to			GVSLRPIGAS CRDDSECITR LCRKRRCSLS VAQE,	
		LEAP 2.			Corresponding to amino acids 24-77 of human LEAP2	
					(uniprot I D:Q969E1).	
					*POSITIVE CONTROL: Human skeletal muscle.	

PROP	ERTIES.									
S/N	FORM	STORAGE INSTRUCTIONS	STORAGE BUFFER	PURITY	CLONALITY	CLONE NUMBER	ISOTYP E	CONCENTR ATION USED	KONTRI ANTROF RYAN	SPECI FICAT ION
1	Liquid	Shipped at 4°C upon delivery aliquot and store at -20°C. (avoid freeze/thaw cycles).	Preservative: None. Constituent: 50mM Tris HCL pH:7.4	Protien L purified	monoclonal	M11-14b- D10	IgG1	1/500	2 μg/ml	
2	Liquid	Shipped at 4°C, store at ⁺ 4°C short term(1-2) weeks upon delivery aliquot, store at - 20°C long term.	Preservative: None.	Whole antiserum	Polyclonal		IgG	1/500 pancrease.		
3	Liquid	Shipped at 4°C, store at ⁺ 4°C.	Preservative: 0.02% Sodium oxide. Constituent: PBS.	Immunogen affinity purified	Polyclonal		IgG	1/200	15 μg/ml	
4	Liquid	Shipped at 4°C, store at ⁺ 4°C short term(1-2) weeks upon delivery aliquot, store at - 20°C or -80°C (avoid freeze/thaw cycles).	Preservative: 0.1% Sodium Oxide. Constituent: 99% PBS. pH:7.4.	Protein G purified	Monoclonal	4c4	IgG1	12µg/ml —> 1/100	5-20 μg/ml	
5	Liquid	Shipped at 4°C, upon delivery aliquot, store at - 20°C or -80°C (avoid repeated freeze/thaw cycles).	Preservative: None. Constituent: PBS. pH:7.2.	Protein G purified	Monoclonal		IgG	KC 1/100	3 μg/ml	Light chain type- kappa.
6	Liquid	Shipped at 4°C, store at ⁺ 4°C short term(1-2) weeks upon delivery aliquot, store at - 20°C (avoid freeze/thaw cycles).	Preservative: 0.1% Sodium Oxide. Constituents: 10mM PBS, 1% BSA. pH:7.4.	Protein G purified ab27478 is a purified IgG. -it was purified for serum collected from a Rabbit prior to immunization	Polyclonal		IgG	Testia bX 1/200		
7	Liquid	Shipped at 4°C upon delivery aliquot and store at -20°C.	Preservative: 0.02% Sodium	Immunogen affinity purified.	Polyclonal		IgG	1/150	1/200	

	(avoid freeze/thaw cycles).	oxide.				
		Constituents:				
		59% PBS, 40%				
		Glycerol.				
		pH:7.20.				

APPENDIX 5: DEMOGRAPHIC DATA, CLINICAL CHARACTERISTICS AND LABORATORY FINDINGS OF BOTH PFAPA GROUP AND GAβHS GROUP.

Cases.	S/ N.	Protocol number.	Fever onset year.	Tonsillecto my year.	Gender. Male (M). Female (F).	Age. Years.	Age in month.	Period ic fever.	Apthous stomatitis.	Pharyng itis.	Cervical adenitis.	Throat culture.
			5									
Recurrent	1	140	2013	2014	F	4.2	50.00	Yes	No	Yes	No	-ve
tonsillitis due	2	247	2014	2014	F	3.7	43.00	Yes	Yes	No	No	-ve
to PFAPA	3	539	2014	2014	М	5.4	64.00	Yes	No	Yes	No	-ve
(PFAPA	4	1083	2014	2014	F	4.6	54.00	Yes	No	Yes	No	-ve
GROUP).	5	199	2014	2015	М	5.2	62.00	Yes	No	Yes	Yes	-ve
Residence	6	147	2015	2016	F	3.2	38.00	Yes	No	Yes	No	-ve
(Northern Cyprus).	7	725	2016	2016	М	9.8	116.00	Yes	No	Yes	Yes	-ve
Recurrent	S /	Protocol	Fever	Tonsillecto	Gender. Male	Age.	Age in	Period	Upper resp.	Pharyng	WBC/CRP.	GHAβ-HS. Throat
tonsillitis due	Ν	number	onset	my year.	(M). Female	Year.M	month.	ic	tract	itis	Normal (N).	culture.
to group A			year		(F).	onth.		fever.	infection.	(sore	High (H).	
beta-									(Snoring)	throat).		
streptococci												
(GABHS	1	46	2008	2012	F	13.0	156.00	Yes	No	Yes	N/N	+ve
GROUP).	2	90	2015	2015	М	6.1	73.00	Yes	Yes	Yes	7.42(N)/nil	+ve
Residence	3	92	2015	2015	М	9.2	110.00	Yes	Yes	Yes	6.00(N)/nil	+ve
(Northern	4	432	2015	2015	F	7.10	94.00	Yes	Yes	Yes	16.59(H)/2.09(+ve
Cyprus).											H)	
	5	829	2015	2015	М	6.2	74.00	Yes	No	Yes	8.92(N)/2.59(H)	+ve
	6	841	2015	2015	М	8.5	101.00	yes	No	yes	11.76(H)/N	+ve

APPENDIX 6: ORIGINAL IMMUNOHISTOCHEMISTRY RESULTS OF TONSIL'S ANTIMICROBIAL PEPTIDE EXPRESSION

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.		Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
ANTI-BETA-	1	140	2014	F	4.2	50.00	1.00	1.00	1.00
DEFENSIN 1.	2	247	2014	F	3.7	43.00	1.00	1.00	1.00
(PFAPA GROUP).	3	539	2014	М	5.4	64.00	1.00	1.00	1.00
	4	1083	2014	F	4.6	54.00	0.00	0.00	0.00
	5	199	2015	М	5.2	62.00	1.00	1.00	1.00
	6	147	2016	F	3.2	38.00	0.00	0.00	0.00
	7	725	2016	М	9.8	116.00	2.00	1.00	2.00
ANTI-BETA-	1	46	2012	F	13.0	156.00	2.00	2.00	4.00
DEFENSIN 1.	2	90	2015	М	6.1	73.00	0.00	0.00	0.00
(GAβHS GROUP).	3	92	2015	М	9.2	110.00	0.00	0.00	0.00
	4	432	2015	F	7.10	94.00	2.00	1.00	2.00
	5	829	2015	Μ	6.2	74.00	0.00	0.00	0.00
	6	841	2015	Μ	8.5	101.00	2.00	2.00	4.00

LEVELS IN BOTH GROUPS.

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.		Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
ANTI-BETA-	1	140	2014	F	4.2	50.00	3.00	3.00	9.00
DEFENSIN 2 .	2	247	2014	F	3.7	43.00	3.00	3.00	9.00
(PFAPA GROUP).	3	539	2014	М	5.4	64.00	3.00	3.00	9.00
	4	1083	2014	F	4.6	54.00	3.00	3.00	9.00
	5	199	2015	М	5.2	62.00	3.00	3.00	9.00
	6	147	2016	F	3.2	38.00	2.00	2.00	4.00
	7	725	2016	М	9.8	116.00	2.00	2.00	4.00
ANTI-BETA-	1	46	2012	F	13.0	156.00	3.00	3.00	9.00
DEFENSIN 2 .	2	90	2015	М	6.1	73.00	2.00	2.00	4.00
(GAβHS GROUP).	3	92	2015	М	9.2	110.00	1.00	3.00	3.00
	4	432	2015	F	7.10	94.00	3.00	3.00	9.00

5	829	2015	М	6.2	74.00	3.00	3.00	9.00
6	841	2015	М	8.5	101.00	3.00	3.00	9.00

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.		Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
ANTI-	1	140	2014	F	4.2	50.00	3.00	3.00	9.00
CATHELICIDIN	2	247	2014	F	3.7	43.00	3.00	3.00	9.00
(LL-37).	3	539	2014	М	5.4	64.00	2.00	3.00	6.00
(PFAPA GROUP).	4	1083	2014	F	4.6	54.00	2.00	2.00	4.00
	5	199	2015	М	5.2	62.00	2.00	2.00	4.00
	6	147	2016	F	3.2	38.00	1.00	3.00	3.00
	7	725	2016	Μ	9.8	116.00	3.00	2.00	6.00
ANTI-	1	46	2012	F	13.0	156.00	3.00	3.00	9.00
CATHELICIDIN	2	90	2015	М	6.1	73.00	2.00	3.00	6.00
(LL-37).	3	92	2015	М	9.2	110.00	3.00	2.00	6.00
(PFAPA GROUP).	4	432	2015	F	7.10	94.00	3.00	3.00	9.00
	5	829	2015	M	6.2	74.00	3.00	3.00	9.00
	6	841	2015	Μ	8.5	101.00	3.00	2.00	6.00

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.		Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
ANTI-	1	140	2014	F	4.2	50.00	3.00	3.00	9.00
RIBONUCLEASE	2	247	2014	F	3.7	43.00	3.00	3.00	9.00
(RNASE7).	3	539	2014	М	5.4	64.00	3.00	3.00	9.00
(PFAPA GROUP).	4	1083	2014	F	4.6	54.00	2.00	3.00	6.00
	5	199	2015	М	5.2	62.00	3.00	3.00	9.00
	6	147	2016	F	3.2	38.00	2.00	2.00	4.00
	7	725	2016	Μ	9.8	116.00	3.00	2.00	6.00
ANTI-	1	46	2012	F	13.0	156.00	3.00	3.00	9.00
RIBONUCLEASE	2	90	2015	Μ	6.1	73.00	3.00	1.00	3.00
(RNASE7).	3	92	2015	Μ	9.2	110.00	3.00	2.00	6.00

(GAβHS GROUP).	4	432	2015	F	7.10	94.00	3.00	3.00	9.00
	5	829	2015	Μ	6.2	74.00	2.00	2.00	4.00
	6	841	2015	М	8.5	101.00	3.00	3.00	9.00

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.		Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
ANTI-HEPCIDIN.	1	140	2014	F	4.2	50.00	3.00	3.00	9.00
(PFAPA GROUP).	2	247	2014	F	3.7	43.00	3.00	3.00	9.00
	3	539	2014	М	5.4	64.00	3.00	3.00	9.00
	4	1083	2014	F	4.6	54.00	3.00	2.00	6.00
	5	199	2015	М	5.2	62.00	3.00	2.00	6.00
	6	147	2016	F	3.2	38.00	3.00	2.00	6.00
	7	725	2016	М	9.8	116.00	3.00	2.00	6.00
ANTI-HEPCIDIN.	1	46	2012	F	13.0	156.00	3.00	3.00	9.00
(GAβHS GROUP).	2	90	2015	Μ	6.1	73.00	3.00	3.00	9.00
	3	92	2015	М	9.2	110.00	3.00	3.00	9.00
	4	432	2015	F	7.10	94.00	3.00	3.00	9.00
	5	829	2015	М	6.2	74.00	3.00	3.00	9.00
	6	841	2015	М	8.5	101.00	3.00	3.00	9.00

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.	-	Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
RABBIT IgG.	1	140	2014	F	4.2	50.00	0.00	0.00	0.00
(NEGATIVE	2	247	2014	F	3.7	43.00	0.00	0.00	0.00
CONTROL).	3	539	2014	М	5.4	64.00	0.00	0.00	0.00
(PFAPA GROUP).	4	1083	2014	F	4.6	54.00	0.00	0.00	0.00
	5	199	2015	М	5.2	62.00	0.00	0.00	0.00
	6	147	2016	F	3.2	38.00	0.00	0.00	0.00
	7	725	2016	Μ	9.8	116.00	0.00	0.00	0.00
RABBIT IgG.	1	46	2012	F	13.0	156.00	0.00	0.00	0.00
(NEGATIVE	2	90	2015	М	6.1	73.00	0.00	0.00	0.00

CONTROL).	3	92	2015	М	9.2	110.00	0.00	0.00	0.00
(GAβHS GROUP).	4	432	2015	F	7.10	94.00	0.00	0.00	0.00
	5	829	2015	М	6.2	74.00	0.00	0.00	0.00
	6	841	2015	М	8.5	101.00	0.00	0.00	0.00

Antibody used.	S/N	Protocol	Case year.	Gender.	Age.	Age in	+VE cell	Staining	Final expression
		number.		Male (M).	Year.Month	month.	distribution	intensity	level=(PCD*SI).
				Female			(PCD).	(SI). (%).	(%).
				(F).			(%).		
ANTI-LEAP-2.	1	140	2014	F	4.2	50.00	0.00	0.00	0.00
(PFAPA GROUP).	2	247	2014	F	3.7	43.00	0.00	0.00	0.00
	3	539	2014	М	5.4	64.00	0.00	0.00	0.00
	4	1083	2014	F	4.6	54.00	0.00	0.00	0.00
	5	199	2015	М	5.2	62.00	0.00	0.00	0.00
	6	147	2016	F	3.2	38.00	0.00	0.00	0.00
	7	725	2016	М	9.8	116.00	0.00	0.00	0.00
ANTI-LEAP-2.	1	46	2012	F	13.0	156.00	0.00	0.00	0.00
(GAβHS GROUP).	2	90	2015	М	6.1	73.00	0.00	0.00	0.00
	3	92	2015	М	9.2	110.00	0.00	0.00	0.00
	4	432	2015	F	7.10	94.00	0.00	0.00	0.00
	5	829	2015	М	6.2	74.00	0.00	0.00	0.00
	6	841	2015	М	8.5	101.00	0.00	0.00	0.00

APPENDIX 7. DATA VIEW OF THE ANTIMICROBIAL PEPTIDES FINAL AVERAGE EXPRESSION EVELS IN BOTH GROUPS.

S/N	Group.	Protoco	Gend	Age.	Anti.	Anti.	Anti.	Anti.	Anti.	Rabb	Anti.
		I INO.	er. M/F.	mont h.	нрD -1.	нвD -2.	LL- 3.	e7.	din.	it IgG.	LEA P-2.
1	1	140	2	50	1	9	9	9	9	0	0
2	1	247	2	43	1	9	9	9	9	0	0
3	1	539	1	64	1	9	6	9	9	0	0
4	1	1083	2	54	0	9	4	6	6	0	0
5	1	199	1	62	1	9	4	9	6	0	0
6	1	147	2	38	0	4	3	4	6	0	0
7	1	725	1	116	2	4	6	6	6	0	0
8	2	46	2	156	4	9	9	9	9	0	0
9	2	90	1	73	0	4	6	3	9	0	0
10	2	92	1	110	0	3	6	6	9	0	0
11	2	432	2	94	2	9	9	9	9	0	0
12	2	829	1	74	0	9	9	4	9	0	0
13	2	841	1	101	4	9	6	9	9	0	0

Group 1=>PFAPA group. Group 2=>GAβHS group. Gender 1=>Male. Gender 2=>Female. Age (month). AMPs (%).

	anti.HβD-1	anti.HβD-2	anti.LL-37	anti.Rnase7	anti.hepcidin	Rabbit.IgG	anti.LEAP-2
Mann-Whitney U	18.500	19.000	12.000	17.500	9.000	21.000	21.000
Wilcoxon W	46.500	40.000	40.000	38.500	37.000	42.000	42.000
Z	374	352	-1.365	548	-2.138	.000	.000
Asymp. Sig. (2- tailed)	.708	.725	.172	.584	.033	1.000	1.000
Exact Sig. [2*(1- tailed Sig.)]	.731 ^b	.836 ^b	.234 ^b	.628 ^b	.101 ^b	1.000 ^b	1.000 ^b

APPENDIX 8: STATISTICAL ANALYSIS OF MEAN AND SEM (Test Statistics^a).

a. Grouping Variable: Group

b. Not corrected for ties.

MEANS TABLES=anti.HβD1 anti.Hbβ2 anti.LL37 anti.Rnase7 anti.hepcidin Rabbit.IgG anti.LEAP1 anti.LEAP2 BY Group

/CELLS=MEAN STDDEV.

Group		anti.Hβ D-1	anti.Hβ D-2	anti.LL- 37	anti.Rnase 7	anti.hepcid in	Rabbit.Ig G	anti.LEA P-2	anti.LEA P2
PFAP	Mean	.86	7.57	5.86	7.43	7.29	.00	.00	.00
A	Std. Deviation	.690	2.440	2.410	2.070	1.604	.000	.000	.000
GAβH	Mean	1.67	7.17	7.50	6.67	9.00	.00	.00	.00
S	Std. Deviation	1.966	2.858	1.643	2.733	.000	.000	.000	.000
Total	Mean	1.23	7.38	6.62	7.08	8.08	.00	.00	.00
	Std. Deviation	1.423	2.534	2.181	2.326	1.441	.000	.000	.000

APPENDIX 9. STATISTICAL ANALYSIS FOR THE MEAN AND STANDARD DEVIATION OF BOTH GROUPS.

MINE VARIABLES=anti.HβD1 anti.HβD2 anti.LL37 anti.Rnase7 anti.hepcidin Rabbit.IgG anti.LEAP1 anti.LEAP2 BY Group

/PLOT BOXPLOT STEMLEAF HISTOGRAM

/COMPARE GROUPS

/STATISTICS DESCRIPTIVES

/CINTERVAL 95

APPENDIX 10. AVERAGE SEMI-QUANTITAION OF TONSIL AMPs FINAL EXPRESSION LEVELS VALUES SCORED FOR THE MEAN AND SEM IN BOTH PFAPA AND GAβHS GROUP FOR STATISTICAL ANALYSIS IN REGARDS TO AGE AND GENDER.

S/N	AMPs	AGE	RANGE	FEMALE: MALE.		MEAN/AV	/ERAGE	STANDA	ARD
	ANTIBODIE	(YEARS)).			EXPRESS	ION	DEVIAT	ION
	S USED.					LEVELS (%).	(SEM).	
		PFAPA	GAβHS	PFAPA	GAβHS	PFAPA	GAβHS	PFAPA	GAβHS
		GROU	GROUP	GROUP	GROUP	GROUP	GROUP	GROU	GROUP
		Р						Р	
1	ANTI-HβD-1	3-10	6-13	4:3	2:4	0.86	1.67	0.67	1.97
2	ANTI-HβD-2	3-10	6-13	4:3	2:4	7.57	7.17	2.44	2.86
3	ANTI-LL-37	3-10	6-13	4:3	2:4	5.87	7.50	2.41	1.64
4	ANTI-	3-10	6-13	4:3	2:4	7.42	6.67	2.07	2.73
	RNASE7								
5	ANTI-	3-10	6-13	4:3	2:4	7.29	9.00	1.60	0.00
	HEPCIDIN								
6	RABBIT IgG	3-10	6-13	4:3	2:4	0.00	0.00	0.00	0.00
7	ANTI-LEAP-	3-10	6-13	4:3	2:4	0.00	0.00	0.00	0.00
	2								

APPENDIX 11. FINAL STATISTICAL ANALYSIS RESULTS OF THE SIGNIFICANT VALUES OBTAINED FOR BOTH GROUPS AMPS SIGNIFICANT LEVEL.

Antimicrobial peptides. \rightarrow	нβD-1	нβD-2	LL-37	RNase7	Hepcidin	Rabbit IgG	LEAP- -2
Significant levels \rightarrow (P value)	0.708	0.725	0.172	0.584	0.033	1.000	1.000

APPENDIX 12: NON-PARAMETRIC TEST FOR SIGINFICANT DIFFERENCE IN AGE OF PATIENT'S IN BOTH GROUPS.

					Std.
	GROUF	>		Statistic	Error
AGE.MONT	PFAPA	Mean		61.00	9.829
Н		95% Confidence	Lower Bound	36.95	
		Interval for Mean	Upper Bound	85.05	
		5% Trimmed M	ean	59.22	
		Median		54.00	
		Variance		676.333	
		Std. Deviation		26.006	
		Minimum		38	
		Maximum		116	
		Range		78	
		Interquartile Ra	nge	21	
		Skewness		1.947	.794
		Kurtosis		4.346	1.587

GAβH	Mean		101.33	12.473
S	95% Confidence	Lower Bound	69.27	
	Interval for Mean	Upper Bound	133.40	
	5% Trimmed M	lean	99.87	
	Median		97.50	
	Variance		933.467	
	Std. Deviation		30.553	
	Minimum		73	
	Maximum		156	
	Range		83	
	Interquartile Ra	ange	48	
	Skewness		1.267	.845
	Kurtosis		1.897	1.741

NPar Tests. Mann-Whitney Test for age.

-

			Ranks			
		GROUP	Ν	Me	ean Rank	Sum of Ranks
AGE	E.MONTH	PFAPA	7		4.71	33.00
		GAβHS	6		9.67	58.00
		Total	13			
	1	est Statis	stics ^a			
			AGE.MON	TH		
	Mann-Wh	itney U	5.0	000		
	Wilcoxon	W	33.0	000		
	Z		-2.2	86		
	Asymp. S tailed)	ig. (2-	.0	22		
	Exact Sig. tailed Sig.	. [2*(1-)]	.02	22 ^b		
	• •					

a. Grouping Variable: GROUP

b. Not corrected for ties.

EXAMINE VARIABLES=AGE.MONTH BY GROUP /PLOT NONE /STATISTICS DESCRIPTIVES /CINTERVAL 95 /MISSING LISTWISE

APPENDIX 12: NON-PARAMETRIC TEST (Chi's Squqre) FOR SIGINFICANT DIFFERENCE IN GENDER OF PATIENT'S IN BOTH GROUPS GENDER . Crosstabulation

			GRO	OUP	
			PFAPA	GAβHS	Total
GENDER	MALE	Count	3	4	7
		Expected Count	3.8	3.2	7.0
		% within GENDER	42.9%	57.1%	100.0%
		% within GROUP	42.9%	66.7%	53.8%
		% of Total	23.1%	30.8%	53.8%
	FEMALE	Count	4	2	6
		Expected Count	3.2	2.8	6.0
		% within GENDER	66.7%	33.3%	100.0%
		% within GROUP	57.1%	33.3%	46.2%
		% of Total	30.8%	15.4%	46.2%
Total		Count	7	6	13
		Expected Count	7.0	6.0	13.0

% within GENDER	53.8%	46.2%	100.0%
% within GROUP	100.0%	100.0%	100.0%
% of Total	53.8%	46.2%	100.0%

CROSSTABS

]

/TABLES=GENDER BY GROUP

/FORMAT=AVALUE TABLES

/STATISTICS=CHISQ

/CELLS=COUNT EXPECTED ROW COLUMN TOTAL

Cases Valid Missing Total Ν Percent Ν Ν Percent Percent GENDER * GROUP 13 100.0% 100.0% 0 0.0% 13

Case Processing Summary

	Value	df	Asymp. Sig. (2- sided)	Exact Sig. (2- sided)	Exact Sig. (1- sided)
Pearson Chi- Square	.737 ^a	1	.391		
Continuity Correction ^b	.090	1	.764		
Likelihood Ratio	.746	1	.388		
Fisher's Exact Test				.592	.383
Linear-by-Linear Association	.680	1	.409		
N of Valid Cases	13				

Chi-Square Tests

a. 4 cells (100.0%) have expected count less than 5. The minimum expected count is 2.77.

b. Computed only for a 2x2 table

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