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Proteomic analysis of salivary inflammatory biomarkers of developmental gingival enlargements in patients with West and Noonan syndromes: a preliminary pilot single-center retrospective study

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Abstract. – OBJECTIVE: The aim of the preliminary pilot single-center retrospective cross-sectional study was to analyze and compare the presence of non-secretory salivary inflammatory biomarkers in pediatric patients with West syndrome, Noonan syndrome, and a healthy control group.

PATIENTS AND METHODS: A total of 60 saliva samples were collected during dental check-ups. The saliva samples collected were analyzed by liquid chromatography. The results were analyzed with a *t*-test, and the statistical significance was given by a *p*-value lower than 0.05.

RESULTS: We found statistical significance for defensin α1 (*p*=0.006) and thymosin β4 (*p*=0.025) in the Noonan syndrome. In the West syndrome, only the defensin α1 had a statistically significant difference with the other groups (*p*=0.022). Proteomic analysis revealed an overexpression of peptides related to the innate (thymosin β4) and acquired (defensin α1, α3) immunity.

CONCLUSIONS: West and Noonan's syndromes showed the overexpression of molecular biomarkers involved in the pathogenesis of chronic periodontitis. The inflammatory status is triggered and amplified by the abnormal overgrowth of gingival tissues, the amplified release of proinflammatory cytokines from the immune cells, and the poor cooperation in maintaining adequate oral hygiene.

Key Words:
Proteomics, Noonan syndrome, West syndrome.

Introduction

The studies concerning proteomics aim to investigate and establish the identity, quantity, structure, and biochemical and cellular functions of all proteins present in a tissue, in a cell, or in a sub-cellular compartment. Describing how these properties are variable in space, time, or in a specific physiological status[1]. Therefore, the scientific community is committed to the study of specific potential biomarkers that can provide diagnostic, prognostic, and efficacy information on the treatment of certain pathological conditions[2,3]. The implementation of notions related to saliva allows a clearer picture of its composition and, therefore, more information on the biological meaning of the proteomic variations found in pathological situations[4].

Noonan syndrome is an autosomal dominant disorder with a prevalence of 1-5/10,000 characterized by short stature, facial dysmorphism, congenital heart defects, cardiomyopathy, and an increased risk of developing cancer during childhood[5]. The pathognomonic clinical features consist of a broad forehead, hypertelorism, palpebral ptosis, downward slanting palpebral fissures, a high-arched palate, gingival hyperplasia, and low-set, posteriorly rotated ears[6,7]. Treatment requires a multidisciplinary approach; cardiovas-

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cular anomalies are usually treated with standard approaches like calcium channel blockers associated with gingival hyperplasia.

West syndrome, instead, is a rare disorder with an estimated birth prevalence of 1-9/100,000, characterized by the association of clusters of axial spasms, psychomotor retardation, and an hypsarrhythmic interictal EEG pattern[8]. It is the most frequent type of epileptic encephalopathy with onset in infancy and early childhood[8]; it may occur in otherwise healthy infants and in those with abnormal cognitive development[9]. The first-line pharmacological treatment for West syndrome is represented by anticonvulsants or a combination of corticosteroids and anticonvulsants. Major oral clinical features in those patients were generalized tooth wear and gingival enlargement, altered chronology, and sequence of dental eruption[10].

Gingival hyperplasia/enlargement is a heterogeneous clinical condition characterized by slowly progressing outgrowth of keratinized gingiva. It causes a distortion of the gingival contour, and it can be localized to interdental papillae or involve the marginal gingiva. In severe cases, the gingival enlargement may cover the tooth surface, thus determining a sulcus depth greater than normal on probing (the so-called pseudo pockets or false pockets). The development of pseudo pockets is frequently associated with key symptoms of gingival inflammation (redness, swelling, bleeding)[11]. By definition, it affects the masticatory mucosa, but it does not spread beyond the mucogingival junction. The etiology varies and often is multifactorial; however, local, and systemic conditions, disease, and idiopathic factors may contribute to gingival enlargement. The latest classification by the 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions distinguished "gingivitis – dental biofilm induced" and "gingival diseases – non-dental biofilm induced".

Noonan patients can be listed in both categories, as their gingival enlargement is primarily a "genetic/developmental disorder" (listed in the section "gingival diseases - dental biofilm induced"), and "drug-induced" (are treated with calcium blockers, responsible for gingival overgrowth)[12]. West patients can develop gingival enlargements after the use of anticonvulsants.

The consequent pseudo pockets may represent a "local risk factor" for "dental biofilm-induced gingivitis"[13]. As reported by Beaumont et al[14], a drug-induced gingival enlargement is characterized by a pink and stippled appearance and firm consistency. Whereas in the presence of secondary inflammation, the clinical appearance is smooth, shiny, and reddened.

However, the quantification of inflammatory salivary biomarkers can be a diagnostic tool for quantifying and monitoring the status of secondary inflammation of the "genetic" and "drug-induced" gingival enlargements.

The aim of this work was to quantify the non-secretory inflammatory salivary biomarkers for staging the gingival hyperplasia in West and Noonan patients with mild-to-moderate gingival inflammation.

Definitive diagnosis and treatment strategy were identified as both West and Noonan patients shared combined gingival hyperplasia: genetic and drug-induced gingival diseases.

Patients and Methods

Patient Enrollment

This preliminary pilot single-center retrospective cross-sectional study was carried out thanks to the collaboration between the operative unit of pediatric dentistry and the Institute of Clinical Biochemistry at the "University Cattolica del Sacro Cuore" in Rome. The protocol has been reported in compliance with STROBE guidelines and was approved by the Internal Review Board of Policlinico Fondazione Agostino Gemelli (Protocol ID n°3452, acceptance number: 0043903/20). This study was conducted in compliance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the Helsinki Declaration in 1975.

Data Collection

Sixty-one patients voluntarily participated in a "saliva screening day" - dental check-ups where saliva samples were collected free of charge for patients. One patient was excluded from saliva collection due to poor compliance. The pediatric patients were recruited from October 2020 to June 2021. According to the hospital regulatory requirements for minors, a written information consent form for the procedure and the use of personal data was obtained from both patient's parents before the saliva sampling. All the patients underwent a dental check-up with an experienced dentist (F.G.). Patient information was gathered from their medical history and present risk factors (diabetes, metabolic diseases, nutritional de-

ficiencies, and – for only adult patients – tobacco and alcohol consumption). In addition, the dental history provided information on the presence/onset of gingival overgrowth, the occurrence of pain/discomfort, as well as abscesses, bleeding, and tooth mobility.

Then, the patients went through a clinical examination, including the assessment of plaque and periodontal indices, probing pocket depth, gingival bleeding, caries, and tooth mobility. In addition, the extent of gingival overgrowth was graded according to the semiquantitative classification for drug-induced gingival overgrowth introduced by Inglés et al[12].

Sixty anonymized samples were retrieved from the database: fifteen samples were collected from patients with West syndrome (8 male; 7 female), fifteen from patients with Noonan syndrome (8 male; 7 female), and others from randomly selected patients belonging to Noonan's control group (8 male; 7 female) and West's control group (8 male; 7 female-). Each control group consisted of West, or Noonan patients matched with the test groups for gender and age, DMFT (Decayed – Missing – Filled – Teeth) index (< 4.5), gingival index (< 2), and plaque index (< 2)[15]. The inclusion criteria for the test groups were the following: the presence of mixed dentition, age between 6±1 years and 12±1 years, 4 to 6-month follow-up after nonsurgical periodontal therapy, presence of moderate gingival hyperplasia (gingival overgrowth score between 2 and 3) due to the use of drugs, including anticonvulsants (for West syndrome) and calcium channel blockers (for Noonan syndrome), and willing to participate to the "screening day".

If the selected patients did not meet the inclusion criteria, they were invited to an oral hygiene appointment and re-scheduled after the normalization of periodontal indices. In compliance with the rights and protection of the subjects involved in the work, prior consent, free and informed, was requested. All the procedures were in accordance with the ethical standards of the responsible committee for human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. For the saliva collection, a specific protocol was developed for sampling and subsequent treatments. Patients were instructed to stop eating from the day before and drinking for at least two hours before the appointment[4]. Saliva samples were collected between 10:00 A.M. and 12:00 P.M. Saliva was intraorally collected from the floor of the mouth, in front of the tongue-like caruncles, by carrying out 0.5 ml with a sterile plastic 1 ml Pasteur pipette (the minimum amount of saliva required for the mass spectrometry is 1 μg)[16]. The saliva withdrawn was immediately mixed with 0.2% TFA (trifluoracetic acid) in a ratio of 1:1 and centrifuged at 9,000 rpm at 4°C for 5 minutes. Finally, the acidic supernatant was separated from the precipitate and stored at −80°C until the analysis was carried out by HPLC-ESI-MS (High-performance liquid chromatography–electrospray ionization mass spectroscopy) (100/l, corresponding to 50/l of saliva) (Thermo Fisher Scientific – Waltham, MA, USA). This acid treatment was aimed at inhibiting the enzymatic activity of the proteases contained in the fluid, with the aim of making the original composition of the sample stable. It also had the purpose of inducing the precipitation of high molecular weight proteins (mucins but also lactoferrin and albumin) to make the sample more suitable for chromatographic analysis. The fluid turned out to be enriched with peptide and protein fraction with a low molecular weight soluble in a medium and less viscous acid[17].

At the end of the treatment, the supernatant, separated from the precipitate, was analyzed by RP-HPLC-ESI-MS (Reversed phase HPLC-ESI-MS) and stored at a low temperature (–80°C), guaranteeing the reproducibility of the profile chromatographic for a few months.

Data Analysis

The samples underwent HPLC-ESI-Orbitrap Elite analyses through an UltiMate 3000 System, complying with an Orbitrap Elite MS with an EASY-Spray nano-ESI source (Thermo Fisher Scientific - Waltham, MA, USA). The top-down analysis employed an EASY-Spray column 15 cm × 50 μm ID (Thermo Fisher Scientific – Waltham, MA, USA), PepMap C18 (5 μm particles, 300 Å pore size) (Thermo Fisher Scientific – Waltham, MA, USA) coupled to an Acclaim PepMap 100 cartridge (5 μm particles, 300 Å pore size, 300 μm × 5 mm) (Thermo Fisher Scientific - Waltham, MA, USA). Nano-HPLC-ESI-Orbitrap Elite analyses were conducted using two eluents: eluent A consisted of an aqueous solution of Formic Acid (0.1%, v/v) (Thermo Fisher Scientific – Waltham, MA, USA). Acetonitrile/water (0.1% v/v) (Thermo Fisher Scientific – Waltham, MA, USA) was employed as eluent B in a gradient elution as follows: (1) 5% B (2 minutes), (2) from 5% to 60% B (30 minutes), (3) from 60% B to 99% (10 minutes), (4) 99% B (10 minutes), (5)

from 99% to 5% B (2 minutes), and (6) 5% B (10 minutes). The injection volume and the flow rate were set at 5 µL (corresponding to 0.5 µg of total protein amount per sample) and 0.3 µL/min, respectively. In order to obtain peptide trapping and concentration, the study samples were loaded for five minutes into the Acclaim PepMap nano-trap cartridge (Thermo Fisher Scientific - Waltham, MA, USA) using eluent A at 10 µL/min. The temperature for chromatographic separations was set at 35°C. In positive ionization mode, the Orbitrap Elite instrument performed an MS/MS fragmentation by collision-induced dissociation (CID) of the ten highest signals of each spectrum. The measurements were performed in data-dependent scan (DDS) mode (120.000 resolution, and 150-2.000 m/z acquisition range) (Thermo Fisher Scientific – Waltham, MA, USA). The minimum cut-off signal was set at 500.0, the isolation width at 2 m/z, the default charge state at +2, and the activation Q at 0.25 MS/MS acquisition was conducted in the Orbitrap at 60.000 resolution.

The protein sequenced using the Orbitrap Elite instrument was identified with the databases (Thermo Fisher Scientific – Waltham, MA, USA) for all known peptides of each protein, by matching the theoretical masses obtained from the fragmented peptides[15,16,17].

Statistical Analysis

The sample size of this study was calculated based on the results obtained by Mattar et al[18], concerning the difference in concentration of defensins between affected and no-affected hepatitis type C patients[17]. Using the formula proposed by Rosner[19], the minimum number of patients for each group was 11, maintaining a minimum power of 80% and a Type I error of 0.05%: $N = \sigma^2(z_1\text{-}\beta+z_1\text{-}\alpha/2)^2/(\mu_0\text{-}\mu_1)^2$ (N: sample size of the study population; σ: variance of the study population; z: critical Z value for a given α or β; α: the probability of type I error; β: the probability of Type II error; μ_0: population mean; μ_1: mean of the study population).

Furthermore, assuming that the difference between the control and test group could be smaller than the defensin values declared by Mattar et al[18] (infected *vs.* healthy patients), the sample size was increased by 35%, with 15 patients per group.

To determine the relative abundances of the various peptides of interest, the XIC method (eXtracted Ion Current - Thermo Fisher Scientific – Waltham, MA, USA) was used, which allows to selectively "extract" only the m/z values corresponding to the protein of interest. This method allows to obtain an extracted ionic current peak whose area is proportional to the concentration of the peptide within the sample under examination. These values were used for the relative quantification of peptides and, therefore, for statistical analysis.

An Excel data collection form and data management system were used (Microsoft Excel 365, ver. 16.67; Microsoft Corp., Redmond, WA, USA). Prior to entry, all data were evaluated in terms of accuracy and completeness, and they were entered by a single-blinded operator. For each continuous variable, the mean and the standard error were reported.

This study was based on the hypothesis that Study Groups (West and Noonan syndrome patients) would be superior to Control Groups, respectively, in relation to the defensin α1 (primary outcome) expression. In accordance with the Consolidated Standards of Reporting Trials (CONSORT) Statement, a statistical analysis was performed with a superiority approach on all variables. The null hypothesis (H0) was that there was no true difference in terms of protein expression between the study and control groups. Alternative hypothesis (H1) states that there is a difference between the groups.

The hypothesis of normality was tested with the Skewness/Kurtosis tests (normal distribution if p-value > 0.05). The statistical significance of the differences between the variables was evaluated by t-test, and Wilcoxon matched-pairs signed-ranks test where necessary. The threshold value decided for determining the statistical significance corresponded to a p-value of 0.05 (5%). The statistician was blinded and external to the working group. Data analysis was performed with STATA/IC 17 software (StataCorp LLC, College Station, TX, USA).

Results

In order to verify the presence of statistically significant changes, the values of the areas of the XIC peaks belonging to the syndromic patients were compared with those of healthy controls, selected for age and sex (Table I).

Noonan Syndrome

As it is possible to observe a histogram graph relating to the average values of the XIC peaks obtained for each protein in patients with Noonan

Table I. Defensins' and thymosins' levels.

	Noonan 15 patients	Control group (Noonan) 15 patients	West 15 patients	Control group (West) 15 patients
Defensin α1	*1.0×10^9 (p-value = 0.0006)	*1.2×10^8	*8.7×10^8 (p-value = 0.022)	*2.2×10^8
Defensin α3	2.5×10^9	1.5×10^7	5.0×10^8	2.5×10^8
Defensin α4	4.8×10^8	1.0×10^7	1.7×10^7	1.5×10^7
Thymosin β4	*5.2×10^8 (p-value = 0.025)	*1.9×108	3.1×10^8	1.1×10^8
Thymosin β10	1.2×10^8	1.0×10^8	2.4×10^8	1.0×10^8

*: p-value < 0.05.

syndrome, the defensin α1, α2, α3, and α4 showed different levels of expression: defensin α3 was the most expressed in this case (Figure 1).

All the defensins, albeit with different levels of expression, showed a higher presence in syndromic patients than in subjects belonging to the control group.

In this study, we found statistical significance for defensin α1 (p=0.006). In addition, two members of the thymosin class (thymosin β4 and thymosin β10) were also quantified.

As can be seen in Figure 2, thymosin β4 was the most expressed, and its concentration was higher when compared to the control group with a statistically significant difference (p=0.025). On the other hand, the expression of thymosin β10 was comparable to that of the controls, with no significance between the two groups.

West Syndrome

Subjects with West syndrome showed a different trend in defensin expression values compared

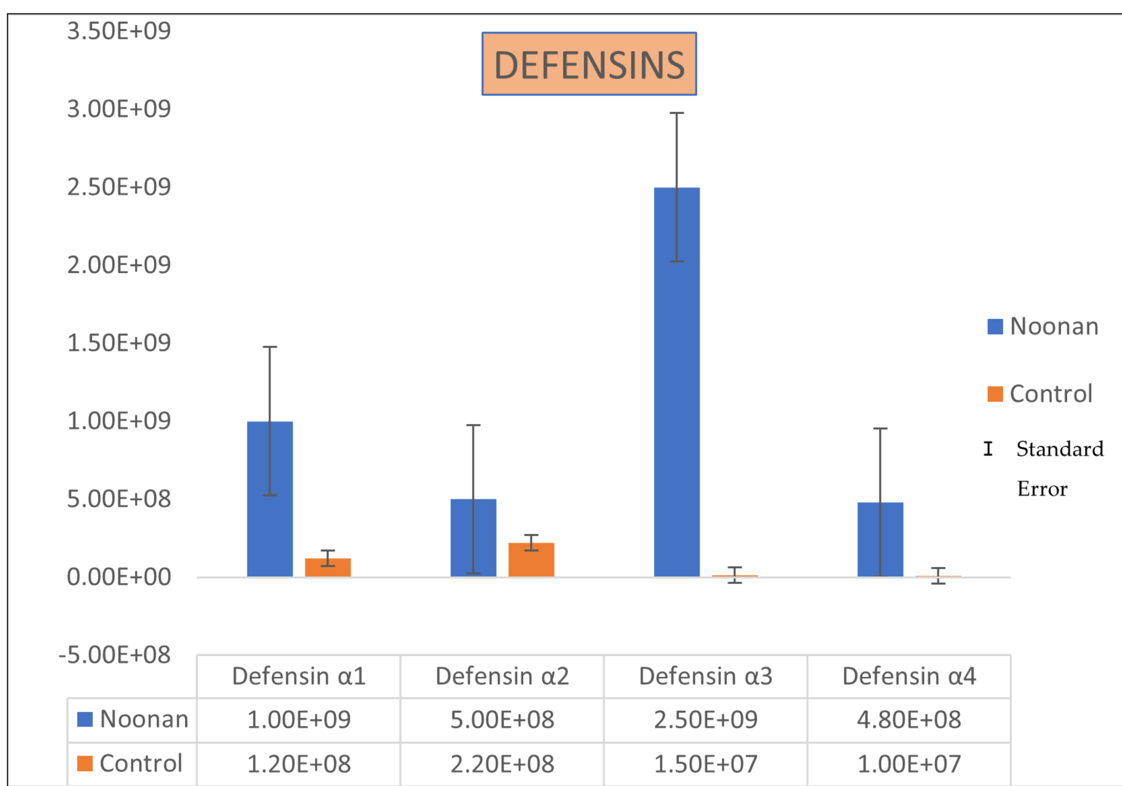

Figure 1. Defensins' XIC peaks in Noonan syndrome (p-value=0.05).

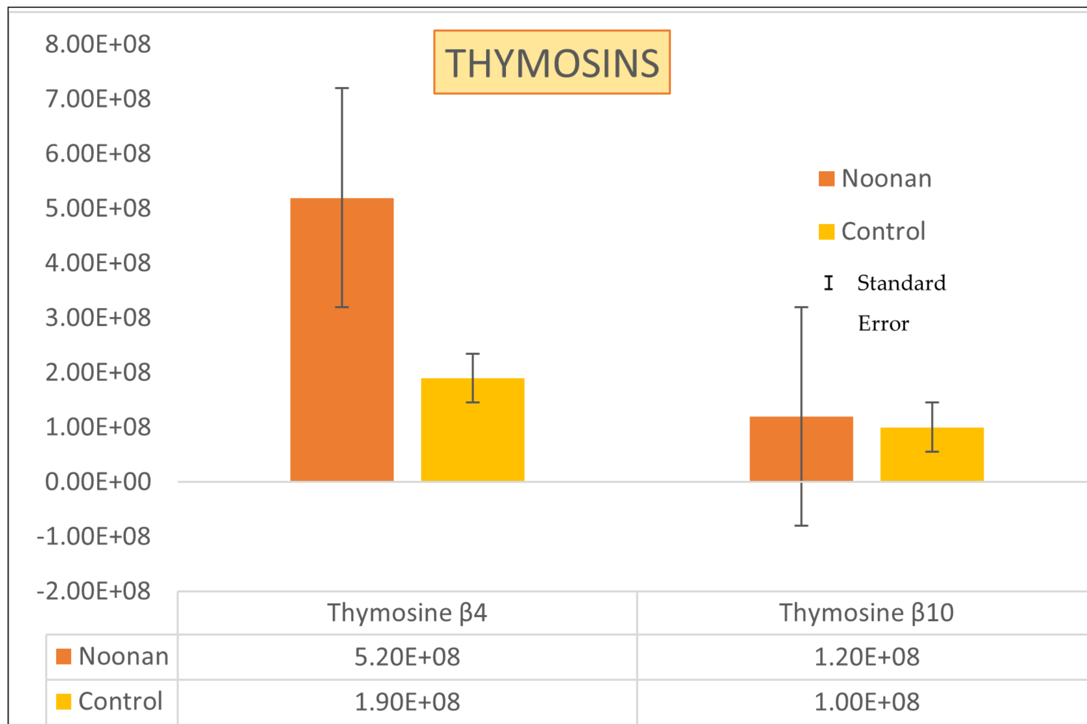

Figure 2. Thymosins' XIC peaks in Noonan syndrome.

to those with Noonan syndrome. In fact, defensin α1was the most expressed protein (p-value=0.022) (Figure 3).

As for the Noonan syndrome, thymosin β4 was the most expressed, followed by thymosin β1, but the values were not statistically significant (Figure 4).

Discussion

We found that the relative concentrations of defensins and thymosins were increased in both the syndromic patient groups compared to healthy controls, albeit with different levels and not al-

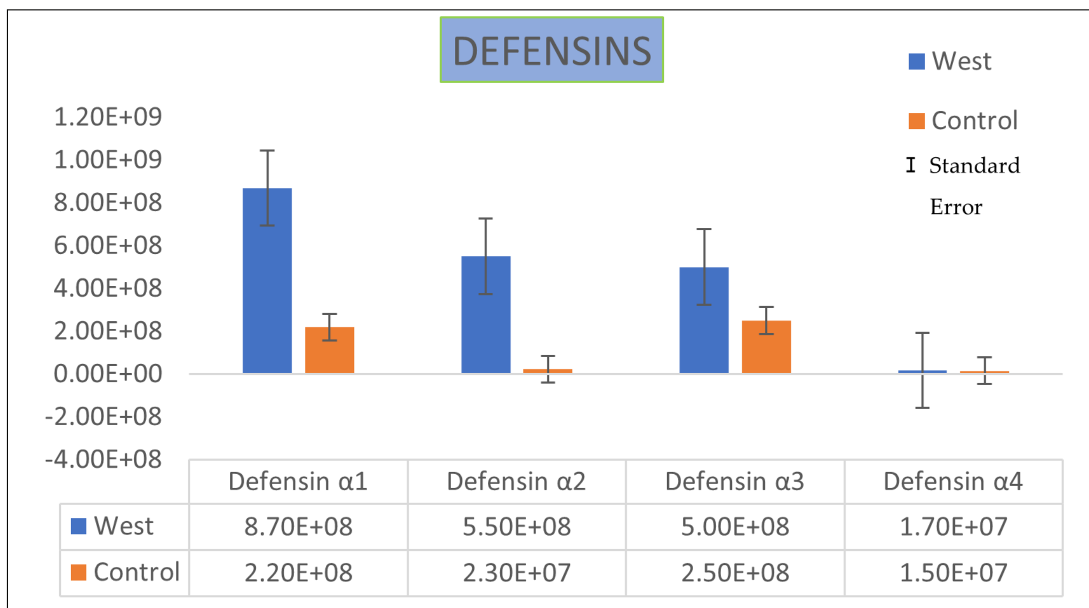

Figure 3. Defensins' XIC peaks in West syndrome.

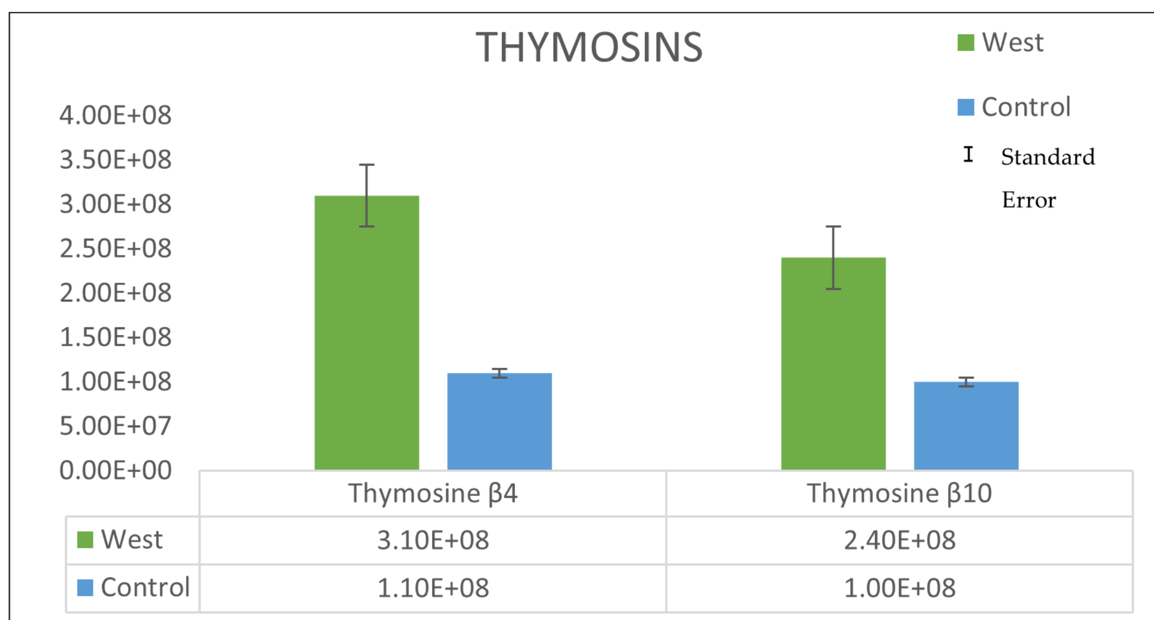

Figure 4. Thymosins' XIC peaks in West syndrome.

ways with statistical significance. The reason for the choice of the two biomarkers is related to their availability in the saliva and the presence of a correlation between these biomarkers and the inflammatory status of periodontal tissues[20,21]. Alterations in the expression levels of defensins and thymosins in saliva have been previously linked[22] to oral disease. Defensin levels in the saliva of patients with oral disease are higher than those in the saliva of normal individuals, as described in literature by Abiko and Saitoh[22]**.** Since gingival overgrowth has an inflammatory component that involves neutrophils, in our study, this phenomenon may be due to neutrophil infiltration associated with the inflammatory periodontal condition that affects mostly Noonan and West syndrome patients[23]. defensin α1in saliva may be a marker of inflammation associated with oral disease since its concentration in saliva was significantly higher in patients with oral inflammation[24].

To the best of our knowledge, no studies had demonstrated the presence of defensins and thymosins in Noonan and West syndrome's saliva before this research study. In addition, there is a lack of evidence on the proteomic expression in the combined form of gingival overgrowths[25,26]. From a dental point of view, it was interesting to compare the two syndromic groups to a control group to analyze the differences in the appearance of periodontal tissues and inflammatory levels since in patients with West and Noonan syndrome is often present gingival hyperplasia, mostly drug-related[27]. Clinically, the defensins have an inflammatory and immune significance: they manage to insert themselves into the membranes and induce the formation of pores with consequent death by cell lysis. They are involved in the signaling route of the innate immune response, being part of the specific and most immediate way through which the organism reacts against pathogens[28]. Thymosin β4 is statistically increased in syndromic patients, while its values are lower in samples from healthy subjects[29]. Thymosin β4 plays a significant role in innate antibacterial immune responses in addition to *in vitro* bacteriostatic activity: it regulates the production of laminin, a protein that acts in the wound healing process[1]. In patients with Noonan syndrome, thymosin β4 and thymosin β10 were quantified, while in patients with West syndrome, thymosin β4, which is the most expressed, was followed by thymosin β1.

We also noted that for West and Noonan syndrome the defensin levels α1, α2, α3, and α4 were higher than those observed in healthy subjects; in addition, in patients with Noonan syndrome, α3-defensin was the most expressed, while in patients with West syndrome the α1 protein was more expressed. The defensins (human neutrophilic peptides, HNP) are anti-microbial peptides (AMPs) released from polymorphonuclear neutrophil (PMN) degranulating upon bacterial

stimulation in the salivary fluid contribute to the maintenance of general oral healthy homeostasis, playing an important role of activating the acquired immunity and stimulating the opsonization of bacteria[30].

In the classification of the progression of gingival inflammation, according to Page and Schroeder[31], a gum condition called "clinically healthy" was recognized. Clinically healthy gingiva does not present any alteration at the clinical level, but histologically, it is characterized by the presence of neutrophils and macrophages in the junctional epithelium and of lymphocytes, especially polymorphonuclear cells (PMN), in the underlying connective tissue[32]. Thanks to the defense due to the phagocytic action of macrophages and neutrophils, the clinically healthy gum faces the attack of microbes, thus avoiding the onset of the disease[33].

Strengths and Limitations

The present study has the strength to propose salivary proteomics as an inexpensive and non-invasive test, representing a source of information; however, its limitation is that the methodology does not provide a biological basis to make a differential diagnosis between a structural diversity in the salivary fluid of different syndromes and a secondary inflammatory change due to bacterial colonization of pre-existent non-inflammatory gingival enlargements. The absence of a follow-up recall appointment makes it impossible to perform a longitudinal analysis of protein concentration in the salivary fluid. Further studies are required to transfer genomic and proteomic knowledge into clinical practice, thus providing answers in terms of prevention, diagnosis, prognosis, and prediction of increasingly accurate and personalized treatments.

Conclusions

Abnormalities in the salivary protein profile have been found in West and Noonan patients with developmental gingival enlargements. The commitment of the scientific community is now to identify which biochemical parameters could be specific for non-inflammatory gingival enlargements.

Conflict of Interest

The authors declare that they have no conflict of interests.

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Authors' Contribution

Conceptualization, P.G., E.S., and F.G.; methodology, E.S.; software, F.I. and A.F.; validation, F.G. and E.S.; formal analysis, F.I. and A.F.; investigation, F.G; data curation, F.K., A.F. and F.I.; writing – original draft preparation, F.K.; writing – review and editing, F.G. and E.S.; supervision, P.G.; project administration, P.G.; funding acquisition, E.S. All authors have read and agreed to the published version of the manuscript.

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Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics Approval

The study was approved by the Internal Review Board of Policlinico Fondazione Agostino Gemelli (Protocol ID n° 3452, acceptance number: 0043903/20). All procedures were conducted in compliance with the Ethical Principles for Medical Research Involving Human Subjects outlined in the Helsinki Declaration in 1975.

Informed Consent

Written informed consent was obtained from all subjects involved in the study.

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