Oral fluids and auxiliary diagnostic techniques in autoimmune and neuropathic disorders of the oral cavity. Local and systemic implications

Fluidi orali e tecniche ausiliarie diagnostiche nei disordini autoimmuni e neuropatici del cavo orale. Implicazioni locali e sistemiche

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1. INTRODUCTION TO THE EXPERIMENTAL WORK

The oral cavity is characterized by an environmental moderate temperature and high humidity. These features allow the growth of aerobic and anaerobic microorganisms which establish a complex ecosystem [1].

Saliva is the most important element to maintain balance within the oral ecosystem.

It performs:

- **digestive** functions, through the presence of α-amylase;
- **emollient and lubricant** functions, thanks to water and mucins;
- **protective** functions for dental elements, oral mucosa and oesophagus with the contribution from antiviral, antibacterial and antifungal substances.

The *crevicular fluid*, also known as *gingival liquid*, is produced by the epithelium localized in the gingival sulcus surrounding teeth crowns. It is produced in very small amounts by healthy gingiva, but in large amounts when gums are inflammed [2]. It performs protective functions for gingival and dental tissues, but it may also be involved in the build-up of dental deposits due to its high protein and calcium content.

1.1 SALIVARY PRODUCTION

Saliva represents the first digestive juice which is encountered along the gastrointestinal tube and is produced by the salivary glands.
There are three major salivary glands which lie extra-orally: the parotyd gland, the submandibular gland and the sublingual gland. However, buccal, palatine, labial and lingual mucosae contain many minor salivary glands.

The parotyd gland weighs 25-30 g and shows a racemose architecture until Stensen’s duct.

The submandibular gland weighs 8-10 g. Like the parotyd gland, it shows a racemose structure to its duct, known as Wharton’s duct.

The sublingual gland, instead, weighs only 2-3 g and shows multiple ducts, known as Rivinus ducts and Bhartolin’s duct.

Under normal conditions, human beings produce between 800 and 1,500 ml of saliva per day, with each gland contributing in a different manner. At rest, 70-75% of saliva is mainly produced by the submandibular gland, while only 20% relies on the parotyd gland.

When a mechanical or chemycal stimulus acts on the oral cavity, like during chewing or after the application of citric acid, over 50% of stimulated saliva comes from the parotyd gland, when only 30-45% relies on the submandibular gland.

At night, the sublingual glands are responsible for the production of 14% of salivary amount during sleep.

Less data have been collected about the contribution of minor salivary glands to saliva production, but they are thought to provide constant lubrication of oral mucosa during the day, allowing the presence of antimicrobial substances [3](Fig. 1).
Fig. 1: Salivary production rates. The salivary glands contribute with different rates according to the physiologic state.

The salivary glands consist of a dead-end duct system, starting from *intercalated ducts* and developing through ducts of higher caliber known as *striated ducts* to *secretory ducts*.

In salivary glands, saliva secretion is a two-phase process: firstly, primary saliva is produced by acinar cells localized in the secretory pole together with intercalated ducts.
Secondly, saliva flows along the striated and secretory ducts where it is modified. Thus the ionic properties of saliva depend on this mechanism [4].

The mechanisms underlying the production of saliva are very complex; a key element is certainly represented by an increasing concentration of calcium ions within acinar cells when they are stimulated by their associated nerve fibers.

Both parasympathetic and sympathetic fibers contribute to salivary production. In this district the two systems work synergistically and not in opposition like in other structures.

Acetylcholine released by parasympathetic fibers activates the inositol triphosphate (IP$_3$) intracellular signaling pathway. The same do α1-adrenergic receptors. On the contrary, β2-adrenergic receptors activate the adenosine monophosphate (AMP) pathway, which stimulates genic transcription, post-transcriptional modifications and secretory vesicles. Acetylcholine stimulation is more effective in producing primary saliva.

The increased levels of intracellular calcium ions lead to complex mechanisms which result in an augmented production of saliva [5].

The autonomous nervous system regulates the secretory activity; moreover drugs influence on this system may trigger alterations in the composition of salivary liquid and in its flow rate.

The striated ducts are completely impermeable to water and this results in an always hypotonic fluid if compared to plasma.

The central nervous system is involved in a more sophisticated regulation of salivary secretion.

Chewing and gustatory stimulation activate the parasympathetic system through the trigeminal lower and upper salivatory nuclei, as well as facial, glossopharyngeal
and vagus nerve fibers, while the sympathetic system is activated through the superior cervical ganglion.

Not only do these fibers stimulate the production of saliva in the acinar cells, but also activate the myoepithelial cells, which are responsible for ductal contraction.

The resulting salivary flow does not depend exclusively on the nature of the stimulus, but also on its duration and intensity. Sour flavours and frequent chewing induce higher salivary flows, but while the parasympathetic stimulation leads to a strong watery salivary flow, the sympathetic stimulation triggers a minor stream, but enriched in mucins, and therefore particularly viscous [6].

Salivary secretion is certainly influenced by the bloodstream which reaches the glands, because the liquid which makes up saliva derives from the interstitial fluid and from the capillary blood.

Blood vessels caliber is regulated by the autonomous nervous system, consequently it can contribute to a greater or minor salivary flow.

Conditioned reflexes influence the production of saliva. The most famous experiment in this field was carried out by Ivan Pavlov on dogs at the end of the nineteenth century. Conditioned reflexes are those actions or feelings acquired as the result of experience to a specific situation or stimulus.

Associative cortices allow a smell or a memory to induce the production of saliva thanks to a pairing association, without the real presence of a stimulus inside the mouth.

Emotional and affective attitudes are strictly related to conditioned reflexes. This feature explains how anxiety or stress may lead to a diminished salivary secretion or to an altered salivary composition.
Psychiatric diseases and disorders are known to be linked to xerostomia, which is defined as the sensation of dry mouth in the presence or absence of a lower salivary flow (Fig.2).

![Control of Salivary Secretion by Nerves](image)

**Fig.2:** control of salivary secretion by nerves. Parasympathetic efferent fibers are coloured in blue, while sympathetic fibers are coloured in red (from Proctor GB [7]).

The first study described in this dissertation, entitled “Low basal salivary flow and Burning Mouth Syndrome: new evidence in this enigmatic pathology”, investigates the role of this very common psychosomatic and neuropathic disorder of the oral cavity in the alteration of salivary flow rates and volumes brought about by strong emotional disorders linked to the disease.
1.2 SALIVARY COMPOSITION

The salivary fluid is made up of two components: a serous secretion and a mixed secretion.

The serous secretion is enriched in water and α-amilase (ptyalin), the enzyme produced by the parotid gland and which is responsible for the digestion of starches.

The mixed secretion, both serous and mucous, is produced by the submandibular and sublingual glands and it is very viscous. The key element in this component is mucin, therefore the secretion is responsible for protective and lubricant functions on oral mucosae [1].

Each gland produces its own saliva, thus the result is a mixed saliva, with the addition of flaking cells, white blood cells and bacteria. The salivary liquid is always hypotonic if compared to blood, even when the composition is altered by an increased salivary flow rate.

A group of organic compounds and electrolytes can be recognized inside the salivary fluid.

The most important organic particles that can be detected in saliva are the proteins secreted by acinar and ductal cells. There are several tens of proteins varying from a few kDa to even more than 1000 kDa of weight. The liquid can reach the level of 2,2 mg/ml of proteins, but it is always less than 60-80 mg/ml detected in blood.

Among the most important proteic compounds there are:

- **Cortisol**, a glycoprotein produced by adrenal glands. Its concentration in saliva ranges between 10 and 20 nM. Under a stressful condition, salivary cortisol can reach a concentration of 30 nM, thus measuring
the presence of this hormone in saliva may help to better understand the presence of underlying stressful conditions or events in patients’ life, avoiding blood collection;

- *Sexual hormones* are present in saliva and they may be monitorized during pregnancy;

- *Glucose* shows concentration values less than 0.1 nM, but in non-compensated diabetic patients values may rise up to 1 nM, and this explains how dental caries and periodontitis are sensibly frequent in diabetic patients;

- *Urea*, which is a catabolyc product of proteins, shows a concentration of 2-4 nM, but in renal failure it can reach values over 85 nM;

- *AB0 blood antigen groups* may be used for forensic medicine.

Among the electrolytes sodium, chlorine, calcium and bicarbonate show higher values when the salivary flow rate is increased, as opposed to what potassium and phosphate do. It should be remembered that the circadian rhythm influences salivary composition [8].

### 1.3 Salivary Functions

Saliva performs a lot of important functions that are essential for the maintenance of oral health. Most of these functions depend on the interaction between saliva and oral surfaces of varying texture and polarity.

The first important role that saliva plays in the oral cavity is the maintenance of *good hygienic conditions* in oral tissues.
This function is performed thanks to the presence of different molecules:

- **Thyocianate** ions;
- **Lysozyme** and other enzymes, which mainly attack bacteria and allow tyocianate ions to enter bacterial cells leading to their destruction;
- **Antibodies**;
- **Growth factors** and **cytokines**.

In addition, the salivary flow washes oral mucosae continuously and flushes away oral microorganisms and food debris. This ability together with swallowing is known as oral clearance [9].

Another important function of saliva is its **buffering capacity**.

Salivary normal pH values are 6.0-7.5 and these values are maintained by different buffering systems. When pH values decrease to 5.0-5.5, dental health is jeopardized since bacterial fermentation and tooth decay can start.

The three buffering systems described in saliva are:

- the **bicarbonate** buffering system;
- the **phosphates** buffering system;
- the **proteins** buffering system.

Salivary proteins play an important role in several functions.

Proteins together certainly determine salivary viscosity and consistency, but some specific proteins perform specific functions:

- **Digestive functions**: serous cells in parotyd and submandibular glands secrete salivary α-amilase after parasympathetic stimulation. The
enzyme is responsible for the degradation of starches and 1-4 glycoside bond in general. It is a very important enzyme, which is degradated in the stomach, highlighting thus the fundamental role of saliva in digestion.

- **Lubricant functions**: two types of mucins can be found in human saliva and they form a gelatinous layer. G1 mucin weighs more than 1.000 kDa and it is the biggest protein in saliva. It is secreted by acinar cells in the submandibular and sublingual glands and also by labial and palatine glands. G2 mucin shows a low molecular weigh, 150-200 kDa, and is produced by the serous cells of many glands, with the exception of parotyd glands [10].

- **Calcium homeostasis**: human saliva is generally supersaturated with hydroxyapatite and other calcium phosphate salts. The calcium binding protein amount markedly varies between healthy patients and patients affected by dental caries. *Statherin*, a small 4-5 kDa protein secreted by acinar cells, inhibits formation of aggregates and prevents intraductal calcification and tartar formation. *Proline-rich proteins (PRPs)* also inhibit the formation of aggregates. They account for 30% of proteic formation in submandibular and parotyd glands.

- **Catalysing the formation of carbonic acid**: carbonic anhydrase is an enzyme which catalyzes carbon dioxide into carbonic acid. The VI isoform known as gustin is a zinc-containing metalloprotein which weighs 37 kDa and represents 3% of human salivary proteins. It is also a growth factor for taste buds.

Special attention should be devoted to *antibacterial functions*. 
There are many antibacterial, antiviral and antifungal agents in human saliva. An alteration in their composition or functions may explain how several subjects are more susceptible to infections than others.

*Immunoglobulins A* are large hydrophilic proteins (380 kDa) produced by plasma cells first and then modified and secreted by acinar and ductal cells. Labial glands are highly specialized in producing IgAs, which represent strong antibacterial agents because of their interaction with mucins and aggregated bacteria.

*Lysozyme*, which weighs about 14 kDa, lyses Gram-positive walls.

*Lactoferrin* is instead a larger protein (75 kDa) and it binds to iron, taking away this precious element from bacterial enzymatic functions.

*Histatins* are small peptides (3-4 kDa) mainly secreted by parotyd glands and are especially effective against *Candida albicans, Streptococcus mutans* and *Porphyromonas gingivalis*.

*Peroxydase* is another enzyme which catalyzes oxydation of tyocyanate into isocyanate thus blocking bacterial metabolism.

*Antimicrobial peptides (AMPs)* are small cathyonic peptides of less than 100 amino acids that are found in host defense settings, and that have antimicrobial activity at physiological concentrations. They can be divided into *cathelicidin LL-37, α-defensins* and *β-defensins*. First described in neutrophils and Paneth cells, they have been recently detected in saliva and epithelial cells [11].

Furthermore, it has been shown that AMPs participate in immunomodulation, so an alteration in their quantity may be related to the pathogenesis of several infective or autoimmune disease [12].
The second study which is included in this dissertation, “Human \( \beta_2 \)-defensins in Oral Lichen Planus and Burning Mouth Syndrome”, investigates if the expression of defensins may play a statistically significant role in the pathogenesis of these oral pathologies.

Other proteins show multiple functions, like cystatins, which are endogenous proteases inhibitors and immunomodulatory agents.

Besides, many proteins coming from the bloodstream may be found in saliva, like albumins, Epithelial Growth Factor (EGF), TGF-\( \alpha \), TGF-\( \beta \) and Fibroblast Growth Factor (FGF) which can be helpful in wound healing and tissue repairing.

In conclusion, saliva is an accessible biofluid that contains components derived from the mucosal surfaces, gingival crevices and tooth surfaces of the mouth. Saliva also contains microorganisms that colonize the mouth and other exogenous substances. For all these reasons, it can potentially provide an insight into the relationship between the host and the environment.

**1.4 SALIVARY COLLECTION**

Collecting homogeneous samples of saliva is very difficult because standardized protocols are lacking. What we know for certain about saliva is its constant variability due to circadian rhythms during the day.

There are some independent variables which influence the salivary flow rate and which cannot be under control during collection: sex, age, body weight, salivary glands dimension, physical and mental health, drugs intake. On the contrary, what a dental clinician can do to perform a collecting procedure as accurately as possible,
is to control dependent variables, like previous stimulation, time, exposition to light, body position, temperature. As long as the dental clinician creates homogeneous environmental conditions and removes the influence of dependent variables, he will be able to perform a useful examination of the salivary fluid [13].

- The most important variable to take into account is the time of day at which the sample is collected. Best conditions are reported at morning fasting or between 8.00 and 11.00 a.m. [14]. Patients should avoid eating or drinking during 90 minutes before examination. Once the time for collection has been established, it must remain the same to compare results from different tests.
- Time for collection should be appropriate, for example 15 minutes.
- Patient’s head must be bent forward and maintained in the same position.
- Light and room temperature should be as constant as possible.

There are several procedures which are described in the international literature for salivary collection.

To obtain an appropriate sample of unstimulated saliva there are two main techniques:

- The spitting method: the patient’s head is bent forward and he/she sits on the dental chair. He/she should spit the salivary content in his/her mouth every minute for at least 5-15 minutes.
- The drooling method: patient does not spit but lets his/her saliva drain constantly in a funnel or test tube.
On the contrary, if the clinician needs to examine *stimulated saliva*, there are several methods to obtain a correct stimulation of the fluid.

- **The chewing technique**: patients chew a piece of paraffin wax for 5 minutes and then spit the entire content in a dedicated container.

- **The taste technique**: some drops of citric acid 2% solution are applied in the patient’s mouth stimulating thus the production of saliva from the parotyd gland.

- **The absorbent technique**: a cotton roll or a sponge gauze is placed into the patient’s mouth to passively absorb the fluid stimulated by the presence of a foreign body in the oral cavity.

These techniques are valid to obtain a collection of the entire mixed saliva which is present in the oral cavity, but they do not distinguish from one type of saliva produced by a gland or by another [15].
Some authors have tried to introduce more techniques to collect specific saliva from
a single gland and some ideas have been tested, like for example the Lashley cup
for the parotyd gland, or the Wolff’s device for submandibular and sublingual glands
[16]. However, these devices have not encountered much success due to their high
cost.

Fig.4a: Lashley’s cup in position on the Stensens duct’s outlet; 3b-c: Wolff’s device for saliva collection (from Sreebny LM and Vissink A [15])
1.5 THE CREVICULAR FLUID

The crevicular fluid, also known as gingival liquid, is a fluid which is contained in the gingival crevice, a space located around a tooth between the wall of the unattached gum tissue and the enamel and/or cementum of the tooth. At the base of the gingival sulcus there is the junctional epithelium which adhers to the dental surface.

Gingival crevice fluid flow is a decisive factor in the ecology of the periodontal pocket or sulcus. It creates a flushing action and an isolation effect.

- **Flushing action**: substances put into the periodontal pocket are rapidly washed out. Even before the importance of this liquid was discovered, clearance of carbon particles and of amalgam particles from the gingival sulcus had been reported.

- **Isolation effect**: substances from the outside do not easily penetrate the periodontal pocket. The crevicular fluid is isolated from saliva and this can be demonstrated by the fact that α-amylase, which is highly expressed in saliva, is not identifiable in the crevicular fluid. On the contrary, the immunoglobulin IgG concentration in the crevicular fluid is approximately 100 times that found in saliva [17].

To obtain a useful collection of crevicular fluid, the clinician should stimulate its production mechanically or chemically and then collect samples with a paper cone which gets soaked by capillarity.
It has been calculated that during the day a total amount of 0.5-2.5 ml is produced, being this production greater in molar areas than in incisive areas.

The composition of crevicular fluid is similar to that of the interstitial fluid. It may also contain epithelial exfoliated cells and white blood cells, mostly neutrophils. In case of inflammation, like during gingivitis or periodontitis, the white blood cells amount increases vertiginously.

Measuring the composition of electrolytes in crevicular fluid is more difficult than in saliva due to its very low volume; however, it contains a higher concentration of sodium and potassium than that recorded in plasma.

Different enzymes can be detected inside the liquid, like alkaline phosphatase, ß-glucuronidase, lysozyme and several proteases.

The main role of crevicular fluid is to protect healthy gingiva, but in case of inflammation it becomes a reservoir of dangerous and toxic substances for gingival
tissues. Likewise, an excess in calcium levels may lead to the formation of dental plaque and tartar.

Therefore the crevicular fluid analysis is important to detect periodontal pathogens or to measure genetic indices when a chronic inflammatory disease is present.

**1.6 SALIVA AND Crevicular fluid AS diagnostic tools**

Unstimulated whole-mouth saliva is preferred as a biomarker fluid because the potential variation created by using different types and intensities of reflex stimulation is avoided. One of the drawbacks of using unstimulated whole-mouth saliva is that the volume of fluid obtained can be low, particularly in older subjects and in those taking xerogenic medications [18]. A broad range of components are present in saliva and represent potential biomarkers for different pathological entities.

- **Diabetes** is a metabolic disease caused by insufficient insulin secretion or by insuline resistance, which leads to a glucose metabolism disorder. A positive correlation was found between α2-macroglobulin and HbA1c. Registered levels of α2-macroglobulin in saliva could reflect the glycemic control in patients with type 2 diabetes mellitus.

- **Cardiovascular disease** (CVD) is related to the circulatory system and includes atherosclerosis, myocardial infarction and coronary heart disease. Some authors found that levels of salivary inflammatory cytokines, including IL-1β, IL-6, TNF-α and prostaglandin E2, increased significantly in both atherosclerosis and periodontal disease. These cytokines might be potential biomarkers for the diagnosis of periodontal disease and atherosclerosis.
- **Pancreatic cancer**: it was found that KRAS, MBD3L2, ACRV1 and DPM1 levels enabled the differentiation between patients with pancreatitis and healthy individuals. There is also a correlation between periodontititis and pancreatic cancer onset. Patients with periodontititis show a 64% higher risk of pancreatic cancer.

- **Breast cancer**: the levels of CA15-3 and c-erb.2 were found to be increased in the saliva of patients affected by this cancer.

- **Lung cancer**: EGFR mutations in the saliva of patients with non-small cell lung carcinoma (NSCLC) have been described.

- **Prostate cancer**: miR21 and miR-141 biomarkers can be detected in the saliva of patients affected by this tumour.

- Diagnostic tests for **viral infections** currently rely on salivary biomarkers, such as viral DNA and RNA, antigens and antibodies. *Hepatitis A virus, hepatitis B virus, hepatitis C virus, HIV-1, measles virus, rubella virus* and *mumps virus* could be detected thanks to proteomics.

- **Helicobacter pylori**: *Helicobacter pylori* is a widespread bacterium, which is found within the water and in some animals’ biological fluids, and it is responsible for chronic gastritis and other gastric diseases. Apart from the stomach, HP has also been found in the distal oesophagus, proximal duodenum, colonic contents, and oral cavity, including tonsils and adenoid tissue.

Extensive research on the relationship between the oral cavity and gastric HP infection has been published, with numerous studies providing evidence that the oral cavity may be a potential reservoir for HP [19].
The third study presented in this dissertation, “Helicobacter pylori in periodontal pockets and saliva: a possible role in gastric infection relapses? A preliminary study in Northern Italy”, tries to establish whether the presence of HP within oral tissues can be an extra-gastric reservoir which contributes to gastric relapses after eradication therapy in a determining manner.

A particular subject, which has recently drawn attention in the international literature, is the relationship between salivary, crevicular fluid biomarkers and Periodontal Disease [20].

Periodontal Disease (PD) is a time consuming and expensive condition to treat and therefore prevention and early detection are considered important factors by dental clinicians.

Research is trying to develop biomarkers to early detect periodontal disease and identify its progression, because current diagnostic approaches do not show the disease activity but only assess the clinical evolution of tissue destruction. Furthermore, studies of the salivary mediators associated with the disease may help in the development of novel therapies aimed at controlling cytokines availability or by targeting the intracellular signaling pathways they activate, approaches which have proved successful in the treatment of other chronic inflammatory diseases, such as Rheumatoid Arthritis.

The fourth study reported in this dissertation, entitled “Rheumatoid Arthritis therapies and Periodontal disease: any correlation?”, proposes evaluating the correlation between genetic and microbiological markers detected in the crevicular fluid in a group of 35 patients affected by Rheumatoid Arthritis with those collected from their bloodstream indicating the rheumatic disease activity.
Among the most recognized cytokines which may have a role in periodontal inflammatory disease there are *Interleukin-1* (*IL-1*), *Tumor Necrosis Factor–α* (*TNF-α*), *Interleukin-6* (*IL-6*) and *Interleukin-10*.

- **IL-1** family is produced by a wide variety of cell types and performs critical functions in innate and adaptive immune responses to infection. IL-1 is a proinflammatory cytokine. In periodontitis, IL-1β is associated with neutrophils recruitment and activation of osteoclasts. Furthermore, the association between periodontitis and elevated IL-1β in gingival crevicular fluid is well established.

- **TNF-α** has a role in cell proliferation, apoptosis and morphogenesis, as well as in host immune defense. It is considered as a key proinflammatory cytokine, but it also has numerous homeostatic functions in human physiology, including a role in neurological regulation. This cytokine is mainly produced by macrophages as a primary response to toll-like receptor activation, by T-cells and NK cells. The long-lasting success of anti-TNF-α therapy in Rheumatoid Arthritis has highlighted how this cytokine is important in inflammatory diseases, including periodontitis.

- **IL-6** is produced by innate immune cells as macrophages and dendritic cells, but also by some CD4+ T-cells, as well as by nonimmune cells, like fibroblasts and endothelial cells. IL-6 is increased in many inflammatory diseases and activates B-lymphocytes. It is interesting that some reports showed how IL-6 blockade proved to be efficacious in the treatment of Rheumatoid Arthritis. Also, whereas some studies have shown that salivary IL-6
concentrations are significantly higher in patients with periodontitis than in healthy individuals, other have found no differences.

- **IL-10** is widely produced by innate and adaptive immune cells alike and is a key anti-inflammatory cytokine, involved in inhibiting and regulating proinflammatory immune responses and in promoting resolution of inflammation. Thus IL-10 is considered an anti-inflammatory cytokine, in opposition to IL-6 and TNF-α.

The advantages of saliva as a diagnostic fluid are that it is simple to collect, convenient to store, essentially non-invasive and contains high-quality DNA. Saliva could become a perfect substitute for blood and research in proteomics may have an important role in identifying biomarkers of diseases and potential targets for drugs. Besides, it would be possible to diagnose diseases at their early stages.

However, research on saliva and its applications for the diagnosis of diseases is still at the beginning and progress is limited by the lack of standardized methods and protocols.

In this dissertation four experimental studies conducted between 2014 and 2016 have been described. Each of them analyzed a particular feature of oral fluids and verified whether saliva and/or the crevicular fluid may be helpful in diagnosing underlying pathologies, monitorizing systemic diseases activity or contributing to a better understanding of some idiopathic oral pathologies.

- In the **first study**, saliva was quantitatively analyzed through the spitting method in a group of 111 patients. Among them, 44 were affected by the most common idiopathic psychosomatic disorder of the oral cavity, known as Burning Mouth Syndrome (BMS), a frustrating condition in which patients refer a burning, tingling,
itching chronic sensation in their tongue or oral mucosa in total absence of clinical and laboratory findings. Through the use of the spitting method, a quantitative analysis of basal salivary flow and stimulated salivary flow was performed and interesting findings were found in the BMS group compared to other patients. Actually, patients affected by the syndrome showed a decreased basal salivary flow while the stimulated flow was normal when compared to the other groups. This study showed how saliva is a complex fluid, composed of different elements coming from each one of the salivary glands, and that the central nervous system and emotional attitudes are very strong influencing agents on the oral health.

- In the second study, a total number of 35 patients were recruited and salivary fluid and crevicular fluid samples were collected to measure the presence of Human ß2-defensin, an antimicrobial peptide involved in defensive functions but also in immunomodulatory ones. A group of 17 patients were affected by the most common autoimmune disease of the oral cavity, Oral Lichen Planus (OLP). Data collected from this group were compared to other 18 patients, 9 affected by BMS and 9 as a control group (CTRL). It was observed that HBD-2 was strictly linked to the degree of local inflammation and that it could become a very important index to measure the disease’s activity and its evolution in the future.

- In the third study a total group of 30 patients with a positive diagnosis for Helicobacter pylori gastric infection were analyzed with microbiological examination performed on salivary and crevicular
fluid samples. The aim of this research was to establish whether the oral cavity may represent a possible reservoir for gastric infection relapses after eradication therapy. Data collected were compared with other 30 patients who were negative on gastric examination. This study demonstrated that periodontal pockets and saliva contain *Helicobacter pylori* and that it does not depend on a retrograde path from the stomach to the oral cavity, but relies on oral health and mostly on periodontal conditions, leading thus to claim that oral hygiene is useful in preventing the formation of a reservoir of microorganisms involved in gastritis and gastric cancer.

- In the *fourth study*, a total group of 34 patients affected by Rheumatoid Arthritis were examined both by the rheumatologist, who registered serum values indicating disease activity, and by the dental clinician, who described periodontal conditions and collected gingival liquid samples. Oral fluids were then analyzed both microbiologically and genetically. Several links between rheumatic factors and periodontal indices were found. Furthermore, patients were divided into groups based upon the drug regimen adopted to treat Rheumatoid Arthritis. Patients undergoing treatment with metothrexate showed worse periodontal indeces, confirming that periodontal tissues and oral fluids represent reliable biomarkers in establishing a correlation with systemic diseases.
In the end, a total number of 240 patients were recruited in this experimental work and each study contributed to new topics in the research on saliva and crevicular fluid as potential diagnostic tools for oral pathologies and systemic diseases.

Plot 1: histogram showing the distribution of the recruited 240 patients among the four studies which are discussed in the dissertation.
2. **1st STUDY: LOW BASAL SALIVARY FLOW AND BURNING MOUTH SYNDROME: NEW EVIDENCE IN THIS ENIGMATIC PATHOLOGY**

2.1 **BACKGROUND**

2.1.1 **Definition**

For at least one century, many researchers have reported in their scientific studies and clinical trials a particular symptomatology: oral burning. In the last decades, they have sought to elucidate the aetiology, the pathogenetic hypothesis of oral burning with clinical research, more or less complete and documented. They have identified specific causal factors, local and systemic, able to explain the burning sensation and the oral pain [21].

However, in many patients it has not been possible to pinpoint a specific aetiology because of negative results on clinical examination. These clinical findings have raised obvious questions, often followed by not very well documented and comprehensive responses. A series of terms and definitions has been suggested to the scientific arena. A definition that sums up, synthesizes and anticipates a complex clinical picture of BMS was expressed by Van der Waal: “The Burning Mouth Syndrome (BMS) is an enigmatous condition for both the patient and the clinician” [22].

The terms glossodynia or glossalgia can be used to describe a painful tongue, and the term glossopyrosis to describe only a lingual burning sensation.
When little discomfort is experienced, the term lingual dysesthesia can be used. Likewise, for complaints elsewhere in the oral cavity, outside the tongue, the terms stomatodynia, stomatopyrosis and oral dysesthesia could be used respectively.

Lamb and Lamey in 1988 [23] defined BMS as a distinct clinical entity of multifactorial aetiology, in which no oral mucosa abnormality is evident on clinical examination. Any area of the oral mucosa can be affected, but the tongue and denture bearing areas are most commonly involved. Besides, van der Waal in 1990 specified that the term BMS should be used only in patients with idiopathic complaints, among which the main symptom is usually described as a burning, painful or itching sensation. It is located in the oral mucosa, with or without involvement of the tongue and with or without associated symptoms in the oral cavity or elsewhere in the body [22].

It is well recognized that many patients complaining of a burning mouth also suffer from a dry mouth, xerostomia, with or without an associated loss of taste or a distorsion of the sense of taste, and with or without a number of other symptoms justifying the term of the syndrome.

Zaskin and Moulton believed that BMS involved a strong emotional component, and attributed its cause to psychogenic or psychological factors and psychopathologic components. In fact, psychological causes are frequently postulated when no physical cause is apparent [24].

Some authors classify BMS under the heading of Atypical Facial Pain (ATP). AFP, atypical odontalgia and oral dysesthesia, including BMS, may occur either sequentially or simultaneously in the same patient, and may be associated with facial arthromyalgia in which the temporomandibular joint or its musculature are involved [25].

In most patients clinical examination is normal and no causative factors are found out. Moreover, the International Association for the Study of Pain (IASP) defines it
as a pain of at least 4-6 months duration. In 2004 IASP and IHS (International Headache Society) defined BMS as a distinctive nosological entity, including all forms of burning or stinging sensation in the mouth, pain, associated with an oral mucosa that appears normal in absence of local, dental or systemic conditions [26].

2.1.2 Epidemiology

The true prevalence of BMS is difficult to establish due to the lack of rigorous diagnostic criteria in many of the published series that do not distinguish between the symptom of oral burning and the syndrome itself, regarding BMS as only a symptom of other diseases. Thus, figures vary widely, with prevalence ranging from 0.7 to 15% [27].

In general, the condition principally affects women, with a ratio of approximately 3:1. This difference between genders may perhaps be explained by biological, psychological and sociocultural factors; however such features have not been defined yet. This syndrome is rare in patients under 30 years of age, having never been detected in children or adolescents. Studies into any occupational, educational or social grouping are not available.

Patients are almost exclusively elderly, the mean age of patients being 50-60 years (range 36-84 years)[28]. There is a very strong female prevalence greater than 3:1; this feature is especially noticeable in patients over 50 years. This could be explained by the fact that women seek health care more frequently than men, but it could also be suggestive of menopausal factors if the rising prevalence in older women is taken into account.

In recent years a large sample of 123 BMS patients have been visited in Varese (Department of Surgical and Morphological Sciences, University of Insubria, ASST dei Sette Laghi, Unit of Oral Pathology) and Milano (Department of Biomedical,
Surgical and Dental Sciences, University of Milano, Policlinico Hospital IRCCS Ca’Granda Foundation, Unit of Oral Pathology and Medicine). Epidemiologic data have shown a strong female preponderance with a 4,5:1 ratio to men. The registered mean age is 66, ranging between 37 and 90.

<table>
<thead>
<tr>
<th>PATIENTS</th>
<th>MEN</th>
<th>WOMEN</th>
</tr>
</thead>
<tbody>
<tr>
<td>123</td>
<td>22</td>
<td>101</td>
</tr>
</tbody>
</table>

Table 1: Sex distribution of 123 BMS patients visited during 2014, 2015 and 2016

Plot 2: data showed a 4,5:1 female preponderance to men.

2.1.3 Aetiology

It should be considered that an oral burning sensation with the presence of possible related presentations can have various origins. Several aetiological causes, local and/or systemic, can cause a burning symptom in different oral regions. Local factors, identified by dental and periodontal diseases or soft tissue disorders, and systemic factors, connected with organic disease, should be considered.

Among local conditions oral candidiasis, geographic tongue, hairy tongue, fissured tongue, median rhomboid glossitis, foliate papillitis, fusospirochetal infection, inflammatory oral diseases, Oral Lichen Planus, leukoplakia and erythroplakia could
be associated with an oral burning complaint. Food allergy, smoke and alcohol consumption contribute to oral burning.

Among odontogenic factors, dental treatment, denture-related problems, sensitivity to acrylic resin, galvanism, metal allergy, parafunctional behaviour may be related to the onset of an oral burning sensation.

Among systemic causes, which may be linked to oral burning, hormonal disturbances, iron-deficiency anaemia, pernicious anaemia, vitamin B12 and nutritional deficiencies, hypocalcaemia, hypo or achlorhydria, diabetes mellitus, vascular disturbances, Sjögren’s Syndrome and xerostomia, side-effects of medication, hypothyroidism, immunologic disturbances and rheumatism should be ruled out.

If patients report an oral burning sensation and the clinical examination does not show any clinical sign, the diagnostic hypothesis will be that of BMS. A final diagnosis can only be made after further laboratory tests with negative results.

### 2.1.4 Clinical features

BMS includes a wide variety of complaints. The burning pain is always reported. Other disorders such as prickling, itching or other bizarre sensations may be present. Although patients often describe the burning sensation as being intolerable, it is rarely able to impair the patient’s ADLs. A somewhat similar experience was reported by Hughes, who described the psychiatric disorders seen in 138 consecutive attenders at a psychiatric clinic in a dental hospital. A large number of BMS patients was included into that group [29].

The symptoms of BMS almost always have a bilateral pattern and, in contrast to symptoms in neurological disorders, have a non-anatomic and non-dermatomeric distribution. However, during the clinical exam specific sites of complaints can be
identified. The burning sensation most frequently occurs in the distal structures of the mouth, a pattern similar to that seen in symmetrical polyneuropathies. Most patients with BMS describe the burning sensation as occurring at more than one site in the mouth. In several reports the tongue and especially its tip is the most common location.

Almost without exception, the symptoms of BMS ceaselessly persist over a period of months or years, without distinct periods of remission.

The daily pattern of symptoms is constant for each individual patient. For instance, in some patients the burning is present day and night. In most patients with BMS, the symptoms are not present on waking up but set off and worsen in severity as the day progresses, without preventing the patient from falling asleep.

In 1989, Lamey and Lewis defined three subtypes of oral burning in BMS on the basis of diurnal variation of its symptomatology [30].

**Type 1** is characterized by progressive pain. Patients wake up without pain, which then increases throughout the day and reaches its maximum intensity by early evening. Type 1 affects approximately 50% of patients. This type may be associated with systemic disorders, like hormonal imbalance in women.

In **type 2** the burning sensations are unvaried and continuous throughout the day and patients find it difficult to get to sleep. Type 2 represents 40% of patients. These patients usually present with associated psychological disorders and are the most resistant to therapy.

A burning-free period at night is always present in type 1 and type 2.

In **type 3** symptoms are intermittent and characterized by symptom-free days, with atypical pain and location. It accounts for 10% of patients. It seems that contact with oral allergens and food allergy can play an important aetiologic role in this group. Although in terms of aetiology there are similarities between these subtypes, it is
important to distinguish between them, since each type requires specific medical investigation and have different prognosis.

Various diagnostic criteria for the classification of BMS have been suggested but none of these have been validated [31].

The onset of BMS has been related to a previous dental procedure in 33% of subjects [32]. Other patients relate the onset of the syndrome to family health problems, threatened loss of a loved one, move and adverse life events. Other associate the symptoms with menopause, retirement, depression or multiple somatic complaints. Data from the international literature indicate that although BMS patients are affected by elevated psychological stress, the onset of BMS symptoms is not necessarily correlated with stressful life events.

A number of other oral complaints have been reported in BMS patients. Taste and sometimes olfaction disorders are often described, while xerostomia is among the most commonly reported associated symptom, being noted in more than 60% of patients.

Systemic symptoms often associated with BMS are gastrointestinal disorders, like constipation, heartburn, nausea, vomiting and colitis.

Headache, migraine, neck/back ache, skin disorders, dysfunctional uterine bleeding have been recorded in some papers [25].

2.1.5 Diagnostic management

When a brief history of the nature and duration of symptoms and an inspection of the oral cavity do not clearly reveal the cause of BMS, a more detailed approach is necessary.
Firstly, it is important to collect a detailed description of symptoms, such as their nature, location and distribution, their progress during the day, any possible interference with the individual’s daylife.

Secondly, the clinician should collect data about the patient’s medical history and any previous consultation with other physicians. It is essential to check for hypertension and metabolic diseases. Especially in patients who also complain of xerostomia, taking an accurate drug history is mandatory.

At any rate, regardless of the nature and exact distribution of symptoms, a thorough inspection of the entire oral cavity, including the tongue, should be carried out under the appropriate conditions. A localized burning or itching sensation can be the first sign of a malignant or premalignant mucosal lesion. Ignoring the importance of such symptoms or overlooking minor but ominous mucosal changes may have dramatic sequelae. It is striking that in most patients whose complaints include xerostomia a normal or sometimes even an abundant flow of saliva is found on inspection. The result of the oral examination can be more or less decisive for the future management of the patient.

When no oral mucosal lesions are detected on inspection, a large number of issues and questions need to be focused on. Cytologic or histologic examination of the mucosa at the site of burning or any other oral site does not contribute in any way to the further management of the patient. Even the collection of samples for oral smear tests is not helpful.

At this stage the patient needs detailed information about his/her problem. If possible, the spouse or any other close relative, should be present during any further interview with the patient. Any information about the syndrome should be factual and detailed.

1. BMS is a rather uncommon, but definitely not exceptional, syndrome that affects menopausal and post-menopausal women much more
often than men. Not many doctors and dentists are familiar with the signs and symptoms of the syndrome, which accounts for the possible different advice the patient may have received in the past.

2. In many cases aetiology is unknown, which results in a wide range of beliefs held by the patient, including the fear of an underlying malignant disease. By explicitly mentioning the unlikelihood of the latter, a number of patients will be completely reassured and will not require any further treatment or follow-up.

3. Another widely often-held belief by the patient is a possible allergy to food, toothpaste, chewing gum etc., which is rarely if ever the cause of BMS complaints. The same holds true for smoking habits and alcohol consumption.

4. As in other ailments or diseases for which no distinct cause can be found, the possibility of an underlying psychogenic cause is sometimes brought up. Nevertheless long lasting and intensive symptoms are more likely to affect someone’s psychological status rather than vice versa. A certain amount of caution is advisable at this point, and a referral to a psychologist is to be taken into account.

After the anamnestic procedure and oral examination, it is imperative to rule out any underlying systemic disease which could be associated with oral burning. Blood chemistry and allergological tests should be performed to demonstrate that the oral complaint is an idiopathic burning pain.
2.1.6 Therapy

BMS patients very often report a previous visit to other specialists, such as the general practitioner, who often does not know what BMS is, the gastroenterologist, who focuses his/her attention on gastro-esophageal reflux disease, the psychologist, who only considers the psychosomatic aspects of the syndrome or the general dentist, who is afraid that burning symptoms flare up after a dental surgical procedure or other kinds of treatment.

This confusion among different specialists becomes a frustrating condition for patients, who receive completely different answers and feel misunderstood.

Consequently, a point of reference should be found, and this could be provided by the stomatologist.

The stomatologist is actually the only specialist able to visit these patients, and he/she should be the coordinator of a team work also involving other consultants such as the neurologist, the rheumatologist, the gastroenterologist and the endocrinologist, according to the different needs of each patient.

In the international literature, different therapeutic protocols have been reported. Acupuncture, antihistaminics, inhibitors of gastric acid secretion, multivitamin integrators, antidepressants, antipsychotics (i.e. quetamine), even electroconvulsive therapy have been recommended [33].

As a result, it is imperative to establish a therapeutic protocol which could help the clinician to relieve the burning sensation.

Firstly, topical antifungal therapy can be prescribed.
The topical antifungal therapy is used to clean up the oral cavity from Candida albicans and to exclude the possibility of a burning sensation due to the presence of a subclinical oral candidiasis.

After this first step, the use of salivary substitutes, in the form of mouth rinses to effectuate at least 3 or 4 times a day for a long period, is prescribed.

Salivary substitutes are very important because they restore the natural equilibrium among the different components of oral fluids and help protect and lubricate oral mucosa.

For example, they contain lactoferrin, an enzyme which performs a bacteriostatic, antymicotic and antiviral function, making iron unavailable to microbes which require it as a necessary metabolite for their cycle.

Salivary substitutes also contain an important agent, lysozyme, which is effective in the natural defenses against microbes.

Lactoperoxidase is an important enzyme which catalyzes the oxidation of thiocyanate. The products of this oxidation serve a powerful bactericidal function.

Finally, the salivary substitutes contain whey proteins, which are fundamental to restoring oral cavity’s omeosthatis.

The following therapeutic step is represented by the use of specific substances which can desensitize the small nerve fibers associated with oral burning.

At the end of the nineties Nature published an interesting paper which demonstrated the existence of specific receptors for capsaicin, the molecular principle contained inside red hot peppers and responsible for the characteristic burning sensation in the oral cavity felt after their ingestion [34].

This paper paved the way for further research and the discovery of a family of particular receptors: the Transient Receptor Potential Vanilloid (TRPV).
This is a family of transient receptor potential ion channels. These channels are selective for calcium and magnesium over sodium ions.

The most interesting feature of these vanilloid receptors is that they can be activated at a different thermal threshold, while specific stimulation with chemical substances is converted into thermal sensation, i.e. a burning sensation after the assumption of capsaicin contained in the red hot pepper or a cold sensation after eating a gum or a sweet containing piper mint.

TRPV-1 was the first vanilloid receptor to be discovered and it has been studied because of its specific responsiveness to capsaicin.

Capsaicin is produced by chilly peppers and gives a sensation of burning in any tissue with which it comes into contact. Probably it is produced by red hot peppers as deterrent to certain mammals.

The most interesting clinical feature is that upon prolonged exposure to capsaicin, TRPV-1 activity decreases, a phenomenon called desensitization. This process involves intracellular calcium and specific signaling pathways.

Desensitization of TRPV-1 is thought to underlie the paradoxal analgesic effect of capsaicin.

The process of desensitization of TRPV-1 receptors could be obtained through the use of topical capsaicin, thanks to the successful outcome achieved by the treatment of diabetic peripheral neuropathy reported in the literature [35].

The prescription should include red hot chilly pepper powder mouth rinses 3 times a day. Patients should collect just the tip of a coffee spoon of red hot pepper powder and stir it in half a glass of water. It corresponds approximately to a concentration of 3,54 micrograms/ml of capsaicin [36].
After a mouth rinse of about 30 seconds patients should spit and must not take any food or drink in their mouth for at least 30 minutes.

The results of 99 patients who underwent treatment with topical capsaicin in Varese e Milano confirmed the importance of topical capsaicin.

Patients were subdivided into two groups, cooperative and non-cooperative, by using the psychological multidimension scale questionnaires, like HAM-A, HAM-D and Mc Gill-Pain Questionnaire.

After 1 month the percentage of success was 67, but there was a significant difference between the two groups: 74% of success among cooperative patients, but only 44% of success among non-cooperative patients.

After 3 months, 6 months and 1 year the percentages of success stayed unchanged.
Table 2a and Plot 3a): percentages of success and unsuccess after 1 month of treatment with topical capsaicin followed by a subdivision into the two groups of patients (collaborative vs non-collaborative) based on the results of psychometric questionnaires; Tables and Plots 3b-c-d): percentages of success and unsuccess after 3, 6 and 12 months respectively.

Treatment was regarded as successful if it led to a decrease in oral burning and lasted over time.

Topical capsaicin is very effective in the treatment of burning symptoms in BMS patients, but it is successful in those patients whose complaint seems to have a neuropathic origin, whereas those patients whose condition is likely to be linked to psychogenic factors do not obtain a satisfactory percentage of success.
Another promising therapeutic approach which could be used together with topical capsaicin is Low-Level Laser Therapy (LLLT), also known as Biostimulation.

LLLT acts significantly on NADH reduction and cellular respiration, increasing thus energy levels and producing an antalgic effect.

Moreover, it contributes to activate the serotonergic inhibitory descending pathway.

Several studies described a positive effect of LLLT in the treatment of burning mouth symptoms [37].

If topical therapies do not work as expected, or if the clinician wants to improve the therapeutic effectiveness, he/she may prescribe alpha-lipoic acid (ALA).

ALA is a neuroprotector and helps the trophism and metabolism of nerve fibers. In past years, it was thought of as the therapy of first choice, but now its efficacy has been re-evaluated and it is only prescribable as an adjuvant drug [38].

The percentage of success in the analyzed patients is nearby 20%.

Alpha-lipoic acid is prescribed in the dosage of 400 mg per day for the first 10 days and then 800 mg per day for the next 30 days.

If previous therapies do not bring about any substantial improvement in oral complaints after topical applications, the stomatologist should refer patients to the neurologist who will prescribe anticonvulsant drugs (i.e. Gabapentin tablets or Pre-gabalin capsules).

The last chance lies in psychiatric drugs, like benzodiazepines (i.e. Clonazepam) or SSRI (Fluoxetine or Sertraline).
The international literature has recently suggested the use of Duloxetine as the most effective SSRI in the treatment of Burning Mouth Syndrome [39].

The problem is that very often patients visit a dental clinic with an already positive pharmacological anamnesis for these drugs, and it becomes very difficult to manage a change in their therapy or find a solution to their problems.

In short, a correct diagnosis, which consists in the exclusion of all those local or systemic conditions possibly related to oral burning is the mainstay of any future treatment.

Afterwards, it is fundamental to establish a strict and thorough therapeutic protocol following a “staircase approach” from the peripheral nerve fibers to the central nervous system.

But both diagnosis and therapy require a psychological approach to BMS patients.

Specific questionnaires should be administered to distinguish between predominant neuropathic BMS and predominant psychogenic BMS.

If the clinician is faced with neuropathic BMS, he/she could prescribe topical therapies: antifungal therapy, salivary substitutes, topical capsaicin and LLLT and alpha-lipoic acid; in 75% of cases he/she can treat his/her patients with topical therapies without referring them to a neurologist.

On the contrary, if he/she has to deal with psychogenic BMS, 80% of patients will require a neurological consultation, and only a few patients will notice a slight improvement with topical therapies. Moreover, topical therapies could even worsen patients’ conditions over time, since the situation may become frustrating both for them and the clinician.
So, when a psychogenic BMS is evident, it is highly advisable to refer patients to the neurologist immediately, so as to establish a systemic therapy.

### 2.1.7 Update on the origin of the syndrome

The pathophysiology of primary BMS is a conundrum and has caused a big controversy over the years. The aetio-pathogenesis seems to be complex and in a large number of patients probably involves interactions among local, systemic and/or psychogenic factors. Particular emphasis has been placed on the concurrent symptoms of dry mouth and taste alterations [40].

The role of the **peripheral nervous system**, particularly pertaining to concurrent dysgeusia has been a topic of study. The results in the international literature suggest that BMS oral burning may be a disorder of peripheral pain pathways, accompanied by a variety of positive and negative neurological findings in some patients. Ito et al [41] in 2002 demonstrated in a case-control study that the thermal pain threshold on the tongue and the duration and intricacy of the pain complaint was significantly higher in BMS patients than controls, suggesting a relationship between the pain and peripheral nerve dysfunction at the tongue and/or central dysfunction in patients with BMS.

Interesting links have recently emerged between BMS and peripheral nerve damage. Lauria and co-workers showed that BMS patients had a significantly lower density of epithelial nerve fibers in the biopsy specimens of the anterior two thirds of the tongue than controls. This feature was more evident with a longer duration of symptoms. Epithelial and sub-papillary nerve fibers showed diffuse morphological changes indicating axonal degeneration [42].

A similar experiment is being conducted in Varese, analyzing samples from 8 patients with primary BMS and comparing them with negative controls. 4 of these
patients underwent topical therapy with capsaicin for one month before biopsy, while the other 4 patients started therapy after the procedure. The two subgroups show differences at confocal microscopy examination.

Fig. 6 and Plot 3: biopsy specimens collected from patients affected by BMS. Each sample was processed and stained with Hematoxylin-Eosin, Silver stain and analyzed at confocal microscopy with direct immunofluorescence to detect anti-TRPV1 antibodies directed against receptors for capsaicin. Images A-B-C show the results from a patient who underwent biopsy before starting topical therapy with capsaicin. Images from D-E-F show the results from a patient who underwent biopsy after one month of topical therapy with capsaicin. The plot shows a dycotomic distributions of TRPV-1 signal expressed by the two groups.
Evidence for chorda tympani hypofunction in BMS has recently been presented [43]. Furthermore, an abnormality in the blink reflex has been recorded [44]. Based upon clinical and histopathologic findings, BMS could be regarded as a chronic, slow and burning pain with a neuropathic origin not well explained.

In addition to changes at the peripheral level, data emerge that illustrate the involvement of the central nervous system and the interaction with the peripheral nervous system [45]. Recent data from animal experiments suggest an important role played by the basal ganglia in the processing and sensorimotor rating of nociceptive information. PET scans revealed in some BMS patients that the presynaptic dopaminergic function in brain was significantly decreased in the right putamen compared to control subjects. The finding of decreased striatal FDOPA uptake in the putamen supports the involvement of the nigrostriatal dopaminergic system in pain. Other studies indicated that thermal stimulation in BMS patients was associated with increased cerebral blood flow as observed in functional Magnetic Resonance Imaging [46].

The additional finding that estrogens function as neuroprotectants of the nigrostriatal dopaminergic system and decline with menopause, could help to explain the age and gender predilection of this disorder.

Already in 1920 a possible psychogenic cause of BMS was mentioned by Engman, but even in the most recent literature much attention has been paid to possible psychogenic causes.

Patients with resistant BMS are significantly more easily fatigued and sensitive and tend to be more concerned about their health. With regard to psychologic functioning, BMS patients have much more difficulty taking the initiative, become dizzy more easily and have more sad thoughts. They also complaint of palpitations and/or precordial pain more often. The observed significant differences in personality and psychologic functioning might suggest that burning sensations are
psychosomatic symptoms in these patients [47]. Approximately 21% of BMS patients studied in the literature have psychometric data that show a likelihood of psychologic distress, and further evaluation by an appropriate health professional should be recommended for such individuals.

The role of saliva and local environmental factors have been investigated, including salivary gland dysfunction and altered mucosal blood flow [48].

Some authors deduced that mucosal atrophy may result from altered salivary ionic composition and may be accompanied by a peripheral oral neuropathy originating from dysfunction [49].

2.2 **AIM**

The current study proposes that the basal and stimulated salivary flows in a group of patients with BMS be measured and the outcomes compared with the results of a control group (CTRL) and a group of patients with Oral Lichen Planus (OLP), the most common chronic inflammatory autoimmune disease of the oral cavity. The aim is to check whether there is a quantitative decrease in salivary flow among BMS patients to offer a fresh perspective to better understand the contributory effect of saliva in this misdiagnosed pathology.

2.3 **MATERIALS AND METHODS**

A group of **44 BMS** patients were identified following a thorough diagnostic protocol, relying on a diagnosis of exclusion and the application of Scala’s criteria for the assessment of BMS [45]: the presence of a burning, scalding, tingling and/or numb sensation bilaterally in the mouth for at least 6 months with total absence of
clinical and radiographic findings; the contemporaneous presence of secondary symptoms such as xerostomia and/or dysgeusia; the exclusion of oral pathologies or local irritating factors; negative haematochemical analyses to rule out all systemic factors linked to oral burning sensations such as vitamin B12 or iron deficiency, hepatic enzyme alterations, autoimmune diseases; the exclusion of allergy to dental restorative elements by a specific skin patch test series; and negative results on cytological and/or microbiological examination.

A second group of 27 patients affected by OLP was identified on the basis of clinical and histological examinations. Among these patients, there was an equal distribution of the different subtypes of OLP: papular, reticular, hyperplastic, atrophic, erosive and ulcerative. No cases of bullous OLP or lichen planus pemphigoides were included in the current study.

Finally, 40 healthy patients were chosen as a control group (CTRL) with similar epidemiologic and demographic distributions to the other two groups.

This study was included in a more articulated research programme on salivary volumes in different pathologies: oral candidiasis, OLP, BMS and several conditions such as pregnancy, smoking and drug-related xerostomia. The choice of OLP was made because it is the most representative chronic inflammatory pathology affecting oral mucosae and to highlight the peculiarity of the outcomes registered in the BMS group.

During the first appointment, an informed consent form was provided to patients, followed by a questionnaire to record the participants’ systemic health history and their drugs consumption, as well as their dietary habits and any potentially harmful habits such as smoking.

The inclusion criteria were informed consent to the protocol, good compliance, age between 40 and 80 years, dental restorations or prostheses in good repair and a healthy periodontal condition. Non-cooperative patients, pregnant women and
patients with a history of oral radiation were excluded from the protocol. This study was approved by the Institutional Review Board for research programmes. In 2015 it was published on the *Journal of Oral pathology and medicine*. Authorization for the use of the paper in this PhD dissertation is enclosed.

The basal salivary flow and the stimulated salivary flow in the three groups of patients were measured using the “spitting” method, which is a simple and reliable method reported in the international literature [15]. The patients were asked to spit every minute for 5 min and to avoid swallowing or other confounding factors. The examination started after patients had spat all oral fluid secreted in their mouth. Subsequently, they were asked to repeat the procedure a second time, but a drop of 0.2 ml of citric acid (2%) was placed on their tongues every minute to stimulate salivary secretion. The stimulation was begun soon after the patients had spat all oral fluid in their mouth. Finally, the amount of citric acid added during the procedure (1 ml) was subtracted from the total reported flows.

After 2 weeks, the patients underwent the same procedure but for 15 min, and the basal and stimulate salivary flows were recorded. A total volume of 3 ml of citric acid was added during the procedure and was subtracted from the overall quantity of stimulated salivary flow.

The choice of measuring the salivary flows and volumes twice and for different times was to verify the homogeneity of the outcomes in each patient and their reproducibility during a shorter and a longer stimulation. Furthermore, repeating the procedure was important to help patients be at their case with the procedure and the operator.

The procedure was performed by the same operator, in the same place, at the same time in the morning to avoid hormonal influences, under the same lighting conditions and in absence of potential confounding factors [14]: light’s variations,
changing in dental chair position during the examination, noise, speech and changing in head’s inclination.

The distribution of basal and stimulated salivary flow volume (ml) and rate (ml/min) in each group was summarized by box plots and histograms, respectively, providing mean and total values.

To test the null hypothesis of no difference in salivary flow volumes among the three study groups, a standard analysis of covariance was performed, with age and sex, potential confounders, as covariates. The estimated mean flow volume and confidence intervals by group were reported, as well as the P-value from the F test for overall association (2 degrees of freedom test). The pairwise comparison t-test P-values for testing the difference between each pathological group (OLP and BMS) vs the control group were reported. All the analyses were done by using the SAS software, 9.2 release (SAS Institute Inc., Cary, NC, USA).
Low basal salivary flow and Burning Mouth Syndrome: new evidence in this enigmatic pathology

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BACKGROUND: Burning mouth syndrome remains a puzzling condition. One symptom commonly associated with the burning sensation is xerostomia. The current study measured basal and stimulated salivary flow in a group of burning mouth syndrome patients.

METHODS: Three groups of patients were recruited: 44 burning mouth syndrome patients, 27 oral lichen planus patients and 40 healthy patients. We chose to measure basal salivary flow and stimulated salivary flow in the three groups of patients using the ‘spitting’ method. Thus, the patients were asked to spit every minute for 5 min. Afterwards, they were asked to repeat the procedure a second time, but a drop of citric acid was positioned on their tongue every minute to stimulate salivary secretion. After 14 days, the same procedure was repeated for 15 min.

RESULTS: Although there was no significant difference between the burning mouth syndrome group and the other two groups regarding the stimulated volumes, an important difference was found in the basal volumes, with the burning mouth syndrome patients showing lower values.

CONCLUSIONS: The outcomes of our research demonstrate the presence of very low basal salivary flow in burning mouth syndrome patients compared with the other two groups, but the stimulated salivary flow was equal, if not higher, in the burning mouth syndrome patients. This study contributes new topics for further investigation of a solution to the very mysterious pathology represented by burning mouth syndrome.


Keywords: burning mouth; burning mouth syndrome; saliva; xerostomia

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Introduction

Burning mouth syndrome (BMS) is a condition characterised by a sensation described by patients as stinging and burning that affects the oral mucosa in the absence of clinical or laboratory data to justify these symptoms (1). This condition principally affects women at a ratio of approximately 4:1; this difference between the sexes might be explained by biological, psychological and sociocultural factors; however, such factors have not yet been defined (2).

The pathophysiology of idiopathic burning pain is unclear and has generated controversy over the years. The aetio-pathogenesis seems to be complex, and in a large number of patients, it most likely involves interactions among local, systemic and/or psychogenic factors (3). Particular emphasis has been placed on concurrent symptoms of xerostomia and taste alterations (4).

There are a number of precipitating factors in BMS, and these factors have been comprehensively reviewed (5). One factor associated with BMS is reduced salivary gland function and the consequent complaints of xerostomia (6). Although BMS affects an older age group, it is debatable whether salivary gland function declines with age (7–10). Nevertheless, both resting and stimulated salivary flow rates are reduced in post-menopausal women, which is the population most commonly affected by BMS. These findings therefore support salivary gland hypofunction as a contributory factor in BMS (11), but contradictory findings have also been reported in the literature (12).

The roles of saliva and local environmental factors have been investigated, including salivary gland dysfunction and altered mucosal blood flow (12–16). The current study proposed to measure the basal and stimulated salivary flows in a group of patients with BMS and to compare the outcomes with the results for a control group and a group of patients with oral lichen plaques (OLP) with similar epidemiologic, demographic and set characteristics.

We aimed to verify whether there was a quantitative decrease in salivary flow among BMS patients to establish
Fig. 8: authorization for the use of the published paper in this PhD dissertation.
Fig.9: informed consent which was filled in by patients before undergoing salivary examination
2.4 RESULTS

A total number of 111 patients were recruited in this study. 44 patients were affected by primary BMS, 27 by OLP and 40 were included as healthy.

The mean age in the BMS group was 67 with a F:M=5,6:1 ratio.

The mean age in the OLP group was 64 with a F:M=4,4:1 ratio.

The mean age in the CTRL group was 62 with a F:M=4,8:1 ratio.

Each group underwent a first examination of the basal and stimulated flows for 5 min, followed by a second examination after 14 days for 15 min. The outcomes as to both the salivary flow rate and the total salivary volume were recorded.

Subsequently, the mean salivary flow rates and volumes were calculated for both the basal and stimulated salivation conditions.

In the CTRL group, the mean basal flow rate was 0,34 ml/min for the 5-min examination and 1,73 ml/min for the 15-min examination.

The total basal salivary volumes were 1,7 ml during the first test and 5,9 ml during the second one, while the total stimulated volumes were 8,5 ml during the 5-min examination and 25,9 ml during the 15-min examination. These values corresponded with the values reported in the international literature [5].

In the OLP group, the mean basal flow rate was 0,36 ml/min for the 5-min examination and 0,37 ml/min for the 15-min examination, while the mean stimulated flow rate was 1,56 ml/min for the 5-min examination and 1,68 ml/min for the 15-min examination. The overall basal salivary volumes were 1,8 ml for the first examination and 5,6 ml for the second one, while the total stimulated volumes were 7,8 ml during the 5-min examination and 25,3 ml for the 15-min examination.
There were no statistically significant differences between the CTRL group and the OLP group, with P-values of 0.9 and 0.8 for the basal flow volumes during the 5-min and 15-min examinations, respectively, and 0.5 and 0.8 for the stimulated flow volume results.

In the BMS group, the mean basal flow rate was 0.18 ml/min for the 5-min examination and 0.19 ml/min for the 15-min examination, while the mean stimulated flow rate was 1.78 ml/min for the 5-min examination and 1.73 ml/min for the 15-min examination. The total basal salivary volumes were 0.9 ml for the 5-min examination and 2.9 ml for the 15-min examination, while the total stimulated salivary volumes were 8.9 ml during the first test and 26 ml during the second one.

While there were no statistically significant differences between the three groups with regards to the stimulated volumes (overall F test: P=0.6 for the 5-min examination and P=0.9 for the 15-min examination; BMS vs CTRL group: P=0.7 for the 5-min examination and P=0.9 for the 15-min examination), an important significant difference was found for the basal salivary flow among the three groups (overall F test: P=0.002 for the 5-min examination and P=0.0002 for the 15-min examination). In particular, the salivary rate was half in the BMS if compared with the control group (BMS vs CTRL group: P=0.006 for the 5-min examination and P=0.003 for the 15-min examination). A graphical representation of the salivary volumes is provided as a box plot.
Plot 4a): mean flow rates among the three groups. Both the basal and stimulated salivary flows of the CTRL group corresponded with the values reported in the literature. In the BMS group, a lower basal salivary rate was registered when compared with the other two groups, while the stimulated flow rate was equal; 4b): salivary outcomes among the three groups. The stimulated flow appeared to be equal in the BMS group when compared with the other two groups, while the basal salivary flow was very low when compared with the OLP and CTRL groups.

Table 3: mean salivary flow values and following statistical analysis.
Plot 5: box-plot charts. Evidence of the decreased level of basal salivary flow in BMS patients during both the 5-min (A) and 15-min examination (B). In contrast, the stimulated salivary flow of BMS patients at the 5-min (C) and 15-min (D) examination appeared to be equal when compared with the other groups.
2.5 DISCUSSION

Burning Mouth Syndrome is a very enigmatic pathology of unknown aetiopathogenesis that is characterized by the presence of a chronic burning sensation in the oral mucosa with the absence of related local clinical findings or systemic conditions.

It mainly affects post-menopausal women, and it is associated with secondary symptoms such as xerostomia, which is present in more than 60% of patients.

Many theories have been developed to explain the pathogenesis of this pathology, but no one has been enlightening yet. A group of scientific papers has claimed that the onset of this syndrome is mainly attributable to psychogenic factors [28], while other researchers think BMS is a neuropathic disorder [42].

Nevertheless, xerostomia is one of the main symptoms noted by patients, while hyposcialia and salivary gland disorders are absent.

The current study suggests measuring the basal salivatory flow and the stimulated salivary flow in BMS patients and comparing these rates with rates from a group of OLP patients and a control group.

The OLP group was not included for its statistical significance per se, since a BMS and a CTRL group were sufficient to compare the outcomes. However, OLP is the most frequent chronic inflammatory condition of oral mucosae and the normal values registered in this group corroborates the peculiarity of the outcomes registered in BMS patients. Otherwise, it could be thought that a reduced flow may be a consequence of any condition affecting the oral mucosa or the salivary glands, and that it is not strongly associated with burning mouth syndrome. The outcomes of the research reveal a very low basal salivary flow in BMS patients compared with the other two groups, but the stimulated salivary flow is similar, although not higher, in BMS patients.
This means that salivary function is preserved in burning mouth syndrome. In fact, during stimulation, the salivary glands are able to produce the same quantity of saliva as in the other two groups. However, a low basal flow was registered during the test, which could be a key element to better understand the secondary symptom of xerostomia complained of by BMS patients.

The hypothesis is that chronic assumption of antihypertensive, anxiolytic and antidepressant drugs on one side and the concomital presence of psychological behaviour distress on the other side could influence the basal tone of those salivary glands responsible for the basal flow function, such as the submandibular, sublingual and the minor salivary glands. These glands are innervated by parasympathetic fibers which refer to the upper salivatory nucleus. On the contrary, stimulated flows are produced especially by the parotyd gland, whose innervation relies on the function of those parasympathetic fibers which refer to the lower salivatory nucleus. In fact, patients usually describe a slight or even a dramatic improvement in their oral symptoms while eating or stimulating their salivary function with a chewing gum. During the examination, patients did not report a marked improvement in oral symptoms, as it often happens when they normally eat, but this is probably due to the application of citric acid which can be irritant.

Further research should be carried out to better investigate this phenomenon through the use of more specific salivary tests and functional neurophysiological imaging.

This hypothesis should be verified with more specific salivary volumes collecting methods, for example Crittenden cups, as the spitting method of saliva collection is admittedly easy to use but is open to criticism due to its non-selective nature. It is impossible to distinguish parotyd saliva from submandibular, sublingual and minor salivary gland secretions. Whether this phenomenon is one of the aetiological factors or only a concomitant one in Burning Mouth Syndrome must be investigated.
This study does not contribute to our understanding of the pathogenesis of BMS or to unifying the neuropathic and the psychogenic theories, but it provides evidence and new topics for further investigation to find a solution to the enigma represented by the pathogenesis of Burning Mouth Syndrome.
3. **2nd STUDY: HUMAN BETA-2-DEFENSIN IN ORAL LICHEN PLANUS AND BURNING MOUTH SYNDROME**

3.1 **BACKGROUND**

3.1.1 **ANTIMICROBIAL PEPTIDES (AMPs)**

All living beings are exposed to the attack from different infective agents, such as bacteria, viruses, yeasts, etc.

The Immune system represents the host defense against these agents, distinguishing them from the organism’s own healthy tissue.

In the human being the defensive response to infective agents is carried out by the innate immune system and by the adaptive immune system.

The **innate immune system** is primitive, highly non-specific and thus active against non-recognized agents; it acts in a generic way eliciting an immediate response to infection, but it does not provide long-lasting or protecting immunity to the host.

The innate immune system is an evolutionary ancient defense strategy and it is predominant in plants, fungi, insects and primitive organisms.

Contraversely, the **adaptive immune system** is highly sophisticated: it requires the recruitment of different cell types, the production of antibodies and the activation of complement. It is highly specific and retains “memory” of the dangerous molecules [50].
This process of acquired immunity is the basis of vaccination. Like the innate system, the adaptive system includes both humoral immunity components and cell-mediated immunity components, but it is highly specific to a particular pathogen and the response is enhanced to subsequent encounters with that pathogen.

The innate immune system’s activity relies on the maintenance of physical barriers, which divide tissue from surrounding environment (i.e. cute, epithelia), the phagocytic cell lines, the complement pathway and the Antimicrobial peptides (AMPs).

Fig. 10: the immune system defensive response to infective agents is carried out by the innate immune system and by the adaptive immune system.
The Antimicrobial peptides (AMPs) are polypeptides of less than 100 amino acids that are found in host defense settings and have antimicrobial activity at physiological concentrations under conditions prevailing in the tissues of origin [51].

They represent an ancient arm of the innate immune system whose role is to directly neutralize invading microbes. Over 1500 AMPs have been described from different living beings, including plants, birds, insects, mammals, humans.

The first description of the presence of AMPs was in bacteria and fungi. They were originally regarded as unique defense molecules in unicellular organisms [52]. Some of these molecules seem to be independent from ribosomal translation and derived from enzymatic synthesis. In 1962 the field of research was extended with the description of the hemolytic bombinin peptide from the toad Bombina variegata [53]. Even though the initial focus was on its hemolytic properties, some authors tried to emphasize its antimicrobial properties, but failed [54]. Hence, research in this province stopped for almost twenty years. In 1980 the first AMPs, named cecropins, were isolated from hemolymph of Hyalophora cecropia [55] and they began to provide an explanation of how insects, which do not have the adaptative immune system, defend themselves effectively.

In late 1980s the α-defensins were found in mammaliam cells, including human cells, and this led to consider AMPs associated not only with organisms lacking in an adaptative immune response. Finally the discovery
of AMPs in the African clawed frog *Xenopus laevis* demonstrated how the antimicrobial peptides are widespread among many other species [56].

Even though the number of AMPs in nature is large, there are some general structure features that are shared among them. They are usually small peptides, commonly made up of about 30 amino acids, and are generally positive charged.

They show an amphipathic structure which is crucial for their antimicrobial properties: it seems that the positive charged molecules interact with negatively-charged phospholipids of microbial membranes, while their hydrophobic residues, which are 40-50% on average, aid integration into the microbial cell membrane and lead to its disruption [54].

First named as AMPs due to their antimicrobial properties, other biological effects of AMPs have been described recently: chemotaxis, immunomodulating activity, angiogenesis and wound repair. This is the reason why the ancestral AMPs represent an interesting and challenging field of research [57].

In mammals there are many AMPs, but they can be classified into two families that have been thoroughly characterized, the cathelicidins and the defensins.

*Cathelicidins* are not as widespread as defensins, since they recognize a more ancient origin [58]. They have been described in lizards, birds, fish and certain mammals. They are α-chained peptides composed of a highly conserved N-terminal domain, called the cathelin-propart, and of a C-terminal antimicrobial domain. Therefore, the name cathelicidin derives from cathelin connected with a microbicidal peptide.
In humans only one cathelicidin has been described and called human cationic antimicrobial peptide (hCAMP18), consisting in 170 aminoacids and genetically expressed on chromosome 3 [59].

Initially described as a protein synthesized by the bone marrow, it is now reported to be also excreted by cutaneous keratinocytes during inflammation.

After digestion by the neutrophils’ serine protease a small peptide, called LL-37, is involved in the immune modulation activity. hCAMP18/LL-37 cathelicidin is produced by different cell types, including myeloid cells, neutrophils, mast cells, monocytes, epithelial cells of the colon, urinary tract and respiratory mucous membranes.

Defensins are a family of small-sized β-chained peptides whose molecular weight ranges from 3,5 to 4,5 kDa and include 6 cysteine residues which can create disulfide bridges [60]. There are three different families of defensins according to the position of the disulfide bridges: α, β and φ-defensins [61].

α-defensins are composed of 29 to 35 aminoacids and are characterized by 1-6, 2-4 and 3-5 cystein disulfide bridges. There are 6 Human α-defensins. Four of them are expressed in neutrophil granules, and are also referred to as human neutrophil peptides HNP-1 through -4. The α-defensins 5 and 6 (HD5 and HD6) are found in Paneth cells of the small intestine and in the epithelial cells of the female urogenital tract [62].

β-defensins are characterized by 1-5, 2-4, 3-6 cystein disulfide bridges. There are six human β-defensins, but it is believed that others could be discovered. They are produced by epithelial cells [57].
HBD-1 is constitutively expressed in the respiratory and urinary epithelia; HBD-2 in psoriatic skin but also in urogenital, gastrointestinal and respiratory epithelia.

HBD-3, described for the first time in psoriasis, has been found in all epithelia and particularly in saliva and cervicovaginal fluid. HBD-4 has been detected in testicles, stomach and uterus, while HBD-5 and HBD-6 have been mainly found in epididimus.

α- and β-defensins are codified on chromosome 8.

Ø-defensins are not found in humans and New world primates. On the contrary they are mainly found in Old world primates. They are lectin-like cyclic octapeptides expressed in leukocytes [61].

Fig.11: defensins genes and peptides with three-dimensional structures of α-, β- and Ø- defensins (from Hazlett L et al [61])
As to ß-defensins, while HBD-1 is constitutively expressed, HBD-2 can be induced by IL-1α, interleukin-1β, TNF-α, interferon-γ, Gram-positive and Gram-negative bacteria, *C. albicans*, *M. tuberculosis*, LPS. The activity of HBD-2 is increased by several bacterial products through the interaction with toll-like receptors (TLRs) 2 or 4 [63].

AMPs play their role with a direct antimicrobial activity thanks to their electrostatic interaction with the negatively charged phospholipids on bacterial membranes, but they are also capable of immunomodulatory functions. Both cathelicidin hCAMP18/LL-37 and defensins perform a chemoattractive action on monocytes, neutrophils and CD4+ lymphocytes and stimulate the production of other chemokines.

Fig. 12: many bacterial molecules can activate α- and ß-defensins, which recall innate and adaptive immune cells due to a chemoattractive activity.
They are also able to modulate the activity of the dendritic cells and the expression of their epitopes. Defensins can induce the maturation of the dendritic cells towards TLR-4. Thus AMPs represent a bridge between the innate immune system and the adaptive immune system.

Defensins’ activity has been widely described in the international literature with special regards to plants, insects and humans [64].

Plant defensins, for example, exhibit antifungal activity against a broad range of phytopathogenetic fungi [65], most insect defensins identified so far show antibacterial activity against Gram-positive bacteria, Gram-negative bacteria, while yeast and filamentous fungi are less sensitive to insect defensins.

In humans it is debated whether functional impairment or enhancement of AMPs can influence the clinical evolution of several diseases, among which infective and chronic inflammatory diseases.

Defensins are known for their activity against certain viruses, like for example the Human Immunodeficiency Virus (HIV), the Human Papilloma Virus (HPV), Cytomegalovirus (CMV) or against certain bacteria, like Mycobacterium tuberculosis or Pseudomonas aeruginosa [61].

For antibacterial activities, β-defensins have proved to be effective in fighting S.aureus, E.coli and M. tuberculosis [66].

For antifungal activities, β-defensins have turned out to be especially active against C.albicans and other Candida species, with the exception of C. glabrata [67].
For antiviral activities α-defensins are capable of inhibiting Human Immunodeficiency Virus (HIV) and Herpes Simplex Virus (HSV) [68].

For anti-parasite activities, several studies have reported that defensins are active against *Toxoplasma gondii* and *Tripanosoma cruzi* [69].

A decrease in number or function of defensins could increase the host’s susceptibility to infections.

On the contrary, in autoimmune or disregulated immune responses the AMPs’ activity seem to be altered, as it has been shown in Psoriasis and Atopic dermatitis. In psoriasis LL-37 production is increased and is associated with inflammation of the skin, while in Atopic dermatitis the decreased presence of defensins is linked to infection by *Stafilococcus aureus*.

Among respiratory diseases, the major susceptibility to bacterial infection recorded in Fibrosis cystica may be linked to the production of salts within the alveolar fluid. Alterations of AMPs’ activity is also reported in idiopathic pulmonary fibrosis, alveolar proteinosis, acute respiratory distress syndrome, lung transplantation, allergic rhinitis.

It seems that functional and epigenetic defects of defensins’ expression could be a determining factor in the pathogenesis of some autoimmune diseases, including diabetes mellitus and Crohn’s disease [57].
Fig. 13: functions of AMPs in inflammatory diseases. Various cell types are activated by microbes and inflammatory mediators, causing the production and release of AMPs. These peptides show different functions, including antimicrobial activity and modulation of the inflammatory response. However, an alteration in the activity of AMPs leads to the development of infectious or inflammatory diseases (from Guaní-Guerra et al [57]).

Recently a connection between defensins and hyaluronic acid has been shown, especially in the skin.

Hyaluronic Acid (HA) has an important role in tissue biomechanics and intercellular signaling. The dimension of HA molecule is important, since the high weight polymer (over 500 kDa) shows reparative and protective
functions towards cells, while on the contrary the fragments generated by enzymes (hyaluronidasis) or by free radicals show a pro-inflammatory and pro-angiogenetic effect.

In recent years some papers have demonstrated an in-vitro capability of HA olysaccharydes to induce HBD-2 when in contact with keratinocytes [70]. This discovery has shown how defensins are important in protecting the skin from infective agents after traumatic lesions or tissue damage, which generate HA fragments. HA acts through the toll-like receptors 2 and 4 and it is no more considered to be an inert molecule, but an important biological effector in cell migration and proliferation, angiogenesis, wound healing and perhaps tumour diffusion.

It has been noticed that the lenght of HA fragments is crucial in the definition of their function, since HA is a molecule with rigid dysaccharydes sequence and information can be carried only by the dimension of its polymers. Gariboldi et al have shown how a low-weight polymer of HA can stimulate in vitro and in vivo production of beta-defensins through TLR-2 and TLR-4 in keratinocytes [70].
A particular attention is being now turned to brain and neurological degenerative diseases, like Parkinson’s disease and Alzheimer’s disease, in which a chronic inflammatory response is linked to the apoptotic process of neurons. Since HA is a constituent part in the process which regulates
neuronal hydration and electrolytes exchange, it seems that defensins could have a protective role in preventing neuronal apoptosis.

Recently the international literature has been focusing on the role of defensins in the oral cavity and their association with several mucosal and gingival diseases.

Common mucosal oral lesions include candidiasis, recurrent herpes labialis, erythema migrans, hairy tongue, recurrent aphthous stomatitis and Oral Lichen Planus.

The oral cavity is an environment in which antimicrobial peptides fulfil the important function of maintaining balance between health and disease. A lot of AMPs have been cited in the oral literature, being the $\alpha$-defensins mainly expressed in neutrophils, the $\beta$-defensins in the epithelium and the LL-37 cathelicidin in both epithelium and neutrophils.

Defensins have been found in oral tissues, salivary glands, salivary secretions, crevicular fluid.

In the oral cavity HBD-1 was described for the first time in 1998 [71]. Up to now, only a small group of studies has investigated HBD-1 or HBD-2 in the saliva of patients with OLP or other mucosal conditions [72][73].
3.1.2 ORAL LICHEN PLANUS

Lichen Planus is a common, chronic cutaneous disorder which often affects the oral mucosa. Since skin lesions resemble the lichens growing on rocks, Wilson was the first who named the disease after these vegetable organisms [74]. Even though the word *Lichen Planus* can suggest a fungal condition, it is now accepted that it is a chronic autoimmune disease.

The relationship between the disease onset and the feeling of stress/anxiety experienced by patients has been widely discussed but it is still controversial. Most patients with lichen planus are middle-aged adults and the ratio of women to men is 3:2. It is thought that about 1% of the population may have this condition [75].

The skin lesions of lichen planus have been described as purple, itchy, polygonal papules. They usually affect the flexor surfaces of the extremities. Excoriations may not be visible, despite the fact that the lesions itch, and become sore when the patient scratches them.

A thorough examination of the skin surfaces of the papules will reveal the presence of a fine, lacelike network of white lines known as *Wickham’s striae*. 
The majority of patients with dermal lichen planus show associated oral lesions. On the contrary, only 40% of patients with oral manifestations have skin lesions as well.

In the oral cavity, the disease has a somewhat different clinical appearance from the presentation on the skin: it is typically characterized by lesions consisting of radiating white or gray, velvety, thread-like papules in a linear, annular or retiform arrangement forming classic lacy, reticular patches, rings and streaks over the buccal mucosa and, to a lesser extent, on the lips, tongue and palate.

These lesions do not cause significant symptoms in the majority of patients, while in others there may be a complaint of a burning sensation in the involved area [76].

Reticular lichen planus is the most frequent form of the disease. This variant usually does not trigger any symptom and involves the posterior buccal mucosa bilaterally. Other oral mucosal surfaces may also be involved concurrently, such as the lateral and dorsal tongue, the gingivae, palate, and vermilion border.

Fig.15: the glans penis, the vulva and the nails can be also affected (from Neville BW et al [74]).
Reticular lichen planus is thus named because of its characteristic pattern of interlacing white lines (Wickman’s striae). These lesions are typically variable, they wax and wane over weeks or months.

Fig. 16: reticular OLP; the typical Wickman’s striae are evident on buccal mucosa

A *hyperplastic* form of *lichen planus* may also occur on the oral mucosa, generally appearing as well-circumscribed, elevated white lesion resembling leukoplakia. In such cases biopsy is usually necessary to make a diagnosis.

Fig. 17: bilateral white patches on buccal mucosae are the hyperplastic form of OLP
An **atrophic** form of *lichen planus* occurs with some frequency and clinically appears as smooth, red, poorly defined areas, often but not always with peripheral striae evident.

![Fig.18: atrophic OLP; erythema and epithelial atrophy are evident on buccal mucosa and on the dorsum of tongue](image)

**Erosive lichen planus**, although not as common as the reticular form, is more significant for the patient because the lesions are usually symptomatic. Clinically, there are atrophic, erythematous areas with central ulceration of varying degrees.

![Fig.19: erosive and ulcerative OLP; epithelial erosion with the formation of painful ulcers are distinguishing features of this OLP form](image)
If the erosive feature is severe, epithelial separation from the underlying connective tissue may occur. This results in the relatively rare presentation of **bullous lichen planus**.

![Bullous OLP](image)

**Fig.20:** bullous OLP; this form must be distinguished from vesiculo-bullous diseases like pemphigus vulgaris or mucous membrane pemphigoid

The clinical forms can be grouped into two families: the so-called “**white**” lichen planus, which includes the papular, the reticular and the hyperplastic form, and the “**red**” lichen planus, which comprises the atrophic, the erosive and the bullous forms.

The red forms are those in which inflammation is more represented, they are often symptomatic and may undergo malignant transformation, even though it is uncommon.

The histopathologic features of lichen planus are characteristic but may be not specific, because other conditions, such as lichenoid drug reaction, lichenoid amalgam reaction, oral-versus-graft disease (GVHD), lupus erythematosus (LE), chronic ulcerative stomatitis and oral mucosal cinnamon reaction may also show a similar histopathologic pattern.
Varying degrees of ortokeratosis and parakeratosis may be present on the surface of the epithelium with thickening of the granular layer, depending on whether the biopsy specimen is taken from a red or white lesion.

The thickness of the spinous layer can also vary. The rete ridges may be absent or hyperplastic, but they classically have a pointed or “saw-toothed” shape.

Destruction of the basal cell layer of the epithelium (hydropic degeneration) is also evident. This is accompanied by an intense, bandlike infiltrate of predominantly T lymphocytes immediately subjacent to the epithelium. Degenerating keratinocytes may be seen in the area of the epithelium and connective tissue interface and have been termed Civatte bodies. Degeneration of the basal keratinocytes and disruption of the anchoring elements of the epithelial basement membrane and basal keratinocytes (i.e. hemidesmosomes, filaments, fibrils) weakens the epithelium-connective tissue interface. As a result, histologic clefts (Max-Joseph spaces) may form, and blisters on the oral mucosa (bullous lichen planus) may be seen on clinical examination. B-cells and plasma cells are uncommon findings. Immunoglobulin or complement deposits are not a distinguishing feature of OLP. In some instances, fibrinogen and fibrin are deposited in a linear pattern in the basement membrane zone. The pattern of fibrinogen deposition, in the absence of fluorescence by other reagents, is sufficiently unique to be used as a diagnostic criterion for oral mucosal lichen planus.
Fig. 21: histopathologic features of OLP. A chronic band-like lymphocytic infiltrate is localized at the interface between epithelium and connective tissue, rete ridges are atrophic

There is some controversy surrounding its malignant potential. There seems to be a slightly higher incidence of oral squamous cell carcinoma in patients with Oral Lichen Planus than in the general population. The actual overall frequency of malignant transformation is low, ranging from 0.3 to 3%. The forms that more commonly undergo malignant transformation are the erosive and atrophic ones.

At present there is no cure, although various agents have been tried. Medical treatment of OLP is essential for the management of painful, erythematous, erosive, or bullous lesions. The main aim of current OLP therapy is to relieve painful symptoms, heal oral mucosal lesions, reduce the risk of oral cancer, and maintain good oral hygiene. As it is an autoimmune mediated condition, corticosteroids are recommended. In patients with recurrent painful disease, another goal is to prolong their symptom free intervals. The main concern over current therapies is represented by local and systemic adverse effects and lesion recurrence after treatment is suspended. Patients should be
followed up periodically, particularly those affected by the erosive or atrophic forms and those who also have a history of alcohol and tobacco abuse, because of the risk of malignant transformation.

Although in vitro studies into HBDs in oral epithelium cells are well documented, oral epithelium has only scarcely been the subject of in vivo investigations. Published studies have shown that HBD-2 peptide is expressed mainly in granular and keratinized layers, but is not easily seen in the non-keratinized oral epithelium, including buccal and junctional epithelia. Abiko et al [78] found that in 43% of cases of lichen planus, there was a positive immunohistochemical staining for HBD-2 in the spinous layer. Staining was marked even in lichen planus with non-keratinized epithelium. The border of the area with transition of the non-keratinized normal epithelium into lichen planus separated the positively stained areas from those showing no staining. The immunohistochemical localization of HBD-2 peptide in lichen planus was stronger and wider than in normal epithelium. The site of HBD-2 expression was faint and restricted to the keratinized area and to part of the granular layers in normal oral epithelium. Intense staining of HBD-2 in a lower spinous layer of lichen planus was often observed. The presence of HBD-2 is linked to the presence of inflammatory infiltration. Specific inflammatory conditions related to lichen planus may be involved in the upregulated expression of HBD-2. The inflammatory infiltrate in lichen planus is composed of lymphocytes, predominantly T-cells. These T-cells produce many types of cytokines, which, in turn, stimulate the production of other cytokines in epithelial cells. Expression of HBD-2 is upregulated by stimulation of cytokines, including TNF-α and IL-1. In lichen planus, TNF-α is
increasingly produced in both sub-epithelial inflammatory infiltrate and epithelial cells. Upregulated expression of HBD-2 in lichen planus may be involved in increased production of TNF-α.

In a following paper, Nishimura, Abiko et al [79] reported that the localization pattern of HBD-3 mRNA in oral tissues was almost the same as that of HBD-2. Previous papers had shown that HBD-2 expression was involved in keratinocyte differentiation. HBD-3 mRNA was localized in the upper spinous and granular layers in normal oral epithelium. These results may indicate that HBD-3 expression is also involved in keratinocyte differentiation.

Although both HBD-2 and HBD-3 are upregulated by inflammatory stimulation such as the one triggered by cytokines and bacteria, cytokines that stimulate upregulation are different. In contrast to HBD-2 that was induced by TNF-α, no regulation of HBD-3 was found after stimulation, but HBD-3 was induced by IFN-γ. IFN-γ production has been also confirmed in recent papers about lichen planus. The upregulated expression of HBD-3 in lichen planus in the present study may be involved in the production of IFN-γ.

3.1.3 BURNING MOUTH SYNDROME

Burning Mouth Syndrome has been previously described in this dissertation. It affects mainly women who present with a chronic burning or tingling sensation in their mouth with no local or systemic causes that could explain the symptoms.
Recent theories link the onset of this disease to both a psychosomatic alteration in pain perception and a peripheral trigeminal and lingual nerve fibers dysfunction.

It could be interesting to check if an altered level of defensins may be one of the main causes in the pathogenesis of the alteration of the nerve fibers metabolism inside the oral cavity.

3.2 **AIM**

This work focuses on the possible role of defensins in the pathogenesis and maintenance of several oral pathologies. It is included in a larger departmental project which analyses the role of defensins in dermatology, ginecology, ophtalmology and neurology.

The aim of this research is to investigate if the expression of defensins may play a statistically significant role in the pathogenesis of oral conditions.

The most representative oral pathologies were chosen among different categories: Periodontal disease among infective disorders, Diabetes mellitus and its oral manifestations among metabolic diseases, Oral Lichen Planus for autoimmune diseases and Burning Mouth Syndrome among the neuropathic and psychosomatic disorders of the oral cavity.

This study reports the results for Oral Lichen Planus and Burning Mouth Syndrome.
3.3 MATERIALS AND METHODS

The current study was conducted in the Department of Surgical and Morphological Sciences, University of Insubria, ASST dei Sette Laghi, Unit of Oral Pathology, Dental Clinic, Varese, Italy.

A total number of 35 patients were recruited for this study.

Among them, 17 were affected by Oral Lichen Planus (OLP), 9 by Burning Mouth Syndrome (BMS) and 9 were included as a control group (CTRL).

Inclusion criteria for the OLP group were the presence of a positive histopathologic diagnosis of the disease, age greater than 30, while patients undergoing topical or systemic therapies were excluded from the recruitment.

Inclusion criteria for the BMS group were the final diagnosis of a primary idiopathic burning pain according to the guidelines of the international literature [45], age greater than 45 and female sex, whereas the presence of other concomitant mucosal pathologies, such as a secondary burning pain linked to systemic conditions or other neuropathies, were exclusion criteria.

The control group (CTRL) included healthy patients with demographic similitaries to the other two groups, especially considering age and F:M ratio.

Each patient underwent a dental examination by a clinician who was the same throughout the whole study.

Firstly, a sample of saliva was collected through the spitting method for about 1 minute in a sterile test tube.
Secondly, a sterile paper cone was inserted for at least 30 seconds into the gingival sulcus nearby a dental element chosen through randomized criteria. The procedure was carried out 4 times per patients, each time near a different dental element, in order to collect a sufficient quantity of crevicular fluid. Furthermore, each OLP patients was also evaluated for the clinical appearance of the lesions, subdividing the population into two subgroups: red OLP, when patients showed an atrophic or erosive variant of the disease; white OLP, when reticular and hyperplastic forms were detected.

Collected specimens were sent to the Biochemistry Laboratory to be analysed.

The samples were stored at a temperature of –20 °C before examination.

Human β-Defensin 2 Elisa Kit protocol was used for this study (EK-072-37 Phoenix Pharmaceuticals, Inc.). The immunoplate was precoated with anti-Human β-Defensin 2 Capture Antibody and the nonspecific binding sites were blocked. Human β-defensin 2 in the standard solution or in the sample could bind to the capture antibodies immobilized in the wells.

After washing procedure, the Biotinylated anti-Human β-defensin 2 Detection Antibody which could bind to Human β-defensin 2 trapped in the wells was added. After washing, the Streptavidin-Horse-radish Peroxidase (SA-HRP) which catalyzes the Substrate Solution (TMD) was added. The enzyme-substrate reaction was terminated by the addition of a stop solution. The intensity of the colour was directly proportional to the amount of Human β-Defensin 2 in the standard solutions or samples.
Fig. 22: graphic representation of the HBD-2 ELISA-kit protocol

A standard curve of Human β-Defensin 2 with known concentration could be generated accordingly.

Fig. 23: generation of a standard curve of HBD-2
Comparative statistics among the different groups was performed with T-Student independent variables test for parametric values and the Chi-square test for non parametric values by using the IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA.

3.4 RESULTS

A total number of 35 saliva and crevicular liquid samples were analysed with ELISA protocol.

17 patients were affected by Oral Lichen Planus (OLP group), 11 females and 6 males.

9 patients were affected by Burning Mouth Syndrome (BMS group), only women.

9 patients were included in the control group (CTRL group), 7 women and 2 males.

Plot 6: epidemiologic data of patients recruited in the current study
The **OLP group** ELISA test for detection of HBD-2 in saliva ruled out a mean value of 4341.85 +/- 2748.68 pg/ml, with a registered minimum value of 304.28 pg/ml and a maximum of 10890.56 pg/ml. The mean value of HBD-2 reported among the OLP group in the crevicular fluid was 4157.37 +/- 2620.59 pg/ml with a registered minimum value of 444.65 pg/ml and a registered maximum value of 8552.84 pg/ml.

![LICHEN](image)

**Plot 7: HBD-2 salivary registered values in OLP group**

**Table 4: salivary and crevicular fluid HBD-2 registered values in OLP group**

<table>
<thead>
<tr>
<th>OLP</th>
<th>SALIVA (pg/ml)</th>
<th>CREVICE (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>304.28</td>
<td>444.65</td>
</tr>
<tr>
<td>2</td>
<td>2971.62</td>
<td>2971.62</td>
</tr>
<tr>
<td>3</td>
<td>7081.92</td>
<td>7497.44</td>
</tr>
<tr>
<td>4</td>
<td>10890.56</td>
<td>8211.21</td>
</tr>
<tr>
<td>5</td>
<td>2882.04</td>
<td>2882.04</td>
</tr>
<tr>
<td>6</td>
<td>1706.14</td>
<td>1706.14</td>
</tr>
<tr>
<td>7</td>
<td>6664.23</td>
<td>6664.29</td>
</tr>
<tr>
<td>8</td>
<td>3220.35</td>
<td>2298.73</td>
</tr>
<tr>
<td>9</td>
<td>2625.45</td>
<td>2625.5</td>
</tr>
<tr>
<td>10</td>
<td>2031.01</td>
<td>2317.91</td>
</tr>
<tr>
<td>11</td>
<td>7329.89</td>
<td>8063.02</td>
</tr>
<tr>
<td>12</td>
<td>2968.24</td>
<td>2968.24</td>
</tr>
<tr>
<td>13</td>
<td>2750.83</td>
<td>2750.83</td>
</tr>
<tr>
<td>14</td>
<td>3819.53</td>
<td>2780.58</td>
</tr>
<tr>
<td>15</td>
<td>7532.52</td>
<td>8552.84</td>
</tr>
<tr>
<td>16</td>
<td>3031.94</td>
<td>2544.71</td>
</tr>
<tr>
<td>17</td>
<td>5980.87</td>
<td>5395.46</td>
</tr>
</tbody>
</table>
The **BMS group** ELISA test for detection of HBD-2 in saliva ruled out a mean value of 3789,05 +/- 1565,83 pg/ml, with a registered minimum value of 1703,43 pg/ml and a registered maximum value of 7047,26 pg/ml. The mean value of HBD-2 reported in the BMS group in the crevicular fluid was 3314,94 +/- 1064,27 pg/ml with a registered minimum value of 1703,43 pg/ml and a registered maximum value of 5066,56 pg/ml.

![Plot 8: HBD-2 salivary registered values in BMS group](image)

**Table 5: Salivary and crevicular fluid HBD-2 registered values in BMS group**

<table>
<thead>
<tr>
<th>BMS</th>
<th>SALIVA (pg/ml)</th>
<th>CREVICE (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3444,51</td>
<td>2470,36</td>
</tr>
<tr>
<td>2</td>
<td>3408,29</td>
<td>3408,29</td>
</tr>
<tr>
<td>3</td>
<td>2973,98</td>
<td>2509,84</td>
</tr>
<tr>
<td>4</td>
<td>7047,26</td>
<td>5066,56</td>
</tr>
<tr>
<td>5</td>
<td>3147,48</td>
<td>3147,48</td>
</tr>
<tr>
<td>6</td>
<td>3799,01</td>
<td>3799,01</td>
</tr>
<tr>
<td>7</td>
<td>5474,01</td>
<td>4626,04</td>
</tr>
<tr>
<td>8</td>
<td>1703,43</td>
<td>1703,43</td>
</tr>
<tr>
<td>9</td>
<td>3103,45</td>
<td>3103,45</td>
</tr>
</tbody>
</table>
The **CTRL group** ELISA test for detection of HBD-2 in saliva ruled out a mean value of 3268,95 +/- 1886,90 pg/ml, with a registered minimum value of 428,01 pg/ml and a registered maximum value of 6242,46 pg/ml. The mean value of HBD-2 reported in the CTRL group in the crevicular fluid was 4399,70 +/- 3889,23 pg/ml with a registered minimum value of 530,23 pg/ml and a registered maximum value of 11767,84 pg/ml.

![CTRL](image)

Plot 9: HBD-2 salivary registered values in CTRL group

Table 6: salivary and crevicular fluid HBD-2 registered values in CTRL group

<table>
<thead>
<tr>
<th>CTRL</th>
<th>SALIVA (pg/ml)</th>
<th>CREVICE (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5531,09</td>
<td>11767,84</td>
</tr>
<tr>
<td>2</td>
<td>2862,34</td>
<td>530,23</td>
</tr>
<tr>
<td>3</td>
<td>3770,32</td>
<td>645,68</td>
</tr>
<tr>
<td>4</td>
<td>3499,63</td>
<td>7316,93</td>
</tr>
<tr>
<td>5</td>
<td>3761,12</td>
<td>8527,34</td>
</tr>
<tr>
<td>6</td>
<td>428,01</td>
<td>2626,5</td>
</tr>
<tr>
<td>7</td>
<td>2202,03</td>
<td>3333,28</td>
</tr>
<tr>
<td>8</td>
<td>6242,46</td>
<td>2533,43</td>
</tr>
<tr>
<td>9</td>
<td>1123,54</td>
<td>2316,03</td>
</tr>
</tbody>
</table>
One-way ANOVA test was used to compare the values of salivary HBD-2 registered among the three groups. There was not any statistically significant difference among the groups with regards to the production of HBD-2 (p=0.523).

The same test was performed to compare the values of gingival HBD-2 registered among the three groups. There was not any statistically significant difference among the groups with regards to the production of HBD-2 (p=0.897).

However, the distribution of values registered in saliva and crevicular fluid was strictly correlated. Paired sample correlation test was used to compare the associated variance of salivary and gingival values within patients (p=0.000).

Therefore, the presence of corresponding values in saliva and crevicular fluid showed how the ELISA detection method was correct and that saliva and crevicular fluid contained an equivalent quantity of HBD-2 in the same patient.

Nevertheless, observing the scatter plot and the box plot (Plots 10-11) it could be possible to notice that while the mean values were similar among the groups, even if registered values were slightly higher in OLP and BMS than in the CTRL group, in OLP there was a dycotomic pattern of distribution between a subgroup showing very high values (>3500 pg/ml) and another subgroup with values similar to those detected in the other two groups (<3500 pg/ml).
The clinical evaluation of OLP ruled out the presence of 10 white OLP and 7 red OLP.

A statistical non-parametric comparison was made between the classification of OLP based upon the clinical evaluation and the results of the ELISA test defined as low (<3500 pg/ml) or high (>3500 pg/ml). There was a strong statistical correspondence (p=0.002).
Table 7: contingency table clinical * ELISA groups

<table>
<thead>
<tr>
<th>SALIVA</th>
<th>ELISA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt; 3.500</td>
<td>≥ 3.500</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>1</td>
</tr>
<tr>
<td>Clinical</td>
<td>White</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>Totale</td>
<td></td>
<td>7</td>
</tr>
</tbody>
</table>

The same analysis was made for crevicular fluid and proved to be statistically significant (p=0.000), confirming the association between salivary and gingival values.

Table 8: contingency table clinical * ELISA groups

<table>
<thead>
<tr>
<th>CREVICE</th>
<th>ELISA</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>&lt;3.500</td>
<td>&gt;3.500</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Clinical</td>
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<td>10</td>
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<tr>
<td></td>
<td>Red</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>11</td>
</tr>
</tbody>
</table>

ANOVA analysis was performed by comparing the salivary values in relation to the dycotomic clinical classification. There was a statistically significant
difference between salivary HBD-2 values registered in the red OLP subgroup and those found in the white OLP subgroup (p=0,000).

The same result was obtained performing an ANOVA test to compare crevicular fluid values in relation to the clinical classification (p=0,000).

### 3.5 DISCUSSION

Antimicrobial Peptides (AMPs) represent a recent interesting field of research in immunobiology and medicine, even though they are an evolutionary ancient means of defense by the host through the innate immune system.

In recent years the international literature has shown that not only do they play a special role in fighting off infective agents, but also in immunomodulatory functions and wound healing. A reduced production or function of these small peptides imply the onset of infective disease, wound superinfection or cancer, while on the contrary an excess in their presence or activity seems to be linked to autoimmune diseases.

ß-defensins are mainly expressed in the mucosal barriers throughout the human body, like in the bowel, the cute, the genitourinary tract and oral mucosa.
Several studies tried to establish the role of \( \beta \)-defensins in the pathogenesis of periodontal disease and other oral disorders, but only a few papers can be found in the literature.

In this study the choice was to study the role of HBD-2, which is an inducible and not constitutionally expressed defensin, in the pathogenesis of the most frequent autoimmune disease of the oral cavity, Oral Lichen Planus (OLP).

17 OLP patients, with a positive histopathologic diagnosis of the disease, were recruited into this study in order to measure the relative quantity of HBD-2 in their saliva and crevicular fluid. The values were compared with those collected from a group of 9 patients affected by the Burning Mouth Syndrome (BMS), the most enigmatic neuropathic disorder of the mouth, and with a control group (CTRL) of 9 patients.

There was not any statistically significant difference between the groups \((p=0.523; \ p=0.897)\), both the Burning Mouth Syndrome and Oral Lichen Planus. However, the latter seemed to show increased mean levels of HBD-2. Besides, the study highlighted the role of HBD-2 in the maintenance and intensity of the inflammatory component in Oral Lichen Planus.

Patients affected by OLP showed a dycotomic distribution of values: while 10 of them showed similar values to those found out in the other two groups, 7 patients expressed high levels of HBD-2, and 3500 pg/ml was the threshold to distinguish the subgroups.

During the dental visit the clinician classified OLP patients into two groups according to the clinical presentation of the disease: reticular and hyperplastic OLP forms were considered to be associated with a low level of
disease and inflammation, whereas atrophic and erosive forms were related to a high degree of inflammation.

There was a statistical significant correlation between the clinical and numeric classification of the patients (p=0.004; p=0.001), and the expression of HBD-2 was higher in the red OLP group than in the white OLP group (p=0.000; p=0.000).

In conclusion, this study shows that HBD-2, which is an inducible molecule, represents an index to assess active inflammation and it is probably linked to the presence of the typical band-like CD8⁺ infiltrate in Oral Lichen Planus.

HBD-2 can be used as a parameter to monitorize the degree of disease activity and inflammation. Besides, the use of Hyaluronic acid in the treatment of several inflammatory diseases of the oral cavity should be thoroughly investigated because the different fragments derived from enzymatic digestion or topical products could have completely different effects according to their molecular weight. Therefore prescription should be made after careful evaluation of possible side effects and biological activity.
4. 3rd STUDY: HELICOBACTER PYLORI IN PERIODONTAL POCKETS AND SALIVA: A POSSIBLE ROLE IN GASTRIC INFECTION RELAPSES?

A PRELIMINARY STUDY IN NORTHERN ITALY.

4.1 BACKGROUND

*Helicobacter pylori* (HP) is a widespread bacterium, which is found within water and in some animals’ biological fluids, and it is the responsible for chronic gastritis and other gastric diseases [80].

Helicobacter pylori is known to be associated with chronic gastritis in 90% of cases, but it has also been described in acute gastritis, peptic ulcer and gastric cancer. This bacterium has adapted to thrive in the acidic environment of the stomach and this requires additional virulence factors, such as urease production, which is a direct marker for diagnosis. Despite the fact that infection incidence has decremented in recent years, about half of the world population is still infected. Moreover, treatment resistance and relapses represent a threatening issue to be dealt with [81].

4.1.1 History

In 1886 Prof. W. Jaworski was the first one who described a helical bacterium in gastric washings which was originally called *Vibrio rugula*. He suggested a pathogenic role in gastric infection. Bizzozzero detected the presence of an infectious agent in animals’ stomach.
However, these discoveries were confined to Europe. In 1954 the American gastroenterologist Palmer failed in his attempt to find bacteria in gastric fluid from several patients.

The early tests carried out to demonstrate the presence of HP were doomed to fail because there were not homogeneous culture methods.

In 1979 Warren described the presence of a bacterium in the superficial epithelium from several gastric biopsies. This discovery was sensational, since it had always been thought to be impossible until then the possibility of a bacterial proliferation in the stomach and the gastric lumen was dogmatically considered as fully sterilized by gastric acids.

Warren was strongly opposed by the medical community, but in 1981 Barry Marshall supported him in developing an adequate culture field for the bacterium. After initial failure, they published a paper in Lancet suggesting a first-line therapy with Bismuth salts and amoxicillin or tetracycline [82].

Warren was so strongly convinced that his own theory would be effective that he infected himself by ingesting bacterial cultures, developing a well-documented gastritis that was successfully treated. Originally called Campylobacter-like bacterium, it was quite different from Campylobacter due the presence of numerous flagellae and high expression of urease production. In 1989 Goldwin called the bacterium Helicobacter pylori. In 2005 Robin Warren and Barry Marshall won the Nobel Prize.
4.1.2 *Helicobacter pylori*

*Helicobacter pylori* (HP) is a Gram-negative, microaerophilic bacterium, which shows a helicoidal shape in its active form, while on the contrary it exhibits a coccoid shape during latency. It is 2,5-5 x 0,5mm long and has 5-6 unipolar flagellae.

![Helicobacter pylori](image)

**Fig. 24:** Helicobacter pylori is equipped with multiple flagellae

It shows intense catalase, oxydase and urease activities.

It is equipped with a slightly toxic lipopolysaccharide enriched in Lipid A, while the glucidic portion contains analogous sequencies to Lewis X and Y antigens, widely distributed within cell surfaces in human body: this could explain the autoimmune response which usually contributes to mucosal damage.

The natural habitat for HP is the gastric mucosa, and this is possible thanks to its highly expressed urease activity, which also is a primary marker for diagnosis.
4.1.3 Epidemiology

The prevalence of HP infection has changed in recent years and has diminished with peptic ulcers and gastric cancer. However, about half of the worldwide population is still infected, with about one-third in western countries and over 50% in Southern and Eastern Europe, South America and Asia. In developing countries the infection rate is between 70 and 90% and is generally acquired during infancy, while in Western countries it is between 25 and 50% during infancy [83].

In developed countries, upper-class people with higher and economical standards of life are less affected by this infection, while it seems that individuals migrated from countries in which the infection is diffuse are more hit. This is due the fact that one main way of transmission seems to be oral and/or oro-foecal, thus the lack of primary higienic services and clean water play an important causal role. The second way of transmission seems to be linked to the use of infected endoscopes or instruments.

Geographic factors, ethnic background and age greatly influence the epidemiology of the infection, but the low social and economical status represents the key factor in many cases.

![Table of epidemiologic data from the international literature](image-url)
4.1.4 Pathogenesis

HP infection triggers an inflammatory response which is not able to eradicate bacterial colonization but, on the contrary, becomes persistent and damages the lining mucosa. HP binds to the MHC type II complex of gastric epithelium, causing cell apoptosis. The first site of infection is the gastric antrum, where acid-secreting cells are absent.

Disease progress depends on different factors, like bacterial genomics and adaptability, host defenses and immune status, genetic predisposition, familial susceptibility to gastric cancer, smoke and food habits, especially large consumption of salt and meat.

The first step is the colonization of the gastric mucosa, due the interaction between bacterial adhesins and glycans expressed by the epithelium surface.

Urease activity transforms urea into ammonium and carbon dioxide, neutralizing gastric acids.

Furthermore, the bacterium produces several virulence factors, which are responsible of intracellular pathways alteration and pro-inflammatory cytokines production: CagA, VacA, Hsp-B and duodenal promoting gene A. These factors are associated with increased incidence of gastritis and gastric cancer [84].

4.1.5 Clinical features

HP infection is typically associated with active chronic gastritis, but only 10-15% of infected patients develop a gastric ulcer, because of more severe bacterial virulence factors and individual predisposition.
While in the stomach HP acts directly against gastric mucosa, in the duodenum it exhibits an indirect mechanism.

The infection in the gastric antrum determines an increased production of gastrin and a reduced one of somatostatin. The increased acid production within the fundus causes duodenal ulcer and gastric metaplasia, which leads to an increased colonization of HP. In addition, HP decreases the production of bicarbonate in the duodenal tract.

HP is also associated with acute gastritis: nodular gastritis, follicular gastritis, lymphocytic gastritis, haemorrhagic gastritis, granulomatous gastritis, hypertrophic gastritis and Ménétrier disease.

Other clinical manifestations of HP infection are the non-ulcer dyspepsia, multifocal gastric atrophy and intestinal metaplasia, gastric adenoma and hyperplastic polyps.

In 1994 the WHO classified *Helicobacter pylori* as a cancerogenic agent, and the IARC defined it as a first-class carcinogenic agent [85].

Cancerogenesis begins within the context of a pre-malignant lesion (atrophic gastritis, intestinal metaplasia and dysplasia, with a rate of 0,8%, 1,8% and 32,7%).

A specific role in cancer development is played by the presence of acetaldheyde, which is increased in HP infection and in alcohol consumption.

It should be also underlined that HP is statistically associated with gastric lymphoma [86].
HP infection is also correlated to several extragastric diseases, in which it seems to act as a trigger for their development.

These pathological entities include sideropenic anaemia, idiopathic thrombocytopenic purpura, pancreatic tumours, dermatological diseases (especially chronic urticaria), obesity, cardiovascular and neurological diseases,
gynecological diseases, autoimmune thyroiditis, oropharyngeal diseases, bowel diseases [87].

**Fig.27: extra-gastric manifestations of HP infection**

### 4.1.6 Diagnosis

Two kinds of diagnostic procedures allow to detect HP infection: invasive and non-invasive methods.

Among the invasive procedures, **esophagogastroduodenoscopy** with or without histological evaluation, **microbiological** examination and **rapid urease test** (RUT) are indicated.

The Sydney system is used to obtain 5 topographically determined mucosal biopsy samples evaluated by the Sydney score to determine the gastric atrophy grade [88].
The rapid urease test can be performed, with an accuracy of 90%. However, patients have to suspend PPI for two weeks before the examination and bleeding can alterate the histologic results.

Microbiological examination is only performed in particular cases. Cultures are Gram-negative, urease, catalase and oxydase positive. HP is very fragile outside the gastric environment and analysis should be performed as soon as possible once the procedure has been completed.

In addition, PCR-RT can be used on gastric biopsies.

Among non-invasive diagnostic procedures **serological procedures** can be used: the detection of specific serum IgM with ELISA-test, Latex agglutination test and Western Blotting Test. IgG can be found in serum or urina, while IgA in saliva, these latest two less sensible.

However, the great disadvantage of this technique is that it does not distinguish between active and previous infection.

Nowadays C-Breath Test Urea (C-BTU) measures urease activity in breath. Patients swallow urea labelled with an uncommon isotope, either radioactive carbon-14 or non-radioactive carbon-13. 10 to 30 min after ingestion of the urea, the detection of isotope-labelled carbon dioxide in exhaled breath indicates that the urea was split; this indicates that urease (the enzyme that *H. pylori* uses to metabolize urea) is present in the stomach, and hence that *H. pylori* bacteria are present [89].

For the two different forms of urea, different equipment is required. Carbon-14 is normally measured by scintillation, whereas carbon-13 can be detected by isotope ratio mass spectrometry or by mass correlation spectrometry. For each of these methods, a baseline breath sample is collected before taking the isotope-labeled urea, for comparison with the post-urea sample, with a 20- to 30-minute interval between them. Samples may be sent to a reference laboratory for analysis.
Alternatively, mass correlation spectrometry can be performed as an office-based test since breath samples are continuously collected, and results are provided immediately within minutes.

The difference between pre- and post urea measurements is used to determine infection. This value is compared to a cut-off value. Results below the value are considered negative, those above it positive. The cut-off value itself is determined by comparing the results of patients with two or more different detection methods. The chosen value is the one which gives the best combination of sensitivity and specificity.

The test measures active *H. pylori* infection. If antibiotics lower the amount of HP, or stomach acidity is less than normal, the quantity of urease present will decrease.

Accordingly, the test should be performed only 14 days after stopping acid reducing medication (proton pump inhibitors, PPI) or 28 days after stopping antibiotic treatment.

It is a easily reproducible test with 88-95% sensibility rate and 95-100% specificity.

Tests for foecal antigens are also carried out by using anti-catalase monoclonal antibodies [90]. This kind of test is not influenced by PPI.

Guidelines are available at the Acts of the IV edition of Maastricht/Florence Consensus [91].

### 4.1.7 Therapy

The problem frequent relapses has urged clinicians to issue adequate guidelines to treat HP infection, which were discussed during the Toronto Consensus Conference [92].
Therapy is recommended in the following cases:

- gastric or duodenal ulcer: in this case it prevents further recurrence;
- chronic gastritis: therapy cures gastritis and relieves symptoms, sometimes only partially;
- early gastric cancer and MALT lymphoma: eradication therapy is the preferred initial treatment;
- non-ulcer dyspepsia;
- prevention in patients with a high risk of gastric cancer development;
- metaplasia;
- extragastric manifestations: especially in vitamin B12 deficiency and idiopathic thrombocytopenic purpura.

It should be noticed that there is no correlation between HP infection and gastroesophageal reflux disease [93].

**Triple therapy**, including a Proton Pump Inhibitor (PPI) plus two antibiotics, such as clarithromycin and amoxicillin or metronidazole, is the most common treatment of choice.

In recent years the prevalence of resisting bacterial strains drew the attention of clinicians to the issue of the worrisome increase in unsuccessful rates in the therapeutic protocol. Resistance rate to clarithromycin is around 20%, 20-40% to metronidazole while it has remained only 1-3% to amoxicilline. Taking into account the high prevalence of clarithromycin resistance, **quadruple therapy** (PPI+bismuth+metronidazole+tetracyclin) is used as an alternative strategy. In recent years, there have been reports suggesting sequential therapy (agents administered in sequence) and concomitant therapy (non-bismuth quadruple therapy) as alternative treatment able to eradication rates similar to PPI-triple therapy. However, the results of recent studies have shown that documented eradication rates are at their lowest level in history. This could be due to incomplete
elimination of HP, which results in recrudescence of the same strain, or to a new infection by a new strain [94].

Fig.28: recommended therapeutic regimens in eradication of HP gastric infection

Treatment should last at least 14 days for every patient.

The choice of protocol should take into account regional resistance to antibiotics.

The PPI represent fundamental allies to guarantee a correct drug functioning due the presence of high conditions of acidity in the environment of the stomach.

Fig.29: flowchart of medication protocol in the treatment of HP gastric infection
4.1.8 *Helicobacter pylori and the oral cavity.*

Relapses are not uncommon. This leads to the hypothesis of a possible extra-gastric reservoir of infection.

Investigation on a reservoir of HP which could contribute to relapses of gastric infection becomes crucial.

Apart from the stomach, HP has also been found in the distal esophagus, the proximal duodenum, the colonic contents, and the oral cavity, including tonsil and adenoid tissue [95].

Many papers about the relationship between the oral cavity and gastric HP infection have been published, with numerous evidence-based studies suggesting that the oral cavity may be a potential reservoir for HP [96][97].

The presence of HP in the oral cavity was discovered for the first time in 1989 [98]. Following studies diagnosed the presence of HP in dental plaque, saliva, tongue, tonsil tissue and root canals [99].

Some authors believe that HP can live symbiotically with Candida and yeasts, enhancing the ability to resist in the difficult environment of the oral cavity. The fact that this bacterium has been found in saliva, dental plaques and feces reinforces the opinion that oro-oral and oro-foecal ways of transmission are the most involved.

Conversely, other scientists have claimed that the oral cavity may not be a reservoir of HP. This contention is largely based upon different detection methods for oral HP.

A meta-analysis published in 2011 reported strong connection between the presence of HP in the oral cavity and in the stomach [100].
However, triple eradication therapy has no or little effect on oral HP elimination; studies have recorded eradication rates for oral infection below 40% [101].

The presence of oral HP might diminish the effect of eradication therapy and acts as a causal element in the recurrence of HP infection.

Microbiologic examination is considered the gold standard to evaluate the presence of HP within the oral cavity. However it is a technique with low sensibility and specificity rates. Sometimes the bacterium can be in the coccoid form, which is not detected, and/or with a low number of elements.

The introduction of the PCR-RT technique allowed researchers to detect HP when it is slightly present in saliva or dental plaque.

In recent years various studies have documented the interaction between HP and the oral tissue involved in several oral pathologies.

Some clinicians refer the presence of HP in periodontal pockets, oral squamous cell carcinoma and correlate gastric infection to the insurgence of apthous stomatitis [102]. However, other clinicians tend to underestimate the role of HP in the oral cavity, but no scientific consensus has been reached so far.

### 4.2 Aim

It has been a long time since the scientific community started to speculate upon the presence of HP in periodontal pockets as an extra-gastric reservoir responsible of gastric relapses after eradication therapy.

Firstly, it should be borne in mind that there is a difference between recrudescence and reinfection. Recrudescence is the relapse due to the action of the same
bacterial strain after some months from eradication therapy, while reinfection is due to the action of a new bacterial strain.

In developed countries relapses are mainly due to recrudescence, while in developing countries reinfection is more common.

The international literature reports are very controversial.

Some papers highlight the presence of HP especially in patients with low oral hygienic conditions, as reported in the study by Mohammed Al Asqah [103].

Other authors, like Myriama-Lucrecia Medina have pointed out the presence of the bacterium in the oral cavity in association with dyspeptic symptoms [104].

On the contrary, Nélio Vega et al have rejected the correlation between oral cavity and gastric relapses, claiming that HP in the oral cavity is due to gastro-esophageal reflux disease or contaminated food intake [105]. However, the same author has described a greater prevalence of HP infection in the oral cavity by comparing social and demographic variables, age, geography and economic status.

Another much-discussed issue is whether eradication therapy for gastric infection is capable of eliminating HP from the oral cavity.

Several studies have shown unsuccessful eradication from the oral cavity because of the presence of dental plaque biofilm, which is a very effective barrier against antibiotics and drugs. Only periodontal therapy, through dental debridment can eliminate resident bacteria, including HP [106].

Other studies have stated that periodontal therapy plus eradication therapy could prevent gastric relapses more than eradication therapy alone [101].

The aim of this study is to evaluate the presence of Helicobacter pylori in a group of patients who underwent examination for gastric infection.
Firstly it is necessary to compare the presence of HP in patients with negative diagnosis of gastric infection versus patients with a positive diagnosis of gastric infection.

Then, the role of periodontal health as a predisposing factor for oral HP infection should be thoroughly investigated.

Finally, the main purpose is to ascertain whether the presence of HP within oral tissues can represent an extra-gastric reservoir able to contribute to gastric relapses after eradication therapy in a significant way.

4.3 MATERIALS AND METHODS.

This research was conducted at the Department of Surgical and Morphological Sciences, University of Insubria, ASST dei Sette Laghi, Unit of Gastroenterology, Varese, Italy.

The experimental protocol was evaluated by the Institutional review Board, reference number 19/2015.

60 patients were recruited into the current study, subdivided into two groups: 30 patients with a positive result for HP gastric infection with C-UBT examination (group A) and 30 patients with a negative result for HP gastric infection (group B).

An informed consent (see below) was read, understood and signed by all patients.

A dental clinician performed periodontal examination for every patient.

Inclusion criteria were the presence of the signed informed consent, compliance and good general health conditions; exclusion criteria concerned the intake of antibiotics or proton-pump inhibitors for 60 days prior to the breath test.
Dental probing showed the presence of periodontal pockets in all four quadrants. Afterwards, a paper cone was inserted for 30 seconds into the gingival sulcus near the dental element with the deepest probing value registered for each quadrant and then sent in a sterile tube to the laboratory for evaluation.

Saliva was collected through the spitting method for one minute in a sterile tube.

Specimens were processed to extract and purify DNA by using a method that includes two consecutive phases of incubation with lysozyme and proteinase K. Once extracted, DNA was purified through a silica spin-column (Sigma-Aldrich, St. Louis, MO, USA). Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate the amount sequences of 16S rRNA gene of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1). All sequences were aligned to find either a consensus sequence or less preserved spots, useful to optimize the specificity of primers and dual labelled hydrolysis probes. Absolute quantification assays were performed using a 7500 Sequence Detection System (Applied Biosystems). The thermal cycle included 10 minutes incubation at 95 °C to activate polymerase, followed by a two-step amplification of 15s at 95 °C and 60 s at 57 °C for 40 cycles. Each experiment included non-template controls to exclude reagents contamination and serial diluitions of the specific synthetic template (Eurofin MWG Operon, Ebersberg, Germany).

To obtain standard curves plasmids containing bacterial target sequences were used (Eurofin MWG Operon, Ebersberg Germany). The total amount was calculated by using the Thermo Scientific Nanodrop spectrophotometer. Standard curves were created with serial diluitions between $10^7$ and $10^4$ plasmids copies.

The total quantification of absolute bacterial charges determined the relative amount of *Helicobacter pylori*. Under 10 U the results were considered as negative.

Comparative statistics among the different groups was performed by using the IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA.
Informed Consent

INFORMAZIONE AL PAZIENTE

Correlazione tra salute parodontale e presenza di Helicobacter pylori all’interno del cavo orale.

Gent.ma Signora, Gent.Signore

Le viene chiesto di partecipare a uno studio clinico il cui obiettivo principale è quello di valutare la correlazione tra il suo stato di salute parodontale e la presenza all’interno della cavità orale del batterio Helicobacter pylori, causa principale dell’ulcera gastrica.

Lo studio prevede l’accesso alla clinica Odontostomatologica di Velate (VA) dove verrà effettuata una prima visita di igiene orale, momento in cui verranno valutati gli indici parodontali iniziali. In maniera selettiva verranno utilizzati dei coni di carta per prelevare dei campioni biologici, i quali saranno analizzati successivamente attraverso il metodo della PCR (Polymerase Chain Reaction) per determinare o meno la presenza di Helicobacter pylori.

Presso la clinica Odontostomatologica di Velate (VA), potrà essere inserito in un programma di prevenzione della salute orale.

Le visite presso la clinica Odontostomatologica di Velate (VA) relative alla Sua partecipazione allo studio clinico sono gratuite e non prevedono alcuna spesa da parte Sua.

La sua partecipazione è volontaria; per questo le verrà chiesto di firmare il modulo del consenso informato, il quale attesta la disponibilità a partecipare a questo studio. Tutte le informazioni raccolte durante lo studio saranno considerate strettamente confidenziali ed utilizzate soltanto ai fini dell’elaborazione statistica.

Il suo nome sarà sostituito dalle iniziali e i dati personali saranno trattati in modo da mantenere un assoluto anonimato.

In ogni caso, il trattamento dei dati avverrà nel rispetto di quanto previsto dalla normativa sulla privacy (196/2003).

Fig. 30: an informed consent was filled in by patients before salivary and crevicular fluid examination
4.4 **RESULTS**

A total number of 60 patients underwent the experimental procedure, 24 males and 36 females.

Among them, 30 tested positive for Breath test (group A) and 30 negative (group B).

Age ranged between 16 and 78, with a mean age of 52,88 +/- 14,67.

Among 24 male patients, 10 tested positive and 14 negative for Breath test, while among females 20 tested positive and 16 negative.

Age ranged between 21 and 76 among negative patients (mean age 53,07 +/- 15,72) and between 16 and 78 among positive patients (mean age 52,7 +/- 13,81).

Plot 12: males and female distribution among the two groups
Salivary samples values ranged from 0 to 103 units, with a mean value of 11.73 +/- 20.08.

Among them, 38 tested negative because they showed a value lower than 10 U, while 22 tested positive.

Plot 13: 38 patients showed salivary HP units under 10 U, while 22 resulted as positive.

T-Student test for independent variables was used to compare the absolute values of HP detected in saliva within the two groups of positive and negative Breath test. The result was that there were not statistically significant differences (p=0.372).

The Chi-square test was not statistically significant grouping saliva values into negative and positive groups (p=0.108).
Table 9: contingency table Breath test vs Saliva

<table>
<thead>
<tr>
<th></th>
<th>Saliva</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td><strong>Breath</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Count</td>
<td>16</td>
<td>14</td>
</tr>
<tr>
<td>Estimated</td>
<td>19,0</td>
<td>11,0</td>
</tr>
<tr>
<td>Count</td>
<td>22</td>
<td>8</td>
</tr>
<tr>
<td>Estimated</td>
<td>19,0</td>
<td>11,0</td>
</tr>
<tr>
<td>Total</td>
<td>38</td>
<td>22</td>
</tr>
<tr>
<td>Estimated</td>
<td>38,0</td>
<td>22,0</td>
</tr>
</tbody>
</table>

T-Student test for independent variables was conducted for every quadrant in comparison with group variables of the Breath Test (group A vs group B).

There was not any statistically significant difference as regards the presence of HP in saliva among the two groups of patients (p=0,604; p=0,326; p=0,368 and p=0,731).

Afterwards, the statistical analysis focused on the presence of *Helicobacter pylori* in saliva and periodontal pockets following a new grouping based on the periodontal health conditions.

The *mean probing depth* was calculated for every patient and a patient was considered to be **healthy** from a periodontal point of view when their mean probing depth was **under 3**, while he/she was to be **affected** by periodontal disease when his/her mean probing depth was >=**3**.
31 patients tested **negative** for periodontal disease (**healthy**), while on the contrary 29 tested **positive** (**unhealthy**). This grouping procedure did not take into account the results from Breath Test.

![Plot 14: periodontal classification resulted in two homogeneous groups (negative and positive) in an independent pattern from CBT-U test](image)

The mean load of HP registered among the periodontally healthy patients (negative group) was 10,52 with SD 16,51, while it was 22,78 with SD 55,96 among affected patients (positive group).
Table 10: HP mean load in periodontally healthy vs affected patients.

QM.caric = mean load; Qm.n = probing depth

The result was significant, demonstrating thus a correlation between periodontal disease (mean probing depth probing >= 3mm) and the presence of HP in periodontal tissues.

Another statistical examination was conducted in order to verify if there was any difference within the group of periodontal patients subdivided into positive and negative to Breath test.

There was not any statistical significant difference (p=0.305).

The last analysis was conducted between periodontal patients and healthy patients as regards Helicobacter pylori’s load in the saliva.

There was a significant difference between the two groups: periodontal patients showed an increased level of HP in saliva compared with healthy patients (Chi-square p=0.05)
4.5 DISCUSSION

The current study represents a preliminary approach to the role of oral *Helicobacter pylori* (HP) in the relapses of gastric infection.

60 patients were examined, subdivided into two groups of 30 individuals each according to the Breath test results: 30 were positive (group A) and 30 were negative (group B).

Every patient underwent a thorough oral examination by a dental clinician.

A paper cone was inserted for at least half a minute into the gingival sulcus near the dental element with the deepest probing depth. In addition, a salivary sample was collected with the spitting method.

The PCR-RT technique allowed to evaluate the quantitative presence of HP in the oral cavity.

Even though there was not any statistically significant difference among the two groups (A vs B) as regards the total amount of HP in saliva (t-Student p=0.372; chi-square p=0.108) or in periodontal tissues (I quadrant p=0.604; II quadrant p=0.326; III quadrant p=0.368; IV quadrant p=0.731) it should be considered that Breath test and PCR-RT in oral cavity are two completely different methods of bacterial detection.

The first one measures urease activity and distinguishes between positive and negative results, while the second one is quantitatively more accurate in detecting bacterial cells but requires a sample of liquid near the HP reservoir, a more invasive procedure for gastric mucosae since it needs esophagogastroduodenoscopy.
Besides, performing Breath test and PCR-RT for gastric and oral mucosa evaluation respectively does not allow to determine a causal factor, since these tests provide a picture of two anatomical districts at the same moment with two different methods.

This was also confirmed by the comparison between group A and group B patients within the group of the only periodontal unhealthy patients (p=0.305).

On the contrary, there was an interesting result as to the behaviour of HP in the oral cavity in relation to periodontal condition.

Patients were subdivided into other two groups according to their periodontal health: if the mean probing depth was below 3 mm they were considered to be periodontal healthy, while if it was equal or greater than 3 mm they were classified as affected by periodontal disease.

Comparing the mean amount of HP in periodontal pockets within the two groups, periodontal patients showed higher values, confirming that HP is present in the oral cavity and that it is not related to gastro-esophageal reflux or stomach load, like some authors have claimed, but it has an active role in the affected periodontal tissues.

Periodontal disease represents a threatening disease for oral tissues, since it progressively destroyes the anchorage of dental elements to the alveolar bone through the formation of the so-called periodontal pockets, a reservoir of high dose of colonizing bacteria of different species, among which the most dangerous are Porphyromonas gingivalis, Aggregatibacter actinomycetemcomitans, Tannerella forsythensis, Treponema denticola, Fusobacterium nucleatum and Campylobacter rectus.

Recent findings in the literature show how there is an immunological relationship between Helicobacter pylori and Campylobacter rectus [107], and clearly indicate
that one of the shared antigens is a GroEL protein; the biological activity of this protein might play a role in the onset and progression of periodontal disease. Furthermore, the microaerophilic and acid environment of the periodontal pocket represents a suitable context for growth and proliferation of *Helicobacter pylori*. Considering that *Helicobacter* was originally called *Campylobacter*-like bacterium, it could be advisable to consider it as a colonizing bacterium of the periodontal tissues when they are affected by the periodontal disease and when a biofilm with different species can increase the efficacy of the single species thanks to gene exchanges and signaling pathways among the different types of bacteria.

HP in the periodontal tissues is not confined to periodontal pockets, but migrates from the gingival sulcus into saliva, as it was demonstrated by comparing the presence of the bacterium in the salivary fluid between periodontally healthy and unhealthy patients (p=0.05).

Therefore, periodontal disease provides a suitable environment for HP. Periodontal pockets are the favourable site where the bacterium can thrive in synergy with other bacteria. Besides, once the bacterium has gained enough load, it migrates from the sulcus into the oral cavity and enters saliva. Since saliva is continuously swallowed and descends into the stomach, the oral cavity represents an extra-gastric reservoir of *Helicobacter pylori* for the stomach.

This preliminary study demonstrated that the oral cavity is an extra-gastric reservoir of *Helicobacter pylori* when it is affected by periodontal disease. The increased load of HP detected in periodontal disease is not confined to periodontal pockets, but the bacterium migrates from the gingival sulcus into the saliva and is transported to the stomach through swallowing.

There is no statistical significance instead between the results of the Breath test and the PCR-RT carried out in the oral cavity, because they are two completely different methods with different sensibility and specificity and represent a picture at the
same moment of two different anatomical districts. However, it can be deduced that positivity for gastric infection is not linked to an increased amount of *Helicobacter* in the oral cavity.

This preliminary study is undergoing a second step through a perspective study to better understand whether oral *Helicobacter pylori* can induce gastric infection relapses after eradication therapy.

Patients who are negative for gastric infection and positive for oral infection will undergo evaluation through the Breath test and for oral infection with PCR-RT after 6 months. If patients with a relapse are statistically associated to an increased oral *Helicobacter pylori* load when compared with a group of naive patients, it will be possible to establish the true role of the oral extra-gastric bacterium in gastric infection recurrence, which is a worldwide growing concern.
5. **4th STUDY: RHEUMATOID ARTHRITIS THERAPIES AND PERIODONTAL DISEASE: ANY CORRELATION?**

5.1 **BACKGROUND**

**Rheumatoid Arthritis (RA)** is a systemic, chronic disease which has a multifactorial aetiology and an immunomediated pathogenesis. It affects several tissues and organs, such as the skin, blood vessels, heart, lungs and muscles, but joints are the mainly affected site. At this level the disease is associated with the onset of a non-suppurative proliferative synovitis. Joints are typically affected bilaterally in a symmetric pattern. The disease, if untreated, leads to the destruction of joint cartilages, bone erosion and ankylosis [108].

5.1.1 **History**

In 1800 Augustin Jacob Landré-Beauvais, in his doctoral dissertation, described the disease as a “primitive asthaenic gout” [109]. It was widespread especially among women, affected multiple joints, showed a chronic behaviour and led to joint stiffness and deformity. It should be considered that gout was the most common articular disease at that time, and that all other rheumoarthropaties were compared with this disease.

Jean-Martin Charcot pointed out the typical night pain, the onset of the disease within the small distal joints and described the alternance of flare-up and remission periods. Finally, he called the disease “primary chronic rheumatism”.

Sir Alfred Baring Garrod introduced the name of “Rheumatoid Arthritis” in 1859.
Disease prevalence in western countries has only recently risen, as described by paleoanthropological studies [110].

5.1.2 Epidemiology

About 1% of the worldwide population is affected by RA.

Subsaharian and Caribbean black people appear to be less involved. The female to male ratio is 3:1, even though this difference is reduced among the elderly.

The mean age ranges between 40 and 70 years, with two peaks in young adults and in pre-menopausal women [111].

5.1.3 Aetiology

The disease is idiopathic. Rheumatoid Arthritis is a disease which can be explained by an autoimmune response after exposition to an unknown antigen in a genetically predisposed individual [112].

Mycoplasma, Epstein-Barr Virus, Cytomegalovirus, Parvovirus and Rubivirus have been taken into account, but the scientific literature is lacking in data. However, it seems that the molecular mimicry caused by infective agents could be the underlying cause of the disease, likewise in other autoimmune diseases. Therefore, HLA-DR4 or HLA-BDR1 haplotypes are associated with the disease [113].

5.1.4 Pathogenesis

The autoimmune response, which leads to the activation of T-helper cells and other lymphocytes and the production of cytokines, destroys joint architecture.
The first phase is characterized by the presence of monocytic-macrophagic cells just below the basal layer of the synovial membrane. At this time, IL-1, IL-6 and TNF cytokines are increased.

Following activation of T-helper cells positive for CD154 leads to the polyclonal proliferation of B-lymphocytic cells and the production of autoantibodies. The increased production of cytokines and the recruitment of inflammatory cells is associated with an increased level of RANKL and osteoclastic activity [114]. Thus, bone resorption follows joint cartilage destruction.

### 5.1.5 Clinical features

The clinical course of the disease is variable. Over 50% of patients show an insidious and slow onset of the disease with malaise, fatigue, anorexia and diffused muscular pain before the involvement of joint cartilages [115]. These symptoms may lead to an early diagnosis. On the contrary, about 10% of patients show an acute onset with severe symptoms and the early involvement of multiple joints. In these cases, fever, lymphoadenophaty and splenomegaly accompany the clinical onset of the disease.

RA usually involves the small distant joints most frequently those of the hands and feet, with a symmetrical pattern.

Joints appear swollen and painful. Stiffness, especially in the morning, is evident.

The clinical progress of the disease can be rapid or slow and patients usually go through alternate periods of partial/complete remission and of flare-up. Over time, this chronic condition leads to the impairment of function and deformity, especially of the hands [116].

Extra articular manifestations include several tissues and organs and are usually linked to the intensity of the disease activity [117].
- *Rheumatoid nodules* are asymptomatic lesions containing necrotic tissue and macrophagic infiltration. They are usually found in periarticular structures or in those areas which undergo mechanical pressure, sometimes in pleura and meninges.

- *Rheumatoid vasculitis* is associated with an increased level of circulating Rheumatoid Factor. It can develop anywhere and result in polyneuropathies, cutaneous ulcers, visceral infections and finger canker.

- *Pleuropulmonary manifestations* include pleuritis, interstitial fibrosis and nodules. They are more frequent in males.

- *Neurological symptoms* are related to the entrapment of nerve trunks within the tissutal destruction around joints, especially in hands and in vertebral column due to subluxation. Ulnar, median and anterior tibial nerves are those generally involved.

- *Ocular signs* usually include episcleritis and scleritis, even though they usually affect less than 1% of individuals.

- *Oral symptoms and signs* are xerostomia, Temporo-Mandibular Joint dysfuntion, ulcers due to the administration of metothrexate,
increased predisposition to periodontal disease. Sometimes Sjögren’s Syndrome is associated to Rheumatoid Arthritis.

- **Felty’s syndrome** is a form of chronic RA accompanied by splenomegaly, neutropenia, anaemia and thrombocytopenia sometimes. It usually arises in individuals with long-lasting disease and leads to an increased susceptibility to infections.

- **Osteoporosis** is frequent and is mainly due to the prolonged steroid therapy.

- **Lymphoma**, mainly the large cell B lymphoma, is more frequent in patients with Rheumatoid Arthritis, when the polyclonal proliferation degenerates into a monoclonal one.

### 5.1.6 Risk Factors

Several studies suggest the presence of intrafamiliar genetic predisposition, especially within direct line [118]. Over 70% of patients are positive for HLA-DR4 [119], while others show alterations within the genes for cytokines and TNF.

Among cytokines, **Interleukine-6** plays an important role, since an uncontrolled overproduction may lead to the onset of severe chronic disease with autoimmune response. Some papers showed that the use of a monoclonal antibody directed against IL-6 receptor is effective in the treatment of RA.

**Vitamin D receptor gene** is also involved, especially as regards accompanying osteoporosis and pathological fractures [120].
Among environmental factors smoke is considered the most influencing one in the epidemiology of Rheumatoid Arthritis.

5.1.7 Diagnosis

There is usually a delay of 9 months between the onset of the first symptoms and final diagnosis. This occurs because of the non-specificity of early complaints. When the inflammatory involvement of joints concerns the small and distal joints bilaterally and the vertebral column is not affected, a strong suspicion of RA can arise.

Stiffness in the morning and chronic fatigue, accompanied by the presence of subcutaneous nodules, are characterizing features of the disease.

In 1987 the American College of Rheumatology developed Revised Criteria for the classification of Rheumatoid Arthritis [121].

These criteria provide a sensitivity rate of 94% and a specificity rate of 89%. The final diagnosis is made considering clinical features, radiographic imaging, blood chemistry and synovial liquid examination.
Serum values are important indicators for disease activity and appear as prognostic elements to distinguish between aggressive and non-aggressive forms and to determine the response to treatment.

- High levels of ESR and CRP indicate the presence of inflammation. CRP, especially, is connected with disease severity and appearance of lesions [122].
- Rheumatoid Factor (RF) is an autoantibody directed against the Fc portion of IgG antibodies and is expressed in 60-70% of patients with RA. It is not a highly specific antibody, since it can also be found in Cryoglobulinemia, Sjögren’s Syndrome, chronic hepatopathies. However, it is a valuable prognostic factor, since it is high when RA appears aggressive and with an erosive clinical evolution.
- **Anti-citrullinated protein antibodies (CCP)**, on the contrary, are highly specific and sensible. They are characteristically expressed in Rheumatoid Arthritis, so their value is both diagnostic and prognostic [123].

The **synovial liquid examination** is a routine test which is performed to distinguish between inflammatory and mechanical arthritis or between infective or microcrystal arthritis.

Traditional **radiographic imaging** is still the gold-standard test to evaluate the joint damage over time and also appraise damage to bones due to erosion.

**Ecography** could be performed to obtain a dynamic evaluation of the joint function, but it is still too much influenced by the operator’s skills.

Magnetic Resonance, instead, is homogeneous and can be read by different operators, but it is expensive and less accessible.

In 1970 the epidemiologist Alvam Feinstein introduced the term “Clinicometrics” to better standardize the clinical evolution of symptoms and signs in a chronic disease.

The **DAS28** is a measure of disease activity in Rheumatoid Arthritis (RA). DAS stands for ‘Disease Activity Score’ and the number 28 refers to the 28 joints examined in this assessment [124].

There is a wide range of measures to assess disease activity in RA including:

- examination of joints for swelling and tenderness,
- global scores of pain and overall status,
- blood markers of inflammation (e.g. ESR and CRP),
- questionnaires (e.g. the HAQ),
- X-rays, and newer imaging techniques such as ultrasound and MRI.

The DAS28 is a composite score derived from 4 of these measures. This ‘28’ version is a simplification of the original DAS score, which required 44 joints to be counted.
Other versions of DAS28 allow CRP to be used instead of ESR, or the omission of either. To calculate DAS28 the rheumatologist or a specialist nurse:

1. counts the number of swollen joints (out of the 28),
2. counts the number of tender joints (out of the 28),
3. takes blood to measure ESR or CRP,
4. asks the patient to make a ‘global assessment of health’ (indicated by marking a 10 cm line between very good and very bad).

These results are then fed into a complex mathematical formula to produce the overall disease activity score. A DAS28 of more than 3.2 implies **active disease**, less than 3.2 **low disease** activity, and less than 2.6 **remission**.

**Fig.32: DAS-28 score system to assess Rheumatoid Arthritis clinical activity**

Extra articular manifestations are taken into consideration as prognostic factors of disease severity.
5.1.8 Therapy

Therapy requires a multidisplinar approach which involves the rheumatologist, the physiatrist, the orthopedist, the psycologist and the dentist. After making a diagnosis and assessing disease activity the choice of treatment must be patient-tailored.

Drugs are commonly used as palliative therapy in order to keep the inflammation scores at low levels [125].

NSAIDs are commonly used to treat pain or acute inflammation, while steroids are used to control inflammation, even though they seem to be linked to a delay in bony erosion onset.

However, NSAIDs and steroids are not capable of delaying disease progression.

Disease Modifying Anti-Rheumatic DRUGS (DMARDs) are a class of drugs which reduce the level of reactants in the acute phase and modulate the inflammatory components of the disease. They have a beneficial effect only after several weeks or months. Gold compounds, antimalarials, sulfalazine, penicillamine and especially methotrexate belong to this group.

Methotrexate is a folic acid antagonist, it hinders the nucleic acids synthesis and stops the proliferation of several cell types, among which the inflammatory cells. Adverse effects include leukopenia, thrombocytopenia, anaemia, haemorrhages, myelosuppression, gastroenteric ulcers, stomatitis, dyarrhea.

Biological drugs are inhibitors of cytokines production. They usually are antibodies directed against a molecule or a receptor and are characteristically associated with less side effects since they aim at more specific targets. They are usually composed of big-sized molecules and can’t be administered per os. Etanercept, infliximab,
adalimumab are the most used drugs. **Etanercept** is a biopharmaceutical that treats autoimmune diseases by interfering with Tumor Necrosis Factor (TNF; a soluble inflammatory cytokine) by acting as a TNF inhibitor.

It reduces the effect of naturally present TNF, functioning as a decoy receptor that binds to TNF [126].

Tumor Necrosis Factor-alpha (TNF-α) is a cytokine produced by lymphocytes and macrophages, two types of white blood cells. It mediates the immune response by attracting additional white blood cells to sites of inflammation, and through additional molecular mechanisms which initiate and amplify inflammation. Inhibition of its action by Etanercept reduces the inflammatory response which is especially useful for treating autoimmune diseases.

There are two types of TNF receptors: those found embedded in white blood cells that respond to TNF by releasing other cytokines, and soluble TNF receptors which are used to deactivate TNF and blunt the immune response. In addition, TNF receptors are found on the surface of virtually all nucleated cells. Etanercept mimics the inhibitory effects of naturally occurring soluble TNF receptors, the difference being that etanercept, because it is a fusion protein rather than a simple TNF receptor, has a greatly extended half-life in the bloodstream, and therefore a more profound and long-lasting biologic effect than a naturally occurring soluble TNF receptor.

**Immunosuppressants** and cytotoxic drugs are used when DMARDs or biopharmaceuticals fail in the treatment of RA. Chloroquine, hydroxychloroquine, cyclosporine, azathioprine and cyclophosphamide belong to this group.

**Folic acid** is frequently prescribed to hinder the low absorption of the element due the intake of methotrexate, while **vitamin D** is given to reinforce bones and because a high index of bone resorption is associated with greater disease severity.
Patients with Rheumatoid Arthritis need following for physical exercise in order to move the affected Joints without increasing the inflammatory indices.

Surgery, such as arthroplasty or joint substitution, is performed, while synovectomy does not seem to produce long-lasting results.

5.1.9 Periodontal Disease

With tooth decay Periodontal disease (PD) is one of the two most common diseases of the oral cavity. It affects about 80% of the world population. The disease involves the supporting periodontal tissues which include the gums, periodontal ligament and alveolar bone. The final evolution of the disease is the loss by exfoliation of dental elements. Genetic predisposition may play an important role in determining whether an individual can be affected, but dental plaque is the causal agent. Periodontal disease may have several clinical forms, but chronic periodontitis is the most widespread among adults. It can affect individuals of any age, but prevalence increases with age. It can be confined to a single group of teeth or affect the entire mouth [127].

Sick gingiva appears as erythematous, edematous and painful; the presence of periodontal pockets represents a reservoir of infection which leads to dental mobility and loss. Gingival recession, furcation exposure in the oral cavity and dental migration may also occur as a consequence of the loss of periodontal attachment loss.

Gingivitis is the initial form of the disease; it represents a reversible stage in which tissue destruction does not occur yet. Some cases of gingivitis can advance to a more severe condition, periodontitis. Periodontitis is the non-reversible destruction of periodontal ligament and alveolar bone and is always preceded by gingivitis.
The worsening of the condition or its clinical course depend on the host immunitary defenses.

Chronic periodontitis may only affect a localized region or the whole mouth.

The prerequisite for the onset of the disease is the presence of a supragingival and then subgingival bacterial biofilm around the dental element and inside the gingival sulcus.

Risk factors may worsen the course of the disease. These factors are occlusal trauma, bad habits, oral respiration, atypical deglutition, poor hygienic oral conditions, dental malposition, dental abnormalities, low adherent gingiva, ill-fitting dental restorations, orthodontic appliances, smoke, systemic diseases, drugs [128].

From 1890 to 1930s periodontitis was believed to be a specific infection caused by fusiform bacteria, amoebas, spirochetes and streptococci.

From 1930 to the end of 1960s periodontitis was held to be a non-specific infection caused by the presence of dental plaque. From 1960 the theory of specific organisms responsible for the disease has drawn the attention of scientists and several bacterial species as potentially causal agents have been investigated.

It should be underlined that dental plaque contains more than 300 different bacterial species, but only a few have been associated with a high risk of developing periodontal disease.

The link between biofilm bacteria and periodontitis is not random. Six species have been found to be strictly involved and responsible for the clinical course of the disease: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis* [129], *Tannerella forsythensis*, *Treponema denticola* which belong to the so-called “red-complex” and *Fusobacterium nucleatum* and *Campylobacter rectus*, which belong to the “orange-complex”.
Fig. 33: microbial complexes in subgingival biofilm

However, bacterial infection is a necessary factor but it is not sufficient for the onset of the disease, since host immunologic status and local environment are important too.

Dental plaque accumulation triggers a reaction in the junctional epithelium and epithelial sulcus, which produce proinflammatory cytokines and inflammation mediators. Gingivitis follows the inflammatory response, with oedema and PMN migration in the sulcus due to the chemotactic gradient. PMN accumulation and degradation within the sulcus results in tissue damage and epithelial regeneration which migrates apically with consequent bony loss.

Diagnosis requires clinical, instrumental and radiographic evaluation.

Clinical and instrumental criteria are bleeding on probing, depth probing, clinical attachment level, furcation involvement, teeth looseness.
The different parameters are grouped into several recognized indices which can determine the presence or absence of periodontal disease.

Once a thorough periodontal examination and diagnosis have been performed, aetiological therapy is imperative.

Firstly, professional dental cleaning (scaling) removes dental plaque from the tooth-sites and gingival sulcus.

Afterwards, when periodontal pockets are present, debridement of subgingival plaque is performed through curettage, known as root planing.

If aetiological therapy is not sufficient, the dental clinician can remove infection surgically from those areas too deep to be reached during root planing procedures.

Chlorexidine digluconate 0,2% mouth rinses can improve the efficacy of aetiological therapy, but they can’t be used for more than two weeks.

**5.1.10 Genetic predisposition to Periodontal Disease**

The hypothesis of a genetic predisposition in Periodontal Disease was firstly formulated in 1935.

The discovery that genetics influences the development of Periodontal Disease dates back to 30 years ago [130]. This new evidence has shown that PD, like other diseases (diabetes, cancer, etc.), requires a genetic susceptibility to develop.

Individuals respond differently to the attack of the oral microflora according to their genetic predisposition. Furthermore, genetics determines how each person interacts with environmental factors (such as oral biofilm) in the onset of PD. The relationship between genetic and environmental factors determines the onset of PD. Lifestyle (smoke and poor oral hygiene) affects the onset and progression of PD,
but alone it is not able to cause it [131]. The probability of developing PD related to heredity is about 50%. Clinical studies have shown that genetic factors are jointly responsible with environmental factors and lifestyles for the development of PD [132].

Genetic susceptibility to multifactorial diseases is usually due to several gene polymorphism instead of a single, or few, gene mutations. Common variation in the genetic code may result in an altered expression or in functional changes of the encoded proteins, therefore in an increased disease severity.

Recently, research into susceptibility factors of periodontitis has mainly focused on genes that modulate immune regulation, such as cytokines, chemokines, cell-surface receptors, enzymes and proteins related to antigen recognition. Cytokines, such as IL-1α, IL-1β, IL-10 and IL-6, are key factors which mediate the inflammatory process during periodontitis progression. They have a role in activation, proliferation and differentiation of B cells which are the majority of infiltrating cells in advanced periodontitis lesions. Therefore, common variations in the genetic code can alter the progression of disease because they may be responsible for the repeated cycles of tissue inflammation [133].

In Periodontal Disease, the microbiota accumulated in the subgingival region is the environmental factor which influences the inflammatory response in periodontal tissues. However, cytokines contribute to connective tissue destruction and bone resorption.

Another factor associated with bone resorption in periodontal disease is Vitamin D Receptor (VDR), which has been regarded as a periodontitis susceptibility factor. Recent articles have reported a review of scientific literature as to the genetic association analysis between common polymorphism of candidate genes and periodontitis [134]. Vitamin D Receptor expression may be linked to bone pathology following periodontal destruction.
Interleukin-1 genes (IL-1) are pro-inflammatory agents, since IL-1 is associated with inflammation, bone resorption. TNF-α is also linked to an increased degree of inflammation within periodontal tissues. IL-1 and TNF-α are also associated with Rheumatoid Arthritis, thus these two diseases can be linked on the basis of the role played by pro-inflammatory cytokines could be formulated.

IL-10 is considered to be an antinflammatory cytokine which modulates the activity of IL-1 and TNF-α.

A recent paper has underlined the presence of Interleukin-6 gene methylation both in Rheumatoid Arthritis and Periodontal Disease [135].

5.2 AIM

The current study aims at evaluating the correlation between Rheumatoid Arthritis (RA), with special attention to therapy, and Periodontal Disease (PD).

5.3 MATERIALS AND METHODS

34 patients with Rheumatoid Arthritis were recruited into the current study.

The study was conducted in the Unit of Rheumatology, Ospedale di Gallarate, VA, Italy.

Patients were subdivided into three groups: Group 1 included 13 patients treated with TNF-α biological inhibitors (Etanercept), Group 2 included 14 patients treated with Methotrexate and 7 patients were included in Group 3 as control subjects. The control group consisted of patients affected by Rheumatoid Arthritis but not treated with any drug.
Exclusion criteria concerned patients who were partially or completely edentulous.

The experimental protocol has been evaluated by the Institutional review Board, reference number 127/2016.

The rheumatologist collected data about disease severity and laboratory findings. Among haematochemical analysis, *Rheumatoid Factor (RF)*, *anti-citrullinated protein antibody (CCP)* and *HLA-BDR1* were prescribed. The *DAS28* questionnaire was completed by the physician in order to measure the clinical scores of disease activity.

A dental clinician carried out a periodontal examination, collecting data from periodontal probing with special attention to *Depth probing*.

Afterwards, 1 paper cone was inserted for 30 seconds into the gingival sulcus near the dental element with the deepest probing value registered for every quadrant and then sent to the laboratory for evaluation in a sterile test tube.

Specimens were processed to extract and purify DNA by using a method that includes two consecutive incubations with lysozyme and proteinase K, in order to ensure an indiscriminate Gram positive and negative bacterial lysis. Once extracted, DNA was purified through a silica spin-column (Sigma-Aldrich, St. Louis, MO, USA).

Quantitative PCR of 16S rRNA genes was performed with the hydrolysis probes method to identify and evaluate the amount of 5 bacterial species: *Porphyromonas gingivalis, Tannerella forsythensis, Treponema denticola, Fusobacterium nucleatum* and *Campylobacter rectus*.

The 845 sequences of 16S rRNA gene of the Human Oral Microbiome Database (HOMD 16S rRNA RefSeq Version 10.1) were aligned to find either a consensus sequence or less preserved spots, useful to optimize the specificity of primers and dual labelled hydrolysis probes. PCR oligonucleotide sequences were designed by
Primer3web (http://primer3.ut.ee/) and Primer Express (Life Technologies) software. The specificity of PCR assays was also checked by Primer-Blast (http://www.ncbi.nlm.gov/tools/primer-blast/). Absolute quantification assays were performed by using a 7500 Sequence detection System (Applied Biosystems).

The thermal cycle included 10 min incubation at 95°C to activate polymerase, followed by a two-step amplification of 15 s at 95°C and 60 s at 57°C for 40 cycles. Each experiment included non-template controls to exclude reagents contamination and serial dilutions of the specific synthetic template (Eurofin MWG Operon, Ebersberg Germany). These positive controls were used to plot standard curves, i.e threshold cycle values against the log of the copy number, that were used either to check amplification efficiency and for quantification of targets in each sample.

Data from quantitative PCR included the amount of each of the 5 investigated bacterial species and a measurement of the bacterial load from up to 34 patients.

These amounts matched the number of DNA molecules detected in the real time PCR tubes that was directly related to the number of bacteria in the specimens. In order to enhance data analysis with noise removal, statistical analysis was performed on relative amounts, calculated as ratios between the amount of each species and the total bacterial load. This was able to reduce variability due to random factors such as specimen conservation, DNA extraction efficiency, and purification yield, as well as variability due to systematic factors, for instance the higher amount of bacteria expected in specimens from deeper pockets characterizing periodontitis.

Samples for periodontal tissues were also used to assess genetic polymorphism in IL-6, IL-10 and VDR [136].

Comparative statistics among the different groups was performed using the IBM SPSS version 20.0, SPSS Inc, Chicago, IL, USA.
5.4 RESULTS

A total number of 34 patients affected by Rheumatoid Arthritis were recruited in the current study, 24 females and 10 males. 13 patients were undergoing therapy with Etanercept, an anti TNF-α biopharmaceutical; 14 patients were undergoing therapy with Methotrexate, an immunosuppressant, and 7 did not follow any therapeutic regimen (Control group).

![Sex distribution](image)

Plot 15: sex distribution among the 34 recruited patients

![RECRUITED PATIENTS](image)

Plot 16: composition of Group 1 (Etanercept), Group 2 (Methotrexate) and Group 3 (Control)
Firstly, comparative analysis was performed to verify any correlation between genetic predisposition to Periodontal Disease and Rheumatoid Arthritis laboratory findings, both clinical and genetic.

**IL-6** was regarded as predisposing to Periodontal Disease when genotype GG of the polymorphism rs1800795 (G/C) was expressed, while genotypes GC and CC were considered to be protective against Periodontal Disease.

3 patients expressed the protecting genotype, while 31 expressed the predisposing one.

**IL-10** was considered predisposing to Periodontal Disease when genotypes AA and AC of the polymorphism rs1800872 (A/C) were expressed, while genotype CC was considered to be protective against the disease.

12 patients expressed the protecting genotype, while 22 expressed the predisposing one.

**VDR** was considered as predisposing to Periodontal Disease when genotype TC of the polymorphism rs731236 (T/C) was expressed, while genotypes TC and CC were considered protective against the disease.

<table>
<thead>
<tr>
<th></th>
<th>PREDIPOSING</th>
<th>PROTECTING</th>
</tr>
</thead>
<tbody>
<tr>
<td>INTERLEUKIN-6 GENOTYPE</td>
<td>31</td>
<td>3</td>
</tr>
<tr>
<td>INTERLEUKIN-10 GENOTYPE</td>
<td>22</td>
<td>12</td>
</tr>
<tr>
<td>VIT D RECEPTOR GENOTYPE</td>
<td>28</td>
<td>6</td>
</tr>
</tbody>
</table>
Rheumatoid Factor (RF) was positive in 21 patients and negative in 13 ones.

Anti-citrullinated protein antibody (CCP) was positive in 19 patients and negative in 15 patients.

**DAS-28** ruled out 17 patients with a score of 1 (x< 2.6), 9 with a score of 2 (2.6<x<3.2) and 8 with a score of 3 (x>3.2); class 2 and 3 were united in a unique class so that the division was between 17 patients with **absent** disease activity and 17 with **higher** scores of disease.

**HLA-BDR1** was registered in 10 patients while it did not appear in the remaining 24.

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>CCP</th>
<th>DAS-28</th>
<th>HLA-BDR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>POSITIVE</td>
<td>21</td>
<td>19</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>NEGATIVE</td>
<td>13</td>
<td>15</td>
<td>17</td>
<td>24</td>
</tr>
</tbody>
</table>

Non-parametric Chi-square analysis was performed to compare predisposing and protecting genotypes of Interleukin-6 (IL-6) with Rheumatoid Factor (RF), anti-citrullinated protein antibody (CCP), clinical assessment of Rheumatoid Arthritis DAS-28 and HLA-BDR1 haplotype.

There was not any statistically significant difference when IL-6 was compared with RF, CCP and HLA-BDR1, even though it should be considered that most patients expressed a predisposing genotype and the two groups were not balanced.
- IL-6 vs RF p=0,22;
- IL-6 vs CCP p=0,16;
- IL-6 vs DAS-28 p=0,11;
- IL-6 vs HLA-BDR1 p=0,66.

The same analysis was performed to compare genotypes of Interleukin-10 (IL-10) with Rheumatic parameters.

There was not any significant difference between

- IL-10 vs RF p=0,06
- IL-10 vs DAS-28 p=0,36
- IL-10 vs HLA-BDR1 p=0,22

On the contrary, it seems that IL-10 and CCP are associated in an opposite pattern: when IL-10 expresses protecting genotypes against periodontitis, CCP may be increased, while when predisposing genotypes are expressed, CCP is decreased.

IL-10 vs CCP p=0,02

**Table 13: contingency table IL10 vs CCP**

<table>
<thead>
<tr>
<th></th>
<th>CCP</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>IL10</td>
<td>Protecting</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Predisposing</td>
<td>13</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>15</td>
</tr>
</tbody>
</table>
**VDR** results were compared with those of RF, CCP, DAS-28 and HLA-BDR1.

There was not any significant difference in the Chi-square analysis.

- VDR vs RF $p=0.24$
- VDR vs CCP $p=0.45$
- VDR vs DAS-28 $p=0.33$
- VDR vs HLA $p=0.42$

Table 14: results from comparative statistical analyses are reported. A significant connection was found between IL-10 and CCP

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>CCP</th>
<th>DAS-28</th>
<th>HLA-BDR1</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>0.22</td>
<td>0.16</td>
<td>0.11</td>
<td>0.66</td>
</tr>
<tr>
<td>IL-10</td>
<td>0.06</td>
<td><strong>0.02</strong></td>
<td>0.36</td>
<td>0.22</td>
</tr>
<tr>
<td>VDR</td>
<td>0.24</td>
<td>0.45</td>
<td>0.33</td>
<td>0.42</td>
</tr>
</tbody>
</table>

Non-parametric statistical analysis was performed to compare *periodontal genetic indices* among them.

- IL-6 vs VDR $p=0.07$
- IL-10 vs VDR $p=0.35$

There were not differences with the exception of IL-6 vs IL-10, which turned out significative ($p=0.014$). When the individual is genetically predisposed to
periodontal disease in IL-6 genes, also IL-10 seem to be correlated and both cytokines express predisposing genes.

Table 15: contingency table IL6 * IL10

<table>
<thead>
<tr>
<th></th>
<th>IL10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protecting</td>
<td>Predisposing</td>
</tr>
<tr>
<td>IL6</td>
<td>Prot</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Pred</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>12</td>
</tr>
</tbody>
</table>

Table 16: comparative analysis limited to genetic periodontal indices. A significant correlation was found between IL-6 and IL-10

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-10</th>
<th>VDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>Not applicable</td>
<td><strong>0.014</strong></td>
<td>0.07</td>
</tr>
<tr>
<td>IL-10</td>
<td><strong>0.014</strong></td>
<td>Not applicable</td>
<td></td>
</tr>
<tr>
<td>VDR</td>
<td>0.07</td>
<td>0.35</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>

Comparing Rheumatic factors among them showed strict correlations within the population, according to data from the international literature.
DAS-28 is a parameter which results from both laboratory findings and clinical evaluation of the disease. Thus it is less associated with rheumatic factors than the others do.

Rheumatoid Factor and HLA-BDR1, in particular, do not show significant differences when compared to DAS-28.

Table 17: comparative analyses limited to rheumatic parameters

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>CCP</th>
<th>DAS-28</th>
<th>HLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>Not applicable</td>
<td>0.00</td>
<td>0.14</td>
<td>0.00</td>
</tr>
<tr>
<td>CCP</td>
<td>0.00</td>
<td>Not applicable</td>
<td>0.04</td>
<td>0.00</td>
</tr>
<tr>
<td>DAS-28</td>
<td>0.14</td>
<td>0.04</td>
<td>Not applicable</td>
<td>0.74</td>
</tr>
<tr>
<td>HLA</td>
<td>0.00</td>
<td>0.00</td>
<td>0.74</td>
<td>Not applicable</td>
</tr>
</tbody>
</table>
CCP appears as the only rheumatoid arthritis parameter which is more associated to other factors.

**Sex** was compared to periodontal genetic predisposition and rheumatic parameters.

IL-10 predisposing gene is expressed more in males than females (p=0.05)

Sex vs IL-6 p=0.34

Sex vs IL-10 p=0.046

Sex vs VDR p=0.58

Table 18: contingency table SEX vs IL-10

<table>
<thead>
<tr>
<th></th>
<th>IL10</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Protecting</td>
<td>Predisposing</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>M</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>Total</td>
<td>12</td>
<td>22</td>
</tr>
</tbody>
</table>
In addition, Rheumatoid Factor is more common among females than males.

Sex vs RF $p=0.02$

Sex vs CCP $p=0.056$

Sex vs DAS-28 $p=0.75$

Sex vs HLA-BDR1 $p=0.12$

**Table 19: comparative analyses between sex on one side and periodontal and rheumatic parameters on the other side**

<table>
<thead>
<tr>
<th></th>
<th>IL-6</th>
<th>IL-10</th>
<th>VDR</th>
<th>RF</th>
<th>CCP</th>
<th>DAS-28</th>
<th>HLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEX</td>
<td>0.34</td>
<td><strong>0.05</strong></td>
<td>0.58</td>
<td><strong>0.02</strong></td>
<td>0.056</td>
<td>0.75</td>
<td>0.12</td>
</tr>
</tbody>
</table>

**Microbiological analysis** was performed comparing the mean load of every bacterium with PSR and with Rheumatoid Arthritis parameters using the T-student parametric test for independent values.

Periodontal screening record (PSR) index was calculated for every patient. PSR is a clinical parameter which allows to distinguish between the presence and absence of periodontitis and its clinical severity. 22 patients show a PSR score below 3, while on the contrary 12 reported a PSR greater than 3.
Plot 17: 22 patients reported a PSR score $\leq 3$, while 12 showed a PSR score $> 3$

*Porphyromonas gingivalis* (PG), *Tannerella forsythensis* (TF), *Treponema denticola* (TD), *Fusobacterium nucleatum* (FN) and *Campylobacter rectus* (CR) mean loads were compared to the groups of patients individuated after PSR.

- PG vs PSR $p=0.09$
- TF vs PSR $0.88$
- TD vs PSR $p=0.25$
- FN vs PSR $p=0.241$
- CR vs PSR $p=0.41$

There were not any statistical difference in the composition of oral microbioma between the two groups, being the bacterial infection influenced by other factors and not by clinical scoring of periodontal disease.
Afterwards, **bacterial mean loads** were compared with **Rheumatic parameters** to check whether Rheumatoid disease itself or the activity of the disease may represent a risk factor of aggressive pathogens in periodontitis.

Rheumatoid Factor showed a positive correlation with the presence of *Tannerella forsythensis* and *Treponema denticola*, but not with *Porphyromonas gingivalis, Fusobacterium nucleatum* or *Campylobacter rectus*.

**Table 20: descriptive statistics RFvsTF**

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>N</th>
<th>Mean</th>
<th>St deviation</th>
<th>St error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TFMEAN</td>
<td>NEG</td>
<td>13</td>
<td>3001,5385</td>
<td>4786,3485</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>21</td>
<td>7929,9286</td>
<td>12468,76229</td>
<td>2720,91</td>
</tr>
</tbody>
</table>

**Table 21: descriptive statistics RFvsTD**

<table>
<thead>
<tr>
<th></th>
<th>RF</th>
<th>N</th>
<th>Mean</th>
<th>St deviation</th>
<th>St error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TDMEAN</td>
<td>NEG</td>
<td>13</td>
<td>8635,0000</td>
<td>11811,0645</td>
</tr>
<tr>
<td></td>
<td>POS</td>
<td>21</td>
<td>37832,0476</td>
<td>79611,7628</td>
<td>17372,71</td>
</tr>
</tbody>
</table>

There was a significant link between the anti-citrullinated protein antibody (CCP) and the presence of the red-complex bacteria: *Porphyromonas gingivalis, Tannerella forsythensis* and *Treponema denticola*. On the contrary, there was not relationship with the orange-complex bacteria *Fusobacterium nucleatum* and
Campylobacter rectus.

Table 22: descriptive statistics CCPvsPG

<table>
<thead>
<tr>
<th>CCP</th>
<th>N</th>
<th>Mean</th>
<th>St deviation</th>
<th>St error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGMEAN NEG</td>
<td>15</td>
<td>29131,03</td>
<td>55816,035</td>
<td>14411,64</td>
</tr>
<tr>
<td>POS</td>
<td>19</td>
<td>166058,24</td>
<td>410745,594</td>
<td>94231,50</td>
</tr>
</tbody>
</table>

Table 23: descriptive statistics CCPvsTF

<table>
<thead>
<tr>
<th>CCP</th>
<th>N</th>
<th>Mean</th>
<th>St deviation</th>
<th>St error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>TFMEAN NEG</td>
<td>15</td>
<td>2618,93</td>
<td>4545,14</td>
<td>1173,55</td>
</tr>
<tr>
<td>POS</td>
<td>19</td>
<td>8750,77</td>
<td>12855,93</td>
<td>2949,35</td>
</tr>
</tbody>
</table>

Table 24: descriptive statistics CCPvsTD

<table>
<thead>
<tr>
<th>CCP</th>
<th>N</th>
<th>Mean</th>
<th>St deviation</th>
<th>St error mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDMEAN NEG</td>
<td>15</td>
<td>7718,87</td>
<td>11217,22</td>
<td>2896,27</td>
</tr>
<tr>
<td>POS</td>
<td>19</td>
<td>41628,69</td>
<td>82958,91</td>
<td>19032,08</td>
</tr>
</tbody>
</table>
DAS-28 revealed a positive relationship with the presence of all bacteria of the red and orange complexes

- DAS-28 vs PG p=0.01
- DAS-28 vs TF p=0.02
- DAS-28 vs TD p=0.04
- DAS-28 vs FN p=0.01
- DAS-28 vs CR p=0.01

On the contrary, HLA-BDR1 Genetic predisposition to Rheumatoid Arthritis is not linked to the presence of aggressive periodontal bacteria.

- HLA vs PG p=0.73
- HLA vs TF p=0.91
- HLA vs TD p=0.64
- HLA vs FN p=0.48
- HLA vs CR 0.45

Table 25: summary of statistical analysis between rheumatic parameters and periodontal pathogens

<table>
<thead>
<tr>
<th></th>
<th>PG</th>
<th>TF</th>
<th>TD</th>
<th>FN</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>0.07</td>
<td>0.04</td>
<td>0.04</td>
<td>0.94</td>
<td>0.27</td>
</tr>
<tr>
<td>CCP</td>
<td>0.04</td>
<td>0.01</td>
<td>0.02</td>
<td>0.82</td>
<td>0.17</td>
</tr>
<tr>
<td>DAS-28</td>
<td>0.01</td>
<td>0.02</td>
<td>0.04</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td>HLA-BDR1</td>
<td>0.73</td>
<td>0.91</td>
<td>0.64</td>
<td>0.48</td>
<td>0.45</td>
</tr>
</tbody>
</table>
Patients were finally analyzed on the basis of the therapeutic protocol they were following.

Group 1 was under treatment with Etanercept

Group 2 was under treatment with Methotrexate

Group 3 was not under any treatment

- PG in groups 1,2,3 (ANOVA one way) p=0.28
- PG in groups 1,2 p=0.02
- PG in groups 2,3 p=0.07
- TF in groups 1,2,3 p=0.08
- TF in groups 1,2 p=0.00
- TF in groups 2,3 p=0.03
- TD in groups 1,2,3 p=0.20
- TD in groups 1,2 p=0.01
- TD in groups 2,3 p=0.06
- FN in groups 1,2,3 p=0.35
- FN in groups 1,2 p=0.85
- FN in groups 2,3 p=0.07
- CR in groups 1,2,3 p=0.21
- CR in groups 1,2 p=0.06
- CR in groups 2,3 p=0.09

Statistical differences were registered among patients undergoing therapy with Etanercept (group 1) and those undergoing treatment with Methotrexate (group 2)
in the mean load of bacteria belonging to the red complex, while there were no
differences of bacteria belonging to the orange complex.

Table 26: summary of statistical analyses between therapeutic regimens
(Etanercept-Methotrexate) and periodontal pathogens

<table>
<thead>
<tr>
<th></th>
<th>PG</th>
<th>TF</th>
<th>TD</th>
<th>FN</th>
<th>CR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>0,28</td>
<td>0,08</td>
<td>0,20</td>
<td>0,35</td>
<td>0,21</td>
</tr>
<tr>
<td>Et vs Met</td>
<td>0,02</td>
<td>0,00</td>
<td>0,01</td>
<td>0,85</td>
<td>0,06</td>
</tr>
<tr>
<td>Met vs Ctrl</td>
<td>0,07</td>
<td>0,03</td>
<td>0,06</td>
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The control group appeared as a disturbing factor in the overall statistic, since it
masked the effect of a therapeutic regimen on the composition of oral microbioma
compared to another treatment.

Patients undergoing therapy with Methotrexate reported higher levels of bacteria
belonging to the red group.

Conversely, bacteria belonging to the orange groups were not influenced by the
therapeutic regimen.
5.5 DISCUSSION

Rheumatoid Arthritis (RA) is a systemic, chronic disease which is characterized by a multifactorial aetiology and an immunomediated pathogenesis. About 1% of the world population is affected, especially women aged between 40 and 70 years.

Even though the disease is idiopathic, since it is an autoimmune response without an apparent causal agent, recent findings in the literature have tried to explain how genetic predisposition and environmental factors may play a role in the maintenance of chronic inflammation in the joints and other organs.

The oral cavity represents a reservoir of more than 300 bacterial species which colonize gingival and periodontal tissues leading to a chronic inflammatory state, where bacterial virulence is thwarted by host immunologic defenses.

The chronic stimulation of the immune system due to the presence of bacterial antigens within periodontal tissues has been associated with several systemic autoimmune diseases, like diabetes mellitus or other pathologies, infective endocarditis or cardiovascular atherosclerosis.

Rheumatoid Arthritis and Periodontal Disease (PD) share some common features, in particular many of the cytokines which maintain inflammation (i.e. IL-6, IL-10, TNF-α).

The current study suggests that in a group of patients affected by Rheumatoid Arthritis parameters with periodontal genetic risk factors be compared with the microbiological composition of the bacterial load.

Among rheumatic parameters Rheumatoid Factor (RF), anti-citrullinated protein’s antibody (CCP) and HLA-B27 were collected by blood samples, while DAS-28 questionnaire was fulfilled by the rheumatologist in order to establish a recognized score of the disease activity.
Genetic predisposition to Periodontal disease is mainly due to the presence of several polymorphisms of the genes expressing Interleukin-6 (IL-6), Interleukin-10 (IL-10) and Vitamin D Receptor (VDR).

GG genotype of the rs1800795 polymorphism in IL-6 genes triggers periodontal tissue destruction, while on the contrary genotypes GC and CC seem to act as protective agents.

In IL-10 genes polymorphism rs1800872 CC genotype is protective against PD, while genotypes AA and AC are predisposing factors.

In VDR TT genotype of the polymorphism rs731236 is a predisposing agent, while genotypes TC and CC are protective.

A total number of 34 patients affected by RA were recruited into the current study, 24 females and 10 males. Among them, 13 patients were undergoing therapy with the biopharmaceutical drug Etanercept, an antibody directed against TNF-α and inhibiting it acting as a soluble receptor (group 1).

14 individuals were undergoing treatment with Methotrexate (group 2), an immunosuppressant drug with more side effects than Etanercept, while 7 patients were not under any treatment protocol and were included into the control group (CTRL).

Statistical analysis was performed to compare genetic predisposition to PD in each patient with rheumatic parameters, in order to check any interaction between host susceptibility to periodontitis and genetic predisposition and clinical features of the concurrent RA.

IL-6, IL-10 and VDR genes polymorphisms were compared with collected data on RF, CCP, DAS-28 and HLA-BDR1.
IL-6 did not turn out to be significantly associated with any of the rheumatic parametrs (p=0.22; p=0.16; p=0.11; p=0.66). However, it should be considered that only 3 patients expressed the protective polymorphism of the gene, while 31 showed a predisposing polymorphism. The two groups were not equilibrate, so it was impossible to establish or deny any connection between IL-6 and Rheumatoid Arthritis.

The same consideration should be done for VDR (p=0.24; p=0.45; p=0.33; p=0.42), but also in this case 28 individuals expressed a predisposing polymorphism of the gene while only 6 showed the protective variant, so the two subgroups were quantitatively not homogeneous.

In addition, while there was not any link between IL-10 and RF, DAS-28 and HLA (p=0.06; p=0.36; p=0.22), a statistical significant correlation was detected between IL-10 and the anti-citrullinated protein’s antibody (CCP): the presence of CCP in the bloodstream was related to the presence of protective polymorphism CC in IL-10 genes, showing an opposite tendency in this case (p=0.02).

This connection should be further investigated, since recent papers have shown that IL-10 injected into joints affected by RA seems to decrease inflammation and thus is linked to biopharmaceuticals to strengthen drug efficacy.

Comparison of periodontal polymorphisms among them pointed out that IL-6 and IL-10 are strictly connected (p=0.014), being the predisposing polymorphism GG of IL-6 expressed together with predisposing polymorphisms AC and AA of IL-10.

These two cytokines in periodontal disease are not genetically independent, since they both tend to be active together towards predisposition or protection against PD.

Comparative non-parametric analysis among rheumatic data show obvious correlation between them (RF vs CCP p=0.00; RF vs HLA p=0.00). However, we could
confirm data reported in the literature, even though anti-citrullinated protein antibody (CCP) was the only one expressing statistical significance with all other parameters, including the clinical score DAS-28 (CCP vs HLA p=0.00; CCP vs DAS-28 p=0.04). It means that CCP is the most useful element in the bloodstream which provides information about disease activity or severity. On the contrary, the other parameters did not show any correlation with the clinical features collected by DAS-28 (RF vs DAS-28 p=0.14; HLA-DBR1 vs DAS-28 p=0.74).

Sex represented an important feature of statistical analysis.

RA is known to be more associated with females than males (3:1 ratio). While there was not any correlation with IL-6 and VDR periodontal polymorphisms (p=0.34 and p=0.58 respectively), a correlation was found out with IL-10 (p=0.046), being the predisposing polymorphisms AC and AA expressed more in males than in females.

Considering that females expressed RF (p=0.02) and CCP (p=0.56, almost statistically significant) to a major content, these data confirm those reported before between IL-10 and CCP. It seems than males express more IL-10 and less CCP, while on the contrary females express more CCP and less IL-10, being IL-10 an interesting agent to be used for systemic therapy against RA. Furthermore, IL-10 periodontal polymorphism appears to be useful in this association.

After the analysis of genetic and laboratory data was performed, the statistical analysis focused on the role of periodontal inflammation and microbioma and their role in RA.

Periodontal screening record (PSR) was registered for every patient and PSR=3 was considered the threshold according to which patients were subdivided into two groups: individuals with periodontitis and patients with healthy conditions.

Microbiological analysis was performed to establish the mean load of Porphyromonas gingivalis (PG), Tannerella forsythensis (TF), Treponema denticola
(TD), *Fusobacterium nucleatum* (FN) and *Campylobacter rectus* (CR) in periodontal pockets and gingival sulcus of recruited patients.

There was not any statistically significant difference between the microbioma composition in patients with PSR <3 and PSR > 3 (PG p=0,09; TF p=0,88; TD p=0,25; FN p=0,24; CR p=0,41). It means that the clinical status of gingival tissues is not directly correlated to which bacteria colonize periodontal pockets.

Afterwards, the comparison between the bacterial mean load and rheumatic parameters demonstrated that while there is a strong relationship between the “red complex” bacteria and the systemic condition in RA and disease intensity, “orange complex” bacteria are equally distributed and not influenced by RA.

Rheumatoid Factor revealed a positive correlation with the presence of *Tannerella forsythensis* and *Treponema denticola*, while it did not show significative features of correlation with other bacteria.

Anti-citrullinated protein’s antibody was significantly connected with the presence of *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola*, while it did not show either a relationship with *Fusobacterium nucleatum* or with *Campylobacter rectus*.

CCP turned out to be a very precious parameter in monitoring RA activity and correlating rheumatic conditions to periodontitis. The “red complex” bacteria were influenced by the presence of an increased intensity in Rheumatoid Arthritis, while on the contrary the “orange complex” bacteria were not related to systemic conditions in RA.

DAS-28 was associated with the presence of all bacteria and it showed how an increased clinical and instrumental activity recorded in RA leads to a worsened condition within oral microbioma and consequent infection from pathogens (PG p=0,01; TF p=0,02; TD p=0,04; FN p=0,01; CR p=0,01).
Conversely, HLA-DBR1 was not linked to alteration in oral microbioma composition (PG $p=0.73$; TF $p=0.91$; TD $p=0.64$; FN $p=0.48$ and CR $p=0.45$).

Finally, the last statistical analysis conducted on the microbiologic composition of dental plaque in the three groups of patients undergoing therapy with etanercept (group 1), metothrexate (group 2) and patients who were not on medical treatment (group 3) revealed that therapy with **Methotrexate**, an antisuppressant drug, determines the presence of “red complex” bacteria more than therapy with etanercept (PG $p=0.02$; TF $p=0.00$; TD $p=0.01$) while there was not any difference in the composition of “orange complex” bacteria (FN $p=0.85$; CR $p=0.06$).

At the end of the study, different conclusions can be drawn:

1. a connection between anti-citrullinated protein antibody and periodontal expression of IL-10 genes’ polymorphisms was found, with IL-10 protecting tendency against periodontal disease increasing when CCP are found in bloodstream. This correlation suggests a possible role of IL-10 in Rheumatoid Arthritis.

2. IL-6 and IL-10 polymorphisms are strictly associated: when IL-6 expresses predisposing genes to periodontal disease, the same does IL-10. These two cytokines are not genetically independent.

3. CCP proved the most useful bloodstream parameter to be correlated with Rheumatoid Arthritis activity. CCP appears to be associated with all other rheumatic parameters, including the DAS-28 clinical and instrumental score for measuring disease activity in RA.

4. Males express IL-10 predisposing genes to periodontal disease more than females, while females show a greater tendency to express
Rheumatoid Factor and anti-citrullinated protein antibody. This opposite behaviour corroborates the idea of a correlation between IL-10 and Rheumatoid Arthritis which should be studied in depth.

5) The presence of the bacterial “red” and “orange” complexes are not related to Periodontal Score Recording, thus simple clinical evaluation of gingival tissues is not sufficient to assess the related risk of periodontitis.

6) The “red complex” bacteria Porphyromonas gingivalis, Tannerella forsythensis and Treponema denticola are more expressed when rheumatic indices are found out in the bloodstream, especially CCP. On the contrary, the bacterial “orange complex” composed of Fusobacterium nucleatum and Campylobacter rectus is not associated with rheumatic scores. These findings suggest the role of Rheumatoid Arthritis in favouring more aggressive forms of periodontitis.

7) Therapy with Methotrexate is statistically associated with an increased presence of bacteria from the “red complex” compared with Etanercept.
6. FINAL DISCUSSION AND CONCLUSIONS

6.1 DISCUSSION

The main purpose of this clinical trial was to analyze the role of saliva and crevicular fluid in the pathogenesis, diagnosis and therapy of several systemic diseases, like chronic gastritis and Rheumatoid Arthritis or of the most common oral pathologies, Oral Lichen Planus and Burning Mouth Syndrome.

Salivary and gingival fluids were chosen because recent findings in the international literature have strongly suggested their role as potential diagnostic tools, since they contain many biomarkers and cytokines useful to diagnose several diseases at an early stage [6]. Many authors recommend that oral fluids be thoroughly investigated, as they can become valid substitutes for blood test [10].

However, collecting homogeneous samples of saliva is very difficult and there is a lack of standardized protocols. Furthermore, saliva is strongly influenced by many variables, among which only a few can be directly controlled by the dental clinician, like the time of day when the test takes place, exposition to light, dental chair position and environmental temperature [13].

On the contrary, sex, age, body weight, salivary glands anatomy, physical health, mental health and xerogenic drugs are independent parameters which may lead to alterations in the composition and quantity of oral fluids, but can’t be modified by the dental physician.

Besides, crevicular fluid, which is completely different from saliva and is more similar to interstitial fluid, is more difficult to examine since gingival tissues only produce a small quantity per day when healthy (0,5-2,5 ml) [17].
In this experimental work oral fluids were collected from an overall number of 240 patients for quantitative, molecular and/or microbiological investigation.

Unstimulated whole-mouth saliva was preferred as diagnostic fluid because the potential alteration caused by using different types and intensities of reflex stimulation could be avoided [18].

To obtain a suitable sample of unstimulated saliva the spitting method was adopted. Patients sat on the dental chair and then bent their head forward. They had to spit every minute avoiding swallowing for 5-15 minutes. Ejected saliva was collected in a sterile tube or funnel.

However, in some cases, it was useful to measure the stimulated salivary flow, and this was made by adding some drops of citric acid 2% solution into their mouth and subtracting the total amount of the stimulating solution from the total amount of ejected saliva. This was the case of the first study described in this dissertation: “Low basal salivary flow and Burning Mouth Syndrome: new evidence in this enigmatic pathology”.

Burning Mouth Syndrome (BMS) is a condition characterized by a sensation described by patients as stinging and burning that affects the oral mucosa in the absence of clinical or laboratory data to justify these symptoms [21]. This condition principally affects women with a ratio of approximately 4:1; this difference between the sexes might be explained by biological, psychological and sociocultural factors, even though such aspects have not been defined yet. The pathophysiology of idiopathic burning pain is unclear and has generated controversy over the years. The aetiopathogenesis seems to be complex, and in a large number of patients, is most likely to involve an interaction between local, systemic and/or psychogenic factors.

Particular emphasis has been placed on concurrent symptoms of xerostomia. The role of saliva and environmental factors has been investigated, including salivary
gland dysfunction as a contributory factor in BMS, but contradictory findings have also been reported in the literature.

In the above mentioned study the basal and stimulated flows in a group of 44 patients with BMS were measured and then compared with an oral lichen planus group (OLP) of 27 patients and a control group (CTRL) of 40 patients.

Plot 18: 44 BMS patients were recruited into the 1st study and compared with an OLP group of 27 patients and a CTRL group of 40 patients

The OLP group was not included for its statistical significance per se, as a BMS and a CTRL group were sufficient to compare the outcomes. However, OLP is the most frequent chronic inflammatory condition or oral mucosae (see below) and the normal values registered within this group highlighted the peculiarity of the outcomes registered in BMS patients.

As a matter of fact, the outcomes of the research demonstrated a very low basal salivary flow in BMS patients compared with the other two groups, but the stimulated salivary flow was similar in BMS patients (p=0.002).
This leads to the hypothesis that salivary function is preserved in Burning Mouth Syndrome, but that chronic intake of antihypertensive, anxiolytic and antidepressant drugs on one side and the contextual presence of psychological behaviour distress on the other side could influence the basal tone of those salivary glands responsible for the basal flow function, such as the submandibular, sublingual and the minor salivary glands.

This study non only contributes to a better understanding of the role of saliva in the pathogenesis of this enigmatic pathology, but also points out how the central nervous system and associative cortices may strongly influence the salivary flow rate, which is the main contributory factor to determine salivary composition.

Salivary composition is fundamental to maintain stable healthy conditions in the oral cavity.

Saliva fulfills defensive functions, thanks to the presence of many enzymes, among which lysozyme, lactoperoxidase, lactoferrin and antimicrobial peptides.

The antimicrobial peptides (AMPs) are polypeptides of less than 100 amino acids detectable in host defense settings, and that have antimicrobial activity at physiological concentrations under conditions prevailing in the tissues of origin [51].
In humans AMPs can be classified into two families that have been thoroughly characterized, the cathelicidins and the defensins.

The only cathelicidin described in humans has been named human cationic antimicrobial peptide hCAMP18, which is transformed into a small peptide, known as LL-37, by a serine protease expressed in neutrophils. hCAMP18/LL-37 cathelicidin is produced by different cell types, including myeloid cells, neutrophils, mast cells, monocytes, epithelial cells of the colon, urinary tract and respiratory mucous membranes [58].

Defensins are a family of small-sized β-chained peptides whose molecular weight ranges from 3.5 to 4.5 kDa and include 6 cysteine residues which can create disulfide bridges. β-defensins, especially HBD-2, has been studied in the oral cavity. HBD-2 is an inducible peptide and its production is stimulated by proinflammatory cytokines, such as IL-1, TNF-α and by bacterial products [72].

AMPs perform a direct antimicrobial activity thanks to their electrostatic interaction with the negatively charged phospholipids on bacterial membranes, but they also serve an immunomodulatory function. They act as chemoattractive agents on monocytes, neutrophils and CD4+ lymphocytes and stimulate the production of other chemokines.

It is debated whether functional impairment or enhancement of AMPs can influence the clinical course of several diseases, among which infective and chronic inflammatory pathologies.

The second study in this dissertation, entitled “Human β2-defensin in Oral Lichen Planus and Burning Mouth Syndrome”, focused on the possible role of defensins in the pathogenesis and maintenance of the most common autoimmune disorder of the oral cavity, Oral Lichen Planus (OLP).
OLP is a common, chronic cutaneous disease which often affects the oral mucosa. Most of patients with OLP are middle-aged adults, with a 3:2 ratio of women to men. It is believed that about 1% of the population may have this condition. In the oral cavity, the disease develops with lesions characterized by radiating, velvety, thread-like white/grey papules arranged in a linear, annular or retiform pattern forming typical lacy, reticular patches, rings and streaks over the buccal mucosa and, to a lesser extent, on the lips, tongue and palate. These lesions trigger no significant symptoms in the majority of patients, while in others there may be a complaint of a burning sensation in the involved areas [76].

The clinical forms can be grouped into two families: the “White” OLP, which includes asymptomatic papular, reticular and hyperplastic forms, and the “Red” OLP, which is characterized by a clinical evidence of inflammation and may be represented by atrophic, erosive, ulcerative and bullous forms.

In this study samples collected from the saliva and crevicular fluid of a group of 35 patients were analyzed with ELISA protocol the presence of HBD-2. Among them, 17 patients were histologically diagnosed with OLP, 9 with BMS and 9 were enrolled as a control group (CTRL).

Plot 19: 17 OLP patients were recruited into the 2nd study and registered values of HBD-2 were compared with those of a group of 9 BMS patients and a control group of 9 patients.
Furthermore, a dental physician classified the clinical forms of OLP into 10 “White” and 7 “Red” OLP forms based upon their clinical appearance.

While there were not any statistically significant differences between the groups, both for the OLP and BMS groups compared with the CTRL group, the study pointed out the role of HBD-2 in the maintenance and intensity of the inflammatory component of OLP.

Patients affected by OLP showed a dycotomic distribution of values: 10 of them showed similar values to those encountered in the other two groups, while 7 resulted in expressing increased levels of HBD-2. There was a high correlation between the clinical and biomolecular independent classification of patients (p=0.001), and the expression of HBD-2 was higher in the “red” OLP subgroup than in the “white” one (p=0.000). HBD-2 could be used as a parameter to monitor the degree of disease activity and inflammation, and for this reason research into the role of salivary and gingival defensins in the pathogenesis of autoimmune oral disease requires further investigation.

Saliva in the oral cavity does not merely have a defensive function, but it also acts as a key factor in lubrication of oral mucosae, calcium homeostasis and digestion.

Saliva is the first enzymatic liquid which is encountered in the gastrointestinal tube and acts synergistically with other secretions. Saliva contains α-amylase, which is responsible for degradation of the 1-4 glycosidic bond in starches. When saliva is swallowed and reaches the stomach through the oesophagus, α-amylase is inactivated by gastric secretion.

There are many links between the oral cavity and the stomach, considering that they belong to the same apparatus, the gastrointestinal tube.
One of the most interesting finding is the presence of *Helicobacter pylori* in the oral cavity.

*Helicobacter pylori* (HP) is a widespread bacterium, which is associated with chronic gastritis in 90% of cases, but it has been also described in gastric cancer. Despite the fact that infection incidence has decreased in recent years, about half of the world’s population is still infected. Furthermore, therapy resistance and relapses represent a threatening issue to be taken into account [81].

In 1994 the WHO classified HP as a carcinogenic agent, and IARC has included it in the group 1 list of carcinogens [85].

To make a correct diagnosis of gastric infection, the C-Breath Test Urea (C-BTU) is used, since it measures the bacterial urease activity in breath.

Triple therapy, including a Proton Pump Inhibitor (PPI) plus two antibiotics, such as clarithromycin and amoxicillin or metronidazole, is the first treatment of choice.

In recent years, the prevalence of resistant bacterial strains has worldwide called the attention of clinicians to the worrisome increase in the rates of unsuccessful therapeutic outcomes. In fact, the results of recent studies have shown that documented eradication rates are at their lowest level in history. This could be due to incomplete elimination of HP, leading to recrudescence of the same strain, or to reinfection with a new strain, with recrudescence being the more common cause of relapses.

These features made clinicians put forward the possibility of an extra-gastric reservoir of infection [95]. The presence of HP in the oral cavity was firstly discovered in 1989 and following studies detected it in dental plaque, saliva, tongue, tonsil tissue and root canals [98]. A meta-analysis published in 2011 [100] reported a strong connection between the presence of HP in the oral cavity and in the stomach. However, triple eradication therapy has no or little effect and acts as a
causal factor in the recurrence of HP infection. The introduction of PCR-RT technique allowed researchers to detect HP when it is highly present in saliva or dental plaque.

The third study described in this dissertation, entitled “Helicobacter pylori in periodontal pockets and saliva: a possible role in gastric infection relapses? A preliminary study in Northern Italy” proposed evaluating the presence of oral HP in a group of patients who underwent examination for gastric HP infection.

A total number of 60 patients were recruited into this study, 30 with a positive diagnosis of gastric infection and 30 with a negative diagnosis after the C-BTU examination.

![Plot 20: patients with positive results at C-BTU test were recruited into the 3rd study together with 30 patients who tested negative](image)

A dental clinician performed periodontal evaluation and collected salivary and crevicular fluid samples. PCR-RT analysis was conducted to detect the presence of HP in collected samples.
Even though there was not any statistically significant difference among the two groups as to the total amount of HP in saliva \( (p=0.372) \) or crevicular fluid \( (p=0.604; \ p=0.326; \ p=0.368; \ p=0.731) \), it should be considered that they are two completely different methods with different specificity and sensibility and provide a picture at the same moment of two different anatomic districts. However, a remarkable datum was detected as regards HP behaviour in the oral cavity: patients affected by periodontal disease showed higher values of HP.

These outcomes suggest that HP is present in the oral cavity and that it is not related to gastro-esophageal reflux or stomach load, like some authors have claimed so far, but it has a direct role within the affected periodontal tissues.

Recent findings in the literature show there is an immunological relationship between HP and *Campylobacter rectus*, and clearly indicate that one of the shared antigens is a GroEL protein, able to influence the onset and development of periodontal disease through its biological activity [107]. Furthermore, the microaerophilic and acid environment of periodontal pockets provides suitable conditions for growth and proliferation of HP. Considering that HP was originally called *Campylobacter-like bacterium*, it is advisable to regard HP as a colonizing bacterium of periodontal tissues when they are affected by periodontal disease, and when a biofilm with different species can increase the efficacy of the single species thanks to gene exchanges and signaling pathways among the different bacterial subtypes.

In addition, HP in periodontal tissues is not confined to periodontal pockets, but migrates from the gingival sulcus into saliva, as it was demonstrated by comparing the presence of the bacterium in the saliva of periodontal patients with the values measured in healthy individuals \( (p=0.05) \).

Thus, periodontal disease stimulates a suitable environment for HP, the periodontal pocket. At this site the bacterium finds favourable conditions to thrive in sinergy
with other bacteria. Once it has reached a sufficient amount of load, the bacterium migrates from the sulcus into the oral cavity and enters saliva. Since saliva is swallowed and flows into the stomach, the oral cavity consequently becomes an extra-gastric reservoir of HP which reaches the stomach thanks to deglutition.

Whether salivary content in HP may cause gastric infection relapses should be studied in depth by reassessing the 30 C-BTU positive patients after eradication therapy, and by repeating both C-BTU and periodontal examinations to collect data for statistical analysis.

Another issue which has drawn attention of the scientific community is the relationship between salivary and crevicular fluids biomarkers and periodontal disease.

Periodontal disease is with dental caries one of the two most common diseases of the oral cavity. It affects about 80% of the world’s population. The disease involves the supporting structures of the periodontium, like gingiva, periodontal ligament and the alveolar bone. The final stage of the disease is the loss of dental elements by exfoliation. Genetic predisposition may have an important role in establishing whether an individual is prone to be affected [130], but dental plaque is the causal factor. The clinical course of the disease depends on host’s immunitary defenses and the bacterial load. Dental plaque contains more than 300 different bacterial species, but only a few have been associated with a high risk of developing periodontal disease. The connection among bacteria within the biofilm is not random. Six species have been found to be strictly linked and responsible for the clinical development of the disease: *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythensis*, *Treponema denticola*, which belong to the “red complex” of pathogens, and *Fusobacterium nucleatum* and *Campylobacter rectus*, which belong to the “orange complex”.
Among cytokines the ones which may have a significant role in periodontal inflammatory disease are IL-1, TNF-α, IL-6, IL-10 and VDR [134][135].

Salivary and crevicular fluid biomarkers proved to be useful in diagnosing and monitoring some systemic diseases, like diabetes mellitus, cardiovascular disease, viral infections, pancreatic cancer, breast cancer, lung cancer, prostate cancer and Rheumatoid Arthritis.

In the fourth study presented in this dissertation, “Rheumatoid Arthritis therapies and Periodontal disease: any correlation?” the proposal is to appraise the connection between Rheumatoid Arthritis (RA) and Periodontal disease (PD).

34 patients with Rheumatoid Arthritis were recruited into the above mentioned study, all of them being affected by RA. 13 were under therapy with anti TNF-α biopharmaceutical Etanercept (group 1), 14 were undergoing treatment with immunosuppressant Methotrexate (group 2) and 7 were recruited as control group (group 3) without any course of treatment.

Plot 21: 34 patients affected by Rheumatoid Arthritis were recruited into the 4th study. Among them, 13 were undergoing therapy with Etanercept (group 1), 14 with Methotrexate (group 2) and 7 were recruited as a control group (group 3).
The rheumatologist collected data about the disease intensity and laboratory findings. Among haematochemical analysis, Rheumatoid Factor (RF), anti-citrullinated protein antibody (CCP) and HLA-BDR1 were prescribed. The DAS-28 questionnaire for clinical and laboratory scores was completed to determine the degree of RA severity.

Then the dental clinician performed a dental and periodontal examination, collecting samples of gingival liquid for genetic and microbiologic analyses.

Statistical analysis was carried out to compare the genetic and microbiologic periodontal results with RA systemic indices.

There were some interesting correlations between Rheumatoid Arthritis and Periodontal Disease: a link between CCP and expression of IL-10 genes protecting polymorphisms against PD was found (p=0.02), suggesting a possible role of IL-10 in Rheumatoid Arthritis. In addition, IL-6 and IL-10 polymorphisms were strictly related (p=0.014): when IL-6 expresses predisposing genes to periodontal disease, the same does IL-10. These two cytokines are not genetically independent. Males express IL-10 predisposing genes to PD more than females do (p=0.05), while females show a greater tendency to express RF and CCP (p=0.02). This opposite behaviour confirms the correlation between IL-10 and RA and further research should be carried out. CCP proved to be the most useful blood parameter to be correlated with Rheumatoid Arthritis activity and was associated with all other rheumatic parameters, including DAS-28.

Microbiological analysis revealed that the “red-complex” bacteria *Porphyromonas gingivalis*, *Tannerella forsythensis* and *Treponema denticola* are more expressed when Rheumatoid Arthritis indices are detected in the bloodstream, mostly CCP. On the contrary, the “orange-complex” bacteria *Fusobacterium nucleatum* and *Campylobacter rectus* are not associated with Rheumatic scores. These findings suggest a role of RA in favouring more aggressive forms of periodontitis. Finally,
therapy with methotrexate is statistically associated with an increasing presence of red-complex bacteria when compared to Etanercept (p=0,02; p=0,00; p=0,01).

6.2 CONCLUSION AND FUTURE PERSPECTIVES

The experimental work described in this PhD thesis focused on the role of saliva and crevicular fluids as diagnostic tools useful to detect systemic pathologies, understand the complex mechanism of the correlation between periodontal disease and systemic infective and autoimmune diseases and explain the underlying pathogenesis beyond very common oral disorders, like Oral Lichen Planus and Burning Mouth Syndrome.

At the end of this dissertation, it can be affirmed that oral fluids will certainly substitute for blood test, when standardized procedures and reliable data interpretation are provided by the scientific community.

To sum up, the outcomes of the reported studies demonstrated:

- a very low basal salivary flow in Burning Mouth Syndrome (BMS) patients compared with other patients, but equal stimulated flow. This study suggests new topics for further investigation in order to explain puzzling pathologies such as Burning Mouth Syndrome. Besides, it points out how emotions and psychosomatic distress can influence the production of saliva with consequences on oral health. Additional studies are required to better investigate this phenomenon through the use of more specific salivary tests and functional neurophysiological imaging.
the role of Human β2-defensin in the pathogenesis of Oral Lichen Planus (OLP), showing how its most inflamed forms express a larger amount of HBD-2, thus revealing the significance of this small peptide in chronic inflammation. More studies are necessary to evaluate the role of Human β3-defensin in chronic inflammation and the behaviour of both HBD-2 and HBD-3 in periodontal disease, so as to produce low weight polymers of Hyaluronic acid which could be prescribed to treat periodontitis and strengthen causal and mechanical therapies.

- the presence of Helicobacter pylori (HP) in the oral cavity as an extra-gastric reservoir in periodontally affected gums. In addition, the increased load of HP detected in periodontitis is not confined to periodontal pockets, but migrates from the gingival crevice to the stomach through swallowed saliva. Patients diagnosed as positive for gastric infection through the C-BTU will undergo eradication therapy and will be reassessed again after 6 months both for the gastric and oral infection. If patients with relapses are statistically associated with an increased oral HP load, it will be possible to establish the true role of extra-gastric reservoir of HP in gastric relapses, a topic which is becoming crucial worldwide.

- there are many correlations between periodontal disease (PD) and Rheumatoid Arthritis (RA); IL-10 antinflammatory action should be thoroughly investigated to better understand the possible use in next generation drugs for periodontal disease.
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8. REFERENCES


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