UNIVERSITA' DEGLI STUDI DELL'INSUBRIA

DOTTORATO DI RICERCA IN BIOTECNOLOGIE, BIOSCIENZE E TECNOLOGIE CHIRURGICHE

Curriculum Biotecnologie e Tecniche Chirurgiche

XXXII CICLO

Role of saliva, gingival crevicular fluid and oral microbiota in gastrointestinal diseases

Ruolo di saliva, fluido crevicolare gengivale e microbioma orale nelle patologie gastrointestinali

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1. INTRODUCTION

The oral cavity is a complex ecosystem characterized by an environmental moderate temperature and high humidity, due to the presence of oral fluids.

1.1 Saliva

Saliva is the most important element in the manteinance of equilibrium within the oral mouth. It is an hypotonic fluid produced by three major salivary glands (the *parotyd gland*, the *submandibular gland* and the *sublingual gland)* and several minor salivary glands.

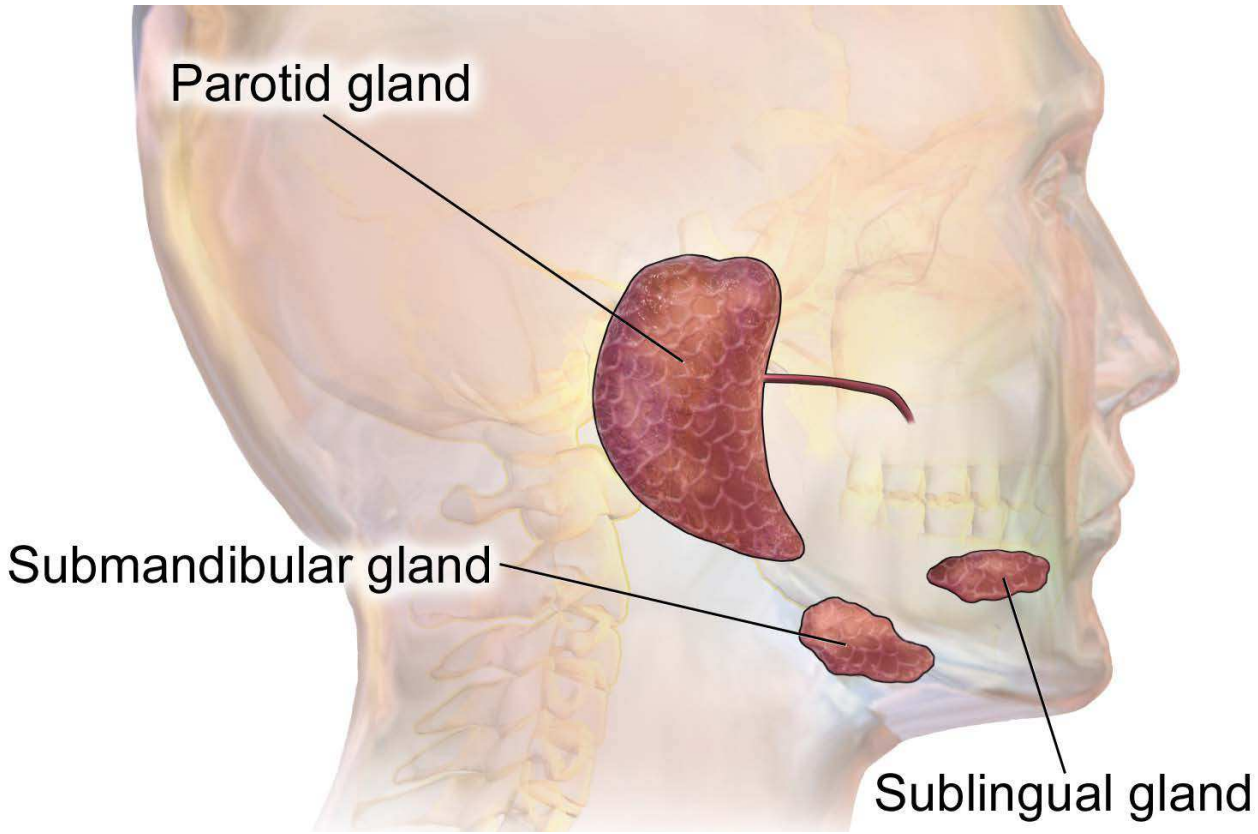


Fig.1 Major salivary glands

Under physiologic conditions human beings produce between 800 and 1.500 ml of saliva per day: during rest conditions most of this liquid is produced by the submandibular gland, while only 20% relies on the parotyd gland. Instead, in case of the presence of a chemycal or mechanical stimulus, over 50% of saliva is produced by the parotyd gland.

The role of minor salivary glands in salivary production seems to be less important in quantitative terms.

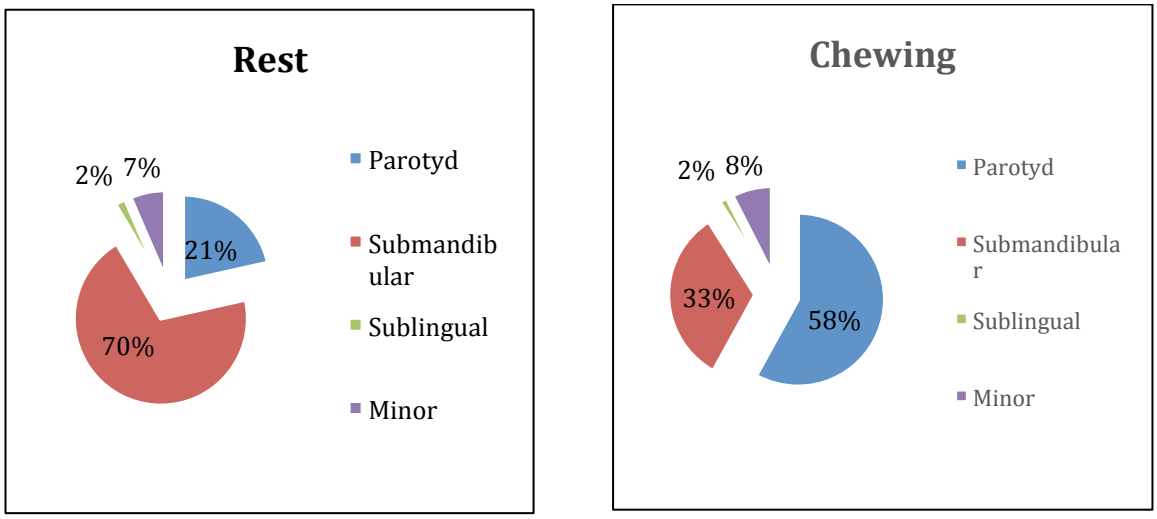


Fig.2 Salivary production rates in different conditions.

Salivary production is regulated by parasympathetic and sympathetic nervous system; within salivary glands, primary saliva is produced by acinar cells localized in the secretory area, then it is passes through secretory ducts where it is modified, and finally it is poured in the oral cavity. The parotid gland and the submandibular gland have a single duct, called Stensen's duct and Wharton's duct respectively . The sublingual gland has multiple ducts, known as Rivinus duct and Bartholin's duct.

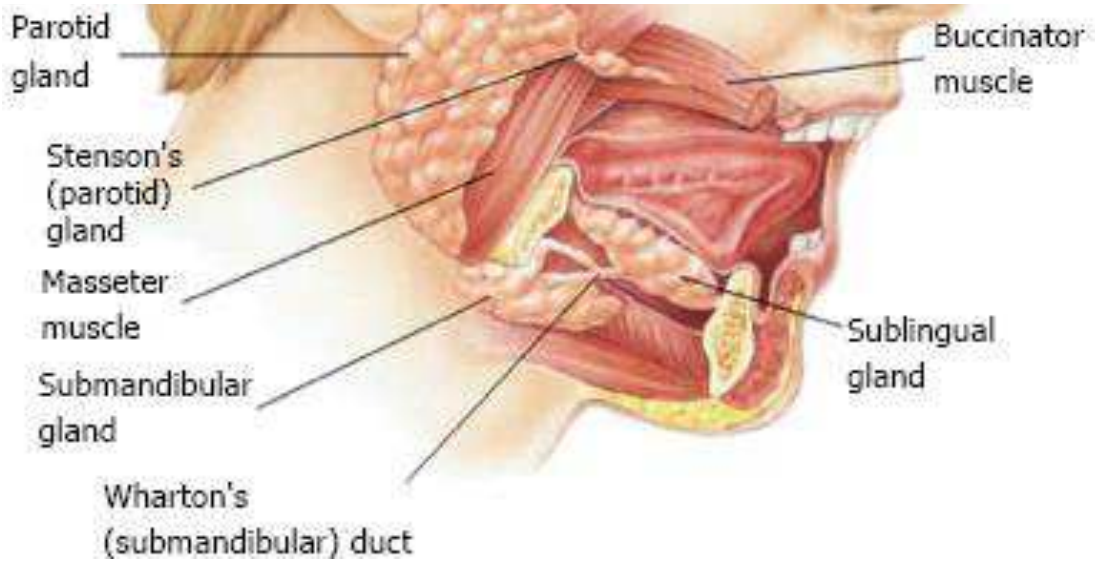


Fig. 3 Salivary glands and ducts

1.2 Salivary Composition and Functions

Saliva contains different elements:

- α-amilase (ptyalin) is an enzyme mainly produced by the parotyd gland which is responsible for digestion
- mucins are high-molecular-weight glycoproteins
- proteic compounds (cortisol, glucose, urea, sexual hormones)
- electrolytes such as bicarbonate sodium, chlorine, calcium

This oral fluid performs several functions which are essential for the maintenance of oral health:

- *digestive* functions, due to the presence of α-amylase;
- *emollient* and *lubricant* functions, thanks to water and mucins;
- *protective* functions for dental elements, oral mucosa and esophagus due to the presence of antiviral, antibacterial and antifungal substances: Immunoglobulins A, Lysozyme, Lactoferrin, Histatins, Peroxydase,

Antimicrobial peptides *(AMPs,* divided in *cathelicidin LL-37*, *α-defensins* and *ß-defensins)* . An alteration in their composition or function may explain how several subjects are more predisposed to infections than others. Besides, it has been shown than AMPs participate in immunomodulation; so an alteration in their quantity could be related to the pathogenesis of several infective or autoimmune diseases.

- *the buffering capacity,* due to the presence of bicarbonate, proteins and phosphates
- many proteins coming from the bloodstream may be found in saliva, such as Albumins, Epithelial Growth Factor (EGF), TGF-α, TGF-ß and Fibroblast Growth Factor (FGF), which could be helpful in *wound healing and tissue repairing.*

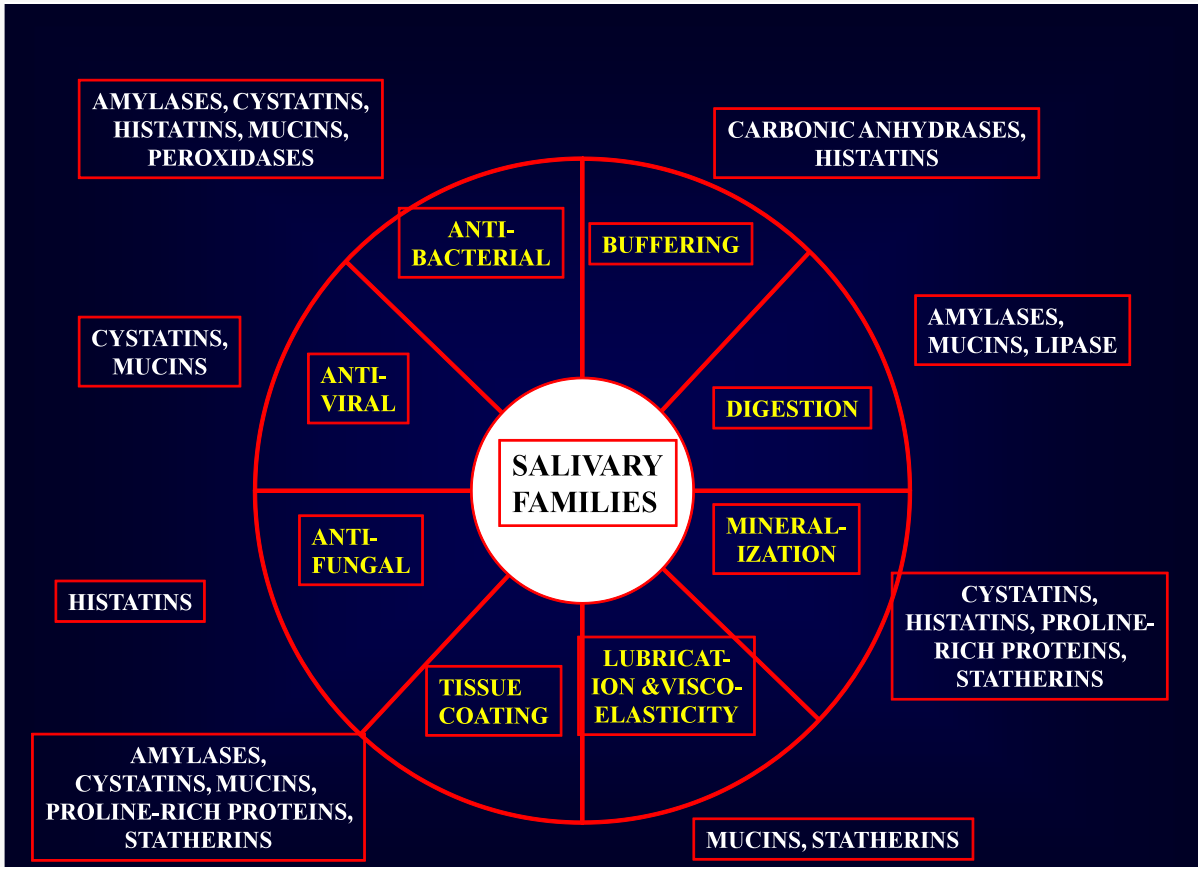


Fig.4 Salivary functions (Adapted from: Levine MJ, Am NY Acad Sci 1993; 694: 11-16)

1.3 Saliva collection

Saliva is an accessible biofluid that can potentially provide an insight into the relationship between the host and the environment.

Only few well-standardized protocols for collecting saliva samples are described. Its constant variabilty during the day, according to circadian rythm, makes the operation hard.

Saliva production is influenced by indipendent variables, which can not be taken under control during collection. They are: age, sex, body weight, drugs assumption, glands dimension and general health. On the other hand, dependent variables, which have to be controlled during sampling are: chemical or physical stimulation, time of the day, temperature, body position and light exposition. A proper collection can be performed only if homogeneous environmental conditions are mantained.

In particular the time of day in which collection is done repesents the most significant variable to be considered; the mornig hours (between 8.00 and 11.00 a.m.) should be preferred [1].

When a precise time is established, it has to be mantained in order to better compare results from different examinations. Also enviroment light and temperature must be kept constant.

In addition, eating or drinking during 90 minutes before examination is not reccomended . An appropriate collection should last about 15 minutes.

Finally, patient's head has to be maintained in the same position as far as possible.

Several techniques described in the international literature for salivary collection are known.

Unstimulated saliva can be collected in two main different ways:

- The spitting method: patient is sitting on a chair and is asked to spit the salivary content of his mouth every minute for at least 10-15 minutes.
- The *drooling method*: this technique is similar to the previous one, however the patient does not spit but lets his saliva drain in a test tube.

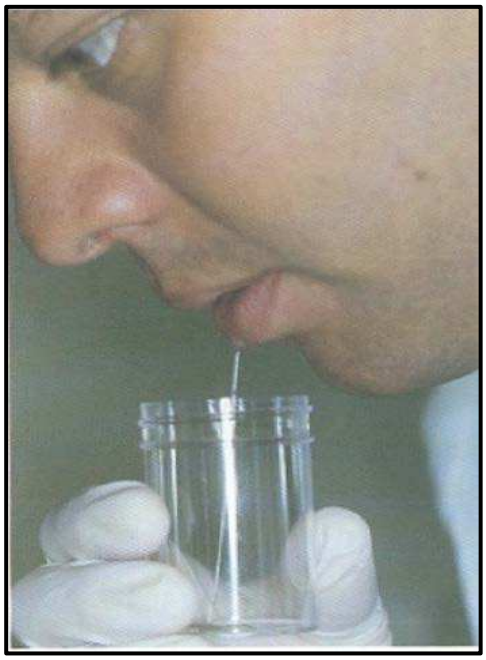
A)  B) 

Fig.5a: the spitting method; 5b: the drooling method, from Sreebny LM and Vissink A [2]

The most used methods for collecting ***stimulated saliva*** are represented by:

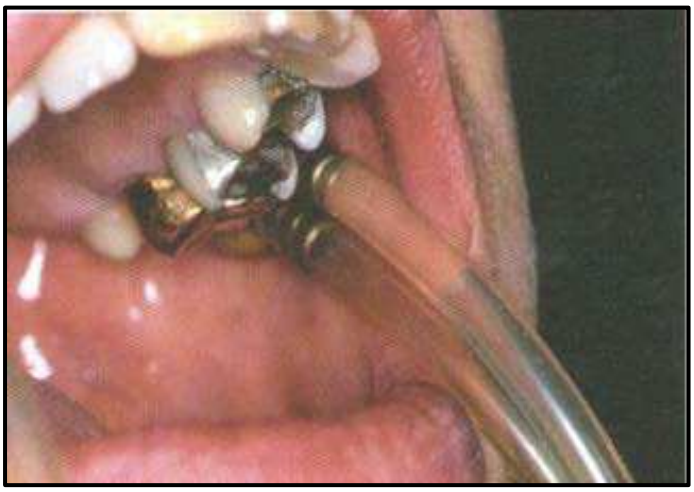
- The *absorbent technique*: a cotton roll is inserted in the oral cavity and left there to be passively wet by saliva (in this case salivary production is stimulated by the presence of a foreign body in the mouth).

- The *chewing technique*: A small piece of paraffin wax is chewed by the patient for 5 minutes; then saliva has to be spitted into a test tube.
- The *taste technique*: few drops of citric acid 2% solution are inserted in patient mouth to stimulate salivary production

All previous methods allow to collect the whole mixed saliva of the oral cavity, but they do not permit to distinguish saliva produced by a single salivary gland from that produced by another one[2].

Some techniques that allow to collect salivary samples from a specific single gland are: Lashley cup for the parotyd gland and Wolff's device for submandibular and sublingual glands [3].
However, these methods are not often used because of their high costs.

A)



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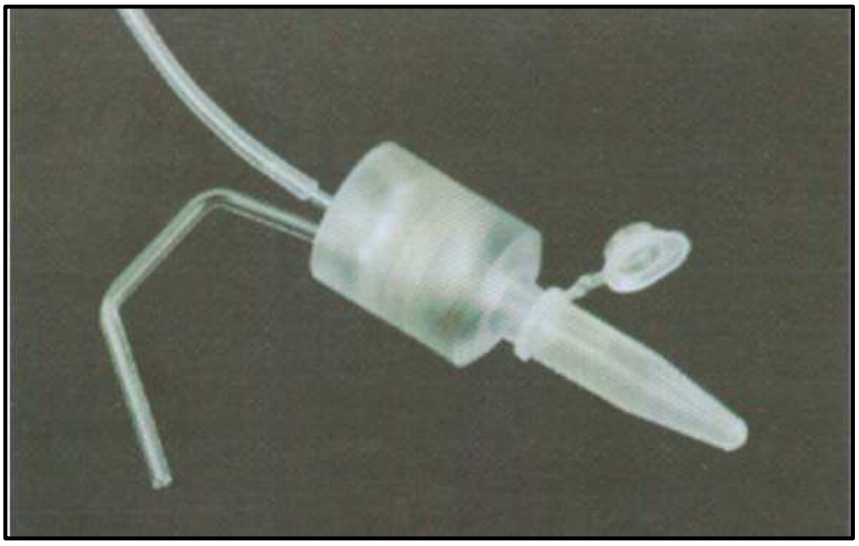

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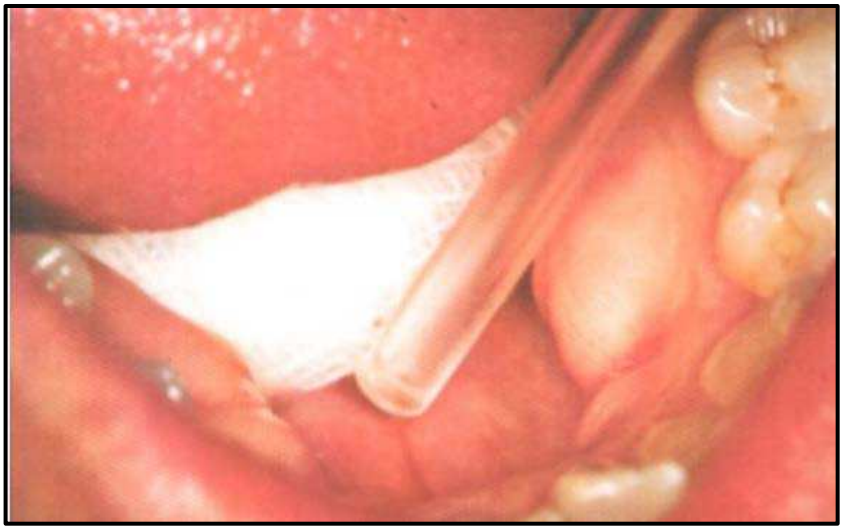


Fig.6a: Lashley's cup in position on the Stensens duct's outlet; 6b-c: Wolff's device for saliva collection (from Sreebny LM and Vissink A [2])

1.4 Crevicular fluid: composition and functions

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 (about 0,5-2,5 ml per day) in healthy conditions and in huge quantities when gums are inflammed.

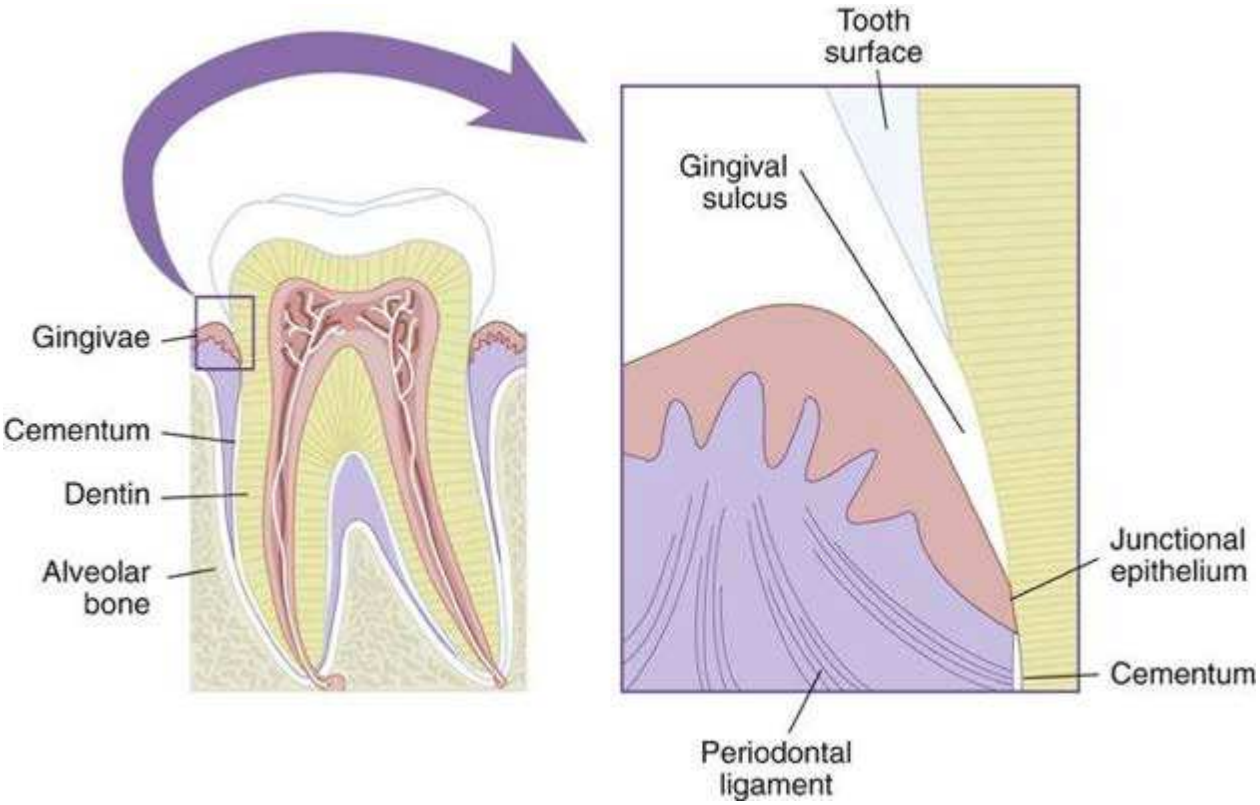


Fig.7 Gingival sulcus anatomy

It cleanses material from the sulcus and contains proteins useful to improve adhesion of the epithelim to the tooth. Furthermore, it possesses antimicrobial properties and exerts an antibody activity in defense of gingiva.

Its composition is similar to that one of insterstitial fluid, in fact it contains:

- Cellular elements: epithelial cells, leukocytes and bacteria
- Electrolyte: sodium, potassium, calcium
- Organic compounds
- Metabolic acid end products: prostaglandines, urea, antibacterial factors

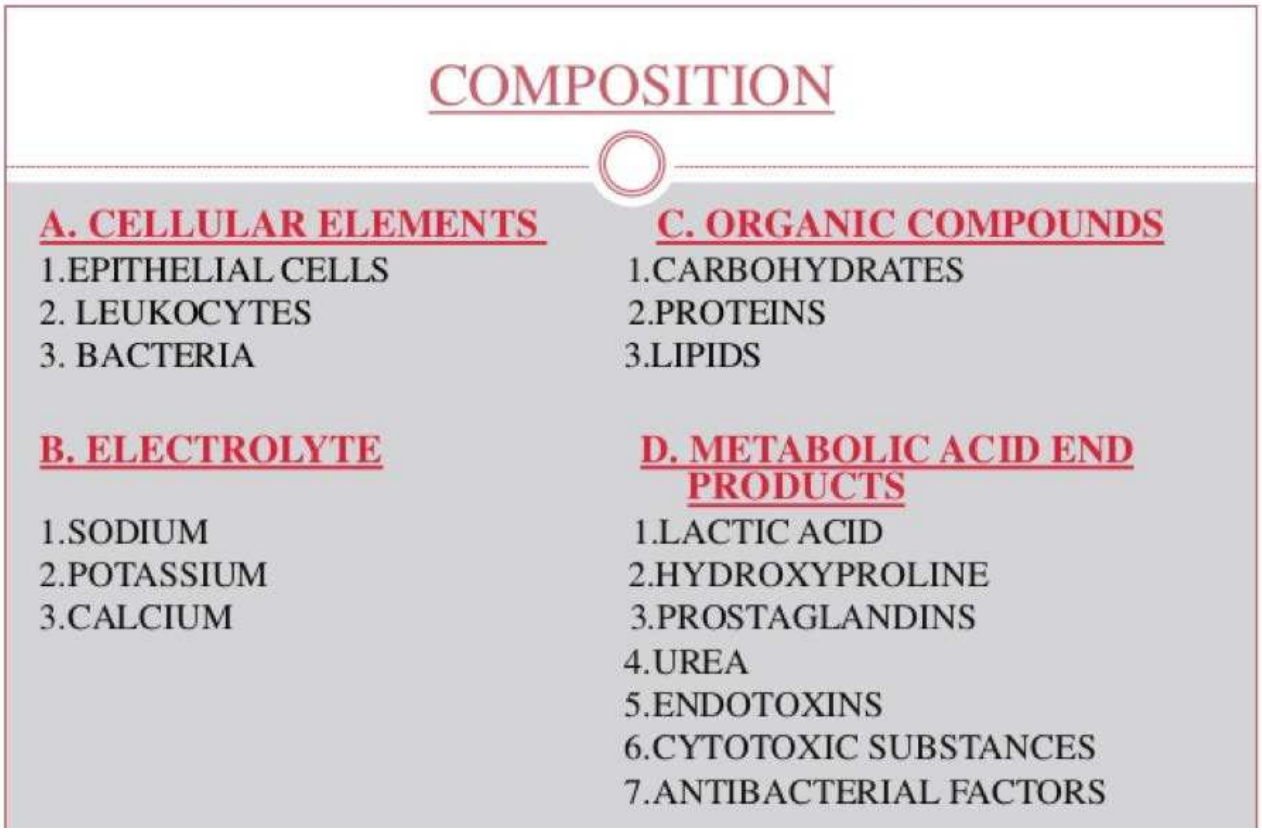


Fig. 8 Crevicular fluid composition

Analysis of crevicular fluid becomes important to detect periodontal pathogens or to measure inflammation indeces in presence of a chronic inflammatory disease.

1.5 Crevicular Fluid collection

The crevicular fluid is generally collected by use of nitrocellulose paper cones or filter paper strips inserted in the crevice for about one minute in order to become soaked for capillarity. These methods are quick, easy to use and not-traumatic. Moreover, these techniques and can be applied for a superficial (in the upper part

of the crevice) or a deep (the cones are inserted and pushed into the crevice until a minimum of resistance is felt) collection[4].

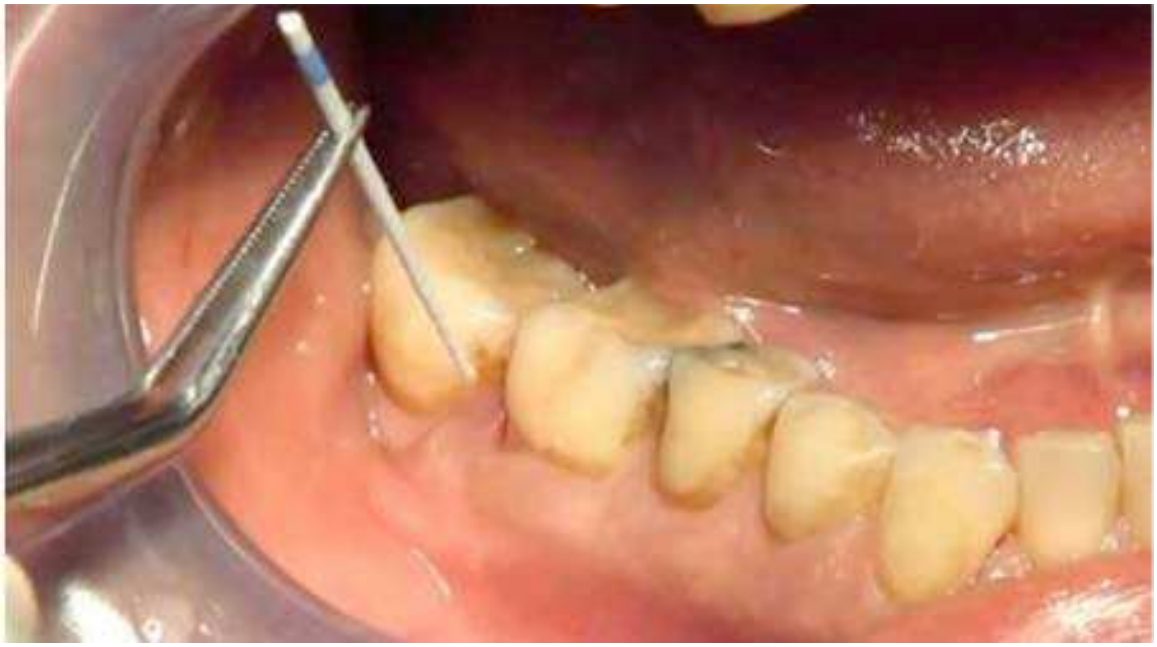


Fig. 9a Crevicular fluid collection with a paper cone

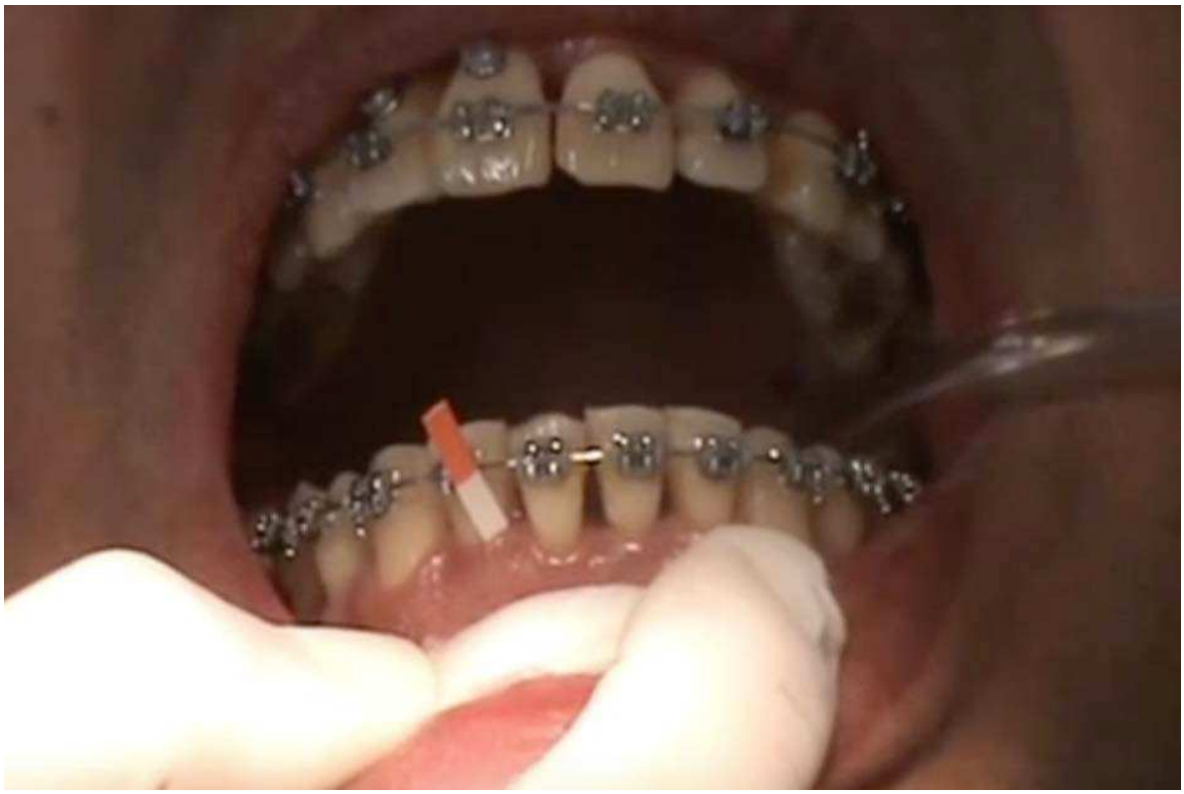


Fig. 9b Crevicular fluid collection with a paper strip

In the present study sterile, endodontic, medium size (diameter 0.30 mm) paper cones were inserted in the gingival sulcus for 60 seconds in order to collect crevicular fluid samples.

An alternative is represented by the “washing method”, which uses the installation and continuous reaspiration of specific solutions (Hanks' balanced salt solution31 or PBS 32 at the gingival crevice); in this case a specific instrument is required: it is composed by two injection needles fitted one within the other. The thinner "ejection needle" is at the bottom of the periodontal pocket and the "collection needle" at the gingival margin. A special solution is ejected into the crevice and immediately drained through the collection needle into a sample tube by continuous suction [5].

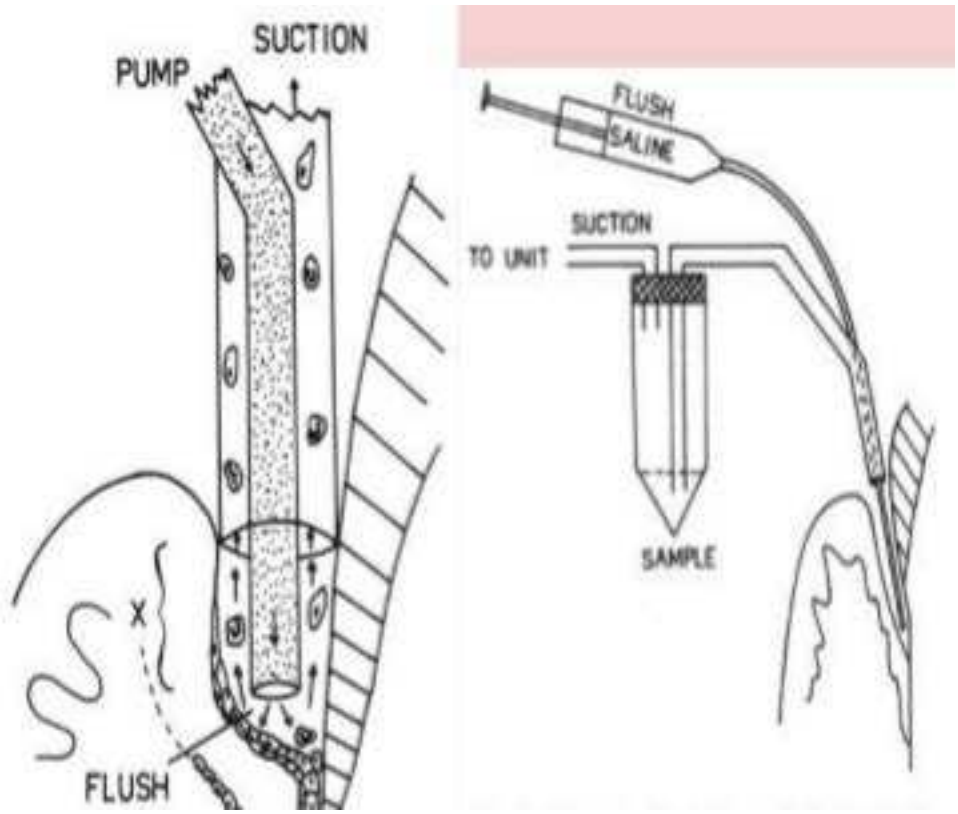


Fig. 10 Schematic illustration of the washing method for crevicular fluid sampling

Although this technique is highly sensitive, it requires training and experience, since it is not easy to be apllied.

1.6 Periodontal Anatomy

The term "periodontium" usually refers to the area around the tooth (Peri = around, Odontos= tooth) whose main function is to to keep the tooth attached to the tissues of the the jawbone, thus allowing the masticatory function.
It is a dynamic structure that is involved in a continuous, longlife, transforming process, which is remodels naturally in relation to age, chewing and oral environment conditions.

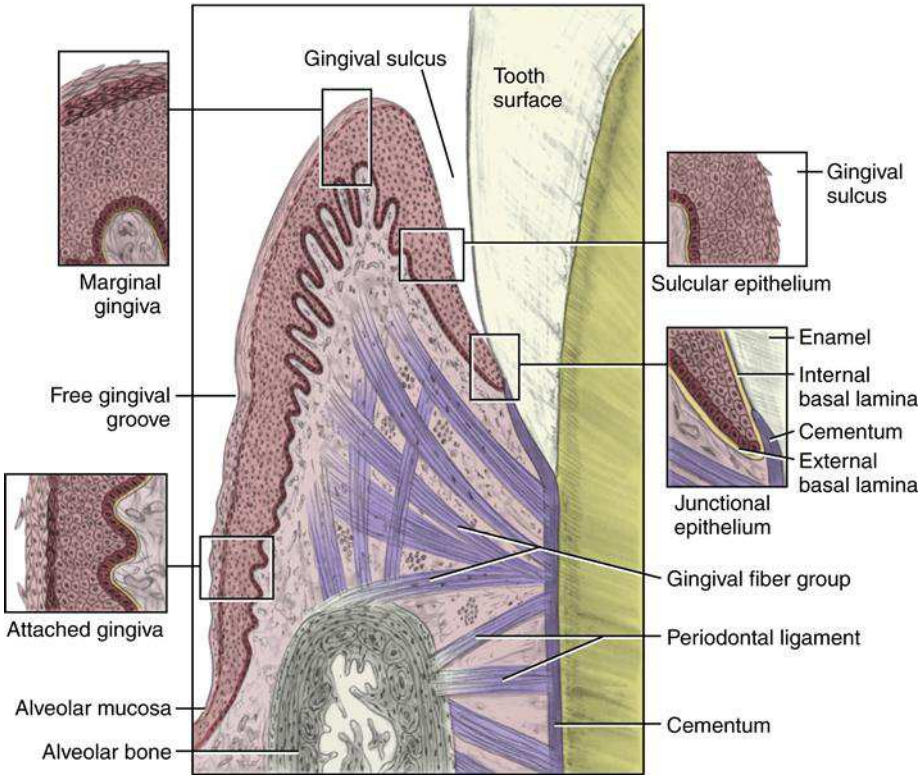


Fig. 11 Schematic representation of periodontum

The periodontium is composed by hard tissues (root cement and alveolar bone) and soft tissues (periodontal ligament and gum).

Gingiva

Gum is only a part of the entire oral mucosa, which cover the oral cavity.

In particular oral mucosa can be divided in:

- Masticatory mucosa (so called because this tissue can withstand the impact of food during chewing) formed by keratinized stratified squamous epithelium, which can be found on the dorsum of the tongue, hard palate and attached gingiva.
- Lining mucosa, non-keratinized stratified squamous epithelium, covering the remaining part of the oral cavity (it can be further divided into buccal mucosa, labial mucosa, alveolar mucosa)
- Specialized mucosa, which can be observed only on the dorsal surface of the tongue in those regions containing taste buds, essential for taste perception.

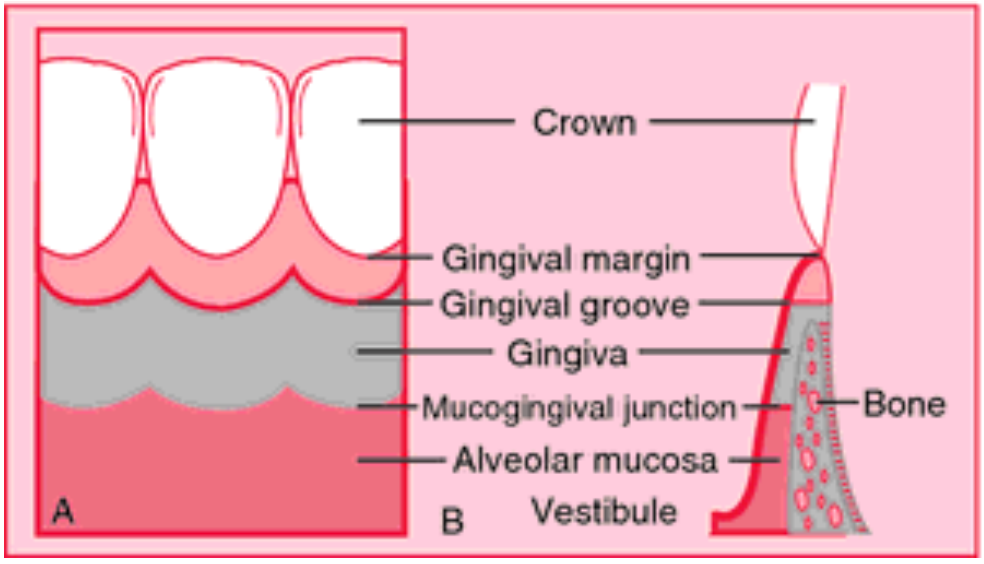


Fig. 12 Anatomical relationship of normal gingiva in facial view (A)and in cross-section (B). From Darby and Walsh, 1994.

Gum is the part of masticatory mucosa which covers the alveolar processes and surrounds the cervical part of a tooth.

In coronal direction it ends next to the tooth surface with the gingival margin, while in apical direction it is separated from the lining mucosa by means of a clearly recognizable boundary line called mucogingival junction (in the palate a mucogingival line can not be observed because the hard palate and the maxillary alveolar process are both covered by masticatory gums).

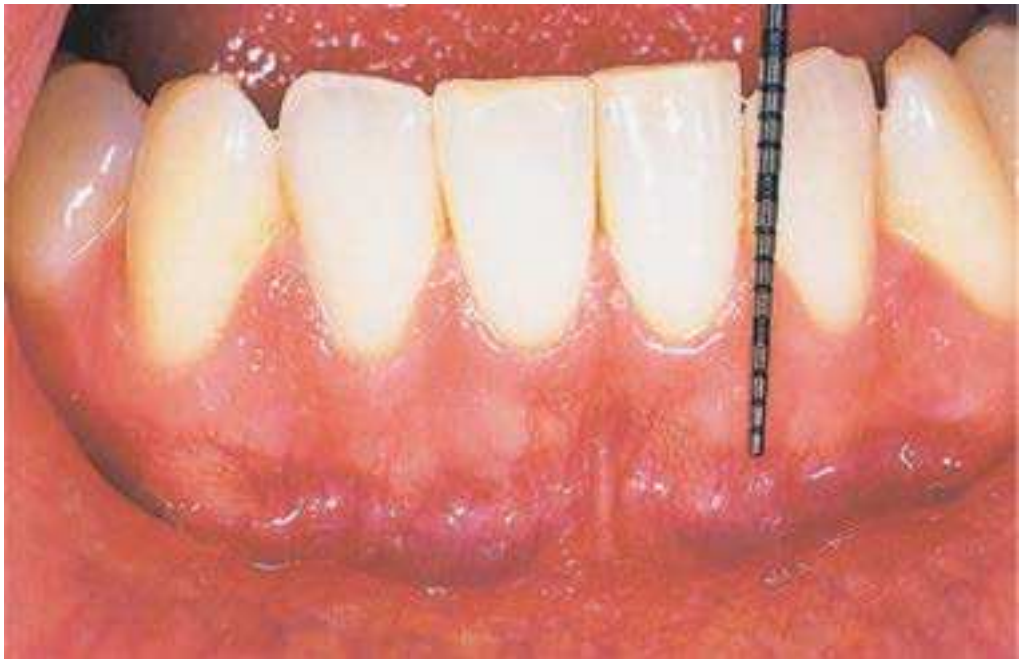


Fig. 13 Dental probe indicating the mucogingival junction

While alveolar mucosa is mobile over deep tissues and is red-colored, gingiva appears pink and can be divided into free gum and adherent gum.

The *adherent gingiva* is delimited by gingival groove in the coronal direction and by mucogingival junction in apical direction.
It shows a compact consistency, coral pink color and is firm and resilient, since it is bound to the underlying cementum and bone.

The *free gum* is delimited in the apical direction by gingival groove and in coronal direction by gingival mrgin. Free gingiva also includes oral tissue in the interdental spaces called papilla.

The shape of the interdental papilla is determined by the contact area between teeth.

Fig. 14 Free gingiva between teeth is called papilla

In the anterior regions the papillae have a pyramidal shape, since the teeth have single contact points in correspondence of the approximal surfaces. However in premolar and molar regions teeth show contact surfaces and papillae have a blunted shape.

Periodontal Ligament

The periodontal ligament is a group of specialized connective tissue fibers, inserting into root cementum on one side and onto alveolar bone on the other [6]. It has an “hourglass shape” with its narrower part positioned at the middle of root height, where its width is between 0.2 and 0.4 mm. This tissue works as a shock absorber; it allows the distribution of chewing forces to alveolar bone: the

pressure exerted on the tooth by chewing leads to a stretching of fibers , converting the masticatory pressure into a traction onto the cement and bone.

It consists of cells, nerve fibers, blood and lymphatic vessels, inserted in an extracellular matrix mainly formed by connective fibers with different orientation:

- Alveolar crest fibers, running from the crestal portion of the root to the alveolar ridge
- Horizontal fibers, located in the coronal portion of the ligament
- Oblique fibers, running from the root in coronal direction towards the alveolar bone
- Apical fibers, running from the apex of the root to the bottom of the alveolus with various orientations.

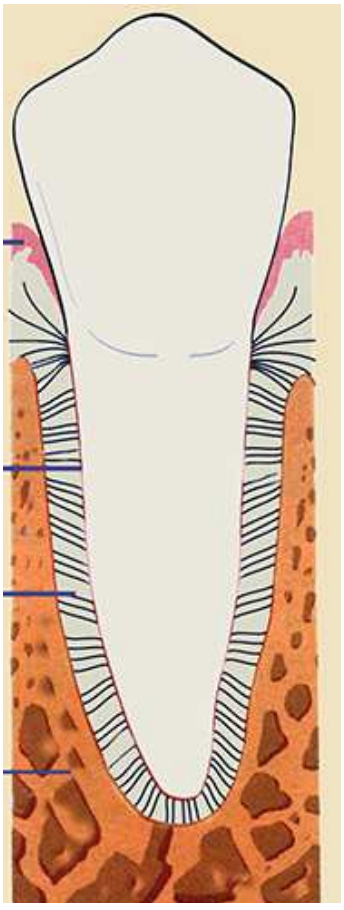


Fig. 15 Periodontal fibres orientation

These main fibers develop simultaneously with the eruption of the tooth and their orientation varies continuously throughout the eruption phase; only at the end of this process a stabilization occurs, however constant remodelling (with reabsorption of the old fibres and the formation of the new ones) is always observed.
The main fibres, penetrating the cement on one side and alveolar bone on the other, are called Sharpey fibres. In addition, some elastic fibres associated with blood vessels and oxitalanic fibres have been described.

Periodontal cells are: fibroblasts aligned along the main fibers, osteoblasts on the bone surface and cementoblasts on the root surface, epithelial cells (Malassez residues), nerve fibres, endothelial cells, macrophages, eosinophils and mastocells.

Radicular Cement

Radicular cement is a calcified tissue that covers the surface of the tooth root; it consists of collagen fibres in an organic matrix and hydroxyapatite (about 65% of its weight). It is linked to the fibres of the periodontal ligament, fixing them to the tooth and contributes to the repair processes of the root. The thickness of this tissue increases with age (it may range from 0.05 to 0.6 mm) . It is thicker apically than cervically.

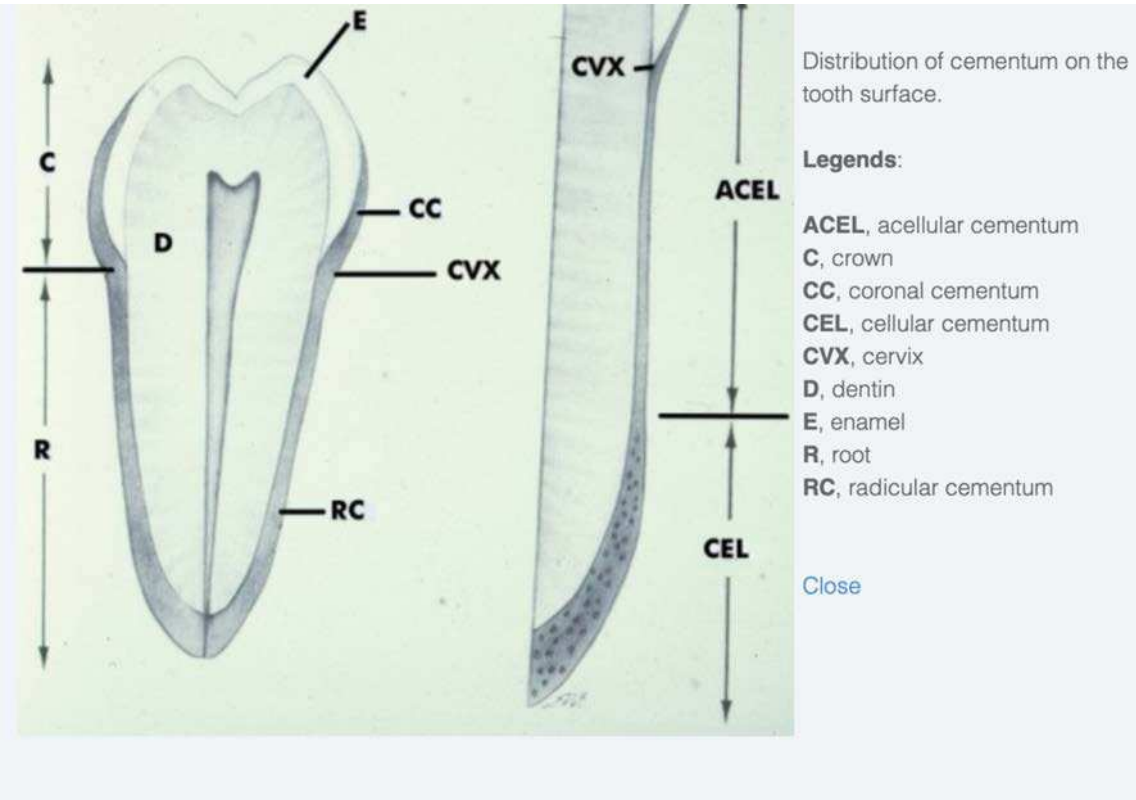


Fig. 16 Distribution of cementum on the tooth surface

Cementum may be classified in the following ways [7]:

1) By location:

- *Radicular cementum*: cementum that is found on the root surface.
- *Coronal cementum*: cementum that forms on the enamel covering the crown.

2) By cellularity:

- *Cellular cementum*: cementum containing cementocytes in lacunae within the cementum matrix
- *Acellular cementum*: cementum without any cells in its matrix

3) By the presence of collagen fibrils in the matrix:

- *Fibrillar cementum*: cementum with a matrix that contains well-defined type I collagen fibrils
- *Afibrillar cementum*: cementum that has a matrix devoid of detectable type I collagen fibrils. Instead, the matrix tends to have a fine, granular consistency.

4) By the origin of the matrix fibers :

- *Extrinsic fiber cementum*: cementum containing primarily extrinsic fibers (i.e. Sharpey's fibers that are continuous with the principal fibers of the periodontal ligament; they are produced by periodontal ligament fibroblasts). Fibers orientation is perpendicularly to the cementum surface; it has a role in tooth anchorage.
- *Intrinsic fiber cementum*: cementum that contains primarily intrinsic fibers, (i.e. fibers produced by cementoblasts and oriented parallel to the cementum surface). It is located predominantly in areas where repairing processes are occurring, after surface resorption.
- *Mixed fiber cementum*: cementum that contains a mixture of extrinsic and intrinsic fiber cementum.

<u>The alveolar bone</u>

The alveolar process is that part of the jaw and the mandible which contains teeth. Only the thin layer of compact bone forming the wall of a dental alveolus is

considered a part of periodontum and it is called “bundle bone”. It is linked with lingual and buccal cortical bones of the alveolar process, while the remaining middle area is occupied by cancellous bone.

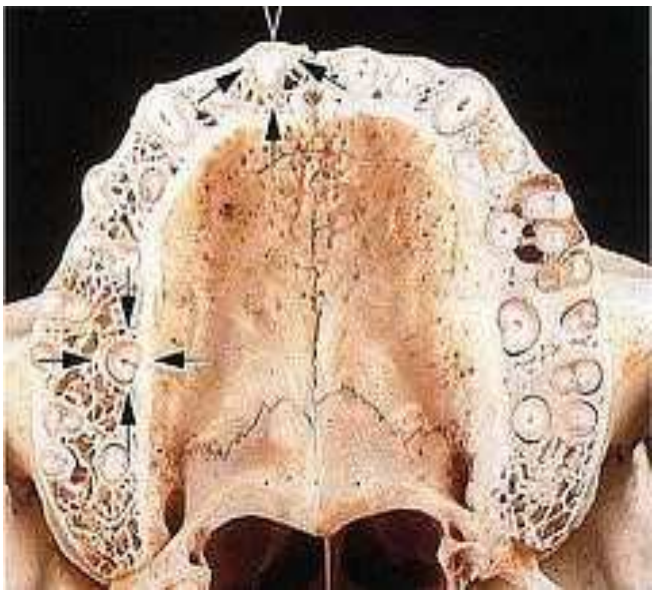


Fig. 17 Alveolar bone of upper jaw, with arrows indicating bundle bone

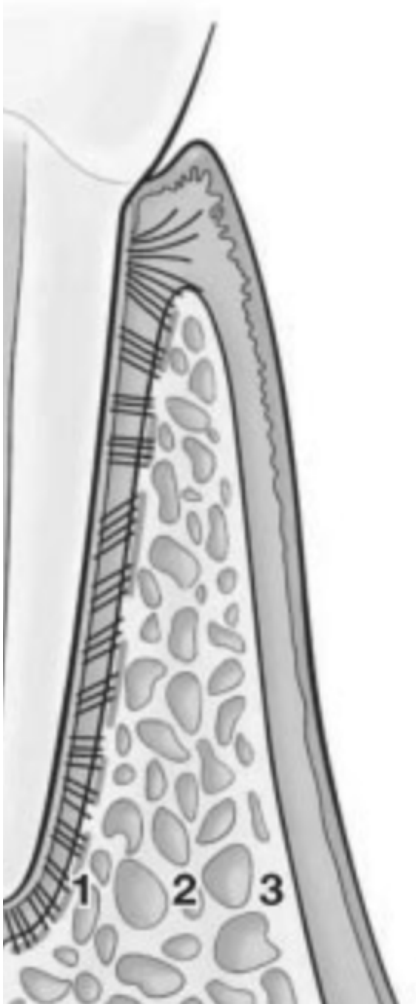


Fig. 18 Alveolar bone. 1 Bundle bone, 2 Trabecular bone, 3 cortical bone

The bundle bone is peforated by several small channels, known as Volkmann channels, through which blood vessels, lymphatics and nerve fibers pass from the alveolar bone to the periodontal ligament. The bundle bone disappears when a tooth is extracted.

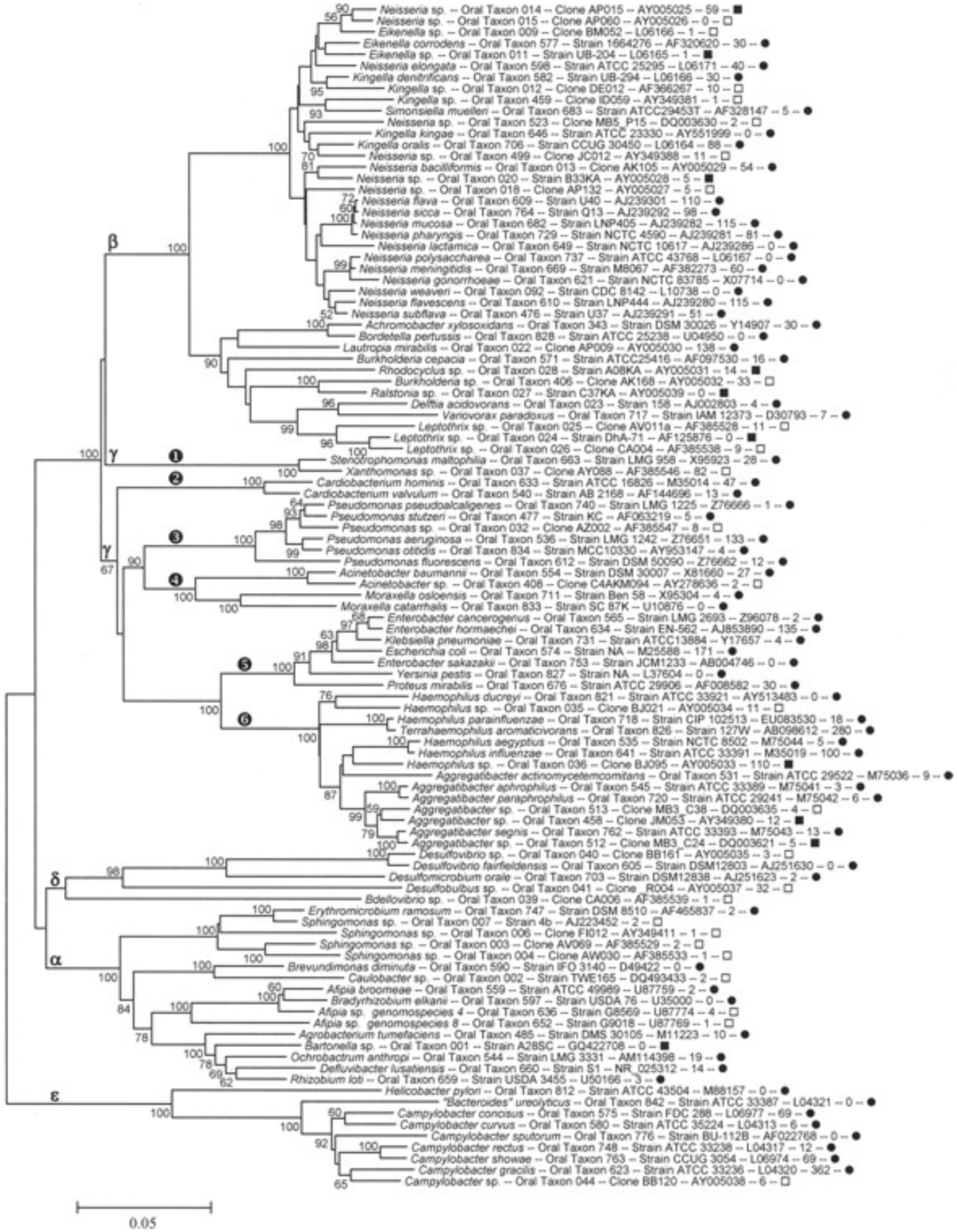
Thickness of the alveolar bone varies from region to region, for exemple it can be thin in the incisal vestibular region, while it is generally thick in molar vestibular region of the mandible .The entire alveolar bone is continually renewed and remodelled by osteobalsts and osteoclasts according to functional needs.

1.7 Oral Microbiota

The oral cavity is a complex ecosystem, characterized by the presence of different habitats, such as teeth, tongue, gums, cheeks , gingival sulcus, tonsils. These areas are populated by opportunistic microorganisms perfectly adapted to the environment, such as Protozoa, Mycetes, Viruses and, above all, Bacteria. Only 280 bacterial species have been isolated in culture from the oral cavity, since they can be cultivated by means of traditional microbiological method, but the total number of species is thought to be between 500 and 700 [8].

The Human Oral Microbiome Database (HOMD) includes 619 taxa in 13 phyla, as follows: Actinobacteria, Bacteroidetes, Chlamydiae, Chloroflexi, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes, SR1, Synergistetes, Tenericutes, and TM7.

According to a recent study, the six major phyla (Spirochaetes , Fusobacteria, Proteobacteria, Actinobacteria, Bacteroidetes and Firmicutes) contain 96% of the taxa. The remaining phyla (Chlamydia, SR1, TM7, Euryarchaeota, Synergistetes, Tenericutes and Chloroflexi) contain the remaining 4% of the taxa [9].


Neisseria sp. -- Oral Taxon 014 -- Clone AP015 -- AY005025 -- 59 --
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Eikenella sp. -- Oral Taxon 009 -- Clone BM052 -- L06166 -- 1 --
Eikenella corrodens -- Oral Taxon 577 -- Strain 1664276 -- AF320620 -- 30 --
Eikenella sp. -- Oral Taxon 011 -- Strain UB-204 -- L06165 -- 1 --
Neisseria elongata -- Oral Taxon 598 -- Strain ATCC 25295 -- L06171 -- 40 --
Kingella denitrificans -- Oral Taxon 582 -- Strain UB-294 -- L06166 -- 30 --
Kingella sp. -- Oral Taxon 012 -- Clone DE012 -- AF366267 -- 10 --
Kingella sp. -- Oral Taxon 459 -- Clone ID059 -- AY349381 -- 1 --
Simonsiella muelleri -- Oral Taxon 683 -- Strain ATCC29453T -- AF328147 -- 5 --
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Kingella oralis -- Oral Taxon 706 -- Strain CCUG 30450 -- L06164 -- 88 --
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Neisseria subflava -- Oral Taxon 476 -- Strain U37 -- AJ239291 -- 51 --
Achromobacter xylosoxidans -- Oral Taxon 343 -- Strain DSM 30026 -- Y14907 -- 30 --
Bordetella pertussis -- Oral Taxon 828 -- Strain ATCC 25238 -- U04950 -- 0 --
Lautropia mirabilis -- Oral Taxon 022 -- Clone AP009 -- AY005030 -- 138 --
Burkholderia cepacia -- Oral Taxon 571 -- Strain ATCC25416 -- AF097530 -- 16 --
Rhodocyclus sp. -- Oral Taxon 028 -- Strain A08KA -- AY005031 -- 14 --
Burkholderia sp. -- Oral Taxon 406 -- Clone AK168 -- AY005032 -- 33 --
Ralstonia sp. -- Oral Taxon 027 -- Strain C37KA -- AY005039 -- 0 --
Delftia acidovorans -- Oral Taxon 023 -- Strain 158 -- AJ002803 -- 4 --
Variovorax paradoxus -- Oral Taxon 717 -- Strain IAM 12373 -- D30793 -- 7 --
Leptothrix sp. -- Oral Taxon 025 -- Clone AV011a -- AF385528 -- 11 --
Leptothrix sp. -- Oral Taxon 024 -- Strain DhA-71 -- AF125876 -- 0 --
Leptothrix sp. -- Oral Taxon 026 -- Clone CA004 -- AF385538 -- 9 --
Stenotrophomonas maltophilia -- Oral Taxon 663 -- Strain LMG 958 -- X95923 -- 28 --
Xanthomonas sp. -- Oral Taxon 037 -- Clone AY088 -- AF385546 -- 82 --
Cardiobacterium hominis -- Oral Taxon 633 -- Strain ATCC 16826 -- M35014 -- 47 --
Cardiobacterium valvulum -- Oral Taxon 540 -- Strain AB 2168 -- AF144696 -- 13 --
Pseudomonas pseudoalcaligenes -- Oral Taxon 740 -- Strain LMG 1225 -- Z76666 -- 1 --
Pseudomonas stutzeri -- Oral Taxon 477 -- Strain KC -- AF063219 -- 5 --
Pseudomonas sp. -- Oral Taxon 032 -- Clone AZ002 -- AF385547 -- 8 --
Pseudomonas aeruginosa -- Oral Taxon 536 -- Strain LMG 1242 -- Z76651 -- 133 --
Pseudomonas otitidis -- Oral Taxon 834 -- Strain MCC10330 -- AY953147 -- 4 --
Pseudomonas fluorescens -- Oral Taxon 612 -- Strain DSM 50090 -- Z76662 -- 12 --
Acinetobacter baumannii -- Oral Taxon 554 -- Strain DSM 30007 -- X81660 -- 27 --
Acinetobacter sp. -- Oral Taxon 408 -- Clone C4AKM094 -- AY278636 -- 2 --
Moraxella osloensis -- Oral Taxon 711 -- Strain Ben 58 -- X95304 -- 4 --
Moraxella catarrhalis -- Oral Taxon 833 -- Strain SC 87K -- U10876 -- 0 --
Enterobacter cancerogenus -- Oral Taxon 565 -- Strain LMG 2693 -- Z96078 -- 2 --
Enterobacter hormaechei -- Oral Taxon 634 -- Strain EN-562 -- AJ853890 -- 135 --
Klebsiella pneumoniae -- Oral Taxon 731 -- Strain ATCC13884 -- Y17657 -- 4 --
Escherichia coli -- Oral Taxon 574 -- Strain NA -- M25588 -- 171 --
Enterobacter sakazakii -- Oral Taxon 753 -- Strain JCM1233 -- AB004746 -- 0 --
Yersinia pestis -- Oral Taxon 827 -- Strain NA -- L37604 -- 0 --
Proteus mirabilis -- Oral Taxon 676 -- Strain ATCC 29906 -- AF008582 -- 30 --
Haemophilus ducreyi -- Oral Taxon 821 -- Strain ATCC 33921 -- AY513483 -- 0 --
Haemophilus sp. -- Oral Taxon 035 -- Clone BJ021 -- AY005034 -- 11 --
Haemophilus parainfluenzae -- Oral Taxon 718 -- Strain CIP 102513 -- EU083530 -- 18 --
Terrahaemophilus aromaticivorans -- Oral Taxon 826 -- Strain 127W -- AB098612 -- 280 --
Haemophilus aegyptius -- Oral Taxon 535 -- Strain NCTC 8502 -- M75044 -- 5 --
Haemophilus influenzae -- Oral Taxon 641 -- Strain ATCC 33391 -- M35019 -- 100 --
Haemophilus sp. -- Oral Taxon 036 -- Clone BJ095 -- AY005033 -- 110 --
Aggregatibacter actinomycetemcomitans -- Oral Taxon 531 -- Strain ATCC 29522 -- M75036 -- 9 --
Aggregatibacter aphrophilus -- Oral Taxon 545 -- Strain ATCC 33389 -- M75041 -- 3 --
Aggregatibacter paraphrophilus -- Oral Taxon 720 -- Strain ATCC 29241 -- M75042 -- 6 --
Aggregatibacter sp. -- Oral Taxon 513 -- Clone MB3_C38 -- DQ003635 -- 4 --
Aggregatibacter sp. -- Oral Taxon 458 -- Clone JM053 -- AY349380 -- 12 --
Aggregatibacter segnis -- Oral Taxon 762 -- Strain ATCC 33393 -- M75043 -- 13 --
Aggregatibacter sp. -- Oral Taxon 512 -- Clone MB3_C24 -- DQ003621 -- 5 --
Desulfovibrio sp. -- Oral Taxon 040 -- Clone BB161 -- AY005035 -- 3 --
Desulfovibrio fairfieldensis -- Oral Taxon 605 -- Strain DSM12803 -- AJ251630 -- 0 --
Desulfomicrobium orale -- Oral Taxon 703 -- Strain DSM12838 -- AJ251623 -- 2 --
Desulfobulbus sp. -- Oral Taxon 041 -- Clone _R004 -- AY005037 -- 32 --
Bdellovibrio sp. -- Oral Taxon 039 -- Clone CA006 -- AF385539 -- 1 --
Erythromicrobium ramosum -- Oral Taxon 747 -- Strain DSM 8510 -- AF465837 -- 2 --
Sphingomonas sp. -- Oral Taxon 007 -- Strain 4b -- AJ223452 -- 2 --
Sphingomonas sp. -- Oral Taxon 006 -- Clone FI012 -- AY349411 -- 1 --
Sphingomonas sp. -- Oral Taxon 003 -- Clone AV069 -- AF385529 -- 2 --
Sphingomonas sp. -- Oral Taxon 004 -- Clone AW030 -- AF385533 -- 1 --
Brevundimonas diminuta -- Oral Taxon 590 -- Strain IFO 3140 -- D49422 -- 0 --
Caulobacter sp. -- Oral Taxon 002 -- Strain TWE165 -- DQ493433 -- 2 --
Afipia broomeae -- Oral Taxon 559 -- Strain ATCC 49989 -- U87759 -- 2 --
Bradyrhizobium elkanii -- Oral Taxon 597 -- Strain USDA 76 -- U35000 -- 0 --
Afipia sp. genomospecies 4 -- Oral Taxon 636 -- Strain G8569 -- U87774 -- 4 --
Afipia sp. genomospecies 8 -- Oral Taxon 652 -- Strain G9018 -- U87769 -- 1 --
Agrobacterium tumefaciens -- Oral Taxon 485 -- Strain DMS 30105 -- M11223 -- 10 --
Bartonella sp. -- Oral Taxon 001 -- Strain A28SC -- GQ422708 -- 0 --
Ochrobactrum anthropi -- Oral Taxon 544 -- Strain LMG 3331 -- AM114398 -- 19 --
Defluvibacter lusatiensis -- Oral Taxon 660 -- Strain S1 -- NR_025312 -- 14 --
Rhizobium loti -- Oral Taxon 659 -- Strain USDA 3455 -- U50166 -- 3 --
Helicobacter pylori -- Oral Taxon 812 -- Strain ATCC 43504 -- M88157 -- 0 --
"Bacteroides" ureolyticus -- Oral Taxon 842 -- Strain ATCC 33387 -- L04321 -- 0 --
Campylobacter concisus -- Oral Taxon 575 -- Strain FDC 288 -- L06977 -- 69 --
Campylobacter curvus -- Oral Taxon 580 -- Strain ATCC 35224 -- L04313 -- 6 --
Campylobacter sputorum -- Oral Taxon 776 -- Strain BU-112B -- AF022768 -- 0 --
Campylobacter rectus -- Oral Taxon 748 -- Strain ATCC 33238 -- L04317 -- 12 --
Campylobacter showae -- Oral Taxon 763 -- Strain CCUG 3054 -- L06974 -- 69 --
Campylobacter gracilis -- Oral Taxon 623 -- Strain ATCC 33236 -- L04320 -- 362 --
Campylobacter sp. -- Oral Taxon 044 -- Clone BB120 -- AY005038 -- 6 --
β
γ
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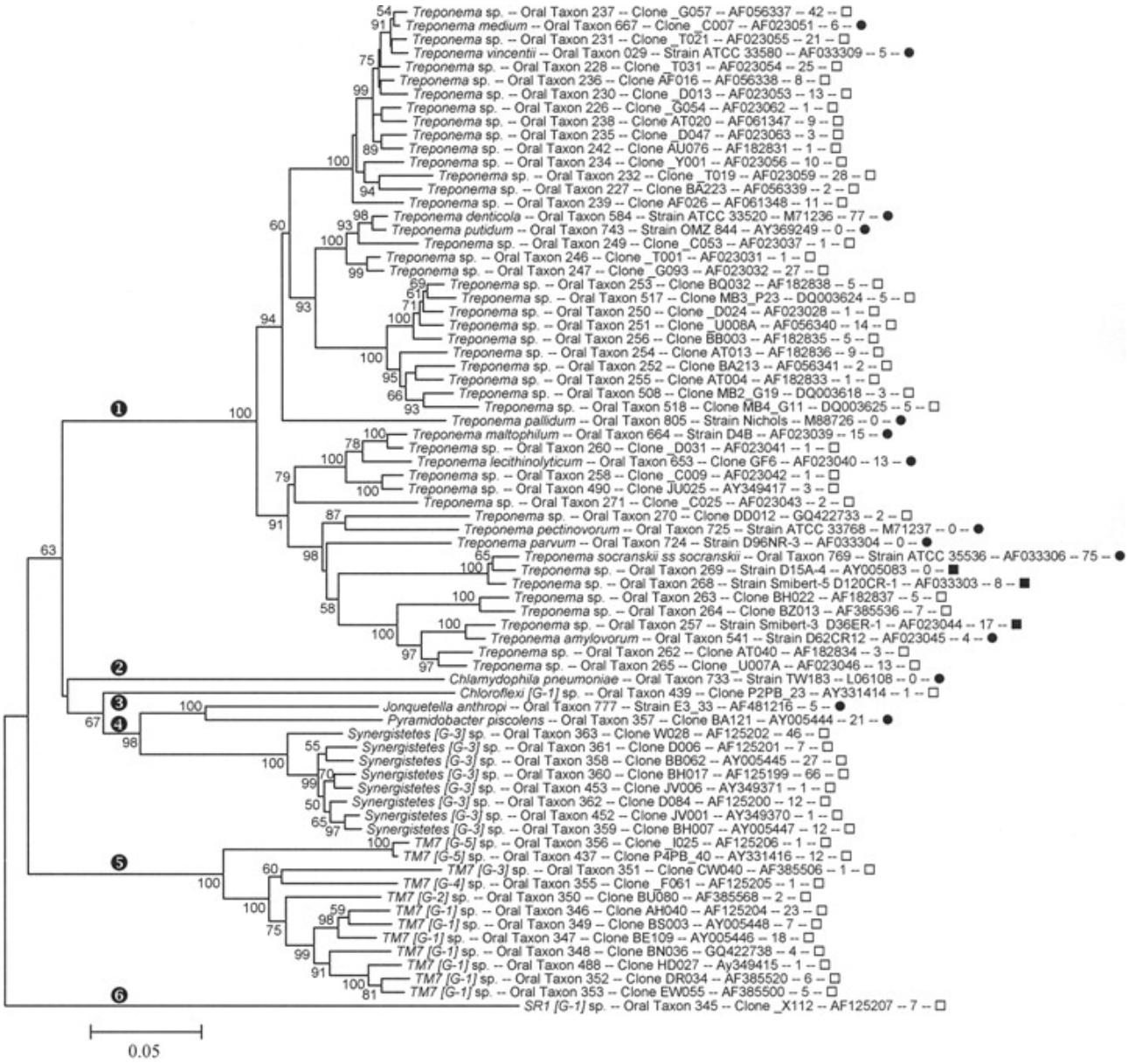


Fig. 19 Part of the Neighbor-joining tree for human oral taxa, from Human Oral DataBase [9]

This complex ecosystem is also characterized by a high dynamism, due to the continuous elimination and introduction of liquids and food. Although a rather constant resident bacterial population is present, some microorganisms that usually colonize other sites of the host can occasionally be detected in this area.

Factors influencing and modifying the oral ecosystem are: presence of plaque, oral hygiene, dental treatments, personal physical conditions and diet.

1.8 Bacterial Biofilm

In the past only individual microorganisms were considered responsible for a specific disease, so they were analyzed in pure cultures, according to well-known Koch's postulates. The discovery of complex biofilms over most of human and inanimate surfaces has completely changed the target of modern research.
Nowadays most of oral diseases, such as caries, periodontitis and other infections are known to be caused by multiple microrganisms, organized in a biofilm rather than by a single bacterium [10].

Bacterial biofilm is a micro-community composed by bacteria within a polysaccharide matrix, that coexist and iteract together.
The biofilm adheres intimately to the dental surface thanks to the presence of the "aquired enamel pellicle", which is a protein film formed on the enamel surface by selective binding of salivar glycoproteins immediatly after a tooth is cleaned or after chewing. This creates the necessary substrate for the adhesion of early-bacterial microorganisms. These first ones are basically aerobic or facultative anaerobic bacteria, such as streptococci, and they begin to subtract oxygen and produce carbon dioxide and waste elements, which become food for new bacteria. Secondary colonizers attach to receptors positioned on already attached bacteria (coadhesion). Furthermore, bacteria start synthesizing several polymers and creating a matrix, which is more than a structural passive scaffold because it can retain and bind molecules and enzymes [11].

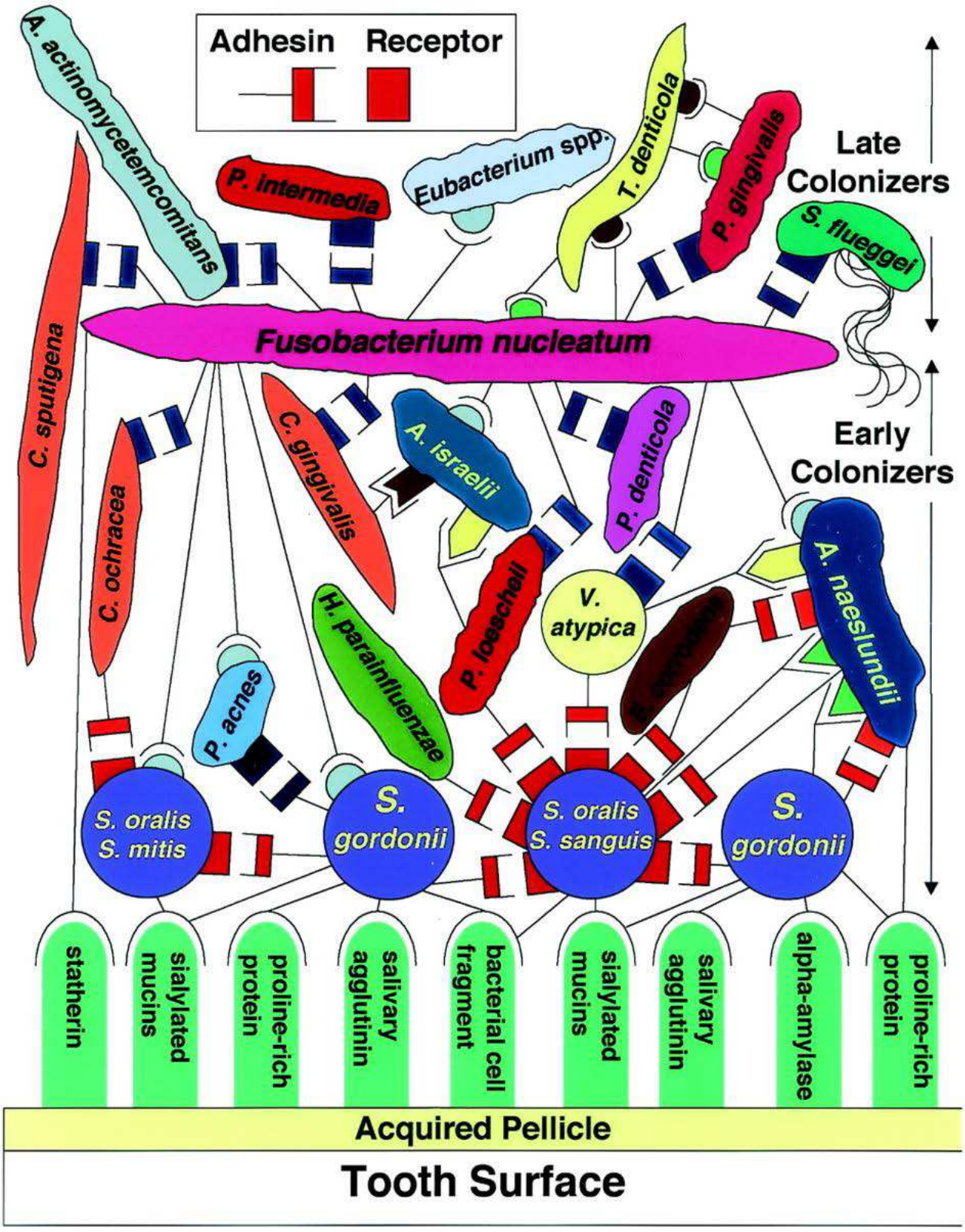


Fig. 20 Oral biofilm over tooth surface

Living in community, some bacteria acquire the ability to synthesize new enzymes capable of breaking down antibacterial molecules and so they become

tolerant to drugs and host defenses.

As the biofilm becomes structurally and functionally organized and the periodontal pocket keeps getting deeper and deeper , favourable conditions stimulate the accumulation of dangerous periodontopathic anaerobic pathogens, such as Prevotella, Porphyromonas, Fusobacterium, Treponema, and Tannerella.

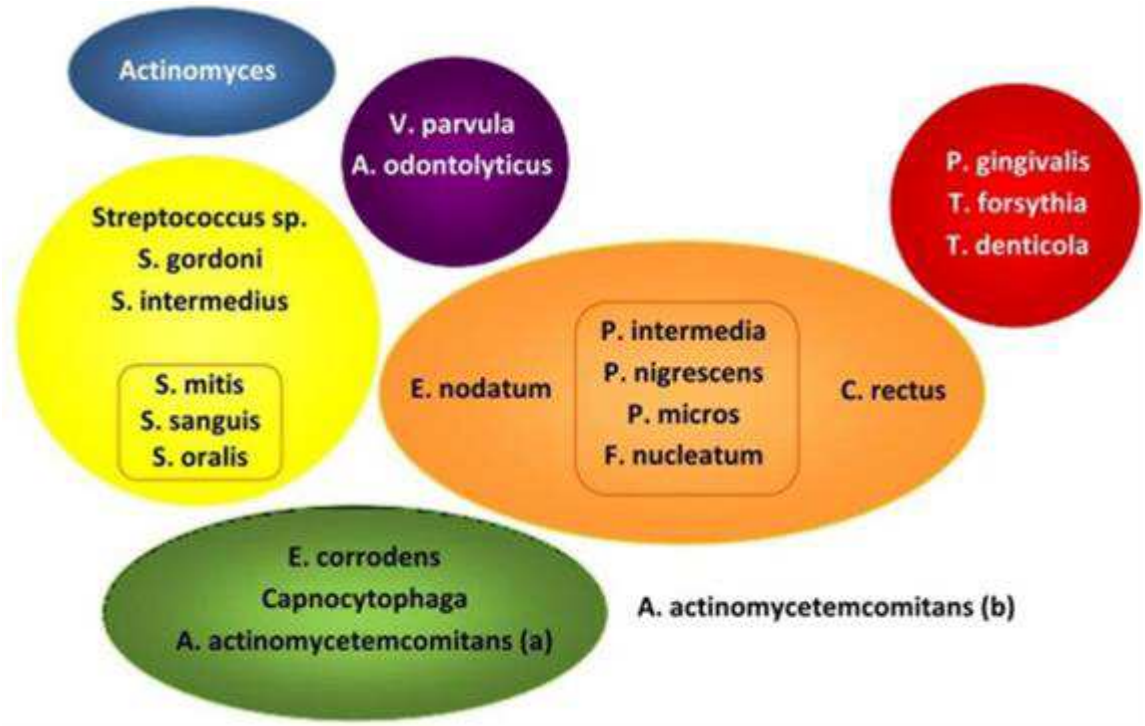


Fig. 21 Subgingival Microbial complexes [12].

1.9 Periodontal Disease

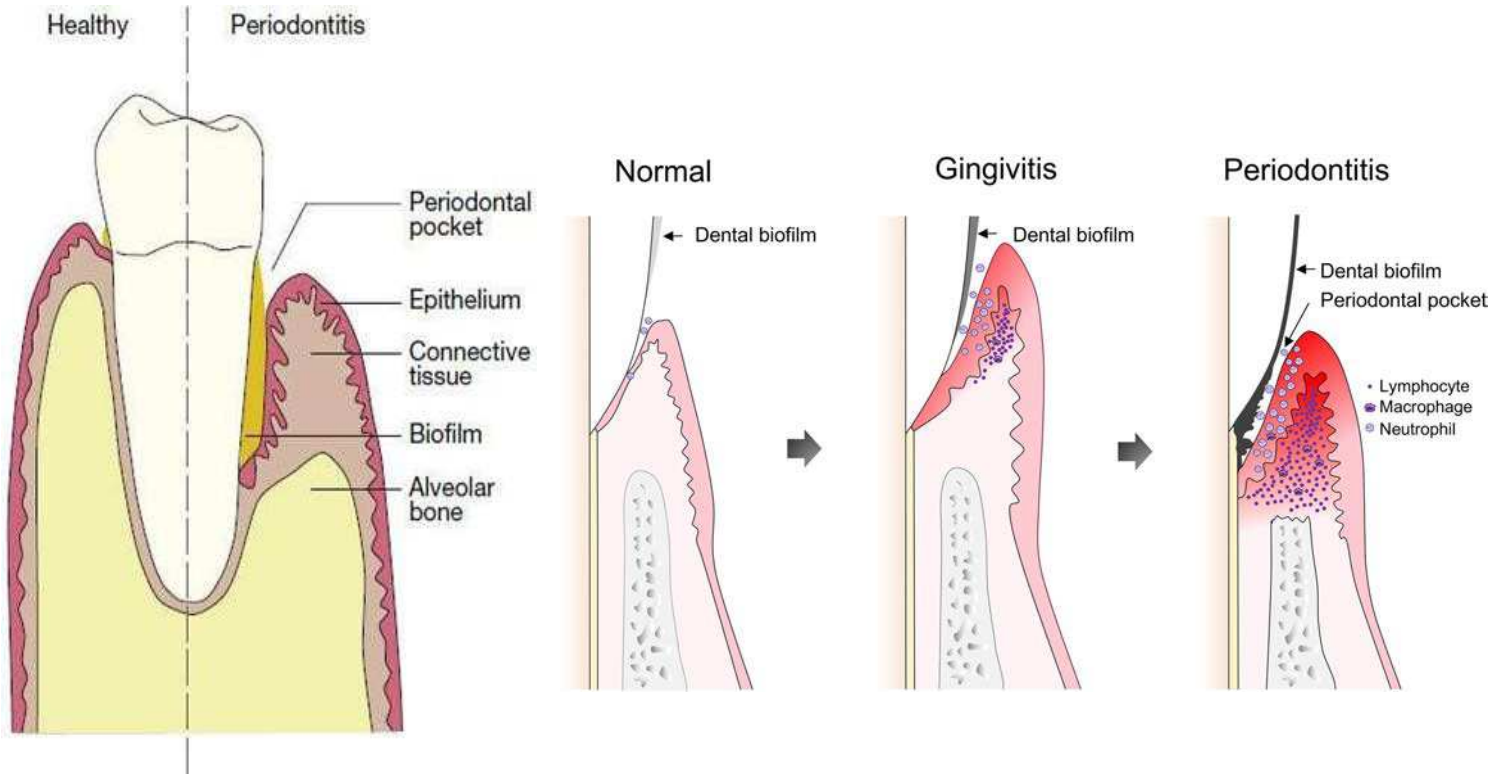
Periodontal disease is one of the most common oral disease, affecting globally about half of the adult population. It is an infectious inflammatory multifactorial disease caused by several bacterial strains present in dental plaque.

In physiological conditions the oral cavity is colonized by a huge number of bacteria, living togheter in polymicrobial communities called biofilm. Although early theories focusing on identifying a single bacterium responsible for oral diseases such as dental caries and chronic periodontitis, it is now generally accepted that these conditions result from the concerted actions of multispecies microbial communities [13].

In case of periodontal disease a microbial shift can be observed: an increase in the number of pathogens from one side and a decrease in the number of symbionts on the other. However it is not possible to identify a single and specific infectious bacterium responsible for this process.

Among oral bacteria , expecially those belonging to "red" complex (Treponema denticola, Porphyromonas gingivalis, and Bacteroides forsythus) and "orange" complex (Prevotella intermedia, Prevotella nigrescens, Peptostreptococcus micros, F. nucleatum subspecies, Eubacterium nodatum, Streptococcus constellatus, and three Campylobacter species) are considered dangerous in development of the disease.

Periodontitis is the major cause of tooth loss in the adult population of industrialized countries with esthetic consequences and damages in masticatory and phonatory functions. The prevalence of periodontal disease in the population seems to vary according to race, geographical area and age (it can be rarely observed in pediatric age but this disease affects about 30% of adult population).



A **B**

Fig. 22 A: Periodotal pocket and healty tissue; B: Gingivitis-periodontitis progression

The classification of periodontal diseases proposed in 1993 by the European Federation of Periodontology was modified in 1999 at the International Workshop in collaboration with the American Academy of Periodontology .

I. Gingival Diseases
   A. Dental plaque-induced gingival diseases
   B. Non-plaque-induced gingival lesions

II. Chronic Periodontitis
   (slight: 1-2 mm CAL; moderate: 3-4 mm CAL;
   severe: > 5 mm CAL)
   A. Localized
   B. Generalized (> 30% of sites are involved)

III. Aggressive Periodontitis
   (slight: 1-2 mm CAL; moderate: 3-4 mm CAL;
   severe: > 5 mm CAL)
   A. Localized
   B. Generalized (> 30% of sites are involved)

IV. Periodontitis as a Manifestation of Systemic Diseases
   A. Associated with hematological disorders
   B. Associated with genetic disorders
   C. Not otherwise specified

V. Necrotizing Periodontal Diseases
   A. Necrotizing ulcerative gingivitis
   B. Necrotizing ulcerative periodontitis

VI. Abscesses of the Periodontium
   A. Gingival abscess
   B. Periodontal abscess
   C. Pericoronal abscess

VII. Periodontitis Associated With Endodontic Lesions
   A. Combined periodontic-endodontic lesions

VIII. Developmental or Acquired Deformities and Conditions
   A. Localized tooth-related factors that modify or predispose to plaque-induced gingival diseases/periodontitis
   B. Mucogingival deformities and conditions around teeth
   C. Mucogingival deformities and conditions on edentulous ridges
   D. Occlusal trauma

Fig. 23 Abbreviated version of 1999 Classification of Periodontal Diseases and Conditions [14]

Gingivitis is defined as gingival inflammation in the absence attachment loss and alveolar bone destruction, in response to biofilm present near the gingival

margin. Clinical signs are redness, swelling, edema, change in tissue consistency. This can lead to bleeding, halitosis and an increase in dental probing. It is a reversible condition and the treatment consists of the control of the dental plaque and its retention factors.

On the contrary, in chronic periodontitis alveolar bone destruction and attachment loss is irreversible and it is consistent with the amount of plaque and other local factors, such as anatomic conditions, overhanging restorations, open contacts and palato-radicular grooves. In general the disease progresses slowly but there may be peaks of destruction. Smoking, systemic diseases and specific local factors can influence disease progression[15] .

In chronic periodontitis both dental arches can be affected and the disease is defined as localized or generalized depending on number of involved theeth (respectively less or more than 30% of dental elements) . Often an irregular and localized spread is described, with involvement of the molars and incisors. Clinically, the gum shows inflammation with edema, erythema, bleeding on probing and sometimes suppuration. Loss of attachment with formation of a periodontal pocket and destruction of alveolar bone are always present. The disease can manifest as gingivitis already in adolescence and slowly progresses. During the course of life, the pathological effects accumulate until destructive effects are revealed. The extent of destruction depends on plaque levels, stress, diabetes and the efficiency of the immune system. Furthermore, the risk is increased in smokers, who show also a more unfavourable prognosis, even though the mitigation of inflammation process induced by this habit tends to hide the real severity of the disease. Periodontal therapy involves the removal of dental plaque (above and below the gum) and instructions to manatin oral hygene and health.

Aggressive periodontitis includes rare forms of periodontitis characterized by rapid progression. Both the localized and the generalized form require a genetic predisposition, but while the localized form appears to arise from an infection with Aggregatibacter Actinomycetmcomitans, in the generalized form the role of Porphyromonas gingivalis and Bacteroides forsythus is more important. Smoking is again a risk factor in this aggressive form. The diagnosis of aggressive periodontitis is done looking at rapid loss of attachment and bone destruction in patients with a positive family history and disproportion between bacterial plaque amount and the severity of periodontal destruction, in absence of other significant systemic diseases. It mainly affects the first molars and incisors in a characteristic way.

Periodontitis as manifestatation of systemic diseases identifies all periodontal diseases associated with systemic conditions causing a reduction in patient's immune response, such as neutropenia and leukemia or Periodontitis associated with genetic diseases such as Down's syndrome and the Papillon-Lefevre syndrome.

Necrotizing ulcerative gingivitis and necrotizing ulcerative periodontitis are the most serious inflammatory and infectious periodontal diseases, showing a very rapid destruction of periodontal attachment and bone loss. Clinically gums are ulcerated and necrotic papillae and gum margins are observed. Ulcers can be covered with soft yellowish-white pseudomembrane. Lesions are rarely associated with periodontal pockets because the rapid extended necrosis coincides with loss of alveolar bone. Treatment of the acute phase involves removal of tartar, as long as possible, associated with chemical plaque control systems and systemic administration of antibiotics. Affected individuals should be monitored daily throughout the duration of the acute phase.

In 2018 during the EuroPerio Conference in Amsterdam, a new classification system was presented, updating the previous one. For the first time, the periodontal health condition was scientifically defined and periodontitis was described and codified in four stages.

A)

Staging and Grading Periodontitis

The 2017 World Workshop on the Classification of Periodontal and Peri-Implant Diseases and Conditions resulted in a new classification of periodontitis characterized by a multidimensional staging and grading system. The charts below provide an overview. Please visit **perio.org/2017wwdc** for the complete suite of reviews, case definition papers, and consensus reports.

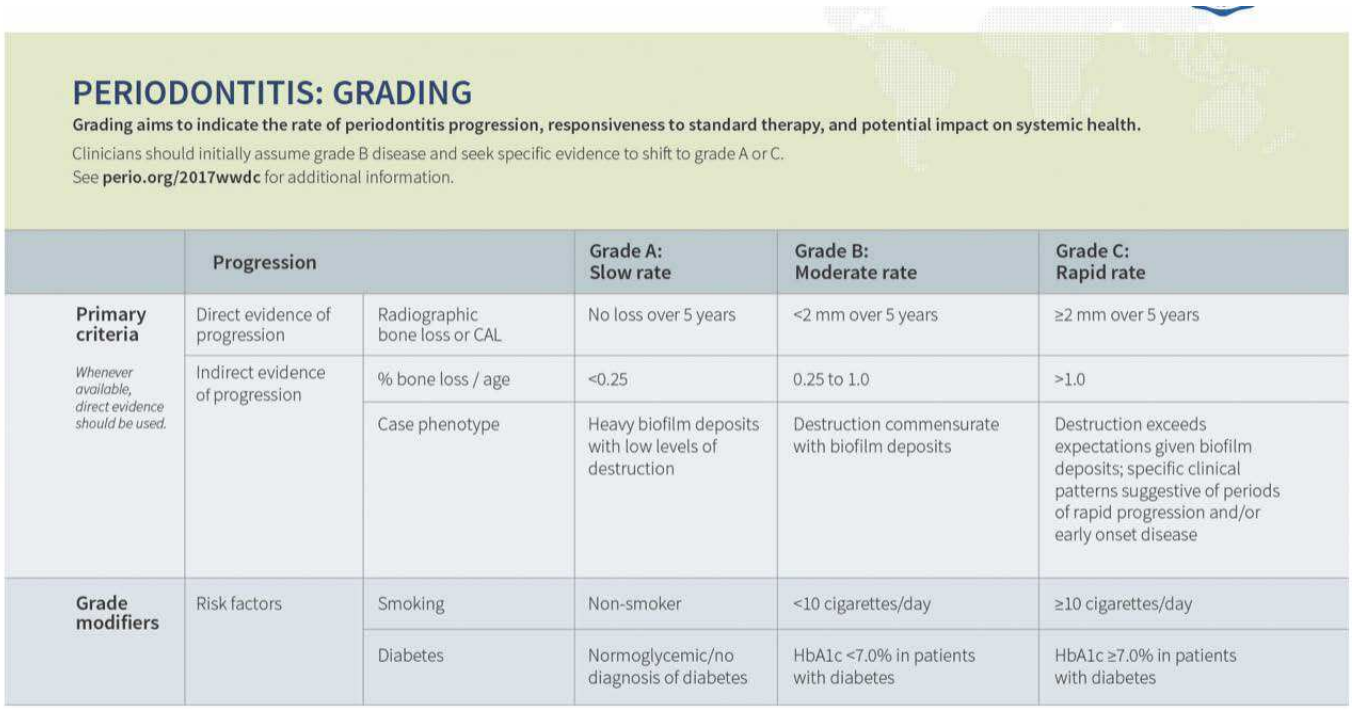
PERIODONTITIS: STAGING

Staging intends to classify the severity and extent of a patient's disease based on the measurable amount of destroyed and/or damaged tissue as a result of periodontitis and to assess the specific factors that may attribute to the complexity of long-term case management.

Initial stage should be determined using clinical attachment loss (CAL). If CAL is not available, radiographic bone loss (RBL) should be used. Tooth loss due to periodontitis may modify stage definition. One or more complexity factors may shift the stage to a higher level. See **perio.org/2017wwdc** for additional information.

	Periodontitis	Stage I	Stage II	Stage III	Stage IV
Severity	Interdental CAL *(at site of greatest loss)*	1 – 2 mm	3 – 4 mm	≥5 mm	≥5 mm
	RBL	Coronal third (<15%)	Coronal third (15% - 33%)	Extending to middle third of root and beyond	Extending to middle third of root and beyond
	Tooth loss *(due to periodontitis)*	No tooth loss		≤4 teeth	≥5 teeth
Complexity	Local	• Max. probing depth ≤4 mm • Mostly horizontal bone loss	• Max. probing depth ≤5 mm • Mostly horizontal bone loss	In addition to Stage II complexity: • Probing depths ≥6 mm • Vertical bone loss ≥3 mm • Furcation involvement Class II or III • Moderate ridge defects	In addition to Stage III complexity: • Need for complex rehabilitation due to: – Masticatory dysfunction – Secondary occlusal trauma (tooth mobility degree ≥2) – Severe ridge defects – Bite collapse, drifting, flaring – < 20 remaining teeth (10 opposing pairs)
Extent and distribution	Add to stage as descriptor	For each stage, describe extent as: • Localized (<30% of teeth involved); • Generalized; or • Molar/incisor pattern			

B)



PERIODONTITIS: GRADING

Grading aims to indicate the rate of periodontitis progression, responsiveness to standard therapy, and potential impact on systemic health.

Clinicians should initially assume grade B disease and seek specific evidence to shift to grade A or C.
See **perio.org/2017wwdc** for additional information.

	Progression		Grade A: Slow rate	Grade B: Moderate rate	Grade C: Rapid rate
Primary criteria *Whenever available, direct evidence should be used.*	Direct evidence of progression	Radiographic bone loss or CAL	No loss over 5 years	<2 mm over 5 years	≥2 mm over 5 years
	Indirect evidence of progression	% bone loss / age	<0.25	0.25 to 1.0	>1.0
		Case phenotype	Heavy biofilm deposits with low levels of destruction	Destruction commensurate with biofilm deposits	Destruction exceeds expectations given biofilm deposits; specific clinical patterns suggestive of periods of rapid progression and/or early onset disease
Grade modifiers	Risk factors	Smoking	Non-smoker	<10 cigarettes/day	≥10 cigarettes/day
		Diabetes	Normoglycemic/no diagnosis of diabetes	HbA1c <7.0% in patients with diabetes	HbA1c ≥7.0% in patients with diabetes

Tab. 1A-1B Staging and Grading Periodontitis; Tables from Tonetti, Greenwell, Kornman [16].

The four Stages of periodontitis are based on the amount of damage which has already occurred, including attachment loss, radiographic bone loss, tooth loss and probing depths for Stages I and II. In addition, furcation involvement, ridge defects and bite collapse are involved in Stages III and IV.

Grading allows dentist to determine the risk of patient for further progression of the disease, according to bone loss, age, case phenotype, biofilm deposits, smoking and systemic diseseases.

Diagnosis of periodontitis is obtained on the basis of medical history, clinical examination, RX images. In some cases , such as in the management of patients

with juvenile Periodontitis and refractory forms of periodontal disease, laboratory and microbiological tests can be performed.

The most important factors that have to be considered in medical history include smoking, drugs (ciclosporin, nifedipine, diphenylhydantoin) and systemic diseases, such as diabetes, Ehlers syndrome, Papillon-Lefevre syndrome.

Clinical inspection evaluates Topography, colour and shape of the gingiva, dental migrations, presence of bacterial plaque and of retention factors (tartar, caries, overflowing restorations, dental crowding).

Dental mobility may increase due to periodontal disease associated with plaque. Differential diagnosis must be performed with other causes of hypermobility, such as occlusal trauma, reduction of supporting bone, orthodontic treatment. Dental mobility is assested by tapping the thooth between two instrument handles, evaluating the movement of the tooth between the two extreme positions.
Three grades of mobility are defined: grade 1 shows horizontal movement of no more than 1 mm, grade 2 is characterized by horizontal movement between 1 and 2 mm, degree 3 shows horizontal mobility higher than 2 mm or vertical mobility.

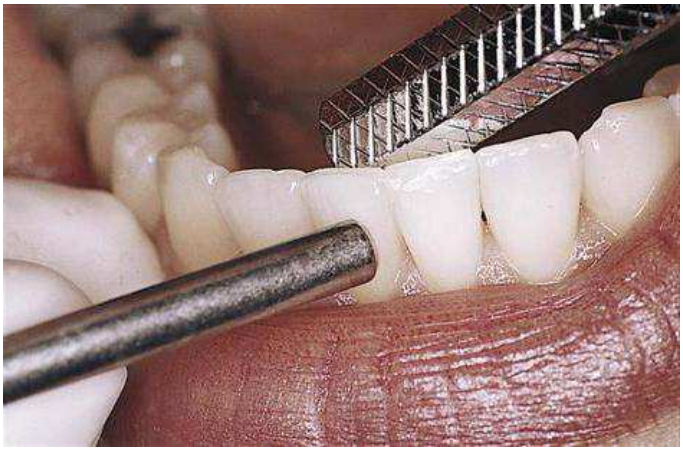


Fig. 23 Technique for assessing dental mobility

Dental probing is carried out by means of a periodontal probe, applying a force of about 30 grams, along the entire circumference of each tooth between the tooth and the gum. It allows the detection of periodontal pockets, level of clinical attachment, involvement of forcations, presence of subgingival tartar and/or incongruous restorations. Presence of bleeding during dental probing is an important risk factor for periodontitis progression.

The *PSR system* is a clinical method created to evaluate the severity of periodontal disease by introducing a specific calibrated dental probe into gingival sulcus of each tooth (Six measurements for each tooth are obtained along the entire circumference). A score between 0 and 4 is assigned to each quadrant of the mouth, considering the deepest periodontal pocket of each quadrant:

0	The lower black band of the WHO probe completely visible when the probe is inserted into the base of the pocket and there is no bleeding	Indicates pockets less than 3.5 mm, no bleeding on probing, no calculus
1	The lower black band of the WHO probe completely visible when the probe is inserted into the base of the pocket and bleeding is noted	Indicates pockets less than 3.5 mm, bleeding on probing present
2	The lower black band of the WHO probe completely visible when the probe is inserted into the base of the pocket and calculus deposits are seen or felt	Indicates pockets less than 3.5 mm, calculus and plaque retentive factors present
3	The lower black band of the WHO probe partially visible when the probe is inserted into the base of the pocket	Indicates pockets between 3.5 mm and 5.5 mm, may have bleeding/calculus
4	The lower black band of the WHO probe not visible when the probe is inserted into the base of the pocket as it is submerged in the pocket	Indicates pockets more than 5.5 mm, may have bleeding/calculus

Fig. 24 PSR score system

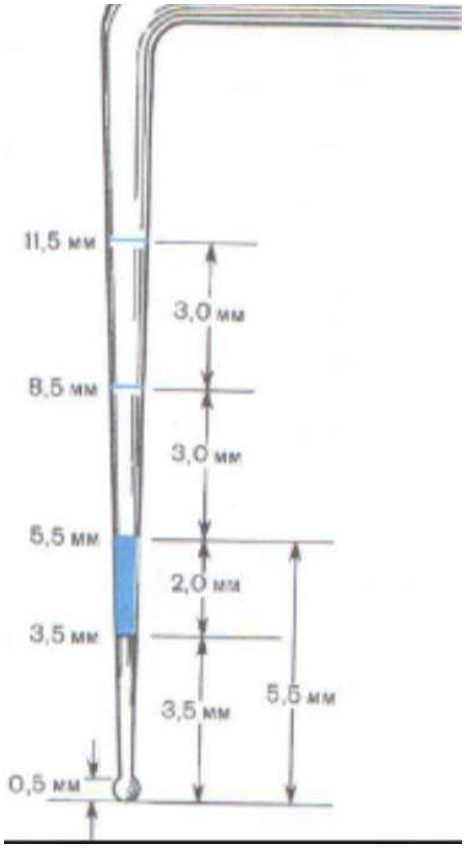


Fig. 25 The specific dental probe used in PSR analysis

Periapical radiography and cone-beam computed tomography (CBCT) are nowadays used to observe, measure and evaluate bone loss around the theet.

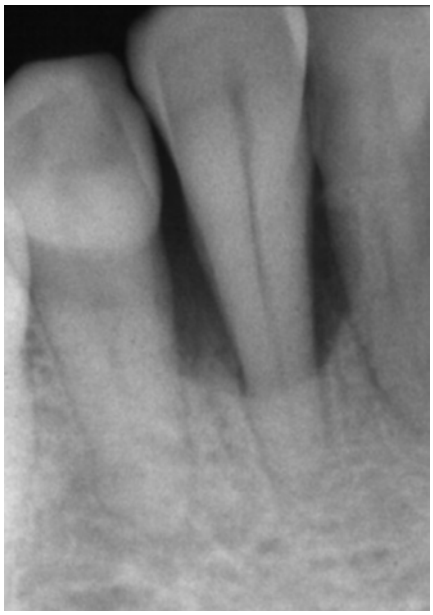


Fig. 26 Periapical radiography of a tooth with alveolar bone loss caused by periodontitis

2. FIRST STUDY: COLORECTAL CANCER AND PERIODONTAL DISEASE

2.1 INTRODUCTION

2.1.1 Colorectal cancer: incidence and prognosis

Colorectal cancer is the third most common cancer and the fourth most common cancer cause of death globally, accounting for roughly 1.2 million new cases and 600 000 deaths per year [17].

Incidence is low among young peolpe (less than 50 years old) but strongly increases with age.

Median age at diagnosis is about 70 years in developed countries [18].

Incidence is higher in Europe, North America, and Oceania than in Asia and Africa. Despite this, incidence in those countries which were considered at low-risk a few years ago (for exemple Spain and west Asia), has been increasing more and more, probably due to changes in dietary patterns and lifestyle habits. On the other end, in the USA incidence started to decrease, probably thanks to improved early detection and treatment techniques (for example an increased use of colonoscopy with polypectomy) [19,20].

In 2008 mortality ranged from 2.7 per 100 000 people in central Africa to 12.2 in central and eastern Europe in women, and from 3.5 to 20.1 in men.

Prognosis has been improving in the last decades with a 5-year relative survival rate of 65% in developed countries, but it is still less than 50% in less developes areas of the world.

Similarly to other cancers, the most critical prognostic factor seems to be the represented by the stage at the moment of the diagnosis.

Colorectal cancer rates: both sexes

Hungary had the highest rate of colorectal cancer in 2018, followed by South Korea.

Rank	Country	Age-standardised rate per 100,000
1	Hungary	51.2
2	South Korea	44.5
3	Slovakia	43.8
4	Norway	42.9
5	Slovenia	41.1
6	Denmark	41.0
6	Portugal	40.0
8=	Barbados	38.9
8=	Japan	38.9
10	Netherlands	37.8
11	Australia	36.9
12	Singapore	36.8
13	Serbia	36.7
14=	Belgium	35.3
14=	New Zealand	35.3
16=	Uruguay	35.0
16=	Brunei	35.0
18	Moldova	34.2
19	Croatia	34.1
20	Ireland	34.0

Tab. 2 Age-standardised rates for coloncancer in both sexes, World Cancer Research Fund

Colorectal cancer rates in men

Hungary had the highest rate of colorectal cancer in men in 2018, followed by Slovakia.

Rank	Country	Age-standardised rate per 100,000
1	Hungary	70.6
2	Slovakia	60.7
3	South Korea	59.5
4	Slovenia	58.9
5	Portugal	54.0
6	Barbados	50.3
7	Japan	49.1
8	Serbia	49.0
9	Moldova	47.3
10	Norway	46.9
11=	Denmark	45.9
11=	Croatia	45.9
13	Netherlands	45.3
14	Spain	45.2
15=	Uruguay	43.8
15=	Belgium	43.8
17	Brunei	43.4
18	Latvia	42.6
19	Czech Republic	42.5
20	Ireland	42.4

Tab. 3 Age-standardised rates for coloncancer in men, World Cancer Research Fund

Colorectal cancer rates in women

Norway had the highest rate of colorectal cancer in women in 2018, followed by Hungary.

Rank	Country	Age-standardised rate per 100,000
1	Norway	39.3
2	Hungary	36.8
3	Denmark	36.6
4	Singapore	34.0
5	Australia	32.4
6	South Korea	31.3
7	Slovakia	31.2
8	Netherlands	31.1
9	New Zealand	30.8
10	Japan	29.6
11	Barbados	28.8
12	Portugal	28.7
13	Uruguay	28.3
14	Jamaica	28.2
15=	Canada	28.0
15=	Belgium	28.0
17	Latvia	27.7
18	Brunei	27.4
19	UK	27.0
20	Ireland	26.4

Tab. 4. Age-standardised rates for coloncancer in women, World Cancer Research Fund

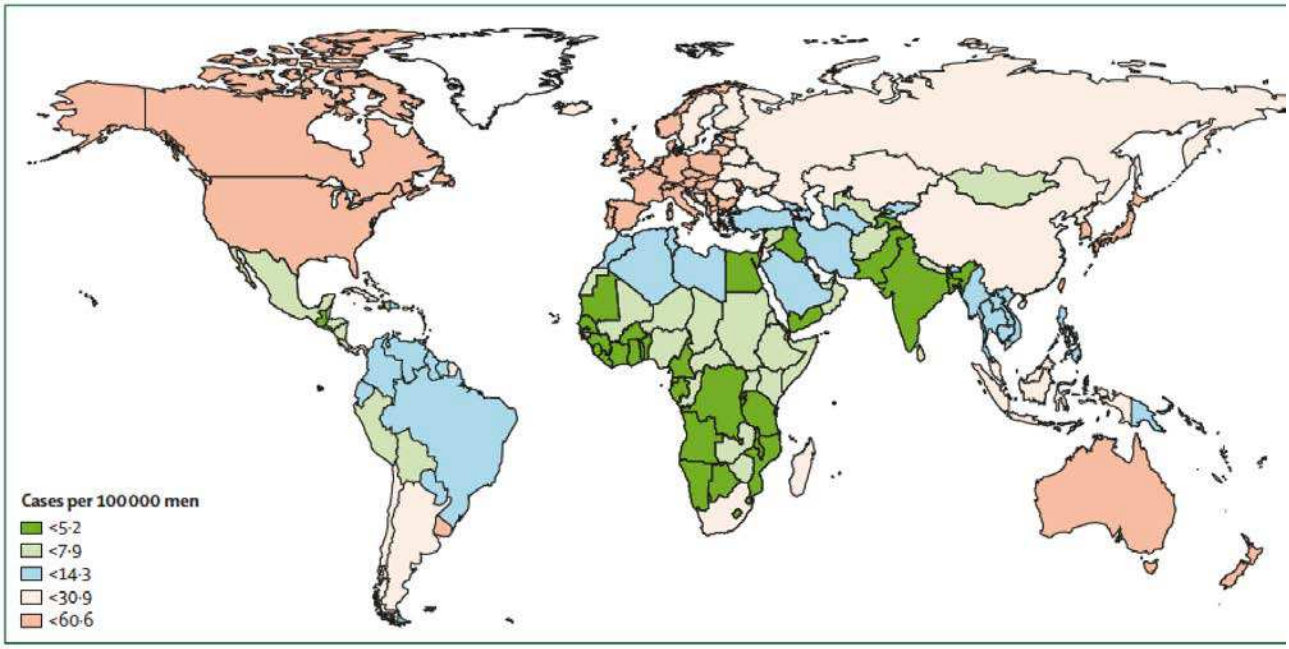


Fig. 27 Estimated age-standardised incidence for colorectal tumor in men in 2008, Globocan 2008. [17]

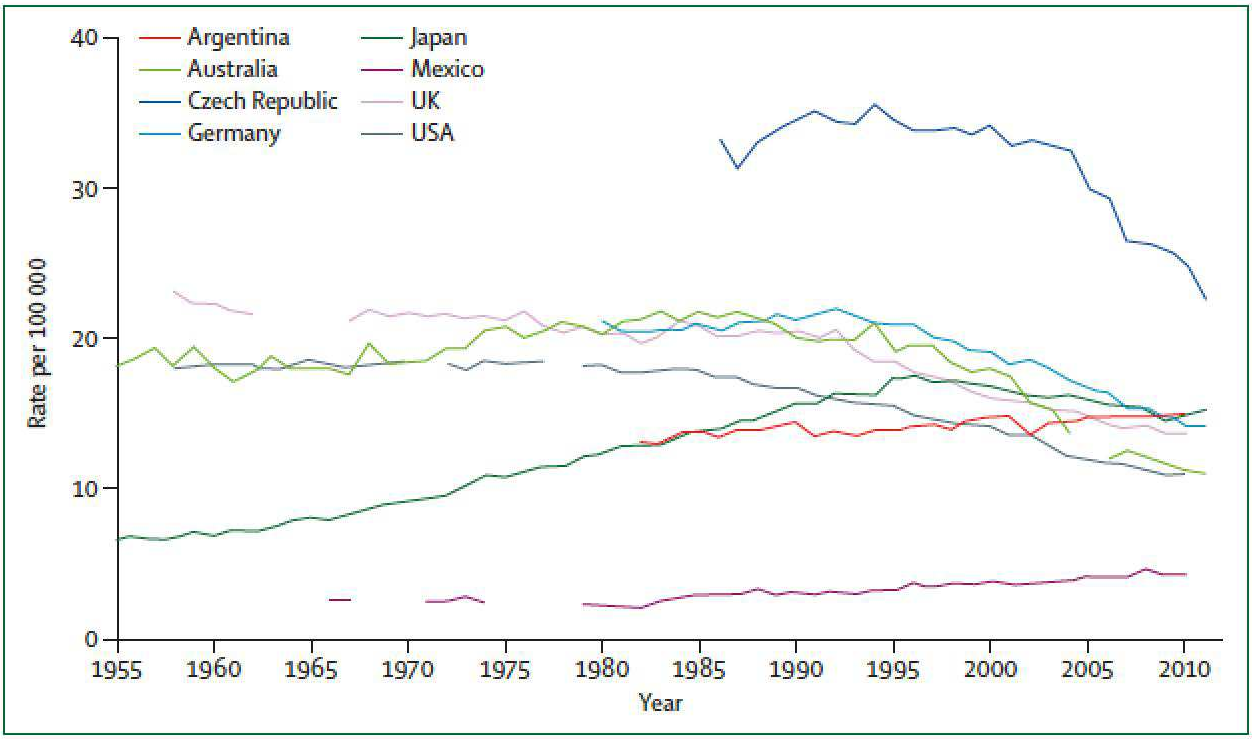


Fig. 28 Colorectal cancer mortality trends for men, 1955–2010, WHO mortality database.

2.1.2 Carcinogenesis: risk and preventive factors

Carcinogenesis is a complex, multiphase process in which genetic alterations and enviromental factors can lead to the transformation of healty mucosa into adenocarcinoma.
Genetic predisposition is definetly significant: some authors stated that in up to 30% of cases, malignant lesions develop in patients with a strong family history [21].
In colorectal cancer malignant transformation is generally slow (it takes more than 10 years) and is characterized by parallel changes in the molecular and histological side.
This tumor is often preceded by precursor lesions like dysplastic adenomas (intestinal adenomatous polyps).
Intestinal polyps are esophitic lesions rising from the intestinal mucosa, which protrude towards the intestinal lumen. Most of them are benign and asymptomatic. They are generally classified as adenomatous (adenomas), hamartomatous and hyperplastic polyps. Adenomas can be ulteriorly divided into tubular, tubulovillous and villous, with approximately 87% of adenomas being tubular, 8% tubulovillous and 5% villous [22].

Altough generally benign, the probability of tumoral transformation increases with polyp size: a diameter higher than 1 cm is considered dangerous [23].

In addition to dimension, the number of polyps could be crucial.
Polyposis is a pathological condition characterized by a high number of polyps in different segments of the entire digestive tract. Some genetic syndromes, such as Familial Adenomatous Polyposis (FAP) and its variants (Gardner's syndrome, Turcot's syndrome and Zanca's syndrome) have been related to a higher

probability of developing colorectal cancer; according to some authors about 2%–5% of all colorectal cancers can develop from a defined inherited cancer syndrome [24].

Familial Adenomatous Polyposis (FAP) is a hereditary condition characterized by multiple (usually more than 100) colorectal adenomatous polyps. Incidence is approximately 1 in 10,000 cases and it is responsible for 1% of all colorectal cancers in the United States [25].

In this condition, specific mutations occur in the APC gene, located on chromosome 5. It is precisely this gene that is thought to play an important role in tumor development, in fact APC gene mutations occur in more than 70% of adenomas as early events [26].

Furthermore mutations of the KRAS oncogene and TP53 tumour suppressor gene can be appreciated [27].

In FAP the risk of malignant transformation is estimated at 60-80% and it is related to the number of polyps and to the age of the patient.

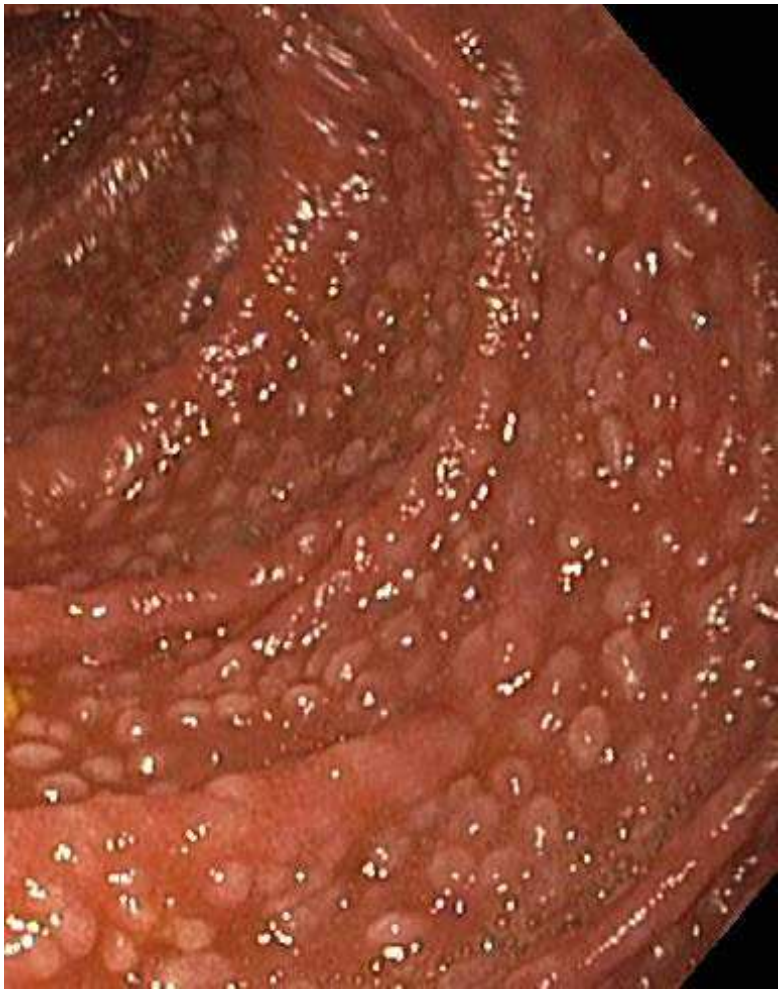


Fig. 29 Endoscopic image of the sigma colon in a patient with FAP

A different genetic condition not related with intestinal polyposis is Lynch Syndrome or Hereditary Nonpolyposis Colorectal Cancer (HNPCC), an autosomal dominant genetic disease, showing a 50%–70% lifetime risk of colorectal cancer, 40%–60% risk of endometrial cancer and increased risks of several other malignancies. It is caused by mutations in important DNA mismatch repair genes such as *MLH1*, *MSH2*, *MSH6* and *PMS2* [28].

In HNPCC about 90% of colorectal cancers and 80% of adenomas have microsatellite instability,too . In the type I variant, the most affected area is the right colon and the neoplasms tend to be multiple, synchronous or metachronous.

In the type II variant a predisposition for the development of extra-intestinal tumours (such as ovary, renal pelvis, stomach, ureter and endometrium) can be observed.

Peutz-Jeghers syndrome is a less less frequent condition (incidence is between 1:10,000 and 1:100,000 births), characterized by the appearance in first decade of life of multiple hamartomatous polyps (affecting the entire gastrointestinal tract), mucocutanous melanosis, luminal gastro-intestinal cancer and extraintestinal cancer.

Similarly, Juvenile Polyposis Syndrome (JPS) is characterized by the appearence of several juvenile polyps in the gastro-intestinal tract. The incidence is estimated between 1:100.000 to1:160 000 [29].
In this case alterations in the transforming growth factor (TGF-beta) pathway are often observed: a mutation in BMPR1A gene or SMAD4 gene is present in 20-30% of patients.
Despite these polyps are not generally malignant, both these last two syndromes have been proved to be a predisposing conditions for the development of colorectal cancer.

Although genetic seems to play a major role in carcinogenesis, and, as some authors said, 35% of colorectal cancer risk might be attributable to heritable factors,[30] other parameters which have to be considered in the analysis: age, sex, inflammatory bowel disease, smoking, excessive alcohol consumption, high consumption of red and processed meat, obesity and diabetes.

Age and sex: colorectal tumor are more diffused among elderly people; in particular incidence rate increases after 60 years, expecially in male. Among people younger than 50 years old, when the disease is less diffused, the male-

female ratio is about 1:1.

Ethnic factors: colorectal neoplasms are frequent in industrialized countries such as Europe, North America and Japan, while they are rare in Africa, South America and Asia. Ethnic factors are particularly evident considering citizens of different ethnicity, residing in the same country: in the United States of America, for example, the highest incidence and mortality is found in the African American population, vice versa the lowest rates are recorded in the Hispanic community [31].

Food habits: obesity, a high-calorie diet and excessive consumption of animal fats and red-and-processed meat have been related to an increased risk of cancer [32] .

Studies have shown that individuals from low-risk areas can develop, over time, the same rate of incidence of the host country polulation, due to new eating habits. On the contrary it is widely established that a diet that favours the consumption of fresh fruit and vegetables can play a preventive role and reduces the risk of occurrence.

Excessive alcohol consumption and smoking: both these habits can be considered risk factors. Some authors support the concept that ethanol, under certain experimental conditions, could represent a cocarcinogen and/or tumour promoter for upper alimentary tract and large intestine malignant lesions [33].
In addition a recent meta-alaysis stated that cigarette smoking is significantly associated with colorectal cancer incidence and mortality [34].
Similarly to oral squamous cell carcinoma, the association of these factors appears to be extremely dangerous: it seems that the risk in patients exposed to both, smoking and large alcohol consumption, could be greater than additive.

Hormone therapy and anti-inflammatory drugs: data on the possible role of female hormones, in particular hormone replacement therapies, in the development of colorectal neoplasms are reassuring: various studies have shown that hormone replacement therapy reduces the incidence of adenomas and colorectal carcinomas in women in menopause [35].
Similarly, data obtained from observational studies on the role of acetyl-salicylic acid and non-steroidal anti-inflammatory drugs in carcinogenesis have confirmed that assumption of these drugs is associated with a reduction in the risk of colorectal cancer of about 20-30% [36].

Physical activity: poor physical activity is considered an independent risk factor and a statistically significant association has been found with the development of colon cancer (especially of the proximal tract); on the contrary regular exercise seems to represent an important preventive factor [37].

2.1.3 Prevention

Early detection is the key objective of modern clinical practice. Primary prevention aims at reducing exposure to etiological factors or the susceptibility of the host to them. The main goal is to prevent the onset of genetic damage and to oppose its progression. However the difficulty of changing life habits (such as nutrition and physical exercise) represents a real challenge. Recently, chemo-prevention has been proposed as an alternative primary preventive method. It can be defined as the use of natural or synthetic substances which have demostrated to be free of toxicity and capable of preventing the processes of initiation, promotion and progression of cancer, before or during the preneoplastic phase, in animal models or in vitro studies. To be precise, different

kinds of chemoprevention are known: primary chemoprevention, directed to the general population or to population groups at risk, which represents a public health intervention and aims to inhibit the onset of cancer. Secondary chemoprevention is applied when the tumour is already present, in order to block progression (or possibly induce regression of lesions) or prevent recurrences. Finally tertiary chemoprevention is performed in association with chemotherapy and aims to reduce its toxic effects and possibly increase its effectiveness. Among the synthetic substances, a possible chemo-preventive role of non-steroidal anti-inflammatory drugs (NSAIDs) has been noted in those patients who have been taking them for a long time, reporting a reduction in incidence and mortality of gastrointestinal malignant tumours of 40-50% [38].

The likelihood of the onset of new adenomas after treatment for previous colon cancer also seems to decrease with these medications. Their efficacy is reported both when the therapy is administered at an extremely early stage of the disease (intact colic mucosa) and when it is performed at a later stage (presence of adenomas), regardless of age, sex and colic segment involved. The problem is that the protective effect is closely linked to a continuous and long intake and stops at the suspension of the drug.

Among medicines particular attention has been paid to selective inhibitors of COX-2 (such as celecoxib and rofecoxib), an inducible enzyme responsible for the synthesis of prostaglandins, thromboxanes and leukotrienes from arachidonic acid, whose activity is increased in both inflammatory and neoplastic diseases. In fact in colon carcinomas a greater expression of COX-2 and its products has been demonstrated than in healthy tissues.

Secondary prevention includes those methods aiming at identification of the disease at an early stage, when it is not yet clinically manifest.

At present the most effective measure to reduce mortality from this disease is still

early diagnosis, in fact the recognition of cancer lesions in the early stages allows a 5-year survival rate of about 90%. The transformation from adenoma to carcinoma is generally quite slow (it can last 10 years): this period gives the patient the possibility to diagnose an asimptomatic lesion at an early stage and eliminate it. These so important screening methods are based on search for occult blood in the stool (Hemoccult), sigmoidoscopy with flexible instrument, colonoscopy and double-contrast clism. Other tests, such as research in stool of altered DNA and virtual colonoscopy, are also being tested, but further data are needed.

Today in many countries medical guidelines recommend colorectal cancer screening programs from age of 50 for people at average risk: they generally consist of annual or biannual guaiac faecal occult blood or faecal immunochemical tests, flexible sigmoidoscopy every 5 years, or colonoscopy every 10 years [39,40].

A positive faecal immunochemical test or guaiac faecal occult blood requires a mandatory colonoscopy. If adenomas and hyperplastic polyps are detected, they have to be removed and an histologiacal analysis has to be performed.

Individuals with increased risk (for exemple first-degree relatives of young patients diagnosed with colorectal cancer, familial adenomatous polyposis, hereditary nonpolyposis colon cancer, or inflammatory bowel disease) should start screening programs at least 10 years before.

2.1.4 Diagnosis and staging

Diagnosis of colorectal cancer is initially based on medical history and search for clinical signs and symptoms. The suspicion is mainly posed by the appearance of rectal bleeding and/or alvus alterations in elderly patients with risk factors. In

Italy, screening programs are based on the detection of occult blood in stools. Peolpe of both sexes, aged between 50 and 69 years, are invited to perform the exam every 2 years without dietary restrictions. If the test result is positive, it is indicated to perform further investigations (colonoscopy or opaque clism) in order to identify the main cause of the bleeding.

The clinical examination can help to make an initial assessment of rectum, since this district represents one of the most frequent locations of large intestine neoplasms. However, the accuracy of the exam can change according to clinician experience. Furthermore, it is not clinically possible to distinguish between an inflammatory disease and a tumor.

Among instrumental examinations the endoscopic analysis is essential and represents the gold standard for diagnosis. This procedure allows to explore all segments of the colon and gives the possibility to take biopsy samples of a suspected lesion in order to analyse it from an histological point of view. Furthermore, this colonscopy has a possible preventive role, allowing the definitive removal of precancerous lesions like polyps. A complemetary examination is represented by double-contrast barium enema, which is a form of contrast radiography : a liquid containing barium and air are put into the rectum and colon to evaluate the morphology of the intestine and detect the presence of even small alterations (up to one centimeter in diameter). This method, as well as the most recent virtual colonoscopy, is useful especially in the presence of stenotic lesions that not allow a complete preoperative evaluation of the whole colon.

Other instrumental diagnostic investigations, such as Computed Tomography (CT) of the abdomen and eco-endoscopy, are mainly used for cancer staging purposes, as they document possible dissemination at a distance, however they play a limited role in diagnosis.

Laboratory tests can also help diagnostic procedures; tumour markers, such as CEA (carcinoembryonic antigen), gastrointestinal tumour antigen CA 19.9 and CA 125, which are generally present at low levels in samples taken from healthy subjects, can raise in patients affected by cancer, so they may represent an indicator of the presence of neoplastic disease. Unfortunately, the value of these substances is often influenced by other non-neoplastic factors such as drug therapies (some antihypertensive drugs) and/or the presence of other diseases (colon chronic inflammatory diseases) or smoking habits. Because of their low specificity, they are not sufficient on their own to make a diagnosis, but they play a role both at a prognostic level and in post-surgical follow-up, representing the indicator of a possible resumption of asymptomatic tumour disease.

Despite these considerations, final diagnosis of this cancer is made only by histological analysis from biopsy samples.

Synchronous malignant lesions are present in about 2–4% of cases and a Complete colonoscopy or CT colonography is essential to find them.
By means of Endoscopic ultrasononography determination of the T-stage of rectal cancer and is possible, even though the most accurate method to define advanced T-stages is MRI [41,42].
Distant metastases can be discovered in about 20% of patients newly diagnosed with colorectal cancer, expecially in liver (thus liver imaging should be mandatory in case of diagnosis of colorectal cancer); less frequent metastases can be found in lungs, bone and brain.

Several classification systems can be used. Although modern TNM method is generally adopted, Dukes classification, proposed by Dr. Cuthbert E. Dukes in 1932, is still diffused as it is simple, essential and reproducible and focuses on tissue infiltration, lymph node involvement and the presence of distant

metastases.

According to this method three stages can be defined A, B and C and each of them is associated with a different survival rate.

A = Tumour confined within the intestinal wall

B = Tumour extending beyond the intestinal wall

C = Any tumour with lymph node metastases

In 1954 Astler and Coller modified stages B and C by dividing them into two sub-groups each: B1 and B2, C1 and C2 [43].

Turnball in 1967 added category D to indicate the presence of distant metastases[44] .

Stage	Description
A	Lesion not penetrating submucosa
B1	Lesion invades but not through the muscularis propria
B2	Lesion through intestinal wall, no adjacent organ involvement.
B3	Lesion involves adjacent organs
C1	Lesion B1 invasion depth; regional lymph node metastasis
C2	Lesion B2 invasion depth; regional lymph node metastasis
C3	Lesion B3 invasion depth; regional lymph node metastasis
D	Distant metastatic disease

Fig. 30 Modified Classification of colorectal cancer according to Astler-Coller.

Finally in 1978 the American Joint Committee for Cancer (AJCC) developed the modern TNM system, valid for both colic and rectal tumours. This establishes the stage of neoplasm on the basis of the extension of the primary tumour (T), the presence of lymph node involvement (N) and distant metastases (M)

Stage	Characteristics
Tumor	
T1	Tumor invades submucosa
T2	Tumor invades muscularis propria
T3	Tumor invades through muscularis propria into subserosa or nonperitonealized pericolic or perirectal tissues
T4	Tumor directly invades other organs or structures and/or perforates visceral peritoneum
Regional nodal metastasis	
NX	Regional lymph nodes cannot be assessed
N0	No nodal metastasis
N1	Metastasis in one to three pericolic or perirectal nodes
N2	Metastasis in four to more pericolic or perirectal nodes
N3	Metastasis in any node along course of a named vascular trunk and/or metastasis to apical node
Distant metastasis	
MX	Presence of distant metastasis cannot be assessed
M0	No distant metastasis
M1	Distant metastasis

Fig. 31 TNM classification of colorectal cancer.

Three different levels of classification are also recognized:

- c-TNM: clinical-diagnostic evaluation;
- s-TNM: surgical evaluation;
- p-TNM: post-surgical evaluation, antomopathology.

However, the first two types of classification have proved to be inadequate with regard to their prognostic value, so the focus is expecially posed on the anatomopathological staging p-TNM.

In the 1980s, Jass and his collaborators developed a way of assigning a score to intestinal lesions, depending on four histological variables: depth of tumour invasion, tumour growth pattern, peritumoral lymphocyte infiltration, lymph node involvement. For each one of these, a score is assigned by the pathologist : the sum of the scores determines the stage of the tumor [45].

Stage I: score 0-1
Stage II: score 2
Stage III: score 3
Stage IV: score 4-5

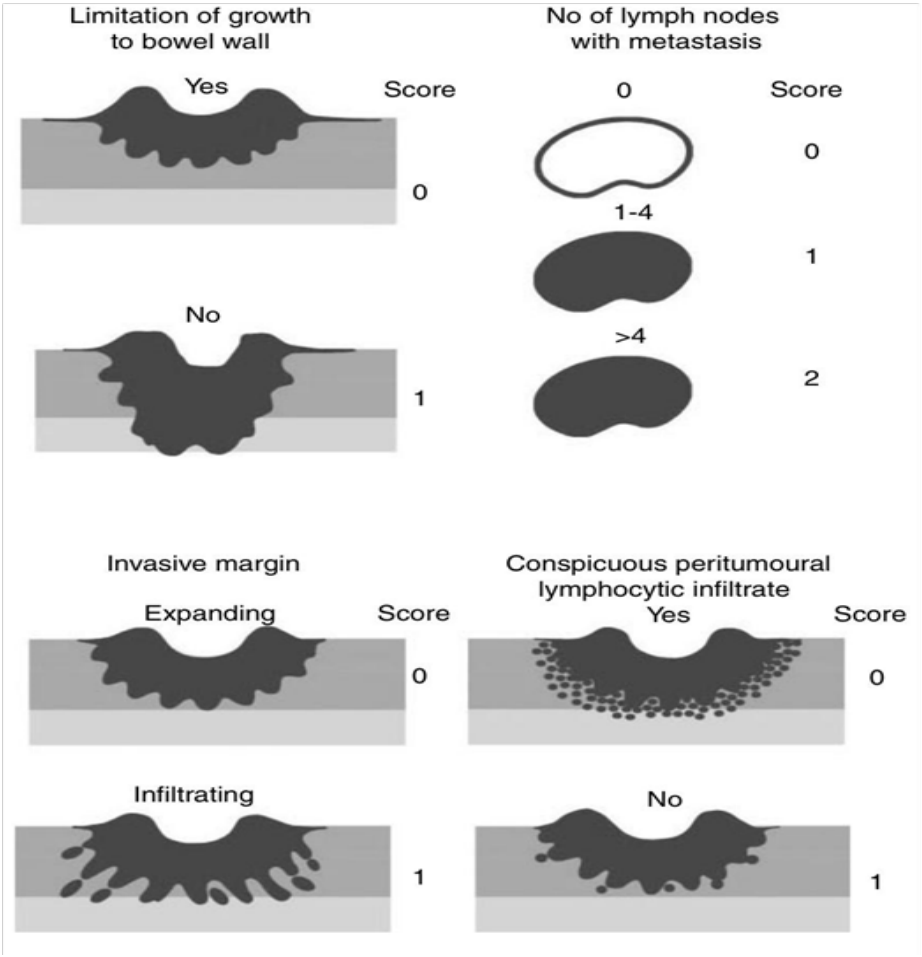


Fig. 32 Parameters and method of assignment of the score in Jass classification

2.1.5 Surgery

The surgical procedure for rectal cancer includes removal of the rectum together with the mesorectum and the mesorectal fascia [46].

The surgeon should leave at least 1mm clear circumferential margin (distance of more than 1 mm between the tumour border and the resection margin) in order to reduce tumor recurrence.

In case of colon cancer tumor, the lesion together with its corresponding lymph vessels should be removed.

Open surgery tecnique has been the only option available for many years, but laparoscopic technique is a valid alternative nowadays. This method lets the

patient achieve similar long-term results as open surgery ,with a reduced need for blood transfusions (3.4% vs 12.2%), faster return of bowel function and a shorter hospitalization (9.1 days vs 11.7 days); despite these advantages, costs are higher and operating times are longer (208 min vs 167 min) [47,48].

2.1.6 Neoadjuvant and Adjuvant therapy

Data for the role of neoadjuvant radiotherapy in locally advanced colon cancer are not so clear. The main goal of this treatment is to reduce the probability of local recurrence but several side effects have to be considered. Stage I patients should not be treated in addition to surgery because of the the low recurrence rate (less than 3%) [49].
Stage III patients may benefit from additional treatment, whereas opinions for patients with stage II are controversial [50].

Otherwise, adjiuvant chemoterapy is reccomendef for patients with stage III disease. Fluorouracil and capecitabine are the most common drugs. In stage II colon cancer patients the survival benefits from adjuvant chemotherapy are not so many, so it is reccomended only in cases with higher risk of relapse.
Considering distant metastases, patients with resectable liver or lung lesions should undergo surgical resection while those with irresectable metastases might benefit from palliative chemotherapy, depending on age, comorbidities, and extent of the tumor.

2.1.7 Human microbiota and colorectal cancer: Fusobacterium nucleatum and Phorpyromonas gingivalis

Similarly to the link between H. pylori infection and gastric cancer, a connection

between human microbiota and other gastrointestinal tumours could be hypotized. Recent data suggests some bactaeria can have a role in the pathogenesis of colorectal carcinoma, in fact they have been detected in tumor microenvironment; in particular an association between Fusobacterium nucleatum (FN) and the colonic mucosa of colorectal cancer has been hypotized [51,52].

Although oral microbiota has been proven to be responsible for different dangerous pathways [53,54] (production of DNA damaging genotoxins and superoxide radicals, induction of cell proliferation mediated by T-helper cells , induction of procarcinogenic pathways due to Toll-like receptor action), a direct relationship between human microbioma colonization of intestinal lumen and colorectal cancer has not be demostrated yet .

Fusobacterium Nucleatum is a common gram-negative anaerobic bacterium of the oral cavity playing an important role in human periodontal disease, even tough it can be detected in extra-oral diseases.

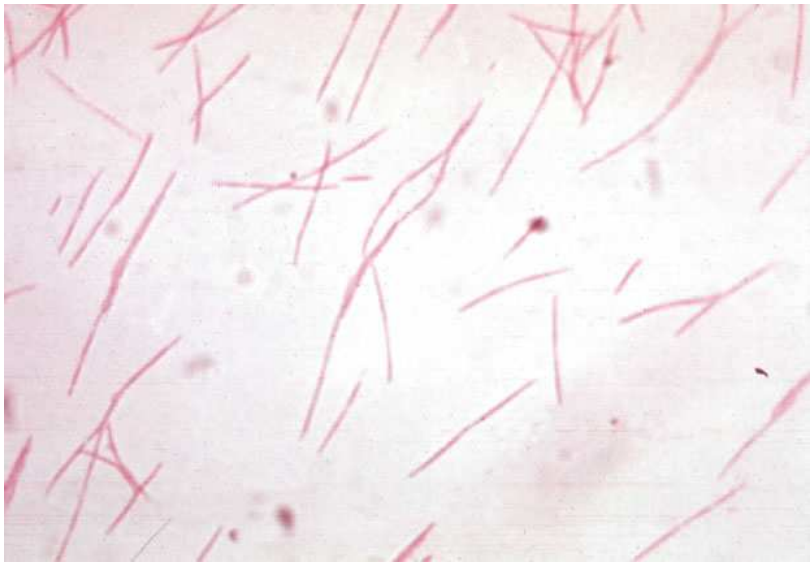


Fig. 33 Fusobacterium nucleatum culture

For exemple It has been associated with oral cancer and premature

stillbirths[55], pancreatic cancer.
In addition, a correlation with liver abscess, appendicitis, sinusitis, mastoiditis and tonsillitis has to be considered [56,57,58].
In this specific case, some recent studies found Fusobacterium nucleatum rates to be highly inceased in colorectal cancer and also in benign precancerous polyps [59].

This microrganism has the ability to colonize and spread throgh tissues expecially due to its intrinsic aptitude to adhere and invade human epithelial and endothelial cells.
That is the reason why the hypotesis that human oral microbiota is not confined to the mouth but is able to move in the body, causing extra-oral infection and inflammation, is plausible.
It is true that the presence of this bacterium in the colorectal adenomas and carcinomas is not sufficent to demostrate a direct causality, but the fact that oral cavity is the starting point of the digestive tract has to be considered: some bacteria could be able to move towards the bowel.
The surface adhesin FadA expressed by *this micoorganism is essential* in cell attachment and invasion processes [60].
Furthermore, FadA can bind to endothelial-cadherin on endothelialn vascular cells, increasing their permeability and allowing bacteria to penetrate. This could result in an increased bacterium penetration and may represent a strategy for sistemic dissemination. This adhesin is unique to and highly conserved among *Fn*, but is absent in non-oral fusobacteria [60].
FadA exists in 2 forms: the intact pre-FadA (about 129 amino-acid residues) and the mature FadA (mFadA, consisting of 111 amino-acid residues) . Both forms are essential to create the active complex required for binding and invasion processes [61].
Furthermore, *Fn* is not only invasive by itself, but is can facilitate tissue invasion

by other species, such as *Streptococcus cristatus* and *E. coli.* [62].
That is probably the main reason baceause this bacterium is frequently detected in mixed infections.

Some authors are convinced that Fusobacterium nucleatum represents a driver of colorectal cancer, in fact they showed that FadA (Fusobacterium Nucleatum adhesin A) adhesion on epitelium and endothelial cells can induce human colorectal cancer tissue growth [63].
F. nucleatum can binds to both normal and tumoral epitelium, but tissue growth is promoted only in the second case. The cancerogenetic pathway could be the following: FadA binding to E-cadherin on epitelial tumoral cells activates b-catenin-regulated transcription; this consequently increases the expression of oncogenes (cyclin D1 and c-Myc), Wnt signalling genes (Wnt7a, Wnt7b, and Wnt9a) and inflammatory genes (NFk-B, TNF-alpha, IL-6, IL-8, and IL-18; the result is the creation of an inflammatory microenvironment which helps the progression of tumor). If FadA binding site on E-cadherin is blocked by a a synthetic peptide the cancerogenic pathway can be stopped and tumor growth can be slowed both in vitro and in xenograft mice [63].

Kostic and al found that short-chain fatty acids and short-peptides (formylmethionyl-leucyl-phenylalanine) released by the bacterium, can attract myeloid-derived suppressor cells (MDSCs) [64].
In addition, it seems that infected cells increase the expression of microRNA-21 (miR21) by activating TLR4 signaling to MYD88, which leads to a further activation of NF-κB, inducing the oncogenic pathway [65].
Furthermore, it has been found that the interaction between bacterial Fap2 (a galactose-sensitive adhesion protein) and the human inhibitory receptor TIGIT can induce human lymphocytes cell death and generate a tumor immunosuppressive microenvironment which stimulates colorectal tumor progression [66,67].

TIGIT is an inhibitory receptor expressed on T cells and natural killer (NK) cells, so this interaction may defend malignant cells from host immune system[67].

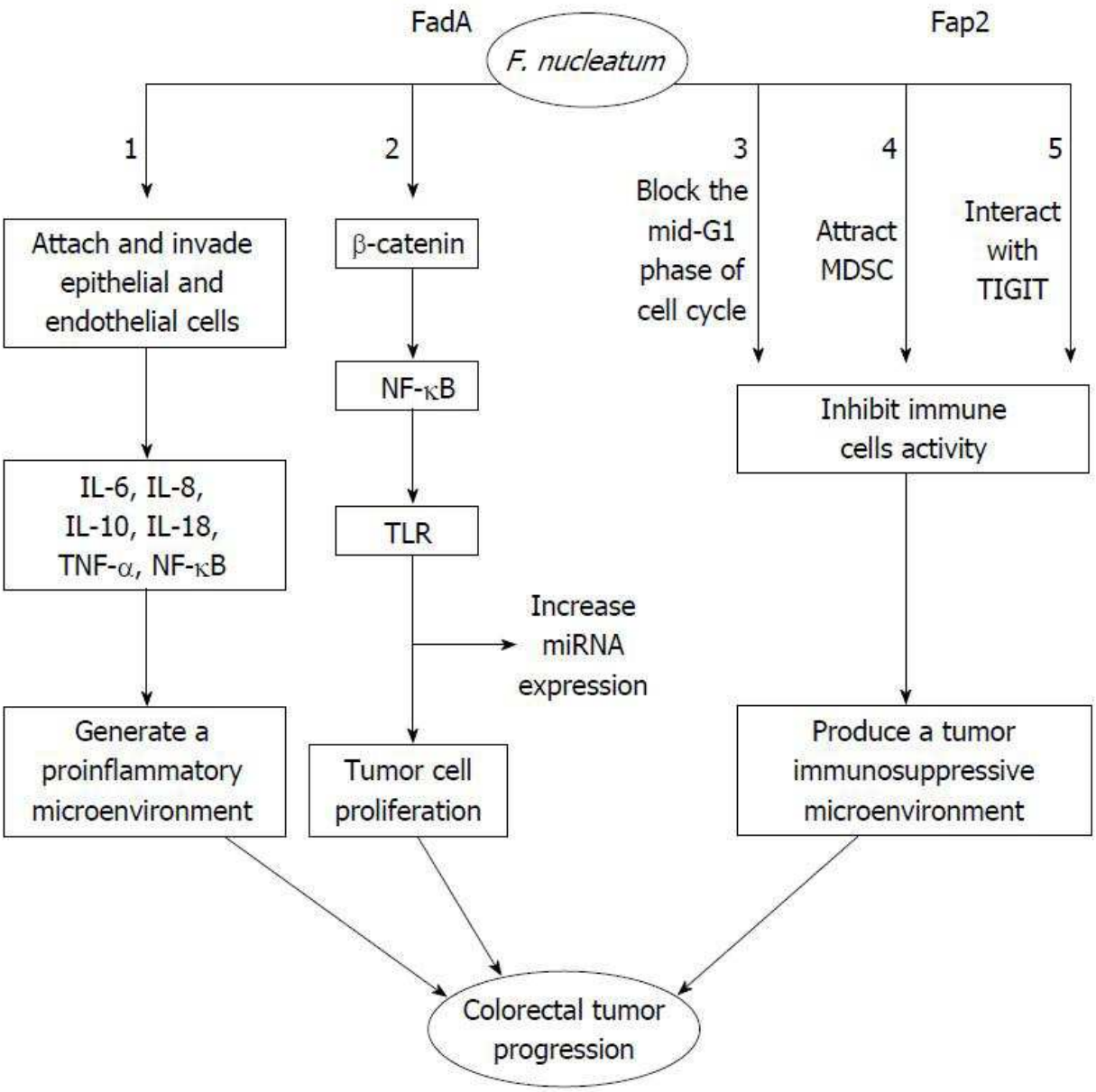


Fig. 34 Fusobacterium nucleatum cancerogenetic pathway

Porphyromonas gingivalis is a Gram-negative, rod-shaped, anaerobic pathogenic bacterium, which has a role in periodontal disease, as well as it has been detected in gastrointestinal tract, the respiratory tract and the colon.

Its main virulence factor are represented by Arg-gingipain (Rgp) and lys-gingipain (Kgp) , Capsular polysaccharide (CPS) and Fimbriae.

In particular Arg-gingipain (Rgp) and lys-gingipain (Kgp) are endopeptidase enzymes, essential for bacterial collection of nutrients, adhesion, invasion and colonization. Furthermore, these enzymes are able to protect the bacterium from the host immune response.

P. gingivalis plays a major role in the onset of chronic periodontitis [68].

Though it can be detected in low abundance in the mouth, it is able to cause a microbial shift of the oral cavity, allowing for huge growth of the miocrobiota. The consequence is represented by alteration of the local host homeostasis and distruption of perioddontal tissues due to adaptive immune response[69].

In addition to its well-known role in periodontal disease it is interesting to notice that *Pg* is able to alter host-microbe equilibrium and cause inflammatory responses by modulating the complement system in extra-oral tissues.

A study suggested a role of PG heat-shock protein in infection-triggered autoimmune diseases [69]. By means of peptidylarginine deiminase (PAD), which converts arginines into peptidylcitrulline, this bacterium can induce anti-citrullinated antibodies and trigger autoimmune inflammation in the host [70].

In some patients this mechanism can induces antibody response in the joints, so a role in development of chronic arthritis has been hypotized [71].

Furthermore, colocalization of *P. gingivalis* with CD4+ T cells was observed, even though the mechanism is still unknown [72].

The virulence mechanisms of *Pg* in systemic infections has been recently studied by means of different animal models. For example in a rat model the consequence of subcutaneous infection with *Pg* at different gestation periods was lower maternal weight gain, lower fetus weight and lower placenta weight [73].

Virulence may vary according to bacterium strain: in a mouse periodontitis model, some authors found that different *Pg* strains can induce different systemic responses and degrees of periodontum distruction [74].

A similar strain-dependent situation was described in infection of murine placentas [75].

Differently from Fusobacterium Nucleatum, the role of PG in colorectal cancer and other gastrointestinal diseases has never been analyzed specifically.

2.2 AIM

This research project aimed to verify the presence of a possible correlation between oral microbiota and colorectal carcinoma. In particular the role of two specific sub-gingival bacteria, Fusobacterium nucleatum and Porphyromonas gingivalis, two pathogens which are present in the oral cavity and increase their load in case of periodontal disease, was analized in order to detect possible correlations with the tumoral changes leading to colorectal cancer development.
This discovery of such a relation would represent an important breakthrough for the prevention of this dangerous disease, which is so widespread nowadays.

2.3 MATERIALS AND METHODS

2.3.1 Study population

The population investigated in this study was represented by patients potentially at risk of developing colorectal cancer, who needed to undergo a diagnostic colonscopy at the SC Gastroenterology of ASST Sette Laghi- Circolo Hospital Fondazione Macchi of Varese.

Inclusion and exclusion criteria

The inclusion criteria were represented by the informed consent to the protocol, good compliance, age not under 18 years. Patients diagnosed with metastatic colorectal carcinoma from other anatomical sites were excluded from the study.

The number of investigated patients was 110.
Firstly, patients were provided with specific information and informed consent, (attached), in accordance with the Regulation on the protection of personal data:

Sistema Socio Sanitario
Regione Lombardia
ASST Sette Laghi
Polo Universitario

Scheda di Informazione per il volontario
(e per il Medico di famiglia)

Gentile Signora/e,

Lei è invitata/o a partecipare ad uno studio che viene effettuato presso questo centro cui Lei si è rivolta/o per motivi di diagnosi o cura. Si prenda tutto il tempo necessario per leggere questo foglio. Se qualcosa non le è chiaro, non esiti a porre tutte le domande che vorrà al Dott. Sergio Segato.

Il titolo dello studio è: "*Fusobacterium* nel contesto gengivo-parodontale come fattore di rischio correlato a insorgenza di adenocarcinoma del colon-retto. Studio microbiologico e istologico".

Lo scopo di questo studio è l'identificazione di una possibile correlazione tra la flora batterica orale e l'adenocarcinoma del colon-retto, in modo da facilitare la prevenzione e diagnosi precoce di questa neoplasia.

Perché questo studio clinico viene proposto?

Tale tipo di ricerca si basa su un innovativo approccio sperimentale incentrato sull'analisi microbiologica della flora orale il cui ruolo nell'insorgenza dei tumori gastro-intestinali è supportato da sempre più numerose evidenze scientifiche internazionali. Il presente progetto propone di analizzare la flora microbica orale mediante prelievi di saliva e fluido crevicolare gengivale e di confrontarla con quella dei tessuti intestinali prelevati durante colonscopia diagnostica. L'individuazione di una correlazione tra alcuni specifici microbioti orali e l'insorgenza dell'adenocarcinoma del colon-retto potrebbe portare ad un miglioramento nella diagnosi, nella prevenzione e nella terapia di questa neoplasia.

Chi propone lo studio?

Lo studio è proposto dall'Azienda Ospedaliera ASST Sette Laghi, S.C. Gastroenterologia ed Endoscopia Digestiva, S.C. Odontostomatologia (U.O. Patologia Orale), e dall'Università degli Studi dell'Insubria- Dipartimento di Medicina e Chirurgia.

Altri centri partecipano allo studio?

Al momento non vi sono altri centri che partecipano allo studio.

Perché sono invitato a partecipare allo studio?

Lo studio è rivolto a pazienti potenzialmente affetti da questo tipo di neoplasia che, come lei, devono sottoporsi a colonscopia diagnostica.

Come è progettato lo studio?

Lo studio è di tipo osservazionale prospettico caso-controllo: i dati anamnestici, i campioni micorbiologici orali e i campioni bioptici verranno raccolti senza cambiare il modo con cui i reparti curano normalmente i propri pazienti, attenendosi alla buona pratica clinica. Successivamente questo verrà sottoposto alle indagini molecolari.

Cosa comporta la partecipazione allo studio, rispetto al normale percorso diagnostico-terapeutico per la mia malattia?

La partecipazione allo studio non comporta alcuna variazione alla modalità di approccio previsto per la sua terapia, ad esclusione del prelevamento di un piccolo campione di tessuto sano di mucosa intestinale per poterlo confrontare con quello eventualmente patologico.

Nel caso di assenza di lesioni, verrà prelevato solo un piccolo campione di tessuto sano per le indagini di laboratorio (gruppo controllo).

Prima dell'esecuzione della colonscopia diagnostica riceverà una visita odontoiatrica di controllo, durante la quale verranno prelevati campioni di saliva e fluido crevicolare gengivale mediante inserimento di piccoli coni di carta sterile all'interno del solco gengivale di alcuni elementi dentari. Questa procedura risulterà assolutamente priva di rischi per la sua salute. Normalmente, parte dei frammenti tessutali prelevati in sede chirurgica sono impiegati in un percorso diagnostico-terapeutico. La partecipazione allo studio implica che una minima parte di questi tessuti venga destinata ai fini di questa ricerca.

Quali rischi o inconvenienti potrei avere dalla partecipazione a questo studio?

La partecipazione allo studio non comporterà nessun rischio aggiuntivo alla sua salute.

Sono obbligato a partecipare allo studio?

No. La decisione di partecipare è assolutamente libera. Se lei acconsente, ha la possibilità di contribuire alla ricerca medica attraverso questo studio. Se però non vuole partecipare, non deve fornire alcuna spiegazione. Il suo rifiuto non influenzerà in alcun modo il trattamento che le verrà proposto, e riceverà comunque tutte le terapie previste dalla buona pratica clinica per la sua patologia.

Se in qualsiasi momento lei volesse ritirare il consenso, potrà farlo senza problemi.

Potrò cambiare idea dopo aver accettato di partecipare?

Sì. La decisione di partecipare allo studio è volontaria e libera, e lei ha il diritto di revocare il suo consenso in qualunque momento lo desideri, senza fornire spiegazioni e senza che questo

influenzi in alcun modo il trattamento che le verrà proposto, che sarà comunque il migliore disponibile.

Se partecipo allo studio, i miei dati personali e clinici saranno noti a tutti?

No. I suoi dati clinici saranno resi anonimi. Il suo nome e cognome saranno sostituiti da un codice che solo i responsabili dello studio conosceranno.

Quali vantaggi potrei avere nel partecipare a questo studio?

Lei non riceverà beneficio diretto dalla partecipazione, tuttavia questo studio potrà contribuire a migliorare la diagnosi e la comprensione del carcinoma del colon-retto e portare alla scoperta di nuove cure. Inoltre lei potrà beneficiare gratuitamente di un completo esame specialistico atto a verificare la salute parodontale della sua bocca e di una analisi microbiologica dei suoi tessuti intestinali, strumenti che potranno risultare utili nel monitoraggio della sua salute odontoiatrica e gastro-enterologica. Per la partecipazione allo studio non è previsto alcun costo aggiuntivo.

Trattamento dei dati

Se Lei deciderà di partecipare allo studio, tutti i dati raccolti (età, sesso, origine etnica e altri dati che La riguardano) saranno trattati in via manuale e/o con il supporto di mezzi informatici per le finalità indicate in maniera rigorosamente anonima, ai sensi dell'art. 13 del Regolamento UE 2016/679 in vigore dal 25 Maggio 2018 sulla tutela dei dati personali e saranno trattati in modo assolutamente riservato.

La persona responsabile della gestione dei suoi dati per questo studio è il Dott. Sergio Segato.

L'accesso diretto alla sua documentazione sarà consentito agli addetti al monitoraggio e alle autorità regolatorie nella misura permessa dalle leggi senza violare la sua riservatezza.

Le informazioni mediche personali saranno mantenute conformemente a tutte le leggi applicabili, compreso "Safe Harbor Act" (2000/520/CE) ed "European Union Data Protection Directive (95/46/EC 24 ottobre 1995)."

Il medico della ricerca le consegnerà una lettera rivolta al suo medico di base, per informarlo della sua partecipazione allo studio. Il protocollo dello studio è stato redatto in accordo con la dichiarazione di Helsinki sull'etica della ricerca in medicina ed è stato approvato dal Comitato Etico di questo ospedale.

A chi posso rivolgermi se ho dei problemi durante lo studio?

Il medico referente per questo studio è il Dott. Sergio Segato.

Nome in stampatello del partecipante allo studio _____

Firma del partecipante allo studio _____
Data_____

Nome in stampatello del Medico ricercatore _____

Firma del Medico ricercatore _____
Data_____

Nome in stampatello del rappresentante legale*_____

Firma del rappresentante legale_____
Data_____

*In caso sia designato quale rappresentante legale un amministratore di sostegno, il medico sperimentatore avrà cura di verificare che l'ordinanza di affidamento da parte del giudice tutelare comprenda anche la tutela della salute dell'amministrato

DICHIARAZIONE DI AVVENUTA INFORMAZIONE DEL VOLONTARIO

Io sottoscritto _____ nato a _____ il _____, dichiaro di aver ricevuto una spiegazione chiara e completa da parte del Dott. _____circa la natura, lo scopo e le modalità di esecuzione dello studio sopra descritto.

Ho chiaramente compreso le informazioni che mi sono state fornite con la scheda di informazione dello studio ed ho avuto modo di discutere tutti i dubbi relativi allo studio.

Con la presente dichiaro di aver compreso le finalità dello studio ed in particolare che è necessario il mio consenso per il trattamento dei miei dati personali nonché per la conservazione dei dati clinici biologici. Acconsento inoltre che sui campioni istologici a me prelevati a scopo diagnostico e conservati presso il servizio di anatomia patologica dell'Ospedale di Circolo di Varese, possano essere effettuati ulteriori approfondimenti con revisioni, colorazioni aggiuntive e immunoistochimiche e tutto ciò che possa servire a completamento del progetto di studio, riservando, qualora vi fosse sufficiente quantità, materiale per indagini future diagnostiche. Mi impegno ad informare questo reparto di un'eventuale ritiro del mio consenso.

Mi impegno a consegnare al mio medico di base la lettera fornitami dal Medico Sperimentatore, per informarlo della mia partecipazione allo studio.

Prendo atto che ricevo una copia firmata del presente Modulo di Consenso, unitamente al Foglio Informativo.

Nome in stampatello del partecipante allo studio _____

Firma del partecipante allo studio _____
Data_____

Nome in stampatello del Medico ricercatore _____

Firma del Medico ricercatore _____
Data_____

Nome in stampatello del rappresentante legale*_____

Firma del rappresentante legale_____
Data_____

*In caso sia designato quale rappresentante legale un amministratore di sostegno, il medico sperimentatore avrà cura di verificare che l'ordinanza di affidamento da parte del giudice tutelare comprenda anche la tutela della salute dell'amministrato

Fig. 35 Informed consent to the study

2.3.2 Collection of saliva and crevicular fluid samples

Then patients were asked to provide information about their medical therapies, medications, diseases, allergies, smoking, eating habits (consumption of red and processed meat, consumption of vegetables and fruit, consumption of alcohol), physical activities, BMI (body mass index). Data were recorded in individual registers and in a table.

At this point a dental examination was performed, by means of a dental probe, in order to evaluate the presence or absence of pathological periodontal pockets in all four quadrants (measurements ranging from 1 mm to 3 mm generally indicate an healty gum; pockets deeper than 3 mm may signify gum disease, particularly if bleeding is associated; larger numbers, from 3,5 to 12 mm, reveal the presence of periodontal disease).
PSR index was calculated in each quadrant and registered in a tab.

Gingival crevicular fluid was collect by inserting 4 sterile paper cones (one for each quadrant) into the deepest periodontal pocket of each quadrant, for 30 seconds. Furthermore, 4 sterile paper cones (one for each quadrant) were

inserted into healty gingiva. These paper cones were then placed in separated sterile tubes and stored at -20°C until processing.

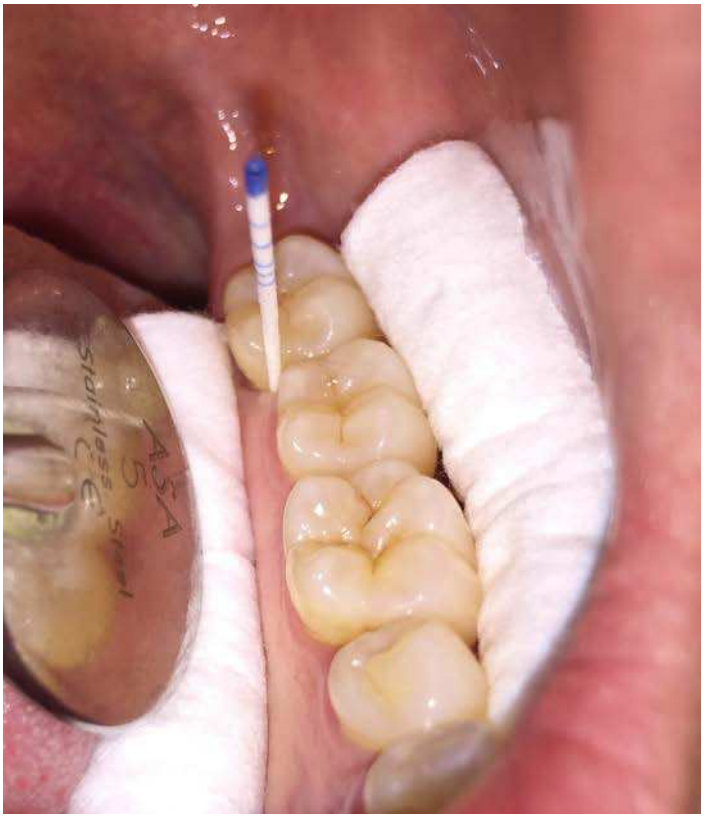


Fig. 36 Crevicular fluid sampling by means of sterile paper cones

2.3.3 Endoscopic examination and colorectal biopsy

A diagnostic colonscopy was performed on each patient in order to verify the prescence of colorectal lesions. All lesions were biopsied in order to perform a histological analysis at the Unit of Pathological Anatomy- Ospedale di Circolo – ASST Sette Laghi.

So patients were divided into 3 groups:

- Patients with a positive histological diagnosis for colorecatl cancer (group 1)
- Patients with a positive histological diagnosis for colorectal adenoma (group 2)
- Patients with colorectal lesions negative at histological examination (control group or group 3)

In group 1 a 2 two intestinal samples (about 5 mm^3) were collected (in addition to the one used for histological analysis): one from pathological mucosa and one from healty mucosa (at a distance of 10 cm from the lesion).
In group 3 only one intestinal sample was collected from healty mucosa.
Intestinal samples were placed into separate sterile tubes containing a stabilizing solution of RNAlater and stored at -20°C until processing.
Finally they were sent to laboratory for microbiological analysis.

2.3.4 DNA isolation

The isolation of the total DNA (human and bacterial) from the samples involved an initial phase of sample digestion, which varied according to the nature of the sample, and a subsequent phase of purification, common to all two types of sample, which was conducted by means of purification kit QIAamp 96 DNA QIAcube HT (Qiagen), using an automated extraction system, the robot QIAcube HT (Qiagen).

Isolation of DNA from paper cones soaked in crevicular liquid

The paper cones were directly processed through incubation for 1 hour at 55 °C, with the lysis solution containing proteinase K. As described below for intestinal biopsy samples, automated purification using QIAcube HT was carried out.

Isolation of DNA from intestinal biopsies

After removal of the RNAlater Preservative Solution, the biopsy specimens were washed with a 1x PBS saline solution.
The tissue was then incubated for 1 hour at 55 °C with a lysis solution containing proteinase K (20 mg/ml) in order to degrade the tissue and digest the nuclear membrane, thereby isolating the total DNA (human and bacterial).
The samples were then processed automatically using the QIAcube HT instrument. This instrument uses an extraction kit that binds the DNA to the resin of a purification column, two successive washes with solutions containing ethanol, and finally the elution of the purified DNA that can be used for subsequent tests.

2.3.5 DNA amplification by PCR-RT

Each sample was amplified by PCR-RT to detect and quantify the presence of the bacterium Fusobacterium nucleatum and Porphyromonas gingivalis.
For this purpose, specific amplification assays weree designed using the sequences contained in the "Human Oral Microbiome" database (HOMD 16S rRNA RefSeq Version 10.1).

Absolute quantification was performed by the Applied Biosystems 7500 Sequence Detection System. The amplification profile involved a 10-minute incubation at 95°C to activate polymerase, followed by two amplification steps of 15 seconds at 95°C and 60 seconds at 57°C for 40 cycles.
Plasmids containing the Fusobacterium nucleatum and Porphyromonas gingivalis sequences were used as standard to construct the dilution curves necessary for absolute quantification by interpolation of the tested bacteria.

2.3.6 Histological analysis of biopsy samples

The fragments of intestinal mucosa subjected to biopsy, taken from all three groups of patients, were analyzed histologically to verify the presence of any architectural and cytological changes in the tissue under examination, necessary for effective diagnosis of colorectal adenocarcinoma.

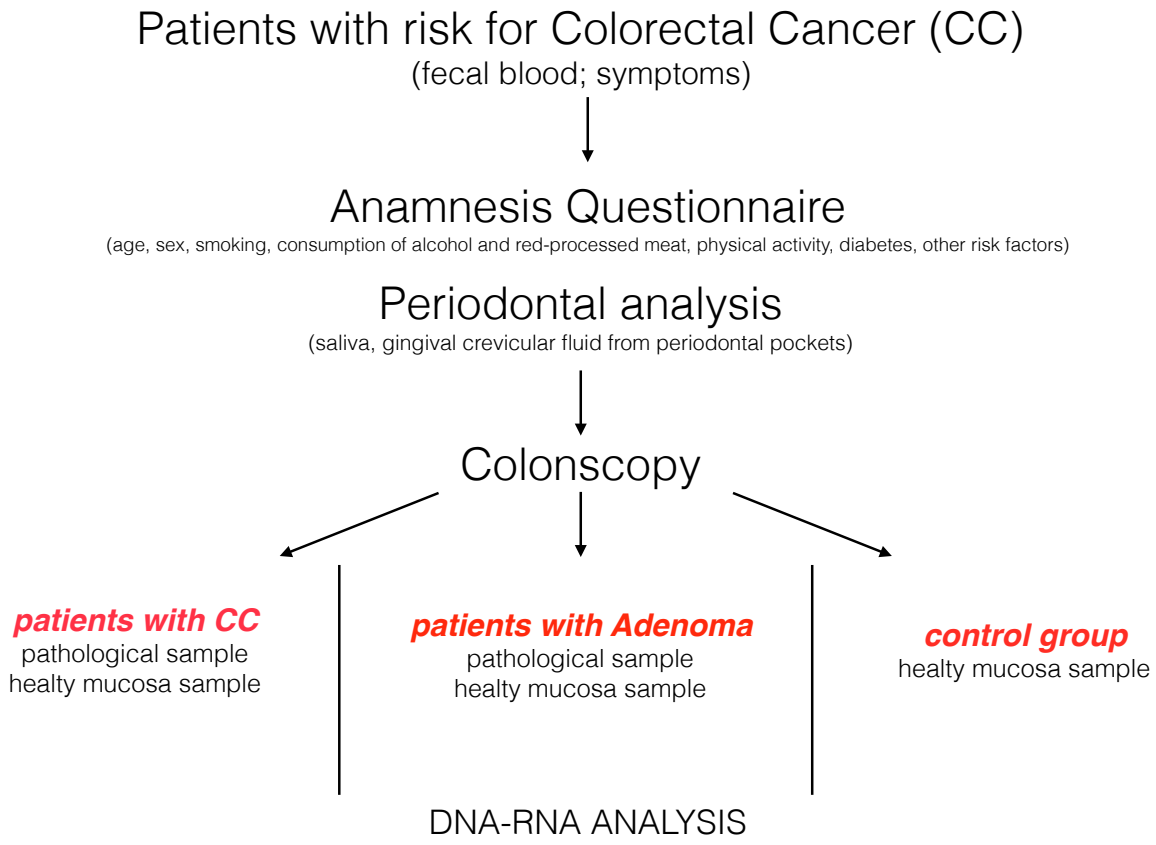


Fig. 37 Study flow-chart

2.3.7 Statistical analysis

Data were elaborated with the SPSS v.20 software by means of a PC with Window 10 operating system. Descriptive statistics of continuous variables were presented as mean ± standard deviation (SD) whereas the categorical variables were presented as numbers and frequencies.

Bacterial load comparison between patients groups was performed by ANOVA and Dunnet post hoc test. Data were also processed with the non-parametric Kruskal-Wallis statistics because data did not always fit the Gaussian distribution.

In order to establish in this study if there were any possible connections between oral and intestinal microflora, correlation analysis was performed to statistical

evaluate the strength of a relationship between oral and intestinal bacterial load in different groups of patients.

Results were considered statistically significant at p value less than 0.05.

2.4 RESULTS AND DISCUSSION

A total number of 110 patients underwent the experimental procedure: 61 males and 49 females.

According to coloscopy and histological analysis patients were divided in 3 gropus:

- Group 1: patients with colorectal cancer
- Group 2: patients with colorectal adenoma
- Group 3: healty patients (control group)

Ages registered assessed between 41 and 89, with mean age of 64 years.

Descriptive Statistics

DIAGNOSIS		N	Minimum	Maximum	Mean	Std. Deviation
Cancer	AGE	10	58	89	74.30	10.781
	Valid N (listwise)	10				
Adenoma	AGE	50	49	82	64.50	7.192
	Valid N (listwise)	50				
Healthy	AGE	55	41	77	61.00	7.604
	Valid N (listwise)	55				

Tab 5 Age in 3 groups

In group 1 ages registered assessed between 58 and 89, with mean age of 74 years. In group 2 ages registered assessed between 49 and 82, with mean age of 64,5 years. In group 3 ages registered assessed between 41 and 77, with mean age of 61 years.

SEX * DIAGNOSIS Crosstabulation

			DIAGNOSIS			Total
			Cancer	Adenoma	Healthy	
SEX	Male	Count	6	34	29	69
		% within SEX	8.7%	49.3%	42.0%	100.0%

	% within DIAGNOSIS	60.0%	66.7%	50.9%	58.5%
	% of Total	5.1%	28.8%	24.6%	58.5%
Female	Count	4	17	28	49
	% within SEX	8.2%	34.7%	57.1%	100.0%
	% within DIAGNOSIS	40.0%	33.3%	49.1%	41.5%
	% of Total	3.4%	14.4%	23.7%	41.5%
Total	Count	10	51	57	118
	% within SEX	8.5%	43.2%	48.3%	100.0%
	% within DIAGNOSIS	100.0%	100.0%	100.0%	100.0%
	% of Total	8.5%	43.2%	48.3%	100.0%

Tab 6 Sex of patients in the three groups

Among patients belonging to group 1, 6 (60%) were male and 4 (40%) were female.

Among patients belonging to group 2, 26 (66%) were male and 17 (34%) were female.

Among patients belonging to group 3, 29 (51%) were male and 28 (49%) were female.

Fusobacterium nucleatum load and Phorphyromonas gingivalis load and the total bacterial load was calculated for all the patients, in healty gingiva, in the deepest periodontal pocket, in healty intestinal mucosa and in pathological intestinal mucosa:

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
CTRLGINGFN	105	0	618315	15574.13	64257.936
CTRLGINGPG	105	0	1389416	26321.28	154800.531
CTRLGIGTOT	105	2828	8471355	686900.42	1275358.242
PATHGINGFN	105	0	4005968	135482.55	505242.319
PATHGIGPG	105	0	5426336	119166.04	667728.682
PATHGINGTOT	105	1795	11266334	1402977.73	2368375.350
CTRLBOWFN	118	0	2041008	19854.31	187967.399
CTRLBOWPG	118	0	9654	173.73	1139.774
CTRLBOWTOT	118	5959	2209906	168561.93	287797.752
PATHBOWFN	64	0	4278689	68739.33	534621.843
PATHBOWPG	64	0	3469	78.52	447.350

PATHBOWTOT	64	2547	13841040	353473.39	1723649.678
Valid N (listwise)	54				

Tab. 7 Bacterial load in different tissues

The same loads were calculated dividing the patients in the three group in order to observe the differences within each group and among groups:

Descriptive Statistics

DIAGNOSIS		N	Minimum	Maximum	Mean	Std. Deviation
	CTRLGINGFN	5	0	1949	389.80	871.619
	CTRLGINGPG	5	0	2130	499.40	925.282
	CTRLGIGTOT	5	2935	74367	18147.00	31440.195
	PATHGINGFN	5	0	302936	60664.20	135434.156
	PATHGIGPG	5	0	29469	6076.40	13081.980
	PATHGINGTOT	5	2940	1872731	377555.60	835829.180
Cancer	CTRLBOWFN	10	0	2041008	209356.80	643769.750
	CTRLBOWPG	10	0	45	4.50	14.230
	CTRLBOWTOT	10	23631	2209906	414710.70	653272.680
	PATHBOWFN	10	0	4278689	431133.40	1351905.131
	PATHBOWPG	10	0	359	40.10	112.399
	PATHBOWTOT	10	26088	13841040	1492580.30	4339640.495
	Valid N (listwise)	5				
	CTRLGINGFN	46	0	618315	20350.20	91261.136
	CTRLGINGPG	46	0	17619	1917.61	4291.729
	CTRLGIGTOT	46	2828	5166896	491986.15	905009.373
	PATHGINGFN	46	0	4005968	129797.41	591322.450
	PATHGIGPG	46	0	4182093	107644.50	615512.182
	PATHGINGTOT	46	1795	9963334	1157202.09	1937110.075
Adenoma	CTRLBOWFN	51	0	96270	4239.55	14798.358
	CTRLBOWPG	51	0	21	1.69	3.987
	CTRLBOWTOT	51	5959	1398011	236417.57	289746.108
	PATHBOWFN	51	0	31494	1725.16	5214.099
	PATHBOWPG	51	0	3469	90.63	499.070
	PATHBOWTOT	51	2547	1255353	133558.27	202949.469
	Valid N (listwise)	46				
	CTRLGINGFN	54	0	153604	12911.59	31338.028
	CTRLGINGPG	54	0	1389416	49500.50	214194.385
Healthy	CTRLGIGTOT	54	2850	8471355	914860.11	1538863.003
	PATHGINGFN	54	0	2462323	147253.07	449379.692
	PATHGIGPG	54	0	5426336	139451.94	742613.442

	PATHGINGTOT	54	2101	11266334	1707288.67	2742633.218
	CTRLBOWFN	57	0	12320	579.37	1819.122

DIAGNOSIS		N	Minimum	Maximum	Mean	Std. Deviation
Healthy	CTRLBOWPG	57	0	9654	357.35	1627.183
	CTRLBOWTOT	57	18877	299456	64665.00	59204.433
	PATHBOWFN	3	0	0	.00	.000
	PATHBOWPG	3	0	2	.67	1.155
	PATHBOWTOT	3	29135	602340	295007.33	288842.885
	Valid N (listwise)	3				

Tab. 8 Bacterial loads in different groups

PSR index was calculated in 104 patients (5 belonging to group 1, 45 to group 2 and 54 to group 3). In 6 patients it was not possible to measure all periodontal pockets of the tooth so a valid PSR could not be registered.

Condidering all patients mean PSR value was 2,83.

In group 1 mean PSR value was 2,60.

In group 2 mean PSR value was 2,93.

In group 3 mean PSR value was 2,76.

Periodontal pocktes were measured in all 4 quadrants.

Considering all patients mean periodontal pocket depth was 4, 28mm in the first quadrant, 4, 52 mm in the second quadrant, 4,49 mm in the third and 4,68 in the fourth.

Descriptive Statistics

	N	Minimum	Maximum	Mean	Std. Deviation
POCKET1	82	2	8	4.28	1.552
POCKET2	81	2	9	4.52	1.811

POCKET3	87	2	10	4.49	1.776
POCKET4	85	2	10	4.68	1.907

Tab. 9 Periodontal pocket depth in 4 quadrants considering all patients

The same measures were calculated in the 3 groups in order to observe differences in each group and among groups.

Descriptive Statistics

DIAGNOSIS		N	Minimum	Maximum	Mean	Std. Deviation
Cancer	POCKET1	3	2	4	3.00	1.000
	POCKET2	2	2	3	2.50	.707
	POCKET3	4	3	5	3.38	.750
	POCKET4	4	2	5	3.25	1.258
Adenoma	POCKET1	33	2	7	4.47	1.541
	POCKET2	33	2	9	4.59	1.839
	POCKET3	38	2	9	4.41	1.766
	POCKET4	36	2	10	4.68	1.852
Healthy	POCKET1	46	2	8	4.23	1.570
	POCKET2	46	2	9	4.57	1.797
	POCKET3	45	2	10	4.67	1.831
	POCKET4	45	2	10	4.81	1.975

Tab. 10 Periodontal pocket depth in different groups

Looking at intestinal samples, Fusobacterium nucleatum load, Phorpyromonas gingivalis load and the total bacterial load was calculated in the three groups on healty and pathological mucosa and Oneway ANOVA was performed in order to find statistically significant differences between mean values considering the 3 intestinal conditions :

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
CTRLBOWFN	Between Groups	392723628199.908	2	196361814099.954	6.036	.003
	Within Groups	3741090307847.491	115	32531220068.239		
	Total	4133813936047.398	117			
CTRLBOWPG	Between Groups	3717786.859	2	1858893.430	1.442	.241
	Within Groups	148275262.463	115	1289350.108		
	Total	151993049.322	117			
CTRLBOWTOT	Between Groups	1456006549710.848	2	728003274855.424	10.167	.000
	Within Groups	8234816333956.609	115	71607098556.144		
	Total	9690822883667.457	117			
PATHBOWFN	Between Groups	1556505775642.964	2	778252887821.482	2.886	.063
	Within Groups	16450186679037.143	61	269675191459.625		
	Total	18006692454680.105	63			
PATHBOWPG	Between Groups	40420.496	2	20210.248	.098	.907
	Within Groups	12567255.488	61	206020.582		
	Total	12607675.984	63			
PATHBOWTOT	Between Groups	15452395922518.312	2	7726197961259.156	2.745	.072
	Within Groups	171718601435668.900	61	2815059039928.999		
	Total	187170997358187.220	63			

Tab. 11 ANOVA on intestinal samples

A P value of 0,003 was found in Fn load on healty intestinal mucosa.
Similarly a P value of 0.000 was found considering the total bacterial load on healty mucosa.
On the other hand the Pg load on healty mucosa is not significative.
Both, single bacterial load (Pg and Fn) and total bacterial load on pathological mucosa do not show significative values.

These differences are due to higher mean values of Fusobacterium nucleatum load on healty mucosa in patients with diagnosis of carcinoma compared to control group patients. This result is similar to that one emerging from Kostic analysis [53], who found that Fusobacterium sequences were significantly enriched in the colorectal cancer tissues, if compared with other bacteria.

It is very interesting to note that intermediate values (fewer than cancer group but higher than control group) can be observed in patients with a diagnosis of adenoma, which can actually be considered a potential precancerous lesion.
The same was observed on healty bowel mucosa considering the total bacterial load:

Multiple Comparisons

Dunnett t (2-sided)

Dependent Variable	(I) DIAGNOSIS	(J) DIAGNOSIS	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
CTRLBOWFN	Cancer	Healthy	208777.432*	61837.247	.002	69105.01	348449.86
	Adenoma	Healthy	3660.181	34764.784	.993	-74863.39	82183.76
CTRLBOWPG	Cancer	Healthy	-352.851	389.301	.590	-1232.17	526.47
	Adenoma	Healthy	-355.665	218.864	.198	-850.02	138.69
CTRLBOWTOT	Cancer	Healthy	350045.700*	91744.072	.000	142822.42	557268.98
	Adenoma	Healthy	171752.569*	51578.345	.002	55252.03	288253.11
PATHBOWFN	Cancer	Healthy	431133.400	341846.822	.290	-313883.78	1176150.58
	Adenoma	Healthy	1725.157	308511.717	1.000	-670641.87	674092.18
PATHBOWPG	Cancer	Healthy	39.433	298.790	.979	-611.75	690.61
	Adenoma	Healthy	89.961	269.654	.881	-497.72	677.64
PATHBOWTOT	Cancer	Healthy	1197572.967	1104472.235	.379	-1209501.60	3604647.54
	Adenoma	Healthy	-161449.059	996769.908	.969	-2333798.37	2010900.25

*. The mean difference is significant at the 0.05 level.

a. Dunnett t-tests treat one group as a control, and compare all other groups against it.

Tab. 12 Dunnett test used to evaluate the ANOVA analysis

Among non parametric tests Kruskal-Wallis Test and Median Test were used to compare independent samples concerning the total bacterial load:

Ranks

	DIAGNOSIS	N	Mean Rank
CTRLBOWTOT	Cancer	10	79.40
	Adenoma	51	74.06
	Healthy	57	42.98
	Total	118	

Tab. 13 Non-parametric Kruskal-Wallis Test considering the total bacterial load

A chi square test confirmed the results (p=0.000):

Test Statistics[a,b]

	CTRLBOWTOT
Chi-Square	25.912
df	2
Asymp. Sig.	.000

a. Kruskal Wallis Test

b. Grouping Variable: DIAGNOSIS

Frequencies

		DIAGNOSIS		
		Cancer	Adenoma	Healthy
CTRLBOWTOT	> Median	8	36	15
	<= Median	2	15	42

Tab. 14 Non-parametric Median Test considering the total bacterial load

A chi square test confirmed the results:

Test Statistics[a]

	CTRLBOWTOT
N	118
Median	69709.00
Chi-Square	25.037[b]
df	2
Asymp. Sig.	.000

a. Grouping Variable: DIAGNOSIS

b. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 5.0.

Similarly as done before, considering gingival samples, Fusobacterium nucleatum load, Phorpyromonas gingivalis load and the total bacterial load was calculated in the three groups on healty and pathological gums and non parametric tests Kruskal-Wallis Test and Median Test were performed:

Ranks

	DIAGNOSIS	N	Mean Rank
CTRLGINGFN	Cancer	5	34.80
	Adenoma	46	53.26
	Healthy	54	54.46
	Total	105	
CTRLGINGPG	Cancer	5	51.00
	Adenoma	46	52.76
	Healthy	54	53.39
	Total	105	
CTRLGIGTOT	Cancer	5	58.69
	Adenoma	46	49.78
	Healthy	54	21.20
	Total	105	
PATHGINGFN	Cancer	5	45.30
	Adenoma	46	56.42
	Healthy	54	50.80
	Total	105	
PATHGIGPG	Cancer	5	49.00
	Adenoma	46	53.82
	Healthy	54	52.68
	Total	105	

PATHGINGTOT	Cancer	5	26.80
	Adenoma	46	52.70
	Healthy	54	55.69
	Total	105	
CTRLBOWTOT	Cancer	10	79.40
	Adenoma	51	74.06
	Healthy	57	42.98
	Total	118	

Test Statistics[a,b]

	CTRLGINGFN	CTRLGINGPG	CTRLGIGTOT	PATHGINGFN	PATHGIGPG	PATHGINGTOT	CTRLBOWTOT
Chi-Square	2.196	.040	7.847	1.347	.135	4.125	25.912
df	2	2	2	2	2	2	2
Asymp. Sig.	.334	.980	.020	.510	.935	.127	.000

a. Kruskal Wallis Test

b. Grouping Variable: DIAGNOSIS

Tab. 15 Non parametric Kruskal-Wallis Test

A significative difference have been detected among groups considering the total bacterial load in gingival tissues (P=0.020).

Frequencies

		DIAGNOSIS		
		Cancer	Adenoma	Healthy
CTRLGINGFN	> Median	1	22	29
	<= Median	4	24	25
CTRLGINGPG	> Median	2	20	26
	<= Median	3	26	28
CTRLGIGTOT	< Median	0	20	32
	>= Median	5	26	22
PATHGINGFN	> Median	1	27	24
	<= Median	4	19	30

PATHGIGPG	> Median	2	25	25
	<= Median	3	21	29
PATHGINGTOT	> Median	1	21	30
	<= Median	4	25	24
CTRLBOWTOT	> Median	8	36	15
	<= Median	2	15	42

Test Statistics[a]

	CTRLGINGFN	CTRLGINGPG	CTRLGIGTOT	PATHGINGFN	PATHGIGPG	PATHGINGTOT	CTRLBOWTOT
N	105	105	105	105	105	105	118
Median	.00	.00	162727.00	385.00	94.00	399975.00	69709.00
Chi-Square	2.174[b]	.287[c]	7.626[b]	3.849[b]	.835[b]	2.805[b]	25.037[d]
df	2	2	2	2	2	2	2
Asymp. Sig.	.337	.866	.022	.146	.659	.246	.000

a. Grouping Variable: DIAGNOSIS

b. 2 cells (33.3%) have expected frequencies less than 5. The minimum expected cell frequency is 2.5.

c. 2 cells (33.3%) have expected frequencies less than 5. The minimum expected cell frequency is 2.3.

d. 0 cells (0.0%) have expected frequencies less than 5. The minimum expected cell frequency is 5.0.

Tab. 16 Non-parametric Median Test

The previous data were confirmed , in fact a significative difference have been detected among groups considering the total bacterial load in gingival tissues (P=0.022) and a certain trend is observable looking at Fusobacterium nucleatum.

Finally, the presence of correlations between intestinal mucosa and gingival tissue were analysed, firstly considering all patients:

Correlations

		CTRL GINGFN	CTRL GINGPG	CTRL GIGTOT	PATH GINGFN	PATH GIGPG	PATH GINGTOT	CTRL BOWFN	CTRL BOWPG	CTRL BOWTOT	PATH BOWFN	PATH BOWPG	PATH BOWTOT
CTRLGINGFN	Pearson Correlation	1	-.025	.056	.004	-.021	.032	-.038	-.027	-.041	-.029	-.037	-.032
	Sig. (2-tailed)		.803	.570	.968	.834	.745	.698	.781	.680	.834	.788	.820
	N	105	105	105	105	105	105	105	105	105	54	54	54
CTRLGINGPG	Pearson Correlation	-.025	1	.398**	-.031	.680**	.339**	-.040	.215*	-.042	-.062	-.075	-.075
	Sig. (2-tailed)	.803		.000	.750	.000	.000	.689	.028	.669	.657	.588	.590
	N	105	105	105	105	105	105	105	105	105	54	54	54
CTRLGIGTOT	Pearson Correlation	.056	.398**	1	-.011	.313**	.581**	-.108	.093	-.085	-.073	-.098	-.057
	Sig. (2-tailed)	.570	.000		.908	.001	.000	.274	.344	.390	.602	.480	.682
	N	105	105	105	105	105	105	105	105	105	54	54	54
PATHGINGFN	Pearson Correlation	.004	-.031	-.011	1	.453**	.517**	-.010	-.037	-.029	.047	.010	.046
	Sig. (2-tailed)	.968	.750	.908		.000	.000	.922	.706	.769	.735	.945	.741
	N	105	105	105	105	105	105	105	105	105	54	54	54
PATHGIGPG	Pearson Correlation	-.021	.680**	.313**	.453**	1	.555**	-.033	-.020	-.044	-.016	-.031	-.020
	Sig. (2-tailed)	.834	.000	.001	.000		.000	.737	.840	.658	.911	.822	.888
	N	105	105	105	105	105	105	105	105	105	54	54	54
PATHGINGTOT	Pearson Correlation	.032	.339**	.581**	.517**	.555**	1	-.033	-.043	-.022	.060	-.088	.062
	Sig. (2-tailed)	.745	.000	.000	.000	.000		.735	.663	.825	.664	.527	.654
	N	105	105	105	105	105	105	105	105	105	54	54	54

CTRLBOWFN	Pearson Correlation	-.038	-.040	-.108	-.010	-.033	-.033	1	-.016	.666**	.011	-.025	-.002
	Sig. (2-tailed)	.698	.689	.274	.922	.737	.735		.862	.000	.932	.843	.988
	N	105	105	105	105	105	105	118	118	118	64	64	64
CTRLBOWPG	Pearson Correlation	-.027	.215*	.093	-.037	-.020	-.043	-.016	1	-.062	-.041	.146	-.031
	Sig. (2-tailed)	.781	.028	.344	.706	.840	.663	.862		.504	.749	.251	.807
	N	105	105	105	105	105	105	118	118	118	64	64	64
CTRLBOWTOT	Pearson Correlation	-.041	-.042	-.085	-.029	-.044	-.022	.666**	-.062	1	.063	.043	.057
	Sig. (2-tailed)	.680	.669	.390	.769	.658	.825	.000	.504		.619	.736	.654
	N	105	105	105	105	105	105	118	118	118	64	64	64
PATHBOWFN	Pearson Correlation	-.029	-.062	-.073	.047	-.016	.060	.011	-.041	.063	1	-.023	.993**
	Sig. (2-tailed)	.834	.657	.602	.735	.911	.664	.932	.749	.619		.857	.000
	N	54	54	54	54	54	54	64	64	64	64	64	64
PATHBOWPG	Pearson Correlation	-.037	-.075	-.098	.010	-.031	-.088	-.025	.146	.043	-.023	1	-.029
	Sig. (2-tailed)	.788	.588	.480	.945	.822	.527	.843	.251	.736	.857		.818
	N	54	54	54	54	54	54	64	64	64	64	64	64
PATHBOWTOT	Pearson Correlation	-.032	-.075	-.057	.046	-.020	.062	-.002	-.031	.057	.993**	-.029	1
	Sig. (2-tailed)	.820	.590	.682	.741	.888	.654	.988	.807	.654	.000	.818	
	N	54	54	54	54	54	54	64	64	64	64	64	64

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

Tab. 17 Correlations between intestinal and gingival conditions considering all patients

Then correlatations were evaluated dividing the three groups; firstly in cancer group:

Correlations[a]

		CTRLGINGFN	CTRLGINGPG	CTRLGIGTOT	PATHGINGFN	PATHGIGPG	PATHGINGTOT	CTRLBOWFN	CTRLBOWPG	CTRLBOWTOT	PATHBOWFN	PATHBOWPG	PATHBOWTOT
CTRLGINGFN	Pearson Correlation	1	-.302	-.256	-.249	-.260	-.250	-.255	-.250	.662	-.248	-.250	-.258
	Sig. (2-tailed)		.622	.678	.687	.673	.685	.679	.685	.224	.687	.685	.675
	N	5	5	5	5	5	5	5	5	5	5	5	5
CTRLGINGPG	Pearson Correlation	-.302	1	-.057	-.302	-.275	-.301	-.304	.985**	-.618	-.302	.985**	-.297
	Sig. (2-tailed)	.622		.927	.621	.654	.623	.619	.002	.266	.621	.002	.627
	N	5	5	5	5	5	5	5	5	5	5	5	5
CTRLGIGTOT	Pearson Correlation	-.256	-.057	1	-.271	-.277	-.271	-.255	-.228	-.548	-.271	-.228	-.274
	Sig. (2-tailed)	.678	.927		.659	.651	.659	.679	.713	.339	.659	.713	.655
	N	5	5	5	5	5	5	5	5	5	5	5	5
PATHGINGFN	Pearson Correlation	-.249	-.302	-.271	1	1.000**	1.000**	1.000**	-.250	.508	1.000**	-.250	1.000**
	Sig. (2-tailed)	.687	.621	.659		.000	.000	.000	.685	.382	.000	.685	.000
	N	5	5	5	5	5	5	5	5	5	5	5	5
PATHGIGPG	Pearson Correlation	-.260	-.275	-.277	1.000**	1	1.000**	.999**	-.223	.495	1.000**	-.223	1.000**
	Sig. (2-tailed)	.673	.654	.651	.000		.000	.000	.719	.397	.000	.719	.000
	N	5	5	5	5	5	5	5	5	5	5	5	5
PATHGINGTOT	Pearson Correlation	-.250	-.301	-.271	1.000**	1.000**	1	1.000**	-.249	.507	1.000**	-.249	1.000**
	Sig. (2-tailed)	.685	.623	.659	.000	.000		.000	.687	.384	.000	.687	.000
	N	5	5	5	5	5	5	5	5	5	5	5	5
CTRLBOWFN	Pearson Correlation	-.255	-.304	-.255	1.000**	.999**	1.000**	1	-.114	.968**	-.083	-.129	-.094

	Sig. (2-tailed)	.679	.619	.679	.000	.000	.000		.753	.000	.819	.723	.795
	N	5	5	5	5	5	5	10	10	10	10	10	10
CTRLBOWPG	Pearson Correlation	-.250	.985**	-.228	-.250	-.223	-.249	-.114	1	-.174	-.112	.997**	-.101
	Sig. (2-tailed)	.685	.002	.713	.685	.719	.687	.753		.630	.758	.000	.781
	N	5	5	5	5	5	5	10	10	10	10	10	10
CTRLBOWTOT	Pearson Correlation	.662	-.618	-.548	.508	.495	.507	.968**	-.174	1	.013	-.196	.003
	Sig. (2-tailed)	.224	.266	.339	.382	.397	.384	.000	.630		.972	.588	.994
	N	5	5	5	5	5	5	10	10	10	10	10	10
PATHBOWFN	Pearson Correlation	-.248	-.302	-.271	1.000**	1.000**	1.000**	-.083	-.112	.013	1	-.126	1.000**
	Sig. (2-tailed)	.687	.621	.659	.000	.000	.000	.819	.758	.972		.728	.000
	N	5	5	5	5	5	5	10	10	10	10	10	10
PATHBOWPG	Pearson Correlation	-.250	.985**	-.228	-.250	-.223	-.249	-.129	.997**	-.196	-.126	1	-.116
	Sig. (2-tailed)	.685	.002	.713	.685	.719	.687	.723	.000	.588	.728		.750
	N	5	5	5	5	5	5	10	10	10	10	10	10
PATHBOWTOT	Pearson Correlation	-.258	-.297	-.274	1.000**	1.000**	1.000**	-.094	-.101	.003	1.000**	-.116	1
	Sig. (2-tailed)	.675	.627	.655	.000	.000	.000	.795	.781	.994	.000	.750	
	N	5	5	5	5	5	5	10	10	10	10	10	10

**. Correlation is significant at the 0.01 level (2-tailed).

a. DIAGNOSIS = Cancer

Tab. 18 Correlations between intestinal and gingival conditions cancer group

Then in adenoma group:

Correlations[a]

		CTRLGINGFN	CTRLGINGPG	CTRLGIGTOT	PATHGINGFN	PATHGIGPG	PATHGINGTOT	CTRLBOWFN	CTRLBOWPG	CTRLBOWTOT	PATHBOWFN	PATHBOWPG	PATHBOWTOT
CTRLGINGFN	Pearson Correlation	1	.547**	.043	-.017	-.011	.014	-.057	.416**	-.078	-.061	-.041	-.018
	Sig. (2-tailed)		.000	.777	.911	.944	.924	.706	.004	.608	.689	.786	.906
	N	46	46	46	46	46	46	46	46	46	46	46	46
CTRLGINGPG	Pearson Correlation	.547**	1	-.065	-.057	-.046	.033	-.104	.381**	-.099	-.085	-.085	-.130
	Sig. (2-tailed)	.000		.669	.706	.763	.826	.491	.009	.513	.575	.574	.389
	N	46	46	46	46	46	46	46	46	46	46	46	46
CTRLGIGTOT	Pearson Correlation	.043	-.065	1	-.036	-.024	.439**	-.128	-.112	.012	-.109	-.104	.019
	Sig. (2-tailed)	.777	.669		.811	.873	.002	.398	.460	.934	.469	.492	.901
	N	46	46	46	46	46	46	46	46	46	46	46	46
PATHGINGFN	Pearson Correlation	-.017	-.057	-.036	1	.986**	.678**	-.025	-.043	-.088	-.022	.008	.004
	Sig. (2-tailed)	.911	.706	.811		.000	.000	.867	.779	.563	.886	.957	.981
	N	46	46	46	46	46	46	46	46	46	46	46	46
PATHGIGPG	Pearson Correlation	-.011	-.046	-.024	.986**	1	.679**	-.038	-.047	-.070	-.027	-.034	-.033
	Sig. (2-tailed)	.944	.763	.873	.000		.000	.803	.757	.642	.861	.822	.827
	N	46	46	46	46	46	46	46	46	46	46	46	46
PATHGINGTOT	Pearson Correlation	.014	.033	.439**	.678**	.679**	1	-.037	-.120	.025	-.095	-.092	-.005
	Sig. (2-tailed)	.924	.826	.002	.000	.000		.808	.426	.871	.528	.543	.972
	N	46	46	46	46	46	46	46	46	46	46	46	46
CTRLBOWFN	Pearson Correlation	-.057	-.104	-.128	-.025	-.038	-.037	1	.060	.104	.392**	-.052	-.126
	Sig. (2-tailed)	.706	.491	.398	.867	.803	.808		.675	.470	.004	.716	.379

	N	46	46	46	46	46	46	51	51	51	51	51	51
CTRLBOWPG	Pearson Correlation	.416**	.381**	-.112	-.043	-.047	-.120	.060	1	.334*	-.124	.133	.046
	Sig. (2-tailed)	.004	.009	.460	.779	.757	.426	.675		.017	.385	.354	.749
	N	46	46	46	46	46	46	51	51	51	51	51	51
CTRLBOWTOT	Pearson Correlation	-.078	-.099	.012	-.088	-.070	.025	.104	.334*	1	-.022	.084	.050
	Sig. (2-tailed)	.608	.513	.934	.563	.642	.871	.470	.017		.878	.559	.725
	N	46	46	46	46	46	46	51	51	51	51	51	51
PATHBOWFN	Pearson Correlation	-.061	-.085	-.109	-.022	-.027	-.095	.392**	-.124	-.022	1	-.061	-.112
	Sig. (2-tailed)	.689	.575	.469	.886	.861	.528	.004	.385	.878		.670	.433
	N	46	46	46	46	46	46	51	51	51	51	51	51
PATHBOWPG	Pearson Correlation	-.041	-.085	-.104	.008	-.034	-.092	-.052	.133	.084	-.061	1	-.070
	Sig. (2-tailed)	.786	.574	.492	.957	.822	.543	.716	.354	.559	.670		.628
	N	46	46	46	46	46	46	51	51	51	51	51	51
PATHBOWTOT	Pearson Correlation	-.018	-.130	.019	.004	-.033	-.005	-.126	.046	.050	-.112	-.070	1
	Sig. (2-tailed)	.906	.389	.901	.981	.827	.972	.379	.749	.725	.433	.628	
	N	46	46	46	46	46	46	51	51	51	51	51	51

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

a. DIAGNOSIS = Adenoma

Tab. 19 Correlations between intestinal and gingival conditions in adenoma group

And finally in healty control group:

Correlations[a]

		CTRLGINGFN	CTRLGINGPG	CTRLGIGTOT	PATHGINGFN	PATHGIGPG	PATHGINGTOT	CTRLBOWFN	CTRLBOWPG	CTRLBOWTOT	PATHBOWFN	PATHBOWPG	PATHBOWTOT
CTRLGINGFN	Pearson Correlation	1	-.080	.134	.070	-.055	.091	.192	-.064	.079	.[b]	1.000**	-.124
	Sig. (2-tailed)		.566	.334	.617	.692	.513	.164	.645	.570	.	.000	.921
	N	54	54	54	54	54	54	54	54	54	3	3	3
CTRLGINGPG	Pearson Correlation	-.080	1	.434**	-.055	.862**	.390**	-.062	.198	.105	.[b]	1.000**	-.124
	Sig. (2-tailed)	.566		.001	.693	.000	.004	.654	.152	.448	.	.000	.921
	N	54	54	54	54	54	54	54	54	54	3	3	3
CTRLGIGTOT	Pearson Correlation	.134	.434**	1	-.009	.455**	.619**	-.100	.080	-.041	.[b]	-.495	.924
	Sig. (2-tailed)	.334	.001		.949	.001	.000	.473	.568	.770	.	.671	.250
	N	54	54	54	54	54	54	54	54	54	3	3	3
PATHGINGFN	Pearson Correlation	.070	-.055	-.009	1	-.018	.432**	-.065	-.064	.237	.[b]	.[b]	.[b]
	Sig. (2-tailed)	.617	.693	.949		.897	.001	.640	.644	.084	.	.000	.000
	N	54	54	54	54	54	54	54	54	54	3	3	3
PATHGIGPG	Pearson Correlation	-.055	.862**	.455**	-.018	1	.498**	-.053	-.031	.038	.[b]	.[b]	.[b]
	Sig. (2-tailed)	.692	.000	.001	.897		.000	.706	.825	.782	.	.000	.000
	N	54	54	54	54	54	54	54	54	54	3	3	3
PATHGINGTOT	Pearson Correlation	.091	.390**	.619**	.432**	.498**	1	-.096	-.075	.098	.[b]	-.501	.921
	Sig. (2-tailed)	.513	.004	.000	.001	.000		.488	.591	.482	.	.666	.254
	N	54	54	54	54	54	54	54	54	54	3	3	3
CTRLBOWFN	Pearson Correlation	.192	-.062	-.100	-.065	-.053	-.096	1	-.068	.064	.[b]	.[b]	.[b]
	Sig. (2-tailed)	.164	.654	.473	.640	.706	.488		.616	.637	.	.000	.000

	N	54	54	54	54	54	54	57	57	57	3	3	3
CTRLBOWPG	Pearson Correlation	-.064	.198	.080	-.064	-.031	-.075	-.068	1	-.053	.[b]	.[b]	.[b]
	Sig. (2-tailed)	.645	.152	.568	.644	.825	.591	.616		.694	.	.000	.000
	N	54	54	54	54	54	54	57	57	57	3	3	3
CTRLBOWTOT	Pearson Correlation	.079	.105	-.041	.237	.038	.098	.064	-.053	1	.[b]	.097	-1.000[*]
	Sig. (2-tailed)	.570	.448	.770	.084	.782	.482	.637	.694		.	.938	.018
	N	54	54	54	54	54	54	57	57	57	3	3	3
PATHBOWFN	Pearson Correlation	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]	.[b]
	Sig. (2-tailed)	.	.	.	.	.	.	.	.	.		.	.
	N	3	3	3	3	3	3	3	3	3	3	3	3
PATHBOWPG	Pearson Correlation	1.000[**]	1.000[**]	-.495	.[b]	.[b]	-.501	.[b]	.[b]	.097	.[b]	1	-.124
	Sig. (2-tailed)	.000	.000	.671	.000	.000	.666	.000	.000	.938	.		.921
	N	3	3	3	3	3	3	3	3	3	3	3	3
PATHBOWTOT	Pearson Correlation	-.124	-.124	.924	.[b]	.[b]	.921	.[b]	.[b]	-1.000[*]	.[b]	-.124	1
	Sig. (2-tailed)	.921	.921	.250	.000	.000	.254	.000	.000	.018	.	.921	
	N	3	3	3	3	3	3	3	3	3	3	3	3

**. Correlation is significant at the 0.01 level (2-tailed).

*. Correlation is significant at the 0.05 level (2-tailed).

a. DIAGNOSIS = Healthy

b. Cannot be computed because at least one of the variables is constant.

Tab. 20 Correlations between intestinal and gingival conditions in control group

The aim of the intestinal samples analysis was to evaluate the presence of a statistically significative difference among the bacterial load in patients with

different histological conditions: colorectal cancer, healty mucosa and colorectal adenoma, which can be considered a pre-malignant lesion. Considering colorectal carcinogenesis the development of a tumor is often precedeed by asymptomatic growing polyps, whose early detection represents a fundamental aim of screening colonscopy.

Significative differences among the groups were found considering Fusobacterium nucleatum load (P=0,003) and the total bacterial load (P=0,000) on healty intestinal mucosa. The Dunnett Test confirmed higher Fusobacterium nucleatum loads and total bacterial loads on healty intestinal mucosa of patients with cancer compared to the control group (healty patients). It is significant to observe that patients with adenomas show intermediate values. The stepwise growing pattern of this specific bacterial loads from normal mucosa to the precancerous lesions (adenomas) and from polyps to malignant tumoar tissues reflects the results of Rubinstein and Wang analysis [63].

Fusobacterium nucleatum is a typical oral bacterium that is responsible for periodontal disease, however it has been found in high loads in this extra-oral area, which can be reached by means of ingestion of food and swallowing from the oral mouth. The higher load in patients with cancer and adenomas (pre-malignant lesions) compared to control group makes us reflect on the role of this specific bacterium (and of the oral miocrobiota in general) in carcinogenesis, similarly to data found by McCoy and Araujo-Perez in their analysis [59].

On the other hand the presence of Phopyromonas gingivalis does not seem to be particulary relevant at the moment, looking only at intestinal mucosa.

The same differences can not be appreciated if we consider the analysis of pathological mucosa, but this may be due to the alterations already occurred on these tissues, which do not let the detection of a specific microrganism. In addition the role of oral microbiota could represent a risk factor in the first

phases of tissutal alterations, when healty mucosa starts to mutate, stimulating the beginning of the carcinogenic process.

Looking at gingival samples, data suggest the presence of a significative difference among the three groups considering the total bacterial load (P=0.020 in Kruskal-Wallis Test and P=0.022 in the Median test).

In addition the higher is the bacterial load in healty gingiva, the higher it is in periodontal pocket (P=0.000).

Morover, interesting assessments are evaluable in these bacteria behaviour from the correlations tables : the presence of a single bacterium seems to foster the development of the other, in a kind of cooperation, which is typical of the oral biofilm, but is detectable also in the intestinal mucosa.

Other differences emerge looking at correlations performed within the group of patient with diagnosis of adenoma compared to those of the control group.
The presence of Porphyromoas gingivalis in intestinal mucosa is significatively linked to that one of the same bacterium in gingival tissue only in patients with diagnosis of adenoma (P=0.009), compared to the situation of the control group, where such a correlation is not detectable. This may be due to the fact that a high bacterial load in oral gums, representing a natural reservoir for the microrgnaism, could be related to a major probability of transition through the digestive tract by means of swallowing. The colonization of intestinal mucosa by oral bacteria might be one of the causes of development of polyps in these patients, even thogh more data are needed to confirm this hypothesis, which is anyway plausible.

In addition the same Phorphyromonas gingivalis intestinal load seems to be related also to the presence of Fusobacterium nucleatum in periodontal gums of patients with diagnosis of adenoma (P=0.004), compared to the situation in healty ones.

Again a certain cooperation between these two bacterial pathogens seems to be detectable, but in this case it is observable not only in the same area (as seen before in gingival tissues and in intestinal tissues) but also in two districts which are very far one from the other.

2.5 CONCLUSIONS

In conclusion, the results of the study suggest that oral microbiota and in particular a specific bacterium, Fusobacterium nucleatum, so important in pathogenesis of periodontal disease, can have a role in colorectal cancer; in particular this microrganism may participate in that complex and still non compleatly clear process which is tumoral progression from healty mucosa to adenoma and finally to carcinoma. The bacterial load of this oral pathogen seems to be different on the intestinal mucosa of patients with a diagnosis of colorectal cancer and adenoma, compared to the healty control group.

The role of Phorpyromonas gingivalis, another typical periodontal bacterium, although detected in both, oral and intestinal mucosa, does not seem to be directly correlated with carcinogenesis but further analysis are needed since its behaviour in extra-oral tissues has been analysed only in few studies at the moment. However this last particular pathogen could promote the development of Fusobacterium nucleatum, as observed from correlations on gingival samples and, above all, from those emerged between intestinal and gingival samples on patients who have already developed an adenoma, compared to healty patients.

A cross sectional study let us observe the oral and intestinal conditions of non-affected and affected patients, however further prospective cohort studies will be necesarry in order to enstablish the presence of a clearer causal link in the pathogenetic mechanism of this tumor. Anyway, it seems increasingly evident that emerging correlations between oral microbiota and extra-oral systemic diseases, especially gastrointestinal diseases, are conceivable, since the oral mouth represents precisely the beginning of the digestive tract.

3. SECOND STUDY: HELICOBACTER PYLORI AND PERIODONTAL DISEASE

3.1 INTRODUCTION

3.1.1 Helicobacter pylori

Helicobacter pylori is a widespread, Gram-negative, microaerophilic bacterium, generally associated with chronic gastritis but also with acute gastritis, peptic ulcer and gastric cancer. It was firstly described in 1886 by Prof. W. Jaworski, but only in 1979 Warren found the presence of this microorganism in gastric epithelium samples collected during gastric biopsies. Robin Warren and Barry Marshall won the Nobel Prize for the discovery of this bacterium and its role in chronic gastritis in 2005.

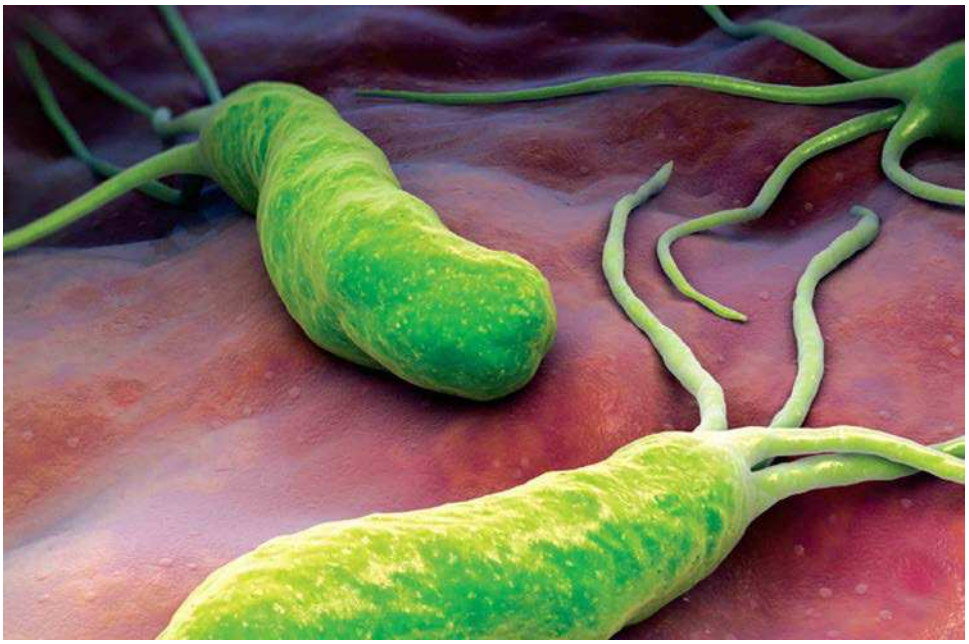


Fig. 38 3D representation of Helicobacter pylori on gastric mucosa

Bacterium structure

The motility of *H. pylori* is permitted by the presence of 5-6 unipolar flagella (molecular weight of 50,000-62,000). The shape is helicoidal in active form and coccoid in latency. It shows a slighty toxic lipopolysaccharide containing Lipid A and a glucidic portion which stimulates autoimmune response in humans. This bacterium shows intense oxydase, catalase and, above all, urease activities.

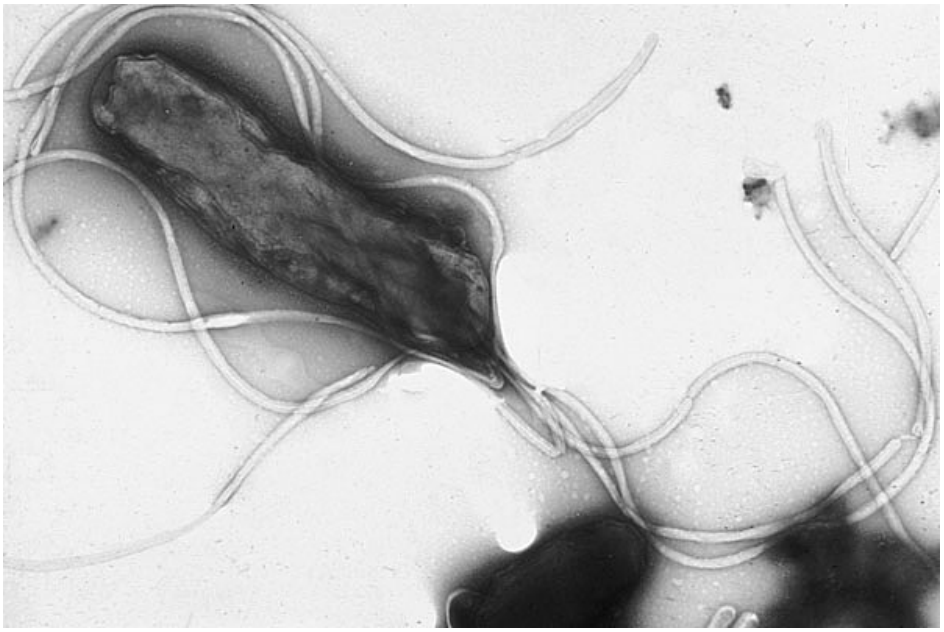


Fig.39 Optical Microscope image of Helicobacter pylori with its flagella

It is precisely the presence of Urease that lets the microorganism colonize the gastric mucosa, in fact, by converting urea into ammonia and bicarbonate, it can counteract the acidity gastric enviroment. Ammmonia is an acceptor for the H+ ions and increases the local pH. Killer T cells and white cells cannot easily survive in this area, so defense of the body from this bacterium is hard.

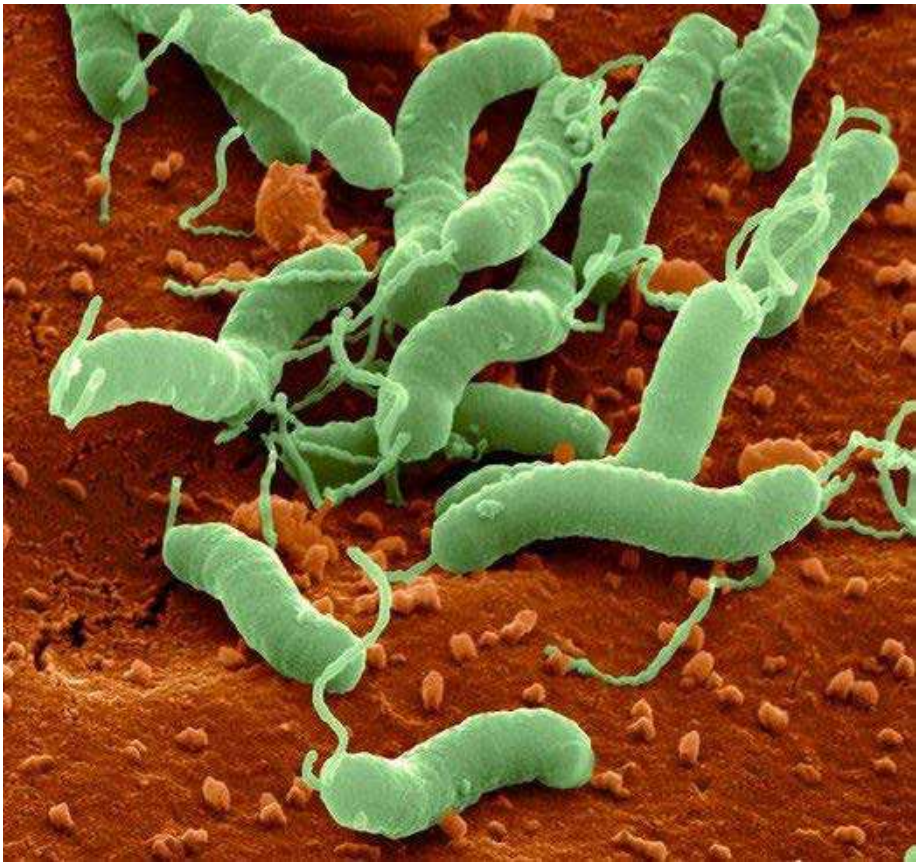


Fig.40 H.pylori colony on the gastric mucosa, observed by SEM and processed with digital techniques.

3.1.2 Epidemiology

Although prevalence is reducing, about half of the worlwide population is still infected, expecially in Southern and Eastern Europe, South America and Asia.

The rate of infection in developing countries is between 70 and 90% and here the bacterium is generally acquired during childhood, before the age of 10. In Western countries the prevalence of infection ranges from 25 to 50% [76].

In developed countries, most of infected individuals are those with poorer social and economical conditions. In fact the way of transmission is mainly oral and/or oro-fecal, so the lack of primary hygienic services and drinking water represents an important risk factor.

In industrialized countries incidence approximately coincides with age (for example, in the age group between 40 and 50 years, the incidence is estimated at

around 40-50% of the population) but after the age of 60-65 this trend changes, maybe due to the increase in older people of atrophic gastritis , a disease which creates unfavourable conditions for HP.

Country (Reference)	Setting	Number	Diagnostic method	Prevalence of *Helicobacter pylori* (%)
Western Europe				
The Netherlands [3]	Blood donors	1550	Serology	31.7
The Netherlands [4]	Pregnant women	6837	Serology	46
Portugal [5]	General population	2067	Serology	84.2
Eastern Europe				
Cyprus [35]	Patients with dyspepsia	103	PCR	39.8
Turkey [6]	General population	4622	UBT	82.5
America				
Canada [7]	Aboriginal population	203	Histology	37.9
Mexico [8]	Pregnant women	343	Serology	52.2
Asia				
Saudi Arabia [17]	Healthy individuals	456	Serology	28.3
Korea [10]	Routine health check-up	10796	Serology	54.4
India [12]	Patients with dyspepsia	2000	Histology RUT	58
India [13]	Patients with dyspepsia	530	Histology Urease test	62
China [11]	Healthy individuals	5417	UBT	63.4
Bhutan [15]	Volunteers	372	Histology RUT Culture Serology	73.4
Bhutan [16]	Patients with dyspepsia	244	Serology	86
Kazakhstan [14]	Asymptomatic and patients with dyspepsia	835	Serology	76.5
Africa				
Ethiopia [21]	Selected population	1388	Serology	65.7
Morocco [20]	Patients with dyspepsia	429	Histology RUT Culture	75.5
Nigeria [22]	Patients with dyspepsia	125	Serology Histology	93.6 80

UBT, urea breath test; RUT, rapid urease test.

Fig. 41 Prevalence of Helicobacter Pylori in different countries

3.3.3 Pathogenesis and carcinogenesis

H.pylori can survive in water for several days, so contaminated water could represent an important reservoir for infection.

Another way of trasmission is the oro-oral one, in fact the bacterium has been detected in dental plaque and saliva.

A further way of trasmission is the iatrogenic one, caused by use of endoscopes or other infected instruments in hospitals.

After the infection, the bacterium can survive in the stomach, maybe in asymptomatic stage , if an effective therapy is not performed.

Helicobacter Pylori, due to the presence of flagelli, can move across the stomach mucosa, where it binds to the MHC type II complex expressed on gastric antrum epitelial cells, by means of bacterial adhesins. In this way it determines inflammation and apoptosis. Howevere the inflammatory response is not able to eradicate the infection but can damage the stomach mucosa.

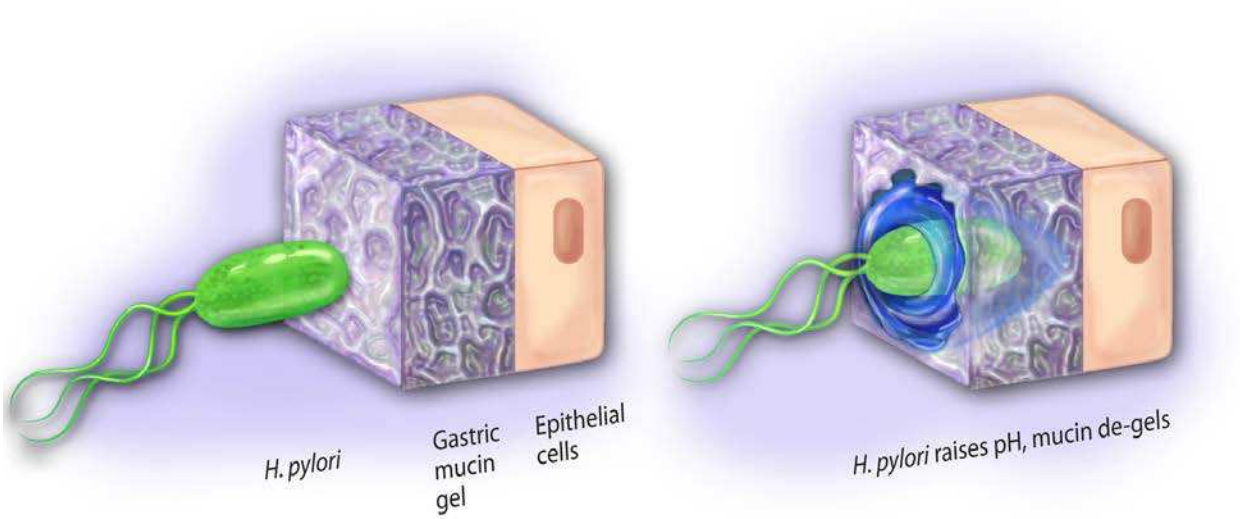


Fig. 42 Schematic representation of Helicobacter pylori crossing mucus layer of stomach

The disease's evolution could be very slow and it depends on different factors: host's defenses, bacterial genomics , genetic predisposition, smoking and food habits. Only 10-15% of patients with active chronic gastritis develop a gastric ulcer. Other manifestations include duodenal ulcer, non-ulcer dyspepsia, gastric adenoma (2%), hyperplastic polyps, multifocal gastric atrophy and intestinal metaplasia.

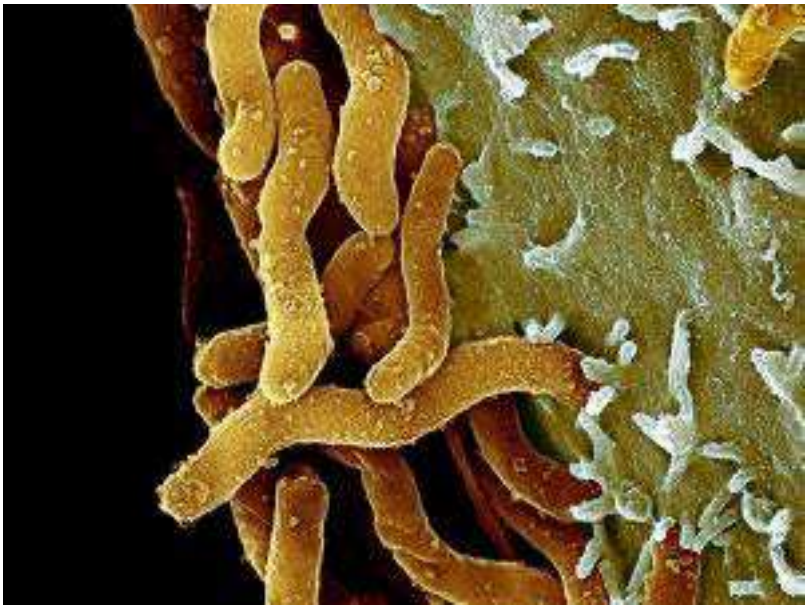


Fig.43 Electron microscopy image representing a colony of H.pylori near the gastric mucosa

In addition to its Urease activity, the bacterium produces dangerous virulence factors, which can play an important role in cytokines production and intracellular pathways alteration: the most important are CagA, VacA, Heat Shock Protein-B and Duodenal promoting gene-A. It is precisaly these factors which are supposed to be associated with increased incidence of gastric cancer and gastritis [77].

In 1994 *Helicobacter pylori was classified* as a cancerogenic agent by the World Health Organization [78].

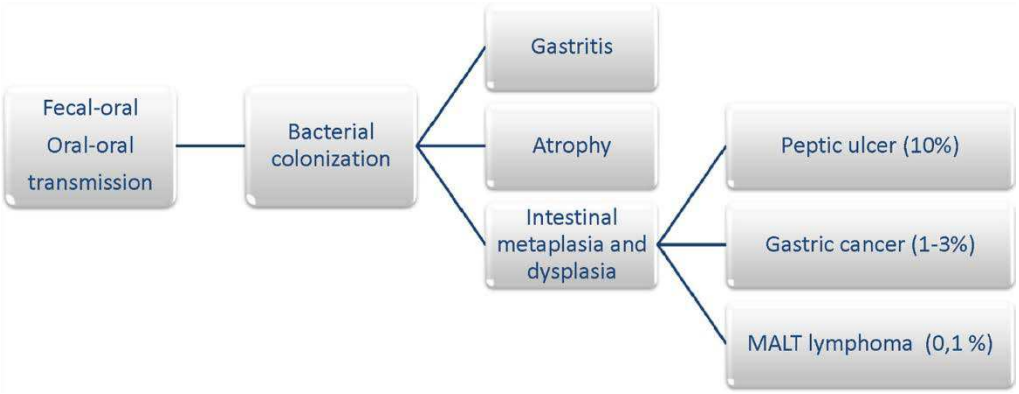


Fig.44 Pathogenesis of gastric after Helicobacter pylori infection

Gastric adenocarcinoma of the intestinal type1 often develops from pre-cancerous lesions (atrophic gastritis, intestinal metaplasia and dysplasia, respectively associated with a risk of developing a tumor within 10 years of 0.8%, 1.8%, 32.7%) .

Correa et al proposed the following model of gastric carcinogenesis in 1975 [79]: normal gastric mucosa→ superficial gastritis (later renamed non-atrophic gastritis, NAG) → multifocal atrophic gastritis (MAG) without intestinal metaplasia → intestinal metaplasia of the complete (small intestine) type → intestinal metaplasia of the incomplete (colonic) type →low-grade dysplasia (low-grade noninvasive neoplasia) →high-grade dysplasia (highgradenoninvasive neoplasia) → invasive adenocarcinoma

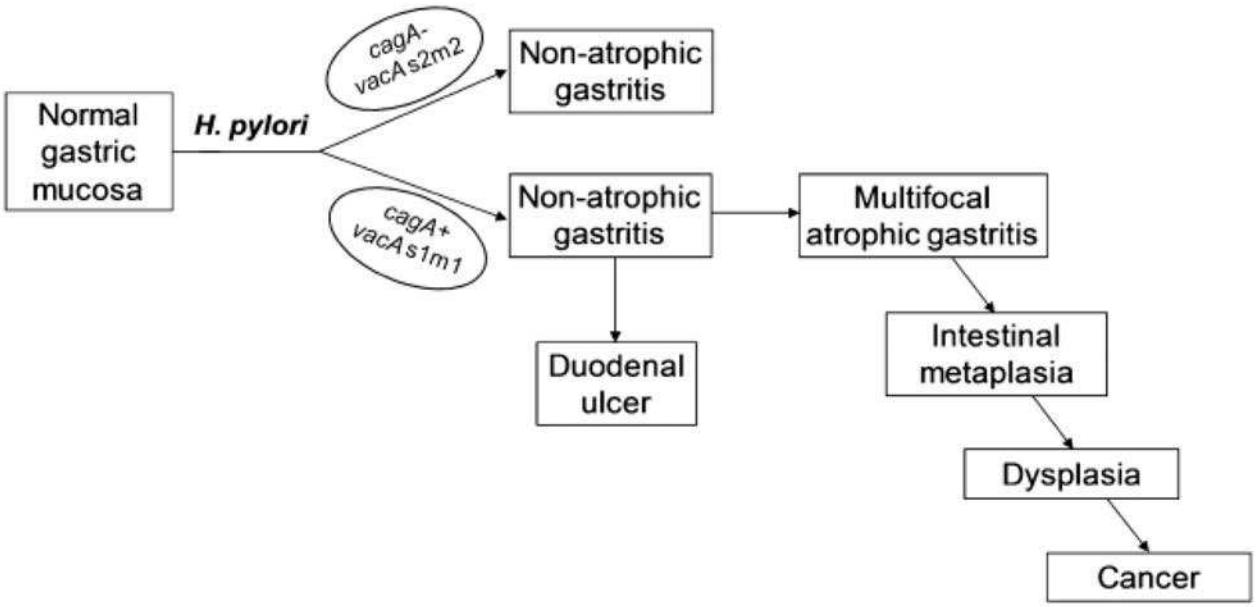


Fig. 45 Sequential steps of the precancerous cascade [79]

Gastritis stage is characterized by infiltration of the lamina propria with mononuclear leukocytes and polymorphonuclear neutrophils, toghter with increase of cytokines , interleukin (IL)-1b , tumor necrosis factor (TNF)-a and interferon (IFN)-g .

If gastritis is not treated, it may evolve in two ways: either gastritis remains as non-atrophic or it progresses with several damages of gastric glands, which could finally disappear (in particular the cag-positive vacA s1m1 strains seems to be associated with galds loss and precancerous lesions) [80].

Fibrosis of the lamina propria is the following step, leading to a phenotypic change of normal epithelial cells, which start showing an intesinal pattern. Two main morphological kinds of Intestinal metaplasia are known: the complete type and the incomplete one. In complete type goblet cells interspersed among absorptive enterocytes with apical microvilli (conferring a typical brush border feature)can be observed. Paneth cells are present, too.

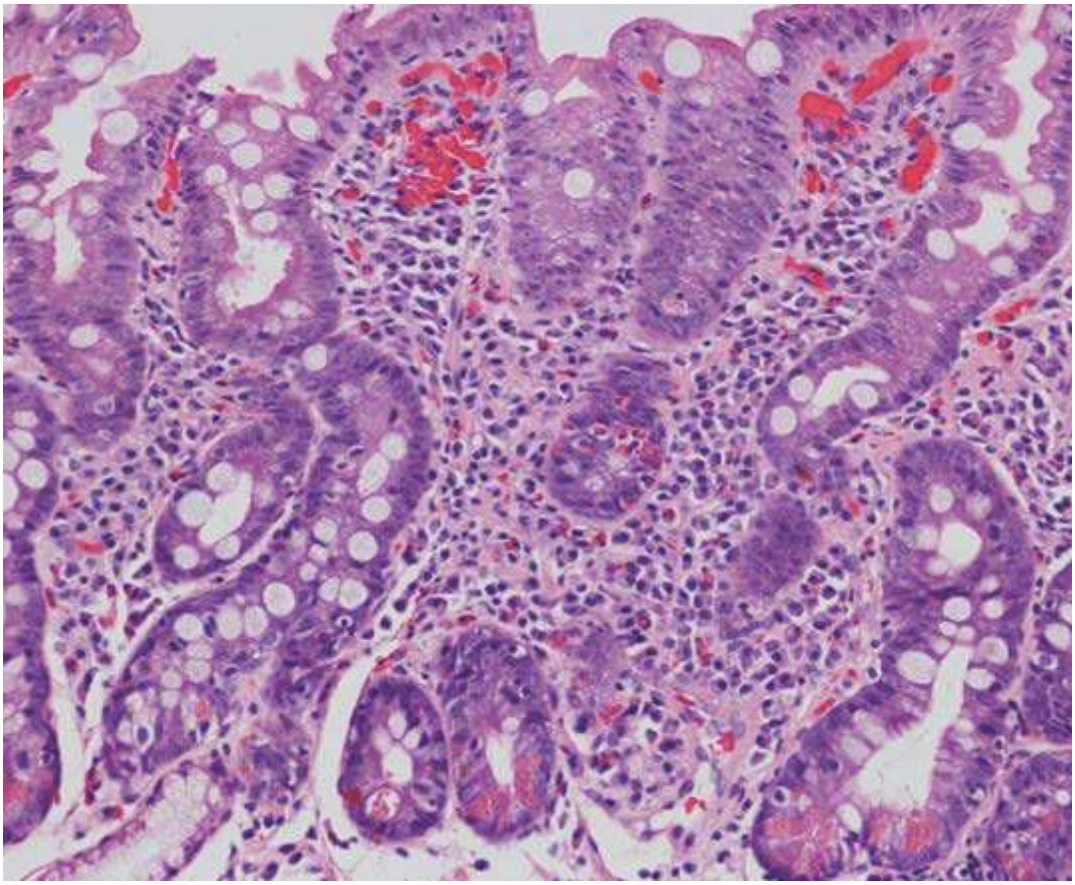


Fig. 46 Complete intestinal metaplasia, Hematoxylin and Eosin (HE) stain image

As the metaplastic transformation progresses a new phenotype can be detected. It is called incomplete or colonic because it may resemble the large bowel phenotype epitelium. Incomplete type is generally associated with a higher risk of developing a gastric cancer [81].

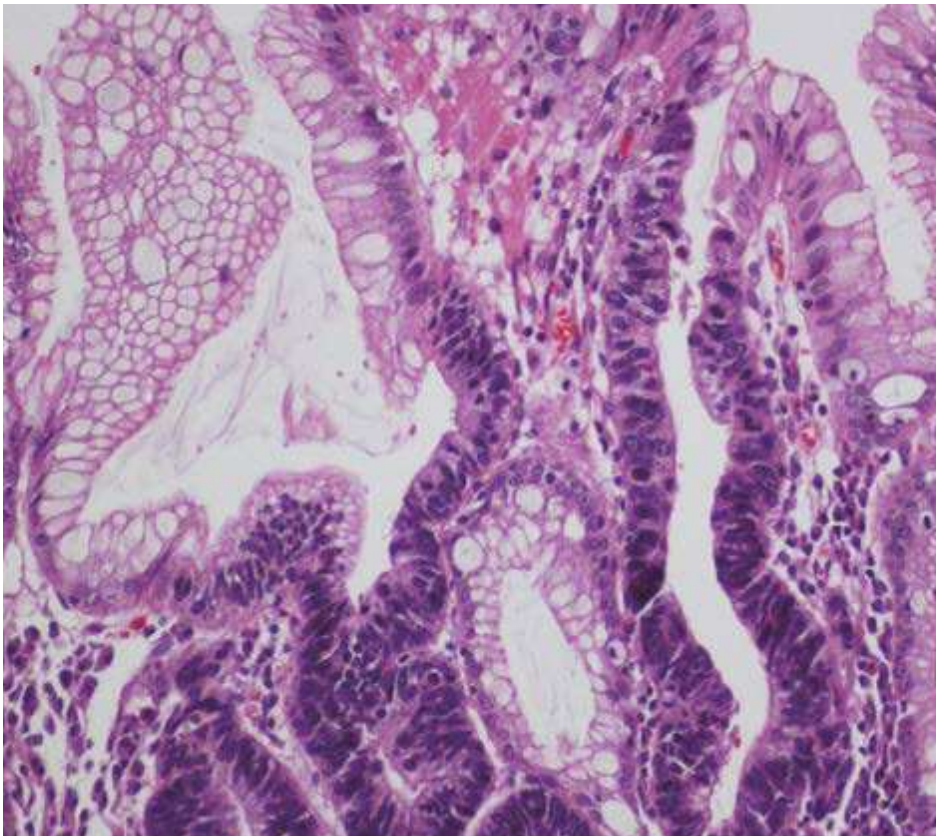


Fig. 47 Dysplasia starting from incomplete intestinal metaplasia, HE stain image

An alteration in cell morphology and architectural organization characterize dysplasia (or non invasive neoplasia). Epithelium appears enlarged, mitoses are frequent, hyperchromatic and crowded nuclei can be observed, glands show irregular shapes. Different grades of dysplasia are known, according to several classifications. In any case cells remain within the bounds of the basement membrane. When this borders are crossed and degradation of intracellular matrix occurs, the disease becomes an invasive carcinoma.

Furthermore, Helicobacter pylori infection is correlated with MALT lymphoma, syderopenic anaemia, gynecological and dermatological diseases, idiopathic thrombocytopenic purpura, cardiovascular and neurological diseases, autoimmune tyroiditis, oropharingeal diseases, obesity and bowel diseases [82].

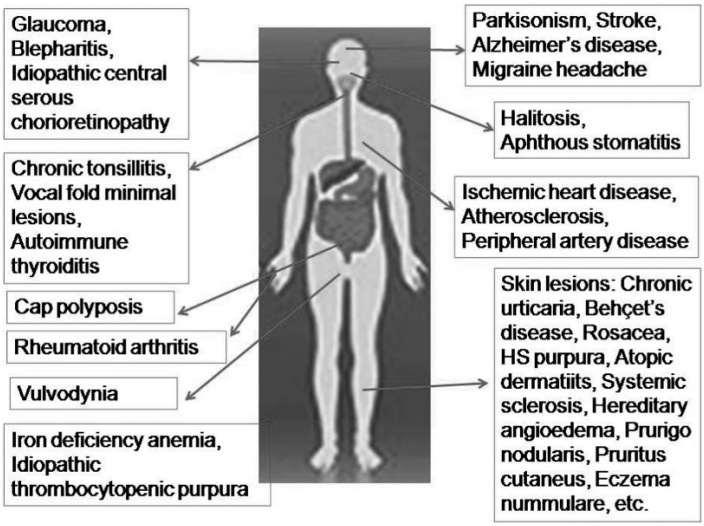


Fig.48 Extra-gastric manifestations of HP infection

3.3.4Diagnosis

HP infection can be dectected by means of both, invasive and non-invasive methods.

Invasive procedures includes esophagogastroduodenoscopy (associated with biopsy for histological evaluation), microbiological examination and urease rapid test.

Non-invasive diagnostic methods are based on serological analysis ,in order to detect specific serum IgM by means of Latex agglutination test, Elisa-test and Western Blotting Test.

C-Breath Test Urea (C-BTU) measures the urease production of Hp. Enzyme urease metabolizes urea to ammonia and carbon dioxide. The carbon dioxide produced diffuses into the blood vessels and is transported into the lungs where is expelled as CO_2. So the test principle is the following:

the patient swallows carbon labeled urea (either radioactive carbon-14 or non-radioactive carbon-13) on an empty stomach. If Helicobacter pylori is present, labeled CO_2will be collected in exhaled air. In the absence of microorganism, labeled urea is absorbed from the gastrointestinal tract and subsequently voided [83].

In addition, a pre-urea baseline breath sample is required in order to compare it with the post-urea sample, with at least 25 minutes duration between them.
The difference between the pre- and post measurements is necessary to determine infection.

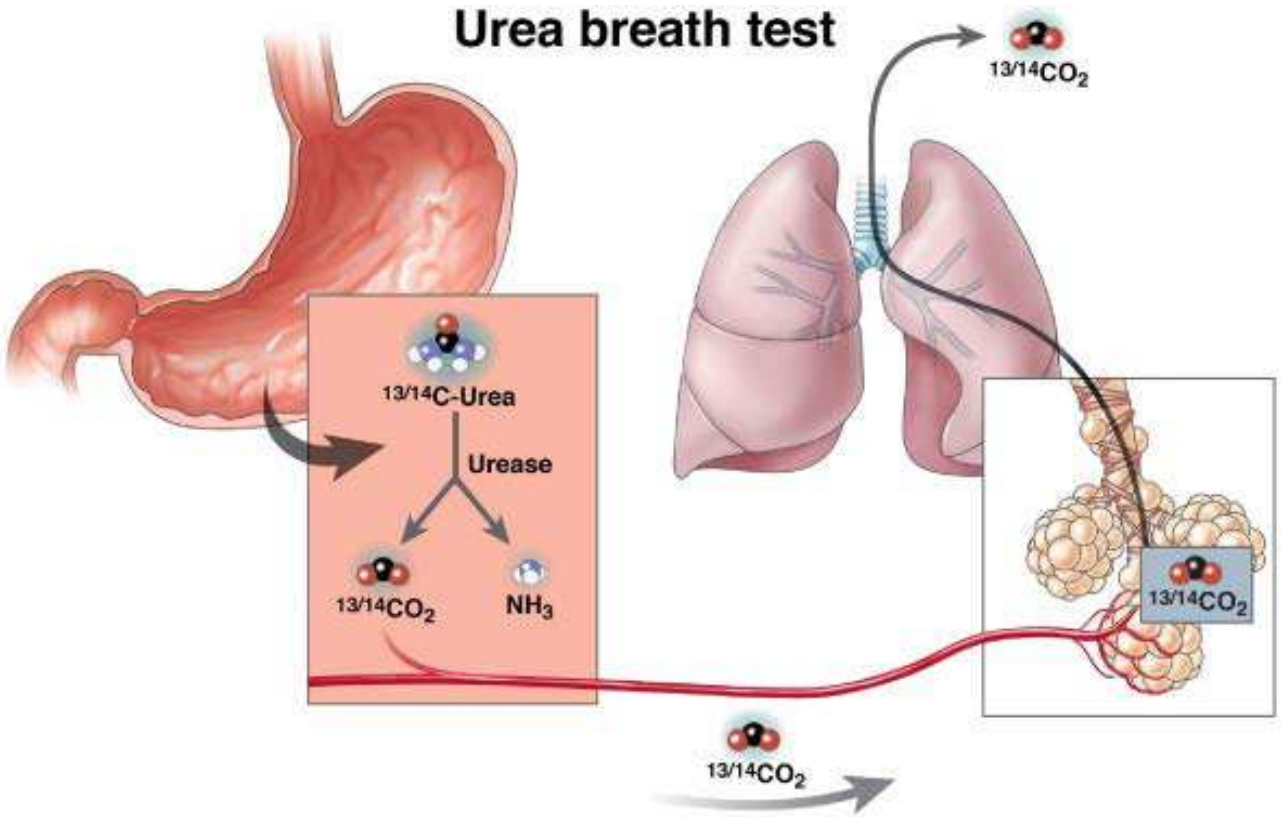


Fig. 49 Schematic representation of C-UBT

The test measures active *H. pylori* infection with high sensibility and specificity. Antibiotics can reduce the amount of *H. pylori* present, as well as proton pump inhibitors (PPI) can reduce the Urease reaction, so this test should be performed only 14 days after stopping PPI medication or 28 days after stopping antibiotic treatment, as indicated by guidelines (Acts of the IV edition of Maastricht/Florence Consensus) [84].

3.1.5 Therapy

Triple therapy is the most used intervention regimen and it consists of double antibiotic medication (clarithromycin plus amoxicillin or metronidazole) and a Proton Pump Inhibitor (PPI) . Duration should be at least 14 days.

According yo guidelines elaborated during the Toronto Consensus Conference, [85] therapy is reccomended in case of: gastric or duodenal ulcer, gastric cancer and MALT lymphoma, chronic gastritis, non-ulcer dyspepsia, metaplasia, extragastric manifestations (idiopathic thrombocytopenic purpura) and for prevention in patient at high risk of developing gastic cancer.

Considering recent prevalence of clarithromycin and metronidazole resistances (20% and 30% of cases respectively), a quadruple therapy (metronidazole ,bismuth ,tetracyclin, PPI) can be performed. Despite this, recent studies have shown that today eradication rates are at their lowest level in history, maybe due to incomplete elimination of the microorganism [86].

Recommendation	Regimen	Definition (see dose table)
First line		
Recommended option	Bismuth quadruple (PBMT)	PPI + bismuth + metronidazole[a] + tetracycline
Recommended option	Concomitant nonbismuth quadruple (PAMC)	PPI + amoxicillin + metronidazole[a] + clarithromycin
Restricted option[b]	PPI triple (PAC, PMC, or PAM)	PPI + amoxicillin + clarithromycin PPI + metronidazole[a] + clarithromycin PPI + amoxicillin + metronidazole[a]
Not recommended	Levofloxacin triple (PAL)	PPI + amoxicillin + levofloxacin
Not recommended	Sequential nonbismuth quadruple (PA followed by PMC)	PPI + amoxicillin followed by PPI + metronidazole[a] + clarithromycin
Prior treatment failure		
Recommended option	Bismuth quadruple (PBMT)	PPI + bismuth + metronidazole[a] + tetracycline
Recommended option	Levofloxacin-containing therapy (usually PAL)	PPI + amoxicillin + levofloxacin[c]
Restricted option[d]	Rifabutin-containing therapy (usually PAR)	PPI + amoxicillin + rifabutin
Not recommended	Sequential nonbismuth quadruple therapy (PA followed by PMC)	PPI + amoxicillin followed by PPI + metronidazole[a] + clarithromycin
Undetermined	Concomitant nonbismuth quadruple therapy (PAMC)	PPI + amoxicillin + metronidazole[a] + clarithromycin

[a]Tinidazole may be substituted for metronidazole.
[b]Restricted to areas with known low clarithromycin resistance (<15%) or proven high local eradication rates (>85%) (see statement 5).
[c]There is some evidence that adding bismuth to this combination may improve outcomes.
[d]Restricted to cases in which at least 3 recommended options have failed (see statement 13).

Fig. 50 American College of Gastroenterology Guideline in eradication of HP gastric infection, 2017

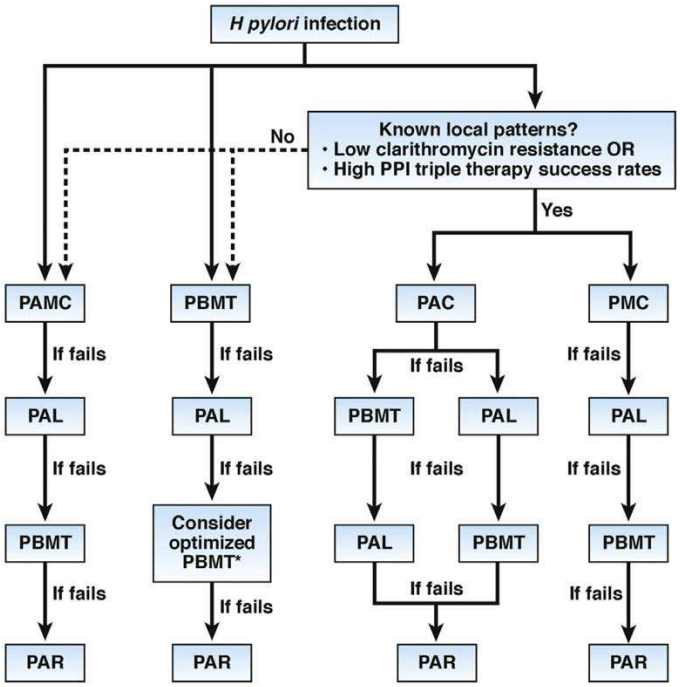


Fig.51 Flowchart of medication protocol in Helicobacter pylori infection

3.1.6 Helicobacter pylori and periodontal disease

Considering the recurrence rate, some authors hypotized the presence of a possible extra-gastric reservoir of infection, which could contribute to relapses of gastric infection.

"Reinfection" (relapse of the disease caused by infection of new bacterium strain) has to be distinguished from "recrudescence" (relapse due the presence of the same bacterial strain some weeks after the eradication therapy ended); the first one is typical of developing countries while the second one prevails in developed areas.

Several studies have been conducted in order to understand if oral cavity and tonsils may represent a reservoir for HP and so a potential element for

recrudescence of the disease [87,88], but opinions are very different. In 2011 A meta-analysis reported the presence of a significant link between HP found in the stomach and in the oral cavity [89].
On the other way, Nélio Vega et al refused the hypotesis of a correlation between gastric and oral cavity relapses, saying that the presence of the bacterium in the mouth could be a consequence of gastro-esophageal reflux disease [90].

Anyway, the presence of this bacterium in the oral cavity was firstly described in 1989 [91]; later it has been found in saliva, tongue dental plaque and tonsils [92].

In case of oral presence of HP, the classical triple eradication therapy is not very effective, as shown by eradication rates which are below 40% [93], and it is precisely this situation which could cause recrudescence of Hp infection. In fact the presence of an organised dental plaque biofilm represents a strong barrier against antibiotics and drugs and only a mechanical removal of this element could destroy resident bacteria, including Hp [94].

Even though the microbiologic examination is still considered the gold standard analysis in order to valuate the presence of this bacterium in the oral cavity, sesibility and specificity rates are not so high; that is why nowadays PCR-RT technique is getting more and more important to detect Hp presence in saliva and dental plaque.

3.2 AIM

Firstly, the aim of this study was to evaluate the relatioship between the presence of Helicobacter pylori in the stomach and in the oral cavity in patients with and without gastric infection, in order to verify the role of oral health and oral hygene in this gastric disease. Secondly, the goal was to verify if periodontal pockets could represent a reservoir of bacteria, involved in gastric disese recrudescence, after a proper eradication therapy.

3.3 MATERIALS AND METHODS

This study 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 has been evaluated by the Institutional review Board.

Phase1

102 patients with age between 18 and 80 were recruited in the current study.
Inclusion criteria were: the presence of the signed informed consent, good cooperation, good general health conditions, presence of theeth in the four quadrants of the oral cavity for periodontal analysis; exclusion criteria regarded the assumption of antibiotics or proton-pump inhibitors (PPI) during the 60 days before the breath test, abscence of theet in oral cavity quadrants.
An informed consent was read, understood and approved by all patients.

INFORMAZIONI 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 ad uno studio clinico il cui obiettivo principale è quello di valutare una 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 all'A.O Ospedale di Circolo e Fondazione Macchi di Varese dove verrà effettuata una prima visita del cavo orale, momento in cui verranno valutati gli indici parodontali iniziali. In maniera selettiva verranno utilizzati dei coni di carta sterile per prelevare dei campioni biologici di saliva e di fluido crevicolare dalle tasche parodontali piu profonde, i quali saranno analizzati successivamente attraverso il metodo della PCR (polymerase chain reaction) per determinare la presenza o meno dell'helicobacter pylori.

Indicaticativamente a 6 mesi dal primo campionamento, le sarà richiesto di sottoporsi nuovamente ad un Breath Test di controllo (indipendentemente dal risultato del primo esame) e ad un nuovo prelievo salivare; in modo tale da tener sotto controllo la salute del Suo stomaco e del cavo orale, potendo prevenire o individuare precocemente eventuali nuove infezioni.

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

Le due visite, comprendenti i prelievi salivari, e il secondo Breath Test di controllo, relativi alla Sua partecipazione allo studio clinico sono gratuiti e non prevedono alcuna spesa da parte Sua.

La sua partecipazione è volontaria; per questo le verrà chiesto di firmare il modulo di 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).

MODULO PER IL CONSENSO INFORMATO ALLA SPERIMENTAZIONE CLINICA:

Correlazione tra salute parodontale e presenza di helicobacter pylori all'interno del cavo orale

Io sottoscritta/o ..... dichiaro di aver letto le informazioni per il paziente e di averne ricevuto copia.

In particolare, ho avuto la possibilità di fare domande e mi sono stati spiegati lo scopo, la durata ed i possibili rischi connessi a questo studio.

SI Acconsento a partecipare alla ricerca sopra indicata

apponendo la mia firma al presente modulo di cui ricevo copia.

Data:

Firma del paziente:.....

Firma del medico: .....

NO Non acconsento a partecipare alla ricerca.

Data:

Firma del paziente .....

Firma del medico: .....

2

Fig. 52 Informed consent to the study

A questionnaire was completed for each patient in order to register medical history (in particular previous infection with HP was investigated), medicines, smoking and oral hygene habits. Data were collected in individual registers.

QUESTIONARIO PAZIENTE	Paziente n° .....
Nome	
Cognome	
Data di nascita	
Sesso	
Indirizzo	
Telefono	
E' sottoposto a terapie mediche?	
Prende regolarmente farmaci? Se si quali?	
Per quale motivo assume questi farmaci?	
Soffre di qualche Patologia?	
Soffre di Gastrite o altri disturbi Gastrointestinali?	
Ha mai avuto in passato l'Helicobacter pylori?	
L'ultima volta che ha fatto l'igiene professionale?	
Quante volte spazzola i denti?	
Fuma? Se si quanto?	

Fig. 53 Individual register for each patient

C- Urea Breath test was performed: the patient swallowed carbon labeled urea (with non-radioactive carbon-13) on an empty stomach and exhaled air was

collected in two tubes. The exam was repeated after 30 min. A mass spectrometer analysis was performed.

Then a dental clinician proceeded with periodontal examination for every patient, by use of a dental probe, in all the four quadrants, in order to detect the presence of periodontal pockets.

Afterwards, in each quadrant, 2 sterile paper cone were inserted for 30 seconds in the gingival sulcus of the theeth with the deepest periodontal pockets . Cones were then put in a sterile tube.
In addition, saliva was collected through the spitting method: the patient was sitting on a chair and was asked to spit the salivary content of his mouth every minute for at least 10 minutes in a sterile tube.
Tubes were sent to the laboratory for a microbiological evaluation.
Specimens were processed to extract and purify DNA using a method that includes two consecutives incubations with lysozime 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-steps 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).

Plasmids containing bacterial target sequences were used to obtain standard curves (Eurofin MWG Operon, Ebersberg Germany). The total amount of bacterial charge was estimated using the Thermo Scientific Nanodrop spectrophotometer. Standard curves were created with serial diluitions between 10^7 and 10^1 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, with the same methods of Fusobacterium nucleatum study.

Phase 2

Among 102 total patients of phase 1, on the base of UBT results and periodontal disease results, 60 patients were selected in order to perform a second perspective phase of the research. The goal was again to evaluate the role of oral Hp (present in oral cavity), in gastric primary infection and gastric reinfection (after a proper eradication therapy); in addition, the preventive role of oral hygene procedures in gastric infection/reinfection is evaluated.

Patients were divided into two groups:

- Group 1 : UBT negative, Oral Hp positive (on the base of Hp load in saliva;)
- Group 2 (control): UBT negative, Oral Hp negative

The presence of oral Hp was evaluated according to the bacterium load in saliva, since it is precisely this oral fluid that can reach the stomach by swallowing.

Patients were contacted again after some months (a minimum of 6-8 months and maximum of 2 years) from the phase 1 analysis. Data concerning medical

therapies, previous BT result, pharmacological therapies, gastrointestinal symptoms, prfoessional and home oral hygene procedures, smoking habits changes were collected.

QUESTIONARIO PAZIENTE RICHIAMO	Paziente n° ..... n° richiami .....
Nome	
Cognome	
Data di nascita	
Sesso	
Telefono	
E' sottoposto a terapie mediche?	
Risultato del primo Breath Test?	
Se positivo, l'infezione è stata trattata con successo?	
Quali farmaci ha assunto / Quale terapia ha fatto? (nomi antibiotici)	
Soffre ancora di Gastrite o altri disturbi Gastrointestinali?	
L'ultima volta che ha fatto l'igiene professionale? Ha fatto un igiene professionale dall'ultimo prelievo?	
Quante volte spazzola i denti?	
Fuma? Se si quanto?	

Fig. 54 Individual register for phase 2

With regard to oral hygene information both professional and home oral hygene habits were investigated: patients were asked how many times a day they are used to brushing their teeth, how many times a year they are used to undergoing a professional oral hygene and when the last professional oral hygene procedure was performed.

A new U- BT (time 1) and new salivary and crevicular fluid analysis (time 1) were performed with the same methods used in the first phase.

At the moment this second phase of the study is still in progress:
17 patients has been recalled and divided into the groups: 11 patients were inserted in Group 2 (BT negative and Oral Hp negative) while 6 patients were inserted in Group 1 (BT negative and Oral Hp positive).

The microbiological statistical analysis were performed in the same way and with the same methods of phase 1.

3.4 RESULTS AND DISCUSSIONS

Phase 1

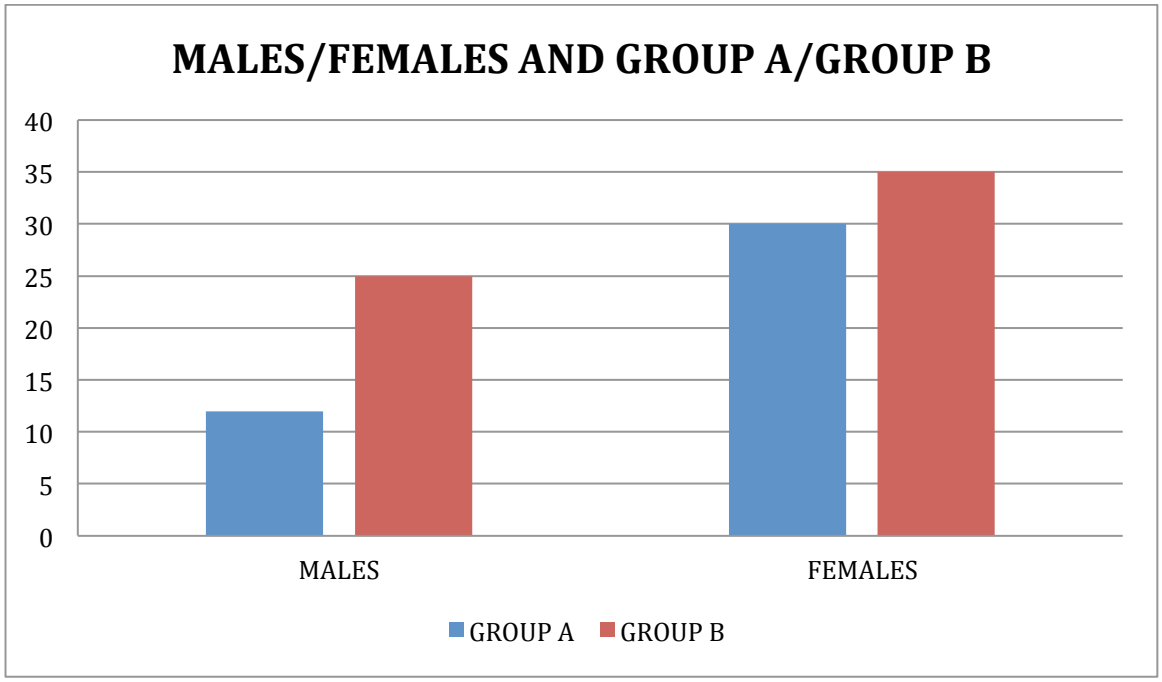
A total number of 102 patients underwent the experimental procedure: 37 males and 65 females.

According to Breath test, executed in that moment at the beginning of this study (time 0) , 42 were found with a positive result (group A) and 60 who were negative (group B).

Ages registered assessed between 16 and 78, with mean age of 52 years.

Among the 37 males, 12 resulted positive and 25 negative at the Breath test, while among 65 females 30 resulted positive and 35 negative.

Age ranged between 16 and 76 among negative patients (mean age of 50 years) while it was between 21 and 78 among positive patients (mean age of 55 years).



Plot 1 Males and female distribution among the two groups

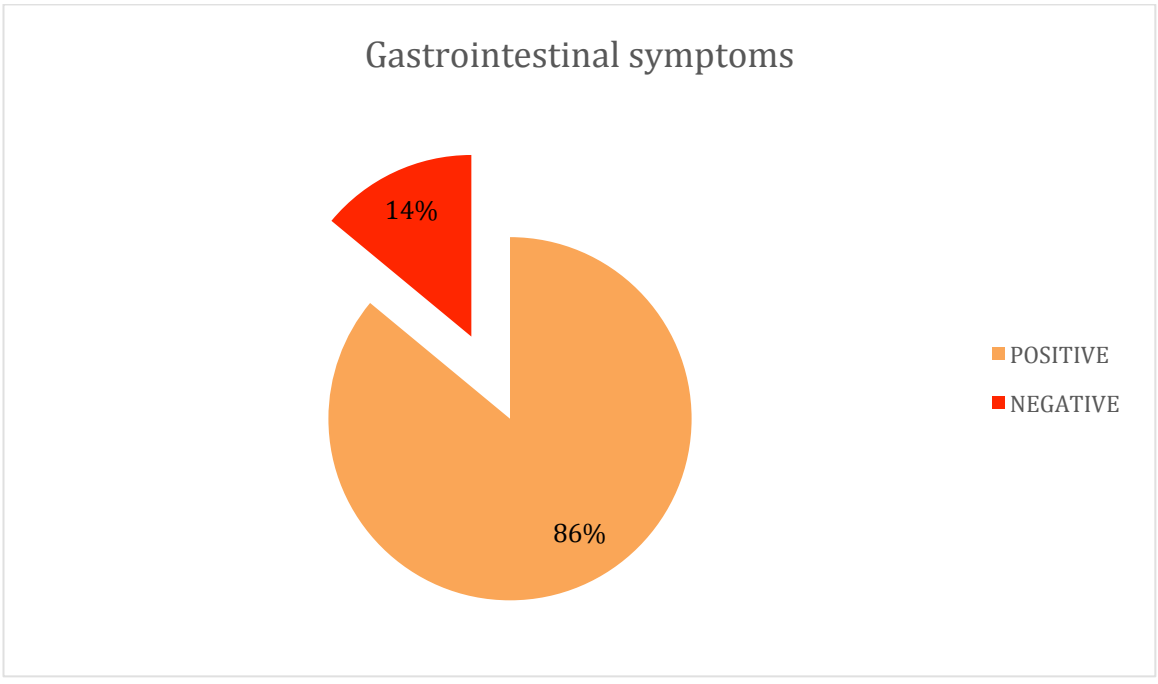
Of the 102 patients selected only 11 were smokers, 5 were ex-smokers and the the remaining 86 ere non-smokers.

Considering oral hygene, patients reported an average tooth brushing frequency of 2 times a day, with a minimum value of 1 and maximum of 3.

Avarage time since the last oral professional hygiene was estimated 14 months, with a minimum value of 1 month and a value of maximum of 120 months.

Further data regarded previous experience of infection by Helicobacter pylori, previous eradication treatment and the presence of gastrointestinal symptoms at the moment of the first sampling.

Only 14% of patients had no symptoms clinically associated with gastroesophageal disorders, while 86% of the patients reported at least one of the following: nausea, gastritis, gastroesophageal reflux, acidity, dyspepsia.



Plot 2 Gastrointestinal symptoms distribution among the two groups

Of the 88 patients with gastro-esophageal clinical symptoms, 38% (33) was positive for C-UBT, while 63% (55) was negative for C-UBT.

Of the 14 patients who did not report significant symptoms, 64% (9) were positive for C-UBT and only 36% (5) were negative.

Previous Helicobacter pylori infection (Time "-1") analysis:

This part of the analysis was performed dividig the patients into 2 main groups, on the base of their previous infection with Hp, reported during the initial interview.

Of a total of 102 patients, 62 had a history of previous infection with Helicobacter pylori (positive medical history, i.e. time -1), and only 40 patients did not report it (negative medical history, i.e. time -1).

All 62 patients with confirmed previous infection underwent eradication therapy with the intake of 1, 2 or 3 antibiotics and IPP. The efficacy of the eradication therapy was evaluated on the basis of the C-UBT performed immediately after the interview (time 0).

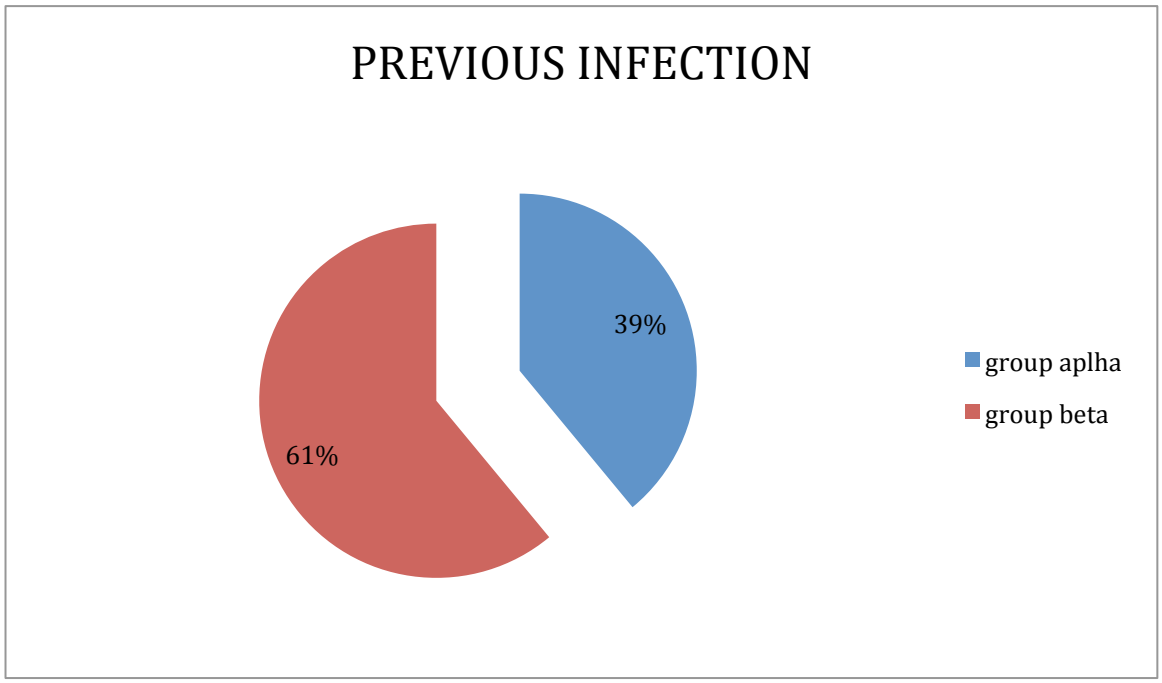
On the basis of these data (UBT results at time -1, UBT results at time 0 and gastrointestinal symptoms), it was possible to divide the patients into 2 different groups and 4 subgroups:

1) Group α : 40 patients without previous Hp infection:
   - Group α1 : 22 patients with negative C-Urea Breath Test result at time 0
     - All reported gastroenteric symptoms
   - Group α2 : 18 patients with positive C-Urea Breath Test result at time 0
     - 13 with gastrointestinal symptoms
     - 5 without gastrointestinal symptoms

2) Group β : 62 patients with previous Hp infection (UBT positive at time -1):
   - Group β1 : 38 patients with previous infection treated with success (UBT changed from positive to negative, in fact UBT at time 0 is negative):
     - 32 patients with reported gastric symptoms

- 6 patients without reported gastric symptoms

- Group β2 : 24 patients with previous infection treated with failure (UBT remained positive also at time 0):
  - 20 patients with gastric symptoms reported
  - 4 patients without reported gastric symptoms

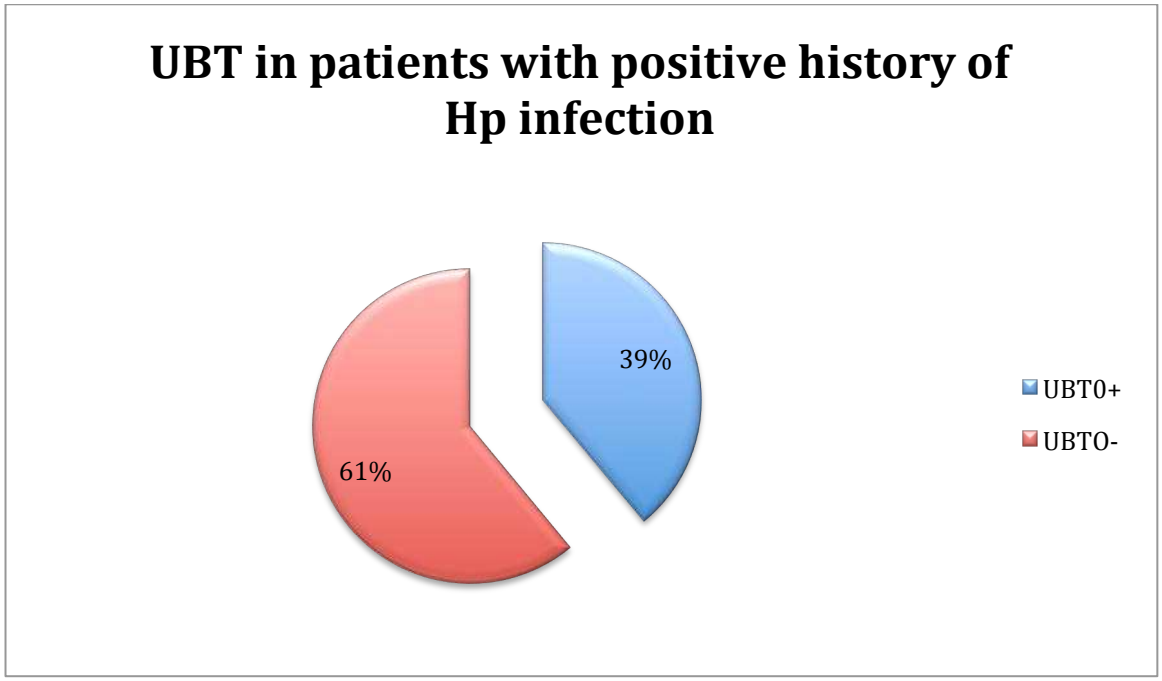


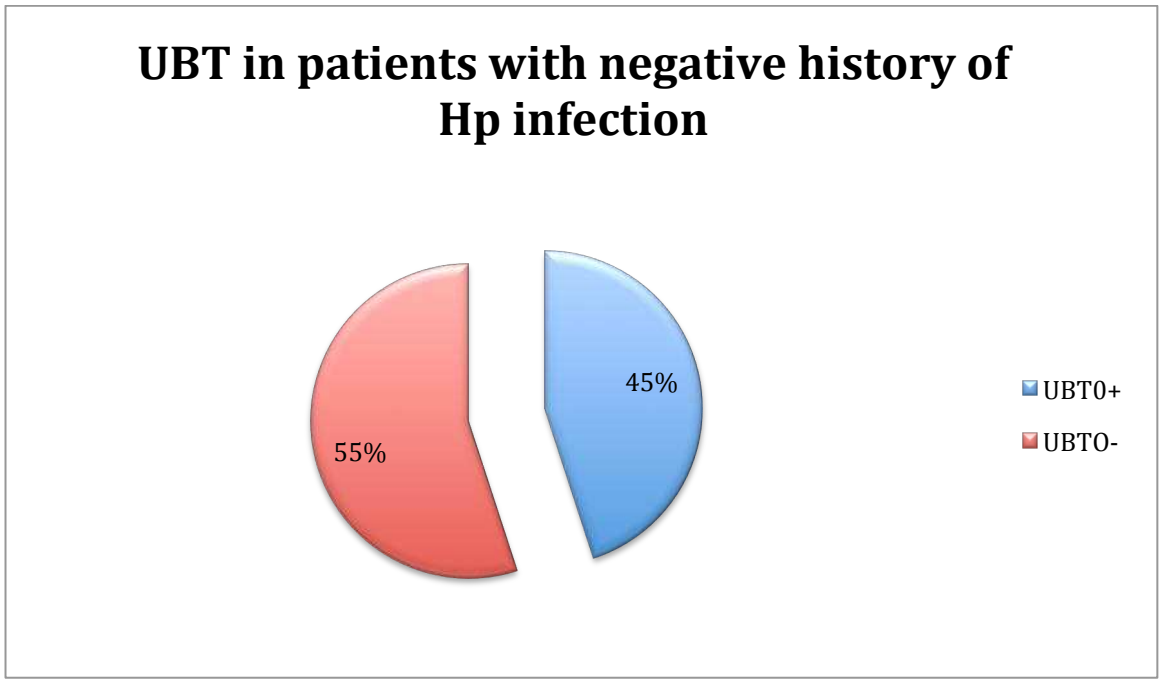
Plot 3 Previous infection with Hp among the two groups

So a comparison has been made between the current situation, recorded at time 0 -that is the time of sampling (UBT and salivary samples)- with the previous history situation reported by the patient (time -1) :

Of 62 Patients who reported a history of prior infection (time -1), all treated with eradication therapy, only 38 resulted negative and 24 positive at time 0 C-UBT, highlighting a percentage of gastric recurrence of 38.71% .

Of 40 patients whithout history of previous infection, only 22 were negative at time 0 and 18 resulted positive.





Plot. 4 UBT results at time 0 in patients with positive and negative history of Hp infection.

In order to evaluate a statistical correlation between the two groups a Chi-Square test was applied, comparing the result of the UBT at time -1 (anamnesis) with the UBT executed at time 0.

contingency table PREGHP*BTZERO

		BTZERO		Total
		,00	1,00	
PREG	,00	22	18	40
HP	1,00	38	24	62
TOTAL		60	42	102

chi-square PREGHP*BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	0,397[a]	1	,529		
Continuity correction	0,180	1	,671		
Likelihood ratio	0,396	1	,529		
Fisher exact test				,544	**,335**
Lin-lin association	0,393	1	,531		
Valid cases	102				

Tab. 21 Contingency table and Chi-square test between UBT at time -1 and UBT at time 0

The test confirmed that there is no statistically significant correlation (P=0.529) between the previous infection and the current infection: the fact that a patient is postitive at UBT at time 0 is independent from his/her medical history.

Separated statistical analysis were performed for each group:

<u>Statistical Analysis Group α (patients without previous Hp infection):</u>

This group consists of 40 patients with a negative medical history for infection with H.pylori. Among them 22 were negative to time 0 BT and 18 were positive.

1 - Microbiological Analysis - Breath Test, Salivary and Periodontal analysis: the aim was to compare the bacterial load of Hp in the oral cavity, saliva and crevicular fluid (samples collected at time 0) in those patients with a negative history of gastric Hp infection.

Firstly the presence of Helicobacter pylori in saliva was evaluated in all 102 patients: results ranged from 0 to 103 HP units (average 11 U). In particular 67 of these had less than 10 U and were classified as negative, while 35, with more than 10 U, were considered positive.

Then T-Student test was performed comparing the prescence of Hp in saliva (detected at time 0) and the results of the BT (performed at time 0), in alpha group (those patient without a medical history for Hp infection).

T-student independent variable BT and HPSALIVA Time 0

	BTZERO	N	Mean	St deviation	St error mean
HPSALIVAZERO	NEG	22	11,3182	18,31311	3,90437
	POS	18	10,2222	17,76499	4,18725

		Levene's Test		T-test						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
									Lower	Upper
HPSALIVAZERO	Equal variances assumed	,013	**,910**	,191	38	,850	1,09596	5,74301	-10,53015	12,72202
	Equal variances not assumed			,191	36,857	,849	1,09596	5,72513	-10,50578	12,69770

Tab. 22 T-student and comparative analysis of salivary Hp load values and UBT

The result is P=0.850, so there is no statistically significant difference between the salivary load of Helicobacter pylori and the Breath Test results in patients with a negative medical history for Hp infection.

In order to confirm this data, a Chi-Square test has been performed (this test represents a qualitative analysis):

contingency table ORALSALIVAPOSNEG*BTZERO

		BTZERO		Total
		,00	1,00	
ORALSALIVAPOSNEG	,00	14	13	27
	1,00	8	5	13
TOTAL		22	18	40

chi-square ORALSALIVAPOSNEG *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	0,333[a]	1	,564		
Continuity correction	,056	1	,812		
Likelihood ratio	,335	1	,563		
Fisher exact test				,737	**,408**
Lin-lin association	,324	1	,569		
Valid cases	40				

Tab. 23 Contingency table and Chi-square test between UBT at time -1 and salivary bacterium load

In this case P= 0, 564, so there is not a stastically significant difference between the two groups. The previous data are confirmed.

The same analysis was performed on crevicular fluid samples, collected from periodontal pockets:

T-student independent variable BT and HPMEAN Time 0

	BTZERO	N	Mean	St deviation	St error mean
HPMEANZERO	NEG	22	31,5455	43,25641	9,22230
	POS	18	15,3889	24,69375	5,82037

	Levene's Test	T-test

		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
									Lower	Upper
HP MEAN ZERO	Equal variances assumed	,3,571	**,066**	1,406	38	,168	16,15657	11,48928	-7,10226	39,41540
	Equal variances not assumed			1,482	34,332	,148	16,15657	10,90539	-5,99795	38,31108

Tab. 24 T-student and comparative analysis of crevicular Hp loads and UBT

This test confirmed, with a P=0.168, that there is no statistically significant difference between the periodontal load of Helicobacter pylori and the Breath Test (performed at time 0) in patients with a negative medical history for Hp infection.

Again the qualitative Chi-Square test confirmed the data with P=0,119:

contingency table ORALPAROPOSNEG*BTZERO

		BTZERO		Total
		,00	1,00	
ORAL PARO POSN EG	,00	8	11	19
	1,00	14	7	21
TOTAL		22	18	40

chi-square ORALPAROPOSNEG *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	2,431[a]	1	,119		
Continuity correction	1,540	1	,215		
Likelihood ratio	2,454	1	,117		
Fisher exact test				,203	**,107**
Lin-lin association	2,371	1	,124		
Valid cases	40				

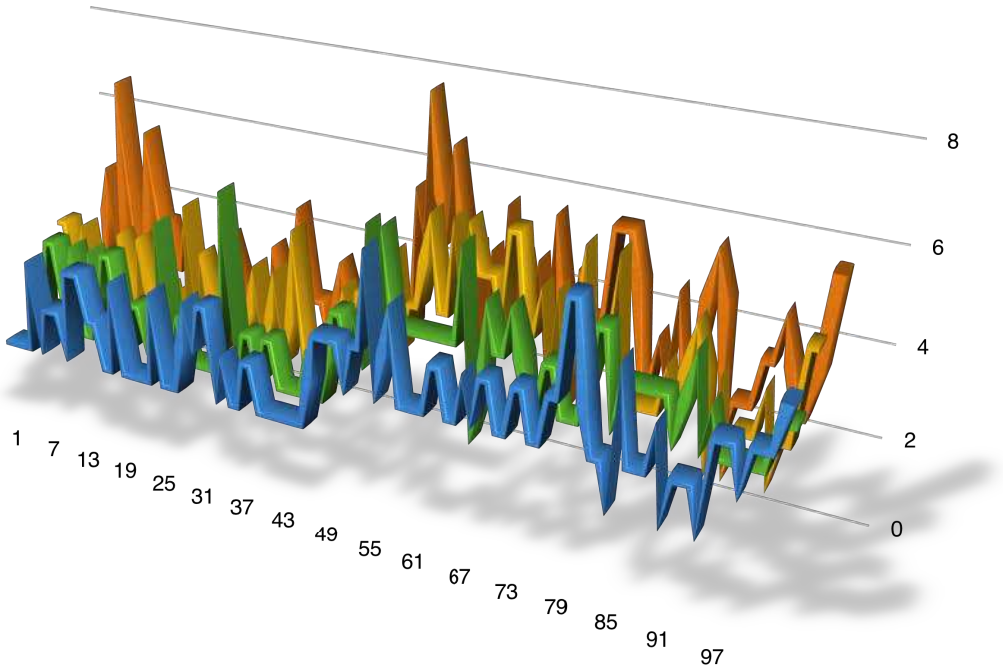
Tab. 25 Conti

ngency table and Chi-square test between UBT at time -1 and crevicular fluid bacterium load

These data are useful to analyse the presence of Helicobacter pylori in saliva and periodontal pockets in relation to the Breath Test result in the alpha group (i.e. patients without previous gastric infection); however the presence of periodontal disease is not considered yet.

2 - *Periodontal Analysis - Helicobacter pylori and Periodontal Pockets*:

Firstly, the average periodontal pocket depth was calculated for each of the 102 patients: a healty condition was represented by a value of average probing depth below 4 mm, while the presence of Periodontal Disease was represented by a value >/= 4 mm. The maximum periodontal pocket depth was also calculated in order to have a parameter showing the severity of the disease.



Plot 5 Periodontal pockets depth in 4 quadrants (blue: I quadrant- green: II quadrant- yellow: III quadrant- orange: IV quadrant)

According to this periodontal analysis of 102 patients it resulted that: 69 patients were considered periodontally Healthy (Negative), while 33 were affected by Periodontal Disease (Positive).

Similarly to the previous analysis, a Chi-Square test was conducted comparing the presence or absence of periodontal disease (based on the **average** periodontal pocket depth) with the result of the Breath Test at time 0, in patients without a positive hisory for Hp infection (alpha group): the result was P=0,131.

contingency table PERIO*BTZERO

		BTZERO		Total
		,00	1,00	
PERIO	,00	15	8	23
	1,00	7	10	17
TOTAL		22	18	40

chi-squarePERIO *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	2,283[a]	1	,131		
Continuity correction	1,415	1	,234		
Likelihood ratio	2,296	1	,130		
Fisher exact test				,200	,117
Lin-lin association	2,226	1	,136		
Valid cases	40				

Tab. 26 Contingency table and Chi-square test between UBT at time -1 and average pocket depth

Furthermore, a Chi-Square test was conducted comparing the presence or absence of periodontal disease (this tame based on the **maximum** periodontal pocket depth) with the result of the Breath Test at time 0, in pathients without a

positive hisory for Hp infection (alpha group): the result was P=0,332.

contingency table MPMAX*BTZERO

		BTZERO		Total
		,00	1,00	
MPMA	,00	8	4	12
X	1,00	14	14	28
TOTAL		22	18	40

chi-squareMPMAX *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	,943	1	,332		
Continuity correction	,390	1	,533		
Likelihood ratio	,959	1	,328		
Fisher exact test				,491	,268
Lin-lin association	,919	1	,338		
Valid cases	40				

Tab. 27 Contingency table and Chi-square test between UBT at time -1 and maximum pocket depth

So in both cases there is no stastistically significant difference between BT results and presence of periodontal disease (represented by periodontal pocket depth).

3- *Symptoms analysis*

A statistical analysis was performed to evaluate the reliability of the reported symptoms in relation to the BT results at time 0, in the group of 40 patients with a negative history for Hp infection (alpha group).

All 22 patients negative for BT at time 0 reported gastrointestinal symptoms, while, within the group of 18 patients positive for BT, only 13 reported symptoms.

Chi-Square test was performed, comparing the presence or absence of symptoms with the outcome of BT at time 0:

contingency table SYMPTOMS*BTZERO

		BTZERO		Total
		,00	1,00	
SYMP	,00	0	5	5
TOMS	1,00	22	13	35
TOTAL		22	18	40

chi-square SYMPTOMS *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	6,984	1	,008		
Continuity correction	4,675	1	,031		
Likelihood ratio	8,871	1	,003		
Fisher exact test				,013	**,013**
Lin-lin association	6,810	1	,009		
Valid cases	40				

Tab.28 Contingency table and Chi-square test between UBT at time -1 and symptoms

A statistically significant value of P= 0,008 is obtained, which represents the reliability of reported symptoms. A patients positive for Hp infection has a major probability to report gastrointestinal symptoms.

4- *Sex*

Chi-Square test was performed in alpha group, comparing the sex of the patients with the outcome of BT at time 0, in order to assess whether the the first could have

some influence on positive rates.

The result of the statistical test is P=0.576, which is a value that indicates the absence of a significant difference between being a woman or a man with respect to the gastric reinfection rates.

5-*Smoking*

Finally Chi-Square test was performed in alpha group, comparing BT results (at time 0) with smoking habits in order to assess the role of smoking in gastric reinfection.
The result was P=0,247 which indicates the absence of such a correlation.

Statistical Analysis Group β (patients with previous Hp infection):

This group consisted of 62 patients with a positive medical history for infection with H.pylori. All of them were treated with eratication therapy, however only 38 resulted negative to BT at time 0 while 24 were positive.
Similarly to the apha group, a statistical analysis was performed in beta group patients, comparing the results of the BT (performed at time 0) with microbiological condition (Hp load in saliva and periodontium), with periodontal disease, with reported symptoms, with sex and with smoking.
The results in beta group showed the absence of a significant correlation between BT results and Hp load in saliva (P=0,650 T-stdent, P=0,409 Chi-square).

Considering crevicular fluid samples, T-student result was P=0,906:

T-student independent variable BT and HPMEAN Time 0

	BTZERO	N	Mean	St deviation	St error mean
HPMEAN ZERO	NEG	38	31,0526	59,87281	9,71265
	POS	18	33,6667	113,93693	23,25728

		Levene's Test		T-test						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
									Lower	Upper
HPMEAN	Equal variances assumed	,602	**,441**	-0,118	60	,906	-2,61404	22,10390	-46,82841	41,60034

ZERO	Equal variances not assumed			-0,104	31,134	,918	-2,61404	25,20390	-54,000879	48,78072

Tab 29 T-student and comparative analysis of crevicular Hp loads and UBT at time 0

However, the Chi-square test between UBT and Hp load in crevicular fluid, showed a statistically significant value(P=0,027), differently from t-student result:

contingency table ORALPAROPOSNEG*BTZERO

		BTZERO		Total
		,00	1,00	
ORALPAROPOSNEG	,00	16	17	33
	1,00	22	7	29
TOTAL		38	24	62

chi-square ORALPAROPOSNEG *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	4,876[a]	1	,027		
Continuity correction	3,791	1	,052		
Likelihood ratio	4,990	1	,026		
Fisher exact test				,037	**,025**
Lin-lin association	4,798	1	,028		
Valid cases	62				

Tab. 30 Contingency table and Chi-square test between UBT at time 0 and crevicular fluid bacterium load

The meaning of this last result seems to be paradoxically inverted: patients with a positive periodontal load of Hp are those with a negative BT at time 0.
This could indicate that the H.pylori in the mouth may play a protective role

against the stomach reinfection or this significant value is due to the case without any correlation to explain it. At the moment it is not possible to confirm with certainty one of these hypotheses; it could be done by a prospective analysis of the patients.

Considering periodontal conditions, in beta group data showed again the absence of a significant correlation between periodontal pocket depth and BT results at time 0 (P=0,191 considering average pocket depth and P=0,806 considering maximum pocket depth).

Similarly, the absence of a stastisically significant correlation between BT results and sex (P=0,126) and between BT and smoking habits (P=0,550) was confirmed also in beta group.

Considering gastrointestinal symptoms, the chi-square test, comparing gastrointestinal symptoms with BT results, showed the abscence of a valid correlation (P=0,702), differently from the alpha group.

Current Helicobacter pylori infection (Time "0" analysis):

The aim of this second statistical analysis is to compare BT results performed at time 0 with the current periodontal condition, without considering patients' history of previous infection.

In order to reduce possible byas a specific preliminary statistical analysis was performed:

- Age: A quantitative statistical T-Student test was conducted to exclude the role of age in BT results. A value of P=0,125 confirmed the absence of such a correlation.
- Sex: chi-square test was conducted to exclude the role of sex in BT results. A value of P=0,176 confirmed the absence of such a correlation.
- Smoking habits: chi-square test was conducted to exclude the role of smoking habits in BT results. A value of P=0,199 confirmed the absence of such a correlation.
- Professional oral hygene procedures: T-Student test was conducted to exclude the role of Professional oral hygene procedures in BT results; in particular BT data were compared with number of months elapsed from the last oral hygene. A value of P=0,798 confirmed the absence of such a correlation.
- Home oral hygene proedures: T-Student test was conducted to exclude the role of Home oral hygene procedures in BT results. A value of P=0,985 confirmed the absence of such a correlation.
- Previous BT: chi-square test was conducted to evaluate a correlation between results of BT performed at time -1 with those of BT performed at time 0. A value of P=0,529 confirmed the absence of such a correlation.
- Gastrointestinal symptoms: chi-square test was conducted to evaluate a correlation between gastrointestinal symptoms, reported by the patients, and BT results. During the visit, only 14 patients reported the absence of symptoms, while 88 patients resulted symptomatic. Of the 88 symptomatic patients, 33 showed a positive C-UBT, while the 55 a negative one. Of the 14 asymptomatic patients 9 were positive for C-UBt and only 5 negative.

contingency table SYMPTOMS*BTZERO

	BTZERO	Total

		,00	1,00	
SYMP	,00	5	9	14
TOMS	1,00	55	33	88
TOTAL		60	42	102

chi-square SYMPTOMS *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	3,578	1	,059		
Continuity correction	2,557	1	,110		
Likelihood ratio	3,525	1	,060		
Fisher exact test				,080	**,056**
Lin-lin association	3,543	1	,060		
Valid cases	102				

Tab. 31 Contingency table and Chi-square test between UBT at time 0 and symptoms

The result was a value of P=0,059 (near to 0,05 limit). Similarly to the previous analysis at time -1, the reported symptoms are almost reliable.

1 - Microbiological Analysis - Breath Test, Salivary and Periodontal analysis: in this case the aim was to compare the bacterial load of Hp in the oral cavity (saliva and crevicular fluid, both collected at time 0) with time 0 BT results (60 negative and 42 positive at UBT at time 0).

Firstly, T-Student test was performed comparing the prescence of Hp in saliva (detected at time 0) and the results of the BT (performed at time 0).

T-student independent variable BT and HPSALIVA Time 0

	BTZERO	N	Mean	St deviation	St error mean

HPSALIVAZERO	NEG	60	11,8500	18,62095	2,40395
	POS	42	10,0476	18,01348	2,77954

		Levene's Test		T-test						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
									Lower	Upper
HPSALIVAZERO	Equal variances assumed	,000	**,995**	,488	100	,627	1,80238	3,69667	-5,53171	9,13647
	Equal variances not assumed			,490	90,204	,625	1,80238	3,67489	-5,49821	9,10297

Tab. 32 T-student and comparative analysis of salivary values and UBT at time 0

The result is P=0.627 , i.e. there is no statistical difference with regard to the presence of Hp in saliva and BT test performed at time 0.

In order to confirm this data, a Chi-Square test has performed:

contingency table ORALSALIVAPOSNEG*BTZERO

		BTZERO		Total
		,00	1,00	
ORALSALIVAPOSNEG	,00	37	30	67
	1,00	23	12	35
TOTAL		60	42	102

chi-square ORALSALIVAPOSNEG *BTZERO

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	1,045[a]	1	,307		
Continuity correction	,656	1	,418		
Likelihood ratio	1,056	1	,304		
Fisher exact test				,397	**,209**
Lin-lin association	1,034	1	,309		

Valid cases	102				

Tab. 33 Contingency table and Chi-square test between UBT at time 0 and salivary bacterial load

In this case P= 0, 307, so it is confirmed that there is not a stastically significant difference between the two groups.

The same analysis was performed on crevicular fluid samples, collected from periodontal pockets.
T-student test, with a P=0,701, showed that there is no statistically significant difference between the periodontal load of Helicobacter pylori and the Breath Test performed at time 0 in all patients.
Again a qualitative Chi-Square test was performed: in this case, as occurred in time -1 analysis, a significative value of P=0,008 was achived.

2- *Periodontal Analysis – Gastric Helicobacter pylori and Periodontal Pockets*:

Similarly to the previous analysis, a Chi-Square test was conducted comparing the presence or absence of periodontal disease (based on the **average** periodontal pocket depth) with the result of the Breath Test at time 0, in all patients: the result was P=0,859.

Furthermore, a Chi-Square test was conducted comparing the presence or absence of periodontal disease (this time based on the **maximum** periodontal pocket depth) with the result of the Breath Test at time 0: the result was P=0,675.
So in both cases there is no stastistically significant difference between BT results and periodontal disease (represented by periodontal pocket depth).

3- *Oral mouth Analysis -Oral Helicobacter pylori and Periodontal Pockets depth*:

In this further statistical analysis the goal was to evaluate the relationship between periodontal conditions and the oral load of H.pylori, particularly referring to pocket depth. First of all, a statistical comparison (t-student) was made between the average value of bacterial load of Hp in the periodontium, measured at time 0, with average pocket depth (the cut-off value for periodontal disease was an average pocket depth >/= 4mm). Among all 102 patients analysed, 33 were positive for periodontal disease and 69 negative. Of the 33 patients with periodontal disease 19 resulted positive for periodontal Hp infection (14 were negative); of the 69 patients without periodontal disease, 31 resulted infected with Hp (38 were negative).

T-student independent variable BT and HPMEAN Time 0

	MALATTIA PARO	N	Mean	St deviation	St error mean
HPMEAN ZERO	NEG	69	17,2754	33,33162	4,012265
	POS	33	53,5455	108,98942	18,97262

		Levene's Test		T-test						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
									Lower	Upper
HPMEANZERO	Equal variances assumed	15,231	**,000**	-2,539	100	,013	-36,27009	14,28703	-64,61515	-7,92504
	Equal variances not assumed			-1,870	34,894	,070	-36,27009	19,39231	-75,64286	3,10267

Tab 34 T-student and comparative analysis of crevicular Hp loads and average pocket depth

The result would be significant (P=0.013), however, considering correction of ANOVA, a value of P=0.070 is achived ; it is no longer significant but very close to the significance limit which is 0.050.
Nevertheless, it is possible to observe that the average Hp load in the patients who have periodontal disease is 53.54, while that of patients without periodontal disease is 17.27; therefore there is a high difference, showing how Hp is related to the presence of disease. So patients with pockets deeper than 4mm (i.e. patients with periodontal disease) show higher loads of Hp than those with healty gums.

To confirm this evaluation, a Chi square test was performed: the result of the test was: P=0,232.
Proportionally to periodontal pockets depth ,H.pylori load increases its value and this area becomes a reservoir for the micrornganism.

Finally, a statistical comparison (t-student) was made between the average value of bacterial load of Hp in the periodontium, measured at time 0, with maximum pocket depth: a significant result (P=0,004) was again achived, so the deeper is a periodontal pocket , the higher is Hp load in that area.

T-student independent variable Time 0

	MPMAX	N	Mean	St deviation	St error mean
HPMEAN ZERO	NEG	29	8,8621	8,30544	1,54228
	POS	73	37,0137	80,50647	9,42257

	Levene's Test		T-test						
	F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
								Lower	Upper

HP ME AN ZE RO	Equal variances assumed	8,043	**,006**	-1,874	100	,064	-28,15163	15,02566	-57,96211	1,65885
	Equal variances not assumed			-2,948	75,770	,004	-28,15163	9,54796	-47,16986	-9,13429

Tab 35 T-student and comparative analysis of crevicular Hp loads and maximum pocket depth

4- *Periodontal Analysis - Helicobacter pylori and Saliva*:

In this analysis the average bacterial load of Hp in the periodontium was compared to the load detected in saliva.

	ORALPAROPOSNEG	N	Mean	St deviation	St error mean
HPSALIVAZERO	NEG	52	4,7500	7,79360	1,08078
	POS	50	17,7200	23,23618	3,28609

		Levene's Test		T-test						
		F	Sig.	t	df	Sig. (2-tailed)	Mean difference	Std Error difference	Confidence interval	
									Lower	Upper
HP ME AN ZE RO	Equal variances assumed	21,898	**,000**	-3,809	100	,000	-12,97000	3,40502	-19,72547	-6,21453
	Equal variances not assumed			-3,749	59,505	,000	-12,97000	3,45926	-19,89073	-6,04927

Tab 36 T-student and comparative analysis of crevicular and salivary Hp loads

The result was P=0.000. So the higher is the bacterial load in periodontum , the higher it is in saliva. Hp can move from periodontum to the saliva, and, from there, it can reach the stomach by swallowing.

In order to confirm the data a Chi-Square test, was performed (the cut off value for bacterial load was 10U):

contingency table ORALSALIVAPOSNEG*ORALPAROPOSNEG

		BTZERO		Total
		,00	1,00	
ORALSALIVAPOSNEG	,00	42	25	67
	1,00	10	25	35
TOTAL		52	50	102

chi-square ORALSALIVAPOSNEG * ORALPAROPOSNEG

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	10,707	1	,001		
Continuity correction	9,385	1	,002		
Likelihood ratio	10,963	1	,001		
Fisher exact test				,002	**,001**
Lin-lin association	10,602	1	,001		
Valid cases	102				

Tab. 37 Contingency table and Chi-square test between crevicular and salivary Hp loads

The result is confirmed: P=0,001.

Phase 2

At the moment 17 patients has been recalled and divided into the groups: 11 patients (4 males and 7 females) were inserted in Group 2 (BT negative and Oral

Hp negative) while 6 patients (1 male and 5 females) were inserted in Group 1 (BT negative and Oral Hp positive).

The total average age of the 17 patients is 56 years, with a minimum value of 37 and a maximum of 56 years.

The average age of group 1 is 51 years, while the avergae age of group 2 is 59 years.

All patients had a negative result at UBT, performed at time 0. All of them underwent a professional oral hygene procedure between phase 1 and 2.

UBT was repeated at time 1 (second phase): one patient of group 1 had a positive result while all the other had a negative result.

Periodontal analysis were repeated at time 1 in saliva and crevicular fluid: 6 patients resulted positive for the presence of Hp in the saliva (average load higher than 10U) and 11 negative. On the other hand 10 patients resulted positive for the presence of Hp in the crevicular fluid (average load higher than 10U) and 7 negative.

A chi-square test was performed to compare Hp load in saliva and crevicular fluid:

contingency table SALIVAPOSNEGUNO*PAROPOSNEG

		BTZERO		Total
		,00	1,00	
ORALS ALIVAP OSNEG	,00	7	4	11
	1,00	0	6	6
TOTAL		7	10	17

	Valore	df	Asynt Sig.. (2 ways)	Exact. Sig. (2 ways)	Exact. Sig. (1 way)
Pearson's Chi-square	6,491	1	,011		
Continuity correction	4,129	1	,042		

Likelihood ratio	8,614	1	,003		
Fisher exact test				,035	**,017**
Lin-lin association	6,109	1	,013		
Valid cases	17				

Tab. 38 Contingency table and Chi-square test between salivary and crevicular Hp loads at time 1

The presence of a significat correlation between Hp load in saliva and crevicular fluid is confirmed at the moment (P=0,011).

This study represents a preliminary approach on the role of oral *Helicobacter pylori (*present in saliva and periodontal pockets) in gastric infection.

The real problem with this infection is the high rate of relapse, despite eradication protocols, as confirmed by a second Breath Test, performed some months after the antimicrobical therapy.

It is estimated that infection with Helicobacter pylori, the main cause of gastritis and peptic ulcer, affects about 50% of the world's population.

The oral cavity could represents a reservoir of the bacterium, providing an optimal environment for its proliferation and protection against specific antibiotic therapies.

This study was conducted in order to evaluate the role of H.pylori in the oral cavity and its relationship with gastric infection recurrence.

The study was structured in two phases of research, one descriptive (Phase 1)

followed by a perspective (Phase 2).

102 patients were recruited: medical history data were registered, a Breath Test was performed, saliva and crevicular fluid sampes were collected.

The statistical evaluation of the results was divided into 3 stages:

- time -1, on the base of anamnesis reported by patients(regarding previous experience of gastric H.pylori infection and C-UBT at time -1)
- time 0: that is the moment when this study began: C-UBT and oral sampling was performed (both at time 0).
- time 1, which corresponds to the prospective phase of the study: selected patients were recalled and another C-UBT and periodontal sampling was performed (both at time 1).

Time -1:

In this first phase evaluations were performed on the base of medical history:

102 recruited patients recruited were divided into two groups according to their previous infection by Helicobacter pylori .

62 patients reported a positive history of gastric infection (BT performed at time -1 was positive)and they were all treated with eradication therapy; on the other hand 40 patients reported a negative history of infection.

Among the patients without a history of previous Hp infection some of them had a negative result in UBT performed at time -1, others never needed to undergo the test.

Among the 62 patients with a positive history of Hp infection, 38 are healty at the moment when this study began (according to BT result executed at time 0), while 24 remained positive despite the eradication therapy. The infection rate is 38,71% in the first group and 45% in the other. It was possibile to verify that the current BT result (time 0) is not conditioned by the prior infection with Hp (P=0,529).

The objective of the preliminary phase of the study is to find out if there is a difference in medical history of patients and evaluate the effectiveness of the therapy, with regards to periodontal conditions.

In the group of 62 patients with previous H.pylori infection (called beta group), there were no significant differences between BT results and the presence of Hp in the saliva (P=0.650, P=0.409), the presence of Hp in the periodontium (T-student P=0.906), the presence of periodontal disease (pockets >/= 4mm, MP mean P=0.191, MP max P=0.806) and the anamnestic components (sex P=0.126 and smoking P=0.550). Actually, to be precise, one difference was found (Chi-Square test), between BT and the presence of Hp in the periodontium (P=0,027) but this results has an inverse meaning: patients with positive Hp loads in periodontum showed a negative BT result at time 0. The meaning of this data has to be investigated in the perspective phase of the study, in order to clarify if it may be due to chance or the oral presence of bacterium can play a preventive role against gastric reinfection.

In the group of the 40 patients with a negative history of Hp infection (alpha group) , 22 remained negative and 18 became positive, according to BT performed at time 0.

In this group there were no significant differences between BT results and the presence of Hp in the saliva (P=0.850, P=0.564), in the periodontium (P=0,168, P=0,119), the presence of periodontal disease (pockets >/= 4mm, MP mean P=0.131, MP max P=0.332) and the anamnestic components (sex P=0,676 and smoking P=0.247). However it should be considered the difference between PCR and UBT methods, not allowing to detect a proper causal determination (see the following “time 0” paragrapgh considerations about this topic).

Looking exclusively at the oral mouth it was not possible to compare the conditions at time -1 with those at time 0, since we did not have the data of the periodontal initial situation (at time-1).

The most intresting conclusions of this part of the analysis concerned gastrointestinal symptoms reported by patients.

Considering all 102 patients, it emerges that there is a correspondence (although not completely significant: P=0.059, this value is near to 0,05 cut off) between symptoms reported and clinical situation. However the symptoms become completely reliable (P=0.008) in patients with negative history of Hp infection and current positive BT (BT-1 negative and BT 0 positive); on the contrary a value of P=0.702resulted in patients positive both at UBT at time -1 and at time 0.

So symptoms are useful for diagnostic purposes only in patients who developed a infection for the first time, passing from a situation of health to one of disease (from BT-1 negative to BT0 positive): before the symptom was not present and then appears, therefore it corresponds to the clinical reality.

On the other hand, the symptoms have no meaning in monitoring the therapy against the Hp, because patients with a positive history for gastric infection, who managed to eradicate the infection, sometimes keep on beeing symptomatic , even though the infection is absent.

So if a patient develops an infection for the first time or gets sick again the symptoms have a clear meaning, but if the patient had already the infection, the symptoms do not indicate whether or not he/she is still ill.

That is why the only way to evaluate the effectiveness of an eradicating therapy is the C-UBT.

This situation partially explains why so many gastroprotectors are prescribed even in patients who do not have the infection.

<u>Time 0:</u>

The aim of second part of the statistical analysis is the evaluation of the current situation (at time 0), both in the stomach and in the oral mouth, and of the role of Hp in periodontal disease. A preliminar specific statistical analysis, conducted to

reduce byas, states that previous Hp infection (P=0,529), eradication therapy, age (P=0,125) ,sex (P=0,798), smoking habits (P=0,199), drugs (exclusion criteria), professional oral hygene procedures (P=0,798), home oral hygene procedures (P=0,985) do not influence the result of this part of the study.

Firstly, the presence of Hp in saliva was compared with the result of BT in the two groups (BT+ and BT-) showing the absence of a significant correlation, according to T-Student test (P=0.627) and Chi-Square test (P=0.307).

The same analysis was performed considering Hp loads in crevicular fluid (T-student P=0.701). However, similarly to time -1 analysys, a significant correlation (according to Chi-square: P=0,008) between Hp loads in periodontum and BT results at time 0 is confirmed. This significance, in reverse relation, between periodontal infection by Hp and gastric infection could be again interpreted in this way: the presence of the bacterium in periodontal pockets might prevent a gastric infection.

In order to have a confirmation of this thesis it is necessary to wait for the results of the second phase of the study.

It should be considered that Urea Breath test and PCR-RT are two different methods of bacterial detection, with differents specificity and sensitivity.

The UBT measures of the urease activity and discriminates among positive and negative results (in a dicotomic way), while PCR-RT is quantitatively more accurate in detecting bacterial loads; however this last method requires a direct prelevation of the fluid nearby the bacterium reservoir. This procedure could be invasive in the stomach due to the necessity of esophago-gastro-duodenoscopy.

For this reason a less invasive, but equally effective, diagnostic examination as UBT it is preferred for the diagnosis and follw-up of gastric infection, but in the oral cavity PCR analysis is the most suitable and predictable technique for the detection of a specific bacterium.

The detection of an infection in the stomach and in the oral mouth with different methods does not allow to effectuate a causal determination (similarly to the situation in time -1 analysis), as emerged from Navabi's review. [95]

Then the periodontal condition, defined according to the "mean" and "maximum" periodontal pockets depth values of all 102 patients, was compared with UBT results at time 0, in order to evaluate whether the presence of periodontal disease (average or maximum pocket depth >/= 4mm) may play a key role in gastric infection.

The comparison between UBT results and periodontal disease does not show a significant correlation (P=0.859 for mean depth and P=0,675 for maximum depth); however if we compare the load of Hp in periodontum with periodontal pocket depth a significant P value of 0,013 is obtained.

This means that patients who have an average pocket depth of more than 4mm (therefore periodontal disease), showed higher loads of Hp in periodontum than patients with healty gums.

In confirmation of that, a significant relationship between the average Hp load in periodontum and the maximum periodontal pocket depth (P=0.004) was found. As the periodontal pockets become deeper, they represent a real reservoir for this bacterium and an association of the presence of Hp in stomach and oral mouth is likely as Czesnikiewicz-Guzik and Karczewska found out. [96]

Finally it was found that the load of Hp in saliva increases proportionally to the periodontal load (t-student, P=0.000); a confirmation came form chi-sqaure test performed comparing Hp loads in saliva and crevicular fluid (P=0,001).

In the pockets, the bacterium can proliferate in favourable conditions, slipping away from human immune system surveillance; once a periodontal reservoir has been created and a sufficent load is reached, Hp can migrate from the gingival sulcus into the oral cavity and it is distributed in the saliva.

Considering that the saliva is swallowed and goes down into the stomach, the oral cavity, in particular periodontal pockets, can represent an extra-gastric reservoir of Helicobacter pylori and this can be one of the main causes of gastric reinfection. A direct causality has to be evaluated in the second phase of the study but, at the moment, we can exclude the hypotesis of a possibile retrograde path of the Hp from the stomach to the oral cavity by means of gastroesophageal reflux, as was said in literature by some authors [90].

Time 1:

In the second phase of the study, we moved from a descriptive evaluation of the condition to a prospective one, aiming again to evaluate the role of H.pylori in gastric reinfections and trying to find out if oral hygene procedures can reduce the relapse retes.

If patients with gastric recurrence can be statistically associated with an increase load of oral Helicobacter pylori, it will be possible to confirm a new key role of periodontum in human health.

Some of the 102 patients were selected on the base of the results obtained from the time 0 Breath Test and salivary sampling; between oral fluids, saliva rather then crevicular fluid was chosen as fundamental parameter to be considered in the choice of recruited patients, since it is exacty this fluid that can reach the stomach by swallowing, causing a possible relapse of the disease; in addition peridodontal Hp load has been demostrated to be proportional to salivary Hp load in the "time 0 analysis".

So, on the base of these data, patients were divided into two groups: group 1 included 23 patients with negative UBT (time 0) and positive Hp loads in saliva examination (performed at time 0); group 2 (the Control Group) involves 37 Naive patients: they had negative results in both the oral cavity and in the stomach regarding infection with Helicobacter pylori at time 0.

These patients were included in a recall program in order to perform a second BT (time 1) and a new collection of oral fluids samples (both saliva and crevicular fluida at time 1). Minimum time between phase 1 and 2 is 6 months. Currently, this prospective phase of the study is at a preliminary stage; of the 60 patient considered suitable, based on the results of the first tests, 17 have been already recruited: 6 were included in Group 1 and 11 in Control group. A cut off value of 10 U of Hp detected with PCR-RT was chosen.

A professional oral hygene performed within the time 0 and time 1 represents an important paramter in order to understand if this procedure can reduce gastric recurrence rates by eliminating the oral reservoir of the bacterium. The results could be compared with Butt and Kahan anlysis.[97]

If gastric recrudescence was reduced by a proper periodontal therapy (scaling, rooth planing, etc.), then these oral procedures could be associated with conventional antimicrobial theraphy in order to increase its effectiveness by removing extra-gastric bacterial reservoirs, aiming at reducing the problem of relapse. This could determine a reduced need for antibiotic treatments, with less risks for the patients and less costs for the National Health Service.

3.5 CONCLUSIONS

The results of this research project have confirmed the relationship between the oral mouth and Helicobacter pylori, which has been demostrated to be present in the oral cavity and is not related to gastro-esophageal reflux, like some authors adfirmed in the literature. In addition to gastric mucosa, this bacterium can colonize oral periodontum, expecially in patients suffering from periodontal disease. This area represents a favourable enviroment where the microrganism can replicate, slipping away from human immune system surveillance. The more severe are oral conditions, the higher are Helicobacter pylori loads. Furthermore, from this extra-gastric reservoir the pathogen can spread within saliva and reach the stomach during swallowing. Further anaysis in patients suffering from this oral disease but negative at gastric Urea-Breath Test, started in the perspective phase of the current research, may lead to the discovery of a direct causal relationship between oral microbiota and gastric infection recurrence and to the evaluation of the role of preventive oral hygene procedures . From this perspective the manteinance of oral health and professional oral hygene procedures, combinable with standard eradication protocols, acquire greater importance, allowing to reduce the recurrence rate and the widespread use of antibiotics, with less side effects for patients and less costs for the National health Service.

4. FINAL CONSIDERATIONS

Our results suggest that oral microbiota could paly an important role in gastrointestinal diseases like colorectal cancer and Helicobacter pylory gastric infection.

Some oral bacteria have developed the ability to escape the host immune surveillance and to induce inflammatory responses leading to dangerous systemic effects.

Periodontitis represents a wide-spread common disease, caused by dangerous bacteria like Fusobacterium nucleatum and Helicobacter pylori, detected in high loads in pathological gums of affected patients. This conditions is charachterzied by the presence of periodontal pockets, which represent a natural reservoir for these microrganism, where they can replicate away from immune system, be poured into saliva and finally reach the stomach and the intestine by swallowing. Thus the manteinance of good oral hygiene could be essential to prevent bacterial dissemination and profoessional oral procedures should be associated to standard medical protocols, in order to reduce risk of developing dangerous extra-oral diseases.

Further studies are needed to elucidate the specific role of these bacteria in systemic health, in fact new virulence mechanisms and components continue to emerge, even from well-known oral bacteria. Only when a clear understanding of oral bacterial mechanisms in extra-oral infections will be achived new multidisciplinary effective therapies could be designed.

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