A NEW METHODOLOGICAL STUDY FOR THE ANALYSIS OF RISK FACTORS IN ORAL AND LARYNGEAL CANCER

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7-5-1- GASTROESOPHAGEAL REFLUX

1-SUMMARY

The etiology of oral and laryngeal tumors is very complex; genetic mutations, promoted by various risk factor, especially smoking and HPV infection, probably are the main cause. This study believes that genetic mutations may already present, but that the beginning of the carcinogenic event occurs only after the mucous layer that protects the epithelium of the entire oral cavity and larynx has been altered: a large number of internal and/or external factors made permeable epithelial or secreted salivary mucins. These risks factors can affect the oral and laryngeal protective mucous layer, in their main components secreted in saliva, MUC7, MUC5B, or alter the mucins adhering to the epithelium (MUC1). For example, smoking, alcohol, inflammatory processes, produce large amounts of free radicals, and alter the balance of mucins; the aging processes, and ineffective metabolic control, modify the same balance, Chemical compounds present in food, such as polyphenols or certain drugs, such as steroids and NSAIDs, can finally interact with the mucins, precipitating them. Particular psycho-endocrinological conditions (post and pre-menopausal conditions) and other hormonal disorders, can influence the oral defenses, decreasing the gene expression of epithelial MUC1 in the oral mucosa, while diseases such as diabetes are instead responsible for a hyperproduction of the secret mucins, also causing alterations of the mentioned layer. All these processes, such as, promote HPV infection: the highest incidence of which is into the anatomical site, where the layer of mucin is in substance naturally absent, namely in the tonsils. Many factors can cause changes in the two fractions of the salivary mucins, mainly the secret ones, which are the main constituents of the mucous layer that protects the oral cavity and larynx; each change alternates of its rheological characteristics evaluated experimentally and changes the spinnbarkeit value. The

spinnbarkeit measures the ability of the saliva to adhere to the surfaces inside the mouth, and serves as an indication of its protective role, in the process of adhesion and lubrication: Its change, indicates the physical and chemical alteration of this layer, which is normally clear, transparent, elastic and slippery, to the mouth epithelium. The alterations of the values of, at the top or bottom, compared to a normal range, of the spinnbarkeit of the saliva, can cause the loss of adhesiveness or the ability to bind, viruses or carcinogenic chemical compounds.

2-PATHOPHYSIOLOGY OF THE ORAL CAVITY:

- Chewing

- digestion (salivary enzymes)

- Speaking (larynx, tongue, cheeks, lips)

- Gustative (chemoreception)

- Swallowing, in other words swallowing (hard and soft palate)

Cancer of the mouth (oral) is a major neoplasm, widespread throughout the world and accounts for most cancers of the head and neck. Theoretically, it should most often be preventable or detectable at an early stage. About 90% of oral cancers are squamous cell carcinoma (SCC), which is typically visible on the posterior-lateral part of the tongue, usually as a white, red or mixed white and red lump or ulcer. SCC is particularly common in the developing world, especially in older males. There is a concern for a continuous increase in younger patients and women, as well as for the oropharyngeal one. The etiology of SCC appears to is a multi-factorial one and very much related to HPV and tobacco infection, alone or with betel chewing and less

influenced by lifestyle, habits, diet, or alcohol use. Other factors such as repeated trauma, may involve, gums and palate, while some immune defects or immune suppression, metabolic changes, or defects in DNA repair enzymes are at the root of some cases of SCC; even exposure to sunlight predisposes to lip cancer.

3- PATHOPHYSIOLOGY OF THE LARYNX

The larynx is an essential organ that is responsible for the following vital functions:

- Maintaining proper ventilation

-Voice modulation

- Protect the lungs from direct exposure to harmful fumes and gases at unsuitable temperatures.

- Protect the lungs from the intake of solids and liquids

- It allows, closing the glottis, the maneuver of Valsalva,

Malignant laryngeal tumors can influence laryngeal physiology depending on the place and size of the tumor. Supra-glottic tumors cannot alter the way laryngeal functions until they reach a relatively large size when airway obstruction is the first symptom. In contrast, glottic tumors alter the sound quality of the voice at the beginning of their development and are often discovered at an early stage. In addition, malignant laryngeal tumors influence the physiology of swallowing. The swallowing mechanism alters when tumors invade and alter the physiology of the swallowing muscles, and this can lead to dysphagia or

aspiration defects. The growth and progression of malignant laryngeal tumors occur at a molecular and histological level. The molecular steps involved in carcinogenesis have not been fully clarified and probably vary from patient to patient. Histological progression occurs from the normal laryngeal mucosa to the dysplastic mucosa, up to carcinoma in its original place, and then becomes invasive carcinoma. This progression is a multi-step process of accumulated genetic events leading to the growth of laryngeal tumors. Until the complex molecular interactions of all the aetiological agents associated with any cancer are understood, these interactions can only be explained as linked events. Think of intrinsic factors (e.g. Genetics) and/or extrinsic factors (e.g. smoking) as the only causes are too simple. For most people, a cause implies a necessary and sufficient condition to produce a precise result. Potential risk factors related to the growth of laryngeal cancer include:

-Male sex

- Infection with human papillomavirus

- Age of greatest

- Exposure to diesel and petrol fumes.

- Exposure to radiation

- Gastroesophageal reflux

Cancers starting with epithelial tissue (squamous cell tumor) account for about ninety cases out of every 100 laryngeal tumors. Cancer develops like squamous cells covering the surface of the epiglottis, vocal cords and other parts of the larynx. Malignant

laryngeal tumors can influence laryngeal physiology depending on the place and size of the tumor.

4 - GENETIC EVIDENCE IN ORAL CAVITY AND LARYNGEAL CANCER

. Genetic research, regardless of the different risk factors highlighted above, tries to find out which are the genes, from which part of the biochemical signal that transforms a normal cancer cell. The change in some genes generally generates an alteration in membrane receptors, which will act as a "chemical switch in the ON place The result shows an uncontrolled mechanism of DNA duplication ;the identity of these genes, can lead to the discovery of the key to turning off the mechanism of this process of uncontrolled cell proliferation. Our recent review (1) with research in Pub Med and GENE, from 2000, until 2016, using as research key "genetic mutation in oral cancer and genetic mutation in laryngeal cancer" And laryngeal cancer, respectively, 2020 and 364 articles, which shows all studies conducted to detect genes allegedly involved in the development of these types of cancer. The bibliographic analysis, the critical review of the mechanisms by which the genetic alteration, influence, promotion of oral cancer and laryngeal is the scientific base of this study. The analysis's concerns only references that have scientific validity and not articles cited individually, or even with a few references,The first choice in PUBMED and GENE, has identified 22 genes, potentially related to the development of oral cancer and laryngeal; This index of genes, with alleged "genetic influence", has been included in the database "Genetic Home References" that shows, in terms of scientifically

confirmed, the cellular functions and effects of their mutations in all organs in terms of promotion of carcinogenesis): This allows cutting the number of genes to fourteen, (see table 1). Our study identified these fourteen genes, as surely involved in the process of carcinogens and has a valid response with a review recently published by Nature in 2016. This review analyzed the relationship between the most often met variables in the oral cavity and larynx tumors, with the frequency and type of mutations of different genes (missense, frameshift, ecc.) with the smoke, and the level of HPV infection, and to the various anatomical sites. The result of this detailed analysis indicates that the genes involved in the highest percentage of cases are Tp53 and CDKN2A, (1), the type of mutations is the Missense and indicates their certain involvement with the simultaneous presence of the state of smoke and/or of the HPV infection Both genes are suppressor /oncogenic, and are present, after histological analysis, in about 80%-90%, of cases of cervical and head cancers. Their mutations, however, are present in the onset of various other tumors, such as those of the bladder, sinus, and melanoma. This result derives from the fact that their main role is to stop the degenerative cellular process, with other genes that generally act in cascade, but always after the genetic mutation is present on both alleles. The particular recurrent family predisposition to suffer from certain types of cancer indicates that the inheriting one of these mutated genes also in one allele, predisposes, in a certain way, to the risk of carcinogens, but only for the Loss of homozygous condition, (heterozygote for the healthy allele). Only after the loss of heterozygosity (-LOH: Loss of heterozygosity), the gene, e.g. T p53, completely loses its

genetic expression that controls the cell cycle in the key role of cellular reconstitution and repair capacity. We can, therefore, affirm that this development of cancer, for the oral cavity and the larynx, is certain consequence by gene mutations resulting, however, from endogenous and/or exogenous causes, that recent research has identified mainly in smoking, alcohol abuse and In cases of HPV infection. Both genes, are present, as we can see in table 1, in so many pathological situations

TABLE 1.

Results in the questions on genetic influence in oral and laryngeal cancer formation. by genetic home references

gene	coded protein and functions	diseases and pathology
tp53 17p13.1	p53-tumor suppressor	breast, bladder, neck head squamous cancer and others cancers, Li Fraumeni syndrome
tp63 3q21	p63-transcription factor	the amylo-blepharonectodermal defect, neck head cancer
cdkn2a 9q21	p16-p14-tumor suppressor	neck head squamous cancer, melanoma, breast pancreatic cancer
notch1 9q34.3	notch1-receptor for proliferation, differentiation, apoptosis	adam-Oliver syndrome,neck head cancer
pik3ca 3q26.3	p110α-oxidative phosphorylation, the cell division signal	megalencephalic capillary, malformation , colorectal cancer
hras 11p15.5	hras-oncogenic and cell division	costello syndrome, bladder, thyroid, kidney cancer
sat1 xp22.2	acetyltransferase family of polyamides metabolism, detoxification	keratosis deculvans
caspe 2p33-34	family caspse /protease-, apoptosis	autoimmune lymphoproliferative syndrome, breast neck head, and

		lung cancer
fat1 4q35	tumor suppressor-Catherine family-cells adhesion	many cancers
hla/abc 6p21.3	human leucocytes antigen-mhc	autoimmune diseases
cyp101 2p22.2	p 450 cytochrome family - metabolism of detoxification	early –onset, glaucoma, Peters anomaly
xrcc3 14q32.3	reca/ rad 51-stability of chromosomes, DNA repair	breast ,melanoma,head and neck many cancer
ads 4q23	alcohol dehydrogenase - energy metabolism and sugar control	alcohol dependence, diabetes
not212 22q12.1	nuclear erythroid derived/transcription factor with up-regulation of oxidative stress	diabetes, multi-sclerosis

This approach tends to define how the current onset of head and neck cancer is not caused, only by hereditary factors, with the help of smoking, alcohol, and HPV. This hypothesis is in fact related to epidemiological data (3), which show, (see Table 2), such as in the main countries where laryngeal cancer has the highest index (ASR), such as Cuba and Iraq, there is absolutely no high consumption of alcohol and tobacco .The epidemiological data of incidence of oral cancer with the presence of tobacco and alcohol use, (4), (5), allows the same conclusions see Table 3

Table 2 laryngeal cancer epidemiology ASR index in correlation with smoking and alcohol habits

CUBA	7,6	+	+
HUNGARIA	6,4	++	+++
IRAQ	5,6	+	-
KAZAKISTAN	5,5	++	++
UZBEKISTAN	5,5	++	++
MOLDAVIA	5,3	++	+++
ROMANIA	5,0	++	+++
MONTENEGRO	5,0	++	+++
BULGARIA	4,9	++	+
CROAZIA	4,7	++	+++
SIRIA	4,6	+	+
PORTUGAL	4,6	++	+++
TURKMENISTAN	4,5	+	++
VENEZUELA	4,4	+	+
POLONIA	4,3	++	++
ITALY	3,2	++	++

Duca - Menicagli

Legend: ASR index = age standardization rate ;numero di casi /100000 peoples + Normal –moderate habits; ++ high ;+++ excessive

Table 3 Cancer epidemiology; ASR index of oral cancer in correlation with smoking and alcohol habit

COUNTRY	ASR	SMOKING	ALCOHOL
PAPUA NEW GUINEA	25,4	+	+
MALDIVE	11,0	-	-
SHRY LANKA	10,3	+	-
BANGLADESH	10,1	+	-
PAKISTAN	9,9	+	-
UNGHERIA	9,7	++	+++
INDIA	7,6	+	+
PORTOGALLO	6,8	++	+++
SLOVACCHIA	6,5	+	++
AUSTRALIA	6,3	+	+
KAZAKISTAN	6,3	++	++
MYANMAR	6,3	+	+
AZERBAIJAN	6,3	++	++
AFGANISTAN	6,3	++	.
KAMBOGIA	6,0	+	+
ITALIA	3,06	+	+

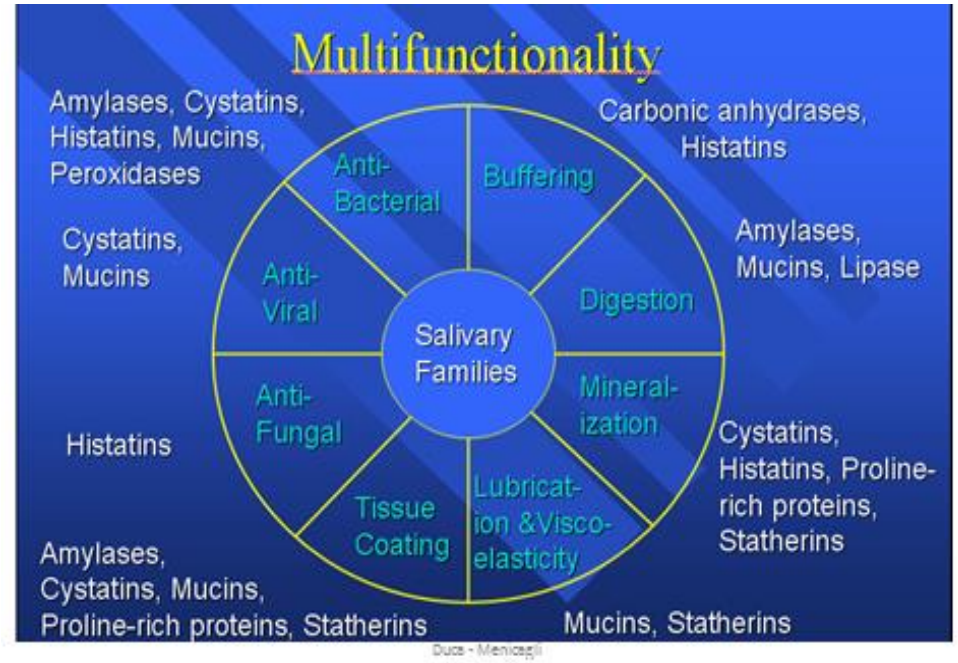
Duca - Menicagli

The above data clearly show that there are other causes to promote cancer and that they involve mutations that alter both alleles of the same gene. These causes are often present in many risk factors, such as tobacco, HPV, alcohol, age, sex, drug use, eating habits, and in some diseases, such as diabetes. Research in the field of oral cancer and larynx cancer should study the mechanisms by which certain biochemical and physical factors alter the cellular, oral and laryngeal defense structure, mainly consisting of salivary mucins (6). Even if laryngeal cancer in some aspects is different from that of the oral cavity, the mucinous layer also protects this anatomical structure and it is foreseeable that the processes of its alterations have in any case the same origin. The purpose of this study is to assume that any risk factor can cause alterations in the oral cavity and larynx, with extremely important and irreversible changes in the salivary mucin layer, and allows, such as smoking and HPV to change genes. Other risk factors can irreversibly influence the normal genetic structure of the cell, allowing the possibility for risk factors to act on cell metabolism without finding an adequate protective barrier.

6- Mucins Protective Layer

The oral mucous, with its protein components, forms a protective coating on moist epithelial surfaces throughout the body that houses the microbiota and plays a key role in the host defense. Mucins are the primary structural components of the mucus that create its viscoelastic properties and have a key role to form the gel and non-gel phases, which protect against invasive pathogens. Alteration of mucin production, are also involved in diseases in other anatomical sites, as well as the oral cavity and larynx, as in ulcerative colitis, asthma and cystic fibrosis, highlighting important of mucin in maintaining homeostasis, in the defense of many organs of the human body (see figure 1).

Figure 1 Multifunzionalità dei componenti salivari



6-1-Mucines in the oral cavity

The most important salivary mucins are MUC5B, MUC7, and MUC1: they consist of a unique domain structure (7), which influences their physical properties, and the localization in the oral cavity. MUC5B is the main mucin forming the gel phase on the mouth epithelium, that the mucous cells in the submandibular, sublingual, Palatine and labial salivary glands secret in the mouth. Other transcriptional mucins have been identified, glycoproteins such as MUC19, salivary mucin forming gel, but MUC5B is the main one to form the gel phase in the oral cavity. MUC7 is also secretory mucin, which exists mainly as a monomer or dimer, and lacks gel-forming properties.

These monomers and dimers are able to self-associate, forming groups of a higher order; the location of MUC7 inside the salivary glands varies in the individuals, with different amounts in the mucous cells of the submandibular and sublingual glands. MUC1 has associated membrane mucin, that is, mucin which aligns the ducts of the parotid, submandibular and minor salivary glands and which may play a role in the transduction of the cellular signal.

6-2-Salive mucin layer structure and secretory mechanism.

MUC7 and MUC5B have different aspects in their primary sequences that determine their ability to form gels and higher order structures, also sharing multiple functions. Both MUC7 and MUC5B consist of a protein spine with carbohydrate chains that radiate outwards to form a "brush": see Figure 2 and Figure 3.

FIGURE 2 - MONOMERIC STRUCTURE OF THE MUCINS

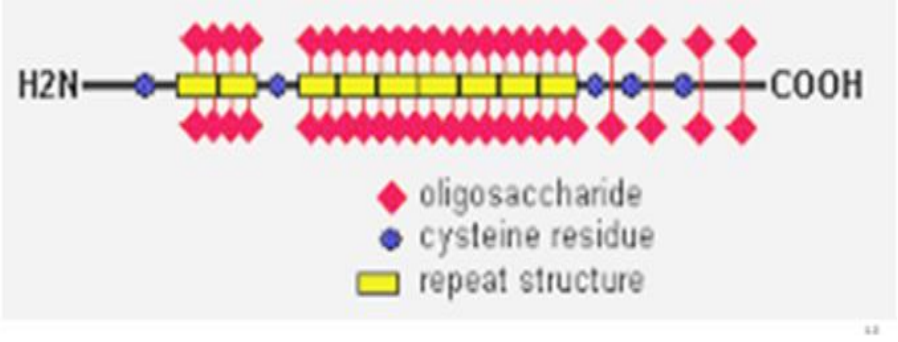
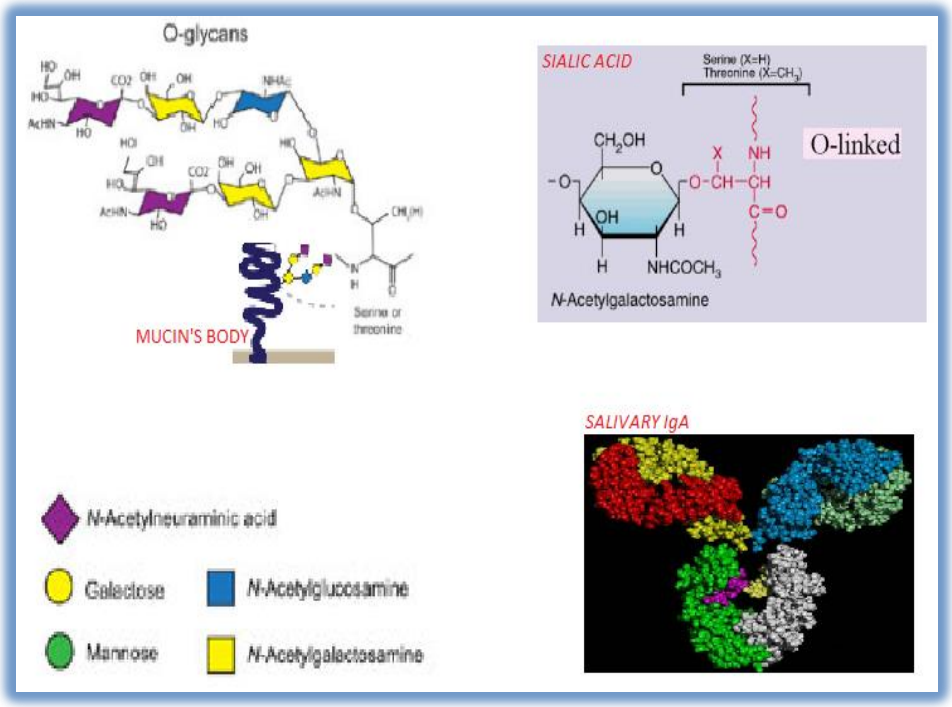


FIGURE 3 The Glycosylated Part of Mucin



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About 5,700 amino acids make up the backbone of MUC5B, widely organized in the N-terminal, central glycosylate and C-terminal region. The central glycosylated region of MUC5B has repeating units of 29 amino acids rich in serine and threonine, which are important, as we will see later, in the process of binding to chemical compounds such as polyphenols. The C-terminal domain participates ta disulfide bonds, which connect each monomer, MUC5B, to dimers and then to polymer chains, to form the disulfide bonds to the N-terminal. After the secretion of the mucin granule, the bivalent calcium ions, which stabilize the folded mucin polymer within the secretive granule, exchange for monovalent sodium ions. The increase in osmotic pressure

leads to a hydration process, which guides to growth the polymers to form a gel phase (8). Cross-linked expanded polymers link together through polymer chains of glycoproteins and/or non-covalent bonds formed by hydrophobic interactions (9). Calcium may also mediate the cross-linking of MUC5B to form higher-order structures. MUC7 has a backbone of 357-amino acid with a central region of repeated units composed of 23 amino acids, and lacks a domain rich in terminal cysteine; therefore, MUC7 mucin is not able to form polymers and exist primarily as monomers. Differences in the MUC7 and MUC5B structure and in their physical place in the oral cavity affect the ways in which they give protection to the oral cavity and larynx

.6-3. The film associated with the mucosa

The oral mucosa form a pellicle extremely hard, to withstand the extreme conditions in which it is susceptible, such as the abrasive action of food, or temperature changes such as hot drinks and meat cooked on fire and ice cream. Hard grasses and vegetables (including various tubers) may contain highly abrasive siliceous particles. The oral cavity has two lines of defense: first, in the parts of the oral mucosa that are under direct action of mechanical forces such as the hard palate have developed mechanically harder keratinized tissues, designed to protect the cells below from damage. Secondly, the lubricating effect of the saliva film protects the oral cavity, tooth enamel and soft tissue, including softer, non-keratinized oral surfaces such as the buccal mucosa. The mucosal film is a supra-molecular film with a complex architecture (10) that includes several structural layers. It includes a complex of many salivary proteins, including: sIgA,

MUC5B, MUC7, carbon dioxide VI (CAVI) and cystatin S. The salivary mucins, MUC5B and MUC7 are essential for the protection and lubrication of the entire oral cavity due to their high molecular weight and the high level of hydration due to be highly glycosylated regions. Both types of salivary mucins adhere strongly to the surfaces of the buccal cells. The MUC5B fraction is the one that dominates within the film adhering to the enamelled surface of the teeth. The process of self-assembly of salivary proteins varies greatly depending on the type of oral surface, with variations in composition, protein content, thickness and speed of reconstitution. This is the key element of the assembly process to form a layer closely bound to the epithelium, and that ensures the adhesion of the film and acts as a model for further protein/mucin associations.The uptake of single salivary proteins and of the saliva on different model surfaces with different model surfaces is the better system to understand the effective protective role of the mucins on the oral cavity (11) . MUC5B has both very hydrophilic glycosylated domains and hydrophobic domains in non-glycosylated areas, MUC5B is also shown to have stronger adsorption to hydrophobic surfaces, unlike other hydrophilic compounds, leading to a higher adsorbed mass and slower resorption times (12). Add calcium also facilitates to deposit the MUC5B on the epithelium and to promote bonds with other proteins. Unlike MUC5B, MUC7 has a much smaller molecular weight (250 kDa than over 2000 kDa of MUC5B) and includes a single glycosylated region surrounded by small non-glycosylated domains. Because of a greater relative size of the glycosylated domains, however, MUC7 has higher levels of hydration that

affect its adsorption. However, MUC7 has a high propensity to self-condensation, or self-assembly, which can counteract its high solubility and increase its incorporation in the film to form complexes with lower molecular weight proteins such as IgA (13). The process, by which the salivary proteins bond with the surfaces is complex, due to the number of proteins present, their size, and concentration. A finely tuned arrangement of electrostatic and hydrophobic forces, as well as specific hydrogen bridge bonding interactions as well as cross-linking phenomena, govern this complex process.Many factors occur to form the salivary films, such as the ionic composition occur to influence and to improve the films, through an increase/decrease of electrostatic interaction and cross-linking of proteins, but the most important factors are the structure and the concentrations of the same Mucins The schematic representation of how this layer form this structure there is below, (fig 4); this interpretation has a good confirmation in the image, in vivo (photo 1), with fluorescence technique, (14. Salivary proteins show a higher affinity to hydrophobic surfaces than hydrophilic surfaces and this is in line with the bare oral mucosa is a largely hydrophobic surface. It then increases its hydrophilic phase, as the protein layer develops on overlapping layers, to form hydrophobic surfaces by the deposition of the saliva (WMS), of parotid origin (PS), sublingual, and sub-mandibular mucins protect the oral cavity through different mechanisms by the nfluence of their unique polymeric structures. Firstly, the mucins can interact with salivary proteins to alter their localization and retention, which could give greater protection for the oral cavity. Moreover,

MUC7 and MUC5B can interact with microbes and oral viruses to ease their removal and/or cut their pathogenicity.

Figure 4 - Hypothetical structure of the mucosal layer (Menicagli .R 2004)

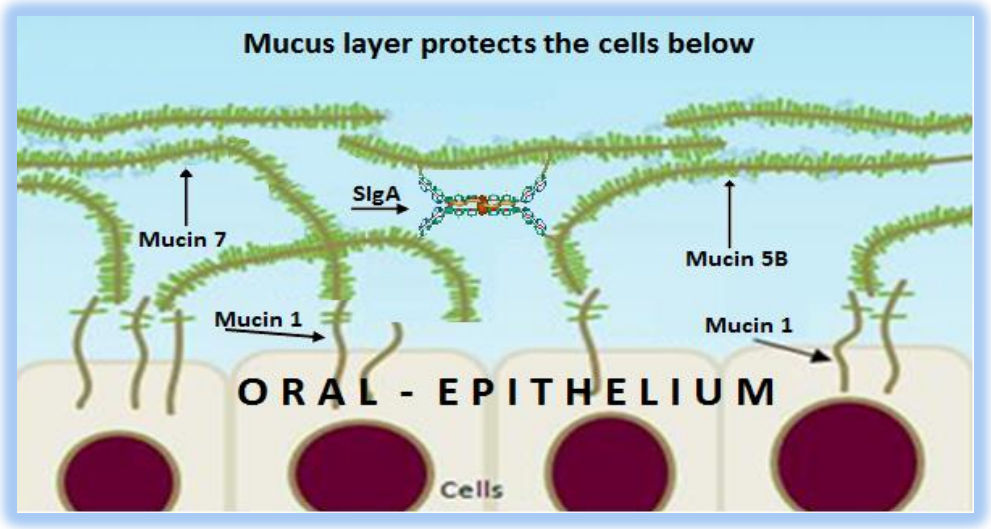
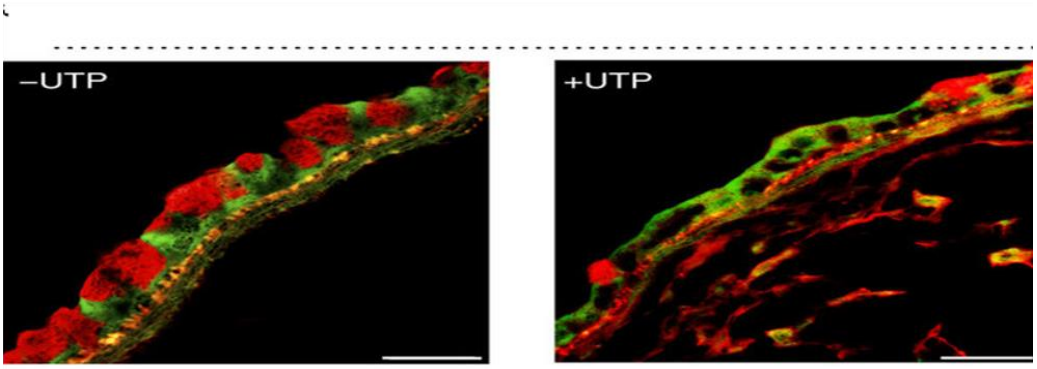


Photo 1 the Fluorescence representation of the Mucinic Layer

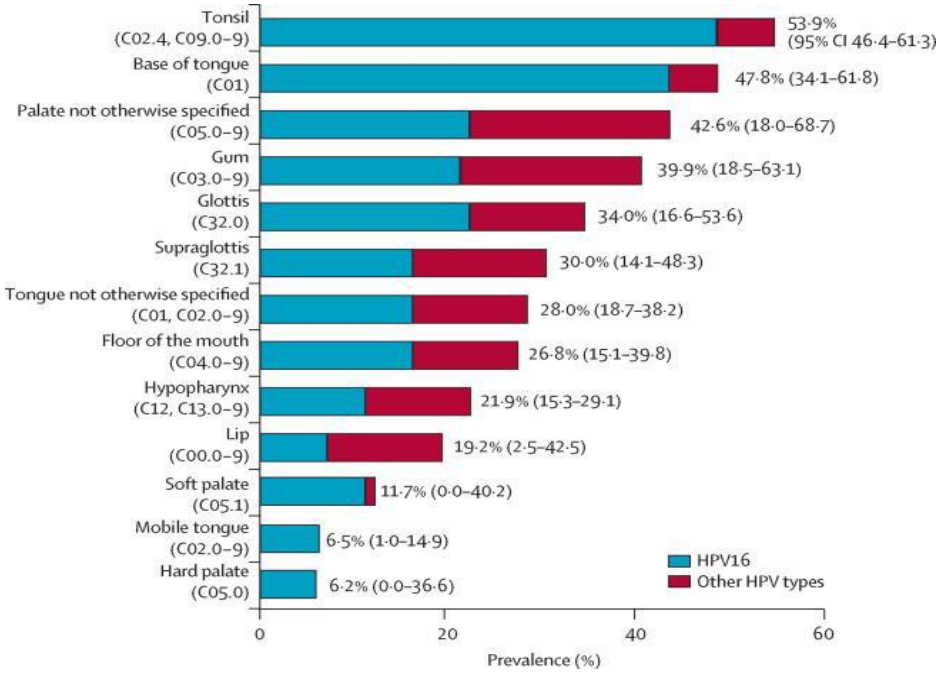


7. RISK FACTORS AND OUR INTERACTIONS WITH MUCOUS MEMBRANES

7-1-INFECTION from HPV

Scientific data (15) show that HPV has little penetrating power compared to the protective layer formed by the mucinae, compared such as to strain A of the influenza virus, but in any case HPV is very common in oral tumours and in the larynx (16) The papilloma virus (HPV), which belongs to human papilloma viruses, are small DNA viruses, belonging to the family Papillomaviridae, which infect the epithelial tissues of the mucosa and skin, to induce proliferative lesions, and warts, in hands and feet. Typical are warts at the level of the genital tract and respiratory lip, as well as carcinomas within the cervical uterus and larynx. Based on their carcinogenic power, oncogenic There are HPV strains with low-risk , particularly the types (6,11,40,42,43,44,54,61,70,72,81) +, CP6108), which mainly cause benign genital injury, and with high-risk types (16,18,31,33,35,39, 45,51,52,56,58,59) associated with genital and anal cancer, (more than 90% of cervical carcinomas Important to develop the oro-laryngeal carcinomas are types 16,18,6. The epidemiological data show the influence of HPV ; through extensive research until the year 2016, about (16), the spread of the HPV virus related to the head and neck and its distribution in various anatomical is very important sites. From the results published in two separate "systematic reviews", it is possible to process the results with the analysis of the real incidence, (17, 18), (fig 5)

FIGURE 5- the anatomical sites for HPV tumors and the incidence of HPV types



1-Epidemiological data, and a recent meta-analysis show that HPV has a signified part only for oropharyngeal tumors.

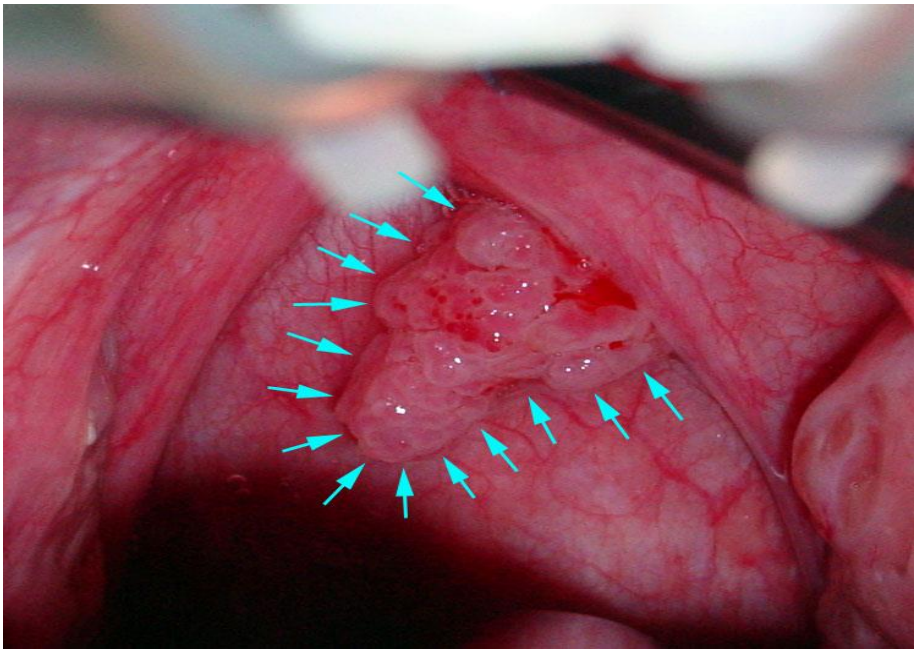
2-Data analysis and conclusions on the real importance of HPV strongly depend on the geographical areas where the researchers carried out the studies.

3-the incidence of HPV cancer, is independent of alcohol and/or tobacco consumption.

4. the anatomical distribution of tumors that exist for HPV is an important issue of an etiological nature. the virus, statistically, propagates almost exclusively in anatomical points not protected

by the mucin layer: palatine and lingual tonsils. layer. (See photo 2 below).

PHOTO 2 -HPV: INFECTION OF THE TONSILS



7-2-SMOKE: KNOWLEDGE AND PREVENTION

7-2-1 – Knowledge

Cigarette smoke is a complex miscellany of over 4700 chemical compounds, (1), with concentrations of highly concentrated oxidizing compounds, both in the gas phase of smoke and in the corpuscular phase. The first phase has high quantities of short-lived oxidants. Such as O2 and nitric oxide (NO). On the other hand, the corpuscular phase of smoke has long-life radicals such as semiquinone radicals, which can react with O2 to

form hydroxyl radicals (OH-) and H2O2. This production of substances (free radicals) can lead conditions of biological stress (oxidative), in organisms. Cigarette smoke is a complex miscellany of over 4700 chemical compounds, (1), with concentrations of highly concentrated oxidizing compounds, both in the gas phase of smoke and in the corpuscular phase. The first phase has high quantities of short-lived oxidants. Such as O2 and nitric oxide (NO). On the other hand, the corpuscular phase of smoke has long-life radicals such as semiquinone radicals, which can react with O2 to form hydroxyl radicals (OH-) and H2O2. This production of substances (free radicals) can lead conditions of biological stress (oxidative).The chemically reactive species, produce the pathological condition definite as the oxidative stress, when is the imbalance between production and elimination of the same. In a state of oxidative stress; it is present an abnormally high quantity of free radicals which exert a damaging action, on the cells and tissues of our organism The main reactive forms of oxygen(ROS) which have a biological interest been: ozone, anion superoxide, hydrogen peroxide, hydroxyl radical, (alkyl-) peroxyl radical, (alkyl-) hydroperoxide Free radicals react with other molecules physiological, for example lipids or DNA, in a chain reaction, as long as two radicals do not combine to produce a neutral species The chain reactions damage important biological molecules: , ROS are traditionally considered as, high-level cellular dangerous.ROS can cause severe oxidative damage especially against DNA, lipids, and proteins These damages may cause a great variety of chronic-degenerative diseases among which, atherosclerosis and cancer.Many metabolic activities, can produce the ROS, but

those are present also as physiological phenomenon: and only the excess of these free radicals is able to find a pathological state. Smoking is among the most important causes of hyperproduction of free radicals. lipids are the most susceptible class of biological molecules to attack free oxygen radicals. oxidation occurs in the fatty acids present in cell membranes or in lipoproteins and the increase in the number of double bonds present in the molecule increases their susceptibility to oxidation.Secondary products such as aldehydes and ketenes recognized as toxic or carcinogenic substances, are themselves the products of these uncontrolled mechanisms The most important lipid oxidation markers is malondialdehyde (MDA). Cigarette smoke also has varying concentrations of NO, in a range of 150000-226000 mg / m3, (2). Cigarette smoke produces a large measure of NO, which is added to the endogenous one, which is necessary for various and important functions of cellular metabolism(3) The nitric oxide, by its nature, is a compound that from the point of view of chemical stability is perennially in an intermediate state of oxidation, so it is able to oxidize and cut the compounds it comes into contact with the same. When it is in excess, may, reacts with each other chemically reactive species, to form peroxy nitric molecules (ONOO-). (2) Producing, in turn, a state of oxidative stress in the body

. Similarly to oxidative stress, nitrosative stress characterizes the imbalance between reactive nitrogen species and the antioxidants. Immoderate/excessive production of nitric oxide (NO) has harmful effects on bio macromolecules.In fact, to its highly reactive nature,.NO reacts principally with. superoxide, leading to the formation of more reactive compounds, (RNS),

and results in cytotoxic effects, (4), as the DNA damage, result of its reaction with carcinogenic nitrosamines, and lack of the same DNA repair mechanism. For these properties hence, nitric oxide is a potent tumor initiator, (5), (6), (7), for many cancer types. Peroxynitrite is also a reactive oxidant which reacts with lipid and leads to peroxidation (malondialdehyde and conjugated dienes and formation of nitrite-, nitro-, and nitroso-peroxide and/or nitrate lipid oxidation adducts (8)

Many studies show (24) that free radicals degrade proteins and mucins where the changes are on both sides, both glucidic and purely made up of amino acids. Mucins are susceptible to attack by reactive oxygen species during which terminal sugars are lost, These reactions fragment both proteins and sugar components. Recent research (26) has studied the interactions of free radicals, with some components of saliva including mucin, and has shown that a lower molecular weight mucin complex forms in smokers' saliva. Important, besides any alteration of the molecular structure of the mucins, is also their quantitative reduction in terms of percentage of the entire protein fraction present in the saliva.
Concentrate mucins are normal in a range from 15% to 20% by weight of total salivary proteins. In this study we present the preliminary results we obtained, on the effects of smoking habits, on total salivary proteins and on the total mucins, in a group of smokers with an age of ≥45 years, compared to a control group, with the same average age, to verify the relationship between tobacco as the same age, on the health effects . Table 4, shows these results, while Table 5 shows the results of the Fisher exact test to verify any differences between smokers and non-smoking groups with the age of ≥ 45 years.

TABELLA 4 I valori di MDA, proteine salivari totali, mucine salivari totali, nei fumatori, non fumatori con età ≥ 45 anni e in non fumatori con età ≤ 45 anni

SMOKERS				**NOT SMOKERS AGE≥45 YEARS**				**NOT SMOKERS AGE ≤45 YEARS**		
AGE	MDA	T.S.P Initial value	T.S.M Effective value	AGE	MDA	T.S.P Initial value	T.S.M Effective value	MDA	T.S.P Initial value	T.S.M Effective value
46	**5,2**	300	27	**54**	3,9	330	32	≤3	330	44
71	5	340	32	**78**	1,9	330	34	≥3	330	50
73	**5,5**	334	31	**77**	4	340	32	≤3	329	52
72	**3,9**	352	34	**55**	3,6	340	33	≤3	340	53
70	**4**	336	40	**55**	2,4	318	32	≤3	338	49
66	**4**	352	31	**52**	3,3	336	37	≤3	316	52
65	**6,1**	350	34	**70**	2,6	356	33	≥3	350	40
66	**2,2**	322	34	**53**	4,2	321	33	≤3	321	52
55	**6,9**	343	38	**59**	2,8	322	32	≤3	346	32*
54	**5**	347	33	**54**	3,2	349	41	≤3	319	49
49	**2,6**	354	39	55	3,3	354	42	≤3	354	49 *
55	**3,9**	342	33	**55**	2,1	322	35	≤3	333	55
46	4,1	322	34	50	2,6	340	35	3,2	333	46
47	4,5	333	29	45	3,7	340	40	3,5	334	39

LEGEND T.S.P ,Total salivary proteins ; T.S.M Total Salivary Mucins; MDA expressed as ngr/ml

Table 5 ; Results of Fisher Exact for the values of MDA in Salivary Mucins in smokers and not smokers group with age ≥ 45 years expressed in table 4

Results FISHER EXACT SMOKERS VS NO SMOKERS AGE ≥ 45 YEARS				
	Category 1MDA	Category 2 TOTAL SALIVARY MUCINS	***Marginal Row Totals***	
Group 1smokers	12	9	21	
Group 2not smokers	9	3	12	
Marginal Column Totals	21	12	33 (Grand Total)	

RESULTS FISHER EXACT TEST p=0.04678 ,

DATA INTERPRETATION

Test T in smokers vs no smokers age ≥ 45 years p=0,01951

Test T results for TOTAL PROTEINS in smokers, vs not smokers age ≥ 45 years, p = 1,19238E-08 ; p≤ 0.05

Test T results for TOTAL MUCINS in no smokers with age ≥45 years ,vs control, p = 9,83628E-08 ;p≤0.05

Table 5 : the values of MDA and SALIVARY MUCINS in smokers and not smokers group with age ≥ 45 years ; p =0,4678

Based on this statistical analysis it may be concluded that:

1) Be smoking involves significant increase in MDA compared with the control.

2) Older age ≥45 years, it has a significant effect on increasing of MDA, both in smokers than non smokers

3) The smoke results in a significant decrease of total salivary mucins vs control

4)table 5 shows that the difference in concentration between salivary mucins in smokers, with MDA ≥ 3 μgr/ml, and non-smokers aged 45 years or older is not statistically significant. Harman's theory (27) or mitochondrial hypothesis, already expressed in 1956, may explain this result. The theory of free mitochondrial radicals of age depends on the fact that mutations in somatic mitochondrial DNA, which accumulate during life, cause excessive production of reactive species of oxygen that damage macromolecules and inhibit the growth of the cells and tissues. In fact, studies have shown that the greater oxidative capacity decreases with age, while the increase of highly reactive species depends on the amount of reactive oxygen. . Harman's hypothesis has been seriously challenged by recent studies showing that reactive oxygen species evoke health and metabolic longevity, perhaps through mechanisms that include autophagy. many studies refute this theory or change it in some respects, but two key arguments stay in its support: first, cellular aging occurs with an imbalance between antioxidant/oxidant. This leads to a great amount of oxidized and damaged macromolecules, which in turn leads to form an aged and degenerated phenotype. The results of our study as the mitochondrial hypothesis is possible , but we must also consider that the same old age reduces the defense mechanism, guaranteed by the oral mucosa, and changes some factors, such as the salivary flow, or the amount of mucins In fact, it is known that the aging process reduces the salivary flow, in specific salivary glands, and with the lower production of salivary proteins (28). These processes of reducing the salivary flow and proteins are also observed with

the use of drugs, the use of which generally increases with age. These findings have important clinical implications for maintaining optimal oral health in older adults, 29), (30), (31)

7-2-2- Prevention

IT ENOUGH TO SMOKE A FEW CIGARETTES, TO SAFEGUARD HEALTH? AN OPEN QUESTION

.The best result for to limit the health effetcts is, in any case, it is only, that of a drastic decrease in the number of cigarettes.But how many fewer cigarettes it is necessary, to smoke to get real health benefits? Is there a threshold value? Make less of the number of cigarettes /day by at least 50 %, allows 27% reduction in the risk of developing lung cancer(9).In any case, even when smoking a few cigarettes, the human organism comes into contact with a large number of free radicals. The purpose of this study is to verify in group of Heavy Smokers, people who smoke 20 or more cigarettes/day, and in Light Smokers, five or fewer cigarettes, their real impact monitoring, both the malondialdehyde and the NO and salivary mucins, to check any differences in the processes that cause total oxidative stress in these two groups of smokers.In this study, saliva samples from volunteers have been after a careful anamnesis concerning the health of the oral cavity to rule out possible diseases that could affect the characteristics of a normal salivary composition excluding that arising from regular cell metabolism. In this study, the samples of saliva, are representative of that production in the secretion from all salivary glands, is called "the whole saliva". This saliva, when secreted under resting conditions, it is referred to as "unstimulated." “In this study,

there are two groups of smokers of ten people each; the first consists of people between the ages of thirty and fifty years, who smoke more than 20 cigarettes a day, (Heavy Smokers); the second group is made up of smokers aged between 31 and 49 years who smoke less five cigarettes a day, (Light Smokers): both groups were matched with a control group of no smokers. Unstimulated salivary sampling was done in every group The groups were asked to collect the saliva, after the exclusion to eat or drink for two hours prior to saliva sampling, seat still, tilt head forward so that saliva accumulates in the anterior part of the mouth. After we collect saliva saliva in a tube; the samples were stored at - 80 ° C until the analysis time. The MDA concentration was measured with the thiobarbituric acid test (TBA); while NO with Griess methods

The thiobarbituric acid test (TBA), concerns the reaction of thio barbituric acid with malondialdehyde (MDA), produced by the decomposition of hydroperoxides in lipid systems, and is linked to the formation of a red-collared chromophore compound with a peak of absorption at 532 nm. The intensity of the color is proportional to the concentration of the aldehydes in the sample. This collared complex results from the condensation reaction of two moles of thiobarbituric acid with a mole of malondialdehyde, under the joint effect of the medium, temperature, and pH...Nitric oxide can be measured using various direct and indirect methods, but the short half- live and low concentrations of NO in-vivo reduce the practicality of these methods for evaluation of biological samples. Additionally, these procedures are generally unsuitable for the clinical laboratory due to instrumentation requirements and inexpedience in processing a large number of

samples. The difficulties inherent to quantification of NO can be eliminated by measuring its stable metabolites, in particular, nitrite and nitrate. The simplest and most frequently applied method employs colorimetric detection with Griess reagent Nitric oxide (NO) analysis The protocol provide to determinate NO levels indirectly with the measure of the nitrite concentration detected by Griess reagent, which is composed of equal volumes of three solutions (A, B and C). Solution A: 0.6 g of sulfanilic acid dissolved in 70 mL of hot distilled water, 20 mL of concentrated hydrochloric acid and distilled water to a final volume of 100 mL Solution B: 0.6 g of alpha-naphthyl- amine dissolved in 20 mL of distilled water, 1 mL of hydrochloric acid and distilled water to a final volume of 100 mL. Solution C: 16.4 g of CH3COONa .3H2O, dissolved in 100 mL of distilled water. After mixing equal parts of the three solutions, Griess reagent was added to the wells of a microplate. Then, we add the same volume of the sample We perform the analysis , using an ELISA reader at 520 nm. Statistical analysis of the values determined in the three groups was analyzed with the Mann-Whitney U Test Calculator. The Mann-Whitney U test is a nonparametric test that allows two groups or conditions or treatments to be compared without making the assumption that values are normally distributed. The results are also statistically analyzed with the Pearson Correlation Calculator The Pearson correlation coefficient is used to measure the strength of a linear association between two variables, where the value r = 1 means a perfect positive correlation and the value r = -1 means a perfect negative correlation.

Below, in table 1 are shown the results of the analyzes, concerning the salivary concentration of MDA(in nM/ml), and the nitric oxide concentration in nM/mL, in the three groups. The results were statistically analyzed with the Mann-Whitney test: In table 2 is shown the comparison of MDA concentration, between the Light Smokers subgroup,age ≥ 40 years, vs Control subgroup, age ≥ 40 years. These results were also statistically analyzed with the Pearson Correlation test. In table 3 are summarized the concentration values of salivary Mucins in the three groups

Table 1 salivary concentration of MDA(in nM / ml), ,and the nitic oxide concentration in nM/mL, in the three groups,

SMOKERS CIGARETTES ≥ 20 /day			SMOKERS CIGARETTES ≤ 5 / day			NO SMOKERS		
AGE - years	MDA (nM7 ml)	NO (µM/ml)	AGE Years	MDA (nM/ml)	NO (µM/ml)	AGE years	MDA (nM/ml)	NO (µM/ml)
30	2.9	83.5	31	2.0	80.5	28	2.1	69.5
44	4.1	93.0	40	3.0	73.0	45	3.0	73.0
34	3.8	88.0	49	3.8	74.0	40	2.6	70.0
28	3.8	78.7	33	2.1	78.7	26	2.4	68.7
29	2.9	80.3	32	2.6	80.3	40	2.4	73.3
40	3.8	99.0	42	3.4	90.0	33	1.9	76.0
50	4.9	107.3	45	4.1	77.3	53	3.3	79.3
35	3.8	96.0	35	3.2	74.0	34	2.8	78.0
41	4.5	91.5	43	3.3	95.5	33	2.0	76.5
43	4.1	99.0	32	3.1	81.0	45	2.8	76.0
Arithmetic average			Arithmetic average			Arithmetic average		
38.0			37.8			37.7		

Table 2- MDA concentration ,(nM/ml), for the Age correlation in Group Ligth smokers vs Control

Light Smokers Group		Control Group	
Age years	MDA	AGE years	MDA
40	3.0	45	3.0
49	3.8	40	2.6
42	3.4	40	2.4
41	4.1	53	3.3
43	3.3	45	2.8

Table 3: The concentration values of Mucins in the three groups

SMOKERS CIGARETTES ≥ 20 /day		SMOKERS CIGARETTES ≤ 5 / day		NO SMOKERS	
AGE years	MUCINS mg/dL	AGE years	MUCINS mg/dL	AGE Years	MUCINS mg/dL
30	37.0	31	33.0	28	33.
44	35.0	40	33.8	45	33
34	35.9	49	33.9	40	34
28	33.0	33	32.9	26	33.8
29	34.0	32	33	39	33.8
40	33.0	42	32.2	33	33.0
50	33.0	41	32.6	53	33.2
35	33.0	35	31.7	34	32.2
41	35.0	43	32.9	33	32.6
43	35.0	32	31.2	45	32.4

RESULTS Table 1

1-MDA in Smokers ≥ 20 cigarettes vs Smokers ≤ 5 cigarettes

The U-value is 23. The critical value of U at $p < .05$ is 23. Therefore, the result is significant at $p < .05$. The Z-Score is -2.00321. The p-value is .0455. The result is significant at $p < .05$.

2- MDA Smokers $\leq$ 5 cigarettes vs No Smokers

. The U-value is 24.5. The critical value of U at $p < .05$ is 23. Therefore, the result is not significant at $p < .05$. The Z-Score is 1.88982. The p-value is .05876. The result is not significant at $p < .05$.

3- NO in Smokers $\leq$ 5 cigarettes vs no Smokers

The U-value is 19.5. The critical value of U at $p < .05$ is 23. Therefore, the result is significant at $p < .05$.The Z-Score is 2.26779. The p-value is .0232. The result is significant at $p < .05$.

4-NO in Smokers $\geq$ 20 cigarettes vs Smokers $\leq$ 5 cigarettes

The U-value is 16. The critical value of U at $p < .05$ is 23. Therefore, the result is significant at $p < .05$.The Z-Score is 2.53236. The p-value is .0114. The result is significant at $p < .05$.

Results table 2

The U-value is 2. The critical value of U at $p < .05$ is 2. Therefore, the result is significant at $p < .05$. .The Z-Score is 2.08893. The p-value is .03662. The result is significant at $p < .05$

1-Pearson Correlation Coefficient Calculator for MDA /AGE in Light Smokers Group

The value of R is 0.3597. Although technically a positive correlation, the relationship between your variables is weak (nb. the nearer the value is to zero, the weaker the relationship).The value of R2, the coefficient of determination, is 0.1294

2-Pearson Correlation Coefficient Calculator for MDA /AGE in Control Group

The value of R is 0.9472. This is a strong positive correlation, which means that high X variable scores go with high Y variable scores (and vice versa).The value of R2, the coefficient of determination, is 0.8972

Results Table 3

1- The concentration values of Mucins in Smokers cigarettes ≥ 20 /day, vs Smoker cigarettes ≤ 5 / day

The U-value is 12. The critical value of U at $p < .05$ is 23. Therefore, the result is significant at $p < .05$.The Z-Score is 2.83473. The p-value is .00466. The result is significant at $p < .05$

2- The concentration values of Mucins in Smokers cigarettes ≤ 5/day, vs Control group

The U-value is 35. The critical value of U at $p < .05$ is 23. Therefore, the result is not significant at $p < .05$. .The Z-Score is -1.0961. The p-value is .27134. The result is not significant at $p < .05$.

The secreted salivary mucins are glycoproteins, which make large complexes with amylase, proline-rich proteins, statherin,

histatin, and other proteins, and form the first line of epithelial protection earlier studies, show that free radicals degrade proteins (10) , with structural changes in their most important fractions, sugar and protein moieties. 11. In addition, surface-exposed cysteine residues of proteins are particularly sensitive to oxidation by almost all forms of reactive oxygen species.The activation in a process of hypersecretion of salivary mucins, induced also by the nicotine, is the last most important result, that works, with a mechanism that is still not clear, even though probably (12) linked with electrostatic and hydrophobic interactions. In addition, to this process ,also the pollutants present in the gaseous and corpuscular phase, to give the activation in an inflammatory process with an excessive production of salivary mucins This not correct production changes the rheological of the saliva ,with an increase of its viscosity ,and consequent partial lack of adhesion of the same ,to oral epithelium Same effect, even if with different metabolic pathways, occurs for an uncontrolled production of nitric oxide :in this case , the effects of the two phenomena can added ,as showed by the results obtained in this study. Heavy Smokers, compared to Light Smokers, are in fact exposed to a greater production of nitric oxide (NO), which can react with O2 to form hydroxyl radicals (OH) and H2O,or with superoxide anions generating highly toxic compounds and principally the peroxynitrite anion, which is responsible for many of its cytotoxic effects,(13), In these conditions, in the presence of an excess of NO, there is a notable lipid peroxidation with formation of MDA, at the expense of the cells, of the first respiratory tract, principally the mouth, that are not sufficiently protected by saliva . Furthermore it knows that ,also in low concentrations, the nicotine, (14), can deepen the viscosity of the mucus, as it happens for example in the pulmonary alveoli, phenomenon, that in the mouth , involves the onset of that feeling, so-called "dry mouth "or a xerostomia,

which is nothing but a clear signal of poor protection of the mouth. In this study, in the "light smokers" group, compared to the control group, there is a statistically significant increase in the concentration of NO, the p-value is 0.0232, but not for MDA and Mucins where the differences of these concentrations are practically negligible: the p-value is 0.5876. And p-value is 0.27134, respectively. These result are relative to the low concentration of salivary MDA, and to the non-alteration of the mucinous layer, found at light smokers may be explained to consider that saliva, on which the test is performed, is present in the anatomical structure directly most affected by the biochemical conditions, respect than the whole organism. The saliva contains mucins, glycosylated proteins, that if not altered and/or decreased ensure perfect protection of the oral cavity Smoking is one of the elements that may deteriorate their structure but in the case of "light smokers intake of it is diluted, over many hours and it can be assumed that the amount of salivary mucins, is little altered so that the physiological turnover of them quickly re-establish the conditions for a 'good oral lubrication, regardless of whether NO can generate MDA,(15). These results, may pose the problem if the same is scientifically acceptable; it 's impossible, in fact, scientifically accept the possibility that there are no "threshold limit values" (doses "safe)" for carcinogens, values underneath which is promoted not the process of carcinogenesis: it is instead correct to affirm that even a single cigarette can promote the cancer. In reality, a process of carcinogenesis can occur even after many years, after the possible genetic damage; the latency time changes depending on many factors, such as the type of promoter, the genetic individuality, the different immune system, and many other parameters. So, considering valid, the results of this study, it may very well happen, that such eventualities, may be manifested, also for every single Light Smoker, also many years far.A first data, to support this possible eventuality, it

shown, by analyzing the results expressed in table 2, where they are compared, the MDA values in two subgroup Light smokers vs Control; both consisting of people over 40 years. The two subgroups were selected to analyze the actual contribution to the increase in MDA, which may already be seen from the examination of the values expressed in table 1, due to the increase in age. The results see Table 2 clearly indicates that this increase is greater and statistically significant. : the p-value is 0.03662., and also the Pearson correlation index in light Smokers group is considerably low, R is 0.3597, showing that the increase of MDA concentration is independent to the age .but since when people have started smoking. On the contrary in the control group, there is a good correlation index between the increase in the concentration of MDA and that of age, as postulated by the theory of Harman (16).

Or mitochondrial hypothesis expressed already in 1956. The mitochondrial dysfunction has long been considered a major cause of aging and age-related diseases.

Mitochondrial free radical theory of aging postulate that somatic mitochondrial DNA mutations that accumulate on the life cause excessive production of reactive oxygen species that damage macromolecules and affect the function of cells and tissues. In fact, studies have shown that the oxidative capacity maximum decreases with age, while increases reactive oxygen species production. Smoking many cigarettes /day causes the excessive formation of free radicals, with a real risk of cancer promotion. In much shorter time there is an activation of a process of hypersecretion of salivary mucins, induced by the free radicals and by the nicotine, with damage of oral defenses Smoking a few cigarettes can actually induce less damage to health, with lower production of MDA, but the chronicity of consumption does not risk the risk of cancer formation, nor any other damage to health

-THE E-CIGARETTES SMOKING

The electronic cigarette is a device designed to simulate and replace, both in use and in appearance, the traditional systems for smoking tobacco derivatives .Electronic cigarettes may or may not contain a variable measure of nicotine together with a chemical mixture typically composed of water, propylene glycol, glycerol, and possible flavoring compounds The mixture passes from the liquid to the vapor state, by an atomizer ,and in this process there is no combustion and no toxic residues such as tar are present. , and polycyclic hydrocarbons In spite of this, the benefits and possible risks associated with these devices are much discussed in terms of safety, efficacy and acceptable quality. Due to the relative novelty of technology, tobacco laws and drug delivery policies, public health surveys and the laws governing the sale and use of electronic cigarettes are now the subjects of heated debate in many countries, including Italy. The scientific knowledge on the efficacy of the electronic cigarette as a quit for smoking cessation or on its potentially harmful effects is still incomplete. It is even clear that e-cigarettes can represent a substitute for the aspects related to smoking gestures, and help the smoker to contain the consumer of cigarettes, but it is difficult, from the literature data, to check this single aspect, like an aspect behavioral, because, the use of e-cigarettes, could even be considered a way of administration of illicit substances (1). The aspect related to the impact of the use of e-cigarettes on human health is much more complex, not only because some liquids may contain more or fewer high concentrations of pharmaceutical nicotine, but also because very little is known about the toxic aspects of various compounds present in the

formulations currently marketed. The e-cig may contain nicotine in a variable amount, (typically, between 2 and 20 mg), in a mixture consisting of water, propylene glycol, glycerol and other substances, including flavoring agents. Some models contain no nicotine, but only a liquid, after vapor flavored with many compounds, of which many investigations are underway to set up the renal toxicity. In fact, in the literature, only on PUBMED, using as a search key: "e-cigarettes health review", identifies 240 items The main context of our research, however, intends to analyze only the problem related to the possible formation of free radicals during the use of e-cigarettes, regardless of whether the liquid has about nicotine The formation of these compounds is important, for their very dangerous consequences, even in the long-term on human health In this context it was very interesting to analyze, the very recent review, year 2018, (2), performed by the "US National Academies of Sciences, Engineering and Medicine Division" , on the consequences on Public Health of E-Cigarettes. In this review, there are not many studies, (3), (4), (5), (6), (7), which address their real impact on health human, linked to the problem of free radicals formation, but in any case we can highlight some important conclusions about the same problem

1-There is enough findings that highlight how the components of e-cigarette aerosols can promote the formation of reactive oxygen species / oxidative stress. In any case, all studies show that reactive oxygen species and oxidative stress induction is generally lower in the e-cigarettes. Than from combustible tobacco cigarette smoke.

2 There is real evidence that some chemicals present in e-cigarette aerosols (e.g., formaldehyde, acrolein) are capable of causing DNA damage and mutagenesis. This supports the biological plausibility that could increase the risk of cancer and adverse reproductive outcomes.

3 There is no evidence available to or not e-cigarette using may cause intermediate cancer endpoints in humans.

A 4-Another important conclusion is that there been evidence suggesting that nicotine and non-nicotine-containing e-cigarette aerosol can adversely affect cell viability and causes cell damage of use e-cigarettes to contain fewer toxic compounds compared with combustible tobacco. The most important conclusion of this review, however, remains the one that, e-cigarettes, the potential risks show less biological activity in a number of in vitro, animal, and human systems; and that apparatus this could be used as a cessation aid to smokers, for who use e-cigarettes exclusively.

Examining these conclusions, and other documents, we can see that in the e-cigarettes there is a much production of free radicals. In reality, it is not enough to clear, what is the main source of production of free radicals, and this fact becomes one of the two main purposes of this study,Another important issue, which according to our opinion, deserves a deepening, and that is the other goal of this research, is that related to damage of the oral cavity, and more precisely to the possible increasing of viscosity of salivary mucous, for the change in the of
salivary mucins concentration that are the main constituent of the mucosa that protects the oral cavity itself

5-Much of the research on e-cigarettes suffers from methodological flaws, and many areas have not yet been

researched. ; The literature anyway suggests that, while there are risks associated with e-cigarettes

.For this study, three groups of seventeen people each were selected, all males, aged between thirty-five and, forty years...The first two groups are made up of people who have quit smoking and have been using e-cigarettes for about a year.The first group (e-Nicotine), smokes e-cigarettes, using liquids commercially defined "cloud chasing" composed of about 70% of Vegetal Glycerine and 30% of Propylene glycol, with 1% aromatizing and with or no pharmaceutical nicotine, (Old Tobacco, Vaporart) equivalent to 10 mg, per 10 ml bottle, while the second, (e-Vapor) uses the same type of liquid, but with zero mg of nicotine The consumption of a 10 ml bottle was expected in two weeks; the third group is the non-smoking group or control group (CG). All the people selected for this study have no cardiovascular disease, oral diseases or diabetes, they don't consume vitamins or antioxidant supplements type of e-cigarettes used is different from each other by brand, but not for the combustion device, i.e. the battery with a voltage of 75 watts. After the bottle has been consumed, MDA concentration, and Total Salivary Mucins was determined in the saliva of people.The salivary concentration of malondialdehyde is determinate with Thiobarbituric acid test. The concentration of the total salivary mucins was determined by the Alcian Blue method, briefly described below. A fraction of the saliva samples diluted (1:10) is incubated for 30 min in a 1% solution of Alcian blue in 50 mM sodium acetate buffer with25 mM of $MgCl_2$ to pH 5.8 under constant stirring at room temperature. The mucin-dye complex is shown with the addition of a dilute solution 1: 2,

Aerosol OT (Sigma Chemical Co., St Louis, MO, USA), bis (2-ethylhexyl) sulfosuccinate, and sodium salt concentration, was determined spectrophotometrically at 605 nm Below, there are the results of the analyzes about the salivary concentration of MDA (in nM/ml), in the three groups,, while and those related to the concentration of the total Mucins are in table 2 The results were statistically analyzed with the Mann-Whitney test: the Mann-Whitney the test is a nonparametric test that allows two groups or conditions to be compared without making the assumptions that are normally distributed

Table 1: salivary MDA concentration in three groups

e- nicotine age years	MDA nM/ml	e-vapor age years	MDA nM/ml	No smokers Age years	MDA Nm/ml
35	3.0	35	2.9	35	2.1
35	2.9	35	2.9	35	2.7
35	2.8	35	2.8	35	2.4
35	2.8	35	2.6	35	2.6
35	2.8	35	2.9	35	2.5
36	2.9	36	2.9	35	2.7
36	2.8	36	2.8	36	2.7
37	3.0	37	2.8	37	3.3
37	3.0	37	3.2	37	2.0
38	3.7	37	3.6	38	2.7
38	3.0	38	2.9	38	2.8
39	3.1	39	3.2	39	2.9
40	3.5	40	3.4	39	3.0
40	3.5	40	3.4	40	3.4
40	3.4	40	3.4	40	2.9
40	3.1	40	3.4	40	2.9
40	4.1	40	3.7	40	3.3

Table 2 : Total Mucins Concentration mg/dL in three groups

AGE	E NICOTINE	AGE	E VAPOR	AGE	CONTROL
years	Salivary mucins mg/dL		Salivary mucins mg/dL		Salivary mucins mg/dL
35	36.1	35	33.0	35	33.
35	34.0	35	33.8	35	33
35	34.8	35	33.9	35	34
35	35.3	35	32.9	35	33.8
35	35.5	35	33	35	33.8
36	33.0	36	32.2	36	33.0
36	31.0	36	32.6	36	33.2
37	33.0	37	31.7	37	32.2
37	35.4	37	31.9	37	32.6
38	35.0	38	31.2	38	32.4
38	34.0	38	30.5	38	32.1
39	32.5	39	32.9	39	31.4
40	35.3	40	30.	40	31
40	30.4	40	30.5	40	31
40	30.0	40	30.8	40	31.5
40	33.0	40	32.7	40	31.7
40	35.9	40	32.2	40	31

Results for table 1 :

Media values MDA nM/ml

e-nicotine =3.14 ; e-vap = 3.11

1-MDA e-Nicotine - vs e Vapor .The U-value is 135.5. The critical value of U at $p < .05$ is 87. Therefore, the result is not significant at $p < .05$..The Z-Score is 0.29277. The p-value is. 0. 77182. The result is not significant at $p < .05$.

2- MDA e-nicotine – vs Control .The U-value is 61.5. The critical value of U at $p < .05$ is 87. Therefore, the result is

significant at p < .05..The Z-Score is 2.84159. The p-value is .00452. The result is significant at p < .05.

.3--MDA e-Vapor vs Control .The U-value is 70.5. The critical value of U at p < .05 is 87. Therefore, the result is significant at p < .05.. The Z-Score is 2.5316. The p-value is .0114. The result is significant at p < .05

Results for table 2

1-Mucins e nicotine - vs e vapor. .The U-value is 62.5. The critical value of U at p < .05 is 87. Therefore, the result is significant at p < .05..The Z-Score is 2.80715. The p-value is .00496. The result is significant at p < .05.

2-Mucins e nicotine - vs control .The value of U is 26.5.The Z-Score is -4.04712. The p-value is < .00001. The result is significant at p < .05

3-Mucins e vapour - vs control : The U-value is 123. The critical value of U at p < .05 is 87. Therefore, the result is not significant at p < .05. The Z-Score is -0.72331. The p-value is 0 .47152. The result is not significant at p <

This study substantially confirms the results obtained in other research. where the users of electronic cigarettes can be exposed to highly reactive ROS. The heating element of the e-cigarette forms a large amount of ROS, during the vaporization phase This study employe a liquid that may contain nicotine, or only glycerine, propylene glycol with the more of the flavoring compound as OLD TOBACCO. Both liquids form

MDA macromolecules with concentrations, almost identical: p-value = 0.77182. A recent study, (4), with the EPR technique, using the spin-trap phenyl-N-tert-butyl nitrone (PBN), it shows that .the aerosols phase generates free radicals from both propylene glycol and glycerol, but there is the PBN radical adducts formation., so to show their different chemical origins In another study, (7) the E-cig vapor has 7x1011 free radicals for puff and elicits to significant increase in oxidative stress. This amount of free radicals, it is nonetheless representing a real number that could be toxic to cells. This concentration is surprising to the fact that E-cig vapor does not contain any combustion products. This process, anyway, in some conditions produces radiation, and furthermore, the battery output voltage generates other toxic chemicals. The age of the heating element influences this process (6).but the oxidant compounds can also derive from the lithium-ion battery of a device, similar to the one used in fuel tobacco cigarette filters, but the most important key point for the free radicals production is due to the chemical reactivity of as glycerol and propylene glycol (8),According to other authors, (9), (10), even the flavoring elements, form the free radicals in some conditions, while other compounds of the mixture, can even perform an antioxidant function. The secreted salivary mucins, which make large complexes with amylase ,proline-rich proteins, statherin , histatin, and other proteins, form the first line of epithelial protection Previous reports show that free radicals degrade proteins (11) and change the mucins in their most important fractions, sugar and protein moieties [12]. In addition, surface-exposed cysteine residues of proteins are particularly sensitive to oxidation by almost all forms of reactive

oxygen species However, the production of free radicals in both group of e-cigarettes is practically identical and greater than the control, This study substantially confirms the results obtained in other research. where the users of electronic cigarettes can be exposed to highly reactive ROS. The heating element of the e-cigarette and after the vaporization of the e-liquid form a large amount of ROS. Our liquid may be containing nicotine, or only glycerine, propylene glycol with the addition of the aromatizing compound as OLD TOBACCO. Both liquids leading to the formation how of MDA with concentrations, almost identical: p-value = 0.77182. In a recent study, (4), with the EPR technique, using the spin-trap phenyl-N-tert-butylnitrone (PBN), it was showed that .the aerosols phase generates free radicals from both propylene glycol and glycerol, but there is the PBN radical adducts formation., so to show their different chemical origins In another study, (7) the E-cig vapor has 7x1011 free radicals for puff and elicits to significant increase in oxidative stress. This amount of free radicals, it is nonetheless representing a real number that could be toxic to cells. This concentration is surprising to the fact that E-cig vapor does not contain any combustion products. iI this process, anyway, is possible to produce radiation, and indeed, the battery output voltage can play a role in the generation of other toxic chemicals. The age of the heating element influences this process (6).but the oxidant compounds can also derive from the lithium-ion battery of a device, similar to the one used in fuel tobacco cigarette filters, but the most important key point for the free radicals production is due to the chemical reactivity of as glycerol and propylene glycol (8),According to other authors, (9), (10), even the

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can contribute to the formation of diseases affecting the oral cavity. In this study, there is the effective demonstration that there are increased salivary mucins, and this causes a greater viscosity of salivary mucus, with a consequent lesser defense of the oral cavity itself. In any case, given the complexity and importance of this topic, more and more research on short- and long- e-cigarettes, as well as their effects on the initiation and cessation of combustible tobacco product use, will bring clarity to the question of whether e-cigarettes will prove to reduce harm or induces harm at the individual and the population levels. E-cigarette product marketplace and user population are changing; there will undoubtedly be new issues which are currently unknown and will require careful surveillance and scientific scrutiny

7-3 - LIFE'S STYLE ,FOOD'HABITS AND DRUGS

The compounds present in food can influence the structure of the components of all salivary proteins as happens when the people consume the betel, that has a notable power to promote oral tumors in large portions of Southeast Asia. Other compounds such as vegetable lectins, found in seeds, nuts, potatoes and beans, bind tthe mucins and O-glycans. In any case, the most important process of precipitation of salivary proteins is the direct result of the presence in many dietary products of an important group of chemical compounds, of vegetable origin, the polyphenols. It is clearly demonstrated that polyphenols are present in high quantities, in betel (up to 30% by weight) and in a range of consumer spices such as paprika or green tea. .polyphenols can interact with the various salivary components,

with the possible result of precipitation of the proteins present in it. Recently, many studies have analyzed,, the effects of polyphenols , such as those contained in green tea, paprika and, in betel, on the main salivary mucins, MUC5B (gel forming), and MUC7 (non-gelling agent), that is, on the main factors constituting the mucous layer, the protective barrier of the oral cavity.

7-3-1-BETEL

The origin of the chewing of the habit, the seed of the Areca catechu (Areca or Betel is the same interchangeably), is typical of south-east Asia, probably Malaysia, where the etymology of the name of the province of Penang indicates exactly "areca-nut". The ancient oriental writers have left evidence about the practice of chewing Betel in China and India is already well established more than two thousand years.Other studie reported (32). a series of benefical effects of this plant, or rather of its seeds,,if applied in Ayurvedic medicine (33),:such as an antimilintic drug, appetite stimulant, breath refresher, diuretic, laxative, nervous system tonic. The areca-nut (Areca catechu fruit or nut) is commonly consumed by people from Asia, (see photo 3), and Asian communities migrated to Europe and North America. In the latter case, consumption seems also related to the religious aspect. The Indian communities in the UK are the main consumers of betel (up to 80% of adolescents and adults in the

community), while the Sikh community consumes less (50% of adolescents and adults); finally, consumption is unknown among Indians and Muslims in Pakistan; where generally these people chew this seed after divided the same into thin portions, combined with a variety of other natural products (including tobacco) and wrapped in the leaf of pepper (betel) (Piper betel), photo

4,5FOTO 3 - Un mercato tipico dove vendono il betel

FOTO 4 - The betel preparation with lime

FOTO 5 –Betel with lime and tobacco

The mouth and teeth of the subjects who chewed the betel continuously, together with or not flakes of slaked lime quickly takes on a color ranging from red to brown see photo 6

FOTO 6 -Gli effetti visibili del betel sulle mucosa orali

These subjects generally show numbness of the tongue and dry mouth (marked xerostomia). New consumers always feel a marked sensation in the throat and esophagus, (34), (35), (36). of constriction. About 0.5%-2 % of betel users develop chronic oral sub-mucosal fibrosis, a disease characterized by epithelial atrophy and accumulation of collagen in the oral mucosa. Catechins and tannins present in betel seem are the responsible for this adverse effect, acting by stabilizing the collagen and making it resistant to both human and bacterial collagenase, becoming the oral mucosa a non-elastic, porous layer easily penetrated by many small molecules. 15% of these subjects have later developed tissue abnormalities (atypical cells), while in 12% of cases the onset of cancer of squamous cells. In this case we can really talk about Betel emergency, especially when this involves risks in young people. The incidence in this group, such as, has increased in Taiwan from 550 to 6,000 cases per year, and six times is the increase in mortality. Taiwan is the world's

leading oral cancer country for the age range 30-54, and the IARC classified betel quid (BQ), (38), chewed with or without tobacco, as a Group I human carcinogen in 2004. People are more interested in this phenomenon, are those in the lower socio-economic class and prefer chewing betel with smoking and drinking alcohol in combination, thus worsening health. Despite this, many people in the world do not see the danger of BQ alone, and understand the synergistic interaction, even more negative between BQ and smoking.The result is that they are also consumed by the 30-40 combined oral associations every day, 15-20 cigarettes and 15 BQ, by almost 2 million adults, and this is proof of a disaster for public health, in the countries of sub-east Asia. Every year a total of 6,000 new cases of oral cancer and 18,000 deaths occur as a result of chewing BQ. The risk of oral cancer with respect to the time life expectancy of a chewer is lower by 42%. Oral cancer has grown rapidly in the last two decades in Taiwan, with a ten-fold increase of one in two chewers. This figure is almost 40 times greater than the average adult life risk in the US, which is 1.08%. While tobacco control is in full swing in Taiwan, the government's attitude is still today, far from aggressive towards controlling BQ, recognizing and responding to the large associations of interest groups related to BQ, namely growers, distributors, retailers and consumers. The scientific literature has long been studying, practically all the chemical components of betel to try to understand their role in the mechanisms of carcinogenesis of the lips and oral cavity. The metabolites of arecoline, "nitrosamine type" resulting from the degradation of this compound with oxygen or "active oxygen" formed in the reaction between the basic substances of

betel and flavonoid type compounds such as catechins present in good quantities, (5-10%) in the seed of Areca Catechu, are the main indications of this process. It 's extremely interesting, in this regard is the monograph IARC (38,where Areca nut ingredients induce the formation of ROS and DNA adducts(6) ,(7) ,with free radicals formation (8) ,and a recent study confirm the a great ROS increasing in presence of areca nut extract in OSCC cells suggesting the key rule of areca nut, in oral cancer promoting. Our hypothesis ,and aim of this study , is show , that the cancer process promoted by areca nut or more common by betel chewing , can happen ,only after that, this risk factor, deprives the oral cavity of its protective layer, precipitating the proteins, and mucins fractions, and ,that this effect probably continues for a while, also after the consume of the betel .Description of in vitro experimental and samples collection; in taking saliva samples during chewing, occur that in each of the same, an amount of the areca-nut ,is certainly subtracted , minimizing its real effect .For this problem ,the operative conditions for the evaluating, the effects of areca-nut chewing on the saliva ,are that ,a single volunteer,(male 35 years old) with negative anamnesis for, oral, cardiac, and diabetes, diseases, provided for saliva samples, for a first study in vitro .The saliva samples, are collected at different stages, in two days, for a total volume of 480 ml, which was then divided into another twenty-four samples . In thirteen, of these , increasing amounts of areca nut in powder will be added, while in the other, the pure commercial tannins . After of this first study , end of a chewing of areca –nut , in two volunteers one of whom was a regular consumer of betel ,were analyzed the concentrations of mucins ,

on six samples of saliva taken at half hour intervals., The concentration of proteins and mucins will be also determined in initial sample of saliva .After the addition of areca nut in powder, and of pure vine tannin, the saliva samples after being briefly mechanically stirred are left to settle, for about two minutes; subsequently, in an aliquot of the supernatant, the concentrations of mucins and total proteins are determined, as described below. Total salivary mucin concentration was determined using the Alcian blue method, briefly described here. The samples of saliva diluted (1:10) were incubated for 30 min in a 1%,solution of Alcian blue in 50 mM sodium acetate buffer with 25 mM Cl2Mg, pH 5.8 under constant agitation at room temperature .After following the dissociation of mucin-dye complexes ,are stripped , with the addition of a 1: 2 dilution of Aerosol OT (Sigma Chemical Co, St Louis, MO, USA) in distilled water, mixing short and ultrasound the extracted samples ethyl ether and after centrifugation the concentration of dye was determined spectrophotometrically at 605 nm in the aqueous phase

- Commercial Tannin : CX -FERVOUR PLUS , produced by Corimplex ,(Italy)

-Areca Nut: commercialized by Zamnesia, (India), pack of 80 gr, (gr 5 a single seed approximately)..A single nut was then reduced to powder, for subsequent experiments on saliva samples and for the determination of tannin concentration

For estimation of tannin in areca nut, is used tannic acid as a standard. Tannin was determined using Folin-Denis reagent , with , spectrophotometric estimation .Tannic acid was taken as a

standard and values were expressed as tannic acid per 100 ml of the sample.

Reagents required:

(a) Folin-Denis reagent: In 750 ml of water, 100 g of sodium tungstate (Na2WO4.2H2O), 20 g of phosphomolybdic acid and 50 ml of 85% phosphoric acid (H3 PO4), are dissolved. The mixture is refluxed for 2 hr, cool to 25 C and dilute to 1000 ml with water.

(b) Saturated sodium carbonate solution: To 100 ml of water, 35 g of anhydrous sodium carbonate is added, dissolved at 70-80 o C and cooled overnight. Clear liquid is decanted before the use.

(c) Tannic acid standard solution: 100 mg of tannic acid is dissolved in 1 litre of water. Fresh solution is prepared for each determination (1 ml = 0.1 mg of tannic acid

The results are statistically analyzed with :Mann-Whitney U Test Calculator and Spearman's Rho Calculator. The Mann-Whitney U test is a nonparametric test that allows two groups or conditions or treatments to be compared without making the assumption that values are normally distributed.Spearman's Rho is a non-parametric test used to measure the strength of association between two variables, where the value r = 1 means a perfect positive correlation and the value r = -1 means a perfect negative correlation, with the condition that the association must be monotonic (i.e.variables increase in value together, or one increases while the other decreases)

b-Results ,Discussion ,Conclusions

In Table 1, are summarized the results on genetic influence in oral cancer formation. by an elaboration review of the recent literature .The analyzes in the sample of the initial saliva, of the proteins and of the total mucins, have determined the following results:

Total Proteins : 1.75gr /dL ; Total Mucins : 0.30 gr /dL ; The Tannin concentration in Areca Nut is resulted in 5% gr/gr

After the addition of pure Vine Tannin ,and powdered Areca nut into the saliva samples are, below showed ,the results of these experiments .In Table 2 are showed the results of proteins and mucins precipitation for the addition of Areca Nut powdered,; in table 3 ,the results of proteins and mucins precipitation for the addition of pure vine tannin .while in table 4 ,and in table 5 ,are reported the salivary mucins concentrations after the areca nut chewing, determinate every half hour, up to the moment, in which, these become equal to the value of the control

Table 2 –Results of proteins and mucins precipitation for the addition of Areca Nut powdered

Saliva (ml)	Areca (mg)	Tannin by Areca (mg)	Surnatant Proteins g/dL	Proteins precipitation (%)	Surnatant Mucins g/dL	Mucins precipitation
20	50	2.5	1.56	11	0.26	13
20	100	5	1.47	16	0.25	16
20	150	7.5	1.42	19	0.24	18
20	200	10	1.35	23	0.23	24
20	250	12.5	1.28	27	0.22	28
20	300	15	1.19	32	0.20	32

20	340	17.0	1.12	36	0.19	36
20	380	19	1.07	39	0.18	40
20	420	21	1.02	42	0.17	42
20	460	23	0.96	45	0.16	46
20	500	25	0.93	47	0.15	49
20	**750**	**38**	**0.36**	**80**	**0.06**	**79**
20	**1000**	**50**	**0.01**	**100**	**0.01**	**100**

Table 3 - results of proteins and mucins precipitation for the addition of pure vine tannin

Vine Tannin mg	**Surnatant Proteins g /dL**	**Proteins % precipitation**	**Surnatant Mucins g/dL**	**Mucins % precipitation**	**Saliva (ml)**
2.5	1.48	22	0.23	23	20
5	1.37	30	0.20	35	20
7.5	1.20	40	0.18	41	20
10	1.1	51	0.15	51	20
12.5	0.90	55	0.14	55	20
15	0.7	62	0.12	61	20
17.0	0.61	70	0.08	73	20
19	0.41	80	0.06	81	20
21	0.19	91	0.02	95	20
23	0.80	95	0.01	99	20
25	0.01	100	0.00	100	20

Table 4- Salivary Mucin Concentration , in the three hours after the chewing of areca –nut in first volunteer

Hour after chewing	Mucins *Control g/dL Areca	
Half hour	0.35	0.30
first hour	0.35	0.30
first hour and a half	0.34	0.30
second hour	0.32	0.30
second hour and a half	0.31	0.30
Third hour	0.30	0.30

Table 5- Salivary Mucin Concentration , in the three hours after the chewing of areca –nut in regular consumer of betel

Hour after chewing	Mucins * g/dL Areca	Control
Half hour	0.24	0.30
first hour	0.24	0.30
first hour and a half	0.25	0.30
second hour	0.24	0.30
second hour and a half	0.25	0.30
Third hour	0.26	0.30

Statistical Analysis

- Comparison for the precipitation of Proteins and Mucins, between Areca Nut powder and pure Vine Tannin :

Mann-Whitney U Test Calculator

1- RESULTS : Total salivary Proteins Precipitation Comparison ,for the first eleven score

Areca Nut vs Vine Tannin

.The U-value is 17. The critical value of U at $p < .05$ is 30. Therefore, the result is significant at $p < .05$.

The Z-Score is -2.8236. The p-value is .0048. The result is significant at $p < .05$.

2-RESULTS :Total salivary Mucins Precipitation Comparison for the first eleven score :

Areca Nut vs Vine Tannin

The U-value is 16. The critical value of U at $p < .05$ is 30. Therefore, the result is significant at $p < .05$.

The Z-Score is -2.88926. The p-value is .00386. The result is significant at $p < .05$.

3- RESULTS : Total salivary Proteins Precipitation by Areca Nut vs Mucins

The U-value is 58. The critical value of U at $p < .05$ is 30. Therefore, the result is not significant at $p < .05$..The Z-Score is -0.13133. The p-value is .89656. The result is not significant at $p < .05$.

4-RESULTS :Total salivary Proteins Precipitation by Vine Pure Tannin ,vs Mucins

The U-value is 57. The critical value of U at $p < .05$ is 30. Therefore, the result is not significant at $p < .05$...The Z-Score is

-0.197. The p-value is .84148. The result is not significant at $p < .05$

Spearman's Rho Calculator

Proteins Areca Nut / Mucins Areca Nut

The value of R, is 1 and the two-tailed value of P, is 0. By normal standards, the association between the two variables would be considered statistically significant

Proteins Vine Tannin / Mucins Vine Tannin

The value of R is: 0.90909 and the two-tailed value of P, is: 0.00011. By normal standards, the association between the two variables would be considered statistically significant

5- RESULTS- Salivary Mucin Concentration Mucins after chewing in first volunteer, see table 4

The U-value is 3. The critical value of U at $p < .05$ is 5. Therefore, the result is significant at $p < .05$...The z-score is 2.32186. The p-value is .02034. The result is significant at $p < .05$

6- **RESULTS**- Salivary Mucin Concentration, after chewing in second volunteer, see table 5

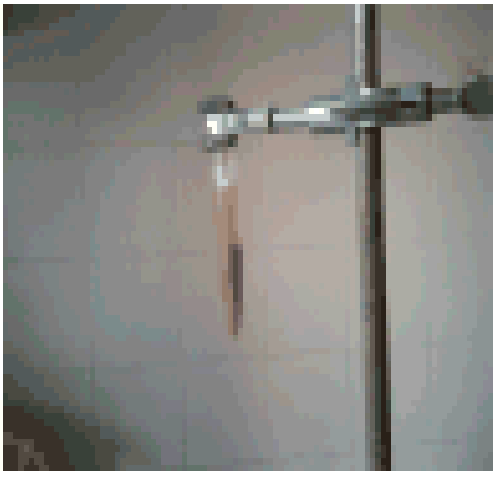
The U-value is 0. The critical value of U at $p < .05$ is 5. Therefore, the result is significant at $p < .05$...The z-score is -2.80224. The p-value is .00512. The result is significant at $p < .05$

This study promises to verify, if tannins decrease the layer of oral mucosa in their principal components secreted in saliva, as the proteins and mucins. The amount(20 ml of saliva) used for

this purpose, represents the theoretical quantity, which should be produced during the chewing time of betel, about 30 minutes, so as to understand what is the probable quantity of proteins and mucins precipitated, from increasing amounts of tannins The results (tables 2,3) show ,as the total salivary Proteins Precipitation in Vine Tannin is greater vs Areca Nut and the difference is statistically significant (p-value is .0048).The same statistical result is obtained for the amounts of total salivary Mucins precipitated by vine tannin that results greater than areca nut:(p-value; .00386). Another important result shows how, with the addition of 50 mg of tannin by vine tannin, or 150 mg by areca-nut, the total precipitation
of proteins and mucins is obtained. In both tests is not statistical evidence ($p \geq 0.05$) for the precipitation of proteins vs mucins These results are also confirmed by the statistical analysis with Spearman's Rho Calculator where the value of R for tannins , by vine vs mucins is 1 and 0.909 for tannin areca-nut vs mucins.. A possible explanation of these results may be that, in the powdered areca nut, there is a considerable concentration of fibres, that binds the tannin components, making the same less soluble; this phenomenon is clearly represented in the two photos ,which depict the precipitation of the protein fraction and mucins, using areca nut (image1), or vine tannin (image 2). In any case, even using powdered areca-nut, the total precipitation of the mucins and salivary proteins is with the use of 1 gr of the same equivalent to use 50 mg of tannins, a concentration about five times lower than that present in the chewing period of betel quid

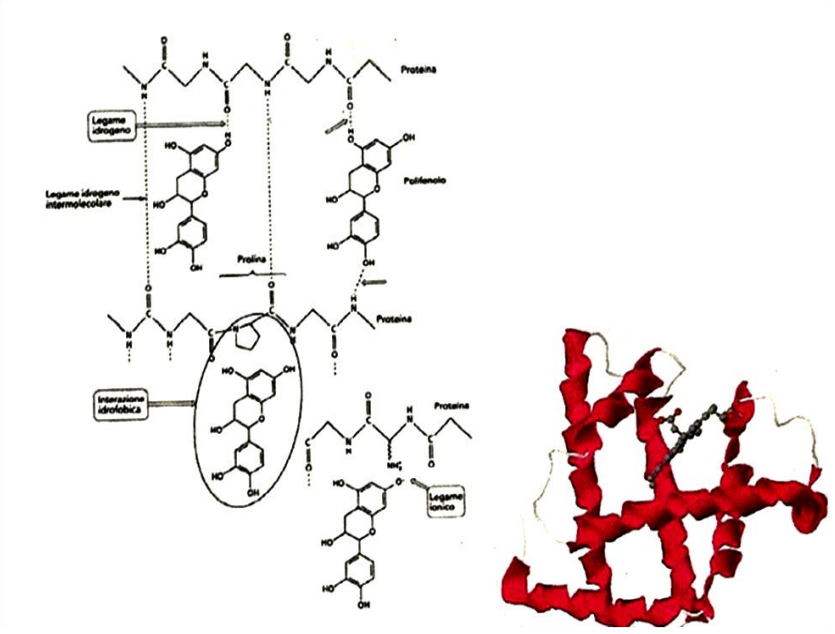
Image 1 Precipitation of the proteins and Mucins by Areca-Nut powdered

Image 2 Precipitation of the Proteins and Mucins by pure vine tannin



That is the fundamental process by which the tannins are able to plunge the whole protein fraction present in saliva, depriving the oral mucosa of his most effective defence in practice, the "seizure" of mucin by tannins is because it is very rich in the amino acid proline and this involves chemically the formation of a very stable aggregate, insoluble in water, following the formation of bonds of the OH groups present - in tannins, on the oxygen of cheto-himide bond -CO-NH- peptide proline. The model for an explanation of the possible interaction between the salivary proteins, the mucin's fraction and the polyphenols compounds is showed (image 3)

Image 3: The hypothesis on the mechanism of the proteins precipitations by the Polyphenol compounds



.In any case, both for the use of vine tannin and for what contained in the areca nut, the amount used was at most a fifth of that which naturally should be released during the chewing of betel quid, with the difference that with the use of areca nut in powder, a certain amount of the same remains in the supernatant, colouring the solution of red that becomes even more evident. As you increase the amount of areca nut (photo 6 above) this amount of tannins is deposited during the chewing on the oral mucosa deprived of the mucous layer. So the result is that the betel nut users develop a chronic oral sub mucosal fibrosis (11) a disease characterized by epithelial atrophy and collagen accumulation in the oral mucosa. Responsible for this adverse effect, in our hypothesis, due to tannins present in the seeds which act by stabilizing the collagen and making it resistant to collagenase both human and bacterial becoming the oral mucosa a non-elastic layer, porous and penetrable by many little molecules. Image 4 clearly shows the oral aspect of this situation as a result for a continue areca-nut chewing. The in vivo results of this study (tables 4, 5) show as these effects are linked to prolong over time of the chewing. In fact for a period of at least two and a half hours, from the cessation of chewing of areca-nut, the oral concentration of salivary mucins is increased in a statistically significant value($p=0.02034$) compared to control. This process of high production of mucins, in reality does not lead, over time, to a protection of the oral cavity, but to a progressive fibrosis of the epithelium, as occurs in the syndrome of Sjögreen, (12) because the hyper secretion of mucins has, as final result, the change in the rheological properties of the saliva that becomes more viscous and adheres

less to the epithelium, diminishing the mechanisms the epithelium, diminishing the mechanisms of defence of the protective layer of the oral mucosa. Contemporaneously the fibrosis of epithelium increases its porosity towards the small molecules. The fibrosis of the oral epithelium is always directly proportional to an inflammatory process and can become chronic, as it probably happens, with the persistence of the chewing of areca-nut for long periods of time. In these conditions the process of keratinisation of the epithelium, in fact progressively increases, and with it, the decreasing of the production of salivary mucins (table 5). The chronic inflammation is maintained in particular by oxidative stress in the cells and causes their malfunction. In this case, substances that continue to activate the immune system, known as cytokines, are produced, causing the non-switching off of the inflammatory response, hence the fibrosis, hyper secretion and final hypo secretion of mucins. In these conditions small molecules can be, the HPV virus, like the free radicals generated in cigarette smoking or better yet those generated by the metabolism of the areca nut during its chewing, to penetrate in oral cells and promote the cancer process .At the end of this study (image 5), we present a a diagram that illustrates the mechanisms of the carcinogenesis process based on this hypothesis. It is very important that in this situation the chemical components of tannins, or polyphenols, lead to the occurrence of a phenomenon known as astringency due to their activity in the precipitation of proteins .It is a taste sensation mediated by the chord tympani nerve, astringency arises primarily

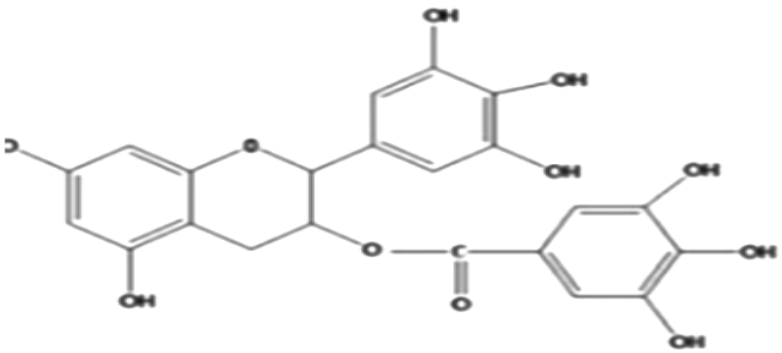
from increases in friction between mucosal surfaces
Evidence is discussed that supports the view that oral astringency results primarily from the precipitation of salivary muco-proteins, which impairs the natural lubrication of oral surfaces The ensuing rise in friction induces sensations of dryness and roughness which, although subtle at first, can grow in magnitude over repeated exposures. This phenomenon could have a fundamental importance in safeguarding the health of the oral cavity: astringency could become the Sentinel Sensation of a process "alteration of the protective oral layer", which is becoming irreversible. Whatever the hypotheses on the real nature of the carcinogenic compounds that activate the tumour process, it is clear that the same, it is allowed by the absence of the oral protective layer that has disappeared due to the precipitation of the protein and mucinous fractions. Under these conditions, i.e. under conditions of prolonged absence of the natural defences of the oral cavity, a mutation can occur for both alleles of the genes involved in the growth or suppression of carcinogenesis. This fact implies that oral cancer can also happen in non-genetically predisposed, for which it is extremely important, an effective campaign of awareness and prevention, on the use of all those compounds or foods, which alter the balance of organic defences

7-3-2-GREEN TEA

The presence of tannic compounds involves the precipitation of proteins and mucins: polyphenol molecules mainly the epigallocatechin-3-gallate (EGCG), fig 8, presents in high quantities in many plant foods is the main agent that bonds the

proteins. Salivary mucins contain MUC5B (gel forming) and MUC7 (which does not form gel phase); these polymeric compounds are the most important components of the protective barrier or layer of the oral cavity. Many experimental studies have shown that a compound present in green tea, epigallocatechin gallate (EGCG), can substantially alter the properties of both the polymeric network of MUC5B and the monometric network of MUC7.

Chemical structure of the EGCG



This compound belongs to the class of polyphenols and is the most abundant of the family in green tea; it also has the most astringent capacities and has been shown to interact with a series of salivary proteins Several research groups explored the first phase when EGCG binds the proteins the and have suggested that EGCG binds of 1-2 amino acids long and that proline, arginine, phenylalanine and histidine have high affinity for EGCG This process is reversible and co-operative and there is the evidence that these interactions involve the hydrophobic interactions and hydrogen bounds , and hydrophobic stacking between EGCG rings and proline side chains is one of the most important mechanism ,while the galloyl ring of EGCG is likely important for protein binding (40).Numerous studies have

studied the mechanism of protein aggregation induced by EGCG, (41), (42), and the main conclusions indicate that the main phase involves the initial association of EGCG to protein to form a complex with hydrogen bonds. Green tea contains high amounts of polyphenols, so it has a good antioxidant power sometimes expressed as epigallocatechin gallate (EGCG) equivalent. However, this property can significantly alter both the characteristics of the polymeric network of MUC5B and the monomer network of MUC7. Studies using particular techniques(43) have in fact verified these interaction processes for the MUC5B fraction present in a sample of human saliva In our recent study, we observed the precipitation of proteins and mucin's in two samples of saliva, after the addition of green tea extract.(see next page) .The sample of green tea extract is a product of the company "MY PROTEIN "Ltd Manchester commercially called "MEGAGREEN TEA EXTRACT" with leaves of superior quality coming from plant Camellia Sinensis. This drawn out powerful contains 98% of polyphenols in total with 45% of EGCG. The protocol provides to add many and different amounts of green tea extract to different of saliva samples. The protocol provides to shake and then to centrifugation the solution; in the surnatant liquid we determinate, the parameters of interest see table. The statistical analysis of the results employs the Fisher Exact test and T-test this phenomenon, however, is not statistically correlated to the increase in additions of tea extract, i.e. the amount of polyphenol compounds in green tea, but still shows a trend, asymptotic type. The results of this trend are that the greatest value of precipitation of proteins is in 32% and 57.6%, for the total mucins. These are the reality concentrations of polyphenols about ten times higher than those that a usual consumer takes every day, drinking a small amount of green tea, can. The study of this process is still very important, with a huge impact on the health of the oral cavity. A recent epidemiological study, in fact, links

the increasing incidence of oral cancer in China with a high consumption of green tea (44)

Table 1. Principal biochemical parameters in first healthy control

Sample	Sex male	Age Years 35	pH	Flow Rate ml/min	SG/POIDS	Total Protein mg/dL	Total Mucins mg/dL	Rate % Mucin proteins
1+2			7.4	1.0	1.005	300	30	
3+4			7.3	1.0	1.01	306	30.6	
5+6			7.4	1.05	1.005	298	29.8	
				AVERAGE				
Final			7.4	1	1.005	301	30.1	10.0

Table 2. Principal biochemical parameters in second healthy control

Sample 2	Sex male	Age Years 60	pH	Flow Rate ml/min	SG/POIDS	Total Protein mg/dL	Total Mucins mg/dL	Rate % Mucins Proteins
1+2			6.5	0.8	1.025	320.5	30.0	
3+4			6.3	0.77	1.025	322.5	30.0	
5+6			6.3	0.83	1.02	312.0	30.0	
				AVERAGE				
Final			6.4	0.8	1.023	318.5	30.0	9.5

Table 3. Effect of the addition of green tea extract on saliva samples first in healthy control

Saliva ml	total proteins conc mg/dl	Protein mg/10cc	Total Mucin mg/dL	Mucins mg/10 cc	green tea mg	Protein Solution mg	Protein Precipit %	Mucin Solution mg	Mucins Precipitate %
10	300	30	38	3.8	50	24.6	18	2.6	30.4
10	300	30	38	3.8	100	22.8	24	2.5	37.0
10	307	30.7	38	3.8	150	22.1	28	2.1	45.6
10	305	30.5	38	3.8	200	21.1	30	1.7	54.3
10	295	29.5	38	3.8	250	20.1	32	1.6	57.6
10	300	30	38	3.8	300	20.2	32	1.6	57.6

]

Table 4. Effect of the addition of green tea extract on saliva samples second in healthy control

Saliva ml	Total Protein Conc mg/dL	Protein mg/10cc	Total Mucins mg/dL	Mucins mg/10 cc	Green Tea mg	Protein Solution mg	Protein Precipitate %	Mucin Solution mg	Mucins Precipitate %
10	315	31.5	30	3.0	50	27.7	12	2.1	28.5
10	326	32.6	30	3.0	100	26.7	18	2.0	34.5
10	330	33.0	30	3.0	150	25.5	22.7	1.8	40
10	315	31.5	30	3.0	200	23.0	26.9	1.7	44
10	310	31.0	30	3.0	250	22.2	28.1	1.5	49
10	315	31.5	30	3.0	300	22.5	28.5	1.5	50

.7-3-3-PAPRIKA

Paprika is a typical Hungarian spice, now often used all over the world. The drying and later pulverization of peppers of the genus Capsicum allow getting a product with the typical characteristics of moderate spiciness and slightly sweet taste. This product has this characteristic because the processing removes the inner membrane that has the largest amount of capsaicin. The paprika serves to prepare a food very known the goulash, and useful as an ingredient that mixes with meat and other foods, even sweet. The amount polyphenols in paprika, according to literature data (very deficient in truth, (45) expressed in mg / gr, is between 1.5 and 4.0 with a greatest value of 7.0 mg / gr: (47) These values show that paprika is a source of polyphenols with rather high values compared to other spices and with very significant consumption. Epidemiological data have shown that (48) Hungary is the leader in Europe, both for morbidity and mortality of oral cancer, and that the mortality rate from oral cavity cancer has increased significantly in the period 1975-2002, both for the male and female population. Our study executes an experiment to simulate as far as possible, the effect of paprika on saliva. The amount of saliva refers to the normal production of saliva during a meal of 25-35 minutes (about 25 cc), a part the most typical dish of Hungarian cuisine: goulash.This study has established that paprika can precipitate salivary proteins can, under experimental conditions that provide for its use in the ratio (2.5 mg / g of saliva). The precipitation mechanism provides that polyphenols, with a small contribution of capsaicin, can bind salivary proteins, so that in the conditions of our study the precipitation reaches values of 30% In these conditions there is a significant decrease

in the structural defences of the oral cavity, probably, accentuated by eating habits that provide for conditions of continuous supply of foods rich in paprika. The Hungarian people, practically use, in all foods, even in desserts), the paprika, and this can cause a state of chronic lack of salivary mucins The results of protein precipitation are not statistically correlated with the increase in concentrations and with the type of both paprika, $p \geq 0.05$, used but show a trend phenomenon with the same values, or rather a strong correlation between the two parameters. Test 1: greatest precipitation 23% (rho = 0.95, test 2: greatest precipitation 23.1% (rho = 0.91). To add the capsaicin alone detects a statistical tendency to precipitate, with a greatest of 10%, but for concentrations fifty times those found in two samples of paprika

7-4-DRUGS AND MUCINS

7-4-1- CORTICOSTEROIDS

The use of inhaled corticosteroids has, particularly for their prolonged use, as is very often the case, side effects on the throat and larynx, such as infection with Candida, cough and hoarseness. These side effects suggest that they may result from a change of the protective layer of the oro-laryngeal mucous membranes. Cortisone drugs have excellent anti-inflammatory properties, and do very good results to treat the respiratory diseases such as asthma and pulmonary fibrosis. The pharmacological activity of corticosteroids is very complex, but

this becomes understood with the study of these main biochemical stages

A- They interact with a glycosylated protein (GR) and form a complex that has the properties of a drug not yet active (CCP), (49), (50), (51), (52), (53).

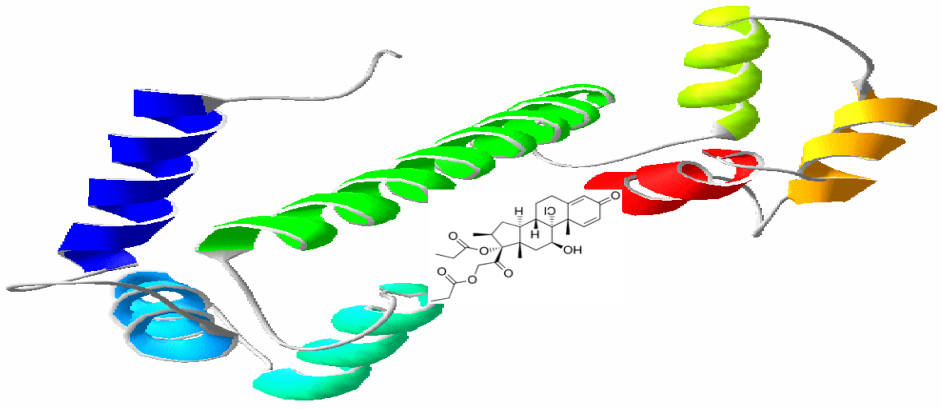
B-transportation of the (CCP) to the target cells

C- (CCP) penetrates the cells and binds the cytoplasm receptor,

D- Activate the anti-inflammatory properties of corticosteroids.

The complex (CCP) penetrates the nucleus and interacts with the DNA that activates or inhibits gene transcription, responsible for the main inflammatory processes. For the particular side effects, in this field of application, we have in our study verified the effects on salivary proteins of two corticosteroids used as aerosols in many respiratory diseases, in two samples of saliva provided by volunteers. We studied these effects at variable dosages of cortisone: at the normal dosage of therapeutic use, the precipitate value of total salivary proteins is about (21%), for beclomethasone, up to a most of 31% for a concentration three times higher than those normally used for therapeutic purposes In Figure 8 we give a possible hypothesis and explanation of the interaction between salivary proteins and corticosteroids. In this figure we can see as the drug inserts, in the protein structure, and forms with it chemical bonds related to hydrophobic interactions, and forces of Van Der Walls

FIGURE 8 the interaction of corticosteroids with proteins



A recent study (55), has studied at different temperatures with different spectroscopic methods, as the mucins interact with three different classes of drugs (theophylline, prednisolone and cefalexin). UV-Vis spectroscopy showed that all three drugs examined can bind to the mucin to form a protein-drug complex. According to thermodynamic parameters (positive DH and DS values), hydrogen forces and van deer Waals forces can play an important role in stabilizing the mucin-prednisolone complex. A similar study (56), conducted in 103 people (49 controls and 54 patients undergoing regular treatment with inhaled corticosteroids (ICs), showed similar results. There are no differences in co-morbidity or smoking habits and patients treated with high doses of IC showed lower levels of salivary MUC5B than those treated with medium doses of IC or untreated. The conclusions of this study are that in patients with asthma, treatment with high doses of CI results in reduced levels

of salivary MUC5B. In one of our studies (57), we analyzed the effects of three corticosteroids, Beclomethasone, Butesomide, and, Fluticasone, used as inhalers, on protein precipitation and total salivary mucins. The results show precipitation, (32%) of the mucin's for beclomethasone, 30% for butesomide, and and 7% for fluticasone. The amount of mucins precipitated by beclomethasone and butesomide, and are in relationship with the average concentration of the drug used is statistically significant $p \leq 0.05$, compared to total proteins, but not for fluticasone, $p = 0.092$. In any case, these data clearly show that there is interaction of corticosteroids with the whole saliva, precipitating mucins. This result is probably due to the form of a mucin-drug complex, with the creation of a new structure, following a possible alteration of the peptide back-bone and the change in polarity around the tryptophan residue. These preliminary results should facilitate the understanding of the type of interaction that occurs between mucin and drugs. Obviously, for a definitive picture of mucin-drug interactions, it would be necessary to investigate a large number of molecules structurally different from each other, because the amount of mucins precipitated by corticosteroids can decrease the natural defences of the oral mucosa, as also another recent study, (58) has shown.

7-5- DISEASES

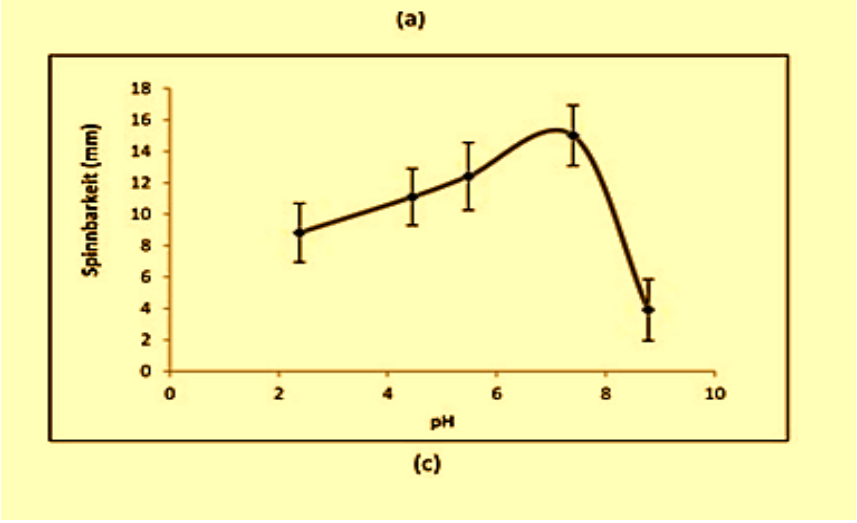
7-5-1- - GASTROESOPHAGEAL REFLUX

Excess gastric acid resulting from gastro-oesophageal reflux disease, also known as acid reflux or heartburn, may contribute to the initiation of scaly cell carcinoma of the head and neck, particularly laryngeal cancer. Previous epidemiological studies, conducted up to the year 2016, have reported mixed results, as shown by a retrospective study, (59). Overall, literature data indicate that an increase in the likelihood of head and neck cancer is not a strong relationship with a history of heartburn symptoms (odds/ratio ratio = 0.85, 95% confidence interval = 0.68-1.06) these data are found to be fewer cases of a precise medical diagnosis, or self-reporting, pathology or previous symptoms. On the contrary, two other studies (60) (61) show a relationship between pepsin increase, induced by laryngeal-pharyngeal reflux and laryngeal carcinoma, for a probable direct action of the same pepsin, where its excessive expression in laryngeal tissue increases in patients with leukoplakia of vocal cords and laryngeal carcinoma, in situ. In any case, such results, in our opinion, could find an explanation, for the fact that, a constant value of very low salivary PH, contributes to the alteration of the fundamental parameter that measures and determines the adhesiveness and elasticity of the protective layer of the mucin that covers the epithelium of the entire oral cavity. This parameter is the spinnbarkeit, and in the saliva, (62), the value of this parameter, reflects the ability of the saliva to adhere to the surfaces inside the mouth and larynx. The spinnbarkeit therefore measures the values of adhesion and lubrication of the particles that make up the mucous membranes and the entire orolaringeal biota. Therefore, alterations of the spinnbarkeit of

the saliva can cause the loss of adhesiveness or the ability to bind to surfaces of the mucins, and the phenomenon involves that they can oral dryness. Experimentally, it appears that the minimum value in mm (cut-off) should not be less than 11-12 mm, even if the optimal value is about 14 mm. The same study showed that there is a negative correlation between the contact angle (this value is directly proportional to wettability) and the spinnbarkeit, for sub-mandibular and labial gland secretions. Saliva samples for which there is an increase in spinnbarkeit, have a decrease in the contact angle (greater wetting capacity and little adhesion of the oral epithelium). The spinnbarkeit of the saliva involves the presence of high molecular weight glycoproteins (mucin's) that aggregate "end-to-end". Mucin glycoproteins and their structures are known as important factors for the rheological properties of saliva. The conformation of mucin depends on factors such as pH and ionic strength, but there are several other rheological properties typical of saliva produced by salivary, submandibular/sublingual glands. These glands are rich in mucin and are more viscous and viscoelastic than those secreted by the parotid glands. These different salivary secretions contribute to the rheology of the mucous layer and contribute to its viscoelasticity and extensive rheology, contributing to the maintenance of normal taste sensations of the mouth. In addition, saliva of submandibular/sublingual origin has variable protein concentrations depending on stimuli such as doors, chewing or taste, compared to unstimulated saliva. The pH value of saliva depends on the bicarbonate buffer system and the concentration of calcium ions and K and Na, influencing the hydrophilic capacity of salivary mucins, and this happens in autoimmune

diseases and diabetes, which generally lead to a lowering of the pH in saliva, up to values of 5.5-6. Since the pH of the saliva decreases from its physiological value, about pH 7.1, the spinnbarkeit shows a proportional decrease within certain limits, to it. However, the spinnbarkeit of saliva shows a steep drop when the pH has been increased from its baseline, as can be seen from Figure three. At these pH values, the relative value of the spinnbarkeit is below the cut-off

Figura 9- Valore Spinnbarkeit per pH



7-5-2- DIABETES

The aim of this study begins with the hypothesis that each risk factor may cause alterations in the oral cavity and larynx protection hat leads to the promotion of cancerogenesis. In this study it is very important to discuss the mechanism whereby the autoimmune diseases induce these alterations, but mostly to understand why only in diabetes there are effective and safe epidemiological findings for its correlation, in the absence of other risk factors, that promote oral and larynx cancer. Our hypothesis is that diabetes involves modifications, extremely important and irreversible, in the salivary mucin layer which allows ,for example smoking, HPV and other risk factors ,to influence irreversibly the normal genetic structure of the cell thus, allowing the possibility to risk factors, acting on cell metabolism without finding an adequate protective barrier.

Materials and Methods

A medical questionnaire was administered to a group of ninety laryngectomees, comprising of sixty-seven men and thirteen women. Included in the laryngectomees group are men and women who have all been subjected to total laryngectomy after their fifty years with a maximum age for this surgery at seventy-two .This medical questionnaire was administrated to a laryngectomees group to see if these patients before surgery,

have autoimmune diseases and/or symptoms of xerostomia .The same questions were posed to the healthy control group which comprised of sixty men and thirty women. This group have the same age range as the laryngectomees group. The results of the proposed questionnaire were statistically analysed with Fisher Exact Test. This study was made under the observation and ethical control of the Laryngectomees Italian Association. Under the control of the same ethical group in this study, we examined the differences in the concentration of total salivary mucin and the pH value in diabetic patients composed of twenty-eight males, non-smokers and no-alcohol users compared to the healthy control group which is also composed of twenty-eight males.Mucin concentration was determined using the Alcian blue method, as briefly described here: The samples of saliva diluted (1:10) were incubated for 30 min in a 1%solution of Alcian blue in 50 mm sodium acetate buffer with25 mm Cl2Mg, pH 5.8 under constant agitation at room temperature. After following the dissociation of mucin-dye complexes are stripped, with the addition of a 1: 2 dilution of Aerosol OT (Sigma Chemical Co, St Louis, MO, USA) in distilled water, mixing short and ultrasound the extracted samples ethyl ether and after centrifugation the concentration of dye was determined spectrophotometrically at 605 nm in the aqueous phase.The pH was determined by the use

of sticks for urinalysis, type Uriscan Roche, reading the values with automatic analyser URIYSIS2400 ROCHE. These data were statistically analysed with Chi-square Test.

Table 2. Results of the Questionnaire Responses by the Two Groups

CONTROL GROUP 60 MEN 30 WOMEN				LARYNX GROUP 72 MEN 18 WOMEN		
RISK FACTOR	MEN	WOMEN	TOTAL M+W	MEN	WOMEN	FISHER TEST P-VALUE BETWEEN CONTROL AND LARYNX GROUP
AGE men ≥65 years women ≥60 years	45	18	63	54	12	1.000
SMOKING	42	15	57	51	9	1.000
ALCOHOL	3	0	3	6	0	1.000
CANCER FAMILY	6	9	15	12	9	0.748
XEROSTOMIA	3	0	3	22	6	0.012
HYPOGEUSIA	3	0	3	12	6	0.103
LIFE STYLE	21	9	10	8	1	1.000
AB GROUPS	12	9	21	12	9	1.000
AUTOIMMUNE DISEASES (DIABETES)	1(2)	4(1)	5(3)	23(18)	6(5)	0.9 (0.0440)

Results of Fisher Exact Test for xerostomia hypogeusia in internal laryngectomees group:

- only in males: XEROSTOMIA p-value = 0.04950 ; HYPOGEUSIA p-value =0.356
- only in females: XEROSTOMIA p-value = 0.183 ; HYPOGEUSIA p-value = 0.183

The result of the incidence of diabetes in men from the laryngectomees group (25%) is statistically significant if compared with the value of the incidence (1.8%) in the males of Italian people: p-value = 0.041,with a proportional test. For an explanation of these results, it is important to analyse the incidence of diabetes for males in Italy.The percentage of people with diabetes in Italy is currently 4.3%, with an incidence between males and females almost identical, and includes all ages .Our sample of laryngectomy patients is a population aged between fifty and seventy-five years, for which the value of comparison of the incidence is related to a percentage of about 1.8%of the Italian population.The Xerostomia in laryngectomees male has a risk sign of$p \leq 0.05$while the autoimmune diseases is a risk factor of$p \leq 0.0491$ for both sexes. The diabetes incidence is 25.6% in laryngectomees male versus 1.8% incidence in Italian female population. With proportional Test the diabetes isa risk factor in laryngectomees male: $p \leq 0.05$.

MUCINS mg/dL		pH	
CONTROL	DIABETES	CONTROL	DIABETES
31	39.7	7.4	6
32	37	7.7	6.4
31	38.9	6.9	6
30	39.6	7.7	6
29.9	39	7.6	6.1
29.8	39	7.3	6.0
28.9	39.6	7..5	6.0
32.1	39.7	7.1	6.4
30.9	39.8	7.4	7
33	38.8	7.4	6.0
33.4	36.1	7.2	6.0
31	38.9	7.6	6.1
31.9	38	7.6	5.9
32.1	39.6	7.6	6.6
31	38.1	7.7	6.3
33.3	37.2	7.9	5.9
31	37.4	7.9	6
31	39.8	6.9	6.1
31.3	39.7	7.4	6.2
32.1	40.1	7	6.4
32.1	39.3	7	5.9
30.9	39.2	7	6
30.4	39	7	5.9
31.1	39	6.9	5.8
30.5	38.9	7	6.2
31.1	38.9	7.9	6.2

Table 3: The salivary mucins concentration and the pH values

Results of the Chi-square Test are:

- Mucin Concentrations: diabetes group vs control group: p =0.032
- pH Values: diabetes group vs control group; p= 0.047

The results of our work, expressed in Table 2,are similar to those of a pilot study conducted at the University of Naples, where a retrospective survey demonstrated that in about one hundred

persons undergone an operation for laryngeal cancer , thirty-six were suffering from diabetes (Iengo M, 2014 data not published) . Diabetes, in fact, can play an important role in the development of laryngeal cancer; this assumption becomes evident from data in countries like Cuba and Iraq (where there is a low alcoholic and tobacco consumption) with a high incidence of laryngeal cancer and a high incidence of diabetes as well.

More certain, even for recent studies, is the link between diabetes and oral cancer. Epidemiological data [3] carried out mainly in Hungary where the incidence of oral cancer is very high (ASR index = 9.0.)show that in this country there is a correlation between the incidence of diabetes mellitus, that affects 7% of the Hungarian population, and in generally glucose metabolic disorders among oral cancer patients and the frequency of different localizations of the same cancer. This study uses data spanning 14 years with two datasets of 1998 to 1999 and from 2012 to 2013 collected first hand by the authors. These datasets have led to examine the blood glucose level in 267 patients with histological confirmed malignant tumours in the oral cavity. Diabetes mellitus was found in 59 of them (22.1%) while our result is 21.5%.A comparative epidemiological study also demonstrates that there was an increased incidence of diabetes mellitus of 17.6% to 22.1% in the dataset 2012-2013.

Our study also achieved very similar results especially considering the comparison between the incidences of diabetes in Laryngectomees group compared to that of the Italian population. Equally significant is the incidence of xerostomia. This symptom is independent of the type of autoimmune disease and the gender. If we consider xerostomia as a symptom that indicates less mucosal oral and laryngeal protection we should consider all autoimmune diseases a real risk factor. However in practice this does not happen and there is no evidence in literature of epidemiological correlation, for example, between Sjogren's syndrome and cancers of the oral cavity and larynx in general however, it must be acknowledged that autoimmune diseases, more than for their genotoxic intervention within cells, induces very substantial alterations in mucin layer allowing other risk factors such as smoking, viruses, alcohol, free radicals, to bring the onset of the cancer process. Such changes may take place mainly according to these possible mechanisms:

a. decrease in salivary flow;
b. defect in glycosylation process of mucins;
c. change in concentration of SIgA;
d. decrease in pH;

The factors and/or processes outlined above led to the "spinnbarkeit" modification of the mucosal layer that lines the

epithelium, which is the clear slippery elastic consistency characteristic of the same. The spinnbarkeit of saliva reflects the ability of saliva to adhere to surfaces within the mouth, thereby serving as a protective role in adherence and lubrication. Therefore, alterations in the spinnbarkeit of saliva may result in the loss in adhesiveness or the ability to bind onto surfaces which may be related to the oral dryness. Experimentally ,the minimum value (cut-off)must not be less than 11-12 mm and the optimal value is about 14 mm[(5)]. The same study has demonstrated the negative correlation between contact angle (this value is directly proportional to the wettability) and spinnbarkeit for the sub-mandibular and labial glands and test saliva samples, where an increase in spinnbarkeit leads to a decrease in the contact angle (greater wetting ability and little adhesion of oral epithelium). The spinnbarkeit of saliva is expected to arise from the presence of high molecular weight glycoproteins (mucins) that aggregate end-to-end. Mucin glycoproteins and their structures are known to be important factors for the extensional rheological properties of saliva [6]. The conformation of mucin depends on factors such as pH and ionic strength, but different rheological properties have been identified in saliva produced by the different salivary glands with mucin-rich submandibular or sublingual secretions being most viscous and viscoelastic, and parotid saliva secretions being

the least viscous and viscoelastic saliva. These various salivary secretions contribute to rheology of the mucosal layer and contribute to its viscoelasticity and extensional rheology, aiding in the maintenance of a normal mouth feel. In addition, submandibular/sublingual saliva have varying concentrations of proteins when stimulated by smell, chewing or taste in comparison to unstimulated saliva.

A. Reduction In-flow Saliva

It is known that the salivary flow may decrease even if not always, in autoimmune diseases [7], and in diabetes [8,9,10], especially in the latter when there is a non-effective glycemic control [11]. A recent study [12] showed that the decrease of the salivary flow is directly proportional to the decrease of the spinnbarkeit (Figure 2), and this implies the low value of flow index; in addition to the occurrence of xerostomia, and it also presents an alteration of the mucin layer unable to adhere sufficiently to protect the oral epithelium.

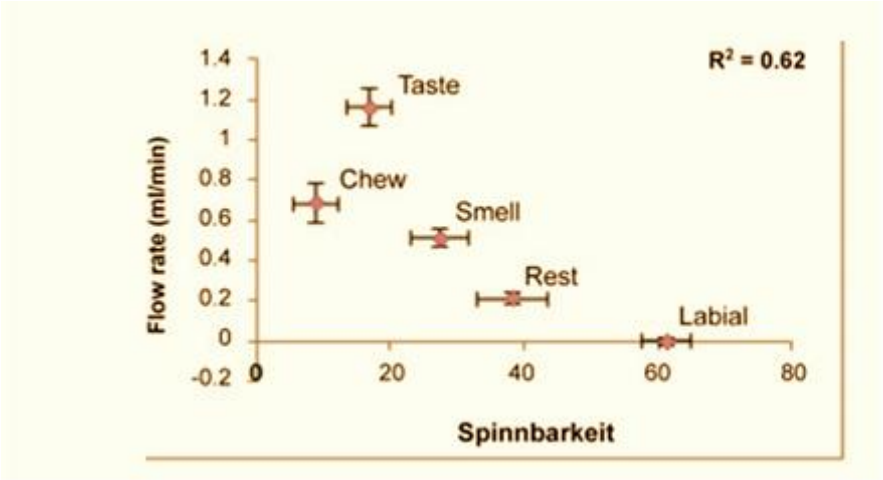


Figure 2. Influence of Flow Rate on Spinnbarkeit Values

B-**Defect in the Process of Mucins Glycosylation**

The glycosylation modification is a wide spread interesting event with about 70% of human proteins and principally characteristic of the cell surface and of those with a secreting function. Recent experimental study on salivary secretion[14] have demonstrated that only a correct glycosylation of mucins can allow in the oral cavity the smooth application of the phonation function of its normal lubrication, the formation of molecular recognition loci needed to protect the same against bacteria and viruses, and a proper hydrophilic process to create a mucin layer with a normal spinnbarkeit value.In many studies, over 90% of patients with dry mouth have a spinnbarkeit below the lower limit cut-off of 11 mm. Further analysis of mucins has revealed that the reduced

glycosylation of mucins led to a sensation of dry mouth. The result of this study indicated that salivary mucin concentrations are not reduced in dry mouth but that the mucin structure (glycosylation) is altered. Incorrect glycosylation can occur due to many intra and extra cellular factors but the most common involves a flaw in the formation of terminal residue of the polymer chain, namely that of glucose and the level of sialic acid. In this regard there are few studies that have analyzed the relationship between the amount of sialic acid in the saliva and autoimmune diseases, and/or diabetes. One of them [15] notes that there is a decrease of concentration in saliva and the hypothesis of the authors is that the decrease of salivary sialic acid level in type 1diabetes may be due to changes in the activities of the enzymes taking part in the synthesis and catabolism of sialic and according to our opinion, it may also involve one linked to mucin.

C-Modification of SIgA Concentration

It is now clear that some protective salivary proteins including mucins and IgA are concentrated on oral mucosal surfaces in the bound pellicle through specific interactions. Concentration of mucins would contribute to lubricate and prevent abrasion damage to soft tissues while an increase at IgA could create an 'immune reservoir' against the mucosal infection. In fact the latest

research highlights another key role of SIgA or one that allows the construction of the mucin layer in defence of oral epithelium; salivary mucins may initiate the formation of the mucosal pellicle through interactions with membrane-bound mucins on cells following the MUC5B MCU7, and form bonds between components of their oligosaccharide terminals with some of the immunoglobulin domains as described in the recent comment in "Nature" [16]. In this study, a possible interaction scheme and subsequent binding which agrees with the crystallographic examination is supposed The interaction process in the saliva , between the SIgA ,and Mucins, is very important because the stability of the mucin layer, also depends, on the concentration of the same immunoglobulin and in consequence the rheology of the mucosal system is susceptible to alterations when the amount, of SIgA , is low or in excess. In autoimmune diseases, such as rheumatoid arthritis, it is frequent , the decrease of SIgA[17, 18], with the increase of xerostomia, symptom even more accentuated if there is present a secondary Sjögreen syndrome Diabetes is a disease with a difficult metabolic control, where the SIgA may be higher [19] or as in many other cases lower[19,20]; in each case the patients are suffering from xerostomia and show a salivary flow rate much lower than the standard [21] and this we know that it is directly proportional to a spinnbarkeit value lower than the normal cut-off.

D-Reduction of the Salivary pH Value

The pH value of the saliva depends on the bicarbonate buffer system and the concentration of calcium ions, K, and Na

affecting the hydrophilic ability of the salivary mucins. This happens in autoimmune diseases and in diabetes in general leading to a lowering of the pH in the saliva [22] up to the values 5.5-6. As the pH of saliva decreases from its physiological value of about pH 7.1, the spinnbarkeit shows a gradual fall. However, the spinnbarkeit of saliva shows a steep fall when pH increased from its baseline as you can see in Figure 3. At these pH values, the related value of the spinnbarkeit is below the cut-off.

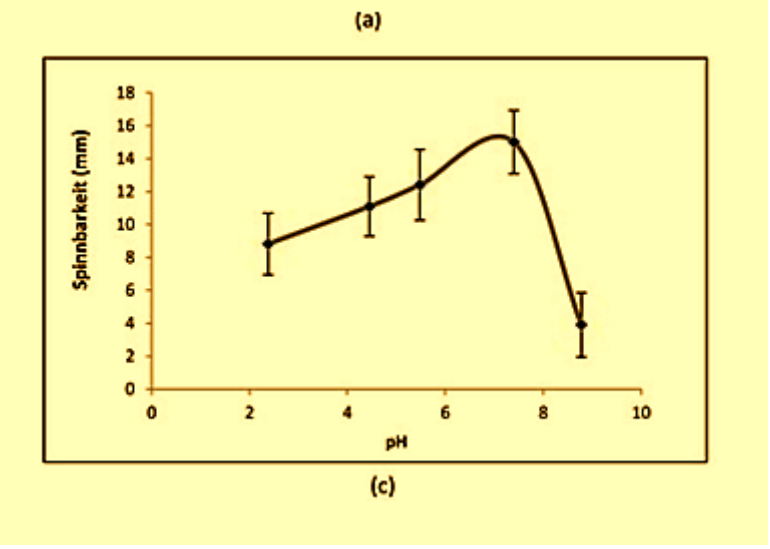


Figure 3: The Influence of pH on Spinnbarkeit Value

Our study shows (Figure 3) a decrease of about one unit from a medium value of 7.3 pH in the control group, to a medium value of 6.4 pH in diabetes group vs control group but this does not necessary implies a reduction of the spinnbarkeit value to go

below the normal cut-off. The possible hypothetical explanation for the presence of the symptoms of xerostomia reported in the questionnaire by the males in the laryngectomees group should be seen within the modification of other parameters such as an increase of the fraction of the mucins secreted in saliva. This fraction is the principal component of a structure formed by proteins, primarily mucinsMUC5B, MUC7,as well as water and mineral salts, amylase, SIgA, and other components. Figure 3 shows a schematic representation of the main functions of these components, which form the protective layer, the oral mucosa and larynx.

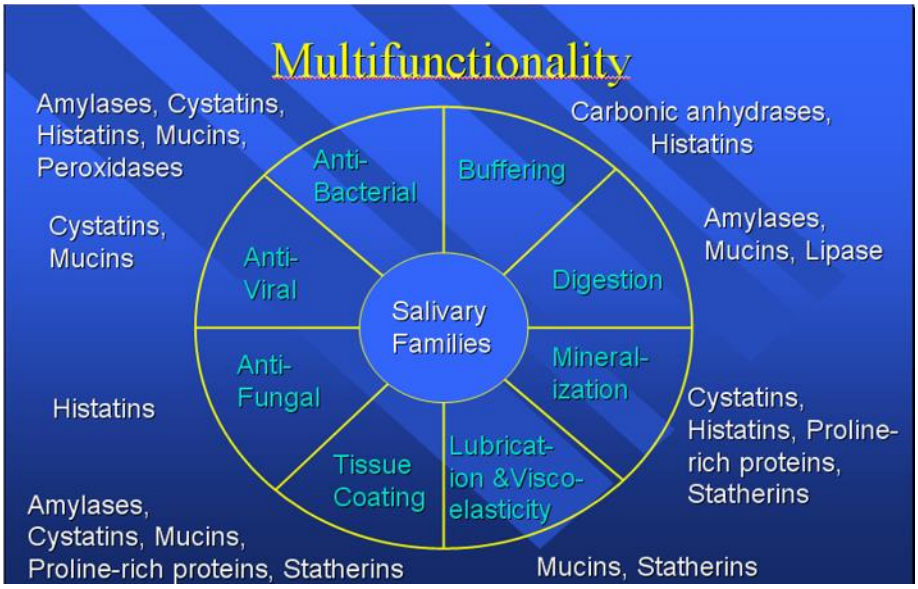


Figure 4. The schematic representation of the components of the Saliva with theirs correlated functions

The Over-Expression of Mucins:

Another fundamental parameter of the mucin layer involved for the presence of diabetes in the normal layer functions of the mucins, is also the concentration of salivary mucins. In diabetes, contrary to other autoimmune diseases, there is a considerable increase of this.Those results could be explained assuming that only in diabetes(with an increase of salivary mucins) there is a mechanism yet to verify with experimental studies that promotes alterations to the saliva layer (always composed by MUC5C and MUC7) with subsequent loss of its protective nature for the larynx. In fact, diabetes greatly increases the concentration in the saliva of the type of secreted mucins, MUC5B and MUC7, but not the MUC1, this linked to epithelial cells and that ensures the anchoring of the protective layer to the oral mucosa.One can reasonably assume that this increase in secreted fraction is excessive compared to that of MUC1 fraction, which entails an equally increasing mutual interactions between the terminal polysaccharide structures of MUC5B and MUC7, with formations of bonds between sialic acid saliva while that in diabetes it is much lower than normal concentrations ,is easily seized in this mucin structure, making it even more compact and highly hydrated. Practically this situation of over-expression in the formation of mucins, especially MUC5B and MUC 7, contributes to a compaction of the type of a bubble. It can be assumed that the saliva present in diabetes is not as a relaxed layer and adhering to the oral mucosa and/or laryngeal, but, as a layer formed from many droplets. Figure 5 shows a didactic representation of the normal protective layer structure of the oral cavity and larynx, as can also be shown by electron microscope

images. In diabetes, the mucin layer that should protect the epithelium, as we hypothesized, becomes a structural assembly, which doesn't look as a stratified epithelium, but for the high concentration of mucins and the high absorption effectof water becomes like a drop of water. A hypothetical representation is shown in Figure 6 where the most obvious difference is the formation of complex, and not more layered regularly on oral and laryngeal epithelium, and therefore less effective in its protection.

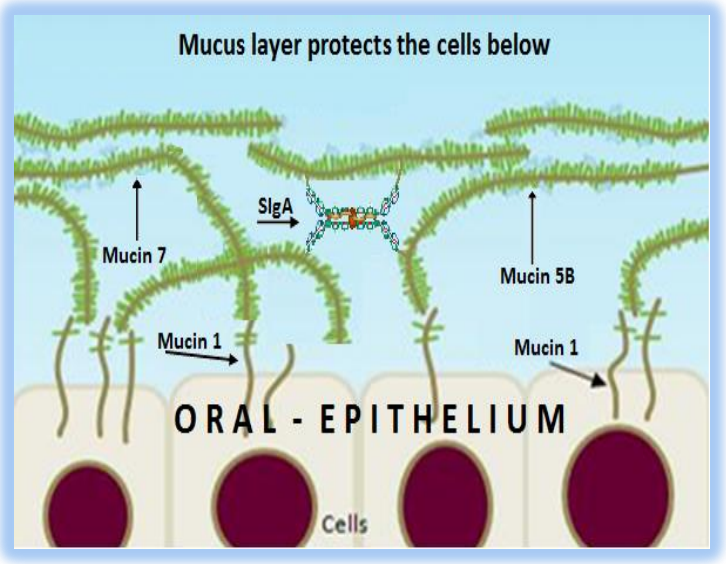


Figure 5: The Hypothetical Structure of Normal Salivary Mucus

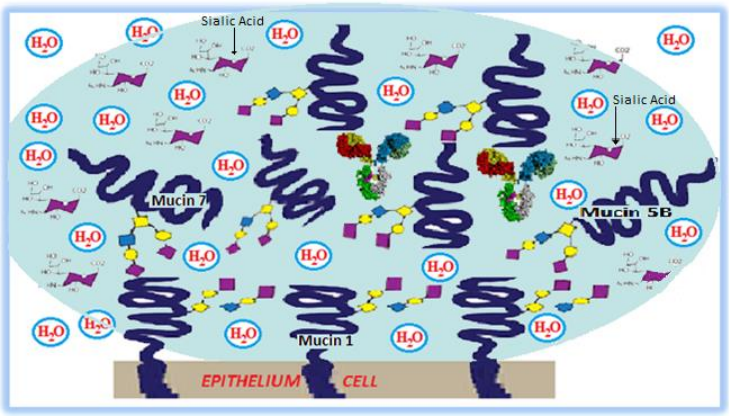


Figure 6: The Hypothetical Structure of Salivary Layer in Diabetes Conditions

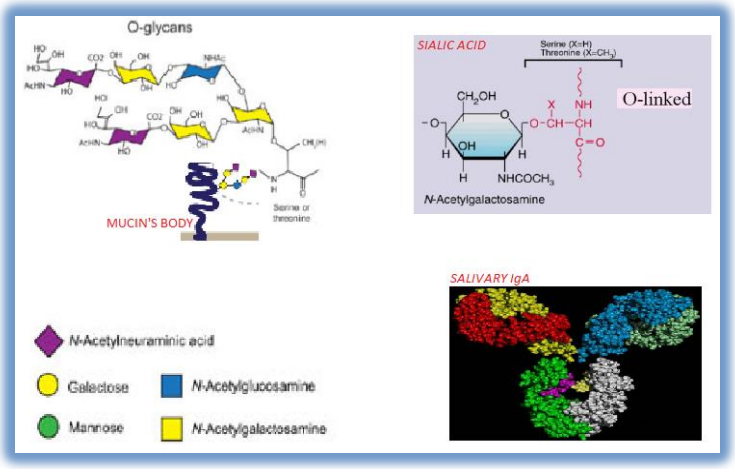


Figure 7. The most important parameters involved in Mucin Layer

In this study we try to demonstrate with sufficient reliability that there could be four distinct reasons why autoimmune diseases and especially diabetes, can alter the mucin layer that protects the

oral cavity. Decreasing the effectiveness of such a protective structure, other factors are able to come into direct contact with oral and laryngeal epithelium, spreading in its cells carcinogens contained in the smoke for example, and/or oncogenic viruses, that can promote the carcinogenic process.These considerations may justify the hypothesis derived from epidemiological data and the relative studies, as there may be a correlation between diabetes, autoimmune diseases, and oral cancer or laryngeal cancer The results of this study are very interesting, because they could explain the high incidence of laryngeal cancer in countries like Cuba and Iraq where in the presence of a low alcohol and tobacco consumption, there is a public health public on diabetes. This study is was undertaken under the observation and ethical control of the Laryngectomees Italian Association.

7-6-SEX AND ORO-LARYNGEAL CANCER

The mechanism of how sex hormones affect cancer risk is probably largely determined by the number and speed, of mitoses, of the epithelial cells of the organ in question. A high mitotic rate may increase the risk of cancer, increasing the likelihood of mutations occurring and replicating before they are repaired, and may also increase the likelihood of early cancer growth. In the case of estrogen, it has also been argued that some estradiol metabolites can cause mutations by directly damaging DNA, but this mechanism has not yet been fully clarified, and it is thought that the major role of sex hormones in cancer is probably linked to cell growth rate, namely mitotic velocity. Androgen, estrogen and progesterone, acting through specific receptors, play an important role in the growth and development of various cancers, including breast, endometrium, prostate and oral cancer. The receptors of sex hormones in vocal tissue have moved from mere theory, to more than a well-founded finding, and with it on the influence of sex hormones in laryngeal diseases..

The ROLE OF EXTERGENS AND PROGESTERN

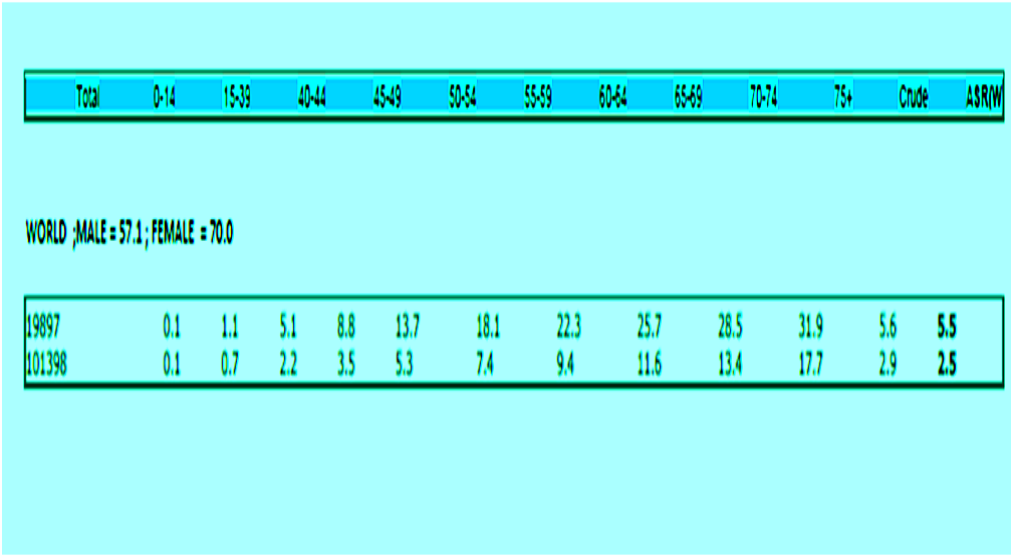
Studies on many of patients with HNC have shown that estrogen levels in women play a protective role to protect to the cancer. Because men have a lower level of estrogen, they are more likely to develop cancer, moreover, the destroy of liver function in alcoholics leads to alterations in the metabolism of sex hormones (estrogen and testosterone), and this is directly related to the increase in tumors. Thus, male alcoholics have a higher risk of developing cancer than non-alcoholics, so that many of patients

with HNC are men, characterized by an endocrine environment, rich in sex hormones and testosterone. Exist estrogens and progesterone receptors in tumours of the oral cavity, larynx or hypopharynx ,and the studies show as in this the level the presencre of of micro RNA (miRNA), and of serum and salivary protein levels. Patients with alcohol abuse, and with chronic liver disease have altered the metabolism of sex hormones involving testosterone and estrogen and the risk a HNC, (72) . They have demonstrated variable expression of miRNA, androgen receptors (AR) and alpha-receptor (ER-α), as well as estrogen receptors, in malignant tissues of the oral mucosa and suggest interference of these sex hormones in HNC. Several estrogen end products have genotoxic, mutagenic, processed and carcinogenic effects. It has also been shown that a number of estrogen metabolism genes live on cells derived from HNC. Another study group, (73), indicated that altered estrogen metabolism in lung tissue following exposure to tobacco smoke may lead to develop airborne digestive tract cancers. Therefore, estrogen is considered one of the imperative factors responsible for to develop HNC.

ROLE OF OTHER HORMONES

Recently, (74), a study has observed, that high levels of prolactin in HNC could indicate a poor prognosis., while other studies, (75) have also shown an increase in levels of follicular hormone (FSH), in (LH) and prolactin ,while decreases the testosterone and estradiol ratio, in patients with tongue cancer. These hormonal fluctuations clearly show a change in the pituitary-surrenal-testicular axis: therefore, it is possible to

suggest that these hormones could play a crucial role to in develop the progress of oral cancer. To explain this possibility, we have reported below the rates of incidence of oral cancer ASR in the world.with its relationship with the age and sex TABLE 10 The variation of the ASR index with age in WORLD



Total	0-14	15-39	40-44	45-49	50-54	55-59	60-64	65-69	70-74	75+	Crude	ASR(W
WORLD ;MALE = 57.1 ; FEMALE = 70.0												
19897	0.1	1.1	5.1	8.8	13.7	18.1	22.3	25.7	28.5	31.9	5.6	**5.5**
101398	0.1	0.7	2.2	3.5	5.3	7.4	9.4	11.6	13.4	17.7	2.9	**2.5**

the table shows at least two relevant data:

A) the ASR index, for males, is constantly increasing

B) the variation of the ASR between 45 and 59 years is much higher in females vs males and in practice, to the overall world data (see tables 10, 11), this index, grows 1.5 times more, than males.

We have statistically analyzed the ASR indices in this age group for the entire world population, and for the African continent, Hungary and India, to calculate the value of the slope of the ASR index, in ralationship to the age. These statistical calculations

return the slope of the linear regression line through the in-y and -x values. The statistical value of the slope indicates the vertical distance divided by the horizontal distance between the two values on the straight line, i.e. the degree of variation along the regression line. As can be seen from the results shown in Table 11, the values in the females, in the age group, taken into consideration, are much higher, which means that for the same range, there is a correlation with respect to age much less linear than for males. Statistical methods "Effect size calculators" can also be used: in simple terms, a measure of effect size provides a standardised measure of the force or magnitude of an effect. A statistical significance test tells us how confident we are that we can have an effect. A measurement of effect size, such as Cohen's D, gives us a standardized way to check the size of the effect. In practice, it is likely that an effect size will always be calculated if we already know that the effect is statistically significant (because it is not possible to calculate the size of an effect, if there is no reason to assume that an effect exists). and the particular way to calculate if the effect size is related to the significance test performed. . To calculate an effect size, you need to know the means and standard deviations of the groups. If you have raw data, you can calculate the means and standard deviations. For the person Z-test sample, Cohen is calculated by subtracting the population average (before treatment, or event, in this case menopause) from the sample average (after treatment) and then dividing the result by the population standard deviation.

TABLE 11 : ASR INDEX FOR AGE

	HUNGARY		WORLD		AFRICA		INDIA	
AGE	ASR	ASR	ASR					
	MEN	WOMEN	MEN	WOMEN	MEN	WOMEN	MEN	WOMEN
44	20	4	5	2	3	2	10	4
49	34	9	9	4	5	3	17	8
54	52	14	14	5	7	4	26	10
59	61	18	18	8	10	5	35	15
64	69	21	23	10	12	8	41	17
PENDENCE	0.39	1.15	1.11	2.45	2,10	3.33	0.62	1.31
Cohen's d	5.926	2.57053	0.44	0.117	0-67	0.26	1.64	1.11

However, there is no epidemiological evidence or meta-analysis to report the difference in the highest incidence of laryngeal cancer in males related to hormonal factors. In a recent study the data show that sex hormone receptors are absent in the vocal cords (76),so other theories should could explain the fact that hormones affect the quality of the voice. This study discusses the possibility that gender- and life-long voice changes may be related to a different expression of some growth factors in the laryngeal tissue and that this expression may in turn be influenced by hormonal changes.

8 - INDICATIONS - PERSPECTIVES - CONCLUSIONS

In all the precedent issue,we reported the arguments, to demonstrate how the etiology of oral and laryngeal cancer, is multifactorial. Oral cancer probably depends by , at least in a large one in particular geographical areas, and environmental or dietary factors (betel). For all types of cancer, both for the oral cavity and for the larynx, we can identify six main risk factors: sex, smoking, age, diabetes, eat habits, HPV and genetic's predisposition, for those genes that have some main genetic mutations, (see Figure 12) In all cases, it is 'possible to assume that whatever the genetic mutation that triggers the tumor event, in most cases this process is possible because the venvironmental factors modifie the protective layer of the mucin layer, Based on the above,we , can considere valid, a "method of analysis",as that we summarize in the flow sheet "genetic" (see Figure 13). For the risk analysis, we show the molecular aspects , within the same, the genes, most susceptible to alterate . The flow sheet, below represente its final target where the same genes, and the risk factors with a cascade process promote the possible pathophysiological consequences.

FIGURE 12, the most important genetic mutations in oral and laryngeal cancer

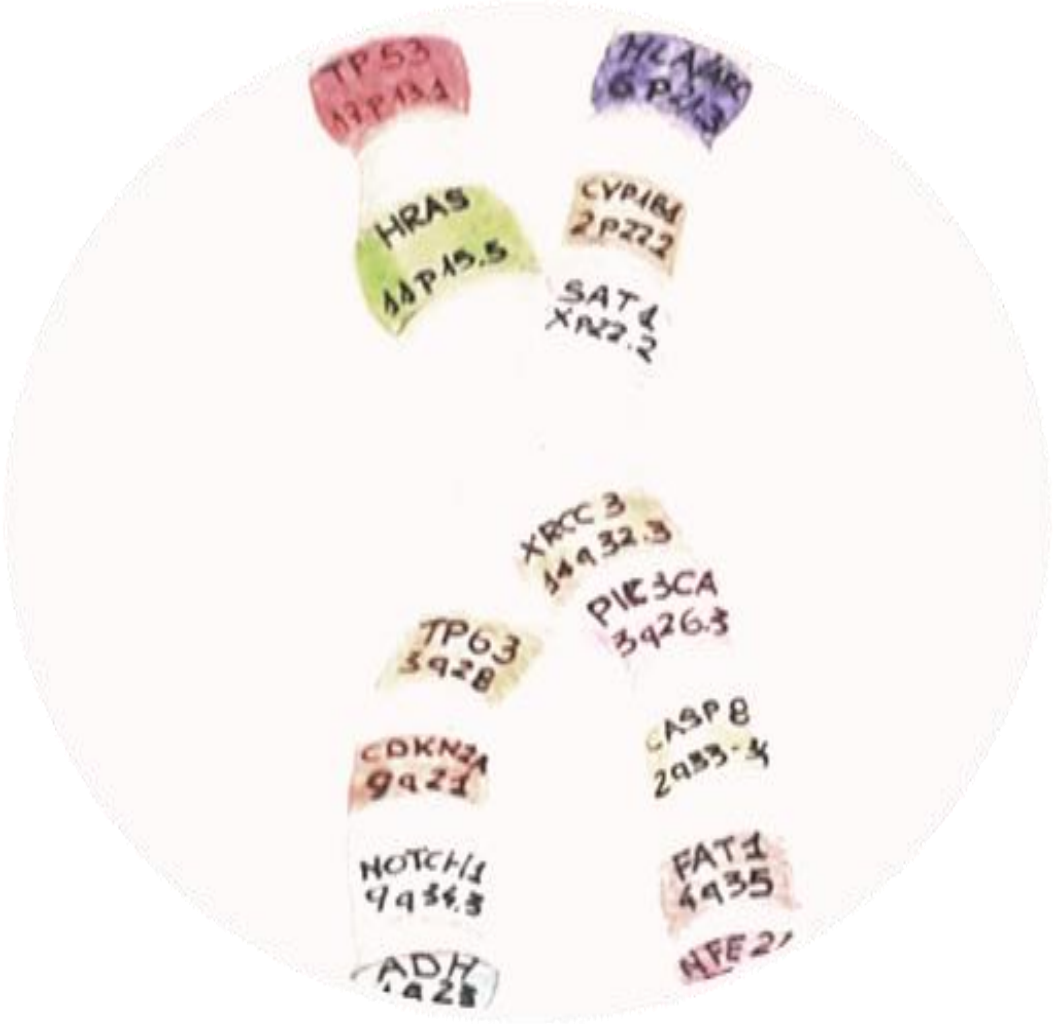
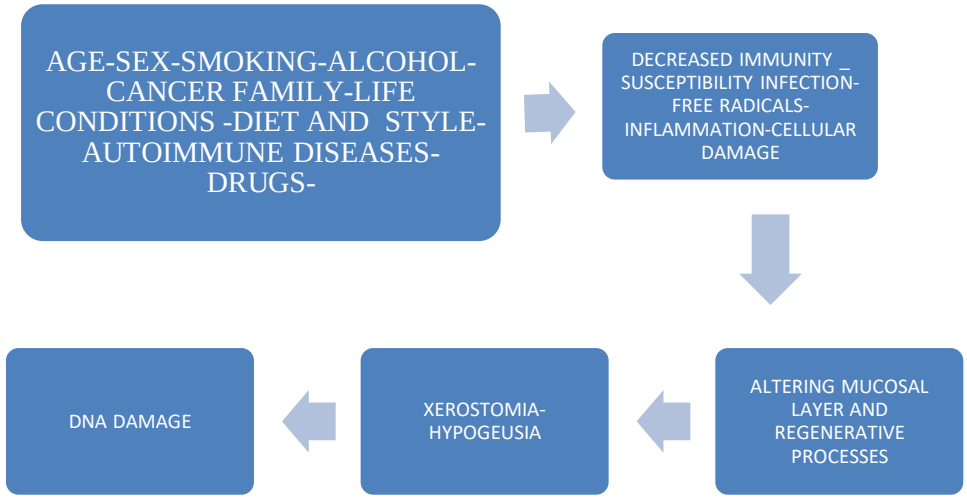


FIGURE 13 hypothetical flow sheet for the promotion of the cancer and his consequence



ANALISI DEI RISCHI E I MARCATORI DELLA CANCEROGENESI

the classic tumour markers (77) in clinical practice are mainly proteins (CEA, CA 19-9, CA 125 etc.), and are particularly important for after the patient's response to pharmacological treatments, as well as for early diagnosis of recurrences. The use of these molecules are very important in the first diagnosis (usually with the contemporaney assess by the radiodiagnostic techniques). An exception is CA125, a marker in ovarian cancer, which is specific only for this particular type of cancer. The appearance and detection of circulating cancer cells (CTCs) in the blood is not particularly frequent to show of probable metastases. Moreover, in most types of tumours, their number reflects their staging: early stage tumours with limited metastatic capacity generally have a low number of circulating cancer cells. Circulating cancer cells, containing intact DNA, or RNA and

proteins, are probably clones that will produce tumors with metastatic potential. These types of cells have significant diagnostic potential, at least in advanced disease. Within the various experiences, for the use of CT-DNA markers there is much literature showing that mutations, including those of relevance to the choice of treatment, can be identified with high sensitivity and specificity, with this type of research, and it also includes the search for gene fusions, while changes in different numbers of copies are more difficult to assess. CT-DNA levels generally say tumour potential and increasing them may also be at pre-malignancy stages: mi RNAs provide promising results for cancer detection, although their ability to predict response to therapy to date is uncertainin contrast to CT, mi-RNA-DNA is a technique that presents very unstable and difficult in the process of molecular sequencing.

- a) With regard to the above considerations, some study groups (for the breast cancer) ,have instead shown that the patients with this cancer have levels in plasma and serum with a good sensitivity and specificity for the diagnosis

- b) mi RNA from exosomes (extracellular vesicles), plasma and ctc can be used to sequencing RNA, allowing to find fusion genes by RNA AND DNA.

- c) mi RNAs from blood leukocytes allow for to find the immunological reactions to cancer and may be of great future importance, particularly for patients treated with immunotherapy (e.g. anti-CTLA4).Of particular interest was the develop of new electromagnetic release technology EFIRM (78), which appears to be more likely to detect single gene mutations when the tumor is in its early stages. The test is based on a new technology that detects very quickly within the "EXOSOMES", genetic mutations; It has found an excellent and reproducible application for the identification and dosage of the gene that encodes the epidermal growth factor (EGFR), a protein of the cell surface that normally in physiological conditions increases and divides cells normally. In non-small cell lung cancer there is an excess of EGFR, which makes them faster. Several drugs can block the growth signal from EGFR and identification of the mutation would allow an early start of therapy. According to Wong, the saliva test could allow screening for a variety of cancers, including those in the oral cavity.

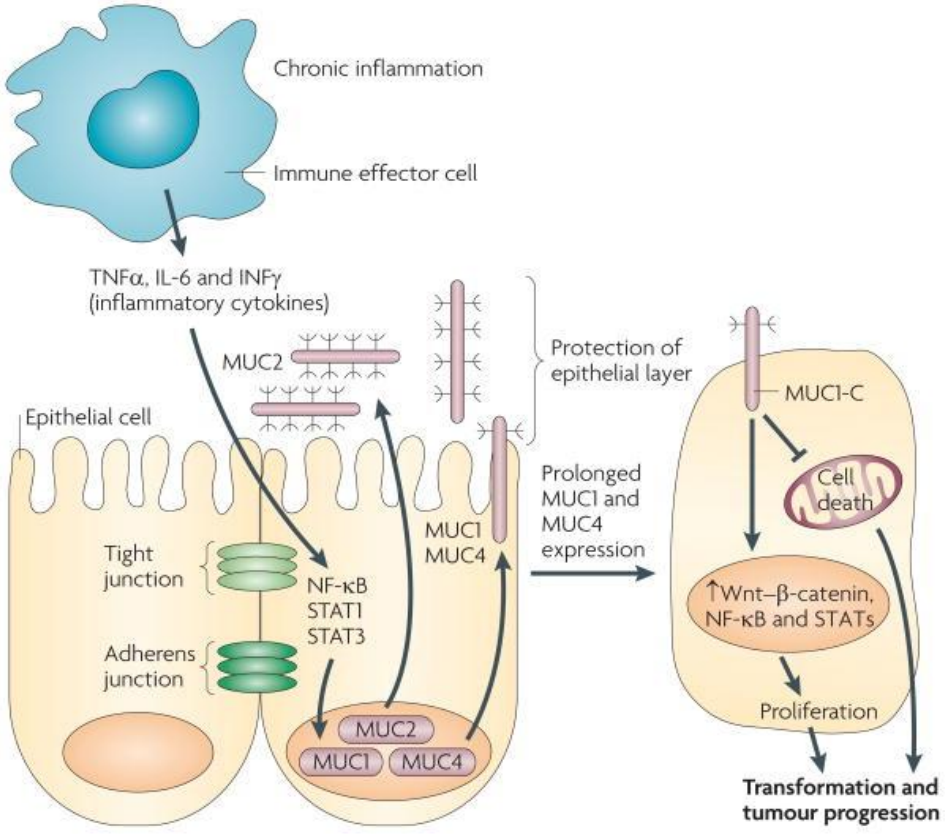
DIAGNOSIS AND TREATMENT PERSPECTIVES CONCERNING THE STUDY ON THE MECHANISM OF MUCIN ALTERATIONS

The best known gene for mucin synthesis encodes up to 20 known proteins, which we classifie into secret forms or associated with the membrane; each class has a typical protein domain structure. Secrete mucinae are synthesized to form transverse connections between them to allow and form an extended mucin networks found in secreted mucus gels. The

mucines associated with the membrane have specific membrane domains that allow their various biological functions as part of the glycolic calyx. All mucines are highly glycosylated and this property is specific to the secretive tissue and is specific to the biological functions of the various anatomical systems. Mucine biology, is dynamic and the degradation and turnover processes well ntegrate their biosynthesis ,to keep up continuous protection of the mucous membranes against all external aggressive forces, as such as happens,when the mucous membranes interacts with the bacterial microflora.Many diseases can modifie the mucins , and this may be due to gene alterations leading to aberrant mucin peptide or changes during glycosylation. Membrane mucins or trans-mucins join the main part of the mucosal barrier, and their variations are largely not recognized as effective indicators of carcinogenesis. However, both types of mucin are intimately involved in inflammation and cancer. In addition, many human malignancies have an excessive expression of trans membrane mucins to exploit their role in signaling cell growth and survival. Major studies and diagnosis protocols find some mucines as markers of adverse prognosis and as attractive therapeutic targets. In particular, analysis of the role and process of trans-membrane mucins in tumour transformation and progression has provided the experimental basis for demonstrating that inhibitors of their function are effective as anticancer agents in preclinical models. For example, MUC1 is aberrantly expressed in ~900,000 of the 1.4 million cancers (carcinomas and certain haematological diseases) diagnosed each year in the United States, making MUC1 the expression of one of the most common alterations in human cancers. The over-

expression of MUC1 may be partly attributed to amplification of the MUC1 loco and/or self-inducing loops involving activation of the MUC1 promoter through NF-κB and other transcription factors that are effects of the inflammatory response (as discussed above). As has recently been reviewed and summarised below, much of the more recent work on MUC1 has focused on the MUC1-C subunit and how it functions as an onco protein

Figure 14 The schematic process of the alteration of the functions of MUC1



There are other examples that prove the role of mucins to develop the cancer improovement The aberrant expression of .Mucin-7, in fact, is present in several types of cancer

Mucins in cancer diagnosis the example of MUC1 in possible applications

Early work showing that MUC1 is above expressed in human breast cancers, ( ) ; this research, in particular, led to the finding that the subunit MUC1-N is present with higher levels in the serum of breast cancer patients. The analysis the circulating levels of MUC1-N, determined according to the protocol of the CA15-3 test approved by the US Food and Drug Administration, is now used in all oncology centers, to check the clinical course of breast cancer patients during treatment and to detect over again early diseases. Several studies have examined the expression of MUC1-N as a marker in breast cancer and show that where exist the aberrant form of localization in non-apical membranes and cell cytosol, gives a worse prognosis. Profiling of gene expression of human prostate cancer has also shown that MUC1 is in relationship with some of its subgroups, and this generally leads to an increased risk of recurrence. As further evidence, comparative genomic hybridization and ct DNA microarray analysis of papillary thyroid tumours find MUC1 as an independent marker of aggressive behaviour and poor survival. In summary, the expression MUC1 gives a prognosis that is not positive over time for different types of carcinomas. Excessive expression of highly glycosylated trans-membrane mucin, particularly in oral and laryngeal cancer, can also provide important elements for early detection. Indeed research shows an

increase, in the oral cavity and laryngeal tumors of certain enzymes such as sialidase which indicates an increase in activity to fragment the mucins. An increase in free sialic acid can be considered one of the most reliable markers, (77), for these types of cancer, see table 12.

TABLE 12 Markers In Oral and Laryngeal Cancer

		Sensitivity	*Specificity*	*AUC*
$Beta_2$-microgobulin	Saliva	90%	90%	0.945
	Blood	100%	100%	1.00
MDA	Saliva	87.6%	100%	0.927
	Blood	73.3%	90%	0.852
Sialic Acid	Saliva	100%	100%	1.00
	Blood	93.3%	100%	0.983
Catalase	Saliva	86.7%	70%	0.765
	Blood	100%	100%	1.00
SOD	Saliva	100%	100%	1.00
	Blood	93.3%	70%	0.858
GSH	Saliva	100%	100%	1.00
	Blood	100%	100%	1.00
Neuraminidase	Saliva	83.3%	80%	0.800
	Blood	63.3%	100%	0.737

METABOLOMICS IN HEAD AND NECK CANCER N

Substances, as sialic acid called as salivary biomarkers, are good indicators of an individual's health status.,but the controversial status of the research don't premise to have a single and real biomark for thtose types of cancer Lack of reliable biomarkers and simple and accurate diagnostic tools for the screening of

early stage cancers are the major obstacles to hurdles in reducing the mortality rates Over the last years, considerable efforts have been made to clarify the potential of salivary metabolomics as an alternative diagnostic tool Salivary metabolites are powerful in elucidating the pathways underlying different diseases and thereby they can be considered as ideal for the early diagnostics of various diseases, such as HNSCC The use of salivary biomarkers is especially attractive in oral cancer, since the tumours communicate with saliva. Furthermore, tumour-derived extracellular vesicles might lead to the development of tumour-specific salivary biomarkers .For this effort the researchers use as main technique the Nuclear magnetic resonance (NMR) spectroscopy ,that is a quantitative technique based on the magnetic properties of atomic nuclei. The nuclei are said to be in resonance with external magnetic field. As the resonance frequencies and chemical shifts are unique or highly characteristic to individual compounds, NMR spectroscopy is powerful method for identification of small molecules in biological fluids such as in saliva . Further, as the area under a signal peak is proportional to the concentration of certain molecule, NMR spectroscopy allows quantitative analysis of salivary metabolites .Identification of new salivary biomarkers would help us to diagnose HNSCC in its early stages, which is highly advantageous and can help in selecting the most appropriate treatment modalities. Here, we have used NMR spectroscopy to assess possible salivary metabolic changes associated with HNSCC. Recently some studies have indentified many metabolites ,but also in this field there is a high scientific discussion .Below ,for example we report, in the table , the results of one of more recent study for the Metabolomics NMR application

Table -Comparison of salivary metabolite concentrations between patients with HNSCC (n=8) and healthy controls (n=30).

Metabolite	HNSCC patients	Controls	P-value
Butyrate	74.2 (33.9–266.4)	58.6 (25.9–128.4)	0.562
Propionate	659.0 (319.9–2,157.6)	527.3 (251.1–1,028.4)	0.428
1,2-propanediol	69.6 (32.7–2,465.4)	30.1 (21.7–54.1)	0.032[a]
Fucose	694.0 (302.0–1,527.2)	189.1 (100.6–284.7)	0.003[b]
Lactate	207.5 (71.5–1,132.9)	197.4 (140.4–324.6)	0.986
Alanine	90.3 (47.1–515.9)	107.4 (53.0–173.0)	0.820
Butanol	59.9 (17.2–190.5)	36.5 (16.8–84.3)	0.428

Metabolite	HNSCC patients	Controls	P-value
Acetate	2916.1 (2,559.8–9,344.8)	3282.4 (1,977.7–5,239.5)	0.428
Pyruvate	27.3 (12.6–73.1)	13.9 (7.2–33.3)	0.148
Succinate	50.6 (24.5–214.3)	58.9 (47.1–71.9)	0.765
Methylamine	5.7 (1.7–66.6)	3.7 (1.9–5.7)	0.445
Choline	17.1 (12.2–43.7)	19.2 (14.2–24.7)	0.765
Taurine	133.8 (72.1–195.4)	170.2 (104.7–205.1)	0.502
Methanol	118.0 (36.6–208.1)	80.4 (51.4–121.5)	0.515
Proline	156.9 (104.1–799.9)	610.1 (318.5–1,244.3)	0.043[a]

Metabolite	HNSCC patients	Controls	P-value
Tyrosine	113.8 (42.3–173.5)	96.8 (55.3–165.5)	0.847
Phenylalanine	100.9 (41.9–147.6)	79.7 (59.1–123.6)	0.880
Formate	229.7 (191.4–426.2)	178.3 (77.0–433.3)	0.428
Glycine	560.8 (103.2–719.1)	494.3 (241.1–923.6)	0.582

Data are expressed as the median (inter-quartile range). P-values are based on Mann-Whitney U test.

[a]P<0.05
[b]P<0.01. HNSCC, head and neck squamous cell carcinoma.

As you can see among all the determined metabolites, the only fucose, plays a fundamental role .We have showed as in previous saliva-based studies aimed to monitor aberrant glycosylation in cancer diagnostics have focused on sialic acid (N-acetyl neuraminic acid) that is a negatively charged nine-carbon monosaccharide. Sialic acids are also important terminal sugars in cell membrane glycoproteins and glycolipids. Previous studies have showed elevated levels of serum and salivary sialic acid in various carcinomas, including oral pre-cancer and OC

,but we can see that sialic acid was not among these 19 metabolites that we managed to identify and quantify in the present study

Our experience in the analysis of biochemical processes related to Mucins, however, leads us to consider that the result does not contradict the validation of sialic acid as predictive markers.

We think that the process of degradation of mucins to fucose, is subsequent to what forms sialic acid for two reasons.

The first reason concerns the position of fucose in the outer chains of the mucins; fucose occupies a more internal position.

Second reason, the methyl group present in the fucose makes it hydrophobic, contrary to the hydrolithylicity of the sialic acid. This characteristic exposes fucose it to enzymatic attacks, when sialic acid is practically absent in the external structure of mucins.

PRINCIPAL CONCLUSIONS :

A)Secrete and trans-membrane mucin fractions represent a family of glycoproteins that play fundamental roles in mucosal protection and biochemical relations with the external environment. (80).

B) The expression "hypersecretion", especially of trans-membrane mucins, may indicate a worse prognosis of cancer.

C) -. The excessive expression of mucins, "over expression" is also a good indicator and / or biological marker, in case of

carcinogenesis. This process, seems to have a dynamic balance in the production and production of the same mucins;
Paradoxically, there may be different biological situations, with lower, or higher concentration of mucin fractions, indicating the same RISK

:

PROPOSALS FOR THE TREATMENT OF THE CANCEROGENESIS PROCESS -

- A - PURIFICATION - EXTRACTION - ACTIVATION OF THE NEURAMINIDASE IN SITU

- B- GENETIC PREPARATION OF AN ONCOGENIC VIRUS (HPV 6,11), - INPUT Tp53 and CDKN2A

- C-INFECTION OF CANCER CELLS

Phase A Neuraminidase, (NA) on the surface of influenza A virus, removes sialic acids from cell glycans before they can be infected. Inhibiting NA with oseltamivir, in fact, suppresses both the viral infection and the release of the virus itself from the epithelial cells of the human respiratory tract in culture. In the infectious phase, the epithelial cells in fact produce a large amount of mucus with a high presence of sialic acid that produces a defensive barrier, however, destroyed by the sialidase present in the virus. These viruses, however, can not be used, because they have no transformations .activities

Step B - use of purified neuroaminidase in the blood

Phase C Engineering of a transforming virus, very low and benign oncogenic (HPV 6.11), with Tp53 and/or CDKN2A

FINAL CONCLUSIONS

There is evidence from epidemiological data, from the most recent literature, from the experiments of researchers and from the considerations that have been made so far on the role of genes, that environmental factors can be considered the true promoters of the carcinogenic process. It is clear that any chemical or physical carcinogen present in food, smoke, drugs, etc., can be in immediate contact with a goldpharyngeal-laryngeal epithelium only when it is practically discovered and under these conditions can promote cancer. This consideration also applies to the HPV virus, which, statistically, takes root almost exclusively in the most impoverished anatomical points of mucin protection, such as the palatine and lingual tonsils.

- The alterations in the formation of the mucines and/or the corresponding layer can be intra and/or extra cellular.

The most evident intracellular alterations are due to changes not yet well defined and related to diseases such as Sjogren's syndrome, but are of little significance. More important are those due to the action of exogenous and/or endogenous, episodic or chronic factors.

In any case, chronic changes involving changes in the chemical-physical properties of the entire saliva, for example, the pH, and the buffer system, in particular conditions leading to high acidity values, of the oral cavity, should be monitored.

It is therefore necessary, in a preventive monitoring or screening operation, to proceed with a protocol that provides for:

1-analysis of the literature and verification of the real effect of the risk factors on the physical and biological equilibrium of the salivary mucines.

2- Assignment of a score, index of danger and dependence, of the various risk factors.

3-formulation of a questionnaire to be proposed to a healthy population such as the one we have already submitted to the laryngectomised patients.

4 - statistical analysis of the results and their validation

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