Introduction

Epidemiological data

Malignant neoplasms of the major salivary glands are uncommon: the annual incidence rates in the world vary between slightly less than 2 and greater than 0.05 per 100,000 [1,2,3]. Tumors are mostly adenocarcinomas of the parotid, the largest salivary glands. These tumors are rare under the age of 40, and incidence at older ages is higher in men than in women.

Etiological and risk factors

The causes of salivary gland cancer are largely unknown. Diet may be effective in preventing salivary gland cancer, by increasing consumption of fruits and vegetables, particularly those high in vitamin C, and limiting food high in cholesterol [4]. A case–control study conducted in the Chinese population revealed a significant protective effect of consumption of dark-yellow vegetables or liver, with about 70% reduced risk of salivary gland cancer among people in the highest intake group of these foods [5]. Irradiation may also be a cause of malignant salivary gland tumors. [6]. The decline in incidence under age 70 in England and Wales is consistent with the reduction of repeated ionizing radiation exposure to medical or dental X-rays [7]. A history of prior cancers, especially those related with ultraviolet radiation, immunosuppression and Epstein-Barr virus, was found to be associated with salivary gland cancers in several studies. Among more than 5000 Swedish patients with Hodgkin's disease, there was a over 4-fold significant increase in cancer of the salivary glands [8]. In a large cohort of southern European men with, or at high risk of, HIV infection, a very high risk to have a cancer of salivary glands (SIR = 33.6) was found [9]. A US and Swedish study revealed an increased risk of second cancer, including salivary gland tumors in more than 1000 children with a diagnosis of medulloblastoma [10]. On a total of about 70,000 Finnish patients with basal-cell carcinoma, the incidence rate to have a subsequent salivary gland carcinoma was 3.3-fold higher than in the general population [11]. Patients with a histologically benign tumor (e.g. pleomorphic adenoma) which occurs at a young age, have a higher risk of developing a malignant parotid carcinoma, since these tumors have the potential for malignant transformation (3–10%) [12]. Chronic inflammation of salivary glands is not clearly defined as a risk factor.

Screening and case finding

Malignant salivary gland tumors are rare; therefore, no screening programme has been developed. Screening is not recommended and clinical case finding has not been evaluated.

Malignant salivary gland tumors are uncommon and therefore it is recommended that treatment be given in experienced institutions, where a multidisciplinary team is available. Neutron radiotherapy, which is not available in every country, is recommended in some particular clinical situations.

Pathology and biology

Histological types

Salivary gland tumors are classified according to the new WHO histological classification published in 2005 [13]. This includes the following histotypes.

- Benign epithelial tumors

Pleomorphic adenoma
Myoepithelioma
Basal cell adenoma
Warthin tumor (adenolymphoma)
Oncocytoma (oncocytic adenoma)
Canalicular adenoma
Sebaceous adenoma
Lymphadenoma
Sebaceous non-sebaceous ductal papilloma
Inverted ductal papilloma
Intraductal papilloma
Sialadenoma papilliferum
Cystadenoma

- Malignant epithelial tumors

Acinic cell carcinoma
Mucoepidermoid carcinoma
Adenoid cystic carcinoma
Polymorphous low-grade adenocarcinoma
Epithelial–myoepithelial carcinoma
Clear cell carcinoma, not otherwise specified
Basal cell adenocarcinoma
Sebaceous carcinoma
Sebaceus lymphadenocarcinoma
Cystadenocarcinoma
Low-grade cribriform cystadenocarcinoma
Mucinous adenocarcinoma
Oncocytic carcinoma
Salivary duct carcinoma
Adenocarcinoma NOS

Myoepithelial carcinoma
Carcinoma ex pleomorphic adenoma
Carcinosarcoma
Metastasizing pleomorphic adenoma
Squamous cell carcinoma
Small cell carcinoma
Large cell carcinoma
Lymphoepithelial carcinoma
Sialoblastoma
Soft tissue tumors
Haemangioma
Haematolymphoid tumors
Hodgkin lymphoma
Diffuse large B-cell lymphoma
Extranodal marginal zone B-cell lymphoma
Secondary tumors

The grade of a tumor (high, intermediate or low) is (aggressive, intermediate or indolent). Salivary carcinomas are classified into histological types or families. Most tumors in a family adenocarcinoma, adenoid cystic carcinoma) have a similar biological nature (although not all of them do). Some families are known to be high grade or biologically aggressive (anaplastic, carcinoma in pleomorphic adenoma, squamous cell carcinoma (SCC), high-grade mucoepidermoid), some are low grade (acinic cell, low-grade adenocarcinoma, polymorphous low grade) or intermediate (adenoid-cystic carcinoma). Besides, in some tumor families histological features may identify a subgroup of tumors with an indolent or aggressive nature. This is the case for mucoepidermoid carcinoma, and to a lesser extend, for adenoid-cystic carcinoma and other groups. Prognosis of salivary gland tumors appears to correlate mainly with histological subtype. A group of neoplasms exists (e.g. salivary duct carcinoma, oncocytic carcinoma, squamous cell carcinoma, large cell carcinoma), which are considered as high-grade tumors with a poor prognosis. These show a high tendency to recur locally and frequently result into distant metastases. In 2005 WHO classification only mucoepidermoid carcinomas are graded by a point score system, as low-grade type (well differentiated), intermediate or high-grade type (poorly differentiated). Differences in tumor grade have been also suggested for adenocarcinoma NOS, salivary duct carcinoma and acinic cell carcinoma. In these cases, prognosis correlates with grading: high-grade tumors

are associated with a poorer prognosis, whereas the prognosis of low-grade tumors is much more favourable. For most of the remaining malignant salivary gland tumors grading schemes do not seem to have any prognostic value.

Tyrosine kinase (TK) and hormonal receptors are currently the most investigated targets [14]. Epidermal growth factor receptor (EGFR) is the most expressed TK receptor in up to 71% of salivary gland cancers and its expression is detected in almost all malignant histotypes. No correlation was found between EGFR expression and gene amplification analysis and activating mutations within EGFR TK domain were very rare. Controversial results were reported about the prognostic role of EGFR expression on disease-free survival and overall survival [15,16,17]. Human

Epidermal growth factor receptor 2 (HER2) is present in particular histotypes derived from the excretory duct, such as salivary duct cancers. A correlation between HER2 3+ and gene amplification is found in at least 57–73% of cases [18,19,20]. Both HER2 overexpression and gene amplification seems to correlate with a worse prognosis . C-kit is expressed mostly in those histotypes originated from intercalated duct, such as adenoid cystic carcinoma, as well as in other malignant histotypes and benign tumors [21,22]. No genetic mutations at exons 11 and 17 were found and an autocrine/paracrine loop seems to be the most probable cause of c-kit activation mechanism [23,24,25]. Androgen receptor expression is rare and mainly restricted to salivary duct cancer and adenocarcinoma . Estrogen and progesterone expression is very rare and it is found both in benign and malignant salivary gland tumors [26,27].

Diagnosis

Signs and symptoms

Major salivary gland tumors

Every painless swelling of a salivary gland must arouse suspicion, especially if there are no signs of inflammation. Malignant tumors comprise 15–32% of parotid tumors, 41–45% of submandibular tumors and 70–90% of sublingual tumors As indicated above, malignant salivary tumors demonstrate a range of biological behaviors. About 40% of such tumors are indolent (especially in young people<40 years of age) and present as slow growing lumps and, if of long duration, they may be associated with pain or early nerve involvement. About 40% of tumors are also aggressive (especially in the elderly) and facial palsy may be a presenting feature but soon an evolving mass is evident. These tumors show frank evidence of malignancy. Clinical indicators suggesting a

malignant salivary gland tumor are: rapid growth rate, pain, facial nerve involvement, and cervical adenopathy. Every sign of facial nerve palsy, either complete or partial, is always a sign of a locally infiltrating parotid cancer [28,29]. Clinical presentation may also be characterized by parapharyngeal fullness, or palatal fullness. Trismus, skin ulceration and fistulas can be present in very advanced malignancies. On the other hand, a slow growth rate of an asymptomatic mass does not exclude a malignant nature [30].

Minor salivary gland tumors

There are between 450 and 750 minor salivary glands in the head and neck. About one half of the tumors that arise in these glands are malignant . The incidence of malignancy depends on the sublingual glands, the incidence increases up to 90% [31,32]. Signs and symptoms depend on tumor size and position and may vary according to tumor location. Minor salivary gland tumors are distributed in the upper aerodigestive tract, in the palate, paranasal sinuses and nasal cavity, tongue, floor of mouth, gingiva, pharynx, larynx and trachea. More than 50% of them are intraoral and usually cause a painless submucosal swelling. The mucosal layer is frequently adherent to the mass, with a small ulcer. Tumors arising in the oropharyngeal area can cause a painless lump. If the nasopharynx or the nasal cavity is infiltrated this may cause facial pain, nasal obstruction or bleeding. If the tumor [33] occurs in the larynx or trachea it can cause hoarseness, voice change, or dyspnoea. site of occurrence. In the palate the rate is similar to that in the submandibular gland, i.e. 40–60%.

Staging

TNM classification [34]

- Primary tumor (T)

TX Primary tumor cannot be assessed
T0 No evidence of primary tumor
T1 Tumor 2 cm or less in greatest dimension without extraparenchymal extension
T2 Tumor more than 2 cm but not more than 4 cm in greatest dimension without extraparenchymal extension*
T3 Tumor more than 4 cm and/or tumor with extraparenchymal extension*
T4a Tumor invades skin, mandible, ear canal, or facial nerve
T4b Tumor invades base of skull pterygoid plates or encases carotid artery
Note: (*) Extraparenchymal extension is clinical or

macroscopic evidence of invasion of soft tissue or nerve, except those listed under T4a and T4b. Microscopic evidence alone does not constitute extraparenchymal extension for classification purposes.

- Regional lymph nodes (N)

NX Regional lymph nodes cannot be assessed
N0 No regional lymph node metastasis
N1 Metastasis in a single ipsilateral lymph node, 3 cm or less in greatest dimension
N2 Metastasis as specified in N2a, 2b, 2c below
N2a Metastasis in a single ipsilateral lymph node, more than 3 cm but not more than 6 cm in greatest dimension
N2b Metastases in multiple ipsilateral lymph nodes, none more than 6 cm in greatest dimension
N2c Metastases in bilateral or contralateral lymph nodes, none more than 6 cm in greatest dimension
N3 Metastasis in a lymph node more than 6 cm in greatest dimension
Note: Midline nodes are considered ipsilateral nodes.

- Distant metastases (M)

MX Distant metastases cannot be assessed
M0 No distant metastases
M1 Distant metastases

Stage grouping

- Stage I

T1, N0, Mo

- Stage II

T2, N0, M0

- Stage III

T3, N0, Mo T1,T2,T3, N1, M0

- Stage IVA

T1,T2,T3, N2, M0 T4a, N0,N1,N2, M0

- Stage IVB

T4b, Any N, M0 AnyT, N3, M0

- Stage IVC

AnyT, AnyN, M1

Prognosis

Natural history

Malignant tumors of the salivary glands show widely different patterns of growth. The most common ones (adenoid cystic, mucoepidermoid low-grade, acinic cell carcinomas) frequently grow slowly, sometimes so slowly as to be mistaken for benign or non-neoplastic lesions, especially in the major salivary glands Invasiveness usually extends parallel to the histopathological degree of malignancy, which accounts for both local recurrences and spreading. Lymphatic spread is generally less frequent than that of mucosal SCC but it can be very frequent in some particular histotypes, such as ductal carcinomas, high-grade mucoepidermoid carcinomas, carcinomas ex pleomorphic, adenoma squamous cell carcinomas. Lymphatic spread is not frequent in polymorphous low-grade adenocarcinoma, is rare in low-grade mucoepidermoid carcinoma and in adenoid cystic carcinoma. Distant hematogenous metastases which localize most frequently in the lungs (80%) followed by bone (15%), liver and other sites (5%), are the main cause of death in malignant salivary gland tumors and depends on the degree of malignancy. Distant metastases from adenoid cystic carcinoma show a particularly slow evolution with survival reaching up to 20 years. Metastasizing pleomorphic adenoma is a rare histologically benign adenoma characterized by multiple local recurrences and a long interval between development of primary tumor and its distant metastases that usually occur to bone (50%) followed by lung and lymph nodes (30% both) [35].
All these remarks should be taken into consideration for treatment planning. Survival strongly correlates with clinical stage and grade. Histology is also a predictor of the tumor behavior and it contributes to optimize treatment.

Aims of the thesis

The aim of this study was to analyze the role and possible interactions between the gelatinases (MMP-2, MMP-9) and cyclooxygenase (COX-1, COX-2) in different pathologies in salivary gland that represent the progression towards the malignant phenotype.

Materials and methods

This is a prospective study on patients who underwent resective surgery for primary operable SG at the Department of experimental medicine, Division of Otolaryngology, University of Palermo, Italy. According to the criteria of the World Health Organization 2005 (WHO) we selected 14 cases.

Samples. The samples used in this study were provided by the otolaryngology clinic of the University hospital "Paolo Giaccone" in Palermo and include:

a) two normal samples of salivary gland,
b) one case of lymphoadenoma,
c) one case of myoepithelioma,
d) four cases of pleomorphic adenoma,
e) four cases of Warthin's tumor
f) two cases of carcinoma.

The informed consent was obtained from the patients few days before the surgery.

Immunohistochemistry. Samples were dissected and fixed in formalin solution. After fixation the tissue was dehydrated in a graded series of alcohols, cleared in xylene and paraffin embedded. Section of 7μm were cut on to Leica microtome RM2145, dried overnight at 37° C and then stored at R.T. until use. On the day of the experiment slides were dewaxed in xylene and rehydrated in a graded series of alcohols. Slides were then transferred into distilled water for 5 min.

The immunohistochemistry was performed using the"Dako Cytomation EnVision+ System-HRP (AEC)" kit from Dako (Dako, Glostrup Denmark), following the manufacturer's instructions. Briefly: sections were covered with the "Peroxidase block" reagent and incubated 5 min R.T. The samples were rinsed once in PBS buffer pH 7.2. The sections were covered with antibody solution and incubated at 4°C O/N.

Mouse anti human MMP-2 monoclonal antibody (Chemicon, Temecula, California, USA) (1:800 dilution), Rabbit anti human MMP-9 full length polyclonal antibody (Dako Glostrup Denmark) (1:50 dilution) were used. Mouse anti COX-1 monoclonal antibody (Invitrogen, Zymed Laboratories) (1:50 dilution), Mouse Anti-Human COX-2 (Dako Glostrup, Denmark) (1:100 dilution) were used. The antibodies were diluted in a 0.1% BSA solution.

Samples were rinsed twice in PBS pH 7.2 and then incubated with the "Peroxidase Labelled Polymer" reagent. Samples were rinsed twice in PBS pH 7.2, then incubated with the "Substrate-Chromogen" reagent and immediately observed under a light microscope; the reaction was carried

on until the staining appeared (2-10 min). Reaction was stopped rinsing the slides in distilled water. Negative control sample was treated in an identical manner, omitting primary antibody. Slides were coverslipped using the " DakoCytomation Faramount Aqueous Mounting Medium" from Dako (Dako, Glostrup Denmark). The specimens were observed under a Leica DM1000 light microscope.

Total RNA extraction. Samples were frozen in liquid nytrogen immediately after surgical dissection and stored at -80°C until use. Total RNA extraction was accomplished using the "illustra RNAspin Mini Kit" (Amersham Biosciences, Milan, Italy) following the manufacturer's instructions. RNA yield was evaluated spectrophotometrically (A260/A280) and RNA aliquots were stored at -80°C until use.

Reverse-Transcription (RT) Polymerase Chain Reaction (PCR). For RT reaction 2 µg RNA was used. To avoid DNA contamination in the RNA samples DNAse digestion was performed using "AMPD1 kit" (SIGMA). RT reaction was performed using the "Enhanced avian HS RT-PCR kit" (SIGMA) following the manufacturer's instructions. Briefly: 1µl random nonamers and 1 µl anchored oligo (dT)23 were added to the DNAse digestion product and incubated at 70°C for 10 min to denature the sample. Then 2 µl 10x Buffer, 1 µl deoxynucleotide mix, 1 µl RNAse inhibitor, 1 µl Enhanced AMV Reverse Transcriptase enzyme and DEPC water were added to the sample. The RT reaction was performed in 20 µl total volume at 42°C for 50 min, followed by 95°C for 5 min to inactivate the enzyme. The PCR was performed using the "PCR enzyme Selection Kit- High specificity" (Invitrogen) following the manufacturer's instructions. Briefly: 2 µl of template DNA, 0.5 µl of the Primers mix (200 nM final concentration) and 22.5 µl of the "Platinum Super Mix" were mixed together. The reaction was cycled for 94°C 3 min, then 40 cycles of 94°C 60 sec, 56°C 60 sec, 72°C 60 sec, with a final extention at 72°C 10 minutes. GAPDH gene was used as internal positive control. Primers sequences are showed in table 1.

Primer name	Primer sequence	Amplification product size
GAPDHhuman forward	GAG TCA ACG GAT TTG GTG GT	238 base pairs
GAPDHhuman reverse	TTG ATT TTG GAG GGA TCT GT	
MMP-2 human forward	TGA TGG TGT CTG CTG GAA AG	280 base pairs
MMP-2 human reverse	GAC ACG TGA AAA GTG CCT TG	
MMP-9 human forward	CAT TTC GAC GAT GAC GAG TTG	554 base pairs
MMP-9 human reverse	AAG CCC CAC TTC TTG TCG CT	
COX-1 human forward	AAG TAC CAG GTG CTG GAT GG	319 base pairs
COX-1 human reverse	GCT GCA GGA AAT AGC CAC TC	
Cox-2 human forward	CCA CCC GCA GTA CAG AAA GT	196 base pairs
Cox.2 human reverse	CAG GAT ACA GCT CCA CAG CA	

The products of PCR were showed on agarose gel 2%, painted with SYBER

Results

Controls (two cases): The very strong expression of MMP-2 and MMP-9 was observed in ductal epithelial cells in normal salivary gland while the expression of MMP-2 and MMP-9 in acinar epithelial cells is strong, the immunohistochemistry (IHC) data was confirmed by molecular analysis (RT-PCR).

At the same manner the immunohistochemistry analysis shows a very strong positivity of COX-1 and COX-2 protein in epithelial ductal cells of normal salivary gland while the expression of these enzymes is weak or absent in acinar cells. The molecular analysis confirmed these data.

Lymphoadenoma (one case): In this case of lymphoadenoma the data of IHC and RT-PCR showed the expression of MMP-2 and MMP-9 protein in ductal epithelium, while they were not detected in acinar cells.

About the expression of COX-1 and COX-2 protein the data of IHC and RT-PCR showed the expression of these two proteins in ductal epithelium, it was not detected in acinar cells.

Myoepithelioma (one case): In myoepithelioma sample both IHC and RT- PCR showed the expression of MMP-2 and MMP-9, in particular the expression of MMP-2 was observed both in ductal cells and acinar cells while it was not detected the expression of MMP-9 in acinar cells.

The immunohistochemical data and molecular analysis showed a strong expression of COX-1 protein in ductal and acinar cells while the expression of COX-2 was observed only in ductal epithelial cells..

Pleomorphic adenoma (four cases): a moderate expression of MMP-2 protein was observed by IHC in ductal epithelium, it was detected also in acinar cells only in one of the samples.
The RT-PCR confirmed these data.
The IHC showed a strong expression of MMP-9 protein in ductal epithelial cells for all four samples while the expression of MMP-9 was detected also in acinar cells only in one of the samples.
The RT-PCR analysis showed the expression of MMP-9 protein in three of four samples.

By IHC, the expression of COX-1 protein was observed in ductal epithelial cells for all samples; it was detected also in acinar cells only in one case of pleomorphic adenoma.
About the expression of COX-2, the IHC data showed the expression of protein in one case, in ductal epithelial cells while the RT-PCR doesn't show expression of COX-2 protein for all samples.
We found, also, a strong expression of COX-1 in one case in some stromal cells.

Warthin's tumor (four cases): In Warthin's tumor, a strong expression of MMP-2 was observed in ductal epithelial cells for two of the samples, the RT-PCR analysis showed the MMP-2 expression only for one sample.
The expression of MMP-9 protein was observed in three cases, while RT-PCR analysis showed the MMP-9 expression in two of four samples.

The moderate/strong expression of COX-1 protein was observed in ductal epithelial cells for three samples. The RT-PCR analysis confirmed these data.
About the COX-2 protein expression, the immunohistochemistry showed a positivity in all samples while the RT-PCR analysis confirmed these data only in two of four samples.

Carcinoma (two cases): The IHC and RT-PCR analysis showed a moderate expression of MMP-2 protein in ductal epithelial cells, in particular the IHC showed a positivity also in acinar cells in one of two samples.

The strong expression of MMP-9 protein was observed by IHC in epithelial ductal cells, the RT- PCR analysis showed the expression of MMP-9 protein only in one of two samples.

A moderate expression of COX-1 was observed in ductal epithelial cells and in acinar cells, these data were confirmed by RT-PCR analysis.
A weak expression of COX-2 protein was observed in ductal epithelial cells for all samples and the some expression of COX-2 protein was detected also in acinar cells only in one of two cases.

	COX 1	COX 1	COX 2	COX 2
	Ductal epithilum	Acinar epithelium	Ductal epithelium	Acinar epithelium
Control	+++/++++	+	+++	0
Control	++/+++	+	++	0
Lympho-adenoma	+		++	
Myoepithelioma	++/+++	++/+++	++	0
Pleomorphic adenoma	++		+++	
Pleomorphic adenoma	+++	++	+++	0
Pleomorphic adenoma	++ stromalcells++		+	0
Pleomorphic adenoma	+		0	
Warthin's tumor	++		+++	
Warthin's tumor	+++		+	
Warthin's tumor	0		+++	
Warthin's tumor	+++		+++	
Carcinoma	++	+	++	+
Carcinoma	+++	++	+	0

Legend 0 absent
++ discrete
+++ strong
++++ very strong

	MMP 2	MMP2	MMP 9	MMP9
	Ductal epithilum	Acinar epithelium	Ductal epithelium	Acinar epithelium
Control	++++	+++	++++	+++
Control	+++	++	+++	++
Lympho-adenoma	++		+	
Myoepithelioma	++/+++	++	+++	0
Pleomorphic adenoma	++		++++	
Pleomorphic adenoma	+++	+/++	+++/++++	++/+++
Pleomorphic adenoma	++ stromal cells+++		++	
Pleomorphic adenoma	++		+++	
Warthin's tumor	++/+++		+++	
Warthin's tumor	++++		++++	
Warthin's tumor	0		+++	
Warthin's tumor	0		0	
Carcinoma	+++		+++	0
Carcinoma	++	0	++/+++	0

Legend 0 absent
++ discrete
+++ strong
++++ very strong

Discussion

Matrix metallo-proteinases (MMPs) are zinc-dependent endopeptidases, they belong to a larger family of proteases known as the metzicin super-family.[36]. MMPs are involved in the breakdown of extra-cellular matrix in physiological processes such as embryonic development, reproduction and tissue remodelling as well as in disease process such as arthritis and metastasis.

They are capable of degrading all kinds of extracellular matrix proteins but also can process a number of bioactive molecules. MMPs are also though to play a major role on cell behaviour such as cell proliferation, migration, differentiation, angiogenesis, apoptosis and host defence.[37,38,39,40]

The most commonly used classification on based partly on historical assessments of the substrate specificity of MMPs and partly on the cellular localization of the MMPs. These groups are the collagenases, the gelatinases, the stromelysin and the membrane type MMPs.

The main substrates of the gelatinases (MMP-2, MMP-9) are type IV collagen.

MMP-2 (matrix metallo-proteinase 2, gelatinase A, 72KDa) degrades type IV and V collagen and elastin while MMP-9 (matrix metalloproteinase 9, gelatinase B, 92KDa) degrades only type IV e V collagen.

Cyclooxigenases are enzymes that catalyse the first step in prostanoid biosynthesis, infact they catalyse the conversion of arachidonic acid (AA) to the key upstream prostanoid precursor prostaglandin H_2 (PGH_2) which is metabolised into the prostaglandin isoforms PGE_2,PGD_2,PGF_2,PGI_2, or thromboxane A2 (TXA_2). Two isoforms of COX have been identified: COX-1 and COX-2.[41]

COX-1 is constitutively expressed, it is expressed in a broad range of cells and tissues, it is involved in cell homeostasis such as cytoprotection in the G1 tract, platet function and renal perfusion; while COX-2 is normally absent in most cells and tissues but is induced during pathological conditions such as inflammation and cancers; but there are some differences, for example COX-1 is inducible under inflammatory conditions in the kidney and COX-2 is constitutively expressed in tissues such as the kidney and blood vessels, a fact which may contribute to some of the efficacy as well as the side effect profiles of some COX-2 inhibitors.[42,43,44].

In this study we examined the presence and the role of matrix metalloproteinases and cyclooxygenases during the process of

carcinogenesis of salivary glands, in fact, our group has been studying the expression of MMPs[45] and COX[46] in healthy and pathological samples of salivary glands through immunohistochemistry (IHC) and RT-PCR analysis.

In our case of lymphoadenoma by IHC and RT-PCR we detected a presence of both classes of enzymes. Because in literature there aren't data about lymphoadenoma it is not possible compare our data with previous studies is known that MMPs are responsible for a wide range of proteolytic events so in agree with our previous studies [47], we believe that the increased MMPs expression induces an activation of tissutal remodelling's mechanisms.
Likewise, the increased expression of COX-1and COX-2 we detected in our case of lymphoadenoma, is index of inflammatory processes within benign lesion which have alterations of regulatory mechanisms.

Myoepithelioma of the salivary gland is a benign tumor set up almost exclusively for a myoepithelial cells, in fact, it is positive to some specific antibodies (e.g. keratin, vimentina, S-100 protein). These myoepithelial cells are transformed but not differentiated, this tumor it is considered as the terminal form of the histopathologic spectrum of mixed tumor but owing to its monomorphic appearance is considered an aside form.[48, 49,50] Our data by IHC and RT-PCR showed the expression of both MMPs and COXs, unfortunately in literature there are not data about this pathology so we believe that although it is a benign tumor, it is going to transform into a malignant phenotype.
In four cases of pleomorphic adenoma, IHC and RT-PCR analysis showed the expression of MMP-2 and MMP-9, our data agree with other studies which represent a mRNA expression levels of MMP-2 were significantly higher as in stromal epithelium as in ductal epithelium component compared to controls. Likewise, our data by IHC showed a increased amount MMP-2 expression also in some stromal cells. [51]
According to some authors [52, 53]we believe that the presence of MMP-2 protein in stromal cells indicates that stromal myoepithelium may be one of the critical elements that promote the transition from carcinoma in situ to invasive cancer so it has been well known that the tumor stroma is directly related to biological behaviour of pleomorphic adenoma.
Previous studies described consistent MMP-2and MMP-9 expression in ductal cells and only weak expression in acinar cells.[54,55,56,57].

So our data confirmed the hypothesis that MMP-2 protein expression reflects the invasive properties and malignant potential of salivary gland tumors [58].

By immunohistochemistry we detected a presence of MMP-9 protein in all cases of AP while RT-PCR analysis shows the expression of MMPs in three of four cases examined; this discordance may be explained by the discrepancy between mRNA and protein expression of MMP, because, there is a difference between ductal epithelium and stromal myoepithelium in the rates of mRNA translation and capacity for intracellular storage.

Moreover, the translation of mRNAs into MMPs proteins is regulated and protein levels can vary greatly depending on post-translational modification and degradation, protein-protein interaction and stabilization/destabilization.

Another consideration might be that MMP2- and MMP-9 synthesized and secreted mainly by stromal myoepithelium and captured on the membranes of epithelium, facilitating the local invasiviness.

In summary, our results provide preliminary evidence that MMP-2 and MMP-9 are mainly produced by the stromal myoepithelium so the stroma may be more important than epithelium in the development and/or progression of PA.

By IHC, we detected COX-1 protein expression in ductal epithelial cells in all cases examined, also in only one case we detected COX-1 protein expression in acinar cells, RT-PCR analysis confirmed COX-1 protein expression in three of four cases.

About COX-2, IHC showed the expression of protein in ductal cells for three of four cases examined while RT-PCR analysis doesn't show gene expression. We believed that the expression of COX-2 in these cases indicates that COX-2 plays a crucial role in process of malignant transformation of PA.[59].

In Whartin's tumor cases, MMP-2 expression was exclusively observed in ductal cells for two of four samples examined while molecular analysis shows the expression of mRNA for this protein in three of four cases. Probably, the discrepancy between the two technique can be due to the different sensibility of two methods.

Likewise MMP-9 expression was detected by IHC for three of four cases examined while molecular analysis confirmed MMP-9 expression only in two of four cases, it is not possible compared these data with literature, because we didn't found studies about the expression of MMPs in Warthin's tumor.

IHC data and RT-PCR analysis showed the COX-1 expression in ductal epithelial cells, these data agree with literature in fact several authors reported that COX-1 is a constitutive enzyme.

Conversely, the COX-2 protein expression was observed by IHC in all samples, these results are agree with literature, in fact several others authors [60] revealed that COX-2 is up-regulated in the epithelial component of Warthin's tumor, this finding support the hypothesis that Warthin's tumor originate from heteropic ductal epithelial cells of the parotid gland but the role of COX-2 expression in the pathogenesis of Warthin's tumor remains to be determined.

In all cases of carcinoma, IHC and RT-PCR analysis detected the expression of MMP-2 in ductal epithelial cells, this result is in agreement with literature[61] which confirms that MMPs are enzymes capable of degrading all kinds of extracellular matrix proteins, in particular MMP-2 and MMP-9 degrade collagen which is a major component of ECM.

In particular, our experiment showed the presence of expression both MMP-2 and MMP-9 in ductal epithelial cells while the expression of these enzymes is weak or absent in acinar cells.

This expression pattern is in accordance with the observations of some authors [62] who described consistent MMP-2 and MMP-9 expression in ductal cells and only weak expression in acinar cells.

According other author [63] the immunoscore of MMP-2 detected in tumor cells were significantly increased in the malignant tumors compared with benign tumours. Probably about our cases it is not possible to value a trend of protein's expression about rising malignancy due to small and no homogenous number of samples.

By IHC, expression MMP-9 protein was detected exclusively in ductal epithelial cells in all cases examined while RT-PCR analysis shows MMP-9 protein expression only in one of two cases examined, the immunohistochemical data are in agreement with other studies [64,65] which claim that immunostaining of MMP-9 was observed predominantly in the tumor cells and occasionally in the inflammatory stromal cells and that the invasiveness and prognosis of high-grade salivary gland cancers may depend on their MMP-9 expression profile, indicating that MMP-9 contribute to the progression and invasion of malignant tumor.

IHC experiments and RT-PCR analysis evidenced COX-1 and COX-2 protein expression in all cases examined. These results in agree with literature, in fact some studies claim that cyclooxygenase plays a pivotal role in the initiation and progression of many cancers.

As it is well know, COX-2 is probably one of most important agents involved in the development and evolution of inflammation. Also overexpression of COX-2 has been observed with particular reference in carcinomas at high-grade while it is not express in low-grade carcinomas.
These results suggest that over-expression of COX-2 plays a crucial role in the pathogenesis of malignant transformation of carcinoma in the parotid gland.
In conclusion, it is well known that MMPs and COX are enzymes that interact between each other in the initiation's process and tumoral progression even if the mechanism is not well clear.
The inhibition of COX-2 activity suppress the invasiviness of tumor so the action of MMPs is necessary for the maintenance of last step of tumoral.
The new idea of our work unlike previous studies is to analyze in all (not in only group or between two groups) benign and malignant tumoral pathologies the role and possible interactions between the gelatinases and ciclooxygenaes and to identify eventual progression from benign phenotype towards malignant phenotype.
Actually, by our results we didn't found linear progression from benign phenotype towards malignant phenotype about MMPs and COXs, certainly it is necessary to increase the number of cases and make to similar each group about the number of pathologies among them in order to understand the role and the functions of these molecules.

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