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Tesi di dottorato

"Salivary extracellular vesicles microRNAs profiling as suitable non-invasive source for biomarkers in oral cancer."

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Concise Summary

Several studies have investigated the expression of micro RNAs (miRNAs) in saliva as potential biomarkers. Since miRNA associated with extracellular vesicles (EVs) are known to be protected from enzymatic degradation, we decide to evaluate whether salivary EVs of patients with oral squamous cell carcinoma (OSCC) are enriched of selective subsets of miRNAs.

OSCC patients (followed at the Oral Medicine Section of the University of Turin, Department of Surgical Sciences) and healthy controls were matched with regards to age, gender and risk factors. Total RNA was extracted from salivary EVs and the differential expression of miRNAs was evaluated by qRT-PCR array and qRT-PCR. The discrimination power as biomarkers of miRNAs up-regulated in OSCC patients versus controls was evaluated by the Receiver Operating Characteristic (ROC) curves.

In a preliminary qRT-PCR array performed on 5 OSCC patients and 5 healthy controls, we identified a subset of miRNAs differentially expressed. On the base of array results, we later studied miRNAs up-regulated or selectively expressed by patients in a cohort of additional 16 patients and 6 controls. miR-302b-3p and miR-517b-3p, were only found in patients' EVs. miR-512-3p and miR-412-3p were up-regulated in patient's EVs versus controls and the ROC curve showed a good discrimination power for OSCC diagnosis. The Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway enrichment analysis suggested the possible miRNA involvement in pathways activated in OSCC.

In this present work, for the first time to the best of our knowledge, we suggest that salivary EVs isolated by a simple charge-based precipitation technique can be exploited as a non-invasive source of miRNAs for OSCC diagnosis.

Moreover, we identify a subset of miRNAs selectively enriched in EVs of OSCC patients that may be simply used as possible oral biomarkers.

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INTRODUCTION

The identification of molecular signatures in biological fluids could create the so-called *"liquid biopsies"*, which would successfully overcome many of the faces associated with traditional tissue sampling (*e.g.* invasiveness and heterogeneity). To date, the pathogenic role of EVs has been studied in several cancers, but very few data are available for oral cancer. The presence of EVs in sera of patients with head and neck cancer has been studied, with only few patients reported with OSCC; no data are available however for the EVs in saliva of patients with OSCC and for the EVs miRNA expressions. The development of forceful techniques and sensitive capture platforms that use readily available body fluid could offer new approaches for OSCC diagnosis, staging and therapy. Thus, taking all these observations together, we decide to measure salivary changes levels of miRNAs in patients with OSCC and relate them to the severity of the disease.

→ The objectives of this study have been planned to be:

1) to detect differences in salivary EV microRNAs profiling in OSCC patients and healthy subject control samples;

2) to validate the results with RT-PCR.

Actually, to the best of our knowledge, miRNAs expression in EVs in OSCC has never been performed in a prospective and comparative manner.

CHAPTER 1 - GENERAL PARTS

1.1 Oral squamous cell carcinoma

Head and Neck Squamous Cell Carcinoma (HNSCC) is one of the ten most common cancer worldwide and involves carcinoma of several anatomic sites such as oral (lip, oral cavity), pharynx (nasopharynx, hypopharynx, oropharynx), larynx and is associated with high rate of morbidity and mortality. Most of HNSCC patients have a history of tobacco use like smoking, betel quid chewing and snuff dipping and/or alcohol [Braakhuis *et al*, 2003].

Oral squamous cell carcinoma (OSCC) is the most frequent HNSCC, and it is the 15th most common cancer in human, contributing 2.1% of the total number of new cases diagnosed in 2012 (wcrf.org), causing significant morbidity and mortality, especially in low socioeconomic status groups.

The main reported risk factors for developing oral cancer are smoking and heavy alcohol consumption, especially a combination of both in elderly patients. Risk increases dramatically when alcohol use exceeds 6 oz. of distilled liquor, 15 oz. of wine, or 36 oz. of beer/day. The combination of heavy smoking and alcohol abuse is estimated to raise the risk 100-fold in women and 38-fold in men. Squamous cell carcinoma of the tongue may also result from any chronic irritation, such as dental caries, overuse of mouthwash, chewing tobacco, or the use of betel quid. Oral human papillomavirus (HPV), typically acquired via oral-genital contact, may have a role in the aetiology of some oral cancers; however, the role of HPV is not as clearly defined in oral cancer as it is in oropharyngeal cancer. About 40% of intraoral squamous cell carcinomas begin on the floor of the mouth or on the lateral

and ventral surfaces of the tongue. About 38% of all oral squamous cell carcinomas occur on the lower lip; these are usually solar-related cancers on the external surface [Moore *et al*, 2000; Bagan *et al*, 2010].

Oral lesions are asymptomatic initially, highlighting the need for oral screening. Most dental professionals carefully examine the oral cavity and oropharynx during routine care and may do a brush biopsy of abnormal areas. The lesions may appear as areas of erythroplakia or leukoplakia and may be exophytic or ulcerated. Cancers are often indurated and firm with a rolled border. As the lesions increase in size, pain, dysarthria, and dysphagia may result. Any suspicious areas should be biopsied. Incisional or brush biopsy can be done depending on the surgeon's preference. Direct laryngoscopy and oesophagostomy are done in all patients with oral cavity cancer to exclude a simultaneous second primary cancer. Head and neck CT usually is done and a chest x-ray is done; however, as in most sites in the head and neck, PET/CT has begun to play a larger role in the evaluation of patients with oral cavity cancer [Moore *et al*, 2000; Bagan *et al*, 2010].

Surgical therapy remains the main treatment approach, while adjuvant therapy such as radiation or chemotherapy may be used in combination with either treatment in advanced cases [Arduino *et al*, 2008; Blatt *et al*, 2017].

Despite of pronounced outstanding improvements in the diagnostic and therapeutic possibilities in oncology, OSCC holds still a poor prognosis with an estimated 5-year overall survival rate of 56% in the United States and Western Europe [Moore *et al*, 2000; Bagan *et al*, 2010]. In Italy, it has been reported an estimated 3-year and 5-year overall survival rate of 57% and 49%, respectively, the latter dramatically increasing up to 80% if considering advanced or metastatic cases. Although in recent years different prognostic biological and molecular

factors have been described in OSCC, they are far removed from a real impact on routine clinical care and more precise and reliable diagnostic tools are needed, since comprehensive histopathological staging of pathological specimens is still the most important determinant of post-operative management and prognosis prediction [Woolgar JA, 2006]. So far, TNM stage, the grade and the depth of invasion of the tumour seem to have still an important effect on the course of the disease. However, summarizing the survey of the available literature data, it may be stated that the prognostic value of the classical clinicopathological parameters is often uncertain and controversial [Arduino *et al*, 2008].

The gold standard in diagnosis of malignant and potentially malignant oral mucosal lesions is incisional biopsy and histopathological assessment. However, histopathological examination has concerns related to sampling errors, subjective errors in interpretation, and a lack of sensitivity to determine if a lesion is likely to progress to malignancy [Prasad *et al*, 2017].

Although many risk factors associated with OSCC have been well documented, few clinical studies about this topic have been conducted in Northern Italy.

The presence or development over time of lymph node (LN) metastasis significantly affects the survival of patients with OSCC, and successful detection and removal of positive LNs are crucial in the treatment of this disease. Despite an improved understanding of some of the factors influencing metastasis through in vitro and in vivo studies, metastatic disease still remains the principal event leading to death in patients with cancer. In OSCC, metastasis spreads predominantly via a lymphatic route with cervical LNs as the first location, whereas metastasis to distant sites is relatively uncommon [Roepman *et al*, 2005; Zanaruddin *et al*, 2013]. Knowledge of specific predictive factors could be of great expectations in their

clinical application for prognosis and therapeutic response in patients with OSCC. One of the chief challenges in cancer treatment, yet still the most efficient treatment, is to develop personalized cancer prediction, prevention, diagnosis and therapy, in which physicians no longer base their treatment choice on population based statistic, but rather on the specific characteristic of individual patients and their tumour.

1.2 miRNA

In the past few years, there has been a paradigm shift in understanding of non-coding RNAs (ncRNAs) and their role in cancer biology [Leucci et al, 2016; Manikandan *et al*, 2016]. After the discovery of alternative splicing in 1970s, major focus in various pathological and physiological processes shifted to role of proteins and protein coding RNAs and mutations as prominent mechanisms in disease aetiology and pathophysiology. However, in 1977, discovery of introns followed by Ribozymes gave indications for role of ncRNAs as regulatory molecules [Morris & Mattick, 2014]. Further, it was demonstrated that these regulatory RNAs were conserved from nematodes to humans [Pasquinelli *et al*, 2000]. The ncRNAs have been divided arbitrarily into two classes based on their size: small molecules (20 to 200 nucleotide long) are called ‘small ncRNAs’ and molecules larger than 200 nucleotides are called ‘long ncRNAs (lncRNAs)’.

Micro-RNAs represent the best studied small ncRNAs and are thought to regulate 60% of the human genome [Friedman *et al*, 2009].

MicroRNAs (miRNA), small RNA molecules of approximately 22 nucleotides, have been shown to be up- or down regulated in specific cell types and disease states. These molecules have become recognized as one of the major regulatory

gatekeeper of coding genes in the human genome [Bartels *et al*, 2009]. miRNAs are expressed in a tissue-specific manner, and changes in miRNA expression within a tissue type can be correlated with disease status [Lu *et al*, 2005]; they are transcribed by RNA polymerase II or III as longer primary-miRNA molecules, which are subsequently processed in the nucleus by the RNase III endonuclease Drosha and DGCR8 (the “microprocessor complex”) to form intermediate stem–loop structures approximately 70 nucleotides long called “pre- cursor miRNAs” (pre-miRNAs) [Gregory *et al*, 2004; Rosenfeld *et al*, 2008]. These pre-miRNAs fold to form imperfect stem–loop structures that are transported with the help of exportin-5 from the nucleus to the cytoplasm, where they undergo further processing by another RNase III endonuclease, Dicer [Lund *et al*, 2004]. Dicer removes the loop of the pre-miRNA to produce an imperfect duplex made up of the mature miRNA sequence and a fragment of similar size (miRNA*), which is derived from the opposing arm of the pre-miRNA. The miRNA strand of the duplex is loaded onto the RNA-induced silencing complex (RISC); the miRNA* separates from the duplex and is degraded [Lee *et al*, 2002]. miRNAs regulate gene expression by regulating mRNA translation and degradation. The mechanism by which they regulate mRNA is dependent on the degree of miRNA complementarily with the mRNA molecule [Hutvagner *et al*, 2002]. Numerous miRNAs have been associated with different tumours [Liu *et al*, 2010]; however, to date, OSCC is one of the less studied and considered cancer, in term of miRNA expression profile. MiRNAs can be secreted via EVs and by protein-miRNA complexes, such as high-density lipoprotein (HDL) and AGO2, which is part of the RISC. As already reported, circulating miRNAs are sensitive to protease treatment of plasma but are

protected from plasma RNase digestion. [Valdai *et al*, 2007; Arroyou *et al*, 2011; Ther C, 2011; Redis *et al*, 2012].

Differentially expressed microRNAs have been shown to promote tumorigenesis by deregulating different oncogenic pathways. miRNAS upregulated during tumorigenesis act as oncomiRs by down-regulating the tumour-suppressor proteins, resulting in increased cell proliferation and metastasis.

1.3 miRNA in HNSCC: literature review

Evidence suggests that miRNAs play an important role in progression, recurrence, metastasis and postoperative survival of HNSCC. Studies have investigated the utility of miRNAs as diagnostic/prognostic tools and as potential therapeutic targets and biomarkers that may improve the management and outcomes of HNSCC. Tardiest studies have shown differential miRNA expression in HNSCC [Masood *et al*, 2015; Koshizuka *et al*, 2017], and a different miRNA expression profile was observed for cancers of base of tongue, tonsil and postnasal space indicating a tissue and region specific miRNA profiles even if very specific data from only the oral cavity are actually missing.

In a recent meta-analysis of 13 independent microRNA profiling studies on HNSCC, 432 miRNAs were found to be differentially expressed, 90 of them reported in at least 2 studies. Out of these 90 microRNAs, 67 (74.4%) were observed to have a dependable direction of change: 46 (68.7%) were consistently up-regulated, and 21 (31.3%) were found to be down-regulated. Among these: seven miRNAs were up-regulated (miR-130b, miR-31, miR-21, miR-155, miR-223, miR-7, miR-34b), and four were down-regulated (miR-99a, miR-125b, miR-100, miR-375) [Chen *et al*, 2012].

miR-1251, miR-618 and miR-328 have been also suggested to be potential prognostic markers in HNSCC [Hui *et al*, 2016]. Another study described a six microRNA signature consisting of hsa-let-7c, hsa-miR-125b-2, hsa-miR-129-1, hsa-miR-337, hsa-miR-654, and hsa-miR-99a as an independent predictor for HNSCC patient survival [Shi *et al*, 2016]. Decreased expression of hsa-miR-200b-5p and hsa-miR-29c-3p in tumour was found to be associated with higher tumour grade, decreased expression of hsa-miR-29c-3p in adjacent tumour tissue was associated with worse survival and with worse relapse-free survival.

Oral cavity squamous cell carcinoma may be more aggressive at presentation and recurrence in young patients compared with older patients. Dysregulation of microRNAs has been associated with the development and prognosis of oral cavity cancer. In a recent study, aggressive tumours were found to have higher levels of let-7c, miR-130a-3p, miR-361-5p, miR-99a-5p, miR-29c-3p and let-7d-5p as compared to non-aggressive tumours in OSCC suggesting their pro-oncogenic role [Hilly *et al*, 2016].

In addition to onco-miRs, various tumour suppressive miRNAs have been also observed in HNSCC. For example miR-512-5p suppresses tumour growth by targeting hTERT [Li *et al*, 2015], miR-451 acts via targeting c-myc suppressing cell proliferation, miRNA-128 target BMI-1, BAG-2, BAX, H3f3b, and Paip2 inhibiting cell proliferation and viability [Hauser *et al*, 2015]; growth factor receptors like epidermal growth factor receptor (EGFR) and hepatocyte growth factor receptor (c-MET) are directly modulated by both miR-1 and miR-206 [Koshizuka *et al*, 2016] and epithelial-restricted with serine box (ESX), a member of the ETS transcription factor family which regulates EGFR was targeted by miR-124. Many miRNAs were also found to target cancer cell migration and invasion.

For example, miR-26a/b, miR-29a/b/c and miR-218 were found to directly regulate LOXL2 expression, thereby inhibiting invasion in HNSCC cells [Fukumoto *et al*, 2016]; miR-203 suppressed EMT by targeting NUAK family SNF1-like kinase 1 (NUAK1) [Obayashi *et al*, 2016]; Moreover, few miRNAs like miR-7 and miR-34b were found to play a mixed role in HNSCC [Sethi *et al*, 2014].

The increasing incidence of head and neck cancer highlights the need to better understand the role of HPV in the development of these particular types of cancers.

Around 10-20% of HNSCC are due to HPV infection mostly with HPV-16 or HPV-18 [Saulle *et al*, 2015]. HPV+*ve* HNSCC were shown to have a distinct miRNA profile that clearly distinguished them from HPV−*ve* ones [Wald *et al*, 2011; Lajer *et al*, 2012]. In particular, the study by Wald and co-workers (2011) described that 11 miRNAs were shown to be differentially expressed in HPV+*ve* as compared to the HPV-*ve* HNSCC cells or normal oral keratinocytes; among these, three miRNAs (miR-363, miR-33 and miR-497) were upregulated and eight were down-regulated, with miR-155 and miR-181a as the two most down-regulated miRNAs.

Over-expression of c-Myc was found to repress expression of several miRNAs: let-7a-1/f-1/d, miR-34a, miR-15a/16-1, miR-22, miR-26a-2, miR-29b-2/c, miR-30e/30c-1, miR-26b, miR-29a/b-1 and miR-146a [Chang *et al*, 2008b].

E2F was found to induce the expression of many miRNA genes which included let-7a-d, let-7i, miR-15b/16-2, miR-17-92 and miR-106b-25 [Woods *et al*, 2007].

Few studies also suggested that microRNAs may inhibit HPV induced carcinogenesis; for example, overexpression of miR-203 in differentiating suprabasal cells shows inhibitory effect on HPV amplification [Melar-New & Laimins, 2010].

Interaction of miR-125b with late gene of HPV [Nuovo *et al*, 2010], and miR-139-3p and miR-375 have been shown to regulate HPV E6/E7 levels co-ordinately [Sannigrahi *et al*, 2017].

The high risk E6 and E7 onco-proteins also increase expression and activity of methyl-transferase, DNMT1 and thus may affect miRNA expression [Au Yeung *et al*, 2010].

1.4 Extracellular Vesicles

In the last decade, extracellular vesicles (EVs) have gained significant attention as conceivable sources for biomarkers discovery, in different fields of medicine. These small membrane-bound vesicles are categorized into 3 different types: exosomes (60-200 nm diameter), microvesicles or ectosomes (100-1000 nm diameter), and apoptotic bodies (~5000 nm diameter) [Raposo *et al*, 2013].

Over the past decade EVs have been the subject of intense interest by investigators from miscellaneous fields. This has been largely due to their role in intercellular communication and the presence of diverse cargo such as proteins (including oncoproteins, tumour suppressors, transcriptional regulators, splicing factors etc.), RNAs (microRNA, mRNA, lncRNA etc), DNA, and lipids [Quesenberry *et al*, 2015; Xu *et al*, 2016]. It is now recognized that the function of EVs is dependent on the cargo they carry, which upon uptake by recipient cells can induce profound phenotypic changes. This horizontal transfer of bioactive molecules plays a vital role in tumour invasion and metastasis, immune modulation within the tumour microenvironment (TME), inflammation, epithelial-mesenchymal transition, neurobiology, pathogen dissemination – to name a few. It is now widely recognized that there are two major classes of EVs – shed

microvesicles (sMVs), that are also referred to as microparticles, and exosomes [Greening *et al*, 2017]. sMVs are formed by the outward budding and abscission of the plasma membrane, whereas exosomes originate from preformed multivesicular bodies that traffic to the plasma membrane whereupon fusion with the plasma membrane they release their contents (exosomes) into the extracellular environment by exocytosis [Xu *et al*, 2016]. sMVs and exosomes exhibit different protein and RNA profiles and biophysical properties. While sMVs are heterogeneous in size, because include MV released by perfectly healthy cells (100 nm to > 250 nm) and pre-apoptotic MV (300-1200 nm), exosomes are essentially homogeneous with a mean diameter of 60 to 120 nm [Greening *et al*, 2015; Quesenberry et al, 2015]; the buoyant densities of sMVs (1.1–1.2 g/mL) and exosomes (1.1 g/mL), assessed by iodixanol density gradient centrifugation, tend to overlap. However, an interesting development in the EV field over the past 5 years has been the recognition that exosomes and sMVs can be further fractionated into discrete sub-populations, based upon cell polarity [Tauro *et al*, 2013; Ji *et al*, 2014], buoyant density [Rogert *et al*, 2016; Willms *et al*, 2016] and mechanism of biogenesis [Bobrie *et al*, 2012], that display different bioactive molecule compositions. Since the same cell may release both sEV and exosomes, and overlapping mechanism of biogenesis and content are present, it has been suggested to cell them collectively EV [Gould and Raposo, 2013]. At this juncture, it has been difficult to discern functional differences between EV subtypes.

EVs are secreted under different pathophysiologic condition into the extracellular environment by a variety of cell types, also promoting tumour progression, survival, invasion and angiogenesis [van Doormaal *et al*, 2009; Grange *et al*, 2011; Camussi *et al*, 2013]. The biomolecule cargo of EVs is remarkably stable in biological fluids

and protected against exogenous RNases and proteases, owing to its encapsulation, or association with RNA-binding or DNA-binding proteins or lipoprotein complexes [Mitchell *et al*, 2008; Kosala *et al*, 2010; Vicker *et al*, 2011]. Thus, EVs might be firm under hostile physical conditions (*e.g.* extremes in pH, long-term storage and multiple freeze-thaw cycles) making them an attractive source for cancer biomarker development [Mitchell *et al*, 2008]. It is now established that cancer cells release more EVs than normal cells, in order to regulate their micro-environment via the horizontal transfer of bioactive molecules: proteins, lipids, and nucleic acids.

As said, one of the most exciting advances in EV biology has been the demonstration that cancer cell-derived exosomes participate in crucial steps of metastatic spread of primary tumour, from reprogramming of local neoplastic stroma to pre-metastatic niche establishment.

1.5 EVs in OSCC: literature review

Circulating miRNAs are one of the most promising next-generation biomarkers for cancer diagnosis, establishing the novel concept that EV miRNAs could have potential not only as putative biomarkers but also for reflecting cancer progression. The identification of molecular signatures in biological fluids could create the so-called “liquid biopsies”, which would successfully overcome many of the faces associated with traditional tissue sampling (e.g. invasiveness and heterogeneity).

To date, the pathogenic role of EVs has been studied in several cancers [Nawaz *et al*, 2014], but very few data are available for OSCC. The presence of EVs in

sera of patients with head and neck cancer has been studied [Bergmann *et al*, 2009], with only 27 patients reported with OSCC [Kim *et al*, 2005]; no data are available however for the EVs in saliva of patients with OSCC and for the EVs miRNA expressions. The development of forceful techniques and sensitive capture platforms that use readily available body fluid could offer new approaches for OSCC diagnosis, staging and therapy.

Recently, some authors aimed to understand the biological effects of EVs isolated from two cell lines of OSCC (e.g., SCC15 and HSC3) on endothelial cell tubulogenesis [de Andrade *et al*, 2017]. The results have showed that EVs derived from both those cell lines displayed typical spherical-shaped morphology and expressed the EV markers CD63 and Annexin II. Although the average particle concentration and size were quite similar, SCC15-derived EVs promoted a pronounced tubular formation associated with significant migration and apoptosis rates of the endothelial cells, whereas EVs derived from HSC3 cells inhibited significantly endothelial cell tubulogenesis and proliferation. The findings of this study revealed that EVs (derived from different OSCC cell lines by a polymer-based precipitation method) could promote pro- or antiangiogenic effects.

Packaging of small molecular factors, including miRNAs, into small extracellular vesicles has been also reported as a possible contribute to malignant phenotypes; this could facilitate communication between cancer cells and tumour stroma. The process by which some miRNAs are enclosed in EVs is selective rather than indiscriminate, with selection in part governed by specific miRNA sequences [Dickman *et al*, 2017]. Herein, it has been described the selective packaging and removal via EVs of four miRNAs (miR-142-3p, miR-150-5p, miR-451a, and miR-223-3p) in a panel of oral dysplasia and oral squamous cell carcinoma cell lines.

Inhibition of exosome export protein Rab27A increased intracellular concentration of these miRNA candidates and prevented their exclusion via EVs. Increased intracellular miR-142-3p specifically was found to target TGFBR1, causing a decrease in TGFBR1 expression in donor cells and a reduction of malignant features such as growth and colony formation. Contrariwise, increased excretion of miR-142-3p via donor cell EVs and uptake by recipient endothelial cells was found to reduce TGFBR1 activity and cause tumour-promoting changes in these cells in vitro and in vivo [Dickman *et al*, 2017].

Recent study, moreover, has established the increased circulating micro-particles (MPs) and their pro-coagulant activity in OSCC, evaluating different phenotypes of circulating MPs in OSCC patients and exploring their clinical significance and effects on angiogenesis (a critical event in tumour progression). Correlations between circulating MPs and clinicopathologic data, micro-vessel density, and proangiogenic factor levels in patients with OSCC were analysed by immunohistochemistry [Ren *et al*, 2016]. Those results showed that the levels of circulating MPs as well as the subsets of platelet-derived, endothelium-derived, and pan-leukocyte MPs in stages III to IV OSCC were significantly higher than stages I to II and healthy subjects. Moreover, these increased circulating MPs were significantly correlated with tumour size, TNM stages, micro-vessel density, and expression levels of vascular endothelial growth factor (VEGF) and matrix metallopeptidase 9 (MMP9).

It has been also reported that report that exosomes are able to modify the radiation response of head and neck different cancer cell lines (e.g., BHY and FaDu) [Mutschelknaus *et al*, 2016]. Authors had demonstrated that radiation could influence both the abundance and action of exosomes on recipient cells.

Exosomes transmit pro-survival effects by promoting the proliferation and radio-resistance of head and neck cancer cells. Taken together, this study had showed a functional role of exosomes in the response of tumour cells to radiation exposure within a therapeutic dose range and encourages that exosomes could be useful objects of study for a better understanding of tumour radiation response.

CHAPTER 2 - PATIENTS AND METHODS

2.1 Patients' selection and characteristics

The enrolled subjects were attending the Oral Medicine Section of the Department of Surgical Sciences, University of Turin, CIR- Dental School, between January and June 2015.

Patients with biopsy-proven OSCC were involved; the following patients were excluded: 1) <18 years of age, 2) pregnant or breast feeding, 3) psychiatrically or mentally unstable, 4) patients who refused to be part of this study.

Local ethical committee approval (n° 310/2015, "A.O.U. Città della Salute e della Scienza di Torino", Turin, Italy) was obtained and all patients gave written informed consent.

Demographic information, agreement of histological diagnosis between the first biopsy specimen and surgical specimen (considered as the percentage of specimens diagnosed as OSCC on both biopsy, which has to be 100% in total cases), age at the time of diagnosis and gender, smoking (current or former smoker vs. non-smoker), alcohol consumption (current of former drinker vs. non-drinker), tumour site, T classification and neck nodes involvement at the time of diagnosis [Hermanek *et al*, 1988; UICC, 1997], treatment and outcome have been preciously truthfully detailed. Subjects had to be resident in Piedmont region, North-west Italy. Tumour locations have been divided as having occurred in the alveolar mucosa, lateral border of the tongue and dorsum, mouth floor, palate, buccal mucosa, lips, retromolar area and anterior tonsillar pillar. The cases have been also classified according to treatment modality: surgery alone, radiotherapy

alone, surgery in combination with radiotherapy and no treatment. Ablative surgical resection was the main modality of treatment and patients who presented with node positive neck disease underwent also elective neck dissection in the same way as when tumour invasion of the midline structure was observed. Adjuvant radiotherapy with local dose field of 50 to 66 *Gy* was used in patients with positive or close margins, vascular or perineural invasion and extracapsular spread.

Typical follow up schedule will be 1, 2, 3, 6, 9 and 12 months postoperatively in the first year, followed by every 6 months thereafter. Computed tomography scans or resonance imaging of the head and neck region will be performed 6 months postoperatively and afterwards on annual basis. The evolution of the disease will be detailed as follow: 1) healing (H): if, during the follow up period, new lesions will not appear in the same place of the primary disease; 2) oncological event (OE): if during the follow up period a dysplastic or neoplastic lesion will be diagnosed again in the oral cavity.

Healthy subjects presenting no clinically detectable oral lesions were recruited as controls. Cases and controls have been stratified for age, gender, and risk factors.

2.2 Saliva collection

A single research (PGA) recruited all the subjects, obtaining signed informed consent and providing a written assessment that had questions on demographics, social and health behaviours.

As previously reported [Arduino *et al*, 2015], all subjects were asked to refrain from eating, drinking, or oral hygiene for at least one hour prior to collection (usually between 9 and 11 a.m.); they rinsed out their mouths with water and then waited at least 5 min before spitting into a 50 ml Falcon tube.

Participants were instructed not to cough or forcefully expectorate to allow for unstimulated saliva collection.

Each subject was asked to provide at least 5 cc of saliva.

2.3 HPV-16 in situ hybridization

A single operator (LM), unaware of the study and external to the doctoral research study, performed this analysis.

In situ hybridization (ISH) for HPV was performed on haematoxylin and eosin sections using the Bond TM Ready-to-Use ISH HPV Probe (Leica Biosystems, Newcastle, UK) that targets the following subtypes: 16, 18, 31, 33, 51.

ISH was carried out following the manufacturer's instructions on the automated Leica BOND system (BOND-MAX, Leica Biosystems).

2.4 EV isolation

The sample of saliva from patients with OSCC and healthy controls was diluted 1:1 with PBS (phosphate buffered saline) and centrifuged at 3000g for 15 minutes at room temperature to remove cells, debris and bacteria (Refrigerated Microfuge SIGMA 1-15K; SciQuip Ltd, Newtown, Wem, Shropshire, SY4 5NU).

The supernatant was filtered with 0.2 μm filters and transferred to a sterile tube. The precipitation solution (65 μL per 250 μL of saliva) were added to the samples then processed as previously described [Deregibus *et al*, 2016].

The pellet was resuspended in 100 μL of PBS for NanoSight analysis or 600 μL of Lysis/Binding Buffer (mirVana Isolation Kit, Thermo Fisher Scientific, Waltham, MA, USA) and stored at -80°C for subsequent RNA extraction.

2.5 EV characterization

The EV samples isolated from saliva were diluted 1:200 in physiologic solution and analysed by NanoSight LM-10 (Malvern Instruments Ltd, Malvern, UK).

The average number and size of EVs were measured by Nanoparticle Tracking Analysis (NTA) software (Malvern Instruments Ltd).

Analysis results include: mean dimension, standard deviation and concentration of EVs. To obtain EV release index, Nanosight results were normalized basing on culture conditions (culture medium volume, culture time, and EV re-suspension volume after ultracentrifugation and dilution index for Nanosight analysis).

Transmission electron microscopy (TEM) of negatively stained EVs (NanoVan, Nanoprobes, Yaphank, NK, USA) was performed as previously described [Deregibus *et al*, 2016]. EVs were observed by an electron microscope Jeol JEM 1010 (Jeol, Tokyo, Japan).

2.6 Western Blot analysis

Protein concentration in EV samples was measured by Bradford assay. Protein samples were loaded on polyacrylamide gel at the concentration of 30 µg/well and separated by SDS/PAGE, using 4-15% precast gel (Mini-PROTEAN® Precast Gels, Bio-Rad, Hercules, CA, USA).

Proteins were transferred on nitrocellulose membranes by liquid electrophoresis. Membranes were immunoblotted by polyclonal antibodies anti-CD9, CD63, TSG101, and Alix (Santa Cruz Biotechnologies, Dallas, TX, USA). Protein-bands were detected by chemiluminescent Clarity™ ECL Western Blotting Substrate (Bio-Rad) and analysed by ChemiDoc™ XRS+System (Bio-Rad).

2.7 RNA extraction and quantification

miRNAs were extracted from purified EVs by mirVana Isolation Kit (Thermo Fisher Scientific), according with kit instruction. RNA concentration and quality were measured by Nanodrop ND-1000 (Thermo Fisher Scientific).

2.8 Microarray miRNA expression data

To select differentially expressed miRNAs, microarray analysis was performed on EVs from patients with OSCC and healthy controls. The concentration of selected RNA samples was up to 20 ng/µl and the quality was good. 50 ng of total RNA was retro-transcribed to cDNA with TaqMan® MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific) and cDNA was pre-amplified with Megaplex™ RT Primers, Human Pool Set v3.0 and TaqMan® PreAmp Master Mix (Thermo Fisher Scientific) by using Veriti Thermal Cycler (Thermo Fisher Scientific). The expression profile of a panel of 754 human microRNA was evaluated by TaqMan® Array Human MicroRNA Card Set v3.0 (Thermo Fisher Scientific) with the real-time thermal cycler 7900HT (Thermo Fisher Scientific).

2.9 qRT-PCR

Array data was then validated by qRT-PCR on salivary EVs from patients with OSCC and healthy controls. 500 ng of total RNA was retro-transcribed to cDNA with miScript II RT Kit (Qiagen, Hilden, D). We evaluated the expression of miRNAs significantly up-regulated in the patients group compared to the control group, and of miRNAs only expressed by the OSCC patients group. Each sample was run in triplicate and each miRNA-specific primer was run in a separate reaction. SnoRNA RNU6B and miR-191 were used as endogenous control, they were previously

described as stably expressed in saliva samples [Park *et al*, 2009; Momen-Heravi *et al*, 2014; Sauer *et al*, 2014] the and microarray analysis showed a stable expression in tested samples. The qRT-PCR reaction mix was composed of 2 ng of cDNA, 100 nM miScript Universal Primer (Qiagen), 100 nM miRNA-specific primer (Eurofins Genomics, Ebersberg, D), 5 µl QuantiTect SYBR Green PCR Master Mix (Qiagen), RNase free water (Qiagen) to reach the reaction volume of 10 µl. The Real-Time Thermal Cycler Quant Studio 12k (Thermo Fisher Scientific) was used.

2.10 Enrichment analysis

Enrichment analysis for biological pathways was performed for miRNAs that were found to be up-regulated or only expressed by OSCC patients. Kyoto Encyclopedia of Gene and Genomes (KEGG) pathway analysis was performed by DIANA-mirPath v.3.0 [Vlachos et al, 2015] online software. miRNA targets were searched on microT-CDS [Paraskevopopulou *et al*, 2013]. Results were merged by "pathway-union" criteria. The p-value was calculated by DIANA online software with False Discovery Rate (FDR) correction, p value threshold at 0.05 and MicroT threshold at 0.8. Fisher's exact test was used as the enrichment analysis method.

2.11 Discrimination power analysis

The discrimination power of the up-regulated miRNAs as biomarkers for OSCC diagnosis was evaluated by the Receiver Operating Characteristic (ROC) curves [Hajian-Tilaki *et al*, 2013]. The ROC curves were constructed on the base of the expression levels values, expressed as relative quantification (RQ), of controls and OSCC patients by the demo version of GraphPad Prism 6.01 software. Sensitivity,

specificity, area under curve (AUC) and p value were calculated by the software. The optimal threshold value was decided using Youden's index (sensitivity + specificity-1) [Ruopp *et al*, 2008].

2.12 Statistical analysis

Sample size has not been estimated based on the lack of any previously reported changes in EV miRNA expression between OSCC and healthy samples. However, we have been planning a study with 5 case patients and paired control subjects, in order to detect a true difference in the mean response of experimental and control subjects of +-0 .71 standard deviation with an 80% power. The Type I error probability associated with this test of the null hypothesis that the population means of the experimental and control groups are equal is 0.05. If we suppose a Bonferroni setting, the detection of true difference in the mean response is +-1.71 standard deviation. For the replication set, we have considered 15 cases and 15 controls: the detection of true difference in the mean response should be +-0.59 standard deviation (PS Power and Sample Size Calculator V3.0; ref: Dupont WD, Plummer WD: "Power and Sample Size Calculations for Studies Involving Linear Regression", Controlled Clinical Trials 1998; 19:589-601).

For array data analysis, SDS Software v.2.3 (Thermo Fisher Scientific) was used to calculate Raw Ct values, with automatic baseline and threshold. ExpressionSuite Software 1.0.3 (Thermo Fisher Scientific) was used to calculate RQ values with global normalization and to identify miRNAs significantly differentially expressed between patients and controls. Values of Ct > 35 or Amp score < 0.7 was excluded from analysis. For qRT-PCR data analysis, Excel software (Microsoft Office 365 ProPlus) was used to calculate ΔCt, -$\Delta\Delta$Ct, and RQ

(2-ΔΔCt) for patients and controls. Statistical analysis was performed on RQ values by the demo version of GraphPad Prism 6.01 software using unpaired non-parametric Mann-Whitney test. One-sided p value was set, since array data showed that the miRNAs were up-regulated in patients compared to control group. MiRNA up-regulation in patients was validated by setting confidence level as 95% (p value ≤ 0.05).

CHAPTER 3 - RESULTS

3.1 Patient characterization *(Appendix A_Table I)*

Twenty patients with OSCC were analysed, 13 men and 7 women (mean age 65.75 ± 8.34 years). The T staging system identified the following lesion categories: T1 (n=6), T2 (n=9), T3 (n=4), T4 (n=1); histologically, on the biopsy specimen, two patients were identified as well differentiated, ten as moderately differentiated and four as poorly differentiated. The lateral border of the tongue was the most commonly affected site (31.3%), followed by the floor of the mouth (25%), gingiva and palate (18.7% respectively) and lower lip (6.3%); 31.25% of all patients were tobacco smokers. One, out of 20 patients, was positive for HPV (5%). OSCC patients and healthy controls were matched with regards to age, gender and risk factors.

3.2 EV characterization *(Appendix B, C and D)*

EVs isolated from saliva samples by precipitation [Deregiubus *et al*, 2016] appeared as a heterogeneous population of exosomes and microvesicles according to NanoSight, with size ranging from 100 to 300 nm (Fig. 1, left panels). TEM analysis showed the characteristic shape, aspects, and dimensions of EVs (Fig. 1, middle panels). Western Blot analysis demonstrated the expression of the exosome markers CD63, CD9, TSG 101, and Alix (Fig. 1, right panels). We observed that the size and concentration of salivary EVs from OSCC patients were slightly increased compared to healthy controls, however the differences were not significant. The expression of the exosome markers was similar for both OSCC

patients and controls.

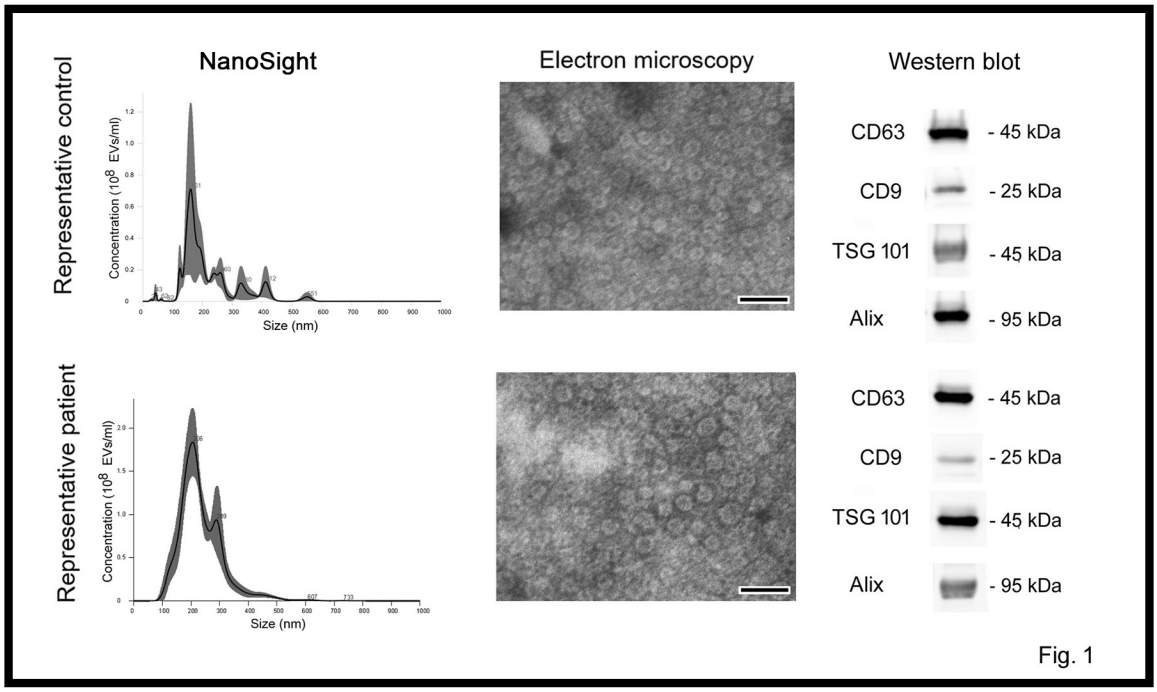

Figure 1: Characterization of salivary EVs. *Representative NanoSight image of isolated EVs showing particle size (nm) /concentration (10^8 EVs/ml) of a representative control (upper-left) and a representative OSCC patient (lower-left) and representative transmission electron microscopy image of purified EVs negatively stained with NanoVan (JEOL Jem-1010 electron microscope, black line=200 nm) of one control (upper-central) and one patient (lower-central). Representative western blots of the expression of the exosome markers CD63, CD9, Tsg101, and Alix, on salivary EVs from one control (upper-right) and one OSCC patient (lower-right). Four experiments were performed with similar results.*

3.3 Microarray for miRNA expression analysis

The analysis of miRNA expression in salivary EVs from five controls compared to five OSCC patients showed that, in tumour EVs, five miRNAs (miR-412-3p, miR-489-3p, miR-512-3p, miR-597-5p, and miR-603) were significantly up-regulated, while six miRNAs (miR-193b-3p, miR-30e-3p, miR-376c-3p, miR-484, miR-720, and miR-93-3p) were significantly down-regulated compared to controls (Tab. 1).

Moreover, eight miRNAs were only detectable in EVs from OSCC patients, while fourteen miRNAs were only expressed in EVs from controls (Tab. 2). Finally, EVs from OSCC patients showed a non-significant up-regulation of sixty-four miRNAs, compared to controls (Tab. 3).

	miRNA	RQ	RQ Min	RQ Max	P Value
	miR-412-3p	9.404	5.855	15.104	0.007
UP	miR-489-3p	35.07	17.47	70.41	0.020
	miR-512-3p	5.13	2.05	12.85	0.031
	miR-597-5p	3.62	1.83	7.14	0.026
	miR-603	2.36	1.30	4.28	0.042
DOWN	miR-193b-3p	0.26	0.05	1.27	0.042
	miR-30e-3p	0.21	0.07	0.65	0.010
	miR-376c-3p	0.17	0.08	0.32	0.035
	miR-484	0.44	0.23	0.85	0.048
	miR-720	0.29	0.09	0.96	0.017
	miR-93-3p	0.39	0.15	1.05	0.044

Table 1: miRNAs differentially expressed in salivary EVs of patients with OSCC compared to healthy controls. *MiRNAs were considered significantly up-regulated (UP) or down-regulated (DOWN) for p value < 0.05.*

	miRNA	Ct mean	St dev
only patients	miR-27a-3p	29.5	1.33
	miR-302b-3p	33.9	0.34
	miR-337-5p	30.0	1.32
	miR-373-3p	30.9	0.40
	miR-494-3p	33.5	1.24
	miR-517b	32.2	1.57
	miR-520d-3p	31.2	1.42
	miR-645	34.2	0.40
only controls	miR-126-5p	32.6	1.86
	miR-127-3p	32.1	1.39
	miR-1276	31.4	0.95
	miR-1289	33.8	1.34
	miR-144-5p	32.4	2.55
	miR-182-5p	32.2	1.11
	miR-30d-3p	31.3	1.22
	miR-520c-3p	32.1	1.70
	miR-550a-5p	32.1	0.29
	miR-628-3p	30.5	0.70
	miR-944	32.9	0.93
	miR-99a-3p	31.1	1.21
	miR-942	31.1	0.85
	RNU48	32.1	1.03

Table 2: miRNAs only expressed in salivary EVs of the OSCC patient group or the control group. *MiRNAs were considered as expressed only by patients or controls when at least 3 sample out of 5 have Ct > 35.*

miRNA	Cт Mean	RQ	RQ Min	RQ Max	Cт Mean	RQ	RQ Min	RQ Max	P-Value
miR-873-5p	30.24	1.00	0.39	2.55	29.29	3.01	1.29	7.01	0.07
miR-218-5p	29.73	1.00	0.19	5.29	27.99	4.15	1.82	9.47	0.08
miR-511-5p	31.12	1.00	0.13	7.61	29.28	5.01	1.80	13.92	0.08
miR-424-5p	31.96	1.00	0.54	1.85	31.70	1.62	1.46	1.79	0.09
miR-155-5p	29.41	1.00	0.54	1.86	28.79	2.15	1.05	4.41	0.11
miR-668-3p	30.28	1.00	0.01	77.34	29.99	1.18	0.41	3.42	0.12
miR-298	32.64	1.00	0.34	2.97	31.52	3.36	0.20	55.58	0.12
miR-130b-3p	27.61	1.00	0.41	2.47	27.04	2.08	0.99	4.35	0.12
miR-383-5p	33.73	1.00	0.02	59.32	30.68	10.48	2.53	43.48	0.14
miR-485-3p	31.23	1.00	0.42	2.38	30.26	3.05	0.20	46.14	0.15
miR-503-5p	31.79	1.00	0.28	3.56	29.41	8.08	0.58	113.26	0.15
miR-372-3p	32.04	1.00	0.45	2.20	30.90	3.07	0.57	16.49	0.16
miR-642a-5p	30.87	1.00	0.24	4.12	30.02	2.37	0.95	5.92	0.16
miR-604	31.34	1.00	0.29	3.40	28.66	6.62	0.33	131.71	0.17
miR-381-3p	32.72	1.00	0.36	2.79	32.03	2.49	0.79	7.86	0.17
miR-570-3p	33.13	1.00	0.15	6.81	30.88	7.41	1.06	51.96	0.17
miR-545-3p	31.69	1.00	0.44	2.26	29.42	7.53	1.83	30.90	0.17
miR-204-5p	29.18	1.00	0.45	2.22	28.79	1.83	0.28	11.99	0.18
miR-222-5p	30.58	1.00	0.44	2.26	29.39	2.35	0.57	9.57	0.19
miR-211-5p	28.28	1.00	0.31	3.23	26.95	3.51	0.40	31.16	0.19
miR-548d-5p	30.94	1.00	0.08	12.97	29.65	3.41	0.21	55.27	0.19
miR-125a-3p	32.17	1.00	0.04	22.80	30.72	3.83	0.52	27.98	0.19
miR-548b-5p	27.84	1.00	0.31	3.26	27.27	2.07	0.21	20.40	0.20
miR-502-5p	31.36	1.00	0.31	3.24	30.32	2.86	0.54	15.23	0.21
miR-422a	31.34	1.00	0.43	2.30	30.86	1.95	1.00	3.78	0.21
miR-212-3p	28.27	1.00	0.62	1.62	28.29	1.37	0.48	3.89	0.22
miR-516b-5p	32.47	1.00	0.09	11.52	31.99	1.95	0.22	17.77	0.22
miR-548c-5p	27.77	1.00	0.22	4.47	27.02	2.35	0.26	21.17	0.23
miR-1290	25.61	1.00	0.50	2.02	24.96	1.56	0.71	3.41	0.23
miR-509-5p	30.93	1.00	0.22	4.45	30.13	2.44	0.19	30.54	0.24
miR-1305	32.32	1.00	0.01	78.93	25.30	129.16	0.44	37,579.14	0.25
miR-34c-5p	31.41	1.00	0.37	2.74	31.21	1.60	0.25	10.26	0.28
miR-31-5p	26.51	1.00	0.68	1.46	26.46	1.30	0.79	2.14	0.29
miR-191-3p	30.87	1.00	0.26	3.82	29.09	3.41	0.70	16.53	0.31

miR-150-5p	27.59	1.00	0.47	2.12	27.52	1.47	0.54	4.02	0.31
miR-450b-5p	30.55	1.00	0.39	2.57	30.73	1.38	0.23	8.15	0.32
miR-518d-3p	31.20	1.00	0.29	3.50	30.94	1.58	0.05	46.90	0.32
miR-135b-3p	30.97	1.00	0.23	4.35	30.14	1.92	0.45	8.12	0.32
miR-1255b-5p	29.35	1.00	0.10	9.87	27.16	4.72	1.12	19.83	0.35
miR-331-5p	29.11	1.00	0.39	2.54	28.87	1.65	0.66	4.13	0.35
miR-1247-5p	29.27	1.00	0.00	295.36	22.19	130.42	0.43	39,287.06	0.35
miR-183-3p	30.81	1.00	0.39	2.58	29.31	2.77	0.41	18.83	0.36
miR-616-3p	30.39	1.00	0.11	9.37	30.10	2.11	0.06	75.56	0.37
miR-323-3p	32.09	1.00	0.19	5.20	31.22	2.56	0.86	7.63	0.37
miR-28-3p	26.91	1.00	0.18	5.55	24.76	6.20	0.06	610.50	0.37
miR-141-3p	26.34	1.00	0.36	2.76	24.24	6.28	0.02	2,169.60	0.37
miR-628-5p	31.97	1.00	0.22	4.60	26.07	87.56	0.05	170,413.84	0.37
miR-486-5p	25.53	1.00	0.00	7,021.08	25.90	1.14	0.00	5,223.24	0.38
miR-618	30.39	1.00	0.56	1.79	30.51	1.21	0.16	8.91	0.38
miR-487b-3p	32.03	1.00	0.04	25.69	28.76	14.69	0.03	7,714.66	0.39
miR-649	32.02	1.00	0.16	6.26	31.54	1.37	0.01	175.83	0.39
miR-29b-3p	30.40	1.00	0.82	1.21	27.45	10.33	0.00	202,876.83	0.39
miR-31-3p	29.08	1.00	0.01	80.43	24.15	33.06	0.00	10,242,902.00	0.39
miR-320a	24.98	1.00	0.50	2.00	24.98	1.40	0.68	2.89	0.39
miR-296-5p	29.03	1.00	0.62	1.61	29.18	1.26	0.41	3.86	0.40
miR-181c-3p	32.16	1.00	0.08	12.68	30.12	4.09	0.53	31.71	0.40
miR-193a-5p	26.24	1.00	0.44	2.28	26.09	1.54	0.67	3.58	0.40
miR-99b-3p	31.92	1.00	0.25	3.99	31.00	1.88	0.37	9.48	0.40
miR-369-5p	33.86	1.00	0.26	3.87	31.30	10.96	0.04	2,836.80	0.41
miR-708-5p	30.05	1.00	0.35	2.86	29.81	1.65	0.36	7.61	0.41
miR-1249-3p	29.91	1.00	0.11	9.07	26.88	9.25	0.00	48,952.13	0.43
miR-636	26.12	1.00	0.01	97.45	26.13	1.24	0.01	184.53	0.44
miR-409-3p	33.14	1.00	0.17	6.03	32.81	1.29	0.02	83.40	0.49
miR-184	31.67	1.00	0.36	2.76	32.04	1.08	0.18	6.36	0.50

Table 3: miRNAs not-significantly up-regulated in salivary EVs of the OSCC patient group compared to the healthy control group. *MiRNAs were selected with RQ > 1 in OSCC patient group and p value between 0.05 and 0.5.*

3.4 qRT-PCR expression analysis

After the initial screening by microarray, we selected eleven miRNAs for the subsequent validation by qRT-PCR on 15 OSCC patients and healthy controls. We chose the five miRNAs significantly up-regulated in OSCC patients (miR-412-3p, miR-489-3p, miR-512-3p, miR-597-5p, and miR-603) and six miRNAs expressed only by OSCC patients (miR-27a-3p, miR-302b-3p, miR-337-5p, miR-494-3p, miR-517b, and miR-520d-3p). The analysis of the up-regulated miRNAs confirmed the significant up-regulation of miR-412-3p and miR-512-3p in OSCC patients with respect to controls (Fig. 2A). The qRT-PCR analysis of miRNAs detected by gene array only in patients showed that miR-27a-3p, miR-337-5p, miR-494-3p, and miR-520d-3p were overexpressed in patients but still present in controls (Fig. 2B). Among these miRNAs, miR-27a-3p and miR-494-3p showed statistically significant up-regulation in OSCC patients (Fig. 2B). MiR-302b-3p and miR-517b-3p were confirmed to be expressed only in patients (data not shown).

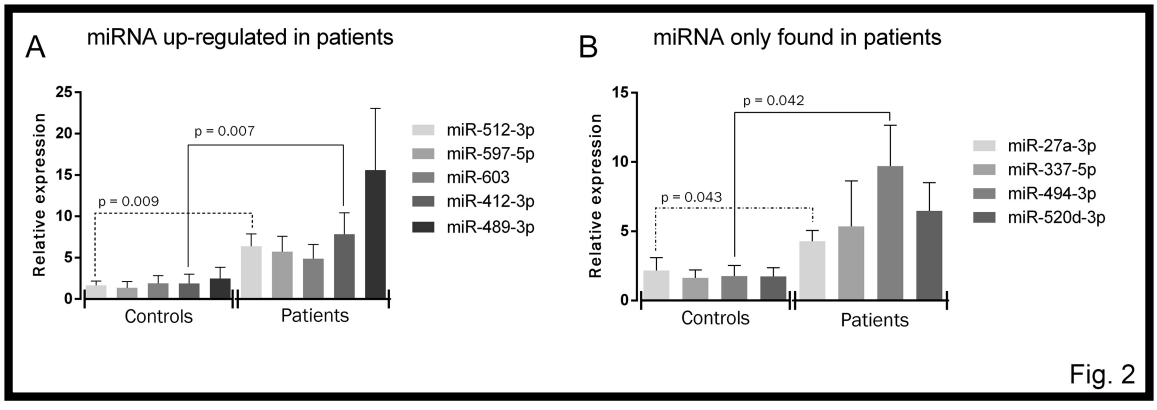

Figure 2: miRNA relative expression detected by qRT-PCR in salivary EV samples from OSCC patients compared to normal subjects. *(A) Expression levels of miRNA found to be significantly up-regulated in patients. (B) Expression levels of miRNA found to be only expressed by patients. The bars represent mean relative expression (2^-ΔΔCt) of control and patient groups ± SEM, p value < 0.05 (Mann-Whitney test) are reported.*

3.5 KEGG pathway enrichment analysis

The four miRNAs (miR-512-3p, miR-412-3p, miR-27a-3p, and miR-494-3p) confirmed to be up-regulated and the two miRNAs (miR-302b-3p and miR-517b-3p) confirmed to be only expressed in OSCC patients by qRT-PCR were selected for KEGG pathway enrichment analysis. Eight pathways were found to be significantly enriched for at least two of the tested miRNAs (Fig. 3A). Figure 3B shows the number of predicted/functional target genes involved in each pathway (Fig. 3B). Figure 3C shows, for each miRNA, the number of predicted/functional target genes in each pathway and the respective p value (Fig. 3C).

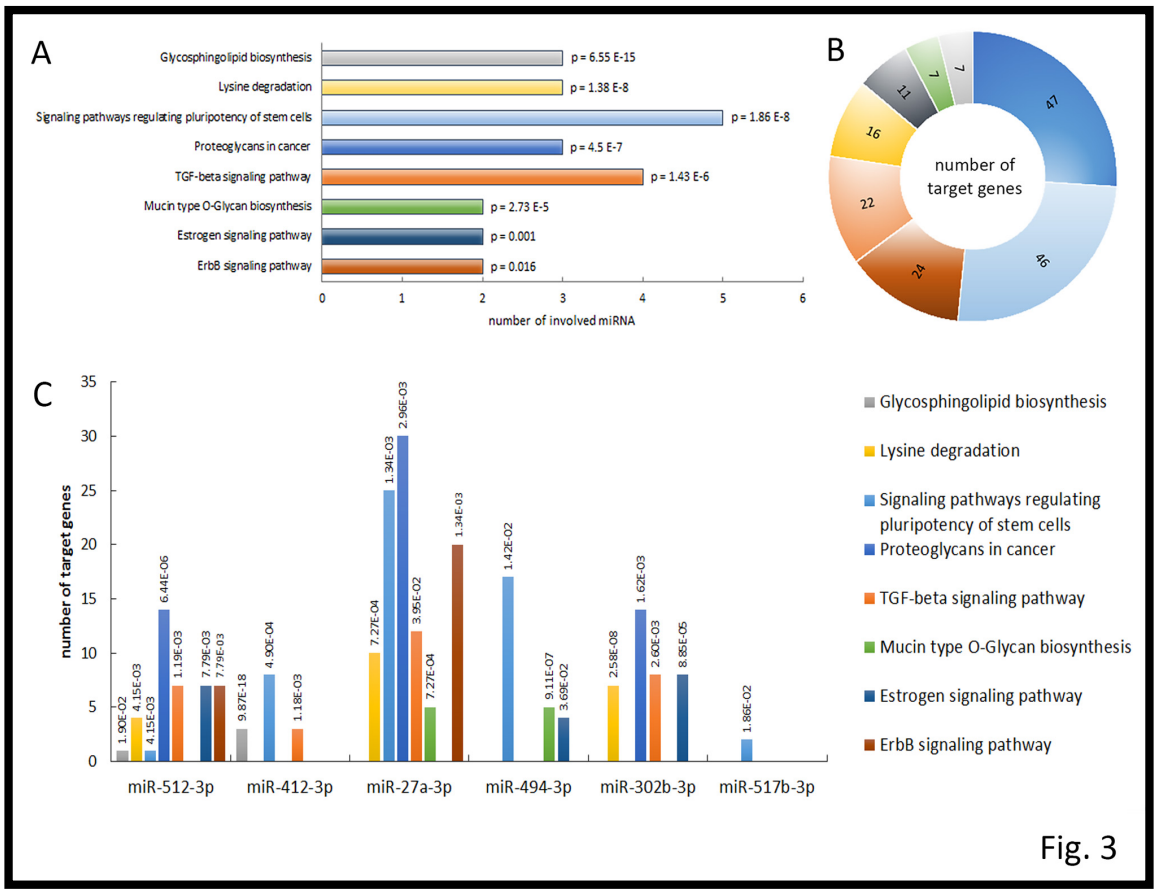

Figure 3: KEGG pathway enrichment analysis for up-regulated miRNA (miR-512-3p, miR-412-3p, miR-27a-3p, miR-494-3p) or miRNA only expressed in salivary EV from OSCC patients (miR-302b-3p, miR-517b-3p). *(A) The graph shows the significantly enriched biological*

pathways labelled with their respective p values. X axis shows the number of miRNAs involved in each pathway. (B) The graph shows the number of target genes for each enriched pathway. (C) The bars represent the number of target genes for each miRNA in each pathway and the respective p value. The legend (lower-right) shows the color assigned to each pathway and is valid for all the figure (A-C). The p value was calculated with FDR correction and threshold was set as 0.05.

3.6 Discrimination power of miRNAs as OSCC biomarkers

ROC curves were constructed to evaluate the discrimination power of the four up-regulated miRNAs as biomarkers for OSCC diagnosis. For each miRNA, figure 4 displays the ROC curves, expressing the sensitivity (true positive rate) versus 1-specificity (false positive rate) at various cut-off values, the AUC, indicating the discrimination power of the biomarker, and the p value. The optimal threshold value was set as the maximum Youden's index (sensitivity + specificity-1) and is represented as a black circle (Fig. 4). MiR-512-3p (Fig. 4A) and miR-412-3p (Fig. 4B) showed high sensitivity and specificity, with high AUC values of 0.847 and 0.871 respectively, and p values lower than 0.02. MiR-27a-3p (Fig. 4C) and miR-494-3p (Fig. 4D) showed AUC respectively of 0.75 and 0.763. The p value was 0.083 for both miRNA, and the maximum Youden's index showed that miR-27a-3p was specific, but not sensitive (50% probability to detect false negatives), while miR-494-3p was sensitive, but not specific (50% probability to detect false positives).

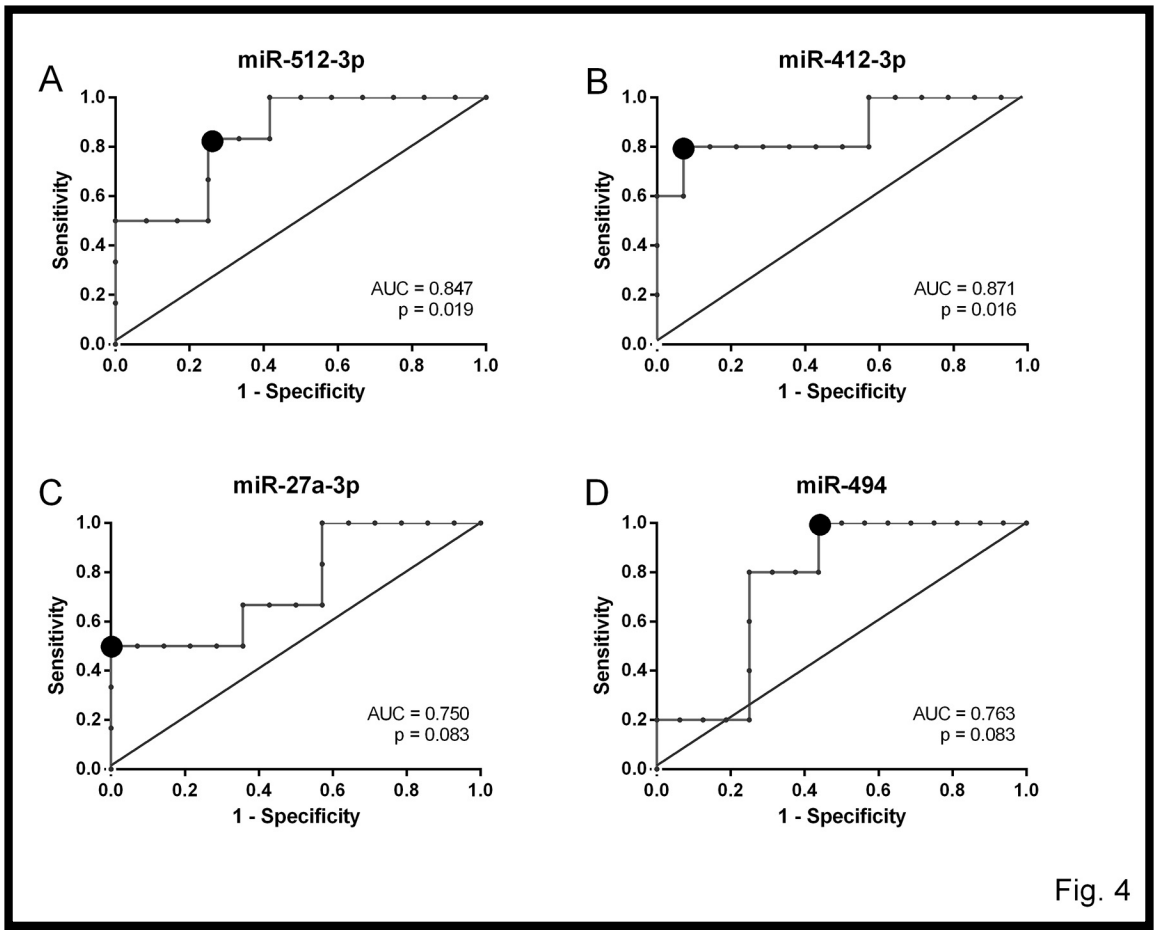

Figure 4: ROC curve describing predictive potency of the up-regulated miRNAs as a diagnostic test. *The curves represent specificity versus sensitivity of miR-512-3p (A), miR-412-3p (B), miR-27a-3p (C), miR-494 (D). Data are derived from miRNAs' expression levels (RQ) of OSCC patients and controls. The big grey dots indicate the optimal threshold value of sensitivity and specificity determined by the maximum Youden's index (sensitivity+specificity-1).*

CHAPTER 4 – DISCUSSION & CONCLUSIONS

The biological significance of ncRNAs as a group is being discovered rapidly with new roles being deciphered each day. The development of high throughput sequencing technology, high resolution imaging and experimental techniques has resulted in advancement of ncRNA research and development of the Cancer Genome Atlas (TCGA) databases has accelerated our understanding of the molecular basis of cancer. Non-coding RNAs, earlier considered as transcriptional noise, have been recently shown to be linked to all six hallmarks of cancer including continuous growth-promoting signalling, evading growth suppressors, resisting apoptosis, enabling continuous proliferation even at limited nutrient conditions, activating invasion and metastasis and inducing angiogenesis. To date, microRNAs appear to be important in pathophysiology of HNSCC but appear to have distinct profile and function depending on the aetiology and state, tissue type and site of tumorigenesis [Sannigrahi *et al*, 2017].

Salivary screening can be the best choice as the primary screening test for the high-risk cases of OSCC, since the collection procedure is non-invasive and low cost. In addition, the specimen is with low background and inhibitory substances and less complex than blood. The use of saliva as a diagnostic bio fluid has been widely recognized, and it has many advantages over other specimens like blood, exfoliated cells and urine [Li *et al*, 2004; Kaczor-Urbanowicz *et al*, 2017]. Salivary biomarkers have the potential to serve as non-invasive, widely accessible screening tools; the collection is inexpensive and can be easily performed. Identifying the proper salivary biomarker profile could contribute to the current screening method of oral cancer, which is limited to physical exam and biopsy of

suspicious lesions [Kaczor-Urbanowicz *et al*, 2017]. If becoming a routine as a diagnostic tool for OSCC, saliva would also comprise a suitable instrument for population screening, monitoring of patients at high risk of recurrences, and subsequently for improving survival rates.

Several works describe the possibility to detect RNA biomarkers of numerous diseases in saliva [Li *et al*, 2004; Michael *et al*, 2010; Kaczor-Urbanowicz *et al*, 2017] and, more specifically, miRNAs associated with oral cancer [Yang *et al*, 2013; Zahran *et al*, 2015; Zhou *et al*, 2017]. Evidence demonstrates that it is possible to isolate EV-associated RNA from saliva and oral samples [Michael *et al*, 2010; Lasser *et al*, 2011; Yap *et al*, 2017]. However, to date, miRNA expression in EVs from OSCC has never been studied.

In this work, according to previous evidence [Marzesco *et al*, 2005; Ogawa *et al*, 2008; Lasser *et al*, 2011; Deregibus *et al*, 2016; Yap *et al*, 2017], we successfully isolated EVs from saliva. A previous study [Deregibus et al, 2016] showed that most of the salivary RNA was associated with EVs and that salivary EVs were enriched in miRNAs.

By the molecular analysis of miRNAs, we observed a significant up-regulation of miR-412-3p, miR-512-3p, miR-27a-3p, miR-494-3p and we found that miR-302b-3p and miR-517b-3p were only expressed in OSCC patients. By the KEGG pathway enrichment analysis of these six miRNAs, eight pathways showed to be significantly enriched. Each pathway was targeted by two or more miRNAs. MiR-512-3p and miR-27a-3p showed to target respectively 7 and 20 genes involved in ErbB signalling pathway, which promotes cell proliferation and survival in several solid tumours [Appert-Collin *et al*, 2015] and was shown to be activated also in OSCC [Li *et al*, 2016; Ohnishi *et al*, 2016; Li *et al*, 2017]. MiR-512-3p, miR-27a-3p,

and miR-302b-3p showed to target respectively 14, 30, and 14 genes in proteoglycan in cancer pathway. Evidence has shown that CD44, which is targeted by both miR-512-3p and miR-302b-3p, and the downstream pathway promote cell invasion and migration upon c-Fos stimulation in OSCC [Dong *et al*, 2015]. Increased CD44 expression has been associated to ERK1/2 phosphorylation, and to increased tumour aggressiveness [Judd *et al*, 2012]; moreover, high CD44 levels have been described as a characteristic feature of cancer stem-like cells in OSCC [Ghuwalewala *et al*, 2016]. In addition, miR-512-3p, miR-412-3p, miR-27a-3p, and miR-302b-3p showed to target several genes of TGFβ signalling pathway, including TGFβR2 gene. Interestingly, it has been previously reported that TGFβR2 is commonly reduced in oral epithelium and stroma in OSCC patients [Meng *et al*, 2011]. Moreover, 46 genes involved in signalling pathways regulating pluripotency of stem cells are targeted by miR-512-3p, miR-27a-3p, miR-494-3p, miR-517b-3p, and miR-412-3p. The overexpression of Bmi1, which is targeted by miR-494 and miR-27a-3p, has been shown to promote formation, growth, migration, and metastasis in a subpopulation of tumour cells of the HNSCC [Liu *et al*, 2012]. Basing on these observations, we may speculate that the increase in salivary EVs of miRNA targeting genes involved in tumour progression might represent a defences mechanism of tumour cells to eliminate anti-tumour miRNAs. However, the relation between miRNAs and target genes should be experimentally proven and these hypotheses should be furtherly studied.

To better evaluate the discrimination power of the four up-regulated miRNAs as OSCC biomarkers, we constructed the ROC curves, expressing the true positive rate (sensitivity) versus false positive rate (1-specificity) at various cut-off values. MiR-512-3p and miR-412-3p resulted to be either sensitive and specific, as shown

by high AUC values (0.847 and 0.871 respectively, with p values < 0.02) and maximum Youden's Index. This indicates that these two miRNAs are good predictors and can be suggested as new candidate biomarkers for OSCC, even if further studies are required on a larger population. On the other hand, miR-27a-3p and miR-494-3p showed AUC of 0.75 and 0.763 respectively, indicating that the prediction is better than random. However, p value was 0.083 for both miRNA, indicating that the discrimination power was good but not sufficient. In fact, miR-27a-3p and miR-494-3p showed to be respectively specific and sensitive, but not both. Therefore, the up-regulation of miR-27a-3p and miR-494-3p is frequent in OSCC and can be used as an indicator, but it is not sufficient as a diagnostic biomarker. Nevertheless, the involvement of miR-494-3p and miR-27a-3p in OSCC is supported by the literature. MiR-494 has been previously isolated from blood of OSCC patients and proposed as a biomarker [Ries *et al*, 2014]. MiR-27a-3p takes part in OSCC progression by targeting YAP1 and inhibiting the epithelial to mesenchymal transition processes [Zeng *et al*, 2016] and by targeting MCPH1, which act as an onco-suppressor gene [Venkatesh *et al*, 2013]. It has also been proposed that high levels of miR-27a increase heat sensitivity in OSCC cells, enhancing hyperthermia-induced-cell death [Kariya *et al*, 2014]. Moreover, miR-27a-3p seems to play a role in progression and metastasis of nasopharyngeal carcinoma [Li *et al*, 2017], gastric cancer [Zhou *et al*, 2106], oesophageal cancer [Wu *et al*; 2015], and it has been proposed as an EV-associated biomarker for colorectal cancer [Ostenfeld *et al*, 2016]. No evidence has been found about the involvement of miR-412-3p, miR-512-3p, miR-302b-3p, and miR-517b-3p in OSCC, thus our work provides new insight about miRNA dysregulation in the tumour. It is worth mentioning that miR-512-3p has been identified as up-regulated

in metastatic prostate cancer [Sadeghi *et al*, 2016], but it has shown a tumour inhibitory activity in non-small cell lung cancer [Chen *et al*, 2010] and hepatocellular carcinoma [Zhu *et al*, 2015].

On the other hand, in our molecular analysis, we observed a not significant up-regulation of several miRNAs that have been reported in the literature as up-regulated in whole saliva or plasma of OSCC patients and have been proposed as biomarkers. For example, several works report the overexpression and involvement of miR-31 in OSCC [Liu *et al*, 2010; Hung *et al*, 2014; Lu *et al*, 2014; Siow *et al*, 2014; Hung *et al*, 2016; Yan *et al*, 2016]. We observed an up-regulation of both miR-31-5p and miR-31-3p in OSCC patients compared with controls (Tab. 3). Then, miRNA-184 has shown a good diagnostic value [Zahran *et al*, 2015] and was up-regulated in our group of patients. Mir-708 is up-regulated in progressing oral premalignant lesions [Yang *et al*, 2013] and was increased in our group of patients. The discrepancies among the miRNAs up-regulated in our study and in other studies in the literature may be due to the relatively small number of recruited patients and controls, to different modalities of miRNA isolations and to the different geographical origin of the group of cases enrolled in each study.

In this work, we confirmed the possibility to use salivary EVs as a non-invasive source of miRNAs for OSCC diagnosis and we identified a subset of miRNAs selectively enriched in EVs of OSCC patients that may be used as biomarkers, even if future study must confirm this preliminary data.

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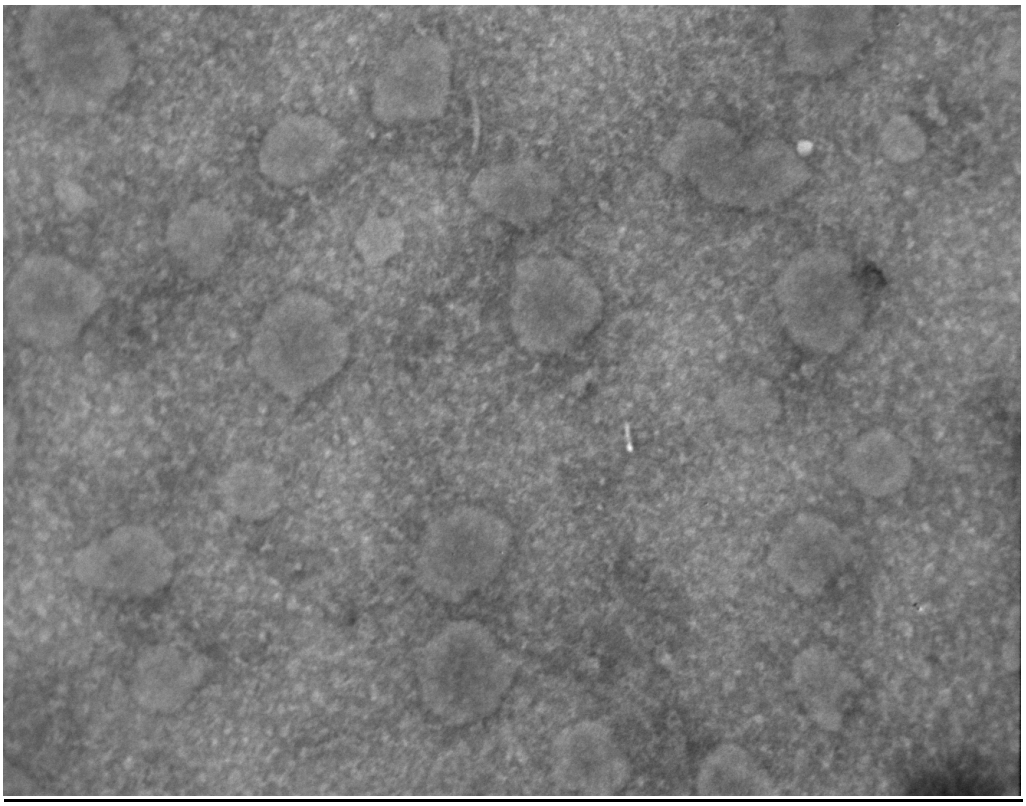
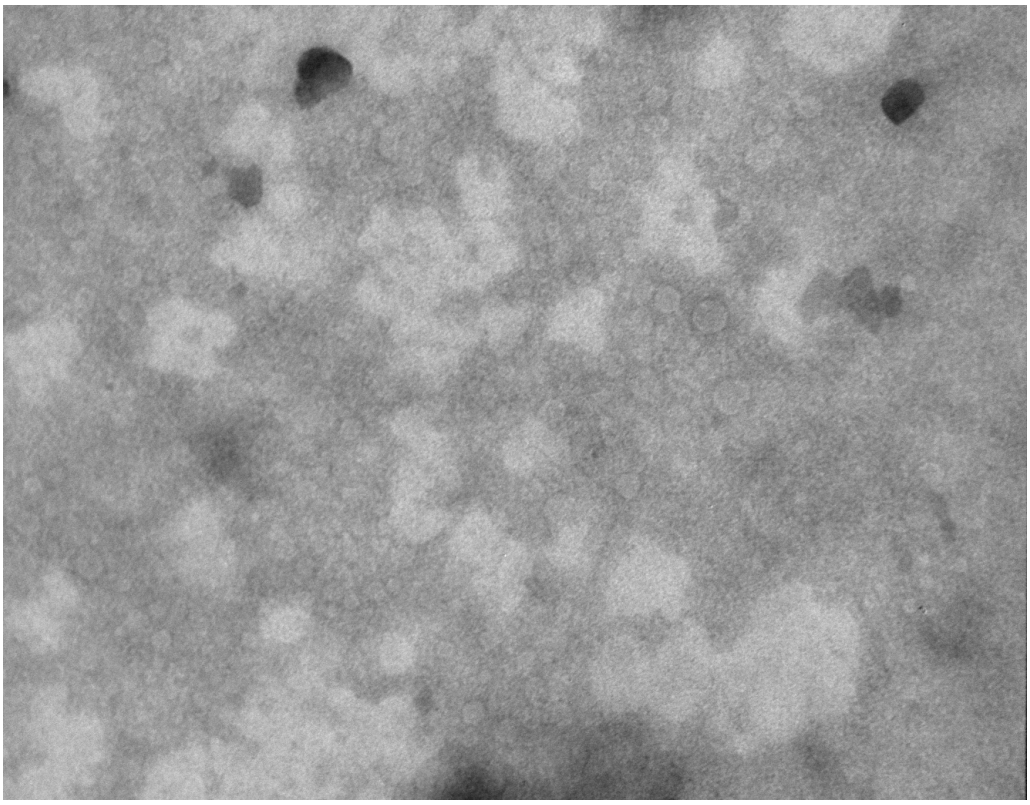
APPENDIXES

APPENDIX A: Data about OSCC patients (Table I)

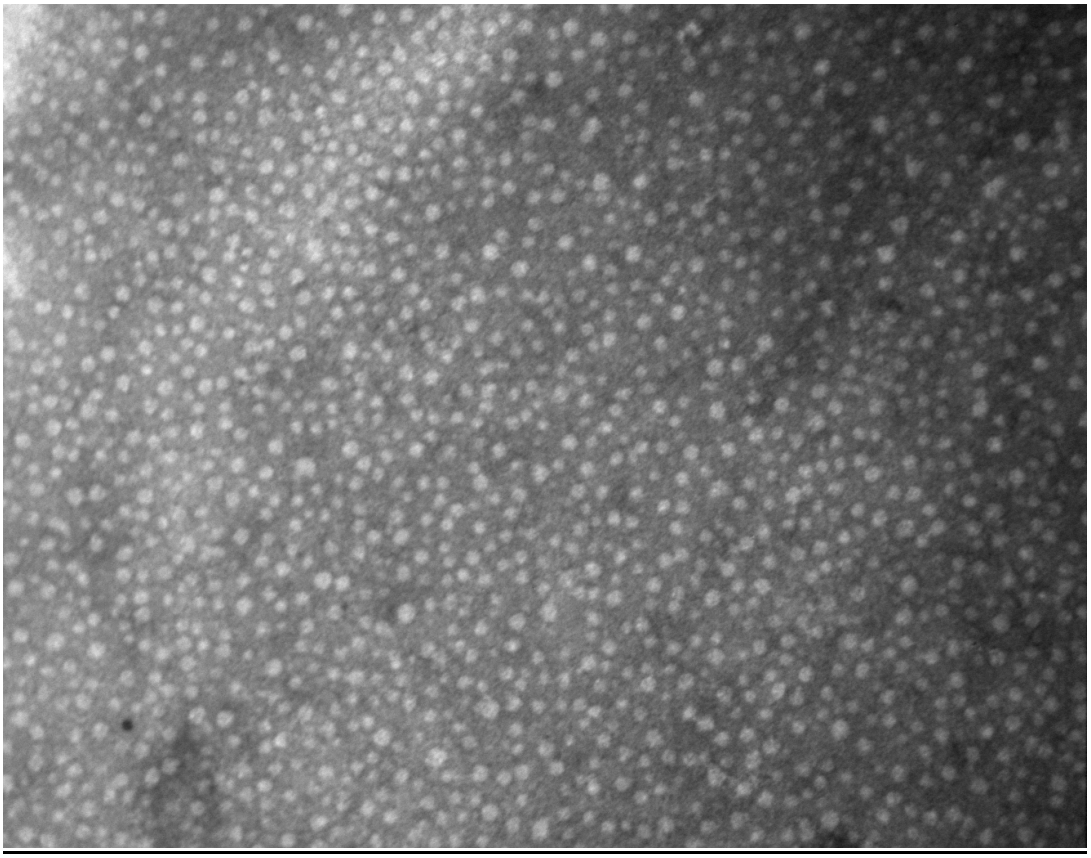
	SESSO	SEDE	Età	G	T	N	M	FUMO	CHIRURGIA	Radiotx	Chemiotx	FOLLOW-UP	RECIDIVA	DECEDUTO
1	M	mucosa geniena	69	2	4	0	0	Ex	Sì	Sì	NO	20 mesi	NO	NO
2	F	pelvi linguale	62	3	3	2	0	Ex	Sì	Sì	Sì	21 mesi	NO	NO
3	M	labbro inferiore	69	1	pT1	0	0	No	Sì	NO	NO	18 mesi	NO	NO
4	M	pilastro	62	2	pT2	0	0	Si	Sì	NO	NO	20 mesi	NO	NO
5	F	Bordo – pelvi linguale	63	2	pT2	cN0	0	Si	Sì	NO	NO	19 mesi	NO	NO
6	F	cresta superiore	73	3	pT4a	pN2b	0	No	Sì	NO	NO	3 mesi	NO	SI
7	F	bordo linguale	46	2	pT1	pN2b	0	No	sì	NO	NO	10 mesi	NO	Sì
8	M	bordo linguale	71	3	3	2	0	Si	NO	Sì	Sì	6 mesi	NO	Sì
9	F	bordo linguale	76	1	pT1	pN0	0	No	Sì	NO	NO	18 mesi	NO	NO
10	M	pilastro palatino	69	3	cT3	cN+	x	Ex	NO	Sì	Sì	16 mesi	NO	NO
11	F	pilastro palatino	70	3	pT2	pN1	0	Ex	Sì	NO	NO	17 mesi	NO	NO
12	F	Tuber-vestibolo	73	2	pT4a	pNx	0	No	Sì	Sì	NO	8 mesi	NO	Sì
13	F	bordo linguale	78	2	pT3	pN0	0	No	Sì	NO	NO	16 mesi	NO	NO
14	M	pavimento orale	71	2	pT1	pN0	0	Ex	Sì	NO	NO	16 mesi	NO	NO
15	F	gengiva superiore	73	1	pT1	pNx	0	No	Sì	NO	NO	17 mesi	NO	NO
16	F	pavimento orale	56	3	pT1	pN0	0	Si	Sì	NO	NO	16 mesi	NO	NO
17	M	Pavimento – cresta inferiore	61	2	3	2	0	Si	NO	NO	Sì	13 mesi	NO	NO
18	F	cresta inferiore	75	2	pT1	pN0	0	Ex	Sì	NO	NO	14 mesi	NO	NO
19	M	bordo e base lingua	73	2	pT3	pN2b	0	No	Sì	Sì	Sì	10 mesi	NO	NO
20	M	bordo lingua	58	2	pT2	pN1	0	No	Sì	NO	NO	12 mesi	NO	NO
21	M	pelvi linguale	38	3	pT1	pN0	0	Ex	Sì	NO	NO	6 mesi	NO	NO
22	M	pavimento orale	60	2	pT1	pN2c	0	Si	Sì	Sì	Sì	9 mesi	NO	NO
23	F	trigono retromolare	60	2	pT2	pN1	0	Si	Sì	Sì	NO	7 mesi	NO	NO
24	F	bordo lingua	73	2	pT1	pN0	0	Ex	Sì	NO	NO	16 mesi	NO	NO
25	F	pavimento orale	71	2	pT1	pN0	0	Ex	Sì	NO	NO	16 mesi	NO	NO

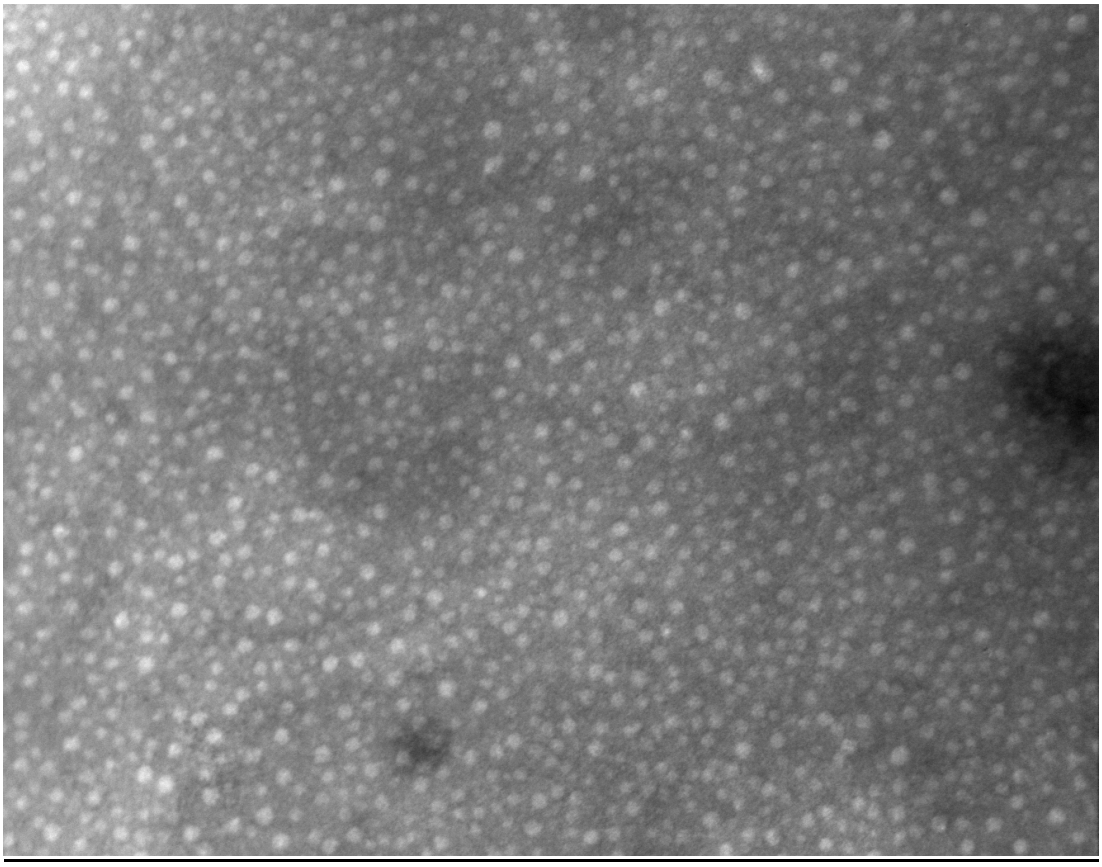
APPENDIX B: representative transmission electron microscopy images (cancers cases #13, #26)





APPENDIX C: representative transmission electron microscopy images (control cases #2, #5)





APPENDIX D: NanoSight analysis

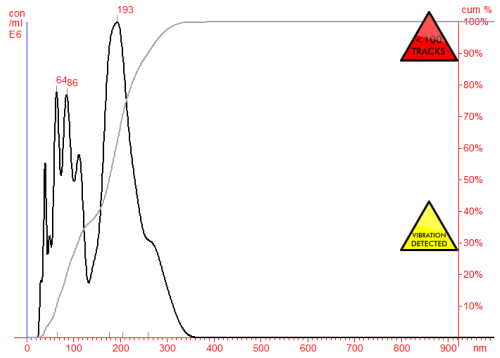
Fig. I. Representative image of NanoSight analysis: saliva-derived vesicles from an exemplificative case of OSSC patient purified by precipitation. The peaks show the relationship between particle distribution (left Y axis) and particle size (X axis); the curve describes the correlation between cumulative percentage distribution of particles (percentile in right Y axis) and particle size (X axis). Mean size and particle concentration values were calculated by the Nanoparticle Tracking Analysis (NTA) software that allows analysis of video images of the particle movement under Brownian motion captured by Nanosight LM10 and calculation of the diffusion coefficient, sphere equivalent and hydrodynamic radius of particles by using the Stokes-Einstein equation.

NANOSIGHT

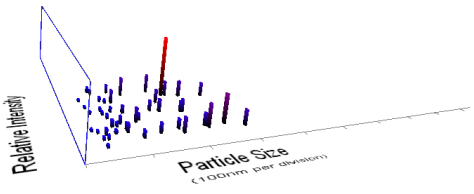
Nanoparticle Tracking Analysis (NTA) Version 2.3 Build 0008 BETA4

ANALYSIS REPORT

Sample:
Date/Time of Capture:
Video File: MATTEJA 11 11 2014.avi analysis no: 001
Operator:
Comments:



Particle Size / Concentration



Particle Size / Relative Intensity 3D plot

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
10	0.000	0.000
30	4.863	2.225
50	11.326	7.407
70	19.914	16.518
90	20.885	26.073
110	16.642	33.686
130	7.623	37.174
150	9.208	41.387
170	23.089	51.950
190	30.758	66.022
210	25.619	77.743
230	16.367	85.231
250	10.888	90.212
270	9.244	94.442
290	6.824	97.564
310	3.721	99.266
330	1.346	99.882
350	0.240	99.992
370	0.018	100.000
390	0.000	100.000
410	0.000	100.000
430	0.000	100.000
450	0.000	100.000
470	0.000	100.000
490	0.000	100.000
510	0.000	100.000
530	0.000	100.000
550	0.000	100.000
570	0.000	100.000
590	0.000	100.000
610	0.000	100.000
630	0.000	100.000
650	0.000	100.000
670	0.000	100.000

Bin Centre (nm)	Concentration (E6 particles/ml)	Percentile Undersize (%)
690	0.000	100.000
710	0.000	100.000
730	0.000	100.000
750	0.000	100.000
770	0.000	100.000
790	0.000	100.000
810	0.000	100.000
830	0.000	100.000
850	0.000	100.000
870	0.000	100.000
890	0.000	100.000
910	0.000	100.000
930	0.000	100.000
950	0.000	100.000
970	0.000	100.000
990	0.000	100.000
1000-2000	0.000	100.000

Results

Mean:	**164 nm**
Mode:	**193 nm**
SD:	**71 nm**
D10:	**65 nm**
D50:	**177 nm**
D90:	**259 nm**
User Lines:	**0 nm, 0 nm**
Concentration:	**2.19 E8 particles/ml**
Completed Tracks:	**48**

Measurement Conditions

Temperture:	24.25 °C
Viscosity:	0.90 cP
Frames Per Second:	28.57
Measurement Time:	30 of 30 s
Drift Velocity:	10730 nm/s
Camera Shutter:	12 ms

Analysis Conditions

Blur:	Auto
Detection Threshold:	10 Multi
Min Track Length:	Auto
Min Expected Size:	100nm