PROTEOMIC ANALYSIS IN NASOPHARYNGEAL CARCINOMA

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PROTEOMIC ANALYSIS IN NASOPHARYNGEAL CARCINOMA: BIOMARKER RESEARCH USING NASOPHARYNGEAL CARCINOMA FORMALIN-FIXED PARAFFIN-EMBEDDED SAMPLES FROM PATIENTS TREATED AT IPO LISBON BETWEEN 2009-2013

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To:

Affonso Netto *(in memoriam),*

Carmem Vianna,

Carmem & Manuel Moreira,

Esmeralda Poli.

This work has been developed according to the current Legislation (DGS, ACSS, CNPD and CDHB included) and with approvals from Administration Boards and Ethics Committee from the institutions:

- Radiation Oncology Department (Instituto Português de Oncologia de Lisboa Francisco Gentil, Director Margarida de Abreu Roldão, MD, PhD).

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INTRODUCTION

Nasopharyngeal carcinoma (NPC) is a particular type of head and neck cancer with a strong ethnic and enigmatic epidemiology that long puzzles the scientific society (Ho 1978; Chang & Adami 2006a).

Despite present all over the world, around 80% of all cases are in Asia where it is endemic, followed by low incidence regions in all other continents. The Mediterranean basin is a well-known non-endemic region. Portugal has the second incidence in Europe for women, and third for men (Globocan 2012) among all European countries.

Many efforts have been made to clarify and understand this unique geographic and epidemiologic distribution. Of particular interest, is the UICC Symposium for Nasopharyngeal Carcinoma held in Singapore in 1964 (Muir, 1967) that brought light over four decades of Epidemiology data on Southeastern Asia NPC.

The onset and evolution of nasopharyngeal carcinoma is a complex multi-stage process and may take a long time to occur (Hu). Although the molecular basis remains uncertain, we know that environmental factors (e.g. intake of preserved food, exposure to several carcinogens), Epstein-Barr virus (EBV) infection and genetic susceptibility are considered to be the three major contributors.

Despite investigations from early events of NPC, genetic changes in pre-invasive lesions have been documented in several studies particularly the deletions of 3p and 9p, overexpression of BCL-2, and activation of telomerase (Hu, Wei, Chen, Ciaran B Woodman, et al. 2012a).

Expression of latent EBV genes such as LMP1, LMP2A and EBNA1 may lead to abnormal regulation of a number of signaling pathways, for example, the NFκ-B, and the TGFβ pathways that are involved in regulation of cell apoptosis and proliferation(Hu, Wei, Chen, Ciaran B Woodman, et al. 2012a; Lee et al. 2013; Tulalamba & Janvilisri 2012; Plieskatt et al. 2014; Aga et al. 2014).

Oncogene activation including BCL-2 overexpression and telomerase activation has also been observed in a high proportion of dysplastic lesions, suggesting that their functions in anti-apoptosis and cell immortalization occur from the early stages and contribute to NPC development (Hu, Wei, Chen, Ciaran B Woodman, et al. 2012a).

Multiple genetic changes are accumulated in the later stage of NPC development, such as deletions of 11q, 13q, 14q and 16q, and gains of 3q, 8q and 12q. These genetic aberrations are correlated with more TSGs inactivation and oncogene activation (e.g. inactivation of THY1, ATM, TSLC1 and E-cadherin, and activation of Cyclin D1, PIK3CA and LTβR) that will either speed up NPC development or contribute to NPC invasiveness and metastasis (Hu, Wei, Chen, Ciaran B Woodman, et al. 2012a; Lee et al. 2013; Tulalamba & Janvilisri 2012; Plieskatt et al. 2014; Aga et al. 2014).

Further examination of these genes' expression in each tumor revealed that around 93 oncogenes are up regulated in each tumor, while the mean number of TSGs down regulated was 109. A sample possessing the combination set of up or down-regulated tumor-associated genes, in various proportions, creates an extensive list[6-12].

In Portugal, the works of Souza & Breda have identified an important polymorphism markers that may play a role in the onset and NPC development on the Portuguese northern region population (Breda, Catarino, et al. 2008; Sousa et al. 2011).

Despite the efforts made, the molecular mechanisms of NPC carcinogenesis and progression remains to be understood. In this regard, current omics methodologies (e.g. proteomics, genomics etc.) offer a different approach to identify unique miRNAs and proteins that expression signatures associated with the cancer phenotype, reflecting the biological and pathological grade of the disease. Thus, the discovery of useful NPC biomarkers will lead to new diagnostic and prognostic tools (Jiang et al. 2009; Shi et al. 2010).

Meanwhile, the treatment of NPC has evolved considerably in the past two decades. NPC is very sensitive to radiotherapy (RT). In fact, since Intergroup 0099 trial, concurrent chemoradiotherapy with or without adjuvant cisplatin-based chemotherapy is the current standard of care for nasopharyngeal carcinoma at most stages (Al-Sarraf et al. 1998; Blanchard et al. 2011; Ribassin-Majed, Marguet, Anne W M Lee, et al. 2017; Langendijk et al. 2004).

Historically, conventional 2D RT can offer almost 95% of local control for initial tumors (T1 and T2). Nevertheless, more advanced stages (T3 and T4) have control rates dropped to 44-68% due to limitations in tumor coverage while protecting critical neurological structures (Chu et al. 1984; Mesic et al. 1981; Hoppe et al. 1976; Sanguineti et al. 1997; Vikram et al. 1984; Teo et al. 2004; Bailet et al. 1992).

Since 2000, intensity modulated radiation therapy (IMRT) has been widely used to treat nasopharyngeal carcinoma. IMRT provides better dose delivery to the target while sparing the surrounding normal tissues while local control reaches 98% at 4 years (Butler et al. 1999; Nutting et al. 2011). At least one prospective randomized controlled trial showed its benefit in salivary protection in HNC (Nutting et al. 2011). One recent meta-analysis confirmed these findings (Nader et al. 2013). However, despite the excellent local control, 43% of patients will develop distant metastasis before 5 years and die.

In Portugal, IMRT is used at the Instituto Português de Oncologia de Lisboa (IPOLFG) since 2009 and represents the current standard of care for HNC RT, including NPC. Our initial experience has already been presented and confirmed the high control rate (90%) even with 84% of T4 lesions, however 42% of patients still developed distant metastasis before 4 years (Netto et al. 2015).

Until now, several prognostic markers were identified and investigated for screening, and prognostic tools for NPC, have been purposed, particularly in Asia (Hu, Wei, Chen, Ciaran B Woodman, et al. 2012a; Lee et al. 2013; Tulalamba & Janvilisri 2012; Plieskatt et al. 2014). However, to our knowledge, there are no validated identified markers to predict distant metastasis or outcome. Moreover, the studies exploring this subject have identified a population-based variety of biomarkers stressing an omic translation of NPC ethnic distribution (Chang & Adami 2006b; Sousa et al. 2013; Breda, Breda, et al. 2008; Sousa et al. 2011; Hu, Wei, Chen, Ciaran B Woodman, et al. 2012a).

At the present work, the author studied formalin-fixed paraffin-embedded samples of biopsied nasopharyngeal carcinoma tumors via proteomic analysis. The aim is to investigate the presence of tumor profiling from the studied cohort and discover biomarkers to predict distant metastasis. Secondary endpoints are the discovery of biomarkers related to tumor radioresistance and treatment toxicity.

This thesis is divided in four parts. At the first part, the disease will be reviewed along its natural history and characteristics. Its prognostic factors may be patient related, tumor related, environmental or treatment related. A deep emphasis will be given to the radiation oncology technique as it plays a role in all stages of this disease treatment.

At the second part, it will be described the principles of proteomic analysis and its applications in oncology, particularly in nasopharyngeal carcinoma.

Then, at the third part, follows the experimental work developed, describing the process, sample separation, analysis and obtained results. Finally, the data will be presented along the study conclusion and future work for this line of research.

1. Nasopharyngeal carcinoma (NPC)

The aim of this part is to review NPC, its unique geographic distribution, and factors related to its etiology. It's frequently impossible to distinguish epidemiological, geographical, ethnical to cultural behaviors specially food habits and environmental exposures from epidemiology to etiology.

1.1. Epidemiology

1.1.1.Geographic and Racial Distribution

It's a rare cancer allover the world but extremely prevalent in Asia, where almost 80% of all cases occur, but with a wide variation in incidence across the continent. (Anon 2002)

The Cantonese Southeastern China Guangdong Province, including Hong Kong area, can reach incidences of more than 27 cases per 100.000 habitants / year whereas Beijing area have incidences of 1 case per 100.000 habitants / year, very close to western countries. In fact, Cantonese-speaking Chinese nationals may have 20-fold incidence than other dialects. (Chang & Adami 2006b)

Western countries like the USA have low incidence about less than 1 case for 100.000 habitants per year. Nevertheless, the Chinese descendent population in Hawaii State has over 10 cases per 100.000 habitants per year (Sousa et al. 2011; Hu, Wei, Chen, Ciaran B Woodman, et al. 2012b; Chang & Adami 2006a).

1.1.2.Gender and Age Distribution

Across every continent studied, the incidence in male patients occurs in a 2 to 3 fold increased than in females. In low risk areas, there is a constant increase of incidence with age whereas it has been described a peak of incidence in the sixth decade declining thereafter (Adham et al., 2012; Jia et al., 2006; Lee & Ko, 2005).

1.1.3.Eastern and Western Migration Studies

The Asian studies following the AJCC/UICC Symposium for Nasopharynx Carcinoma held in Singapore resulted in almost four decades of important data to understand the disease distribution.

Data from high incidence areas population migrating to low incidence area shows that the migrant population keeps the risk as high as its original provenance. However, the risk seems to decrease constantly within generations in the West. The contrary has not been described, since low incidence nationals who migrate to high endemic areas still have the same incidence of their native land. (Anon 2002)

Authors speculate where there are aspects of Asian lifestyle that may change with migration and is not incorporated by non-native migrants. There is also a self selection bias since migrants may reflect a different socioeconomic status (Anon 2002; Chang & Adami 2006a).

1.1.4.Portuguese Epidemiologic Data

According to the Globocan (2012), Portugal has the second gender-adjusted incidence of NPC for women in Europe (0.5 cases per 100.000 hab/year) and the third incidence in men (1.5 cases per 100.000 hab/year) just after Romania and Malta, accounting for 132 cases in 2012.

1.2. Etiology

The complex etiology of NPC rises from a balance between three major factors: Epstein-Barr virus infection, environmental factors and genetic susceptibility. Nevertheless, other factors may play a role on its onset and development.

1.2.1.Epstein-Barr Virus (EBV)

1.2.1.1. Natural history

Epstein-Barr virus has first been described in 1964. By 1970, had already been related to NPC disease (Henle et al. 1970).

It is estimated that 90% of the world populations is infected with EBV. The infection occurs typically at childhood. Hong Kong data demonstrates that, by the age of 10 years old, almost 100% of children are seroconverted.(Kangro et al. 1994)

1.2.1.2. EBV infection in humans and its role in NPC carcinogenesis

After inoculation, B-lymphocytes are the primary target of EBV infection. Its pathway entry into epithelial cells is unclear but EBV replication occurs in epithelial cells of the pharyngeal wall, as well as in B-lymphocytes in both normal and malignant tissues.

Anti-EBV antibodies were observed to be higher in NPC patients than in controls. NPC patients have elevated IgG and IgA antibody titers to the EBV viral capsid antigen lgA and early antigen, as well as increased IgG against the latent viral nuclear antigens 1 and 2 (EBNA-1, EBNA-2).

Nevertheless, EBV infection alone is not enough to cause NPC. Despite all adults are infected, yet only a small number of individuals will develop NPC in their lifetime. Therefore, it seems that environmental associated with/and/or host's genetic profile contribute to NPC risk(Lu et al. 2016).

1.2.2.Environmental factors

Previous data from IARC studies documented that chronic ear, nose and throat infections are associated with doubling the risk of NPC. Theoretically, chronic infections would make the nasopharynx mucosa more susceptible to NPC (Geser et al. 1978). Another explanation relies on the fact that some bacteria can reduce nitrate to nitrite, which can then form carcinogenic N-nitroso compounds (Bartsch et al. 1992).

The Hong Kong epidemiologic study from Geser et al. has described following factors were found to be positively associated with NPC: belonging to the four lowest social classes; practicing Buddhism or any ancestor worship having religious altars at home; and having a history of previous illnesses of the ear or nose after the age of 15 years. The following factors were found to be negatively associated with NPC: eating bread; eating of tinned food; and use of spices (Geser et al. 1978).

Infectious mononucleosis it is long to be considered as a late childhood or young adulthood EBV infection but its relation to NPC remains unclear. It is claimed that a late infection with EBV is rare NPC endemic areas (Niederman et al. 1970; Chang & Adami 2006b).

Vaughan et al. reported that infectious mononucleosis decreased NPC development by 60% (Vaughan et al. 1996).

Betel nut chewing is associated with increased risk of oral cancer, but there are conflicting information regarding its association to NPC (Jeng et al. 2001). Yang et al, on behalf of the Chinese and American Genetic Epidemiology of NPC Study Team published their data evaluating more than 2600 individuals. They found that betel chewing for more than two decades was associated with higher risk of NPC in families with more than two affected relatives (Yang et al. 2005). Others haven't found the same association (West et al. 1993).

1.2.3.Other factors:

1.2.3.1. Salted food

The carcinogenic effect of salt-preserved fish was demonstrated by experiments in mice (Zheng et al. 1994).

It is the most consistently non-viral exposure strongly associated with risk of NPC, a common tradition in NPC-endemic regions. In China, the relative risk of NPC associated with weekly consumption, compared with no or rare consumption, generally ranged from 1.4 to 3.2, whereas that for daily consumption ranged from 1.8 to 7.5 (Yuan et al. 2000).

Salt-preserved foods are a dietary tradition in NPC-endemic populations (Poirier et al. 1987). In Southern China, intake of salted fish and preserved foods is high among

boat-dwelling fishermen, the so called *Tankas*, a subgroup at highest risk of developing NPC (Chang & Adami 2006a).

Childhood exposure seems more strongly related to NPC. Furthermore, increasing duration and frequency of consumption are independently associated with higher risk of NPC. (Yu et al. 1989; Jia et al. 2010).

1.2.3.2. Tobacco and Alcohol

Data regarding the association of various kinds of smoking have been conflicting. Studies evaluating cigarette smoking (including second hand smoke) and risk of NPC reported an increased risk of 2- to 6- fold (Zhu et al. 2002), although some studies found no association (Tsao et al. 2014; Chang & Adami 2006a)

Vaughan et al. have described that an estimated two thirds of grade I NPC, but not grades II or III, in America was attributable to smoking, (Vaughan et al. 1996). There is a declining prevalence of smoking seen in the United States in the past three decades (Giovino et al. 1994) that help understand the recent decreasing trend in the incidence of type I NPC (Sun et al. 2005).

There is also divergence in studies examining burning incense or anti-insect devices. Some series report a risk up to a 6-fold with use of anti-insect devices (West et al. 1993), and other with domestic religious altars (Geser et al. 1978), but most studies finding no evidence to support this statement (Chen et al. n.d.).

Alcohol consumption is not associated with NPC risk, because the vast majority (Chang & Adami 2006a; Friborg et al. 2005; Yang et al. 2005; Tsao et al. 2014) epidemiological studies were negative.

1.2.3.3. Hereditary and familial susceptibility

Studies also accessed the relation between polymorphism of some genes and NPC risk like homozygous variant derived from cytochrome P4502E1 (CYP2E1), null allele of glutathion S-transferase M1 (GSTM1), T cell receptor polymorphism (TCR), poly immunoglobulin receptor (PIGR), tumor suppressing gene GX6, DNA repair gene hOGG1,

and XRCC1 (West et al. 1993; Chang & Adami 2006a).

A Chinese study demonstrated that might exist an NPC susceptible site on chromosome number 3 (Hunan Province, Southern China). A locus on 3p21 was identified to link to NPC with a maximum logarithm of odds for linkage score of 4.18. Fine mapping located the locus to a 13.6-cM region on 3p21.31-21.2, where a tumor suppressor gene cluster resided (Xiong et al. 2004).

In a Western non-endemic region, the Portuguese data from Sousa et al. revealed that 31.2% of NPC patients were IL-1RN A2*A2, compared with 9.7% observed in the control group. The statistical analysis revealed that IL-1RN*A2 homozygosity for the A2 allele was associated with a 4-fold increased risk for NPC development ($p < 0.001$). Furthermore, cumulative hazard analysis showed that an estimated median age of onset of NPC is significantly ($p < 0.001$) different for A2*A2 homozygous versus non-A2*A2 (57.0 vs. 74.0, respectively). According to Sousa et al., IL-1RN*A2 homozygosity is a significant risk marker in the Portuguese population and may help identify a susceptibility profile for NPC onset (Sousa et al. 2011).

1.2.3.4. HLA Genes

Human leukocyte antigen (HLA) genes encode proteins required for the presentation of antigens to the immune system for lysis, including viral peptides. HLA alleles with a reduced ability to present EBV antigens may have an increased risk of developing NPC. On the contrary, individuals with HLA alleles that present EBV efficiently may have a lower risk. The association between HLA-A2 with NPC to HLA-A*0207 may explain reported associations between HLA-A2 with NPC among Chinese, but not Caucasians. It was suggested that haplotypes associated with NPC might explain the high rates of NPC in Chinese population (Hildesheim et al. 2002).

The work of Lu et al. has demonstrated a gene linked to the HLA locus associated with a 21-fold higher risk of NPC (Lu et al. 1990).

In a meta-analysis of studies in Southern China, evidence suggested a positive association of NPC risk (2-3 fold higher risk) with HLA-A2, B14, and B46, and a negative association (up to 50% lower risk) with HLA-A11, B13, and B22 (Goldsmith et al. 2002).

1.2.3.5. Chinese medicine

Any association with use of herbal drugs may be difficult to separate from other aspects of a traditional Eastern lifestyle, like diet. There is a rationale for Chinese herbal plants in NPC development since several such commonly used plants can induce viral lytic antigen expression by activating EBV (Zeng et al. 1994).

EBV-inducers were found in extracts of soils, vegetables and from areas in southern China where NPC is endemic (Zeng et al. 1984).

Herbal medicines was associated with elevated NPC risk in the Philipines, especially herbal drugs with high anti-EBNA antibody titers individuals, suggesting a direct proliferative effect of these herbs on EBV-infected cells (West et al. 1993; Hildesheim et al. 1992).

1.2.3.6. Professional exposure

A Scandinavian Meta-analysis with more than 30 epidemiological studies showed that exposure to formaldehyde was significantly associated with NPC, and a dose–response (exposure) relationship was demonstrated (Partanen 1993).

In 1995, IARC ruled formaldehyde to be an etiological factor for NPC. Subsequently, IARC did find sufficient evidence of carcinogenicity (Cogliano et al. 2005).

Dust particles like wood dust of 5–10 um are easily inhaled to pharynx. Epidemiological studies have found that the risk factor for NPC was increased in people exposed in wood dust. A recent meta-analysis concluded that there is low-to-moderate quality evidence that supports a causal association between the incidence of cancer and occupational exposure to wood dust (Alonso-Sardón et al. 2015) .

Nickel is one of the carcinogens to human. Surveys performed in high-incidence areas found that nickel concentration on water, and hair of local inhabitants was significantly higher than that in low-incidence areas. On those areas, nickel content in NPC patients was also higher than in healthy population. Epidemiological surveys also found that trace elements like zinc and cadmium were positively associated with NPC (Bolviken et al. 1997).

2. Pathology Classification

2.1. Different pathological types of NPC and WHO Grade System.

NPC can be classified as non-keratinizing and keratinizing. The non-keratinizing group can be further divided into undifferentiated carcinoma and differentiated carcinoma groups.

2.1.1. Undifferentiated non-keratinizing carcinoma (WHO Grade 3): is the major and most important pathological type of NPC, although the exact percentage varies among populations worldwide. In endemic regions, undifferentiated carcinoma can represent between 47% and 92% of all cases of NPC (Shanmugaratnam et al. 1979; Tan & Putti 2005). In a important non-endemic region series (Western), this subtype represented only 44% of all NPCs (Al-Sarraf et al. 1998). It is widely recognized as the most radio and chemo sensitive histological type with consistent data referring to a better prognosis. It is microscopically containing tumor cells with spindle-to-oval vesicular or hyperchromatic nuclei with prominent nucleoli and which also feature mitotic activity. Variable lymphocytes and plasma cells numbers are seen.

2.1.2. Differentiated non-keratinizing carcinoma (WHO Grade 2): it is very similar to undifferentiated carcinoma, except that the tumor cells have a pavemented display with cell borders being readily discernable. Tumor cells may have a plexiform arrangement, a growth pattern that remembers the transitional cell carcinoma of the urinary tract. In series from Asia (i.e. Singapore), it represents between 7% and 49% of cases of NPC (Shanmugaratnam et al. 1979; Tan & Putti 2005). Despite histological similarities, differentiated non-keratinizing and undifferentiated NPCs have comparable prognosis. Whether their distinction have clinical significance remains to be proven.

2.1.3. Keratinizing Squamous Cell Carcinoma (WHO Grade 1): keratinizing squamous cell carcinoma (SCC) is uncommon in NPC-endemic regions. In Asia endemic areas, it accounts between 1% and 20% of all cases of NPC (Shanmugaratnam et al. 1979; Tan & Putti 2005). In contrast, the proportion of this subtype in non-endemic western populations has been reported to be up to 67% of all cases. Histologically, the tumor shows abundant keratinization, with squamous pearl formation and intercellular bridges. The tumor can be graded as well, moderately and poorly differentiated, as well as any other SCCs from elsewhere in the body. It can be described to have the worst prognosis of all NPC subtypes The 5-year survival is reportedly 20%–40% as compared with about

65% for non-keratinizing NPC subtypes. Recently, in Caucasians, SCC have been related to HPV infection, which holds a strong worse prognosis in NPC (Stenmark et al. 2014). Basaloid SCC is the most unusual variant of NPC with only a handful of cases reported in the literature and very similar to other basaloid SCCs that occur in the rest of the upper aerodigestive tract (Müller & Beleites 2000).

2.2. NPC and EBER

NPC has important and unique clinic-pathological features in the field of head and neck pathology. NPC is pathologically classified as non-keratinizing or keratinizing. The former is further subdivided into undifferentiated carcinoma and differentiated carcinoma subgroups and is the predominant form of NPC encountered worldwide, particularly in NPC-endemic regions.

Microscopically, non-keratinizing NPC is characterized by tumor cells growing in either a cohesive or reticulated pattern with a typical admixture of lymphocytes, while keratinizing NPC shows the features of a well-differentiated SCC. While light microscopy of H and E-stained sections remains the cornerstone of NPC diagnosis, EBV encoded early RNA in situ hybridization (EBERISH) and cytokeratin immunohistochemistry are useful adjuncts in diagnostically challenging cases.

EBER-ISH is almost always positive in non-keratinizing NPC, while it is negative in benign nasopharyngeal epithelium and most other malignant differential diagnoses of NPC. Cytokeratin immunohistochemistry helps highlight the irregular infiltrative nature of NPC, contrasting with the regular surface and cryptal epithelium of normal biopsies. Malignant differential diagnoses that need to be distinguished from NPC include lymphoma, sinonasal carcinoma, and melanoma, while benign mimics include reactive lymphoid germinal centers, crush artifacts, and post radiation changes. For the assessment of suspected metastatic sites, FNAC has been shown to be a convenient and fairly accurate technique.

The most relevant RNAs in EBV-infected cells are small nuclear EBER RNAs that are present at approximately 105 copies per cells (Arrand & Rymo 1982; Swaminathan et al. 1991).

EBER1 and EBER2 have 167 and 172 nucleotides, respectively. The EBERs are expressed in most of the malignancies related to EBV infection and its presence may contribute to the maintenance of latency in vivo (Raab-Traub 2002).

2.3. EBV and miRNA

Viral microRNAs (miRNAs) were first described following the cloning of RNAs from a B cell line infected with EBV (Pfeffer et al. 2004). EBV may also use cellular miRNAs to regulate gene expression. Viral miRNA have been extensively studied. A manually curated list can be found on Annex 1.

A recently described EBV property is that it encodes about 30 mature miRNAs from 20 pre-miRNAs, which represents a 1,000-fold enrichment relative to those in its human host (Landgraf et al. 2007; Pfeffer et al. 2004).

At least two cellular miRNAs have been described to have pleiotropic cellular effects that may be induced by EBV proteins. LMP1 activates the promoter for mir-146a, which down-regulates interferon-responsive genes. Thus, EBV may affect a cellular miRNA pathway involved in modulating the interferon response to enhance EBV replication (Lo et al. 2007).

The mir-155 is another cell miRNA derived from BIC, a noncoding RNA whose expression is up-regulated in a variety of B cell malignancies including diffuse large B cell lymphomas, CLL, and Hodgkin's lymphoma (Kluiver et al. 2005; Eis et al. 2005). Transgenic mice carrying an miR-155 transgene develop B cell lymphomas. This could indicate that EBV induces cell miRNAs with effects on immune responses and oncogenesis (Costinean et al. 2006).

3. Disease Staging

NPC workup includes complete examination of all head and neck sites in order to describe the full extent of the tumor. This will be critical to plan optimal radiotherapy since submucosal extension may not always be detected on imaging studies. Therefore, multidisciplinary consult with close cooperation with ENT specialist may yield endoscopic staging.

NPC commonly arises from the lateral pharyngeal recess and spreads widely into the surroundings along well-defined routes. Primary tumor volume is a significant independent prognostic factor of the disease, but technical complexity often limits its use

in daily practice. Cervical lymphadenopathy is very common in NPC and is usually the initial presenting complaint.

NPC has a relatively high incidence of systemic metastasis, and the risk increases in individuals with parapharyngeal tumor extension and supraclavicular lymphadenopathy.

Diagnosis of NPC is often achievable through endoscopic examination and biopsy, but imaging is essential for accurate staging of the tumor, delineating the full scale of submucosal, osseous, or intracranial tumor spread, the detection of which eludes clinical and endoscopic examinations. Multi-planar contrast-enhanced MRI is the best tool for full evaluation of the disease extent, while high-resolution bone algorithm CT is of value in assessing cortical bone erosion. A thorough understanding of the complex anatomy of the nasopharynx and the natural history of the disease facilitates accurate tumor mapping and treatment planning, which are crucial for favorable therapeutic outcome.

As EBV is an important etiologic factor, pretreatment analysis must include tissue testing for EBV-encoded RNA (EBER) and IHC staining for LMP1. The first is usually more sensitive than LMP1 for carcinomas. Serum analysis of PCR can detect EBV DNA load.

Some centers in the East and USA have been investigating the role of EBV serum load as a prognostic marker for post treatment recurrence. A least one meta-analysis including 13 studies demonstrated that pre-treatment serum EBV DNA levels were associated with risk of death (HR, 2.81; 95% CI, 2.44–3.24; $P < .001$) and distant metastasis (HR, 3.89; 95% CI, 3.39–4.47; $P < .001$). Plasma EBV DNA has also been studied as an indicator of disease response to chemotherapy as induction therapy prior to chemoradiation and in the setting of distant metastases.

3.1. AJCC-UICC 8th Edition Grading system

The American Joint Committee on Cancer (AJCC) TNM Staging System for the Nasopharynx has recently been updated in its 8th edition (2017). Despite its importance neither EBV viral load nor WHO grading are part of the current staging, but are important as prognostic factors. The following types of cancer are not included along NPC and have their own staging and guidelines: mucosal melanoma, lymphoma, and sarcomas of the soft tissue, bone and cartilage. Group staging can be seen on Table 1. The AJCC-UICC 2017 8th Edition stages NPC as follows:

Primary Tumor (T) :

- **TX** Primary tumor cannot be assessed
- **T0** No tumor identified, but EBV-positive cervical node(s) involvement
- **Tis** Carcinoma *in situ*
- **T1** Tumor confined to the nasopharynx, or extension to oropharynx and/or nasal cavity without parapharyngeal involvement
- **T2** Tumor with extension to parapharyngeal space, and/or adjacent soft tissue involvement (medial pterygoid, lateral pterygoid, prevertebral muscles)
- **T3** Tumor with infiltration of bony structures at skull base, cervical vertebra, pterygoid structures, and/or paranasal sinuses
- **T4** Tumor with intracranial extension, involvement of cranial nerves, hypopharynx, orbit, parotid gland, and/ or extensive soft tissue infiltration beyond the lateral surface of the lateral pterygoid muscle

Regional Lymph Nodes (N):

- **NX** Regional lymph nodes cannot be assessed
- **N0** No regional lymph node metastasis
- **N1** Unilateral metastasis in cervical lymph node(s) and/or unilateral or bilateral metastasis in retropharyngeal lymph node(s), 6 cm or smaller in greatest dimension, above the caudal border of cricoid cartilage
- **N2** Bilateral metastasis in cervical lymph node(s), 6 cm or smaller in greatest dimension, above the caudal border of cricoid cartilage
- **N3** Unilateral or bilateral metastasis in cervical lymph node(s), larger than 6 cm in greatest dimension, and/or extension below the caudal border of cricoid cartilage

Distant Metastasis (M):

- **M0** No distant metastasis
- **M1** Distant metastasis

Histologic Grade (G): A grading system is not used for NPC staging.

Table 1: Anatomic Group stage by combined prognostic factors (Foote et al. 2018).

Stage 0	Tis	N0	M0
Stage I	T1	N0	M0

Stage II	T0, T1 T2	N1 N0, N1	M0 M0
Stage III	T0, T1, T2 T3	N2 N0, N1, N2	M0 M0
Stage IVA	T4 Any T	N0, N1, N2 N3	M0 M0
Stage IVB	Any T	Any N	M1

4. Treatment of NPC

4.1. Treatment Overview

Unlike other head and neck sub sites, nasopharyngeal carcinoma, anatomy, disease behavior, richness of lymphatic drainage makes surgical attempts less useful in an upfront scenario.

Since long ago, radiation therapy has become the cornerstone of NPC treatment, yielding local and regional controls that allow symptom relief and enduring disease control (Marks et al. 1982; Vikram et al. 1985).

Several techniques may be used to optimize therapeutic ratio. In the past two decades, intensity-modulated radiation therapy (aka IMRT) has dramatically changed outcomes for this entity, and became the preferred technique to enhance local control and reducing long-term severe toxicities.

Other techniques, such brachytherapy, stereotactic radiosurgery (SRS) and fractionated stereotactic radiotherapy (SRT) also brought opportunities for the small proportion of patients that will locally recur.

Not a novelty among the radiotherapy community, particle therapy via protons, neutrons, or heavy ions have become more available and their unique Bragg's Peak physical property draw attention to new capabilities like never before. Emerging data confirms their ability to spare normal tissue in head and neck radiotherapy. Clinical trials are ongoing.

Advances in surgery with new tools and robotic assistance are increasing in other head and neck sub sites (i.e. TORS in oropharyngeal cancer) and may represent another option for recurrent patients.

Nevertheless, the majority of patients will succumb due to distant metastases. This behavior has always challenged the scientific community and makes a line of research for several types of systemic therapies to date.

4.2. Early Stage Treatment (Stage I)

The definition of early stage disease in NPC varies enormously in the literature regarding the Staging system used and in the Eastern and Western countries.

In Western countries, NPC is a rare cancer in most of countries and regions and it is presented typically in advanced stages. Since, the majority of patients will receive concurrent chemo-radiation with or without adjuvant chemotherapy (Intergroup 0099 trial). This will be further discussed in the next section (4.3).

There is scarce evidence that local disease (i.e. T1 or T2 N0 patients) need chemotherapy. On the other hand, there is solid historical evidence that radiotherapy alone is able to yield excellent outcomes.

A review of the literature indicates that consider all patients stages I and II as early stage group is not appropriate.

There is a higher incidence of WHO grade I NPC in the western world, strongly associated with poorer prognosis of this subgroup. Even in small tumors, these patients could be considered to CCRT.

In addition, variations in the staging systems used lead to some ambiguity in the definition of early or advanced local disease. As a result, patients with bulky tumors that are not purely T3 should be considered for more aggressive therapy.

Randomized trials to investigate the benefit of concurrent chemotherapy in early stage NPC and refine an appropriate regimen are needed.

4.3. Loco-regionally Spread Disease (Stages II-IVA)

Management of loco-regionally advanced NPC has evolved enormously and fast during the past two decades. While radiation therapy alone was generally accepted to be the only curative treatment for this entity only not a long time ago (Lu et al. 2016).

The cornerstone of this practice-changing paradigm was the publication of Intergroup 0099 trial, which provided the highest level of evidence to support chemotherapy as an integral part of curative NPC treatment.

The Intergroup trial 0099 randomly assigned 193 patients for chemoradiation plus chemotherapy versus radiotherapy alone. The trial closed prematurely when an interim analysis showed a significant survival advantage favoring combined chemoradiation (Al-Sarraf et al. 1998). The addition of chemotherapy had also a positive impact in decreasing local, regional, and distant metastases rates.

Three posterior Asian trials from Hong Kong, Taiwan and Singapore, confirmed the benefit of adding chemotherapy to radiotherapy for locally advanced NPC (Chan et al. 2005; A. T. Chan et al. 2004; Lin et al. 2003).(Wee et al. 2005). Overall survival at 5 years reached 70% for the chemoradiation group versus 59% for the RT group in the Hong Kong trial.(A. T. Chan et al. 2004)

The Singapore trial, designed after the Intergroup 0099, also showed the benefit of chemoradiation to RT alone. The striking difference was the addition of more chemotherapy after the combined modality phase. Patients received adjuvant cisplatin with 5-FU with a more favorable toxicity profile (Wee et al. 2005).

Subsequently, a randomized phase III trial from Sun Yat-sen University Cancer Centre (Guangzhou, China) comparing concurrent chemoradiation with or without adjuvant cisplatin/5-FU did not found significantly improved survival with adjuvant chemotherapy following chemoradiation ($P = 0.13$) (Chen et al. 2012).

The Meta-Analysis of Chemotherapy in Nasopharyngeal Carcinoma (MAC-NPC) from Blanchard et al. pooled 4806 patients from 19 trials showed that both chemoradiation with or without adjuvant chemotherapy were associated with improvement in overall survival and progression-free survival. Unfortunately, there were imbalances in trials from East and West, specially regarding the number of patients with stage II receiving adjuvant chemotherapy, as well as different AJCC staging editions used, jeopardized the analysis' ability to conclude efficacy between modalities.

In 2017, a network meta-analysis based on this individual patient data meta-analysis (including 5144 patients from 20 trials) confirmed that the addition of adjuvant chemotherapy to chemoradiation was associated with better progression-free survival compared to chemoradiation alone (Ribassin-Majed, Marguet, Anne W.M. Lee, et al. 2017).

Neoadjuvant or induction chemotherapy (before concurrent chemoradiation) has also been evaluated for loco-regionally advanced NPC. At least one phase III, randomized, multi-institutional Chinese trial with 480 patients stage III-IVb, with neck disease, demonstrated a better 3 year failure free survival rate of 80% for patients who received induction chemotherapy (TPF) plus chemoradiation versus 72% for those randomized to receive chemoradiation alone (P = .034)(Sun et al. 2016).

Nevertheless, toxicity was significantly more pronounced in the experimental arm. Grade 4 toxicity occurred in 18% of patients who received induction chemotherapy versus 1% of who received chemoradiotherapy alone (P < .001).

In another Chinese randomized trial, 476 stage III-IVb NPC patients were randomly assigned to receive either induction cisplatin with 5-FU followed by chemoradiation, or chemoradiation alone. Three-year disease-free survival was 82% for induction versus 74% to chemoradiation alone (P = .028). Multivariate analyses showed a difference between treatment arms for disease-free survival (P = .023) and distant metastasis-free survival (P = .038). However, OS was not significantly better in patients receiving the induction chemotherapy group (Cao et al. 2017).

Recently, a Singaporean systematic review and meta-analysis was performed containing data from 6 randomized controlled trials (RCTs) and 5 observational studies enrolling 2802 patients. The authors concluded that the addition of IC improved both PFS and OS substantially, but was associated with more toxicity for patients with locally advanced NPC. The consistency of treatment effect between RCTs and high quality OBS suggests that IC is likely to confer real and substantial improved cancer outcomes, at the cost of increased moderate to severe acute toxicities. Nevertheless the authors acknowledge that their conclusion have not been drawn on the basis of individual patient data so it was not possible to recommend it for a specific subgroup (Tan et al. 2018).

Considering the highest level of evidence, results to date suggest that induction chemotherapy before chemoradiation to this subset of patients with locally advanced NPC may potentially impact tumor control, compared to chemoradiation alone. Nevertheless, it

remains unclear whether to administer chemotherapy before or after chemoradiation (Ribassin-Majed et al. 2016; Zhang et al. 2017).

So, administering more chemotherapy to patients with locally advanced NPC, as induction or adjuvant, along concomitant chemoradiation, achieves a reduction in recurrence rates. The choice of the most suited regimen for a given patient must include a consideration of the risk–benefit ratio. Taking into consideration the significant grade 4 rates, the option of induction chemotherapy must be done judiciously.(Ribassin-Majed, Marguet, Anne W.M. Lee, et al. 2017)

Once indicated, it is clear that cisplatin for chemoradiation is recommended for patients who do not have a contraindication to the drug, because the majority of randomized trials support the use of cisplatin-based chemotherapy in this setting. If using adjuvant chemotherapy, adjuvant carboplatin/5-FU is a widely accepted option (Ribassin-Majed et al. 2016; Blanchard, Lee, Marguet, Leclercq, Ng, Ma, Chan, Huang, Benhamou, Zhu, Chua, Chen, Mai, Kwong, Cheah, Moon, Tung, Chi, Fountzilas, Zhang, Hui, Lu, Bourhis, Pignon, et al. 2015).

Several trials under way may bring more light to relevant questions in NPC management. The NRG-HN001 trial (NCT02135042) is in progress and investigates the role of adjuvant chemotherapy following chemoradiation in patients with locoregionally advanced NPC. In this trial patients are selected for adjuvant treatment regarding EBV DNA plasma levels. As EBV is used in Eastern countries as a prognostic factor, this is the first Western trial to incorporate EBV as a patient selection tool.

Additional trials investigate the role of different schemes of induction chemotherapy along accelerated radiotherapy and should be reported soon and might help clarify this matter (NCT01245959, NCT01536223, NCT01872962, and NCT02512315).

4.4. Post-treatment Evaluation and Work Up

Since the deep areas of the skull base may be inaccessible to clinical examination, periodic cross-sectional imaging may be necessary. The clinical benefit of blood EBV DNA monitoring is currently uncertain (see *Epstein-Barr Virus*, above).

In Europe, at least one Italian group has recommended the use of viral copies screening during follow up (Ferrari et al. 2012).

In two large Asian studies, post-treatment level of circulating EBV DNA has shown to be the most significant prognostic factors for progression-free survival and overall survival (Lin et al. 2004; A. T. C. Chan et al. 2004). Post-treatment persistently elevated or rising EBV DNA has been shown to predict and clinical recurrence 3 to 7 months in advance.(A. T. C. Chan et al. 2004; Lin et al. 2003) (Lo et al. 2000; Ngan et al. 2001)

There is sufficient and strong evidence that circulating EBV DNA level is a reliable marker of the extent of subclinical disease and generated the hypothesis that EBV DNA could be used to select patients for more intense therapy or de-escalate aggressive treatments (Lin et al. 2004; Wang et al. 2010). There is an ongoing phase III trial accessing this issue (NRG-HN001 trial - NCT02135042).

Nevertheless, the EBV DNA levels associated with a complete response may vary regarding the center or method used. While the data from Chan et al. reported a levels below 500 copies/ml as marker of complete response in patients treated with chemoradiation, others like Lin et al. reported that undetectable plasma EBV DNA via PCR were strongly correlates with complete response (Lin et al. 2004; A. T. C. Chan et al. 2004).

It seems that quantitative PCR detection is very sensitive method subjected to several variables that may affect the final results. As in other areas, quality assurance is essential to warrant reproducibility and reliable comparison of results. These include the calibration of the PCR instrument, the DNA extraction method, the reference standard and center/operator experience (N. Y. Lee et al. 2012a).

In Portugal, the ongoing trial VEBINASO by Costa et al. (abstract) is currently accessing the role of EBV loads and clinical outcomes. Results are pending and may clarify the viral behavior in our cohorts.

4.5. Metastatic Disease

4.5.1.Metastatic Disease at Presentation

Due to its sensitivity to radio and chemotherapy, there is a rationale to offer radical treatment to patients that present themselves with distant metastasis at diagnosis.

In fact, Asian data from Japan (Yeh et al. 2006) have already described the positive effect of radiotherapy even on Stage IVc with results comparable to chemotherapy alone. In 2017, Rustoven et al. have reviewed the outcomes of 718 metastatic NPC patients and concluded that definitive radiotherapy to the primary site had a positive impact in disease-free survival and overall survival. (Rusthoven et al. 2017). In fact, their conclusion acknowledges that long-term survivors for this subgroup (i.e. IVc) were only possible within radical radiotherapy group.

The 2018 NCCN Guidelines have already been written to incorporated radical chemoradiation as an option for metastatic NPC (Foote et al. 2018).

4.5.2.Metastasis After Definitive Treatment

Consider radiotherapy to pain sites or sites where progression may affect quality of life (i.e. asymptomatic bone metastasis for fracture reduction risk). Several clinical trials ongoing accessing optimal therapy for stage IVc NPC, including immunotherapy.

4.6. Locally Recurrent Disease

It is from vital importance to adequately rule out the possibility of persistent disease or late-responding patterns already described in the literature. It has already been described that late responders may represent up to 20% of patients treated with NPC (Wei & Kwong 2011; Chua et al. 2001).

It its reasonable to detect real progression or recurrence of a tumor previously treated and which has been observed response before indicating further treatments.

Once local recurrent disease is confirmed, options may include: surgery, several ways of re-irradiation (brachytherapy, fractionated stereotactic radiotherapy, radiosurgery or proton therapy) with or without chemotherapy, palliative chemotherapy or, more recently immunotherapy clinical trials.

Asian surgeons have reported impressive local control rates for salvage surgery for NPC. Several centers have used maxillary swing and trans-palatal techniques of conventional surgery to access selected small recurrences (Chua et al. 2009; Wei & Kwong

2011; Wei & Kwong 2010; Stoker et al. 2013a).

Wei et al (2011) have reported a series of 256 patients who underwent maxillary swing surgery for recurrent NPC. Skull base or carotid artery involvements were exclusion criteria. They achieved local control of 75% at 5 years and disease free survival of 56%. Carotid bleeding occurred in 1% of patients, fistulae in 1 % and *trismus* (< 2cm) in 21% of patients (Wei & Kwong 2011).

Most of the Western literature in recurrent NPC offers re-irradiation as the most frequent option. Re-irradiation via IMRT, FSRT or brachytherapy is a valuable choice. Patient selection is key to achieve enduring control.

Another option is photodynamic therapy (PDT). The Dutch group has published their experience of 22 patients treated with PDT. For recurrent T1 and T2 tumors with less than 10 mm depth, their local control rate at 3 years was 100%. Nevertheless, only 17 out of 22 had tumor evaluation with effective complete response. As there is no cumulative effect, PDT can be repeated making an valid alternative for small superficial recurrences (Nyst et al. 2012).

Because local recurrences can be treated with various modalities of surgery, radiation or PDT, chemotherapy for local recurrences is only indicated when further local treatment is ruled out. Moreover, since most of studies addressing the effect of chemotherapy for locally recurrent patients, included patients with failures elsewhere in the body, it is difficult to reach a significant level of evidence to support its role in exclusive local recurrences.

So far, the small series that addressed its use have shown disappointing results. Since patients receive platinum-based chemotherapy at the primary treatment, further platinum regimens exist but depend on a patient-basis evaluation of toxicity and recovery. Alternatives with non-platinum components include taxanes, or capecitabine with response rates ranging from 20-48%. Other agents like Gemcitabine have been used with limited utility (Ngan et al. 2002) Stoker et al., 2013b).

Combination with EGFR for NPC has been already been published, not very satisfactory, though, with an overall response rate of 11 %. A phase II study using the EGFR inhibitor gefitinib in 19 patients with recurrent or metastatic NPC, resulted in no objective responses (Chua et al. 2008).

Currently, several clinical trials are open evaluating the role of immune therapy for

recurrent and/or metastatic NPC. Initial results presented in abstract form at ASTRO 2017 are promising and may represent a new paradigm to this disease.

4.7. Childhood NPC Treatment

To date, children and adults are not submitted to the same NPC clinical trials. Nevertheless, chemoradiation treatment remains the same in a slight different integration. Two published prospective multi-centered trials are available on the management of pediatric NPC.

In the German GPOH Study NPC-91, accrual accounted for 58 high-risk patients less than 25 years-old treated with 3 cycles of induction multi-agent chemotherapy (methotrexate, cisplatin, and 5-FU) followed by radiotherapy. All patients received recombinant IFN-b for 6 months after radiotherapy. The rationale for post RT immunotherapy was: suppressed immunological response in patients with NPC, the strong role of EBV infection on NPC, antiangiogenic effect of interferon, and a 10%–15% response of IFN-b as a single agent in patients with recurrent NPC. Their results were superb with 91% DFS and 95% OS rates. Among their cohort, one patient showed tumor progression during chemotherapy, and another patient had only 1 cycle of treatment because of acute cardiotoxicity. Distant and local relapses were reported in 3 and 1 patients, respectively. It was concluded that the combination of induction chemotherapy, intermediate to low RT doses (59.4 Gy to primary tumor, 45 Gy for cervical lymph nodes), and post RT IFN improved the outcome. The German group has since launched a new treatment protocol, more likely the standard adult approach, to investigate the role of concurrent cisplatin-based chemoradiotherapy followed by 6 months treatment of interferon after three cycles of induction cisplatin and 5-FU(Mertens et al. 2005).

The other prospective multi-centric study (POG 9486) from the U.S.A, included 16 high-risk patients less than 22 years-old were treated with 4 cycles of the same induction chemotherapy with methotrexate, cisplatin, and 5-FU followed by RT alone. Only one patient had progression during induction chemotherapy and the overall response rate to 4 cycles of induction chemotherapy was 93.7%. The overall 4-year EFS and OS rates were lower than the German's, with 77% and 75%, respectively. All failures occurred in systemic sites regardless more induction chemotherapy. The severity of mucositis and the need for nutritional support was greater than expected, allegedly related to methotrexate.

In their new protocol, the same study group will explore the use of less toxic induction chemotherapy with cisplatin and 5-FU, followed by concurrent chemoradiotherapy. As the German group did, it's an approach closer to the evidence of adult population. The circulating EBV-DNA levels will also be measured to evaluate the prognostic significance in an attempt to create risk-adapted, less toxic but effective therapies. The results from these two multi-centric trials indicated that induction chemotherapy improved the outcome and allowed radiation dose reduction tailored to 60Gy in responsive patients (Rodriguez-Galindo et al. 2005).

The OS and the DFS rates of patients in GPOH study was superior to the American study, although patients in the POG study had more chemotherapy (4 cycles vs 3 cycles) and a higher dose of radiotherapy (70Gy vs 60Gy). The difference may be explained by the incorporation of immunotherapy with interferon in the German GPOH study and/or the severe toxicity profile of 4 cycles of induction chemotherapy used in the POG study, which might have jeopardized the radiotherapy parameters (delay, overall treatment time, etc.)(Lu et al. 2016).

In conclusion, induction chemotherapy improved the outcome of childhood and adolescence NPC by increasing both the locoregional control and also the systemic control. Induction chemotherapy has allowed radiation dose reduction from 70 to 60Gy. Further reductions may be feasible, as it was shown that these patients have superior results and initial response to induction chemotherapy is a strong predictor of the outcome (Lu et al. 2016).

4.8. Ongoing Studies of Immunotherapy in NPC

Since interferon has been used for decades in child or recurrent NPC, immunotherapy is not a novel alternative. Currently, contemporary immunotherapy has already been incorporated in the algorithm of recurrent and metastatic head and neck cancer and several studies addressing its role in the curative setting are under way. There are also two multi-centric trials evaluating the role of immunotherapy in NPC treatment.

"A phase III Trial evaluating chemotherapy and immunotherapy for Advanced NPC patients" is a multi-center, randomized, open label, phase III clinical trial for advanced NPC patients. Drugs used in chemotherapy, such as gemcitabine and carboplatin, work in

different ways to stop the growth of tumor cells, either by killing the cells or by stopping them from dividing. Giving an infusion of cytotoxic T lymphocytes (CTL) that have been treated in the laboratory may help the body build an effective immune response to kill tumor cells. Giving combination chemotherapy together with laboratory-treated T lymphocytes may kill more tumor cells. This Phase III trial is to assess if combined gemcitabine-carboplatin (GC) followed by adoptive T-cell therapy would improve clinical outcome for patients with advanced nasopharyngeal carcinoma (NPC). It is also the world's phase III trial with T-cell therapy ever conducted in NPC. Enrollment is aiming for 330 patients from 29 hospital centers across Asia and North America. This trial is conducted on the basis of a previous phase II NPC trial involving 38 patients at the National Cancer Centre of Singapore. This trial produced the a published 2-year (62.9%), and median overall survival (OS) data (29.9 months) in 35 patients with advanced NPC who received autologous EBV-specific CTL. (Chia et al. 2014)

Another trial is “Safety and Efficacy Study of PDR001 in Patients With Recurrent or Metastatic Nasopharyngeal Carcinoma”. The purpose of this randomized controlled Phase II study is to assess the efficacy of PDR001 versus investigator's choice of chemotherapy in patients with advanced NPC, recurrent or metastatic. By blocking the interaction between PD-1 and its ligands PD-L1 and PD-L2, PDR001 leads to the activation of a T cell mediated antitumor immune response (NCT02605967). Results from both trials are expected to be available not before 2020.

Recently approved by the FDA, Epacadostat® is an orally available hydroxyamidine and inhibitor of indoleamine 2,3-dioxygenase (IDO1), with potential immunomodulating and antineoplastic activities. It targets and binds to IDO1, an enzyme responsible for the oxidation of tryptophan into kynurenine. By inhibiting IDO1 and decreasing kynurenine in tumor cells, epacadostat increases and restores the proliferation and activation of various immune cells, including dendritic cells (DCs), NK cells, and T-lymphocytes, as well as interferon (IFN) production, and a reduction in tumor-associated regulatory T cells (T-regs). Activation of the immune system, which is suppressed in many cancers, may inhibit the growth of IDO1-expressing tumor cells. IDO1 is overexpressed by a variety of tumor cell types and DCs (Komiya & Huang 2018).

5. Part II – Proteomic Principles

The term "proteome" refers to a set of proteins encoded by a genome (Wasinger et al. 1995). The proteome is not constant; it differs from cell to cell and changes over time. The large-scale analysis of the proteome is often called Proteomics.

Several technologies have been developed to investigate proteomes. The most commonly used rely on mass-spectrometric methods for protein identification/ quantification and can be divided in Gel-based proteomics and Shotgun proteomics.

5.1. Protein Identification Technologies

Since mass spectrometry (MS) was possible, protein mining technologies, such as Matrix-assisted laser desorption ionization (MALDI) mass spectrometry and electrospray ionization (ESI) have been developed and allowing identification in of proteins in cancer cells (Karas & Hillenkamp 1988; Dalerba et al. 2007).

Further developments led to identification as a function of particle composition, wavelength, and size with a time-of-flight mass spectrometer (TOF-MS)(Thomson & Murphy 1993). Later in 1996, it was demonstrated that MS had the sensitivity, specificity and throughput for large amount of information (Shevchenko et al. 1996).

MALDI-MS profiling requires the solubilization of proteins/peptides and removal of interfering compounds (lipids and salt). A small amount of solubilized protein mixing is then embedded in a matrix, producing protein ions carrying a single charge for MS (Albalat et al. 2014).

Electrospray ionization, by itself, generates charged droplets producing multiple charged ions allowing examination of hydrophobic membrane-bound proteins via MS (Schey et al. 1992).

Both, MALDI and ESI, are used for ion sources in biological research and it is It is possible to obtain MS by both ways combined with TOF-MS (Chen et al. 2015b).

MS represents the relative presence of ionizable molecules with different mass-to-charge (m/z ratio) values. Any given m/z value in MALDI-MS is, indeed, a molecular mass. Therefore, an intensity of a spectrum for any m/z-ratio, represents the relative presence of a specific substance matching this m/z-ratio (Chen et al. 2015b).

Surface-enhanced laser desorption/ionization time of flight mass spectrometry (SELDI-TOF-MS) was first described by Hutchens et al. in 1993. It is a MALDI modification using a different target to reach biochemical affinity (Liu 2011).

In SELDI-TOF-MS, the sample mixture is placed on a modified surface that allows binding affinity. Then, the matrix is applied for crystallization. After binding and washing off, surface-bound proteins remain for analysis. Finally, spotted samples on SELDI surface are analyzed via TOF-MS (Liu 2011).

Among its advantages, SELDI technology, it's suitable for serum analysis because samples are deposited without been dried over MALDI allowing detection of low weight compounds, already described in oncology for colorectal cancer(Zheng et al. 2006).

SELDI-TOF-MS is able to deliver a selective and fast protein profiling with minimal requirements for purification and separation. This technology has become a promising technology for the early cancer detection and response prediction (De Bock et al. 2010).

5.2. Gel-based proteomics

In gel-based proteomics, the proteome can be separated by one-dimensional polyacrylamide gel electrophoresis (1-DE), two-dimensional gel electrophoresis (2-DE) or differential gel electrophoresis (DIGE).

5.2.1 One-dimensional SDS-polyacrylamide gel electrophoresis (1-D SDS-PAGE, 1-DE): is used for molecule separation by size and charge. SDS is a detergent that denatures and non disulphide-linked tertiary structures and combines them with a negative charge correlated with their length. This method allows molecular weights to be estimated (Laemmli 1970).

SDS is a non-continuous gel that has different pH values and polyacrylamide concentrations. Therefore, small proteins will move through the gel more quickly than large proteins and, depending on the concentration of polyacrylamide and each protein, the latter are separated in bands (Brunelle & Green 2014).

5.2.2 Two-dimensional SDS-PAGE (2-DE): consists of two steps or dimensions of separation: the first dimension, protein molecules are separated depending on their molecular weight (MW) and charge (isoelectric point, pI). The second dimension, separation is performed based only on the MW. Once each protein molecule has its own

MW or/and PI, proteins are more efficiently separated by 2-DE rather than by 1-DE (O'Farrell 1975).

2-DE is a powerful tool that is used for the separation and fractionation of protein mixtures from humans, animals, plants, or other microorganisms, becoming a widely used technology. It has the ability to fractionate, identify, and quantify proteins molecules when used along mass spectrometry (MS) or other immunological tests (Magdeldin et al. 2014).

5.2.3 Two-dimensional difference gel electrophoresis (2-DIGE): 2-DIGE was developed to optimize limitations in 2-DE such reproducibility problems, narrow dynamic range, low-throughput and workload, as well as limitations in separation of hydrophobic and acidic proteins (Friedman et al. 2004). Usually, in 2-DIGE, protein samples are pre-labeled, allowing different samples to run on the same gel in two dimensions. Fluorescence allows protein detection after electrophoresis. At the end, the differences in composition of each protein can be seen without any gel post-processing. 2-DIGE has great sensitivity and accuracy in quantitative proteomics. Nevertheless, both technologies of 2-DE and 2-DIGE are gel-based methods of separation that share the same problem of reducing enzyme accessibility to the protein or missing large peptides extracted from the gel_Even if described in other areas such as cardiology, cross-talk allowed use in a broad range of applications (Warren et al. 2010). Despite several optimization methods such, high-resolution and limited-range isoelectric focusing (IF), liquid isoelectric focusing (LIEF), fluorescent labeling and enhanced detergents may be used, other separation methods were developed (Petriz et al. 2012)(Issaq et al. 2002).

5.3. Shotgun proteomics

Shotgun proteomics is a high-throughput technique that allows for the identification of thousands of proteins in a complex sample by coupling Reverse-phase liquid chromatography (RP-LC) to tandem mass spectrometry via Electrospray ionization (ESI). In a typical shotgun proteomics experiment, the proteome is digested with an enzyme, typically, trypsin and the resulting pool of peptides is then separated in a RP-LC. The peptides are then ionized in the ESI ions source, analyzed and selected, on the flight, for fragmentation in order to retrieve sequence information. A very important issue in proteomics is to determine the protein abundance or at least to determine the fold change between conditions. To that end, several relative quantification techniques have been established, such as Isotope-coded affinity tags (ICATs), stable isotope labeling of amino

acids (SILAC), isobaric tags for relative absolute quantitation (iTRAQ), and label-free quantification.

5.3.1 Isotope-Coded Affinity Tags (ICATs): electrospray-tandem mass spectrometry (ESI-MS/MS), combined with LC, is one of the most common gel-free proteomics methods. And LC-MS/MS has been used successfully for the large-scale identification of proteins without gel electrophoresis separation (Issaq et al. 2002)(Cho et al. 2014). It was based on a class of reagents named isotope-coded affinity tags (ICATs) and tandem mass spectrometry (MS). ICATs consist of three properties or elements: specific chemical reactivity, isotopically coded linkers, and affinity tags. ICATs could be used to detect sufficient amounts of low-abundance proteins via MS. Its limitation relies on that can only be applied to comparative groups of two, limiting several experimental controls (Issaq et al. 2002; Petriz et al. 2012).

5.3.2 Stable Isotope Labeling of Amino Acids (SILAC).

SILAC stands for stable isotope labelling of amino acids and was originally described by Ong et al., 2002 (Ong et al. 2002)._To label proteins via SILAC has several advantages. SILAC requires no peptide labeling steps, so optimizing biological material. Second, incorporation is virtually 100%, eliminating differences in labeling between samples. Proteins are uniformly labeled, so several peptides from the same protein can be compared. The mass differential between two states can be specified more directly. Therefore, SILAC uses most of the devices already available in most laboratories making a simpler, inexpensive, and accurate alternative for quantitative proteomic. It overcomes ICATs' disadvantage in quantitation changes for proteins that may not contain any cysteine residues. Nevertheless, SILAC depends on living biological material.

5.3.3 Isobaric Tags for Relative Absolute Quantitation (iTRAQ).

iTRAQ puts isobaric mass labels at chains of peptides in a digestion mixture. Ions are used to identify and quantify each member of a protein set. The isobaric nature of the labels permits the simultaneous comparison of multiple samples. Its multiplex nature removes the quantitative variability from chromatography that may be seen in sequential 2D-LC-MS analyses of individual peptide mixtures. Peptide coverage is significantly increased relatively to ICAT. The tagging chemistry is global, so any peptide with a free amine can be labeled and measured. It has complemented 2-DE, 2DEIG, ICATs, and SILAC methods within proteome analysis (Ross et al. 2004).

5.3.4 Label-free quantification

Label-free quantification (LFQ) is a mass spectrometry method that aims to determine the relative amount of proteins in different biological samples. Unlike the previous mentioned methods used for protein quantification, label-free quantification does not use stable isotopes to chemically label the proteins (Wei et al. 2008; Chen et al. 2015a).

LFQ may be based on precursor signal intensity using high-resolution data obtained from time-of-flight (TOF), or Orbitrap mass analyzers. The high-resolution power facilitates the extraction of peptide on the MS1 level, uncoupling quantification from the identification process (Chen et al. 2015a).

5.4. Proteomic Research in Nasopharyngeal Carcinoma

Proteomics analyses are useful tools to understand the tumor proteome in cancer such NPC. It has described NPC protein expressions in different stages, allowing comparison between samples, protein interactions and identification of signaling pathways. Different materials may be used for NPC proteomic: cell-lines, xenograph NPC in mice, human fluids or tissues (fresh, frozen or FFPE).

In this review, we'll focus on the evidence regarding FFPE NPC biopsy samples.

5.4.1. Review of Proteomic Analyses in NPC Tumor Biopsies

It has been reported that proteomics research in human tissue or fluid of NPC dates back to 2005 (Chen et al. 2015b) and a NPC proteome map was first described by Chen's group in 2007(Li et al. 2007).

At their work, Li et al. analyzed 20 NPC fresh biopsy specimens via 2-DE, MALDI-TOF-MS and ESI-Q-TOF-MS. They identified 216 proteins that can be used for comparison between WHO grade types of NPC, disease stages or correspondence between normal and tumor tissues or fluids (Li et al. 2006; Li et al. 2007).

Tumor samples from NPC (via FFPE) patients allow us to obtain invaluable information of morphological pathology, WHO grading, immunohistochemistry, EBER positivity and expression and tumor percentage on each sample. In case of heterogeneity, it is still possible to enhance tumor percentage, separating stroma and lymphocyte infiltration using techniques such as laser capture microdissection (LCM) (Ai Lan Cheng et

al. 2008).

In 2008, Cheng et al. analyzed and compared proteins from NPC and normal nasopharyngeal epithelium (NNE) via LCM. Samples were submitted to 2-DE and MALDI-TOF-MS and ESI-TOF-MS. Results showed 36 expressed proteins between the two tissues. Stathmin, 14-3-3 σ and annexin-I identified in NPC samples were related with WHO grading and metastases. The same group has identified up-regulated cathepsin-D as a poor prognosis marker (Ai Lan Cheng et al. 2008).

Keratin-18 up-regulated was also related to worse prognosis as it was down-regulated in NPC but up-regulated in lymph node metastasis. Both cathepsin-D and keratin-18 are since considered biomarkers for NPC prognosis (X.-M. Li et al. 2009).

In 2008, Cheng et al., using 2-DE, MALDI-TOF-MS and ESI-QTOF-MS, demonstrated that Raf kinase Inhibitory protein (RKIP) maybe a NPC metastasis suppressor. Decreased RKIP was associated with invasiveness of NPC cells via Raf-1/MEK/ERK pathway activation. Another 20 proteins were markedly different in NPC and NNE (Chen et al. 2008). RKIP was confirmed as a metastasis suppressor in other series using proteomic approaches (Yuan et al. 2016; Granovsky & Rosner 2008)

Comparing NPC patients with lymph node involvement with 2-DE and MALDI-TOF-MS, Liao et al. identified that HSP27, slCAM-l, cathepsin-G, and other proteins were up-regulated and NM-23-H1 proteins was down-regulated in NPC patients with lymph node metastasis (Liao et al. 2008).

In 2010, Li et al. used LCM 2-DE and MALDI-TOF-MS/ESI-QTOF-MS to demonstrate that Keratin-31, vimentin, and prohibitin were up-regulated in tumour stroma while Rho GDP dissociation inhibitor (GDI) β, superoxide dismutase, keratin-19, and annexin-A2 were down-regulated. CapG was also upregulated in NPC stroma cells (Li et al. 2010). Since 2004, the Ghent data had already demonstrated that active nuclear import of an actin binding protein pointing to a role for nuclear CapG in invasion (De Corte et al. 2004).

Rho GDP dissociation inhibitor (GDI) β, Superoxide dismutase, and Keratin-19 were also described down regulated in another study evaluating tumor stroma, alongside HSP 70. At the same cohort, periostin, keratin-1, and nm-23 protein, were up-regulated in tumour stroma (M.-X. Li et al. 2009).

In 2009, Chen et al. used 2-DE and ESI-Q-TOF-MS to compare NPC to NNE tissues. They found 13 tyrosine phosphorylated proteins level of RKIP, 7 up-regulated and 6

down-regulated in NPC that may be involved in the development and progression of the disease (Chen et al. 2009).

In 2009, Huang et al., using SELDI-TOF-MS compared NPC to normal controls without cancer and demonstrated that the method was able to identify one protein (m/z 13,738) highly present on NPC. The authors conclude that the technique could be useful for population-based studies (Huang et al. 2009).

In 2010, Cao et al. used the same method to detect an early stage profiling of NPC. The SELDI-MS profiling was better than the current EBV capsid antigen IgA antibodies (VCA/IgA). They conclude that their profiling might be a potential tool to identify NPC patients (Cao et al. 2010).

Xiao et al, used iTRAQ to explore FFPE NPC tissues and detected that the expression levels of cathepsin D, keratin-8, 14-3-3σ, and stathmin-1 were correlated to WHO grading histological types of NPC. Their results were consistent with the immunohistochemistry previously acquired (Xiao et al. 2010a).

In 2011, Su et al. compared primary NPC to recurrent NPC. Despite small sample number, the detected changes in phosphorylation levels of c-Jun, histone H2AX, SEK1 and KIT improve DNA damage repair ability and might have important roles in NPC relapse, inhibiting apoptosis and promoting carcinogenesis (Su et al. 2011). Indeed, Guo et al. using cell lines confirmed that c-Jun may be involved in mechanisms of radioresistance of NPC. Its gene knockdown could be a potential strategy to improve radiosensitivity (Guo et al. 2015).

In 2012, the Sun Yat-sen University group (China), used MALDI-TOF-MS to investigate biomarkers for early stage disease. Using 99 samples for validation, 4 proteins were identified resulting in a 100% rate of both sensitivity and specificity (Tao et al. 2012).

At the same year, Li et al. used 2DGE and MALDI-TOF-MS to compare non-metastatic to metastatic NPC. They concluded that nm23-H1 behaves as a metastasis suppressor gene, and its downregulation is a biomarker for poor prognosis in NPC (X. Li et al. 2012).

Wu et al. reported their experience use MS to detect radioresistance biomarkers. Results suggest that ERp29 is associated with radioresistance in NPC, and ERp29 could be a potential biomarker for predicting NPC response to radiotherapy (Wu et al. 2012).

In 2012, Li et al. evaluated stroma proteins involved in NPC, proteins from stroma of NPC and NNE were subjected to quantitative proteomic analysis. Authors concluded that periostin, when over-expressed, was associated with clinical stage lymph node involvement and worse overall survival (M. Li et al. 2012).

5.5. Detected Proteins and Their Role in NPC

The most prevalent proteins found on the studies above are several variants of: keratin, annexin, heat shock proteins, 14-3-3σ, nm-23 protein, cathepsin, heterogeneous nuclear ribonucleoproteins (hnRNP), enolase, triosephosphate isomerase, stathmin, prohibitin, and vimentin (Chen et al. 2015b). Here follows a description of each ones detected role in NPC:

a) *Keratin*: Keratin-1 (KRT1) protein was found to have activity levels higher in cDDP-resistant NPC cell lines compared to NNE. KRT1 could serve as a biomarker for chemotherapy sensitivity of NPC (Tang et al. 2012). It is also related to invasiveness and metastasis. Keratin-8 (KRT8) and 18 (KRT18) were found to be tyrosine-phosphorylation targets of EGFR signaling and may have implications in cancer therapy of NPC (Ruan et al. n.d.). Keratin8 (KRT8), with cathepsin, SFN (14-3-3 σ), and stathmin1 (STMN1) were validated by IHC in primary NPC regarding WHO grading type (Ruan et al. 2010).
b) *Annexin*: annexin is a binding protein related to invasion, metastasis, p53 function and EGFR signaling pathway. Annexin-a3 (ANXA3) was validated as a potential tyrosine-phosphorylation targets of EGFR signaling (Ruan et al. n.d.) and radioresistance (Li et al. 2013). Significant down-regulation of annexin-I was observed in NPC versus NNE, and significant down-regulation of annexin I was also observed in lymph node metastasis. Down-regulation of annexin-I was also significantly correlated with poor histologic differentiation, advanced clinical stage, and recurrence (A L Cheng et al. 2008). Annexin-a5 (ANXA5) was identified as showing higher expression in CNE2/cDDP compared to CNE2 that may be related to drug resistance (Tang et al. 2012).
c) *Heat Shock Protein*: in NPC, heat shock protein (HSP) is related to treatment resistance (low levels of HSP-β-1) or specifically to drug resistance (overexpression of HSP-90) (Tang et al. 2009). HSP-27 also related to

carcinogenesis, radioresistance and metastasis onset (Li et al. 2011)(Zhang et al. n.d.).

d) *14-3-3σ:* it is a unique member of 14-3-3 family, as a negative regulator of the cell cycle. It is triggered by p53 to initiate cell cycle checkpoint control after DNA damage. Although 14-3-3 sigma is linked to p53, its mechanisms of cell cycle regulation by 14-3-3 sigma are still unclear. Decreased expression of 14-3-3 sigma was reported in several types of carcinomas including NPC, suggesting that the negative regulatory role of 14-3-3 sigma is affected during carcinogenesis. 14-3-3σ is also related to invasion, metastasis, drug resistance, and WHO grading (Lee & Lozano 2006; Yang et al. 2008).

e) *Nm-23*: protein was originally described as a metastasis suppressor protein in gastric cancer (Radović et al. 2013). Nm23-H1 behaves as a metastasis suppressor in NPC, and its down-regulation is a biomarker for poor NPC prognosis (X. Li et al. 2012) . It has also been reported as a marker for radioresistance (Li et al. 2013).

f) *Cathepsin*: cathepsin L1 has been labeled as a serological cancer marker as reported for the Human Protein Atlas (Wu et al. 2010). In conjunction with annexin, has been related to cisplatin resistance (Tang et al. 2012). It was also related to WHO grading and metastasis (Xiao et al. 2010a)

g) *Heterogeneous nuclear ribonucleoproteins* (hnRNP): the mechanism for the up-regulation of heterogeneous nuclear ribonucleoprotein remains unclear (Zhang et al. n.d.). hnRNPs and hTra2-betal regulate the genetic expression, which is concerned with estrogen receptor (ER) (Yang et al. 2014).

h) *Vimentin*: vimentin is involved in the apoptosis process but there is conflicting data whether vimentin is up-regulated or down-regulated in NPC (Li et al. 2010; Chen et al. 2008). Yamasaki et al. have published their data studying the role of vimentin in kidney cancer. Data suggest that vimentin may work as an oncogene regulated by tumor suppressive miR-138. The existence of a tumor suppressive miR-138-mediated oncogenic pathway provided evidence for RCC oncogenesis and metastasis. It's unclear if it vimentin plays the same role in NPC (Yamasaki et al. 2012).

i) *Triosephosphate isomerase* (TIM): TIM is an essential component of glycolysis. Tumors are often dependent on glycolysis for energy. Glycolysis inhibitors thus show promise as cancer treatment mechanism. TIM inhibition also produces toxic methylglyoxal targeted to regions of high glycolysis, an effect that might also be therapeutically useful. It is frequently found in NPC (Chen et al. 2015b; Marsh & Shah 2014).

j) *Enolase*: it is associated to NEG1 gene regulation. Expression of NESG1 transcripts and protein is downregulated or absent in NPC tissues and cell lines in comparison to NNE (Liu et al. 2011; Liu et al. 2012)

k) *Stathmin*: stathmin has a role in apoptosis and its different expressions can be related to NPC pathological WHO grading. It has been associated to radioresistance (Hsu et al. 2014; Xiao et al. 2010a; A L Cheng et al. 2008; Li et al. 2013)

l) *Prohibitin*: is usually down-regulated in NPC along annexin and stathmin (Wu et al. 2012; Chen et al. 2015b; Qiu et al. 2012).

As reported in this section, proteomics is widely used for NPC research, specially in Asia. Either searching biomarkers for screening strategies, patients selection, radioresistance, recurrence or metastasis, several Asian groups have explored proteomics techniques to better understand this entity.

On the past decade, a growing body of evidence was presented here shows the most used methods to explore NPC.

We have found very limited data from European cohorts. Hu et al. data extensively investigated genetic expression using cohorts from Asia, Northern Africa and Southern Europe (n=15). There is very little correlation between chromosomal copy number aberrations and expression levels of TSGs and TPGs. The author warns that simple classification of TSGs and TPGs may not be realistic (Hu, Wei, Chen, Ciaran B. Woodman, et al. 2012). No other phenotype or proteomic description has been found.

Taking all together, the most identified proteins are: keratin, annexin, heat shock protein, 14-3-3σ, nm-23 protein, cathepsin, heterogeneous nuclear ribonucleoproteins, enolase, triosephosphate isomerase, stathmin, prohibitin, and vimentin. These proteins consistently appear in NPC in different settings and situations and have potential value for research in screening, patient selection, therapy selection, follow up and outcome prediction.

6. Part III – Results of Proteomic Analysis in Nasopharyngeal Carcinoma

6.1. Study proposition

We proposed a experimental retrospective study exploring formalin-fixed paraffin-embedded samples from nasopharyngeal carcinoma biopsies via label-free quantitative mass spectrometry.

6.2. Objectives

Primary aim was:

- Characterize tumor profiles from formalin-fixed paraffin-embedded NPC, via proteomic analysis and mass spectrometry and correlate them to patients' outcome on a Portuguese cohort of patients.

Secondary objectives were:

- Identify biomarkers related to tumor local relapse (radioresistance);
- Identify biomarkers related to tumor progression and metastases.

6.3. Methods

6.3.1. Study design and statistics

It's a single center retrospective study with experimental application of basic research analysis. After approval from the institutional board (UIC901), we retrieved clinical, image and laboratorial data from 109 patients consecutive patients with biopsy-proven nasopharyngeal carcinoma (NPC) treated between February 2009 and December 2013. All patients were staged accordingly to AJCC/UICC 7th Edition. We performed a pathological review according to WHO classification for this manuscript.

Patients were treated with cisplatin-based chemoradiation as per Intergroup 0099 trial (Al-Sarraf et al. 1998). IMRT volumes were contoured as per RTOG 0615 (N. Y. Lee et al. 2012b).

Complete case datasets were used in the analysis. No imputation methods were used. Survival was estimated with Kaplan-Meier survival analysis and log-rank test to detect differences (SPSS v.23, IBM). For statistical analysis, the day of first treatment was used. Outcome data was calculated as per April 6th, 2018.

6.3.2. Sample size calculation

We used the Sample Size Calculator by Raosoft, Inc (www.raosoft.com/samplesize.html) to estimate the recommended sample size regarding the clinical outcomes.

For calculation, we considered our published cohort of patients (n=109) and the obtained clinical outcomes of local control and distant metastasis. Local control, for all stages, was around 90% (10% of local relapse). On the contrary, distant metastases were around 23%, a little bit lower than the 30-40% described in the other series.

Considering a 95% confidence level, with a margin of error of 5%, and a response distribution of 10% for local relapse from a 109 patient's cohort, the minimum recommended size was 59 samples.

For distant metastases, we used a worst-case scenario of 40% of metastases described in the literature. For a 95% confidence level, with a margin of error of 5%, the recommended sample size was 79 samples.

Out of 109 patients, we manage to use 83 samples. The remaining was excluded due to low percentage of tumor (<60%) and budget limitations.

6.3.3. Protein extraction, quantification and FASP digestion

Formalin-fixed paraffin-embedded (FFPE) NPC samples were deparaffinized using standard procedures as described by Araújo et al. and Donnarumma et al. with minor modifications. Briefly, FFPE slices were deparaffinized three times with xylol for 5 min in a dry bath at 63ºC. Then the tissue material was rehydrated with starting with 100% ethanol for 10 min with gentle shaking followed by incubation with 96%, 80%, 70%, 50%. and water. Samples were centrifuged at 2000 rpm for 10 min and the supernatant was discarded and replaced with fresh Milli-Q water, followed by a final centrifugation step at 3000 rpm for 10 min. Finally tissue sample were washed with Milli-Q water.

Protein extraction was carried out as described by Araújo et al. with minor modifications(Araújo et al. 2014). Tissue samples were solubilized in 200 µL Tris-HCl 20 mM pH 9.0 containing 4% SDS and 0.1 M DTT. Samples were incubated at 100 ºC for 20 min followed by ultrasonication using an ultrasonic probe (1min, 2 mm tip, 100%

ultrasonic amplitude). For protein quantification aliquots of protein extract were diluted 1:5 and 1:10 before measuring protein absorbance at 280 nm using a nanodrop. Protein digestion was performed using filter aided sample preparation (FASP) method as described by Donnarumma et al. (Figure 1). Finally, samples were interrogated by mass spectrometry using label-free protein quantification LC-MS/MS methodology (Donnarumma et al. 2013).

LC-MS/MS data were analysed using Data Analysis 4.2 software (Bruker). Proteins were identified using Mascot (Matrix Science, UK). MS/MS spectra were searched against the SwissProt database S_Prot Human (73,045,382 sequences; 24,698,382 residues. Tandem MS data were searched with MASCOT search engine with the following parameters: precursor mass tolerance of 20 ppm, fragment tolerance of 0.05 Da, trypsin specificity with a maximum of 2 missed cleavages, cysteine carbamidomethylation set as fixed modification and methionine oxidation, as variable modification. False discovery rate (FDR) was estimated by running the searches against a randomized decoy database. Results of the identification step were filtered to proteins with a FDR below 1%.

Label-free quantification was carried out using MaxQuant software V1.6.0.16. All raw files were processed in a single run with default parameters(Tyanova et al. 2015). Database searches are performed using the Andromeda search engine with the UniProt-SwissProt Human Uniprot Proteome database as a reference and a contaminants database of common contaminants. Data processing was performed using Perseus (version 1.5.0.31) (Tyanova et al. 2016). In brief, protein group LFQ intensities were log2-transformed to reduce the effect of outliers. To overcome the obstacle of missing LFQ values, missing values were imputed before fitting the models. Log ratios were calculated as the difference in average log2 LFQ intensity values between the two digestion methods tested (two-tailed, Student's t test). A protein was considered statistically significant if its fold change was ≥ 1.5 and FDR ≤ 0.05 (Cox & Mann 2008). A schematic representation of the protocol can be seen on Figure1.

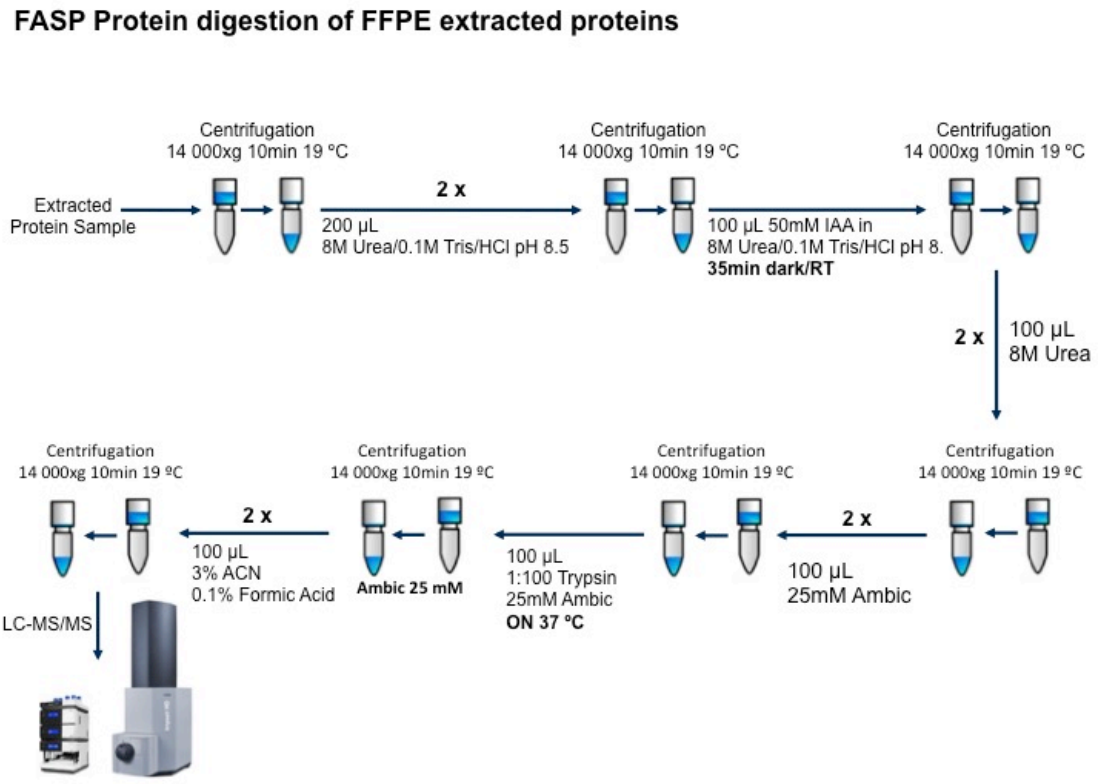


Figure 1: Schematic representation workflow of FASP protein digestion of FFPE extracted proteins

6.3.4. Functional annotation using cytoscape

Integrative analysis with Cytoscape was based in the use of ClueGO plug-in. This plug-in strongly improves biological interpretation, once integrates Gene Ontology (GO) terms and KEGG pathways, creating a functionally organized GO/pathway term network(Bindea et al. 2009).

6.4. Results and Discussions

6.4.1. Clinical Outcomes

We performed a single center retrospective study of tour entire cohort (n=109).

Median follow up in surviving patients was 56 months. Almost 74% were male. WHO grade III was present in 73% of cases. Most patients were Caucasians (97%), with a few other ethnicities. Patients and tumor characteristics are displayed on Annex 2.

The 4-yr local control was 88.5% (86,9% for T1; 100% for T2; 89,5% for T3; and 82,6% for T4). The median time to local recurrence was not reached. From 102 patients evaluable for local control, we found 11 local relapses: 5 on T1; 0 on T2; 2 on T3; and 4 on T4 patients. Seven out of 11 occurred before 2 years of follow up. All 11 local recurrences occurred inside PTV70 volume. There was no difference in local control regarding T stage (p = 0.376; Figure 2A).

The 4-year regional control rate was 95% (100% for N0; 91% for N1; 94% for N2 and 96% for N3). Nodal failure occurred in 6 of the 102 patients: 1 on N1; 4 on N2; and 1 on N3 patients. Three out of 6 regional recurrences occurred less than 2 years after treatment. There was no difference in regional control regarding N-stage (p=0.434) even dichotomizing N0 versus N-positive groups (p=0.276). Only one patient shared both local and regional relapses.

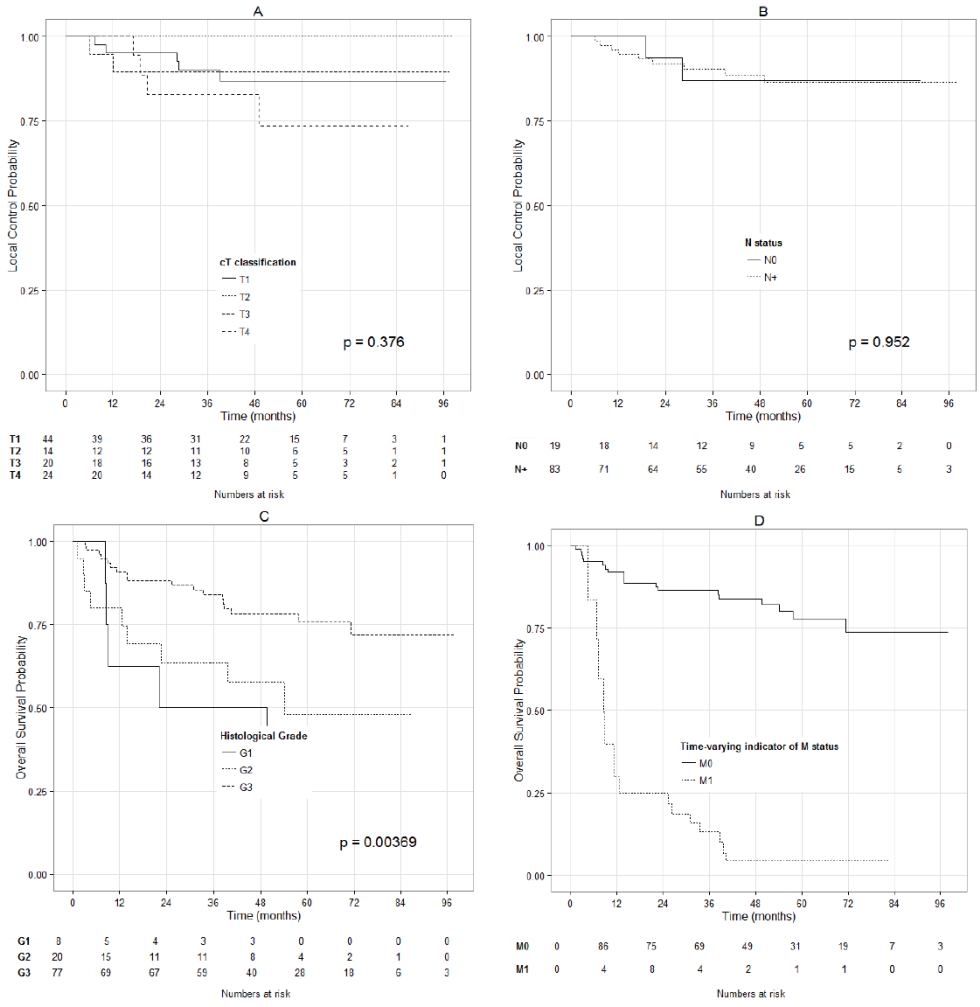


Figure 2: A) local control by T-Stage (p=0.376); B) Regional control by N-Stage (N0 *vs.* N+, p=0.952); C) Overall survival by pathology WHO grade (p=0.003); D) Overall survival by metastases status (M0 *vs.* M1, p <0.001).

Five patients (5%) had distant metastasis at diagnosis and were treated with both concurrent radical treatment to primary site and metastatic disease. Fourteen patients had distant metastasis diagnosed during follow-up, of these 11 occurred within the 2 years after treatment completion. The 4-year distant metastasis free survival was 79.8% and median time to distant metastasis was not reached.

Treatment outcomes stratified by T-, N-category, WHO pathological grade and metastases (M1) are presented on Figure 2(A-D).

Patients with WHO grade 3 had significantly better local control and survival (p=0.001 and p = 0.004 respectively, overall comparison).

From the 36 deaths, 20 were due to distant metastases, 3 to grade 5 toxicity, 2 from local progression, 5 were not cancer or treatment related. In 6 cases, the cause of death could not be determined.

Long-term toxicity profile is presented on Table 2. Xerostomia was the most frequent late toxicity 55% (n=60), but no patient developed grade > 2. Hypothyroidism requiring hormonal replacement occurred in 15.5% (n=17) with no other hormonal deficit in this cohort. With a mean cochlear dose of 49Gy, grade 3 hearing loss or need of aid occurred in 8% (n=9). Persistent peripheral neuropathy was seen in 4.5% (n=5). One patient developed grade 2 renal toxicity, 1 had asymptomatic temporal lobe necrosis and other had skull base osteoradionecrosis requiring hyperbaric oxygen therapy, although none with reirradiation.

Table 2. Long-term toxicity profile

	Grade ≤ 2 (%)	Grade 3-4 (%)
Hearing loss	-	9 (8%)
Xerostomia	60 (55%)	-
Hypothyroidism	17 (15.5%)	-
Peripheral Neuropathy	5 (4.5%)	-
Skull Base Radionecrosis	-	1 (1%)
Temporal Lobe Radionecrosis	1 (1%)	-
Renal	1 (1%)	-
Pituitary dysfunction	1(1%)	-
Any	85 (77%)	10 (9%)

Only 60 patients (55%) had EBV DNA and viral copy load study pre treatment and 36 (60%) of those with titles above detection level (600 copies).

The 4-year outcomes in the present report represent an improvement from our previous published data from the 3DCRT era(d'Espiney Amaro et al. 2009) and are consistent with our preliminary reports from in the IMRT era [5,6].

D'Espiney Amaro series have reported a 5 year overall survival of 65.1% in 2009 using 3DCRT. This cannot be attributed solely to technique alone, since the previous data included a large number of patients (80% n=117) treated with neoadjuvant chemotherapy followed by radiotherapy with or without chemotherapy.(d'Espiney Amaro et al. 2009)

Among 109 patients, we observed 4-year actuarial local or regional similar control rates of 90%, almost the same as the 2-year rate previously reported by our group in a preliminary abstract. By 2015, after a median follow up of 22 months, 2-year local control was 95,9%, regional control 98%, freedom from distant metastases 88% and overall survival 79,8% (Netto et al. 2015).

The present series show distant metastases-free survival of 86% and a overall survival rate of 77% for all stages similarly to other series with IMRT (Spratt & Lee 2012a; Lee et al. 2009a; Blanchard, Lee, Marguet, Leclercq, Ng, Ma, Chan, Huang, Benhamou, Zhu, Chua, Chen, Mai, Kwong, Cheah, Moon, Tung, Chi, Fountzilas, Zhang, Hui, Lu, Bourhis & Pignon 2015; Au et al. 2018).

Table 3. Variables independently associated with overall survival (multivariable Cox regression analysis)

	Hazard Ratio (HR)	95% Confidence Interval	p-value
Local recurrence* Yes vs No	7.52	2.64 – 21.45	<0.001
Distant metastasis* Yes vs No	32.99	12.95 – 84.08	<0.001
N status at diagnosis N+ vs N0	3.03	0.69 – 13.27	0.141
Age at diagnosis Per additional year	1.08	1.04 – 1.11	<0.001

*time-dependent variables

Others have related a poorer local control rate for T4 patients (Setton et al. 2015). A conclusion could only be drawn with a dosimetric and volume evaluation of the patterns of failure. That was not the scope of this manuscript. Treatment outcomes in local control for the overall cohort were excellent, regardless T stage. In fact, we found no statistical difference in local control between T1 to T4 (Figure 2A) or any stratified comparison T4 vs. other T stage. In our cohort, local relapses occurred inside PTV70 volume covered by the prescribed dose per protocol.

Among the 11 local relapses, 5 (50%) were in previous T1 patients, 0 on T2, 2 on T3 and 4 on T4 stages. From these local recurrences, 8 could be successfully salvaged either with fractionated stereotactic re-irradiation or radiosurgery described in the literature (Stoker et al. 2013b).

Regional control was also excellent (90%). In fact, N stage did not affect local or distant failure, even considering that more than half (n=59) of our cohort was N2 or N3 (66%) and it is similar to what has already been described by others. (Setton et al. 2015)

WHO grade 3 patients (73%) experienced a much more favorable prognosis with significant higher local control and survival (p = 0.000 and p = 0.032 respectively) as well in other Eastern and Western series (Figure 2C) [2,8,10].

Surgery was indicated for persistent neck enlarged nodes after treatment, or clinical / imagiological worrisome features (i.e. unhealed neck ulcer on tumor site) in 13 patients. With median number of 13 nodes dissected (range 5-32), only 1 patient had persistent metastatic disease after combined modality. This patient was planned to receive adjuvant CT but indication was withdrawn due to toxicity. All others had fibrosis and post treatment findings in nodes up to 4 cm. As in other head and neck sites, before 2016, PET-CT was not used to select patients for neck dissection (Mehanna et al. 2016)

Five patients with stage IVC (AJCC/UICC 7th Edition) were included in this analysis since they received concurrent chemoradiation as part of the initial approach. Data from Asian and Western cohorts have already been published confirming that intensive treatment incorporating concurrent chemoradiation yields superior results (Yeh et al. 2006; Rusthoven et al. 2017).

From the 36 deaths, 20 were due to distant metastases confirming this feature as the most important cause of death similarly to others in the Eastern and Western reports[2,3,9-11].

On multivariable Cox regression analysis (Table 3) distant metastases had greatest impact on overall survival (p < 0.001; HR = 32.99; 95% CI 12.95-84.08). Nevertheless, despite salvage reirradiation been able to successfully rescue 8 out of 11 local relapses, local recurrence was also correlated with increased risk of death (p<0.001; HR 7.52; 95%CI 2.64-21.45). Nodal involvement was not predictive of regional or distant relapse when analyzed as a single variable or was associated with death (p=0.141; HR 3.03; 95%CI 0.69-13.27).

Treatment with radiation alone was an option for patients with severe comorbidities or frail elderly patients (n=12). CCRT with or without adjuvant CT was the standard treatment for the majority of patients. N-stage was the most frequent reason for this strategy since N2 or N3 patients were 66% (n=71) of all patients. Although controversial, adjuvant chemotherapy was performed with 3 cycles in 52 patients. Only 7 (12%) patients who had planned adjuvant CT did not received it due to toxicity, a percentage equal or superior to other series[17]. Induction chemotherapy was used instead of upfront CCRT in 4 patients for problematic RT planning or immediate treatment (e.g. bleeding).

Our acute toxicity profile have already been presented (Winckler et al. 2015). Late toxicity is displayed on Table 2. Xerostomia was the most frequent late toxicity from chemoradiation late toxicity comparable with other published data as the most frequent late complication from treatment[8-10,17]. Hypothyroidism requiring hormonal replacement was present on 17 patients (15.5%). As all patients received lower neck irradiation, thyroid (as an OAR) was not optimized at planning. Only one other survivor has pituitary dysfunction requiring medication. Two patients developed radiation necrosis, one asymptomatic temporal lobe necrosis and another skull base necrosis requiring hyperbaric oxygen therapy.

Audiogram was not available for our patients prior to treatment, so hearing was accessed by CTCAE recommendations without enrolling in monitoring program. With a median cochlear dose of 49Gy, 9 patients (8%) developed hearing impairment requiring aid devices. Further efforts may also be done to lower cochlear dose whenever possible. The MSKCC group have already reported lower toxicity with a average cochlear dose of 43Gy (Bakst et al. 2011).

This report has several important limitations such as its retrospective single-centered nature. Proper serum EBV DNA and viral copy loads data before and/or after treatment was not available in nearly half of the patients which prevents us to draw conclusions and comparisons to other endemic and non-endemic cohorts. The authors decided to keep the initial staging according to the AJCC UICC 7th edition since all patients were staged and treated between 2009 and 2013.

In conclusion, our matured clinical data confirm excellent local regardless T-Stage, and regional control of concurrent chemoradiation in the IMRT era, with favorable late toxicity, comparable to others and represent a major improvement from our 3DCRT era cohort. In our study, optimal radiotherapy via IMRT yielded important local control

regardless T stage. Biomarkers for local recurrence, toxicity and distant metastases prediction are needed. As in endemic and other non-endemic cohorts, distant metastases are a challenge and desperately need more investigation.

6.4.2. Proteomic Analysis Preliminary Findings

We performed a preliminary study with 28 samples to evaluate our method and its applicability in NPC FFPE tissue. Tumor samples contained a median 75% of tumor material (70-90%). Patients and tumor characteristics can be found on Annex 3. With a median follow up of 37 months, the overall local control is 83% for this cohort of 28 patients (90% for T1, 100% for T2, 57% for T3 and 86% for T4), overall survival is 84% and 6 patients developed distant metastases. All 5 patients that died were due to metastatic disease.

A tumor profiling with up-regulated (n=59) and down-regulated (n=12) proteins was identified in early T-Stage (combining T1 and T2) compared to advanced T-Stage tumors (combining T3 and T4). See Figure 3. Outcomes are displayed on Figure 4. Gene ontology and KEGG pathway were generated for T1+T2 tumors (Figure 5), T3+T4 tumors (Figures 6 and 7). On both groups, different on protein expression were statistically significant ($p<0.01$).

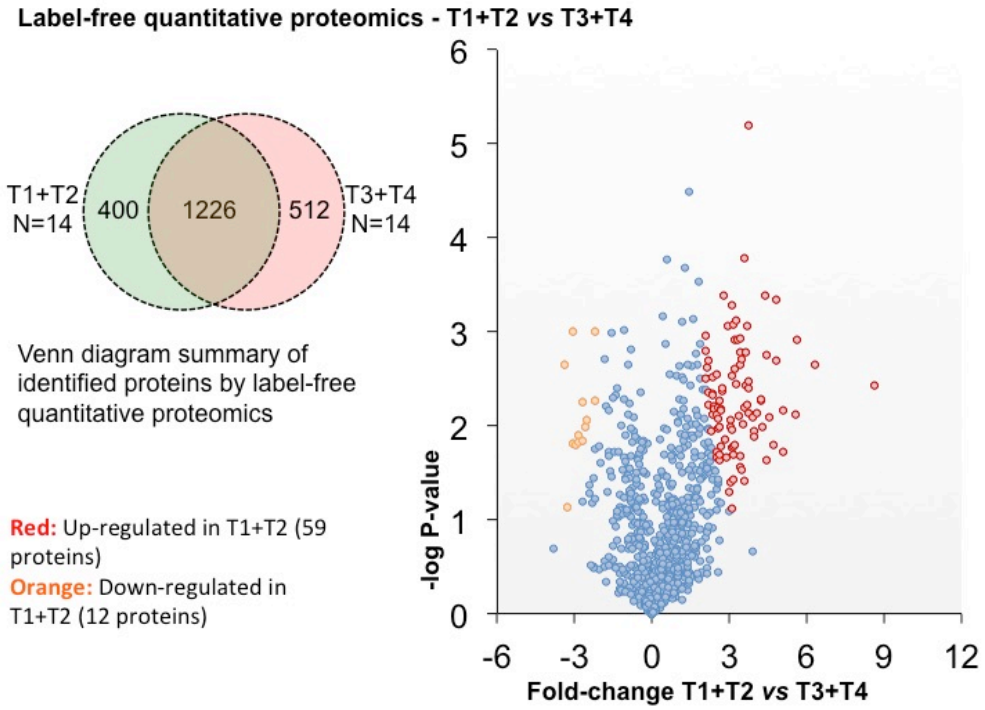


Figure 3: Volcano plot of label-free quantitative protemic results comparing early-stage NPC tumors (T1 and T2 grouped versus T3 and T4).

Local control was not affected by T-Stage (Figure 4A), even dichotomizing T1+T2 vs T3+T4 (Figure 4B) or comparing all T-Stages vs. T4 (Figure 4C). In our cohort, neither local control nor overall survival was affected by N-stage (Figures 4D-E). The presence of distant metastasis was the most important prognostic factor. Patient's outcomes are available in Figure 4(A-F).

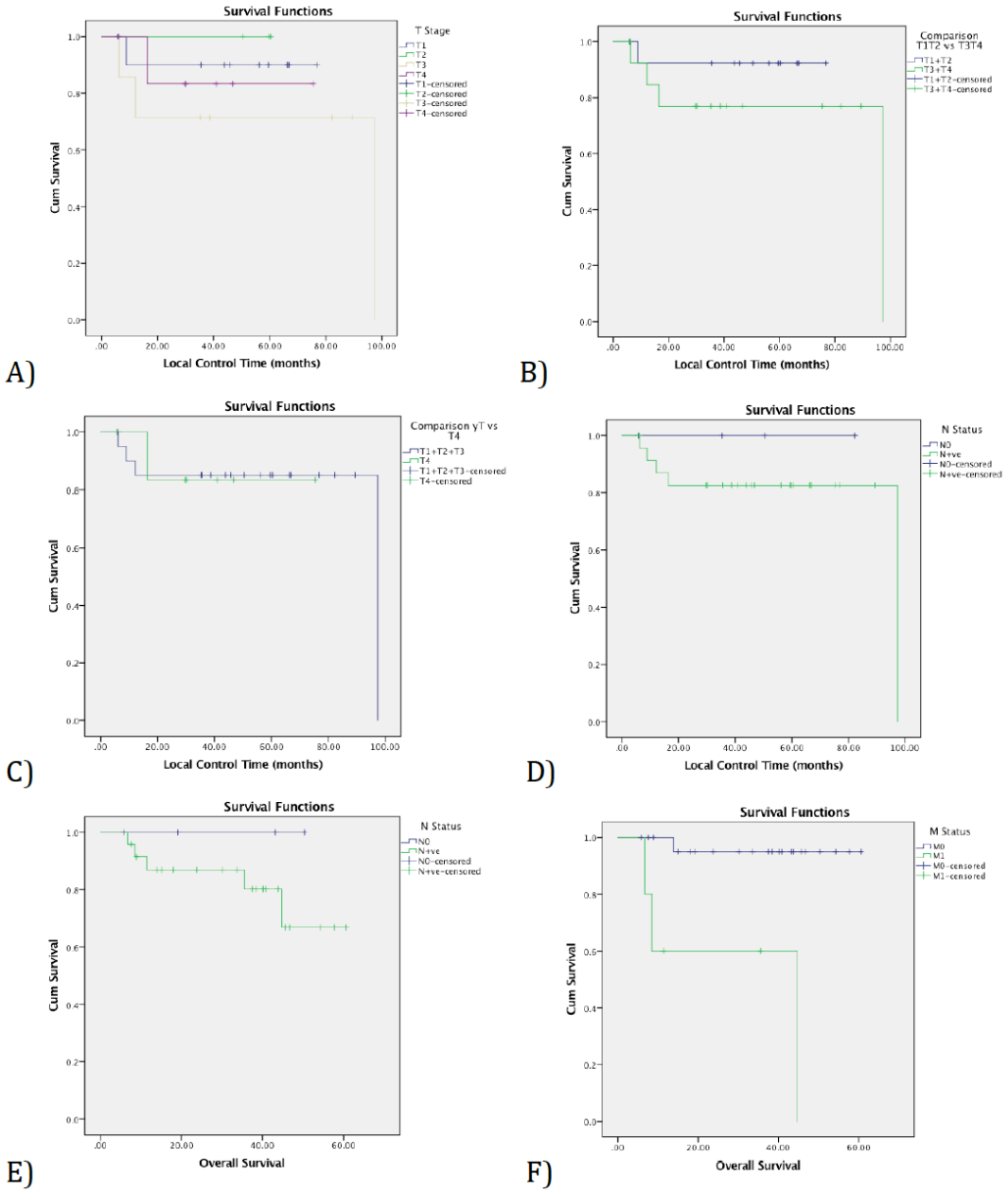


Figure 4 – Outcomes: A) local control by T-stage (p = 0.623); B) local control stratifying T1+T2 vs T3+T4 (p = 0.297); C) local control stratifying all T stages vs T4 (p = 0.983); D) regional control by N stage comparison: N0 vs N+ (p = 0.454); E) overall survival by N-status (N0 vs N+ve, p=0.392); and F) overall survival by M stage (M0 vs M1, p=0.001).

A pool of 10 proteins were statistically up-regulated in M1 patients: tumor protein D52, tumor protein 63, serine/threonine-protein kinase MRCK alpha, tyrosine-protein kinase HCK, EGFR-kinase substrate 8-like protein 2, caspase-1, heat shock-related 70 kDa protein 2, interferon regulatory factor 6, interferon-induced protein with tetratricopeptide repeats 2 and Interferon-induced protein with tetratricopeptide repeats 3. Median survival from this M1 group was less than 1 year ($p < 0.001$; Figure 8).

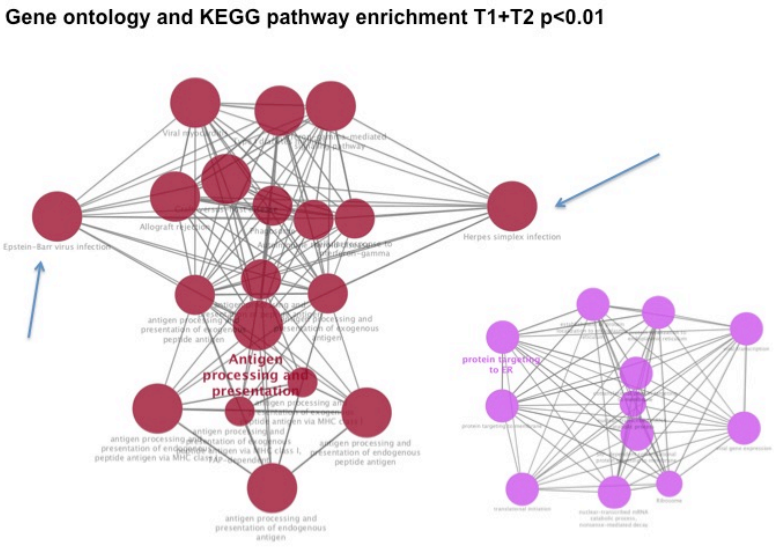


Figure 5: Gene ontology and KEGG pathway enrichment significant present on early-stage NPC tumors (T1 and T2 patients versus T3 and T4 tumors, $p<0.01$). Arrows point to Epstein-Barr virus and Herpes simplex infection proteins identified on the Antigen processing and presentation pathway.

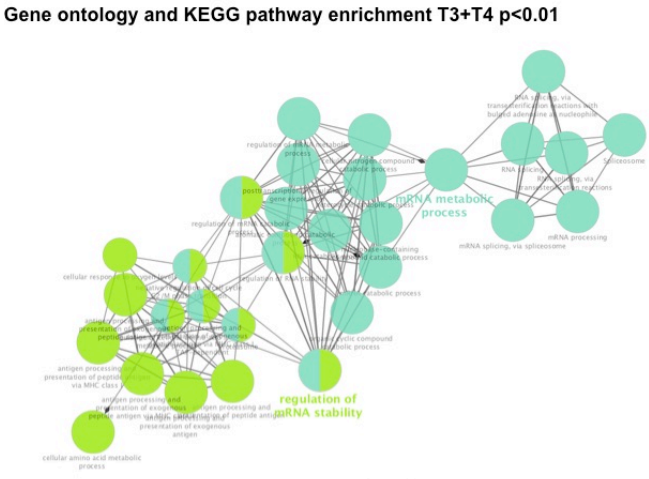


Figure 6: Gene ontology and KEGG pathway enrichment significant present on advanced-stage NPC tumors (T3 and T4 patients versus T1 and T2 tumors, p<0.01).

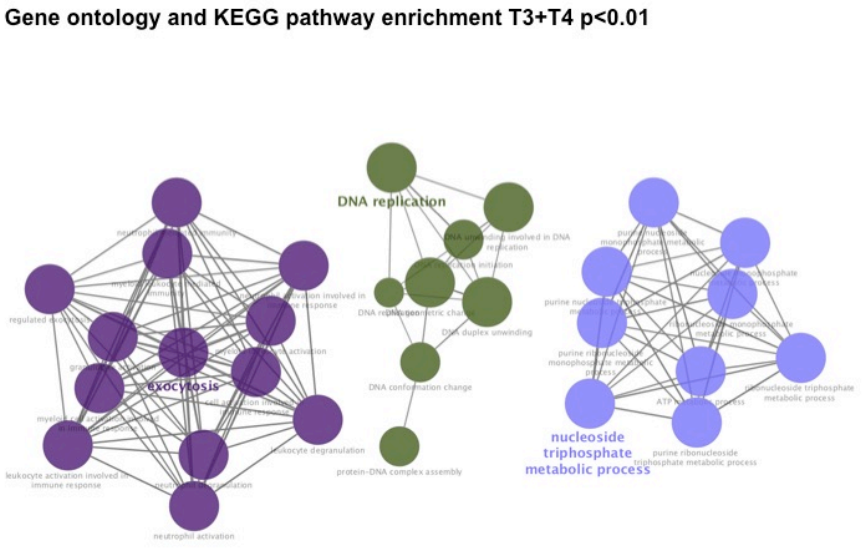


Figure 7: Gene ontology and KEGG pathway enrichment significant present on advanced-stage NPC tumors (T3 and T4 patients versus T1 and T2 tumors, p<0.01).

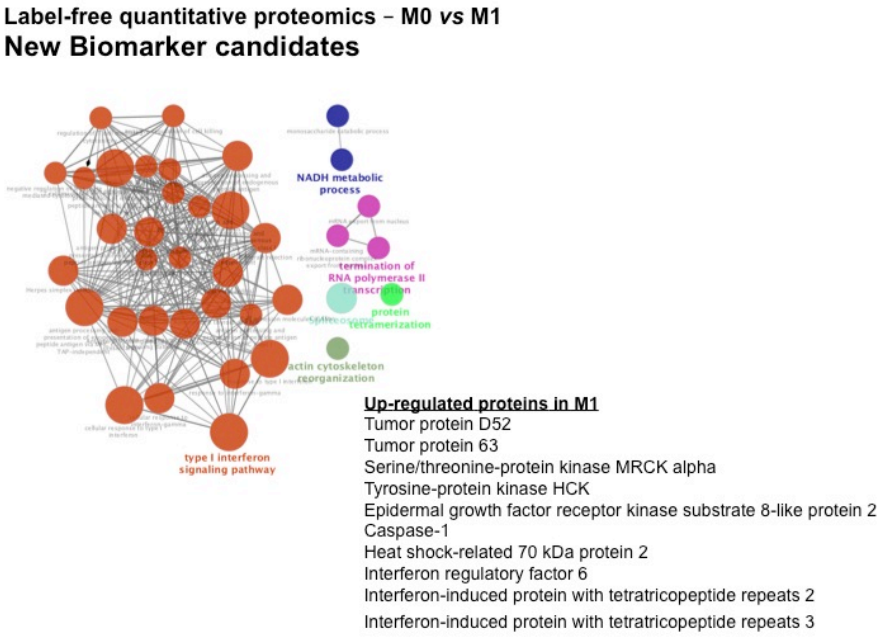


Figure 8: Pool of 10 proteins were statistically up-regulated in M1 patients (p<0.01).

FFPE tissues hold invaluable information regarding patients' outcomes. Nevertheless, its features pose challenging tasks to scientists all over the world. Few series have studied NPC via FFPE samples (Chen et al. 2016; Xiao et al. 2010b).

Our data showed a pool of up (n=59) and down-regulated (n=12) proteins in early T-Stage (T1 and T2) compared to advanced local disease (T3 and T4). All were statistically altered. Despite that, survival curves for local control were identical for any T-stage ($p = 0.623$) even dichotomizing between T1+T2 versus T3+T4 ($p = 0.297$) or comparing all T-stages versus T4 ($p = 0.983$) (Figures 4A-C).

This can be explained by the excellent local control and survival yielded by chemoradiation for all T or N stages ($p = 0.454$). These rates are in agreement to others in Eastern and Western countries (Setton et al. n.d.; Rotolo et al. n.d.; Chan et al. 2012; Lee et al. 2009b; A. W. M. Lee et al. 2012; Kam et al. 2004). It is unclear rather this signature means that an early T-stage could receive a different protocol prescription (i.e. lower doses). At this subgroup, bioinformatics KEGG pathway showed that the most significant difference in proteins were in up regulation of antigen processing and presentation and also protein targeting ER (Figure 5).

On the other hand, a significant pool of 10 proteins were up regulated in M1 patients ($p < 0.01$). This is important information since M1 disease is the most important factor affecting survival for any stage ($p < 0.001$). See Annex 4. At this M1 subgroup, KEGG ontology pathway via bioinformatics showed functioning activity by type I interferon signaling pathway, actin cytoskeleton reorganization, protein tetramerization, termination of RNA polymerase II transcription and NADH metabolic process (Figure 8). These signatures contain known proteins related to head and neck cancer onset and behavior, not only NCP, and potential therapeutic targets (like interferon and EGFR). Adjuvant interferon has yielded superb results in children and young adults with NPC (German GPOH Study NPC91)(Mertens et al. 2005).

As expected in this entity, viral proteins were markedly present on early stage group of patients (T1 and T2) specially EBV. EBV proteins may also trigger miRNAs able to cause pleiotropic effects on cells. It has been described that LMP1 can activate mir-146a, which can down regulate genes related to interferon responsiveness (Lo et al. 2007).

However, not only EBV proteins were present, but also Herpes simplex virus. Whether this co-infection can be interpreted as a cofounding factor or a relevant issue for

early-stage in our cohort remains unclear. Nevertheless, we have not found HPV related proteins, a known marker for poor prognosis among Caucasians (Stenmark et al. 2014).

Asian authors have already published data with the use of FFPE tissue for biomarker discovery or validation (Table 3). Despite different methods used, it was possible to detect different effects on outcome. Chan et al. and Chen et al. identified biomarkers related to metastasis promotion (Chan et al. 2008; Yuan et al. 2016). Xiao et al. (Xiao et al. 2010b) have reported 4 biomarkers related to NPC types (cathepsin D, keratin 8, SFN and stathmin 1). The report of Chen et al., using FFPE as validation for Cell line research identified TRIM29 as a metastasis-promoter using 2D LC-MS/MS. (Table 3).

Recently, Xu et al. published novel biomarkers of NPC metastasis risk identified by reverse phase protein array (RPPA) based tumor profiling with consideration of plasma EBV DNA load. Their study reported a pool of 26 proteins related to metastasis in a Chinese cohort of patients (Xu et al. 2016). Instead of searching a single-based biomarker signature, our report also presents findings compatible with outcome-related protein combination.

Our study has limitations. We acknowledge that there is a high number of missing data from EBV plasma load pretreatment. And, we kept included 2 patients already M1 at diagnosis. Since we wanted to report the profiling related to M1 disease, it's a minor limitation to the final analysis.

6.4.3. Proteomic Analysis Final Results

6.4.3.1. Viral Expression in Proteomic Analysis

Our preliminary results have shown a statistically significant presence of viral-related proteins overexpressed in early stage primary tumor samples (T1+T2) compared advance tumors (T3+T4).

Viral proteins were consistently identified as Epstein-Barr virus (EBV) and Herpes simplex virus (HSV). As our data matured, with a higher number of samples analyzed, these patterns maintained the same expression in early-stage for both virus, regardless metastasis status (Figure 9).

As others, we hypothesize whether EBV has a pivotal role on NPC early stage and onset until a point where cellular signaling pathway triggers other proteins to be more

prevalent on detection. The role of HSV in this setting is less clear. Nevertheless, although sharing a few proteins (n=4) with EBV (since both are from the same family), the proteomic profiling confirms the distinct presence of HSV co-infection with EBV on our cohort. The meaning of this finding remains unclear and generates hypotheses to be studied.

Even in high-incidence countries, EBV vaccination policies are controversial since it has a distinct behavior of other viruses well related to cancer onset and development.

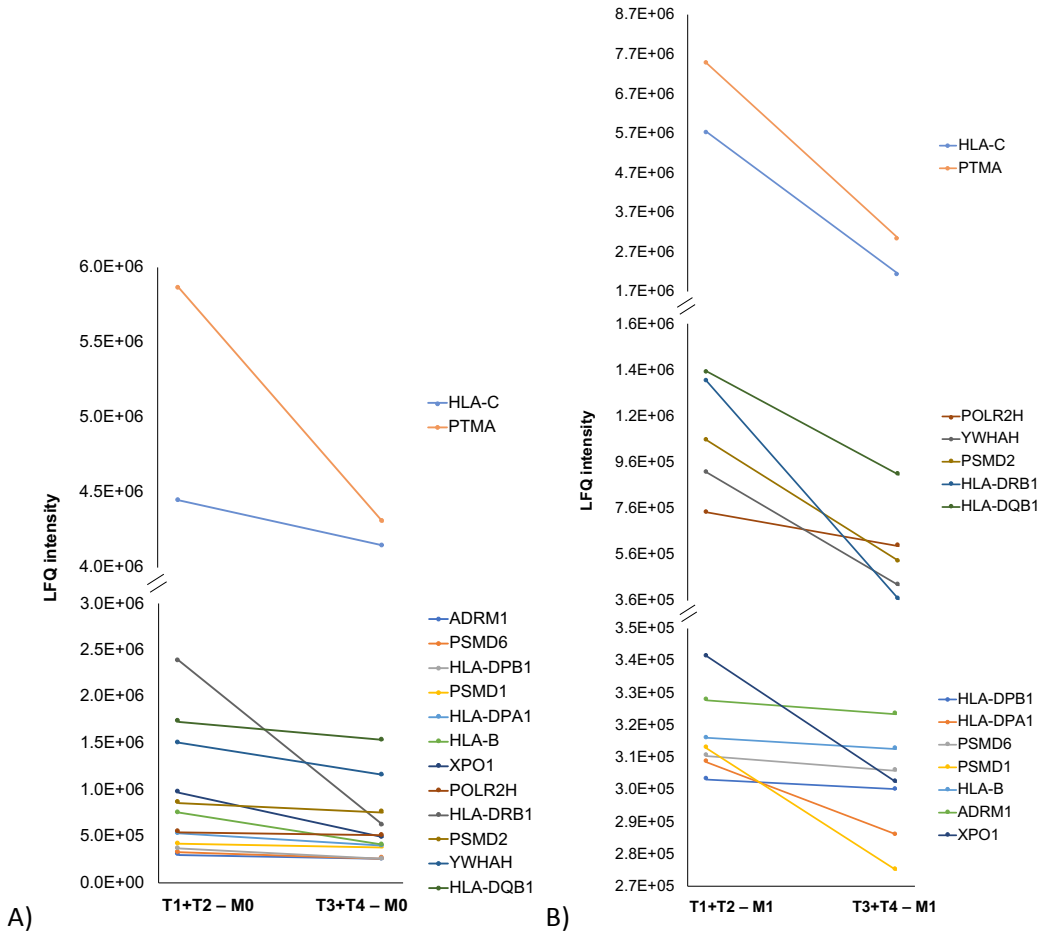


Figure 9: Label-free quantification intensities for the proteins associated with EBV infection detected in T1+T2 – M0 (n=42) *vs.* T3+T4 – M0 (22); B) Label-free quantification intensities for the proteins associated with EBV infection detected in T1+T2 – M1 (n=11) *vs.* T3+T4 – M1 (n=7).

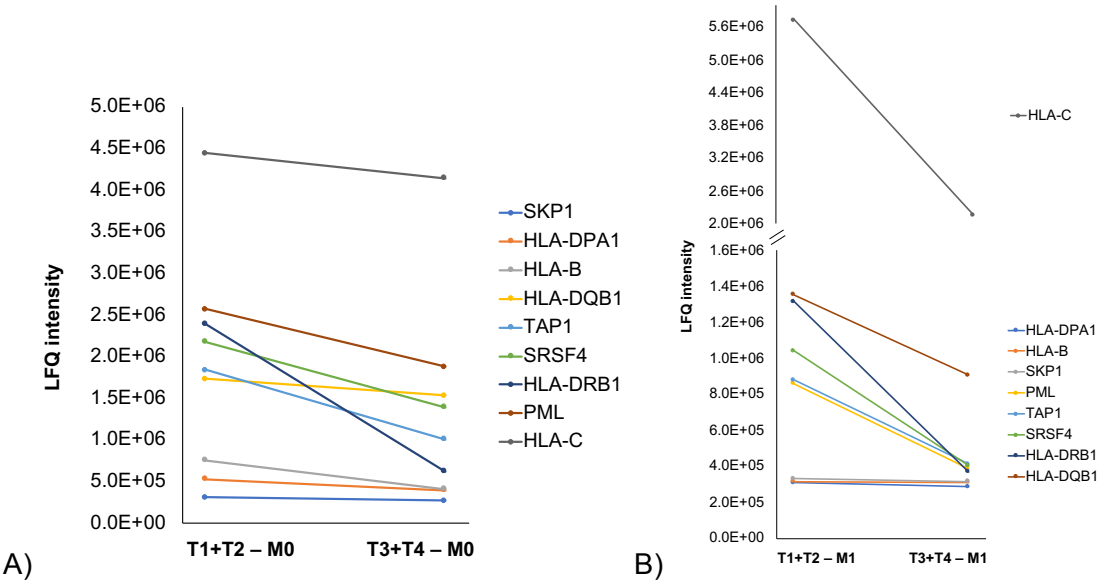


Figure 10: A) Label-free quantification intensities for the proteins associated with Herpes simplex infection detected in T1+T2 – M0 (n=42) *vs.* T3+T4 – M0 (22); B) Label-free quantification intensities for the proteins associated with Herpes simplex infection detected in T1+T2 – M1 (n=11) *vs.* T3+T4 – M1 (n=7).

6.4.3.2. Local Relapse Biomarker Research

For local relapse, we evaluated 53 patients, their characteristics at diagnosis were: 71% male; 96% Caucasian; 73% WHO grade 3; 44% T1, 14% T2, 21% T3 and 21% T4; 23% N0, 15% N1, 37% N2 and 25% N3; 94% M0. Complete stage were: 11.5% I, 11.5% II, 29% III, 19% IVA, 23% IVB and 6% IVC.

The majority of patients were treated with concurrent chemoradiation followed by adjuvant chemotherapy (54%). With median follow up of 41.5 months for the whole cohort, 4-year local control was 83%, distant metastases free-survival was 77%. Out of 53 patients we found 9 patients with local relapse after NPC treatment. All relapses occurred in the highest dose volume (70Gy). Five were on T1, 2 on T3 and 2 on T4. Median time to local relapse was 16.5 months and 8 out 9 patients had relapses before 2 years of follow up. All 9 patients received reirradiation. Two patients died from local progression, three died from distant metastases and the remaining 4 patients are alive with no evidence of disease.

We found 58 differentially expressed proteins on patients with local relapse ($p<0.01$, Figure 11). Forty-nine proteins were up-regulated on patients with local relapse. Another pool with 9 proteins were found to be down-regulated in relapsed patients: profilin-1, interferon-induced GTP-binding protein Mx1, eukaryotic initiation factor 4A-III, eukaryotic translation initiation factor 4H, cathepsin B; interleukin enhancer-

binding factor 2, Ras GTPase-activating-like protein IQGAP1, sorbitol dehydrogenase and cathepsin D (p<0.01). KEGG ontology pathway enrichment was generated (Figure 12).

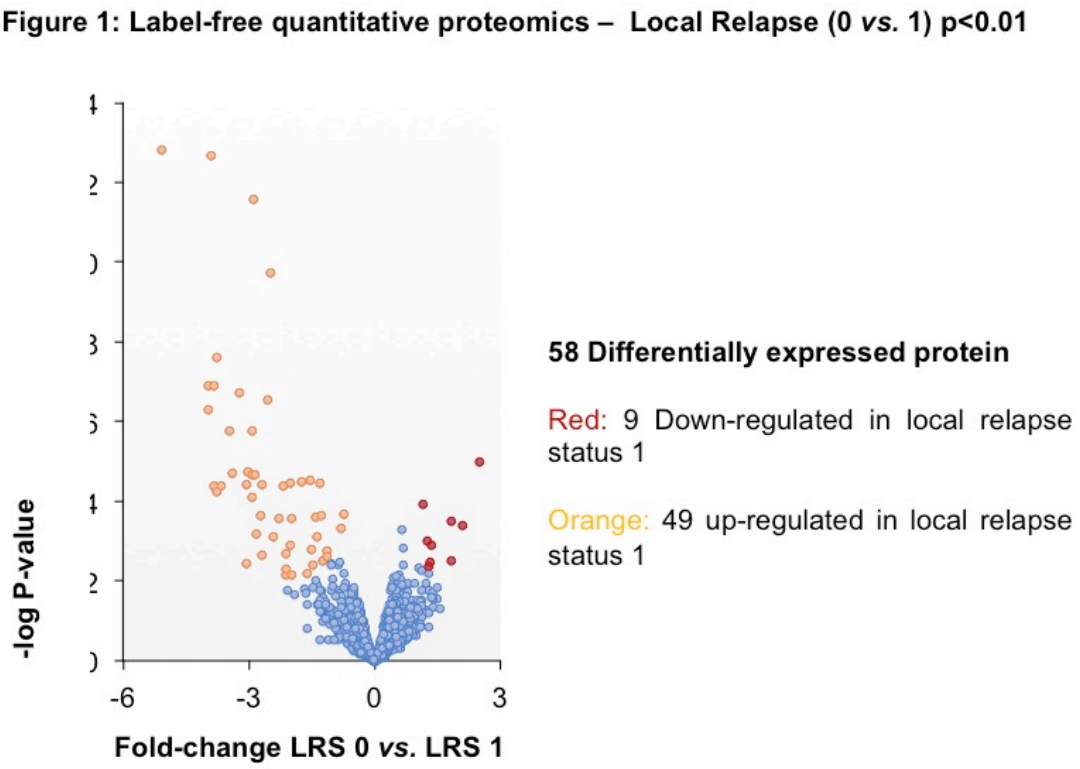


Figure 11: Label-free quantitative proteomics evaluating local relapse (0 vs 1, p<0.01).

Our method was able to identify a tumor profile that could potentially be related to local relapse in FFPE NPC samples. We found a tumor profiling of differentially expressed proteins, including a pool of nine proteins up-regulated as potential proteomic signature of local relapse or radioresistance.

Nevertheless, local relapse was not different between early or advanced stage groups (Figure 13). As in section 6.4.2, we consistently achieved 90% local control rate among all T stages. These results are the same available on the clinical literature by chemoradiation on the IMRT era (Spratt & Lee 2012b; Ribassin-Majed et al. 2016).

Figure 2: Gene ontology and KEGG pathway enrichment in local relapse (p<0.01).

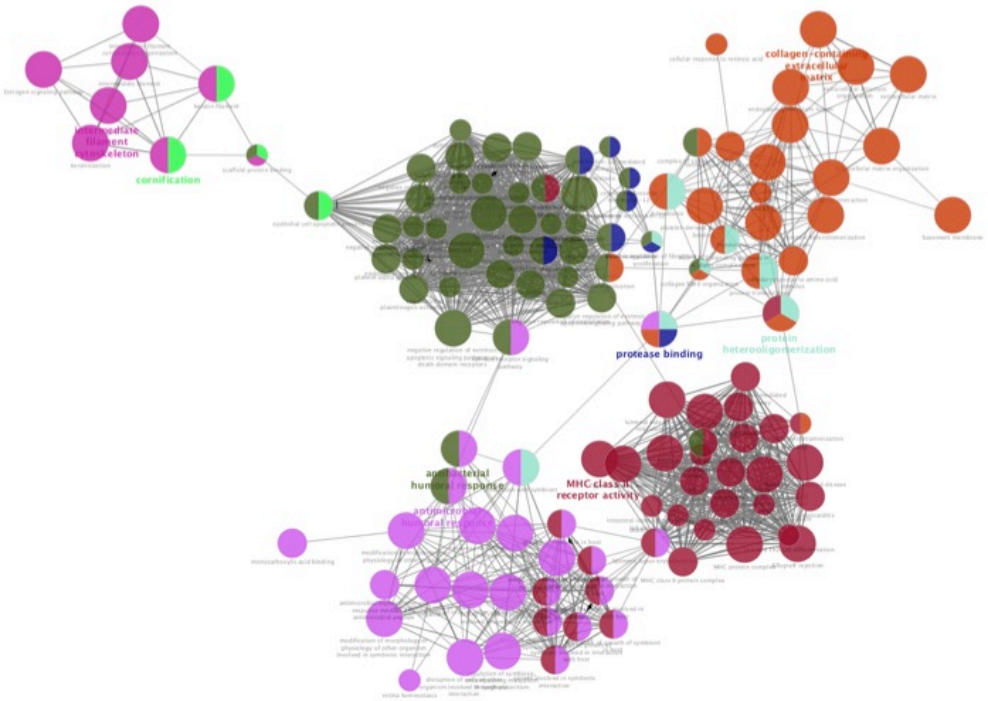


Figure 12: Gene Ontology and KEGG pathway enrichment in local relapse (p<0.01).

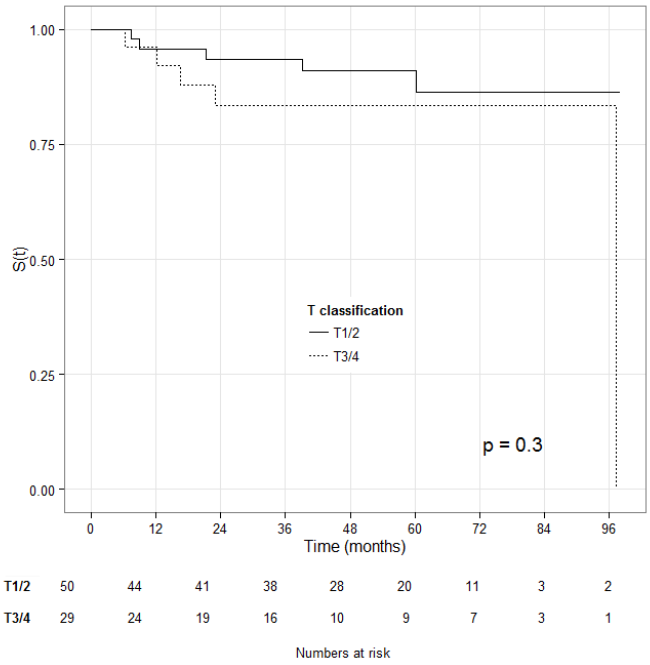
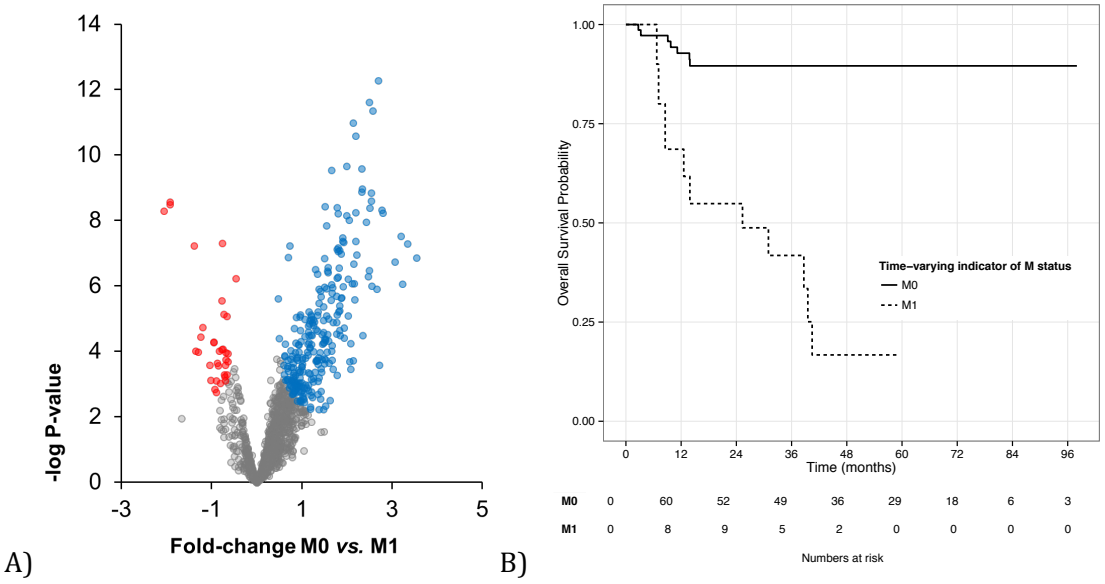


Figure 13: Kaplan-Meier curve of local control stratified by T-Stage (T1+T2 vs. T3-T4, n=79) Log-rank p = 0.3.

6.4.3.3. Distant Relapse (distant metastasis)

In our whole cohort, 18 out of 83 patients developed distant metastasis. As distant metastases are the most important prognostic factor for death risk, outcome-related biomarker research is necessary. A complete list of 303 differentially expressed proteins up- (n=25) and down-regulated (n=268) on M1 patients can be found on Annexes 5 and 6, respectively. Label-free protein quantification analysis of metastasis status (M0 vs. M1) are presented on a volcano plot comparing metastasis status (M0 *vs.* M1, FDR 0.05, S0>0.1, Figure 14A) Overall survival by distant metastases patients from the same cohort is on Figure 14B.



A) B)

Figure 14: A) Label-free protein quantification analysis of metastasis status (M0 vs. M1). Volcano Plot of Metastasis status M0 *vs.* M1, FDR 0.05, S0>0.1: 303 Differentially expressed proteins from which 268 proteins are up-regulated in M0 (Down-regulated in M1) and 35 proteins are down-regulated in M0 (Up-regulated in M1). B) Overall survival by distant metastases status (p<0.01).

A heatmap showing the differentially expressed proteins of Metastasis status M0 *vs.* M1 was generated (Figure 15) with Clustvis: a web tool for visualizing clustering of multivariate data (Metsalu & Vilo 2015). We discuss the roles of the most significantly up-regulated in M1 patients.

a) TPD54:

Among up-regulated proteins, tumor protein D54 or TPD54 (Gene TPD52L2) had the highest significance of detected difference (p<0.01). The tumor protein D52 (TPD52) family includes TPD52, -53, -54 and -55 (Byrne et al. 2014). Reports have described the roles for

TPD52 and TPD53 and have suggested the potential role of TPD54 in physiological effects. In a oral squamous cell carcinoma in vivo study, TPD54 overexpression attenuated tumor volume in vivo. Kato et al. results showed that TPD54 not only down-regulated anchorage-independent growth and cell migration in vitro, but also attenuated tumor growth in vivo. Based on these results, it is considered that TPD54 might behave as a negative regulator of tumor progression in head and neck cancer cells (Kato et al. 2017).

Oral carcinoma cancer cells (OSCC) were subsequently subjected to exogenous over-expression of alternative splice variants (ASVs) of TPD54 and to TPD54 knock-down, mediated by siRNA. Next, the role of TPD54 in cellular growth, apoptosis, invasion, migration and extracellular-matrix (ECM)-dependent migration and attachment was investigated, as also the concomitant expression of integrins and integrin-related proteins by the OSCC-derived cells. Western blot analysis and RT-PCR revealed that TPD54 affects OSCC cell attachment to the extracellular matrix (ECM), cell migration, and Akt/PKB activation by modulating integrin activation via a talin1-mediated inside-out signal of the ECM. The authors suggested that TPD54 may serve as a novel biomarker for OSCC and as a possible target for OSCC therapy (Mukudai et al. 2013).

Kato et al. has described TPD54 acts as a negative regulator of anchorage-independent proliferation and cell migration of OSCC cells in vitro. Moreover, TPD54 decreased body weight gain and tended to attenuate tumor growth in vivo. These combined data suggest that an increase in the expression of TPD54 might improve outcomes in OSCC patients (Kato et al. 2017).

A 2018 study found tumor protein D52 (TPD52) as a direct target of miR-379 by qPCR. Furthermore, silencing of TPD52 significantly inhibited the C666-1 cell line proliferation, migration and invasion suggesting that miR-379 negatively regulates the growth and migration of NPC cells by down-regulating TPD52 expression, while modulation of miR-379 expression may be a therapeutic strategy for this entity (Zhao & Chu 2018).

b) Immunoglobulin

We found several immunoglobulins differentially expressed in M0 or M1 patients. Liu et al. have described other virus-encoded oncoproteins, such as HBX, E6, E7, can also activate many signal pathways including NF-κB and AP-1 pathways. These oncoproteins might induce immunoglobulin gene expression through the mechanism similar to EBV-LMP1. Results suggest that human iEκ is active in Igκ-expressing NPC cells and LMP1-stimulated NF-κB and AP-1

activation results in an augmenting activation of the iEκ. LMP1 promotes the interactions of heterodimeric NF-κB (p52/p65) and heterodimeric AP-1 (c-Jun/c-Fos) transcription factors with the human iEκ enhancer region are important for the upregulation of kappa light chain in LMP1-positive nasopharyngeal carcinoma cells. Their study offered an insight into the mechanisms by which non-lymphoid cancer cells express immunoglobulin (Liu et al. 2009).

c) Calmodulin

A recent study using tandem mass tag (TMT) labeling and high-performance liquid chromatography (HPLC) fractionation followed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) was used to identify differentially expressed proteins. Serums from 40 patients with NPC were evaluated [recurrence (n=20) and no recurrence (n=20)]. Compared to non-recurrent NPC, Meng et al. found 59 proteins to be significantly dysregulated in recurrent NPC. The dysregulation of calmodulin (CALM) was confirmed in 74 new patients [recurrence (n=32) and no recurrence (n=42)]. A preliminary pathway analysis revealed that oxidative phosphorylation was altered in the patients with recurrent NPC compared to those with non-recurrent NPC. These data identified CALM as a potential biomarker for recurrent NPC (Meng et al. 2017).

d) Ribosomal proteins

Ribosomal proteins have already been presented in the previous section strongly related to NPC. In the RPS17 gene protein, it has already been associated to salivary oral squamous cell carcinoma as a marker. Oral cancer associated mRNAs previously identified and validated are highly significantly up-regulated when analyzed after adjusting for reference genes. Salivary gene expression assays also hold promise for the identification of a number of other systemic diseases and for long-term disease surveillance (Martin 2016).

e) Staphylococcal nuclease domain-containing protein 1

Staphylococcal nuclease domain-containing protein 1 is related to SND1 gene syntheses and has been described as a novel gene transcription activator recognizing the conserved Motif domains of Smad promoters, inducing TGFβ1 response and breast cancer metastasis (Yu et al. 2017). Among other detected proteins in M1 patients, protein SGT1 homolog, signal peptidase complex subunit 2, thyroid hormone receptor-associated protein 3, cytosol aminopeptidase, 60S ribosomal protein L13a, 40S ribosomal protein S17 may be

associated in head and neck cancer. (https://www.proteinatlas.org/ENSG00000165416SUGT1/pathology# gene _information).

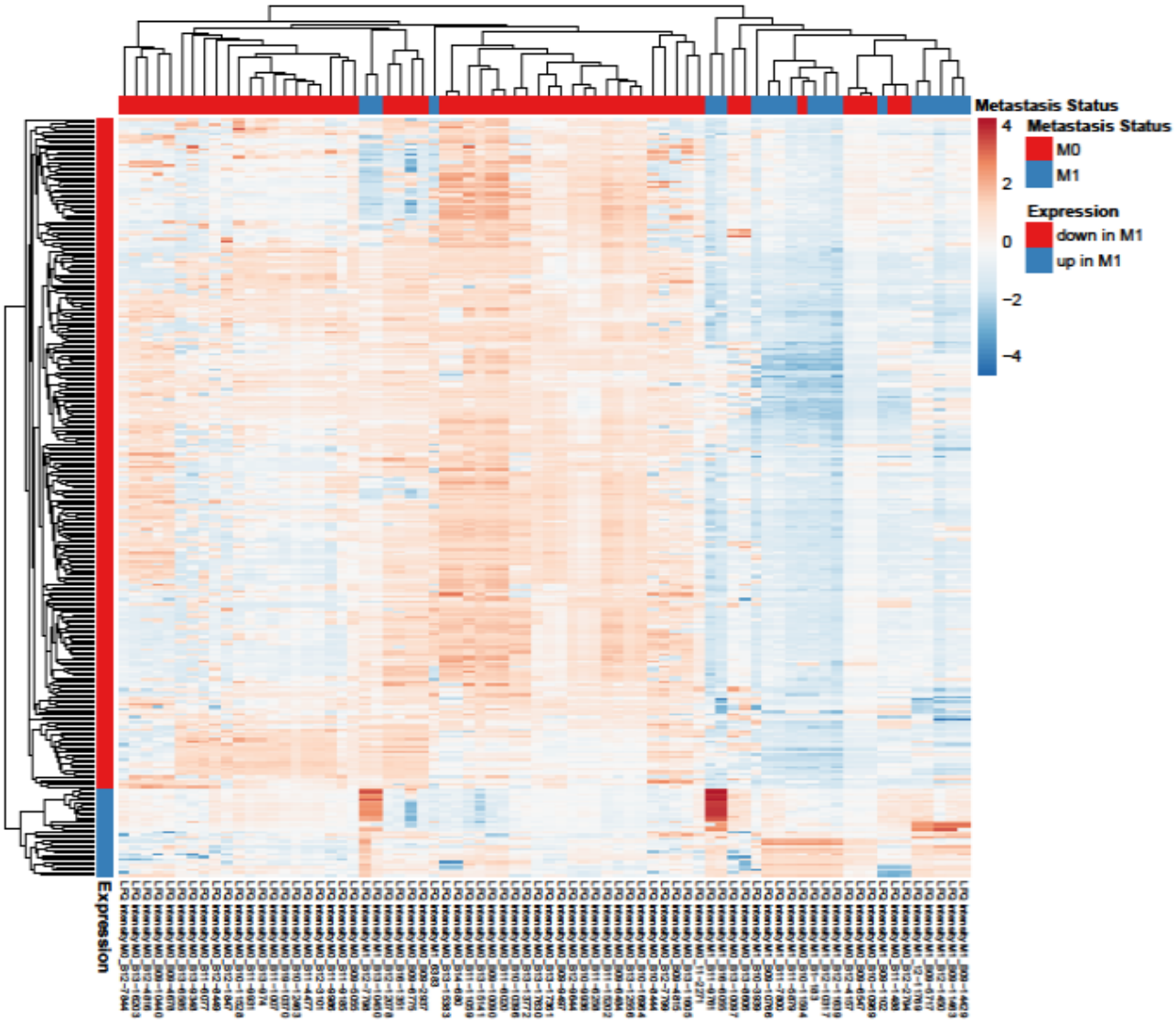


Figure 15: Heatmap showing the differentially expressed proteins of Metastasis status M0 *vs.* M1. Data obtained after MaxQuant and Perseus Analysis. Annotations on top of the heatmap show clustering of the samples. 303 Differentially expressed proteins from which 268 proteins are up-regulated in M0 (Down-regulated in M1) and 35 proteins are up-regulated in M0 (Up-regulated in M1). The heatmap was generated with Clustvis: a web tool for visualizing clustering of multivariate data (Metsalu & Vilo 2015).

6.4.3.4. Signaling pathways found

a) Oncogenic MAPK signaling: the RAS-RAF-MAPK cascade regulates cellular proliferation, differentiation and survival as its components are frequently mutated in

a large number of human malignancies. It is estimated that RAS mutations are found in one third of human cancers, and 8% of tumors express an activated form of BRAF. RAS activation is also detected in a smaller subset of cancers by loss-of-function mutations in negative regulators of RAS signaling, such as the RAS GAP NF1 (Prior & Hancock 2012; Pylayeva-Gupta et al. 2011; Samatar & Poulikakos 2014; Maertens & Cichowski 2014). Our data found Ras-related protein Rab-33A (gene RAB33A) significantly up-regulated on M1 patients ($p<0.01$).

b) DNA Double Strand Break Repair: DNA Double-strand breaks (DSBs) can occur during the processes of DNA replication, meiotic exchange or recombination. It is one of the most deleterious types of DNA lethal damage and are a well-known biological effect of ionizing radiation or drug related effect (i.e. bleomycin). DNA-dependent protein kinase catalytic subunit (gene PRKDC), DNA replication licensing factor MCM3 (gene MCM3), DNA replication licensing factor MCM2 (gene MCM2), single-stranded DNA-binding protein, mitochondrial (gene SSBP1) were down-regulated in M1 patients ($p<0.01$)

c) Adaptive immune system proteins: it refers to antigen-specific immune response to external pathogens targets. The human immune system has B and T lymphocytes carrying receptors able to identify specific antigens or pathogens. During infections, dendritic cells (DCs) work as sentinels in tissues to identify and pick a pathogen as antigenic determinants. DCs have the function to present antigens to T cells, that will kill the aggressor directly or produce cytokines to request B lymphocyte response, in order to stimulate humoral immunity via antibodies production for each antigen.

d) Signaling by B cell receptor (BCR): IgM and IgD immunoglobulin are present at the plasmatic membrane with Ig-alpha (CD79A, MB-1) and Ig-beta (CD79B, B29) to build the BCR in mature cells. BCR plays a pivotal role in ensuring the effective and appropriate B-cell response to antigen (Brezski & Monroe 2008). The complex antigen-Ig activates the tyrosine-based activation motifs (ITAMs) phosphorylation in the cytoplasmic part of Ig-alpha and Ig-beta via Src tyrosine kinases (i.e. LYN, FYN and BLK) (Harwood & Batista 2010; Gauld & Cambier 2004). SYK protein kinase binds the ITAMs phosphorylated immunoreceptor on the cytoplasmic part of Ig-alpha (CD79A, MB-1) and Ig-beta (CD79B, B29) (Wienands et al. 1995; Tsang et al. 2008). This link activates SYK phosphorylation that, along other kinases will phosphorylate BLNK (SLP-65), BCAP, and CD-19 (Bradshaw 2010). These will support the construction of

large complexes (signalosomes), by recruiting PI3K (phosphoinositol 3-kinase), phospholipase C gamma (like PLC-gamma2 in B cells), NCK, BAM32, BTK, VAV1 and SHC (Coggeshall et al. 1992). PLC-gamma (BLNK-linked) hydrolizes phosphatidylinositol-4,5-biphosphate allowing inositol-1,4,5-triphosphate (IP3) and diacylglycerol (Carter et al. 1991; Kim et al. 2004). IP3 binds receptors at the endoplasmic reticulum and releases calcium ions from ER to the cytosol. Calcium depletion from the ER activates STIM1 to interact with ORA1 and TRPC1 channels in the membrane, resulting in extracellular calcium influx (Muik et al. 2008; Luik et al. 2008). PI3K (linked with BCAP and CD19) phosphorylates phosphatidylinositol-4,5-biphosphate allowing phosphatidylinositol-3,4,5-triiphosphate Second messengers like calcium, diacylglycerol, inositol, 1,4,5.triphosphate, and phosphatidylinositol 3,4,5.triphosphate are able to trigger signaling pathways: NF-kappaB is activated via protein kinase C beta; RAS is activated via RASGRP proteins; NF-AT is activated via calcineurin and AKT (PKB) is activated via PDK1 (Stone 2006; Shinohara & Kurosaki 2009). We found 6 immunoglobulins up-regulated on metastatic patients. Ig-lambda variable 3-19 (gene IGLV3-19); Immunoglobulin heavy variable 4-61 (gene IGHV4-61); Ig-kappa variable 2D-26 (gene IGKV2D-26); Ig heavy variable 3-66 (gene IGHV3-66); Ig-lambda constant 2 (gene IGLC2); and Ig-kappa variable 1-5 (gene IGKV1-5), all with $p<0.01$. And also 1 down-regulated immunoglobulin: Ig heavy constant gamma 4 (gene IGHG4), $p<0.01$.

e) Signaling by Rho GTPases: Rho GTPases are well known for their roles in regulating cell migration, and also contribute to a variety of other cellular responses(Haga & Ridley 2016). They are divided into typical and atypical. The typical Rho family members, including RhoA, Rac1 and Cdc42, cycle between an active GTP-bound and inactive GDP-bound conformation. Atypical Rho family members have aminoacidic substitutions that alter their ability to interact with GTP/GDP and hence are regulated by different mechanisms. Both typical and atypical Rho GTPases contribute to cancer progression. In most cancers' expression levels and/or activity of Rho GTPases is altered. Rho GTPase signaling could therefore be therapeutically targeted in cancer treatment (Haga & Ridley 2016). Rho family of guanine nucleotide binding proteins is one branch of the Ras family. They serve as a binary switch controlling several biological processes by cycling between GTP-bound and inactive GDP-bound conformation. RhoA, Rac1 and Cdc42 have been more studied. They can induce rearrangements of the plasma membrane actin cytoskeleton (Aspenström et al. 2004; Govek et al. 2005). Rho GTPases also regulate actomyosin and microtubule dynamics.

Rho has secondary functions on mediated effects on transcription and membrane trafficking. Rho GTPases have been associated in processes like cell growth, cytokinesis, cell motility, extracellular matrix adhesion, cell transformation and invasion, and cell development, important hallmarks in cancer biology (Govek et al. 2005). Our data found Ras GTPase-activating-like protein IQGAP1 (gene IQGAP1) significantly down-regulated on metastatic patients ($p<0.01$).

f) Extracellular matrix organization (ECM): the matrix is a component of all mammalian tissues consisting on fibrous proteins collagen, elastin and associated-microfibrils, fibronectin and laminis among viscoelastic gel. Beyond its structural role, it influences cell behaviors in proliferation, adhesion and migration, and regulates cell differentiation and death (Hynes 2009). Its remodeling is involved in the regulation of cell differentiation processes on maintenance of stem cell niches, branching morphogenesis, angiogenesis, bone remodeling, and wound repair. Abnormal matrix can cause abnormal cell proliferation with invasion, failure of cell death, and loss of cell differentiation. All these processes can lead to several benign or malignant diseases (i.e. cancer). Fibronectin protein (gene FN1) was down-regulated on M1 patients ($p<0.01$).

g) Hemostasis-related proteins: hemostasis is a physiological process that leads to arrest of bleeding. It is achieved by a cascade of complex events that play a pivotal role in coagulation by reduction of some endothelial dilating agents like adenosine, NO, prostacyclin and by direct effect of ADP, serotonin and thromboxane (Becker 2000). Fibrinogen plays an important role in the cascade. It aggregates with platelets, forms bridges between activated platelets initiating the clotting cascade. Negatively-charged phospholipids interact with tissue factor, leading to an insoluble fibrin clot. Regarding hemostasis, fibrinogen alfa- beta- and gamma chains were significantly down-regulated on M1 patients ($p<0.01$).

h) Cell surface interaction at the vascular wall: cell extravasation is a controlled process that leads white cell movement from the vascular lumen to a certain required tissue. The adhesive connections between cells require several steps to be taken so white cells can overcome the vascular wall. Platelets adhered to the vessels make difficult barrier to overcome. So, the rolling and invasion of leukocytes on the injury site is mediated by binding of selectins to cognate cell-surface glyconconjugates. Endothelial cells are strongly bound together via several proteins that regulate the junctions. An

important role for these junctional proteins is to rule the transendothelial migration of cells under normal and abnormal situations. Intercellular adhesion molecule 1 (ICAM1) was down-regulated on M1 patients ($p<0.01$).

7. Conclusions and Future Work

There are several conclusions one can retrieve from this research:

A) Our matured clinical data confirm excellent local and regional control of concurrent chemoradiation in the IMRT era with acceptable late toxicity, comparable to others and represent a major improvement from our 3DCRT era cohort;

B) In our series, optimal radiotherapy via IMRT yielded superb local control regardless T stage. As recurrences were not present in lower neck, de-escalation studies are warranted to decrease the long-term toxicity rates;

C) FFPE is underused as source of clinical information. Our protocol was able to safely extract and present biological material from FFPE samples yielding adequate material to MS analysis;

D) Moreover, with a median tumor percentage of 75% on each sample, using a single slice of 10μm, no patient had its biopsy exhausted in this research;

E) Our preliminary experimental data identified tumor signature profiles strongly associated with early and late T-stages, and also related to distant metastases in a non-endemic cohort of NPC patients;

F) Preliminary results identified 12 up-regulated proteins on early-stage primary tumors that were not present on advanced-stage primaries. Moreover, EBV and HSV co-infection was detected. Clinical outcomes of early and advanced primary tumors were identical, what can be attributed to the effectiveness of intensive chemoradiation. Moreover, the difference in tumor profiling may reflect that those early-stage tumors could benefit from de-escalation strategies;

G) The presence of co-infection EBV and Herpes simplex was found significantly on early-stage tumors. There was no HPV co-infection in our cohort. This co-infection may generate hypothesis for further studies and strategies to identify early-stage tumors;

H) We could not detect EBV-related proteins on advanced stage primary tumors (T3+T4) on our matured data. This has also unclear meaning since there are limitations on detecting low proportion concentration with our method. Nevertheless, even if present, it is also important to stress that it would be in such a low proportion concentration;

I) A tumor profiling with a pool of 10 up-regulated proteins was detected for M1 patients, among several known proteins related to invasiveness and metastasis like heat shock protein, cathepsin, etc. we found an important finding of interferon

induced proteins, interferon regulatory proteins, and tyrosine kinase protein in metastatic disease;

J) Interferon-induced GTP-binding protein Mx1 was down-regulated in another tumor profiling with 9 proteins was found to be significantly down-regulated in patients bearing local relapse;

K) The presence of interferon proteins generates several hypotheses regarding the immune-based targets for our patients. Since the German Group has successfully treated NPC patients with adjuvant interferon, it is reasonable to argue whether this strategy should be evaluated on our cohorts;

L) Tyrosine kinase inhibitors (TKI) play a role in several other cancers. Unlike interferon, to date, there are no data to integrate TKI in NPC treatment.

M) We also find interferon related proteins up-regulated in locally relapsed tumors. As local relapse represents a minority of patients (around 10%) always treated with re-irradiation, a potential biomarker could help select patients for upfront more intensive treatment;

N) Tumor protein D54 was significantly up regulated on M1 patients and was identified as a potential biomarker candidate for metastasis prediction;

O) Rho GTPases have been associated in processes like cell growth, cytokinesis, cell motility, extracellular matrix adhesion, cell transformation and invasion. We detected an important signaling pathway on M1 patients (Ras GTPase-activating-like protein IQGAP1, via gene IQGAP1, significantly down-regulated on metastatic patients ($p<0.01$);

P) Though validation is needed, these findings support the need for further clinical and basic investigation regarding this entity.

This is an ongoing work of our group.

8. Abstract (English)

Nasopharyngeal carcinoma (NPC) is a particular type of head and neck cancer with a strong ethnic and enigmatic epidemiology that long puzzles the scientific society. Despite present all over the world, around 80% of all cases are in Asia where it is endemic, followed by low incidence regions in all other continents. Portugal has the second incidence in Europe for women, and third for men (Globocan 2012) among all European countries.

Many efforts have been made to clarify and understand this unique geographic and epidemiologic distribution. The onset and evolution of nasopharyngeal carcinoma is a complex multi-stage process and may take a long time to occur. Although the molecular basis remains uncertain, we know that environmental factors, Epstein-Barr virus (EBV) infection and genetic susceptibility are considered to be the three major contributors.

Further examination of these genes' expression in each tumor revealed that around 93 oncogenes are up regulated in each tumor, while the mean number of TSGs down regulated was 109. In Portugal, the works of Souza & Breda have identified important polymorphism markers that may play a role in the onset and NPC development on the Portuguese northern region population.

Despite the efforts made, the molecular mechanisms of NPC carcinogenesis and progression remains to be understood. In this regard, current omics methodologies offer a different approach to identify unique miRNAs and proteins that expression signatures associated with the cancer phenotype, reflecting the biological and pathological grade of the disease. Thus, the discovery of useful NPC biomarkers will lead to new diagnostic and prognostic tools.

Meanwhile, the treatment of NPC has evolved in the past two decades. Since 2000, intensity modulated radiation therapy (IMRT) has been widely used to treat nasopharyngeal carcinoma. IMRT provides better dose delivery to the target while sparing the surrounding normal tissues while local control reaches 98% at 4 years[24-28]. At least one prospective randomized controlled trial showed its benefit in salivary protection in HNC. However, despite the excellent local control, 43% of patients will develop distant metastasis before 5 years and die[25-26].

In Portugal, IMRT is used at the Instituto Português de Oncologia de Lisboa (IPOLFG) since 2009 and represents the current standard of care for HNC RT, including

NPC. Until now, several prognostic markers were identified and investigated for screening, and prognostic tools for NPC, have been purposed, particularly in Asia. However, to our knowledge, there are no validated identified markers to predict distant metastasis or outcome. Moreover, the studies exploring this subject have identified a population-based variety of biomarkers stressing an omic translation of NPC ethnic distribution.

At the present work, this research explored formalin-fixed paraffin-embedded samples of biopsied nasopharyngeal carcinoma tumors via proteomic analysis. The aim is to describe a tumor profiling from the studied cohort and discover biomarkers to predict distant metastasis. Secondary endpoints are the discovery of biomarkers related to tumor radioresistance and treatment toxicity.

Label-free quantitative mass spectrometry was able to identify 12 up-regulated proteins on early-stage primary tumors that were not present on advanced-stage primaries. Moreover, EBV and HSV co-infection was detected. No signs of HPV-related proteins were seen. Although the clinical outcomes of early and advanced primary tumors were identical, this can be attributed to the known effectiveness of intensive chemoradiation. Moreover, the difference in tumor profiling may reflect that those early-stage tumors could benefit from de-escalation protocols.

We were able to detect the presence of a pool of 10 proteins related to distant metastases. Among them, the significant presence of interferon and tyrosine kinase proteins generates the hypothesis that they may represent therapeutic targets. Validation is needed.

8. Abstract (Portuguese)

O carcinoma da nasofaringe (NPC) é um tipo particular de cancro da cabeça e pescoço com um epidemiologia enigmática e forte cariz étnico que há muito intriga a comunidade científica. Apesar de presente em todo o mundo, 805 dos casos encontram-se na Ásia, onde é endémico, seguido de regiões de media e baixa incidência em todos os outros continentes. Portugal tem a segunda incidência em mulheres e a terceira em homens dentre todos os países da Europa (Globocan 2012).

Muitos esforços foram realizados para esclarecer e compreender esta distribuição epidemiológica e geográfica. A instalação e evolução do NPC é um processo com múltiplas fases e pode demorar um longo período de tempo para ocorrer. Apesar da base molecular da sua origem permanecer incerta, sabe-se que factores ambientais, a infecção pelo vírus Epstein-Barr e a susceptibilidade genética do hospedeiro são os três maiores contribuidores.

Análise aprofundada da expressão genética revelou que cerca de 93 oncogenes estão sobre regulados enquanto que o número médio de genes supressores de tumor down regulated é de cerca de 109. Em Portugal, o trabalho de Souza & Breda identificaram um importante marcador de polimorfismo que pode estar relacionado com a instalação do NPC na população portuguesa na região norte.

À despeito dos esforços realizados, o mecanismo molecular da carcinogênese do NPC e sua progressão permanecem por ser esclarecidos. A este respeito, as actuais tecnologias de ômica oferecem uma abordagem diferente capaz de identificar miRNAs e proteínas únicos que expressam assinatura com um fenótipo do cancro, refletindo o grau biológico e patológico da doença. Assim, a descoberta de biomarcadores úteis levará a novas ferramentas prognósticas.

Ao mesmo tempo, o tratamento do NPC evoluiu consideravelmente nas últimas duas décadas. Desde 2000, a radioterapia com intensidade modulada do feixe (IMRT) tem sido amplamente utilizada para o tratamento do carcinoma da nasofaringe. IMRT fornece melhor entrega da dose ao alvo enquanto poupa os tecidos adjacentes sadios ao mesmo tempo que o controlo local alcança 98% aos 4 anos (Lee et al, 2002).

Pelo menos uma meta-análise demonstrou o benefício na capacidade de proteção salivar em carcinomas da cabeça e pescoço. Entretanto, apesar do excelente controlo local, até 43% dos doentes vão morrer devido às metástases à distância antes dos

5 anos.

Em Portugal, IMRT é utilizada no Instituto Português de Oncologia de Lisboa (IPOLFG) desde 2009 e representa o actual estado da arte no tratamento da maioria dos carcinomas da cabeça e pescoço incluindo NPC. Até o momento, diversos marcadores prognósticos de NPC foram propostos, particularmente na Ásia. Contudo, até o momento não são utilizados marcadores validades para predizer metástases ou evolução do doente. Estudos avaliando esta questão identificaram uma população variada de biomarcadores distintos a outras regiões geográficas, ressaltando a diversidade étnica da população dos doentes com NPC.

Neste trabalho, esta pesquisa explorou amostras de carcinoma da nasofaringe provenientes de biopsias fixadas em formalina e embebidas em parafina através de análise proteómica. O objectivo é descrever o perfil tumoral da coorte estudada e descobrir biomarcadores capazes de predizer metástases à distância. Objectivos secundários é a descoberta de biomarcadores relacionados ao tumor que determinem radioresistência ou toxicidade ao tratamento.

Espectroscopia de massa por ressonância magnética foi capaz de identificar 12 proteínas sobre expressadas nas em tumores primários iniciais que não estão presentes em tumores primários avançados. Além disso, foi detectada uma coinfecção EBV e HSV sem sinais de proteínas relacionadas ao HPV.

Apesar dos resultados clínicos terem sido idênticos em ambos os grupos, isto pode ser explicado pela efectividade e intensidade do tratamento combinado. Além disso, a diferença em perfil tumorais podem refletir um perfil de doentes que poderia se beneficiar de protocolos de desintensificação.

Esta pesquisa foi capaz de detectar a presença de 10 proteínas relacionadas aos doentes com metástases à distância. Entre elas, a presença de interferon e inibidores da tirosina cinase geram a hipótese de que podem representar potenciais alvos terapêuticos.

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Annex 1 – NPC related miRNA

miRNA	Relationship type	Year
hsa-miR-212	Unspecified	2008
hsa-miR-216	Unspecified	2008
hsa-miR-217	Unspecified	2008
hsa-miR-29c	Causal	2008
hsa-miR-34b	Unspecified	2008
hsa-miR-34c	Unspecified	2008
hsa-miR-151	Unspecified	2008
hsa-miR-192	Unspecified	2008
hsa-miR-100	Causal	2009
miR-BART21	Causal	2009
miR-BART22	Causal	2009

Source: Jiang Q., Wang Y., Juan L., Teng M., Zhang X., Wang G., Liu Y., (2009) miR2Disease: a manually curated database for microRNA deregulation in human disease. Nucleic Acids Res37:D98-104.

Annex 2 - Table 1. Patient, tumor and treatment characteristics (n=109)

Variable		N (%)
Age, years	Median (range)	51 (12-89)
Gender	Male	81 (74%)
	Female	28 (26%)
Histological grade	1	9 (8%)
	2	20 (18%)
	3	79 (73%)
	Missing	1 (1%)
Clinical T Stage	T1	47 (43%)
	T2	15 (14%)
	T3	20 (18%)
	T4	27 (25%)
Clinical N Stage	N0	19 (17%)
	N1	19 (17%)
	N2	42 (39%)
	N3	29 (27%)
Clinical M Stage	M0	104 (95%)
	M1	5 (5%)
Stage	I	7 (6%)
	II	10 (9%)
	III	36 (33%)
	IVA	25 (23%)
	IVB	26 (24%)
	IVC	5 (5%)
BMI	20-25	40 (37%)
	< 20	3 (3%)
	25-30	22 (20%)
	>30	15 (14%)
	Missing	29 (27%)

ECOG performance status	0	81 (74%)
	1	23 (21%)
	2	2 (2%)
	3	2 (2%)
	4	1 (1%)
Ethnic Group	Caucasian	106 (97%)
	African	1 (1%)
	Asian	2 (2%)
Smoking habbits	0 to ≤10 pack-year-units	69 (63%)
	>10 pack-year-units	38 (35%)
	Missing	2 (2%)
Treatment	RT	12 (11%)
	CCRT	28 (26%)
	CCRT + aCT	59 (54%)
	iCT + CCRT	1 (1%)
	iCT + RT	3 (3%)
	Missing	6 (6%)
Radiotherapy Technique	IMRT (70 Gy)	103 (94%)
	Missing	6 (6%)
Adjuvant Chemotherapy Compliance	3 Cycles	52 (88%)
	2 Cycles	4 (7%)
	1 Cycle	3 (5%)
Overall Treatment Time	< 49 days	65 (60%)
	> 49 days	41 (38%)
	Missing	3 (3%)
EBV EBNA serum pre treatment	Undetected	24 (22%)
	Detected	36 (33%)
	Missing	49 (45%)

Annex 3 - Table 1. Patient, tumor and treatment characteristics (n=28)

Variable		N (%)
Age, years	Median (range)	47 (31-74)
Gender	Male	22 (79%)
	Female	06 (21%)
Histological WHO Grade	1	03 (10%)
	2	01 (04%)
	3	24 (86%)
Clinical T Stage	T1	11 (40%)
	T2	03 (10%)
	T3	07 (25%)
	T4	07 (25%)
Clinical N Stage	N0	04 (14%)
	N1	03 (11%)
	N2	13 (46%)
	N3	08 (29%)
Clinical M Stage	M0	26 (93%)
	M1	2 (07%)
Stage (7th Edition)	II	02 (07%)
	III	11 (40%)
	IVA	06 (21%)
	IVB	07 (25%)
	IVC	02 (07%)
ECOG performance status	0	24 (86%)
	1	04 (14%)
Ethnic Group	Caucasian	27 (97%)
	Asian	01 (03%)
Smoking habbits	No	14 (50%)
	≤10 pack-year-units	03 (10%)
	>10 pack-year-units	11 (40%)

Treatment	RT	01 (04%)
	CCRT	03 (10%)
	CCRT+CT	24 (86%)
Overall Treatment Time	< 49 days	26 (93%)
	> 49 days	02 (07%)
EBV serum pre treatment	Undetected (<600)	05 (18%)
	Detected (≥600)	07 (25%)
	Missing	16 (57%)

Annex 4 - Results from Label-free quantitative proteomic analysis between T1+T2 versus T3+T4 patients ($p < 0.01$).

Down-regulated in T1+T2 (12 proteins)	**Up-regulated in T1+T2 (59 proteins)**
Thioredoxin	14-3-3 protein beta/alpha
Complement component 1 Q subcomponent-binding protein	14-3-3 protein sigma
Cathepsin B	26S proteasome non-ATPase regulatory subunit 2
Small nuclear ribonucleoprotein Sm D3	40S ribosomal protein S11
60S ribosomal protein L10	40S ribosomal protein S15a
60S ribosomal protein L23	40S ribosomal protein S25
Ras-related protein Rab-6A	60S ribosomal protein L13a
DNA replication licensing factor MCM2	60S ribosomal protein L22
ADP/ATP translocase 2	60S ribosomal protein L26-like
Transformer-2 protein homolog beta	60S ribosomal protein L34
Interferon-induced GTP-binding protein Mx1	60S ribosomal protein L35a
Hypoxia up-regulated protein 1	60S ribosomal protein L6
	Activated RNA polymerase II transcriptional coactivator p15
	Alanine--tRNA ligase, cytoplasmic
	Annexin A3
	Annexin A8
	Apoptosis-associated speck-like protein containing a CARD
	Barrier-to-autointegration factor
	Bifunctional glutamate/proline--tRNA ligas
	Calponin-3
	Catalase
	Cathelicidin antimicrobial peptide
	Cellular retinoic acid-binding protein 2
	Collagen alpha-1(I) chain
	Collagen alpha-1(IV) chain

	Collagen alpha-2(I) chain Cystatin-A Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase DNA-(apurinic or apyrimidinic site) lyase Erythrocyte band 7 integral membrane protein Exportin-2 Gamma-interferon-inducible lysosomal thiol reductase Glucosidase 2 subunit beta Heterogeneous nuclear ribonucleoprotein F Histidine triad nucleotide-binding protein 1 HLA class I histocompatibility antigen HLA class II histocompatibility antigen gamma chain HLA class II histocompatibility antigen, DQ beta 1 chain HLA class II histocompatibility antigen, DRB1-4 beta chain Ig gamma-2 chain C region Ig gamma-3 chain C region Ig lambda-3 chain C regions Ig mu chain C region;Ig mu heavy chain disease protein Isocitrate dehydrogenase [NADP] cytoplasmic Junction plakoglobin Keratin, type I cytoskeletal 13 Keratin, type I cytoskeletal 16 Keratin, type I cytoskeletal 17 Keratin, type I cytoskeletal 18

	Keratin, type I cytoskeletal 19 Keratin, type II cytoskeletal 6A Keratin, type II cytoskeletal 8 Leukocyte elastase inhibitor Microtubule-associated protein 4 Myeloperoxidase Myristoylated alanine-rich C-kinase substrate Neutrophil defensin 3 Neutrophil elastase Periostin Peroxisomal multifunctional enzyme type 2 Phosphate carrier protein, mitochondrial Prosaposin Proteasome activator complex subunit 1 Protein deglycase DJ-1 Protein S100-A6 Protein S100-A8 Ribosome-binding protein 1 Septin-7 Serine/arginine-rich splicing factor 6 Small nuclear ribonucleoprotein Sm D1 Sulfide:quinone oxidoreductase Synaptogyrin-2 Tapasin Transaldolase Transforming growth factor-beta-induced protein ig-h3 Translocon-associated protein subunit delta Vinculin

Annexx 5 – Proteins differentially expressed up-regulated in M1 patients (p<0.01).

Protein	Gene	Difference
Tumor protein D54	TPD52L2	-0,466002574
Immunoglobulin lambda variable 3-19	IGLV3-19	-0,639203889
Protein SGT1 homolog	SUGT1	-0,645825583
Endoplasmic reticulum chaperone BiP	HSPA5	-0,665652843
Plastin-2	LCP1	-0,666951906
Xin actin-binding repeat-containing protein 1	XIRP1	-0,683019445
Immunoglobulin heavy variable 4-61	IGHV4-61	-0,684808758
Beta/gamma crystallin domain-containing protein 2	CRYBG2	-0,694004047
Threonine synthase-like 1	THNSL1	-0,694256173
Marginal zone B- and B1-cell-specific protein	MZB1	-0,711463588
Ras-related protein Rab-33A	RAB33A	-0,720078639
Immunoglobulin kappa variable 2D-26	IGKV2D-26	-0,730822688
CSC1-like protein 1	TMEM63A	-0,758189867
Calmodulin-1	CALM1	-0,767832245
Signal peptidase complex subunit 2	SPCS2	-0,770994603
Thyroid hormone receptor-associated protein 3	THRAP3	-0,779497658
Staphylococcal nuclease domain-containing protein 1	SND1	-0,814533767
Histone H4	HIST1H4A	-0,830211935
Filamin-A	FLNA	-0,848450419
Immunoglobulin heavy variable 3-66	IGHV3-66	-0,881263059
Cytosol aminopeptidase	LAP3	-0,89371942
60S acidic ribosomal protein P0	RPLP0	-0,897383493
Annexin A6	ANXA6	-0,935187994
60S ribosomal protein L13a	RPL13A	-0,948628452
60S ribosomal protein L21	RPL21	-0,954002804
Immunoglobulin lambda constant 2	IGLC2	-1,015555196
ATP synthase subunit O, mitochondrial	ATP5PO	-1,044366541
Brain acid soluble protein 1	BASP1	-1,20266783
Dipeptidyl peptidase 1	CTSC	-1,248159216
Putative uncharacterized protein NEXN-AS1	NEXN-AS1	-1,300852439
Partitioning defective 3 homolog	PARD3	-1,356528782
HLA class II histocompatibility antigen, DQ alpha 2 chain	HLA-DQA2	-1,390087143
40S ribosomal protein S17	RPS17	-1,923962847
Fibrillin-1	FBN1	-1,924211907
Immunoglobulin kappa variable 1-5	IGKV1-5	-2,058149186

Annexx 6 – Proteins differentially expressed down-regulated in M1 patients (p<0.01).

Proteins	Gene	Difference
Fibrinogen beta chain	FGB	3,542087373
Fibrinogen gamma chain	FGG	3,337652328
Keratin, type II cytoskeletal 8	KRT8	3,230585708
Keratin, type I cytoskeletal 18	KRT18	3,198568825
Histone H2A type 2-C	HIST2H2AC	3,066348121
Nucleoside diphosphate kinase B	NME2	2,792662204
Protein S100-A9	S100A9	2,775364304
Fibrinogen alpha chain	FGA	2,715856704
Non-POU domain-containing octamer-binding protein	NONO	2,697624154
Keratin, type II cytoskeletal 6A	KRT6A	2,664284945
T-complex protein 1 subunit zeta	CCT6A	2,57175866
14-3-3 protein sigma	SFN	2,545406224
Heterogeneous nuclear ribonucleoprotein H	HNRNPH1	2,543989378
T-complex protein 1 subunit alpha	TCP1	2,533769982
Heterogeneous nuclear ribonucleoprotein A1	HNRNPA1	2,510516632
Thioredoxin	TXN	2,494936232
DNA-dependent protein kinase catalytic subunit	PRKDC	2,493104053
Beta-2-microglobulin	B2M	2,470381922
Lamin-B1	LMNB1	2,427210074
Protein S100-A8	S100A8	2,347757926
Adenosylhomocysteinase	AHCY	2,338814785
60S ribosomal protein L27a	RPL27A	2,327121882
T-complex protein 1 subunit delta	CCT4	2,321160562
Eukaryotic initiation factor 4A-I	EIF4A1	2,219520081
Eukaryotic translation initiation factor 5A-1	EIF5A	2,193874548
Splicing factor, proline- and glutamine-rich	SFPQ	2,190487037
Fatty acid synthase	FASN	2,190301891
Fibronectin	FN1	2,165825174
Phosphate carrier protein, mitochondrial	SLC25A3	2,155342613
Vitronectin	VTN	2,152418818
Trypsin	N/A	2,13497479
LIM and SH3 domain protein 1	LASP1	2,132781612
Glutathione S-transferase P	GSTP1	2,118636786
Histone H1.5	HIST1H1B	2,084835056
Keratin, type I cytoskeletal 14	KRT14	2,079015664
Transforming protein RhoA	RHOA	2,063215135
Polypyrimidine tract-binding protein 1	PTBP1	2,04706617
40S ribosomal protein S16	RPS16	2,029570057
Protein S100-A11	S100A11	2,027375134
Protein/nucleic acid deglycase DJ-1	PARK7	1,995393072
Protein PML	PML	1,993481341
Keratin, type I cytoskeletal 19	KRT19	1,936525307
Hemoglobin subunit delta	HBD	1,936502525
Serine/arginine-rich splicing factor 2	SRSF2	1,923535642
Polyadenylate-binding protein 1	PABPC1	1,910415551

60S ribosomal protein L18a	RPL18A	1,907066814
Haptoglobin	HP	1,901418535
RNA-binding protein FUS	FUS	1,881884772
40S ribosomal protein S7	RPS7	1,869420892
Catalase	CAT	1,868886914
HLA class II histocompatibility antigen, DRB1-16 beta chain	HLA-DRB1	1,844888649
Dihydropyrimidinase-related protein 2	DPYSL2	1,839601017
Sorbitol dehydrogenase	SORD	1,836657861
Core histone macro-H2A.1	H2AFY	1,834656193
Ras GTPase-activating-like protein IQGAP1	IQGAP1	1,830925654
40S ribosomal protein S20	RPS20	1,820906056
T-complex protein 1 subunit theta	CCT8	1,814699582
Lamina-associated polypeptide 2, isoforms beta/gamma	TMPO	1,805940897
THO complex subunit 4	ALYREF	1,802459823
Protein SET	SET	1,801704592
Guanylate-binding protein 1	GBP1	1,800779517
Intercellular adhesion molecule 1	ICAM1	1,782856752
Cytochrome b-c1 complex subunit 1, mitochondrial	UQCRC1	1,780412769
60S ribosomal protein L15	RPL15	1,780159231
Heterogeneous nuclear ribonucleoprotein A/B	HNRNPAB	1,780157468
Tubulin beta chain	TUBB	1,777276471
Ferritin light chain	FTL	1,764201149
Heterogeneous nuclear ribonucleoproteins C1/C2	HNRNPC	1,763866845
Glucose-6-phosphate isomerase	GPI	1,76312822
Nucleophosmin	NPM1	1,693388405
Galectin-1	LGALS1	1,690415878
Probable ATP-dependent RNA helicase DDX5	DDX5	1,676693137
Isocitrate dehydrogenase [NADP] cytoplasmic	IDH1	1,671766663
Heterogeneous nuclear ribonucleoprotein F	HNRNPF	1,670826586
Histone H2B type 2-E	HIST2H2BE	1,668221341
Peptidyl-prolyl cis-trans isomerase FKBP4	FKBP4	1,65778749
Peroxiredoxin-2	PRDX2	1,656616374
Alpha-1-antitrypsin	SERPINA1	1,655377097
Ribonuclease inhibitor	RNH1	1,644409327
Lactotransferrin	LTF	1,619342846
Myeloperoxidase	MPO	1,601248889
Clusterin	CLU	1,594447287
Histone H1.4	HIST1H1E	1,586962291
Actin-related protein 2	ACTR2	1,578404461
Acidic leucine-rich nuclear phosphoprotein 32 family member A	ANP32A	1,574166919
Interleukin enhancer-binding factor 3	ILF3	1,573081921
MICOS complex subunit MIC60	IMMT	1,572483369
Tryptophan--tRNA ligase, cytoplasmic	WARS	1,558371347
Bifunctional purine biosynthesis protein PURH	ATIC	1,549457058
Cytoskeleton-associated protein 4	CKAP4	1,548024878
Serine/threonine-protein phosphatase PP1-beta catalytic subunit	PPP1CB	1,541281057
Heterogeneous nuclear ribonucleoprotein A0	HNRNPA0	1,527035497
T-complex protein 1 subunit eta	CCT7	1,526018071
Keratin, type I cytoskeletal 17	KRT17	1,522549932

Matrin-3	MATR3	1,518465076
RuvB-like 1	RUVBL1	1,517313038
Heterogeneous nuclear ribonucleoprotein L	HNRNPL	1,506298765
ATP-dependent 6-phosphofructokinase, liver type	PFKL	1,506250204
Transgelin-2	TAGLN2	1,505753124
Cystatin-A	CSTA	1,502391501
40S ribosomal protein S28	RPS28	1,50185761
Isocitrate dehydrogenase [NADP], mitochondrial	IDH2	1,493390768
40S ribosomal protein S3a	RPS3A	1,480289546
Far upstream element-binding protein 1	FUBP1	1,479759776
DNA replication licensing factor MCM3	MCM3	1,479411265
Spliceosome RNA helicase DDX39B	DDX39B	1,476443548
Prelamin-A/C	LMNA	1,473051824
60S ribosomal protein L23a	RPL23A	1,454963843
60S ribosomal protein L14	RPL14	1,445500052
Poly(rC)-binding protein 2	PCBP2	1,44378983
60S ribosomal protein L26-like 1	RPL26L1	1,436500576
Transformer-2 protein homolog beta	TRA2B	1,427009075
Clathrin heavy chain 1	CLTC	1,426579411
Ferritin heavy chain	FTH1	1,426330048
HLA class II histocompatibility antigen, DR alpha chain	HLA-DRA	1,413302172
T-complex protein 1 subunit epsilon	CCT5	1,409433312
Serine/arginine-rich splicing factor 6	SRSF6	1,405892524
60S ribosomal protein L3	RPL3	1,383666004
Endoplasmic reticulum aminopeptidase 1	ERAP1	1,382582029
WD repeat-containing protein 1	WDR1	1,379514005
40S ribosomal protein S11	RPS11	1,376266495
HLA class I histocompatibility antigen, A-23 alpha chain	HLA-A	1,364652827
Far upstream element-binding protein 2	KHSRP	1,359850762
Proteasome activator complex subunit 2	PSME2	1,357137733
40S ribosomal protein S15a	RPS15A	1,356933881
10 kDa heat shock protein, mitochondrial	HSPE1	1,351473835
Translocon-associated protein subunit delta	SSR4	1,346560925
Stress-induced-phosphoprotein 1	STIP1	1,345131738
CD44 antigen	CD44	1,344058434
Proteasome subunit alpha type-7	PSMA7	1,343251951
Calmodulin-like protein 5	CALML5	1,330523264
Importin subunit beta-1	KPNB1	1,327378773
Proteasome subunit alpha type-3	PSMA3	1,309511601
60S acidic ribosomal protein P1	RPLP1	1,308466336
RuvB-like 2	RUVBL2	1,30249087
Cytochrome c oxidase subunit 2	MT-CO2	1,290830896
Multifunctional protein ADE2	PAICS	1,273958259
ATP-dependent RNA helicase A	DHX9	1,258996687
Cytosolic non-specific dipeptidase	CNDP2	1,256985547
Prohibitin	PHB	1,256365689
Voltage-dependent anion-selective channel protein 1	VDAC1	1,252942785
KH domain-containing, RNA-binding, signal transduction-associated protein 1	KHDRBS1	1,251886864
Peroxiredoxin-4	PRDX4	1,238507543

Cytochrome c	CYCS	1,232151947
DNA replication licensing factor MCM2	MCM2	1,222047193
Histone-binding protein RBBP4	RBBP4	1,211564038
HLA class II histocompatibility antigen gamma chain	CD74	1,211376236
Fascin	FSCN1	1,209699256
Small nuclear ribonucleoprotein Sm D2	SNRPD2	1,209559444
Desmoplakin	DSP	1,209329242
Poly [ADP-ribose] polymerase 1	PARP1	1,20421925
Cytochrome c1, heme protein, mitochondrial	CYC1	1,197277523
Major vault protein	MVP	1,196660186
60S ribosomal protein L4	RPL4	1,193136684
Proteasome subunit beta type-1	PSMB1	1,186006959
60S ribosomal protein L24	RPL24	1,179116128
Ras-related protein Rab-7a	RAB7A	1,176963636
Heat shock protein 75 kDa, mitochondrial	TRAP1	1,172826949
Eukaryotic initiation factor 4A-III	EIF4A3	1,172170174
Probable ATP-dependent RNA helicase DDX17	DDX17	1,165190685
Serine/threonine-protein phosphatase 2A 65 kDa regulatory subunit A alpha isoform	PPP2R1A	1,159920995
Tropomyosin alpha-4 chain	TPM4	1,155771736
Alpha-2-macroglobulin	A2M	1,151650686
ELAV-like protein 1	ELAVL1	1,14805309
40S ribosomal protein S26	RPS26	1,145923512
Nucleosome assembly protein 1-like 1	NAP1L1	1,144649161
Vesicle-trafficking protein SEC22b	SEC22B	1,140120767
Proteasome subunit alpha type-4	PSMA4	1,139268584
Small nuclear ribonucleoprotein F	SNRPF	1,133272954
SH3 domain-binding glutamic acid-rich-like protein 3	SH3BGRL3	1,132707168
Cytoplasmic dynein 1 heavy chain 1	DYNC1H1	1,12133856
Small nuclear ribonucleoprotein Sm D3	SNRPD3	1,110438691
Apoptosis-associated speck-like protein containing a CARD	PYCARD	1,075831565
Antigen peptide transporter 1	TAP1	1,075213273
Elongation factor 1-gamma	EEF1G	1,071216205
60 kDa heat shock protein, mitochondrial	HSPD1	1,070194437
Cell division control protein 42 homolog	CDC42	1,058128558
Sodium/potassium-transporting ATPase subunit alpha-1	ATP1A1	1,053702529
Heat shock 70 kDa protein 4	HSPA4	1,041593627
Small nuclear ribonucleoprotein Sm D1	SNRPD1	1,038914075
Enoyl-CoA delta isomerase 1, mitochondrial	ECI1	1,035315608
Tenascin	TNC	1,023885235
V-type proton ATPase subunit B, brain isoform	ATP6V1B2	1,021231008
Proteasome subunit alpha type-6	PSMA6	1,013473749
Carbonyl reductase [NADPH] 1	CBR1	1,011360074
Proteasome subunit beta type-9	PSMB9	1,006210516
High mobility group protein B2	HMGB2	1,004140816
Vinculin	VCL	1,000543591
Proteasome subunit alpha type-2	PSMA2	0,994235315
Annexin A4	ANXA4	0,993374397
Coatomer subunit gamma-1	COPG1	0,98947989
60S ribosomal protein L10	RPL10	0,979226915

Chromobox protein homolog 3	CBX3	0,97855283
Histidine triad nucleotide-binding protein 1	HINT1	0,976166475
Flavin reductase (NADPH)	BLVRB	0,972151798
60S ribosomal protein L5	RPL5	0,970087699
Protein LYRIC	MTDH	0,968188759
Catenin alpha-1	CTNNA1	0,967526708
Splicing factor 3B subunit 3	SF3B3	0,954095243
Proliferating cell nuclear antigen	PCNA	0,95152254
NADH-cytochrome b5 reductase 3	CYB5R3	0,951490924
Galectin-9	LGALS9	0,948679807
Lupus La protein	SSB	0,938021781
Ras-related protein Rab-10	RAB10	0,937638824
Lamin-B2	LMNB2	0,935448442
D-3-phosphoglycerate dehydrogenase	PHGDH	0,932830693
Puromycin-sensitive aminopeptidase	NPEPPS	0,928569911
Basement membrane-specific heparan sulfate proteoglycan core protein	HSPG2	0,921166768
Erythrocyte band 7 integral membrane protein	STOM	0,9206138
Barrier-to-autointegration factor	BANF1	0,916732701
Apolipoprotein E	APOE	0,90850312
Electron transfer flavoprotein subunit beta	ETFB	0,902557456
eIF-2-alpha kinase activator GCN1	GCN1	0,901860725
Peroxisomal multifunctional enzyme type 2	HSD17B4	0,899610633
60S ribosomal protein L31	RPL31	0,898996262
U5 small nuclear ribonucleoprotein 200 kDa helicase	SNRNP200	0,898770026
Eukaryotic translation initiation factor 3 subunit C	EIF3C	0,880569776
Nuclear mitotic apparatus protein 1	NUMA1	0,878338189
Single-stranded DNA-binding protein, mitochondrial	SSBP1	0,873663278
Actin-related protein 2/3 complex subunit 2	ARPC2	0,872486951
Immunoglobulin heavy constant gamma 4	IGHG4	0,864894178
Delta(3,5)-Delta(2,4)-dienoyl-CoA isomerase, mitochondrial	ECH1	0,862644286
Heterogeneous nuclear ribonucleoprotein H3	HNRNPH3	0,861925897
Deoxyuridine 5'-triphosphate nucleotidohydrolase, mitochondrial	DUT	0,854464909
Small nuclear ribonucleoprotein G	SNRPG	0,852161619
High mobility group protein B1	HMGB1	0,848001321
Heat shock protein HSP 90-alpha	HSP90AA1	0,846960299
Hexokinase-1	HK1	0,839690065
Actin-related protein 2/3 complex subunit 1B	ARPC1B	0,835721304
HLA class II histocompatibility antigen, DQ alpha 1 chain	HLA-DQA1	0,833660118
U1 small nuclear ribonucleoprotein 70 kDa	SNRNP70	0,830727619
Heterochromatin protein 1-binding protein 3	HP1BP3	0,825681505
Leukotriene A-4 hydrolase	LTA4H	0,82316905
Putative protein FAM10A5	ST13P5	0,822023157
Protein RCC2	RCC2	0,821216428
Cell cycle and apoptosis regulator protein 2	CCAR2	0,812156015
Filamin-B	FLNB	0,811852103
40S ribosomal protein S23	RPS23	0,810196475
Catenin delta-1	CTNND1	0,806895324
Receptor of activated protein C kinase 1	RACK1	0,805047467
Myosin light polypeptide 6	MYL6	0,800810095

Methyltransferase-like protein 7A	METTL7A	0,789367093
Eukaryotic translation initiation factor 3 subunit B	EIF3B	0,788811086
Splicing factor U2AF 65 kDa subunit	U2AF2	0,778841972
Cystatin-B	CSTB	0,768337715
60S ribosomal protein L35	RPL35	0,76768905
FACT complex subunit SPT16	SUPT16H	0,767434904
UBX domain-containing protein 1	UBXN1	0,754232709
Galectin-3-binding protein	LGALS3BP	0,747821539
Ras-related protein Rab-2A	RAB2A	0,739722604
Aldehyde dehydrogenase, mitochondrial	ALDH2	0,733392712
Aspartyl aminopeptidase	DNPEP	0,722715669
Heterogeneous nuclear ribonucleoprotein K	HNRNPK	0,699253752
Glutaredoxin-3	GLRX3	0,692094723
Proteasome subunit beta type-4	PSMB4	0,691756809
Inorganic pyrophosphatase	PPA1	0,691752778
Lysine--tRNA ligase	KARS	0,689224936
Low molecular weight phosphotyrosine protein phosphatase	ACP1	0,684725266
Retinal dehydrogenase 1	ALDH1A1	0,674802296
Unconventional myosin-VI	MYO6	0,670195273
26S proteasome non-ATPase regulatory subunit 11	PSMD11	0,642940786
Cold-inducible RNA-binding protein	CIRBP	0,63498511
Keratin, type II cytoskeletal 5	KRT5	0,622570042
Transformer-2 protein homolog alpha	TRA2A	0,615616507
Acetyl-CoA acetyltransferase, mitochondrial	ACAT1	0,606841723
Eukaryotic initiation factor 4A-II	EIF4A2	0,602169525
Elongation factor 2	EEF2	0,500456462
Tubulin beta-4B chain	TUBB4B	0,476159137

11. Acronyms

1-DE - One-dimensional polyacrylamide gel electrophoresis

1-D SDS-PAGE - One-dimensional SDS-polyacrylamide gel electrophoresis

2-DE - Two-dimensional gel electrophoresis

2-DIGE - Two-dimensional difference gel electrophoresis

2-DE - Two-dimensional SDS-PAGE

2DRT – Two-dimensional radiotherapy

3DRT – Three-dimensional radiotherapy

BCR - B cell receptor

CCRT – Concurrent chemoradiotherapy

CT – Computed tomography

CTCAE – Common toxicity criteria adverse events

DIGE - Differential gel electrophoresis

EBNA – Epstein-Barr nuclear antigen

EBER – Epstein-Barr encoded RNA

EGFR – Epidermal growth factor receptor

ESI - Electrospray ionization

ENT – Ear, Nose & Throat

EBV – Epstein-Barr Virus

FASP - Filter aided sample preparation

FDA – Food & Drug Administration

FDR - False discovery rate

FSRT – Fractionated stereotactic radiotherapy

FFPE – Formalin-fixed paraffin-embedded

HLA – Human leukocyte antigen

HNC – Head and neck cancer

hnRNP - Heterogeneous nuclear ribonucleoproteins

IARC – International Association for Research in Cancer

ICATs - Isotope-coded affinity tags

IMRT – Intensity-modulated radiotherapy

iTRAQ - Isobaric tags for relative absolute quantitation

LCM - Laser capture microdissection

LC-MS – Liquid chromatography mass spectometry

LFQ - Label-free quantification

LMP – Latent membrane protein

MALDI - Matrix-assisted laser desorption ionization mass spectrometry

miRNA – micro RNA

MRI – Magnetic resonance imaging

MS - Mass spectrometry

NCCN – National comprehensive cancer network

NPC - Nasopharyngeal carcinoma

PDT- Photodynamic therapy

PDL-1 – Programmed death-ligand 1

PET – Positron-emission tomography

RKIP - Raf kinase Inhibitory protein

RP-LC - Reverse-phase liquid chromatography

RTOG – Radiation Therapy Oncology Group

SCC – Squamous cell carcinoma

SELDI-TOF-MS - Surface-enhanced laser desorption/ionization time of flight mass spectrometry

SILAC - Stable isotope labeling of amino acids

TIM - Triosephosphate isomerase

TOF-MS - Time-of-flight mass spectrometer

TSGs – Tumor suppressor genes

UICC – Union Internationale Contre le Cancer

WHO – World Health Organization