

Original research article

# Label-free quantitative mass spectrometry from formalin-fixed paraffin-embedded samples of nasopharyngeal carcinoma: Preliminary results from a non-endemic European cohort of patients

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## ABSTRACT

**Aim:** Report our results of biomarker discovery in formalin-fixed paraffin-embedded (FFPE) nasopharyngeal carcinoma (NPC) via proteomic analysis.

**Background:** Nasopharyngeal carcinoma (NPC) is a rare cancer in Western countries. Proteomic analysis have already been reported as a useful tool to provide biomarkers. Formalin-fixed paraffin-embedded (FFPE) samples, despite largely underused, can provide invaluable information for biomarker research via proteomic analysis.

**Methods:** FFPE samples of NPC were submitted to protein extraction followed by FASP-digestion and label-free quantitative mass spectrometry (MS). Patients' received concurrent chemoradiation with or without adjuvant chemotherapy as per Intergroup 0099 trial. IMRT was delivered following the RTOG0615 specifications. Toxicity was scored using the CTCAE 4.03 tables. Survival was estimated using Kaplan–Meier curves. Log-rank was used to detect differences. KEGG ontology graphics were generated.

**Results:** 28 FFPE samples from NPC patients were used. Patients were: 79% male, 97% Caucasians, 86% WHO type 3, 40% T1, 10% T2, 25% T3, and 25% T4. With a median follow up of 37 months, local control was 83 (T1, 100% T2, T3 and T4), overall survival was 84%, and six patients developed distant metastases. All five patients that died were due to metastatic disease. Tumor samples contained a median of 75% of tumor material. We found Epstein–Barr (EBV) and Herpes simplex (HSV) viruses' related proteins significantly present in early-stage primary NPC (T1 and T2,  $p < 0.01$ ). A pool of 10 proteins was statistically up-regulated in the metastatic group of patients ( $p < 0.01$ ). Median survival from this M1 group was <1 year ( $p < 0.001$ ).

**Conclusions:** FFPE samples yielded adequate material for MS analysis. We found EBV and HSV related proteins on early-stage NPC, and proteomic profiling associated with distant metastases, potential candidates of disease biomarkers. Validation is needed.

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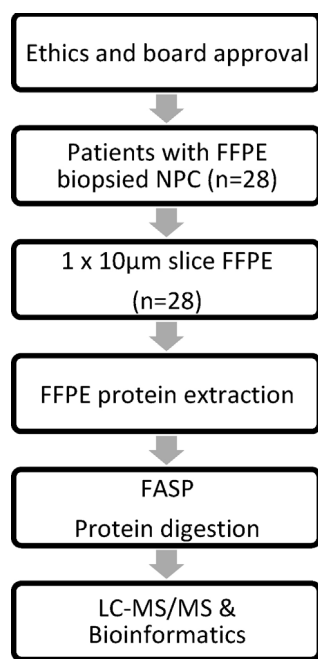
## 1. Background

Nasopharyngeal carcinoma (NPC) is a rare cancer in Western countries. Almost 80% of cases occur in Asia, and the remaining fraction is spread across all continents in non-endemic intermediate and low incidence areas. Its etiology is a complex balance

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between Epstein–Barr virus (EBV) infection, host susceptibility, and environmental factors. Indeed, the past two decades showed a remarkable change in outcomes given to the implementation of widely used concurrent chemoradiation with intensity-modulated radiotherapy (IMRT). Our series, as others, results demonstrate local control of 90% at five years.<sup>1–3</sup> Despite its sensibility to radiation and chemotherapy, nearly half of the patients will die to distant metastases. Given its enigmatic geographic distribution, NPC is not present on the Cancer Genome Atlas, and investigators have actively researched biomarkers to predict behavior and metastases.



**Fig. 1.** Study workflow (FFPE: formalin-fixed paraffin embedded; NPC: nasopharyngeal carcinoma; FASP: filter aided sample preparation; LC-MS/MS: liquid chromatography mass spectrometry).

Proteomics has been used in Asia for biomarker discovery for this purpose. We report our exploratory use of proteomic analysis in a non-endemic European cohort of patients.<sup>4–10</sup>

## 2. Aim

Our aim is to report our results of biomarker discovery in FFPE from NPC samples from a non-endemic cohort of patients using label-free quantitative mass spectrometry proteomic analysis.

## 3. Methods

### 3.1. Study description

After approval from the institutional board (UIC901), we conducted a single-center retrospective study. We retrieved clinical, image and laboratorial data from 28 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 study.

Patients were treated with cisplatin-based chemoradiation as per Intergroup 0099 trial.<sup>11</sup> IMRT volumes were contoured as per RTOG 0615.<sup>12</sup> A study diagram can be seen in Fig. 1.

### 3.2. 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 63 °C. Then the tissue material was rehydrated with starting with 100% ethanol for 10 min with gentle shaking followed by incubation with 96%, 80%, 70%, and 50%. 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.<sup>13</sup> 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 °C for 20 min followed by ultrasonication using an ultrasonic probe (1 min, 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. (Fig. 2). Finally, samples were interrogated by mass spectrometry using label-free protein quantification LC-MS/MS methodology.<sup>14</sup>

LC-MS/MS data were analyzed 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.<sup>15</sup> 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).<sup>16</sup> In brief, protein group LFQ intensities were log<sub>2</sub>-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 log<sub>2</sub> 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  $\geq 1.5$  and  $FDR \leq 0.01$ .<sup>17</sup>

### 3.3. 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.<sup>18</sup>

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.

### 3.4. Treatment Information and follow up

Intensity-modulated radiotherapy (IMRT) was delivered as per RTOG 0615, with a simultaneous-integrated boost of 69.96 Gy in 33 daily fractions to the primary and nodal GTVs, 59.4 Gy to the areas at risk and involved neck levels and 54 Gy to the uninvolved lower neck.<sup>12</sup> Between 2009 and 2012, all treatments were delivered via 5–7 fields arrangement of sliding window IMRT. After 2012, VMAT was also an option depending on dose constraints compliance. Organs at risk were spared by RTOG 0615 recommendations until 2010. After that, QUANTEC tables were added whenever applicable. A 5-mm PTV margin was used until 2011, and 4 mm thereafter since we performed a quantitative study of margins already documented.<sup>22</sup> Concurrent and adjuvant platinum-based

## FASP Protein digestion of FFPE extracted proteins

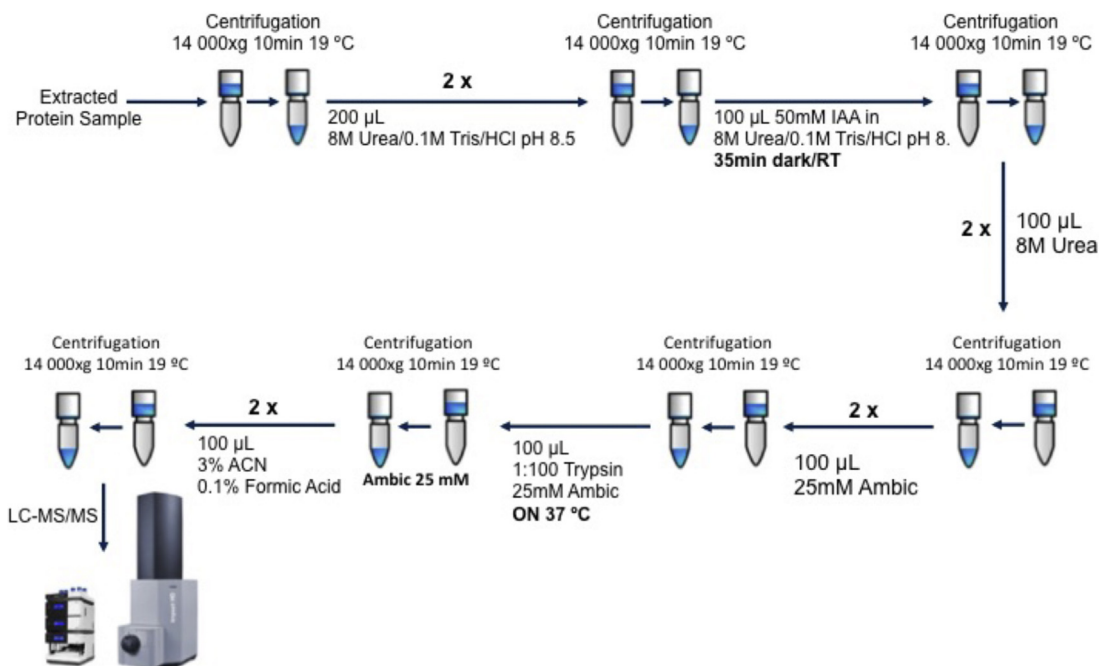


Fig. 2. Schematic representation workflow of FASP protein digestion of FFPE extracted proteins.

chemotherapy was delivered according to the Intergroup 0099 trial fashion. Induction chemotherapy was performed for M1 patients and definitive radiation or chemoradiation was added 3–6 cycles depending on response. Post treatment neck dissection was performed in the presence of worrisome clinical or imaging features. Patients were followed every 3 months with consults and routine blood tests including thyroid function, with ENT, Medical Oncology and Radiation Oncology for the first 2 years with endoscopy and MRI or CT image. Similar follow up was continued every 6 months until 5 years, and yearly thereafter. Toxicity was scored using CTCAE v. 4.03 whenever possible. Audiogram was not available for our patients, so ototoxicity was scored following CTCAE recommendations for non-enrolling patients in audio monitoring.

## 4. Results

Patients and tumor characteristics can be found in Table 1. With a median follow up of 37 months, the overall local control is 83% for the whole cohort (90% for T1, 100% for T2, 57% for T3 and 86% for T4), overall survival is 84%, and six patients developed distant metastases. All five patients that died were due to metastatic disease.

Local control was not affected by T-stage (Fig. 3A), even dichotomizing T1 + T2 vs. T3 + T4 (Fig. 3B) or comparing all T-stages vs. T4 (Fig. 3C). In our cohort, neither local control nor overall survival was affected by N-stage (Fig. 3D and E). The presence of distant metastasis was the most important prognostic factor.

Tumor samples contained a median of 75% of tumor material (70–90%).

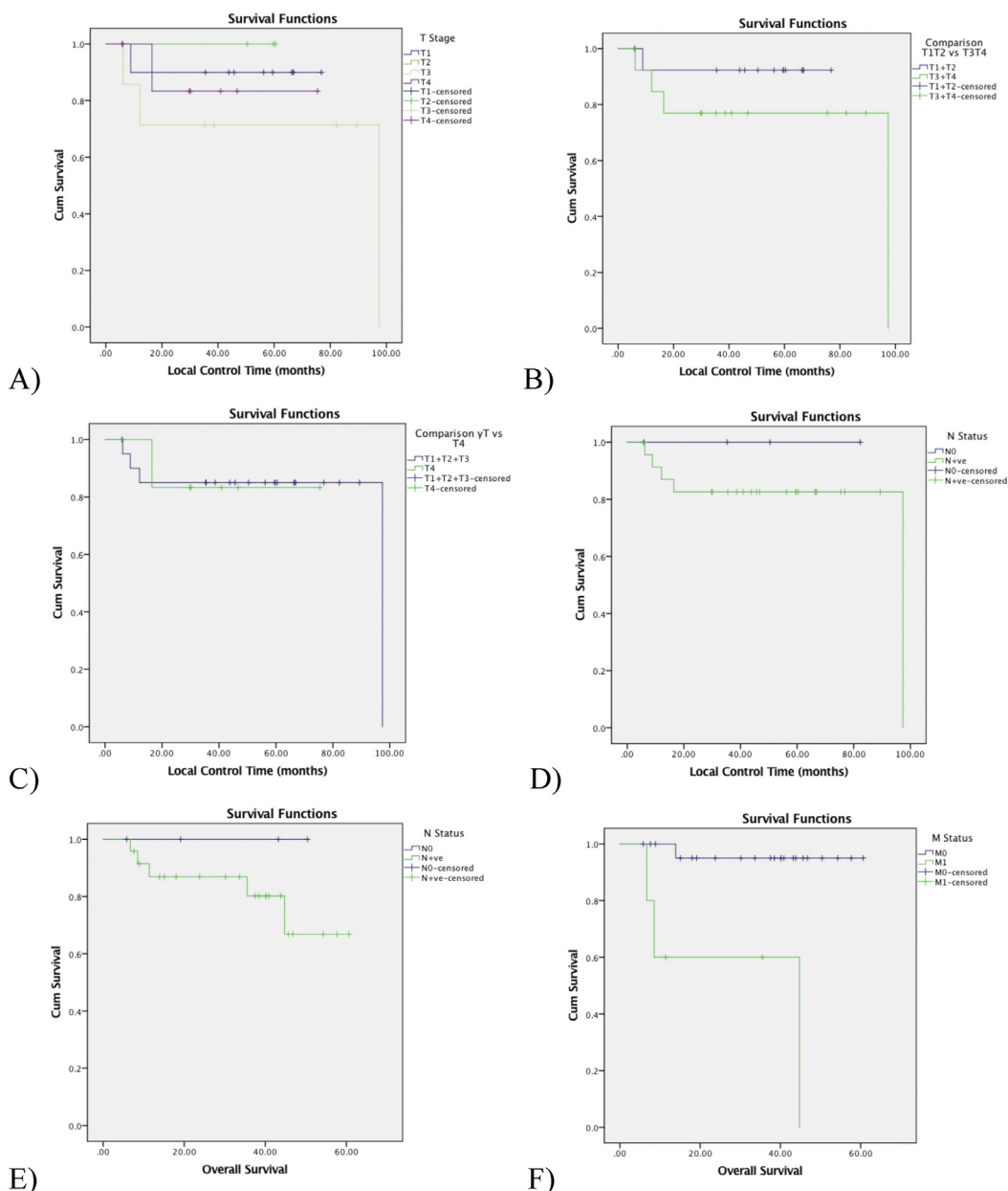
Patient's outcomes are available in Fig. 3A–F.

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 Figs. 4 and 5 and Annex 1. Gene ontology and

Table 1

Patients and tumor characteristics at diagnosis.

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 habits	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%)
	<49 days	26 (93%)
Overall treatment time	>49 days	02 (07%)
	Undetected (<600)	05 (18%)
EBV serum pre treatment	Detected (≥600)	07 (25%)
	Missing	16 (57%)



**Fig. 3.** 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,  $p = 0.001$ ).

KEGG pathway were generated for T1 + T2 tumors (Fig. 4), T3 + T4 tumors (Figs. 6 and 7). On both groups, different on protein expression were statistically significant  $p < 0.01$ .

A pool of 10 proteins was 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 one year ( $p < 0.001$ ; Fig. 8).

## 5. Discussion

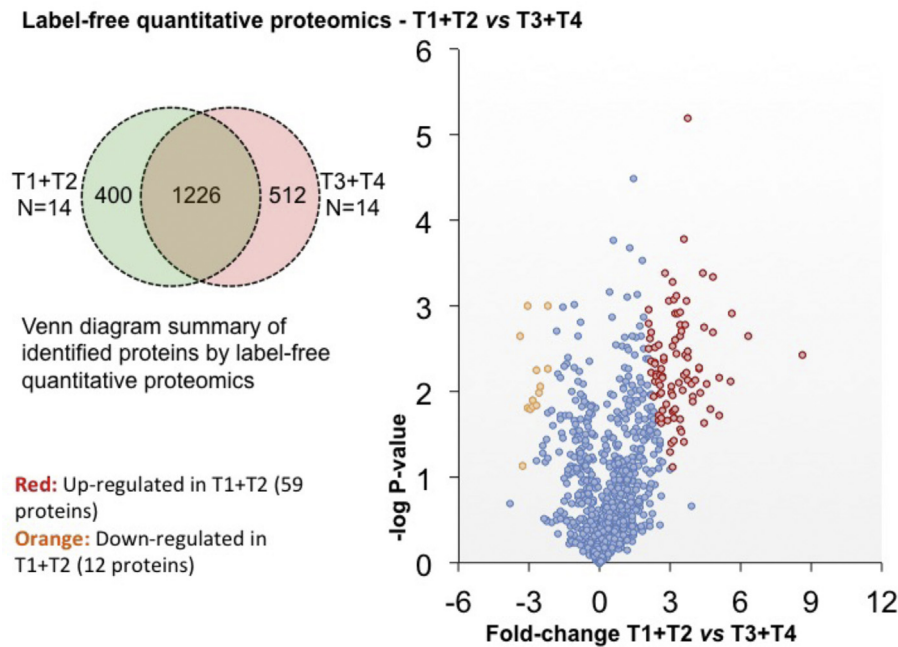
FFPE tissues hold invaluable information regarding patients' outcomes. Nevertheless, its features pose challenging tasks to scientists all over the world. Few series have studied NPC via FFPE samples.<sup>9,19</sup>

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 vs. T3 + T4 ( $p = 0.297$ ) or comparing all T-stages vs. T4 ( $p = 0.983$ ) (Fig. 3A–C).

This can be explained by the excellent local control and survival yielded by chemoradiation for all T or N stages ( $p = 0.454$ ). Nevertheless, future mature data could increase the power to detect differences. These rates are in agreement with others in Eastern and Western countries.<sup>1,20–24</sup> It is unclear rather this signature means that an early T-stage could receive a different protocol prescription (i.e., de-intensified strategies or doses). At this subgroup, bioinformatics KEGG pathway showed that the most significant difference in proteins were in the up-regulation of antigen processing and presentation and also protein targeting ER (Fig. 5).

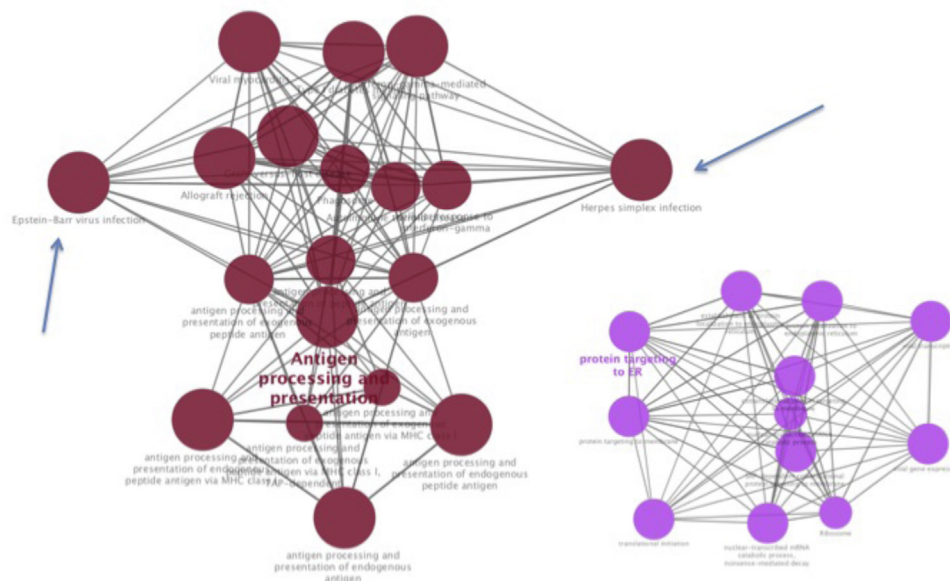
As expected in this entity, viral proteins were markedly present on early stage tumors group of patients (T1 + T2), especially EBV,





**Fig. 4.** Volcano plot of label-free quantitative proteomic results comparing early-stage NPC tumors (T1 and T2 grouped versus T3 and T4).

### Gene ontology and KEGG pathway enrichment T1+T2 $p < 0.01$



**Fig. 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.

ubiquitous in NPC regardless of geographic distribution. The proteins detected and its interplay effect are highly correlated to EBV infection (Table 2). It has already been described that 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<sup>25</sup>. The absence of EBV related proteins in the advanced-stage tumors (T3+T4) proteins group must be interpreted with caution since it can be explained by the method's limitation in detecting proteins with low proportions' concentration. Whether this

absence can represent a different disease stage, it remains to be better understood.

However, not only EBV proteins were present, but also Herpes simplex virus. Whether these signs of co-infection can be interpreted as a cofounding factor or a relevant issue for early-stage in our cohort remains unclear. Nevertheless, in all KEGG pathways run, we have not found any HPV related proteins, a known marker for poor prognosis among Caucasians.<sup>26</sup>

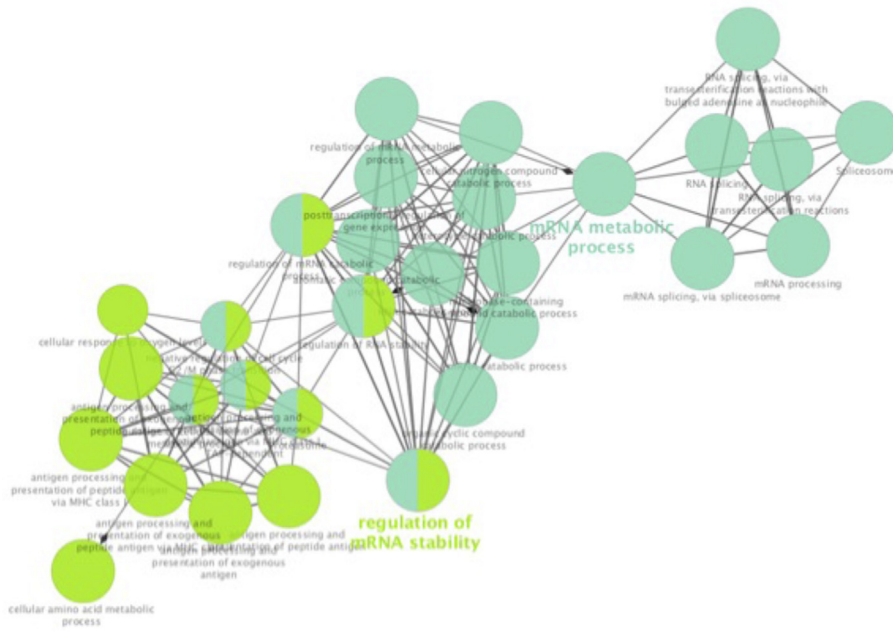
On the other hand, a significant pool of 10 proteins were up-regulated in M1 patients ( $p < 0.01$ ). This is important information

**Table 2**  
Functioning pathway of detected proteins via bioinformatics.

Variables	Function pathway
T1 + T2 (vs T3 +T4, $p < 0.01$ )	Antigen processing and presentation Protein targeting to ER
T3 + T4 (vs T1 +T2, $p < 0.01$ )	Regulation of mRNA stability mRNA metabolic process Exocytosis DNA replication Nucleoside triphosphate metabolic process
M1 (vs M0, $p < 0.01$ )	Type I interferon signaling pathway Actin cytoskeleton reorganization Protein tetramerization Termination of RNA polymerase II transcription NADH metabolic process

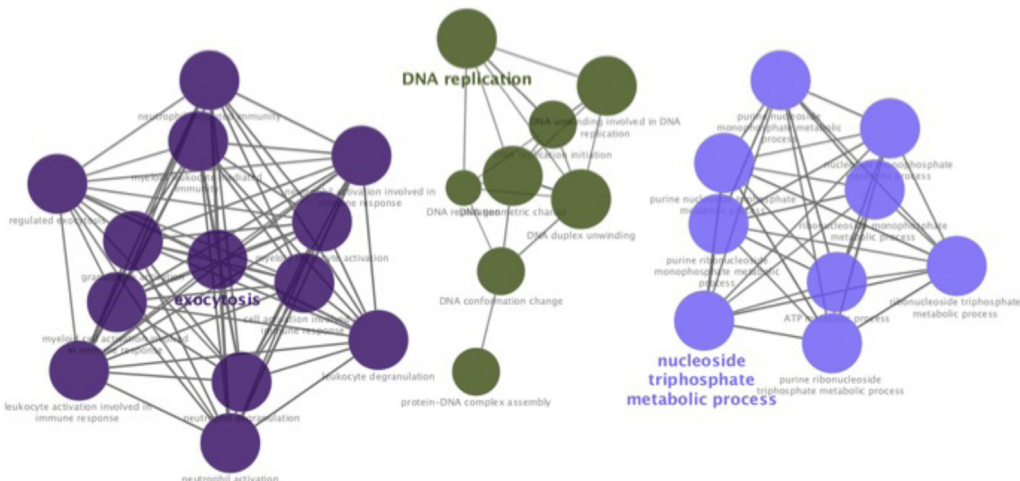
since M1 disease is the most important factor affecting survival for any stage ( $p < 0.001$ ). See Annex 2. At this M1 subgroup, KEGG ontology pathway via bioinformatics showed functioning activity by type I interferon signaling pathway, actin cytoskeleton reorganization, protein tetramerization, termination of RNA polymerase II transcription and NADH metabolic process (Fig. 8). These preliminary results were able to identify signatures containing known proteins related to head and neck cancer onset and behavior, not only NPC, and potential therapeutical targets (like interferon and EGFR). If these results remain consistent, it generates hypothesis whether tyrosine kinase or interferon should be investigated in clinical trials. For example, adjuvant interferon has yielded superb

**Gene ontology and KEGG pathway enrichment T3+T4  $p < 0.01$**



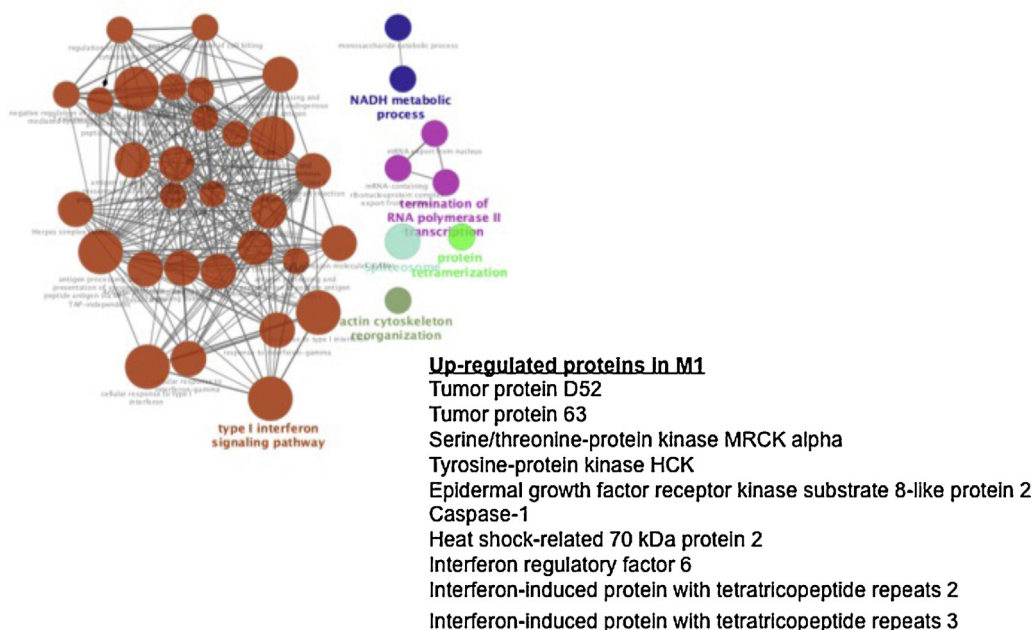
**Fig. 6.** Gene ontology and KEGG pathway enrichment significant present on advanced-stage NPC tumors (T3 and T4 patients versus T1 and T2 tumors,  $p < 0.01$ ).

**Gene ontology and KEGG pathway enrichment T3+T4  $p < 0.01$**



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

## Label-free quantitative proteomics – M0 vs M1 New Biomarker candidates



**Fig. 8.** Gene ontology and KEGG pathway enrichment significant present on M1-NPC patients (M1 vs M0 patients,  $p < 0.001$ ). Graphic shows a pool of 10 proteins up-regulated on M1 patients.

**Table 3**  
Studies using FFPE with methods for biomarker discovery.

Reference	Year	Author	Country	Biomarker	Material	N	Method	Effect
6	2010	Xiao, Z.	China	Cathepsin D Keratin8 SFN Stathmin1	FFPE	40	2D liquid chromatography-tandem MS coupled with iTRAQ	Correlation of differential expression with the NPC types
11	2016	Chen, Z.	China	SQSTM1 RAN TRIM29	Cell line (FFPE for validation)	108	iTRAQ-tagging combined with 2D LC-MS/MS	TRIM29 as a metastasis-promoted protein
19	2007	Chan, C.	China	Annexin II Beta-2-tubulin	Cell-line, FFPE	40	2DGE	Annexin II down-regulation positively associated with node metastasis
20	2016	Yuan, H	China	14-3-3 $\sigma$ Maspin RKIP GRP78	FFPE	149	IHC	RKIP for radiosensitive, and GRP78 for radioresistance

results in children and young adults with NPC (German GPOH Study NPC91).<sup>27</sup>

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.<sup>28,29</sup> Xiao et al.<sup>9</sup> have reported four 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.<sup>30</sup> Our report also presents findings compatible with outcome-related combination of proteins.

Our study has limitations. We acknowledge that there is a high number of missing data from EBV plasma load pre-treatment. And, we kept included two patients already M1 at diagnosis.

To our knowledge, this is the first report of proteomic analysis of NPC from a European cohort of patients. Validation essay is warranted.

## 6. Conclusion

In conclusion, FFPE is largely underused as a source of clinical information. Our protocol was able to safely extract and present biological material from FFPE samples yielding adequate material to MS analysis. These preliminary findings 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. Though validation is needed, these data support further ongoing studies by our group.

## Authors' contribution

Conception and design: Eduardo Netto, Hugo Santos, José Capelo-Martínez.

Method Luís Carvalho, Hugo Santos, José Capelo-Martínez.

Provision of study materials or patients: All.

Pathology Review: Miguel Rito, José Cabeçadas.

Collection and assembly of data: All.

Data analysis and interpretation: Eduardo Netto, Hugo Santos, Luís Carvalho, Margarida Roldão.

Manuscript writing: All.

Final approval of manuscript: All.

## Conflict of interest

None declared.

## Financial disclosure

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## Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <https://doi.org/10.1016/j.rpor.2020.05.007>.

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