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# PREDICTIVE AND PROGNOSTIC ROLE OF BIOLOGICAL MARKERS IN NEUROENDOCRINE NEOPLASIA AND EVALUATION OF ACTIVITY AND SAFETY OF SECOND LINE TREATMENTS IN NEUROENDOCRINE CARCINOMA PATIENTS

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**Abstract**

BACKGROUND

Neuroendocrine neoplasia (NEN) are divided in well differentiated G1,G2 and G3 neuroendocrine tumors (NETs) and G3 neuroendocrine carcinomas (NECs). For the latter no standard therapy in second-line is available and prognosis is poor.

METHODS

Primary aim was to evaluate new prognostic and predictive biomarkers (WP1-3). In WP4 we explored the activity of FOLFIRI and CAPTEM as second-line in NEC patients in a multicenter non-comparative phase II trial

RESULTS

In WP1-2 we found that 4 of 6 GEP-NEC patients with a negative 68Ga-PET/CT had a loss of expression of RB1. In WP3 on 47 GEP-NENs patients the presence of DLL3 in 76.9% of G3 NEC correlate with RB1-loss ($p<0.001$), negative 68Ga-PET/CT($p=0.001$) and a poor prognosis.

In the WP4 we conducted a multicenter non-comparative phase II trial to explore the activity of FOLFIRI or CAPTEM in terms of DCR, PFS and OS given as second-line in NEC patients. From 06/03/2017 to 18/01/2021 53 out of 112 patients were enrolled in 17 of 23 participating centers. Median follow-up was 10.8 (range 1.4 – 38.6) months. The 3-month DCR was 39.3% in the FOLFIRI and 32.0 % in the CAPTEM arm. The 6-months PFS rate was 34.6% ( 95%CI 17.5-52.5) in FOLFIRI and 9.6% (95%CI 1.8-25.7) in CAPTEM group. In the FOLFIRI subgroup the 6-months and 12-months OS rate were 55.4% (95%CI 32.6-73.3) and 30.3% (CI 11.1-52.2) respectively. In

CAPTEM arm the 6-months and 12-months OS rate were 57.2% (95%34.9-74.3) and 29.0% (95%10.0-43.3). The miRNA analysis of 20 patients compared with 20 healthy subjects shows an overexpression of miRNAs involved in staminality , neo-angiogenesis and mitochontrial anaerobic glycolysis activation.

CONCLUSION

WP1-3 support the hypothesis that G3NECs carrying RB1 loss is associated with a DLL3 expression highlighting a potential therapeutic opportunity. Our study unfortunately didn't met the primary end–point but the results are promising

## 1.1 INTRODUCTION

Poorly differentiated neuroendocrine carcinomas (NECs) are very rare malignancies, representing only 5%-10% of neuroendocrine neoplasias (NENs) (1-3). These tumors are characterized by aggressive histological features (high Ki-67 index, extensive necrosis, and nuclear atypia) and are classified as grade (G)3 NECs according to the 2010 World Health Organization (WHO) classification (4). The 2017 WHO classification recognized a further group called G3 NETs as having intermediate

features between NETs and NECs (5).

An etoposide-platinum combination is the gold standard of treatment for G3 NECs and several studies have been published in the 1990s reporting substantial antitumor activity and high response rates (41%-67%). However, prognosis is poor with a median progression-free survival of 9 months and a median overall survival of 15-19 months. When progression occurs after first-line chemotherapy, the disease is usually very aggressive and patients succumb rapidly (6).

Given the rarity of the disease , prospective clinical data are lacking and treatment recommendation are essentially expert-based opinions. A French study focusing on the identification of predictive molecular markers of response to sunitinib in GEP-NECs (NCT01215578) has now closed recruitment and results are eagerly awaited. Another French multicentre prospective phase II trial is currently ongoing to investigate the efficacy of the bevacizumab-FOLFIRI combination after progression on platinum-etoposide (7).

Different second-line chemotherapy combinations have been evaluated but shown poor results (6, 8, 9). In a monocenter retrospective clinical trial, Hentic et al. hypothesized the potential efficacy of FOLFIRI as second-line chemotherapy in patients with G3 extra-pulmonary NECs (10). An objective response rate was obtained in 31% of patients, with a disease control rate (DCR) of 62%. Median progression-free survival (PFS) and overall survival (OS) were 4 and 18 months, respectively.

In another retrospective study, a 71% DCR was obtained with temozolomide-based chemotherapy. A PFS of 12 months (95% CI, 5.5- 24) and OS of 22 months (95% CI, 12-31) was reported in patients who responded to treatment or showed stable disease (SD), whereas OS was only 8 months (95% CI, 0-8) in non-responders. The authors

observed a higher response rate in patients with Ki-67 ≤ 60%. There were also more responders in the group with high uptake in somatostatin receptor scintigraphy (SRS) and in those with positive staining for chromogranin A (CgA). Both factors are often associated with more differentiated tumors (11).

Literature data on lung NECs in progression after first-line chemotherapy are based on small patient series (12). Moreover, there is increasing evidence of some discrepancies in the current grading of NECs, highlighting the need for more accurate biomarkers (4, 5). Recent research has shown that NECs may, in fact, comprise 2 distinct subgroups with different pathogenesis, *i.e.* a highly proliferative group derived from well differentiated neuroendocrine tumors (NETs) and characterized by mutations in MEN1, DAXX and ATRX, and a poorly differentiated group derived from neuroendocrine-differentiated adenocarcinomas and characterized by a mutation in RB1. Both subgroups display a distinct prognosis and different sensitivity to chemotherapy (13-15). Micro(mi)RNAs are a class of small, non-coding, highly conserved single-stranded RNAs involved in the post-transcriptional regulation of cell proliferation, differentiation, survival, and apoptosis (16). They are often associated with resistance to therapy (17, 18). Whilst miRNAs are known to show a specific expression pattern in NETs (19), little is known about differential miRNA profiles in NEC patients. At present, no data are available on the deregulation of specific miRNAs in this setting.

In a study recently published on GEP-NEC patients undergoing first-line platinum-based chemotherapy, median PFS was 19.3 months and 6.3 months ($p$ <0.01) in patients with Ki-67% <50% or >50%, respectively (19). Median (m)OS was 8.1 months in the latter group but was not reached in the former group ($p$ = 0.039). Patients with a

positive $^{68}$Ga-PET/CT had a longer OS than those with a negative scan (75% *vs*. 34.3%, respectively, at 18 months), but the difference was not significant ($p$ = 0.06). Our data highlighted that $^{68}$Ga-PET/CT positivity may be a discriminating factor (20,15) in predicting prognosis, especially important in the metastatic setting where histological material is not always available for evaluation. Also $^{18}$fludeoxyglucose ($^{18}$FDG)-PET/CT could be useful to discriminate patients with different prognosis. (21)

Given the above premises, I decided to investigate the efficacy and safety of second-line FOLFIRI or CAPTEM in patients with GEP and lung NECs in progression after first-line platinum-based treatment. I also aimed to study the serum miRNA profile in relation to the primary mutational status of MEN1, DAXX, ATRX and RB-1, patient prognosis and response to therapy, and to assess the prognostic and predictive role of $^{18}$FDG-PET/CT, $^{68}$Ga-PET/CT and Ki-67 score.

## 1.2 Hypothesis and Aims of the Project

AIM 1: I hypothesize that NECs with mutation in RB-1, might have a significantly worse prognosis and lower responsiveness to chemotherapy than NECs that carry mutation in MEN1, DAXX and ATRX. This task might lead to the identification of prognostic and predictive biomarkers for NEC patient and to the improvement of the actual NEC grading.

AIM 2: The hypothesis is that, as for grade 1 and 2 neuroendocrine tumors, NECs are characterized by a specific miRNA profile and that miRNAs might constitute usefull disease biomarkers. In addition to FDG and Ga68 PET/CT, the site of primary tumor and the Ki67 score these biomarkers might be useful tools to help the physicians in the

treatment choice and prognosis definition.

Other hypothesis is that gene and protein expression analysis of samples would validate potential prognostic biomarkers and treatment targets, e.g. DLL3, and correlate them with immunoprofie in NENs patients

AIM 3 Evaluation of CAPTEM and FOLFIRI regimens as second line therapies for metastatic NECs. Our hypothesis is that FOLFIRI and CAPTEM can be effective treatments for patients progressed after a first line platinum-based chemotherapy

112 mNEC patients will be randomized to 2 arms: FOLFIRI VS CAPTEM. Primary endpoint: DCR at 6 months

## 2.1 METHODS

In order to reach every specific aim, the project was divided into different work packages (WP) characterized by different experimental plans.

### *2.1.1 WP 1-2: Identification of prognostic and predictive biomarkers in NEC patients.*

Experimental Plan: A preliminary study to assess if specific mutations in the primary tumor correlate with Ga-68 PET scan results was conducted. I'll evaluate the

immunohistochemical expression of DAXX, ATRX and RB-1 in patients with NEC. These data will support the hypothesis that NECs with loss of Rb-1 have negative Ga-68 PET that is associated with higher neuroendocrine differentiation and better prognosis.

*2.1.2 WP-2 Material and Methods*

Paraffin-embedded surgical or biopsy specimens of G3 neuroendocrine tumors were sliced with a rotating microtome (Leica Biosystems, Wetzlar, Germany) into 5 µM thick sections and mounted on SuperFrost Plus microslides (Thermo Fisher Scientific, Waltman, MA, USA). Immunolabeling reactions were carried out on a VENTANA BenchMark XT (Ventana Medical Systems Inc., Tucson, AZ, USA) automated slide strainer. The following antibodies were used according to the manufacturer's instructions: DAXX (HPA008736) (Sigma-Aldrich, St. Louis, MO, USA) 1 : 75, one hour at room temperature (RT); ATRX (HPA001906) (Sigma-Aldrich) 1 : 400, one hour at RT; and RB1 (Cell Signaling Technology, Beverly, Massachusetts, USA) 1 : 1000, one hour at RT. The stained sections were analyzed in blind by an expert pathologist in neuroendocrine neoplasms.

68Ga-labeled somatostatin analogs are generally short peptides linked to the positron emitter 68Ga by a bifunctional chelate, normally 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA). 68Ga-DOTA peptides bind to SSTRs, in particular SSTR3 and SSTR5, both of which are usually overexpressed in neuroendocrine cells. There are 3 main 68Ga-DOTA-peptides currently available for imaging procedures on the basis of their affinity for SSTR subtypes. We used 68Ga-DOTA-Phe1-Tyr3-

octreotide (TOC), which has a high affinity for SSTR2 and SSTR5 .

Continuous variables were expressed as mean and standard deviation (SD), while categorical variables were expressed as frequency. Fisher's exact test was used to evaluate the relationship between categorical variables. Median overall survival (OS) was estimated as an exploratory research objective using the Kaplan-Meier method (two-sided 95% confidence intervals (CIs)). Reported P values <0.05 were used as a threshold for significance. Statistical analyses were carried out with STATA/MP 10.1 for Windows (StataCorp LP, College Station, TX, USA).

*2.2.1 WP 3: investigate the role of NOTCH and DLL3 in NEN patients*

Experimental Plan: I analyze DDL3 and NOTCH expression in TRIzol samples of resected neuroendocrine tumors received for primary cultures. We evaluate DDL3 and NOTCH expression in TRIzol samples of resected neuroendocrine tumors received for primary cultures and we'll correlate DDL3, PDL1, TIL and NOTCH expression with clinical data

*2.2.2 WP3 Material and Methods*

We conducted a retrospective study on a case series of 47 patients enrolled at IRST IRCCS, in Meldola, Italy between 2010 and 2019. All patients were required to have GEP-NENs histologically confirmed by an expert pathologist. For each patient, at least one specimen from the primary tumor and/or one from a metastasis had to be available. Physical examination, brain-chest-abdominal CT or $^{68}$Ga- and $^{18}$F-fluorodeoxyglucose (FDG)-PET/CT were included as staging procedures.

Paraffin-embedded or bioptic NEN specimens were sliced into 5-µM-thick sections with a rotating microtome (Leica Biosystems, Wetzlar, Germany) and were mounted on positive-charged microslides (Thermo Fisher Scientific, Waltman, MA, USA). Immunostaining was performed using the VENTANA BenchMark Ultra (Ventana Medical Systems Inc, Tucson, AZ, USA) and the following antibodies were used: DLL3 (SP347) Ventana Assay (Ventana Medical Systems Inc.), prediluted by the supplier; RB1 (Cell Signaling Technology, Beverly, Massachusetts, USA) diluted 1:1000; and PD-L1 (SP142) Ventana Assay, (Ventana Medical Systems Inc.), prediluted by the supplier. All reactions were carried out for one hour at room temperature and sections were counterstained with hematoxylin II (Ventana Medical Systems Inc). Then, stained sections were evaluated by an expert pathologist in a blind fashion. For each section, immunohistochemical (IHC) staining was analyzed if there was a percentage of tumor cells sufficient for a suitable evaluation. Expression values of both DLL3 and RB1 were considered as dichotomous variables (positive/negative). PD-L1 analysis was performed in a subgroup of 42 patients by evaluating the percentage of tumor cells with a positive membranous staining. IHC was scored as positive if more than 1% of the tumor cells showed cytoplasmic or membranous localization of DLL3 and nuclear localization of RB1 (Fig. 1a, b). Stromal cells were used as a positive control for RB1 immunostaining. Median overall survival (OS) was estimated using the Kaplan-Meier method (two-sided 95% confidence intervals [CIs]). Continuous variables were presented as median and minimum-maximum values, while categorical variables were reported as frequency. In order to evaluate the relationship between categorical variables, we used a Fisher's exact test and *P*-values < 0.05 were considered statistically significant. Statistical analyses were performed with STATA/MP 10.1 for Windows (StataCorp LP, College

Station, TX, USA).

2.3.1 WP 4: investigate prospectively the activity and safety of Second Line treatments in NEC patients

2.3.2 Experimental Plan

The SENECA study is a multicentre randomised non-comparative phase II study (Figure 1). Patients with metastatic neuroendocrine carcinomas of different origin (lung or gastroenteropancreatic) in progression after first-line treatment are randomized to receive FOLFIRI every 14 days for a maximum of 12 cycles or until progression or unacceptable toxicity, or CAPTEM every 28 days for a maximum of 6 cycles or until progression or unacceptable toxicity.

The treatments arms are as follows:

## FOLFIRI regimen

- Irinotecan 180 mg/m$^2$, given as a 60-min. intravenous (i.v.) infusion on day 1 every 2 weeks followed by
- Leucovorin 200 mg/m$^2$, given as a 2-h i.v. infusion on day 1 every 2 weeks followed by
- 5-fluorouracil (5-FU) 400 mg/m$^2$ given as bolus, and then 5-FU 2400 mg/m$^2$ given as a 48-h continuous infusion on day 1, every 2 weeks, until progression or for a maximum of 12 cycles.

## CAPTEM regimen

Capecitabine 750 mg/$m^2$ twice a day on days 1-14 in combination with temozolomide 200 mg/$m^2$ daily on days 10-14, every 4 weeks, until progression or for a maximum of 6 cycles.

The study includes patients aged ≥ 18 years with a histological diagnosis of G3 neuroendocrine carcinoma (GEP-NEC and lung NEC), Ki-67 >20% and measurable disease according to Response evaluation criteria in solid tumors (RECIST) 1.1 criteria. All patients must have an Eastern Cooperative Oncology Group (ECOG) performance status ≤2 with a life expectancy > 3 months and must have already undergone first-line treatment for metastatic disease with platinum -based chemotherapy (cisplatin/carboplatin and etoposide, FOLFOX4 or CAPOX). Adequate haematological, liver and renal function is required and effective contraceptive methods must be used by female patients of childbearing age. Written informed consent is obtained from all patients to take part in the study. Exclusion criteria are as follows: metastatic NECs previously treated with an irinotecan regimen, known hypersensitivity to 5-FU, calcium levofolinate, irinotecan or their recipients. All acute toxic effects of any prior therapy (including surgery, radiation therapy and chemotherapy) must have resolved to grade ≤1 according to National Cancer Institute Common Terminology Criteria for Adverse Events Version 4.03 (CTCAE). Patients taking part in another clinical trial with any investigational agent < 30 days prior to study screening or with a history of allergic reactions attributable to compounds of similar chemical or biological composition are excluded Patients who have undergone chemotherapy or radiotherapy < 4 weeks (6 weeks for nitrosoureas or mitomycin C) prior to entering the study, have not recovered from adverse events caused by agents administered > 4 weeks earlier, or have known brain metastases are also not eligible for the study. Patients with other malignancies

with a disease-free interval of < 5 years (with the exception of non melanoma skin cancer or low-grade superficial bladder cancer) are excluded, as are those with any severe and/or uncontrolled medical condition or other condition that could affect their participation in the study such as:

- unstable angina pectoris, symptomatic congestive heart failure, myocardial infarction < 6 months before the start of the study, serious uncontrolled cardiac arrhythmia or any other clinically significant cardiac disease;
- severely impaired lung function (spirometry and DLCO 50% of the normal predicted value and/or oxygen saturation ≤ 88% at rest, in room air);
- uncontrolled diabetes as defined by fasting serum glucose >1.5 x upper limit of normal (ULN);
- any active (acute or chronic) or uncontrolled infections/disorders.

Tumor evaluation by anatomic imaging (multiphase CT and/or MRI) includes chest, abdomen, pelvis, and any additional known sites of disease. These tests are performed at baseline, every three months during treatment and after therapy discontinuation in non-progressing patients until progression. When possible, $^{68}$Ga-PET/CT and $^{18}$FDG-PET/CT is performed at baseline.

*2.3.3 WP4 Patients and Methods*

The primary endpoint of the study is the DCR of each treatment, defined as the percentage of patients who have achieved complete or partial response or stable disease for at least 12 weeks from the start of therapy. DCR will be evaluated using the new international criteria proposed by the RECIST version 1.1. Acute and late toxicity will be evaluated by CTCAE Version 4.03, the latter defined as toxicity occurring at least 30

days after the end of the last treatment cycle. Secondary endpoints are the evaluation of OS, calculated from the start of treatment to death from any cause and PFS, calculated from the start of treatment to the date of the first documented evidence of disease progression or of death from any cause.. The hypothesis for the control arm is based on literature data (22, 23).

An α level of 0.10 (both for toxicity and DCR) and a power of 90% were adopted. A DCR rate ≥60% and a relevant toxicity rate ≤20% are considered acceptable rates while a DCR rate ≤40% and a relevant toxicity rate ≥40% are considered inacceptable rates. Given these hypotheses, the first step of the study will require 25 patients. If ≥10 patients with a DCR are observed and ≥15 patients do not have relevant toxicity, the study will enrol patients in the next step. A total of 53 patients will be enrolled. If ≥25 patients with DCR and ≥36 patients without any relevant toxicity are observed, treatment will be considered active and not toxicTaking into account a 5% dropout rate, 56 patients must be enrolled in each arm (total 112 patients). G3-4 gastrointestinal toxicity, G4 thrombocytopenia, prolonged G3-G4 neutropenia (> 7 days) and drug-related hospitalizations are considered relevant toxicity. The stratification factors of this study are Ki-67 (21%-55 % *vs*. >55%) and site of primary tumor (lung *vs*. GEP).

Complete response, partial response or stable disease for at least 12 weeks will be considered as the DCR. The proportion of patients in this category will be determined and 95% confidence intervals (95%CIs) for the DCR will be calculated. OS and PFS will be estimated using the Kaplan-Meier method (two-sided 95%CIs) (24).
For miRNA analysis was a blood sample was taken from 20 patients enrolled in SENECA study and 20 healthy donors. All the participant signed an informed consent.

Purification of cell-free total RNA, which primarily includes small RNAs including miRNAs, was performed from serum using miRNeasy Serum/Plasma Kit (Qiagen), according to manufacturer protocol. Libraries were then prepared starting from 5µl of RNA using QIAseq® miRNA Library Kit, containing integrated unique molecular indices (UMIs) to enhance differential expression analysis. Briefly, adapters were ligated sequentially to the 3' and 5' ends of miRNAs. Subsequently, universal cDNA synthesis with UMI assignment, cDNA cleanup, library amplification, and library cleanup were performed. Resulting libraries were then checked for quality using Agilent Bioanalyzer 2100, to check the presence of the ≈180 bp peak, and concentration was determined using Qubit Fluorimeter. The samples were pooled in equimolar ratios and the resulting pooled library was then diluted at a final concentration of 1.6pM and sequenced using NextSeq™ 550Dx High Output Reagent Kit v2.5 (75 cycles). Primary data analyses (UMIs count and miRNA sequences mapping) were then performed with proprietary online tool available at geneglobe.qiagen.com.

## 3. **RESULTS**

### *3.1 WP1-2 results*

Expression of DAXX, ATRX, and RB1 in G3 neuroendocrine tumor tissue ex is shown in **table 1**. All the samples were taken from biopsies of a metastatic site.

| | Total (%) | Pancreatic (%) | GI (%) |
|---|---|---|---|
| ATRX | | | |
| Positive | 6 (66.7) | 3 (100.0) | 3 (50.0) |
| Negative | 3 (33.3) | 0 (0.0) | 3 (50.0) |
| Not evaluated | 2 | 2 | — |
| DAXX | | | |
| Positive | 9 (100) | 3 (100.0) | 6 (100.0) |
| Negative | 0 (0) | 0 (0.0) | 0 (0.0) |
| Not evaluated | 2 | 2 | — |
| ATRX + DAXX | | | |
| Positive | 6 (66.7) | 3 (100.0) | 3 (50.0) |
| Negative | 3 (33.3) | 0 (0.0) | 3 (50.0) |
| Not evaluated | 2 | 2 | — |
| RB1 | | | |
| Positive | 4 (44.5) | 2 (66.7) | 2 (33.3) |
| Negative | 5 (55.5) | 1 (33.3) | 4 (66.7) |
| Not evaluated | 2 | 2 | — |

GI: gastrointestinal.

Table 1: IHC expression of DAXX, ATRX and RB1

All markers showed a strong nuclear localization, and stromal cells were used as an internal positive control for immunostaining (**Figure 1**).

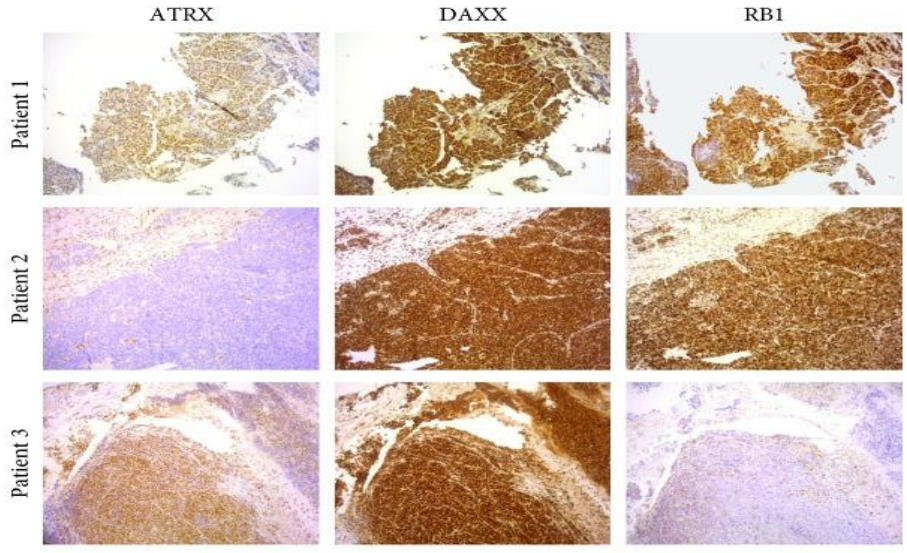



**Figure 1** NEC tissue immunostained for ATRX, DAXX, and RB1. Patient 1 showed positive immunostaining of all 3 markers. Patient 2 showed positive expression of DAXX and RB1 and loss of ATRX expression. Patient 3 showed positive expression of ATRX and DAXX and loss of RB1 expression. Magnification ×10.

DAXX was expressed in 100% of neuroendocrine tumor tissue, and no patient showed loss of IHC expression of this marker. ATRX was expressed in 66.7% of neuroendocrine tumor tissues, and 3 (33.3%) patients showed a loss of expression. Interestingly, all patients with loss of ATRX expression had NECs of gastrointestinal origin. DAXX and ATRX mutations are mutually exclusive. RB1 was expressed in 44.5% of neuroendocrine tumor tissue, and 5 (55.5%) patients showed a loss of expression. Of these, one had pancreatic NEC and 4 had gastrointestinal NECs.

Bioptic material was not evaluable in 2 patients with a positive 68Ga-PET/CT. The other 2 68Ga-PET/CT-positive patients showed expression of ATRX/DAXX. Of the 6 patients with negative 68Ga-PET/CT, 4 showed ATRX/DAXX expression and 2 patients showed a loss of expression. With regard to RB1, patients with positive 68Ga-PET/CT showed expression of this marker. Among those with negative 68Ga-PET/CT, 2 showed RB1 expression and 4 patients a loss of expression.

*3.2 WP3 results*

In WP3 on 47 GEP-NENs patients the presence of DLL3 in 76.9% of G3 NEC correlate with RB1-loss ($p<0.001$), negative 68Ga-PET/CT($p=0.001$) and a poor prognosis.

DLL3 was expressed in 21.7% of tumor samples (10/46 patients; DLL3 was not evaluable in one case). RB1 was expressed in 79.1% of tumor tissues (34/43 patients; RB1 was not evaluable in 4 cases). PD-L1 was expressed in 19.5% of tumor samples

(8/41 patients; PD-L1 was not evaluable in one case and not assessed in 5). The correlation between DLL3, RB1 expression and 68Ga-PET/CT status is reported in **Table 3.**

Table 3. Correlation with biomarkers and 68GaPET/CT

| | **DLL3** | | | |
|---|---|---|---|---|
| | Pos (%) | Neg (%) | Total | **p-value** |
| **Overall** | 7 (16.3) | 35 (83.7) | 43 | |
| **RB1** | | | | |
| Negative | 6 (85.7) | 1 (3.3) | 7 | <0.001 |
| Positive | 1 (14.3) | 29 (96.7) | 30 | |
| **$^{68}$Ga-PET/C** | | | | |
| Negative | 6 (75.0) | 2 (25.0) | 8 | 0.001 |

| | | | |
|---|---|---|---|
| Positive | 1 (5.0) | 18 (95.0) | 19 |

Of the 10/46 DLL3-positive patients, 7 (70.0%) showed loss of RB1, while only 2/36 (6.3%) with negative DLL3 tumors revealed loss of RB1 expression ($p < 0.001$). No correlation was found between DLL3 and PD-L1 expression. Among the patients positive for DLL3 expression, 6 (85.7%) were negative for PD-L1 expression and one (14.3%) was positive, while in patients negative for DLL3 expression, 27 (79.4%) were negative for PD-L1 and 7 (29.6%) were positive. PD-L1 was not associated with any clinical characteristic .

With a median follow-up was 25.6 months (range 0.9-165), median PFS and OS analysis was performed for the different patients' groups according to DLL3 expression (**Figure 2a and 2b**).

Figure 2a

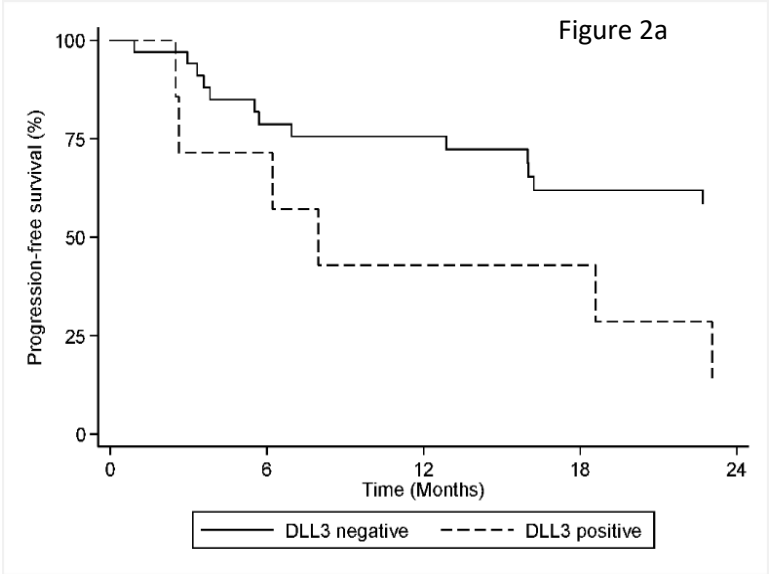
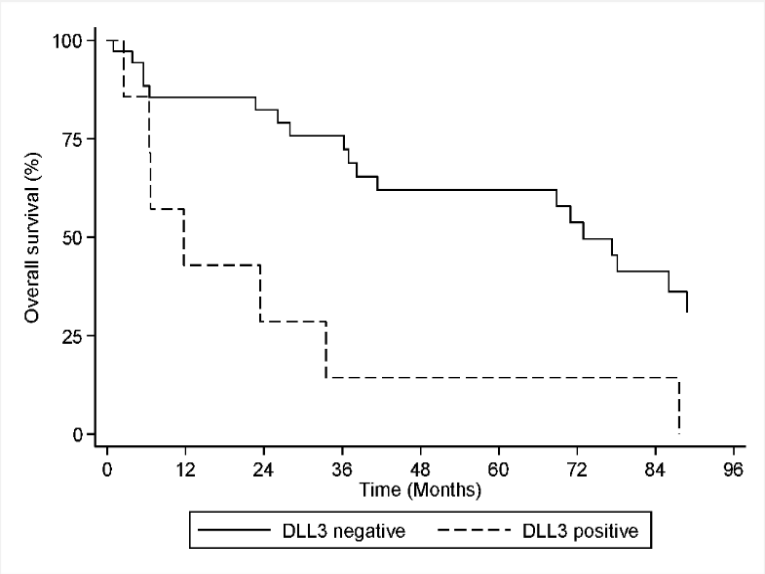



Figure 2b




Expression of DLL3 was negatively correlated with PFS and OS. mPFS was 22.7 months (95% CI: 6.1-68.8) in the group with DLL3-negative tumors with respect to 5.2 months (95% CI: 2.5-18.5) in the group with DLL3-positive disease (p= 0.0083). mOS was 68.8 months (95% CI: 26.0-78.1) in the group with DLL3-negative tumors and 9.5 months (95% CI: 2.5-25.2) in the DLL3-positive group (p= 0.0071).

No survival difference was observed according to gender or age .

*3.3 WP4 Results*

Finally in the WP4 we conducted a multicenter non comparative phase II trial to explore the activity of FOLFIRI or CAPTEM in terms of DCR, PFS and OS given as second line in metastatic NEC patients.

From 06/03/2017 to 18/01/2021 53 out of 112 patients initially planned were enrolled in 17 of 23 participating centers. The main characteristics of patients enrolled have been showed in the table 4

Table 4: patients' characteristics

| Variable | Arm A: FOLFIRI n=28 (%) | Arm B: CAPTEM n=25 (%) | Overall n= 53 (%) |
|---|---|---|---|
| Median (range) | 63 (36-79) | 62 (30-80) | 62 (30-80) |
| Gender | | | |
| Male | 15 (53.6) | 17 (68.0) | 32 (60.4) |
| Female | 13 (46.3) | 8 (32.0) | 21 (39.6) |
| PS ECOG | | | |
| 0 | 14 (50.0) | 15 (60.0) | 29 (54.7) |
| 1 | 12 (42.9) | 8 (32.0) | 20 (37.7) |
| 2 | 2 (7.1) | 2 (8.0) | 4 (7.6) |
| Site of disease | | | |
| Lung | 3 (10.7) | 4 (16.0) | 7 (13.2) |
| Gep-NET | 20 (71.4) | 19 (76.0) | 39 (73.6) |
| Other | 5 (17.9) | 2 (8.0) | 7 (13.2) |
| Lliver metastasis | 18 (64.3) | 18 (72.0) | 36 (67.9) |
| Bone metastasis | 5 (17.9) | 6 (24.0) | 11 (20.8) |
| Lung metastasis | 1 (3.6) | 7 (28.0) | 8 (15.1) |
| Node metastases | 11 (39.3) | 10 (40.0) | 21 (39.6) |
| Other site of metastasis | 4 (14.3) | 5 (20.0) | 9 (17.0) |
| Previous surgery | 15 (53.6) | 15 (60.0) | 30 (56.6) |
| Previous chemotherapy | 28 (100.0) | 25 (100.0) | 53 (100.0) |
| Smoke | | | |
| Yes | 11 (47.8) | 13 (68.4) | 24 (57.1) |
| No | 12 (52.2) | 6 (31.6) | 18 (42.9) |
| Unk | 4 | 6 | 10 |
| 68Ga-PET/CT | | | |

| Variable | Arm A: FOLFIRI n=28 (%) | Arm B: CAPTEM n=25 (%) | Overall n= 53 (%) |
|---|---|---|---|
| Positive | 6 (75.0) | 5 (100.0) | 11 (84.6) |
| Negative | 2 (25.0) | 0 (0.0) | 2 (15.49 |
| Unknown | 0 | 1 | 1 |
| 18FDG-PET/ | | | |
| Positive | 7 (87.5) | 6 (100.0) | 13 (92.9) |
| Negative | 1 (12.5) | 0 (0.0) | 1 (7.1) |
| Morphology | | | |
| Well differentiated | 1 | 1 | 2 |
| Poorly differentiated | 8 | 7 | 15 |
| Not specified | 19 | 17 | 36 |
| KI67 | | | |
| Median (range) | 80 (23-95) | 80 (22-95) | 80 (22-95) |
| Unknown | 2 | 0 | 0 |
| KI67 | | | |
| ≤55 | 8 (30.8) | 7 (28.0) | 15 (29.4) |
| >55 | 18 (69.2) | 18 (72.0) | 36 (70.6) |
| Unknown | 2 | 0 | 0 |
| Patients with comorbidities | | | |
| Yes | 17 (60.7) | 17 (68.0) | 34 (64.2) |
| No | 11 (39.3) | 8 (32.0) | 19 (35.8) |

Median follow up was 10.8 (range 1.4 – 38.6) months.

The 3-month DCR was 39.3% in the FOLFIRI and 32.0 % in the CAPTEM arm. The 6-months PFS rate was 34.6% ( 95%CI 17.5-52.5) in the FOLFIRI and 9.6% (95%CI 1.8-25.7) in the CAPTEM arm.

In the FOLFIRI subgroup the 6-months and 12-months OS rate were 55.4% (95%CI 32.6-73.3) and 30.3% (CI 11.1-52.2) respectively. The mOS was 9.8 (95CI 4.1-13.5) months in the FOLFIRI arm and 7.9 (95%CI 3.7-12.5) months in the CAPTEM arm. In

the latter group the 6-months and 12- months OS rate were 57.2% (95%34.9-74.3) and 29.0% (95%10.0-43.3).

In the pre-planned subgroup analysis according the ki67 value ≤ or > 55% the mPFS in FOLFIRI arm was 8.5 (95%CI 2.0-NE) months with a 6-months PFS rate of 87.5% (95%CI 38.7-98.1) in the first group and 2.9 (95%CI 1.9-3.2) months with a 6-months PFS rate of 6.3% (95%CI 0.4-24.7) in the second one. The median OS was 13.5 (95%CI 5.2-NE) months in the first group and 5.1 (95%CI 1.6-10.7) months in the second one

In the CAPTEM arm patients with a ki67 value ≤ of 55% had mPFS of 4.1 (95%CI 1.1-9.4) months with a 6-months PFS rate of 28.6% (95%CI 4.1-61.1). In patients with ki67 higher than 55% mPFS in CAPTEM arm was 1.9 (95% 1.6-2.9) months. The mOS was not reached in the ki67 ≤ 55% group and 4.3 (2.8-9.8) months in the second group.

Toxicity

Twenty-six (14 in FOLFIRI arm and 12 in CAPTEM arm) of the 53 patients with at least 1 cycle of treatment have at least one G2-G4 adverse event

**Table 5** summarizes the targeted AEs reported by AE type and maximum grade separated by the two treatment arms and was consistent with the already known toxicity profile of both treatments.

Table 5. Targeted AEs reported among patients with at least 1 cycle of treatment

| | FOLFIRI ARM (n=28)<br><br>n of patients (%) | CAPTEM ARM (n= 25)<br><br>n of patients (%) |
|---|---|---|

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | G1 | G2 | G3 | G4 | G1 | G2 | G3 | G4 |
| Neutropenia | 2 (7.1) | 3 (10.7) | 4 (14.3) | 1 (3.6) | 0 (0.0) | 3 (12.0) | 0 (0.0) | 0 (0.0) |
| Febrile Neutropenia | 0 (0.0) | 0 (0.0) | 1 (3.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Leukopenia | 0 (0.0) | 0 (0.0) | 1 (3.6) | 0 (0.0) | 1 (4.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Anemia | 4 (14.3) | 2 (7.1) | 2 (7.1) | 0 (0.0) | 1 (4.0) | 2 (8.0) | 0 (0.0) | 0 (0.0) |
| Thrombocytopenia | 0 (0.0) | 3 (10.7) | 0 (0.0) | 0 (0.0) | 2 (8.0) | 2 (8.0) | 1 (4.0) | 1 (4.0) |
| Asthenia/fatigue | 6 (21.4) | 3 (10.7) | 1 (3.6) | 0 (0.0) | 2 (8.0) | 3 (12.0) | 1 (4.0) | 1 (4.0) |
| Nausea | 5 (17.9) | 3 (10.7) | 0 (0.0) | 0 (0.0) | 3 (12.0) | 1 (4.0) | 0 (0.0) | 1 (4.0) |
| Vomiting | 5 (17.9) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (8.0) | 2 (8.0) | 0 (0.0) | 1 (4.0) |
| Diarrhea | 6 (21.4) | 3 (10.7) | 0 (0.0) | 0 (0.0) | 1 (4.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Loss of appetite | 2 (7.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Erythema/rush | 1 (3.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (4.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Fever | 1 (3.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (4.0) | 0 (0.0) |
| Anorexia | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 2 (8.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Mucositis | 2 (7.1) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Liver toxicity | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (4.0) | 1 (4.0) | 0 (0.0) |
| Renal toxicity | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (4.0) | 0 (0.0) | 0 (0.0) |
| Peripheral edema | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 1 (4.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Nervous system disorder | 0 (0.0) | 1 (3.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Hand-food syndrome | 1 (3.6) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| Other | 6 (21.4) | 0 (0.0) | 2 (7.1) | 0 (0.0) | 3 (12.0) | 1 (4.0) | 0 (0.0) | 1 (4.0) |

Mirna Analysis

At march 2021, 40 subjects ( 20 healthy donors and 20 patients ) were enrolled for miRNA analysis. Of patients enrolled miRNA analysis were performed on 18 patients. The median age was 34.9 years-old in the healty group and 60.5 years old of patients group. Males and females were equally distributed. (table 6)

Table 6. subjects enrolled in miRNA study.

| Variable | Healthy donor N=20 (%) | Patients N=18 (%) | Overall n= 38 (%) | p-value# |
|---|---|---|---|---|
| Age at randomization | | | | |

| | | | | |
|---|---|---|---|---|
| Median (range) | 34.9 (28.6-53.6) | 60.5 (35.8-78.4) | 49.1 (28.6-78.5) | <0.001 |
| Gender | | | | |
| Male | 12 (60.0) | 10 (55.6) | 22 (57.9) | 0.782 |
| Female | 8 (40.0) | 8 (44.4) | 16 (42.1) | |
| Site of disease | | | | |
| Lung | - | 3 (16.7) | 3 (16.7) | - |
| Gep-NET | - | 15 (83.3) | 15 (83.3) | |
| Other | - | 0 (0.0) | 0 (0.0) | |
| Ki-67 value | | | | |
| <55 | - | 7 (38.9) | 7 (38.9) | - |
| ≥55 | - | 11 (61.1) | 11 (61.1) | |

p-value from Wilcoxon rank-sum test for age, and from chi-square for gender

The miRNA analysis of 18 patients enrolled compared with 20 healthy subjects shows an overexpression of the following miRNA involved in staminality , neo-angiogenesis hypoxia induced and NOx downregulation and activation of mitochontrial anaerobic glycolysis: hsa-miR-1246; hsa-miR-1290; hsa-miR- 141-3p; hsa-miR-196a-5p; hsa-miR-200a-3p; hsa-miR-200b-3p; hsa-miR- 200b-5p; hsa-miR-200c-3p; hsa-miR-210-3p; hsa-miR-9-3p; hsa-miR-9-5p. (table 7)

Table 7: MIR expression Median values on crude values

| Variable | Healthy donor N=20 (%) | Patients N=18 (%) | Overall n= 38 (%) | p-value# |
|---|---|---|---|---|
| hsa-miR-1246 (v6) | 41.1 (12.6-92.1) | 483.9 (11.3-9378.1) | 85.3 (11.3-9378.1) | <0.001 |
| hsa-miR-1290 (v9) | 7.8 (3.2-27.6) | 79.1 (2.3-1851.7) | 17.2 (2.3-1851.7) | <0.001 |
| hsa-miR-141-3p (v10) | 1.9 (0.0-36.0) | 8.3 (1.3-222.9) | 4.5 (0.0-222.9) | <0.001 |
| hsa-miR-196a-5p (v13) | 0.0 (0.0-18.0) | 3.5 (0.0-30.8) | 0.4 (0.0-30.8) | <0.001 |
| hsa-miR-200a-3p (v14) | 1.0 (0.0-9.2) | 8.3 (1.3-195.1) | 3.07 (0.0-195.1) | <0.001 |
| hsa-miR-200b-3p (v15) | 1.5 (0.0-12.3) | 9.4 (0.0-192.3) | 3.9 (0.0-192.3) | <0.001 |
| hsa-miR-200b-5p (v16) | 0.0 (0.0-1.7) | 2.4 (0.0-57.2) | 0.7 (0.0-57.2) | <0.001 |

| Variable | Healthy donor N=20 (%) | Patients N=18 (%) | Overall n= 38 (%) | p-value# |
|---|---|---|---|---|
| hsa-miR-200c-3p (v17) | 28.2 (6.3-125.8) | 70.8 (17.9-1813.8) | 41.3 (6.3-1813.8) | <0.001 |
| hsa-miR-210-3p (v18) | 4.0 (0.0-11.7) | 18.9 (7.1-107.4) | 7.2 (0.0-107.4) | <0.001 |
| hsa-miR-9-3p (v32) | 0.0 (0.0-2.2) | 1.2 (0.0-11.9) | 0.0 (0.0-11.9) | <0.001 |
| hsa-miR-9-5p (v33) | 0.0 (0.0-1.7) | 1.3 (0.0-10.8) | 0.0 (0.0-10.8) | <0.001 |

*Minimum and maximum values between parenthesis. P-value based on Wilcoxon rank-sum* test.

The miRNA analysis of 20 patients enrolled compared with 20 healthy subjects shows an overexpression of the following miRNA involved in staminality , neo-angiogenesis hypoxia induced and NOx downregulation and activation of mitochontrial anaerobic glycolysis: hsa-miR-1246; hsa-miR-1290; hsa-miR- 141-3p; hsa-miR-196a-5p; hsa-miR-200a-3p; hsa-miR-200b-3p; hsa-miR- 200b-5p; hsa-miR-200c-3p; hsa-miR-210-3p; hsa-miR-9-3p; hsa-miR-9-5p.

Interestingly patients with an expression of has-miR-1246 less than 648 had a mOS of 10.7 (95%CI 7.4-NE) while patients with an expression ≥ 648 had a mOS of 3.8 (1.2-NE) months (p-value = 0.017). Similarly patients with a hsa-miR-1290 expression < or > than 355 had a mOS of 10.7 (95% CI 7.4-NE) months and 3.7 (95% CI 1.2-NE) months, respectively. Also hsa-miR-210-3p and hsa-miR-9-5p seem to have a similar trend. (Table 8.)

Table 4.4: Overall survival

| | Number of patients | Number of events | Median OS (95%CI) | p-value log-rank test |
|---|---|---|---|---|
| All cases | 17 | 9 | 10.7 (7.4-NE) | - |
| hsa-miR-1246 | | | | |
| <648 | 10 | 3 | 10.7 (7.4-NE) | 0.017 |
| ≥648 | 7 | 6 | 3.8 (1.2-NE) | |

| | | | | |
|---|---|---|---|---|
| hsa-miR-1290 | | | | |
| <355 | 13 | 5 | 10.7 (7.4-NE) | <0.001 |
| ≥355 | 4 | 4 | 3.7 (1.2-NE) | |
| hsa-miR-200b-5p | | | | |
| <2.4 | 9 | 3 | 10.7 (1.1-NE) | 0.0951 |
| ≥2.4 | 8 | 6 | 5.2 (2.8-NE) | |
| hsa-miR-210-3p | | | | |
| <35 | 14 | 6 | 10.7 (7.4-NE) | 0.006 |
| ≥35 | 3 | 3 | 3.8 (3.7-NE) | |
| hsa-miR-9-5p | | | | |
| <6.4 | 14 | 6 | 10.7 (7.4-NE) | <0.001 |
| ≥6.4 | 3 | 3 | 3.8 (1.1-NE) | |

NE→not estimable from statistical software

## Discussion

High-priority unmet needs in neuroendocrine neoplasia include a better molecular characterization of G3 GEP-NEN subgroups (NETs *vs* NECs) and the identification of molecular drivers of the disease that can be used as therapeutic targets. Furthermore the

role of RB1 and DAXX/ATRX is still debate . Despite the limited number of cases analyzed , in the WP1 of the project $^{68}$GA-PET/CT status seems to be helpful to discriminate patients with RB1 loss , that has been previously identified as a biomarker of poor prognosis in Neuroendocrine carcinoma patients. However in a recent published paper, RB1 loss alone doesn't seem to have a prognostic impact on PFS under or OS in patients with metastatic NEC, whatever the primary site. (25)

The negative Notch regulator DLL3 has aroused the interest of researchers for its potential as both a prognostic marker and candidate therapeutic target in neuroendocrine tumors, in particular, small-cell lung cancer, LCNEC and neuroendocrine prostate cancer [26-29]. For the first time, we demonstrated that DLL3 is expressed in GEP-NENs and shows clinical and prognostic significance. Our results revealed that DLL3 expression could distinguish poorly-differentiated NECs from well-differentiated tumors, thus increasing the arsenal of available diagnostic tools. Finally, there is still no truly effective second-line chemotherapy for neuroendocrine carcinoma. The overall prognosis of patients is poor, with an OS of 5 months in the metastatic setting according to the SEER (Surveillance, Epidemiology, and End Results) data (22). Only 5% of all patients are long-term survivors(5).

Despite we didn't reach the primary end-point and the trial was stopped, some consideration could be extrapolated by the results presented. In a recent published meta-analysis second-line therapy for patients with advanced extra-pulmonary NEC had limited efficacy and a high Ki-67 was associated with treatment outcomes, as reported previously in the NORDIC NEC study. Median response rate was 18% (range 0–50; 0% for single-agent everolimus, temozolomide, topotecan; 50% with amrubicin; Table 1). Median PFS was 2.5 months (range 1.2–6.0) and median OS was

7.6 months (range 3.2–22) (30). In our study the mOS was 9.8 and 7.9 months in FOLFIRI and CAPTEM arm, respectively , superior than that reported in this meta-analysis . Furthermore we stratified patients and we pre-planned a subgroup analysis according to ki67 value giving additional information on these categories.

Finally the identification of some circulating miRNAs could be useful to identify new reliable biomarkers for diagnostic and prognostic purpose.

The project has some limitations. For the preclinical part of the project all the samples are taken from a metastatic site and consequently we can't compare the biomarkers expression between metastases and primitive tumor. Furthermore we conducted this part in a retrospective manner. For the clinical part the main limitation of the study is the lacking of a centralized imaging and pathological evaluation.

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