ALMA MATER STUDIORUM
UNIVERSITÀ DI BOLOGNA

DOTTORATO DI RICERCA IN

ONCOLOGIA, EMATOLOGIA E PATOLOGIA

Ciclo 38

Settore Concorsuale: 06/D3 - MALATTIE DEL SANGUE, ONCOLOGIA E REUMATOLOGIA

Settore Scientifico Disciplinare: MED/06 - ONCOLOGIA MEDICA

IDENTIFICATION OF THE GENETIC LANDSCAPE OF NEUROENDOCRINE NEOPLASMS TO IMPROVE DIFFERENTIAL DIAGNOSIS AND IDENTIFY A SIGNATURE FOR TUMOR AGGRESSIVENESS

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Esame finale anno 2026

SUMMARY

ABSTRACT

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Background

Neuroendocrine neoplasms (NENs) are rare and heterogeneous tumors, most commonly arising in the gastroenteropancreatic (GEP) tract and the lung. They vary widely in clinical behaviour. Although WHO classification separates well-differentiated neuroendocrine tumors (NET G1–G3) from poorly differentiated neuroendocrine carcinomas (NECs), the distinction between G3 NET and NEC remains challenging. Molecular markers that refine this taxonomy are needed.

Aims

This project aimed to identify oncogenic alterations associated with tumor aggressiveness; evaluate DLL3 and RB1 expression as prognostic markers; integrate Whole-Exome Sequencing (WES) and RNA-seq data to improve molecular classification.

Material and Methods

A retrospective multicentre study enrolled 104 patients across three Italian centres: IRST, CROB and CSS. Clinical and pathological data were collected. DLL3 and RB1 were evaluated by immunohistochemistry. WES was performed on FFPE samples, while RNA-seq was applied to fresh-frozen tissue. Data were analysed using standard bioinformatics pipelines.

Results

DLL3 was expressed in NECs only and correlated with worse overall survival. RB1 loss was observed in a subset of NECs but was not statistically significant. WES on NETs revealed recurrent mutations in TTN, MUC19, MUC17, MUC4, OBSCN, RYR1, SYNE1, TSC2 and MKI67. Pathway analysis implicated RTK–RAS, PI3K/mTOR, NOTCH, WNT and Hippo signalling. RNA-seq of 37 samples identified 28 pathways upregulated in metastatic tumors, particularly immune response, extracellular matrix remodelling and vesicle transport.

Conclusions

DLL3 represents a marker of aggressiveness in NENs. TSC2 mutations and immune-related transcriptional signatures provide insights into NET progression. Integrating genomic and transcriptomic data refines the molecular classification of NENs and highlights potential biomarkers for patient stratification.

INTRODUCTION

Neuroendocrine neoplasms (NENs) represent a heterogeneous and complex group of epithelial neoplasms that originate from neuroendocrine cells distributed throughout various organs of the body (Diffuse Neuroendocrine System).

These tumors are characterised by the ability to synthesise, store and secrete neuropeptides and amines, while showing remarkable morphological and functional variability [1].

Historically considered rare, their incidence has steadily increased over recent decades, partly due to advances in diagnostic techniques and growing clinical awareness [2].

Recent epidemiological studies estimate an incidence of approximately 6–7 cases per 100,000 individuals, with the gastrointestinal tract and lung being the most common primary sites, although NENs can arise from all body districts such as the urogenital tract, skin, breast, thyroid, etc. [3].

NENs are generally sporadic, but associations with genetic syndromes and familial clustering have been described (multiple endocrine neoplasia type 1 (MEN-1), MEN-2A, MEN-2B, MEN-4, Von Hippel Lindau (VHL) syndrome, neurofibromatosis and tuberous sclerosis) [4, 5].

From a clinical standpoint, NENs display a broad spectrum of biological behaviours: they range from well-differentiated, slow-growing, indolent tumors to poorly differentiated carcinomas that are highly aggressive and associated with rapid progression and poor prognosis [6]. Hormonal secretion is strictly correlated with the clinical picture; this feature allows classification of NENs into functioning and non-functioning forms. Non-functioning NENs generally present with non-specific symptoms or are diagnosed incidentally [7]. In functioning forms, which represent only a minority of cases, serum hormone levels are elevated and associated with hormone-related syndromes due to peptide or amine overproduction [5].

This biological and clinical variability makes diagnosis, staging and therapeutic decision-making complex.

1.1 Classification of Neuroendocrine Neoplasms

The classification of NENs has undergone significant revisions over the years, parallel to advances in histopathological and molecular knowledge.

Gastroenteropancreatic Neuroendocrine Neoplasms (GEP-NEN)

GEP-NENs account for approximately 70% of all NENs [5]. According to the WHO 2022 classification (**Table 1**), NETs are classified based on differentiation grade (G1–G3), Ki-67 proliferation index and mitotic count [8]. NECs, by contrast, are high-grade tumors characterised by marked nuclear atypia, high mitotic activity and extensive necrosis [9]. The category of mixed neuroendocrine–non-neuroendocrine neoplasms (MiNEN), in which each component represents at least 30% of the tumor mass, is also recognised [10].

This classification has relevant prognostic implications: NET G1–G2 generally follow an indolent

course, whereas NECs show aggressive evolution and limited response to systemic chemotherapy [11]. The NET G3 category, introduced more recently, identifies a subgroup of well-differentiated but highly proliferative tumors that differ from NECs both molecularly and therapeutically [12].

Table 1. Classification of GEP-NEN

GRADE	MORPHOLOGY	MITOTIC COUNT/ KI-67
G1 (Low) - NET	Well differentiated	*< 2 mitosis/10 HPF* *< 3% Ki-67*
G2 (Intermediate) - NET	Well differentiated	*2- 20 mitosis /10 HPF* *3 – 20 % Ki-67*
G3 (High) - NET	Well differentiated	*> 20 mitosis /10 HPF* *> 20% Ki-67*
Neuroendocrine Carcinoma (NEC)	Poorly differentiated	*> 20 mitosis /10 HPF* *> 20% Ki-67*
Mixed Neuroendocrine Neoplasm (MiNEN)	Well differentiated (if neuroendocrine component is NET G1-G2-G3) Poorly differentiated	-

Thoracic Neuroendocrine Neoplasms

Lung and Thymus NENs constitute a spectrum of entities ranging from typical carcinoid (TC) to atypical carcinoid (AC), up to large-cell neuroendocrine carcinoma (LCNEC) and small-cell lung carcinoma (SCLC) [13]. The WHO 2021 classification is primarily based on mitotic index and necrosis. Although the Ki-67 proliferation index is not included in the diagnostic criteria, the WHO recognises its utility in the differential diagnosis between low- and high-grade neuroendocrine lesions (**Table 2**). Typically, Ki-67 levels are below 5% for TC and below 30% for AC. However, no threshold value is defined for discriminating TC from AC [14].
Typical carcinoids are defined as low-grade neuroendocrine tumors (NET) with a mitotic count <2 mitoses/2 mm² and absence of necrosis. Atypical carcinoids (intermediate-grade pulmonary NETs) are rarer and more aggressive than typical carcinoids, with a mitotic count of 2–10 mitoses and/or focal necrosis.

A recently described group of thoracic NENs is characterised by high cellular proliferation with a count >10 mitoses/2 mm² and/or a Ki-67 index >30% while retaining well-differentiated morphology.
LCNECs are much more aggressive NENs with a mitotic count >10 mitoses/2 mm² and cytological features of a large-cell carcinoma. SCLCs, the most aggressive of the four subgroups, account for approximately 13% of all lung tumors and generally show a very high mitotic rate and a Ki-67 >70% [15].
Genomic analyses have shown common alterations between LCNEC and SCLC, in particular inactivation of TP53 and RB1 and dysregulation of Notch pathway components [16, 17]. However, the genetic landscape of pulmonary carcinoids is distinct, more frequently characterised by mutations in MEN1, PSIP1 and chromatin remodelling genes [18].

Table 2. Classification of Thoracic NEN

Lung and Thymus	**Classification**	**Criteria**
Well differentiated neuroendocrine tumor (NET)	Typical Carcinoid / NET G1	*< 2 mitosis/2 mm^2 and no necrosis*
	Atypical Carcinoid /NET G2	*2- 10 mitosis/2 mm^2 and/or necrosis*
	Carcinoid/NET with elevated mitotic counts and/or elevated Ki-67 proliferation index	*Atypical Carcinoid morphology and elevated mitotic counts (> 10 mitosis /2 mm^2) and/or Ki-67 >30%*
Poorly differentiated neuroendocrine carcinoma (NEC)	Large-cell Neuroendocrine Lung Carcinoma	*Large-cell morphology, necrosis always present and >10 mitosis/2mm^2*
	Small-cell Lung Cancer	*Small-cell morphology and >10 mitosis/2mm2*

1.2 Disease Heterogeneity and Molecular Alterations in Neuroendocrine Neoplasms

NENs display high inter- and intra-tumoral heterogeneity, which contributes to major diagnostic and therapeutic challenges [19]. Differences between tumors, even within the same histological subtype, include morphology, proliferative activity, receptor expression and genetic profiles [20]. The Ki-67 proliferation index, an essential parameter for classification, is subject to technical and interpretive variability, especially in small biopsy specimens [21]. The distinction between NET G3

and poorly differentiated NEC remains one of the main diagnostic challenges, with important clinical implications [22].

Immunohistochemistry is essential to confirm the neuroendocrine nature of the neoplasm: the most commonly used markers are chromogranin A and synaptophysin [23]. However, in poorly differentiated NECs the loss of expression of these markers may complicate diagnosis [24].
In metastatic cases, identification of the primary site is often complex, as morphological and immunophenotypic features can overlap between different sites [25].
Advances in next-generation sequencing (NGS) techniques have revealed that NENs arising from different organs share few common driver mutations.

In pancreatic and gastrointestinal NETs, the most recurrent mutations affect MEN1, DAXX and ATRX, genes involved in chromatin remodelling and telomere maintenance [26, 27]. These alterations are associated with a relatively favourable prognosis compared with NECs, which more frequently harbour mutations in TP53, RB1 and KRAS [28].

Activation of the PI3K/AKT/mTOR signalling pathway represents a key mechanism in neuroendocrine tumorigenesis, promoting cell growth and survival [29, 30]. The mTOR inhibitor everolimus and the multikinase inhibitor sunitinib have demonstrated significant clinical efficacy in advanced NETs [31].

The low tumor mutational burden (TMB) typical of NETs correlates with their slow-growing biological behaviour and limited response to immunotherapy.

An emerging role is also played by epigenetic regulation, which includes DNA modifications, histone alterations and changes in non-coding RNA expression [32]. In NECs, genomic instability and alterations in TP53/RB1 are almost universal [33]. A subgroup of high-grade NENs displays microsatellite instability (MSI) or high TMB, features that suggest potential sensitivity to immunotherapy [34].

1.3 The Notch Signalling Pathway, DLL3 and RB1 Loss in Neuroendocrine Neoplasms

The Notch pathway is a highly conserved signalling cascade that regulates cell fate, differentiation and tissue homeostasis [35]. In mammals it comprises four receptors (NOTCH1–4) and five ligands (DLL1, DLL3, DLL4, JAG1, JAG2). Ligand–receptor interaction triggers a two-step proteolytic cleavage mediated by γ-secretase, releasing the Notch intracellular domain which translocates to the nucleus and modulates transcription of target genes such as HES1 and HEY1 [36].

Under physiological conditions, Notch maintains the balance between neuroendocrine and non-neuroendocrine cells by inhibiting neuroendocrine differentiation. In NENs, however, the role of this pathway appears context-dependent and biphasic: in well-differentiated NETs Notch inactivation favours tumor growth and maintenance of the neuroendocrine phenotype (a tumor suppressor role) [37, 38], whereas in NECs and other epithelial tumors Notch hyperactivation can promote epithelial–mesenchymal transition, drug resistance and invasive behaviour [39].

Among the Notch family ligands, DLL3 plays a peculiar role. It behaves as a non-canonical inhibitory ligand, preventing Notch activation in adjacent cells and altering the balance of differentiative signalling. Under normal conditions, DLL3 is expressed only during embryonic development and is absent in healthy adult tissues. Conversely, in high-grade neuroendocrine neoplasms, particularly SCLC and LCNEC, DLL3 is strongly overexpressed at the cell membrane [40].

This selective expression has generated considerable clinical interest as a therapeutic target. The antibody–drug conjugate rovalpituzumab tesirine (Rova-T), directed against DLL3, demonstrated antitumor activity in DLL3-positive NECs, albeit with toxicity-related limitations [41]. Next-generation agents — including bispecific antibodies and anti-DLL3 CAR-T cells — currently represent one of the most promising therapeutic research avenues for NEC treatment.

Concurrently, another key molecular alteration in high-grade NENs is RB1 loss, observed at a high frequency in pulmonary and gastrointestinal NECs [16, 17]. The RB1 protein (retinoblastoma 1) is a critical cell-cycle regulator: its inactivation, often associated with concurrent TP53 mutations, results in loss of control over the G1/S transition and promotes uncontrolled proliferation.

The association between RB1 loss and DLL3 overexpression is now recognised as a distinctive molecular signature of NECs, particularly of small-cell-like type, irrespective of the primary site [17, 40]. These alterations, together with Notch pathway silencing, define a biologically aggressive subtype characterised by rapid proliferation, genomic instability and transient response to platinum-based chemotherapy.

Overall, coordinated dysregulation of the Notch pathway, DLL3 overexpression and RB1 loss represent the three central elements of the molecular biology of high-grade NECs, with important diagnostic and therapeutic implications. Understanding the interplay between these mechanisms may help identify novel biomarkers and potential therapeutic targets for personalised management of patients with NENs [42, 43].

1.4 Clinical Unmet Need

Despite the significant progress achieved in recent years, several critical issues remain in the clinical management of NENs:

- The differential diagnosis between grade 3 neuroendocrine tumors (NET G3) and neuroendocrine carcinomas (NEC) is often ambiguous, even when using proliferative and immunohistochemical markers. Similarly, in pulmonary NENs, clear discrimination cut-offs between typical carcinoid (TC) and atypical carcinoid (AC) are lacking.
- Reliable prognostic and predictive molecular biomarkers capable of guiding therapeutic decisions are still missing.
- Treatment strategies remain largely based on histological morphology rather than on the molecular subtype of the neoplasm.

A deeper molecular characterisation integrating genomic and transcriptomic data is therefore essential to refine classification, guide therapeutic choices and identify new potentially actionable molecular targets.

AIMS OF PhD PROJECT

This PhD research project was conceived with the following objectives:

1. To identify genetic and transcriptional alterations associated with neuroendocrine neoplasms (NENs) and with disease aggressiveness.
2. To correlate the expression of DLL3 and RB1 markers with patient clinical outcomes.
3. To integrate Whole-Exome Sequencing (WES) and RNA-sequencing (RNA-seq) data in order to delineate molecular signatures capable of distinguishing indolent from aggressive tumors.

By combining clinical, genomic, and transcriptomic data from multiple institutions, the project aims to provide a comprehensive overview of the molecular landscape of NENs and to define potential biomarkers useful for patient stratification.

MATERIALS AND METHODS

3.1 Study Design and Ethical Approval

A retrospective, multicentric biological study was conducted across three Italian institutions. The coordinating centre was the *Istituto Romagnolo per lo Studio dei Tumori (IRST) "Dino Amadori"*, Meldola (FC). Two collaborating centres participated: the *Istituto di Ricovero e Cura a Carattere Scientifico – Centro di Riferimento Oncologico della Basilicata (CROB)*, Rionero in Vulture (PZ) and *Casa Sollievo della Sofferenza (CSS)*, San Giovanni Rotondo (FG).

The study was approved by the respective Ethics Committees and conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines. All patients provided written informed consent for the use of their biological material and anonymised clinical data for research purposes.

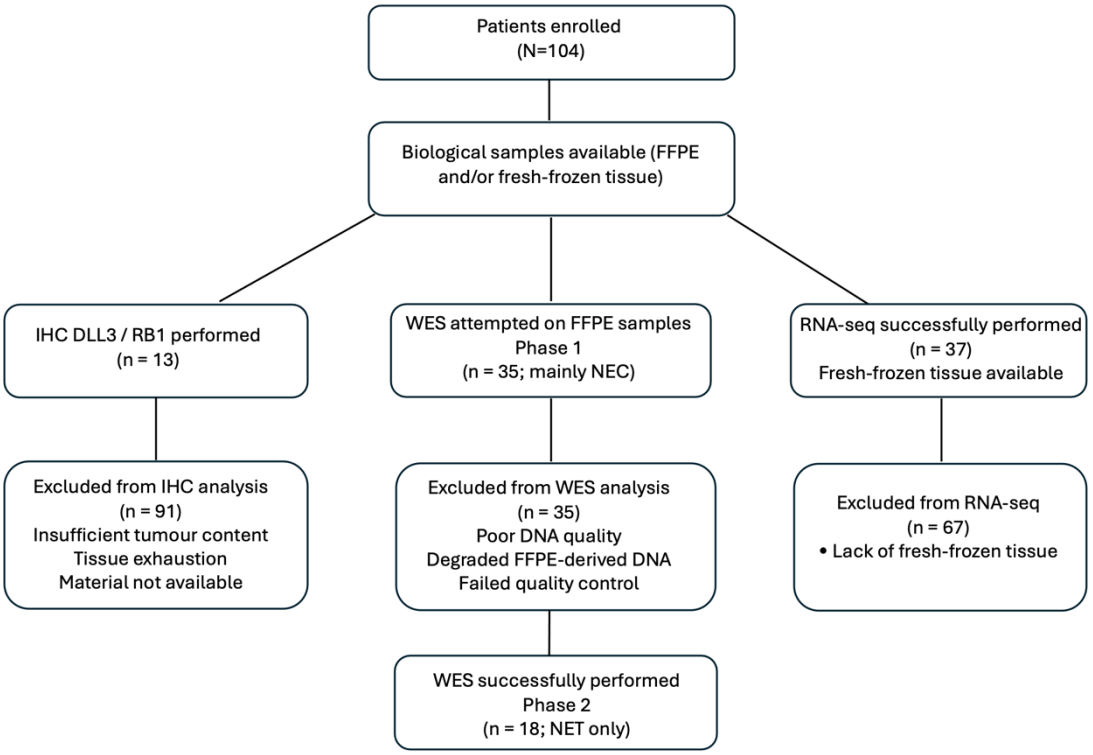
3.2 Study Population

A total of 104 patients diagnosed with neuroendocrine neoplasms between 2018 and 2024 were enrolled in the study.

However, the availability and quality of biological material varied across the different molecular analyses performed. A CONSORT-style flow diagram summarizing patient inclusion, sample availability and attrition across analyses is provided in **Figure 1**.

Although 104 patients were enrolled, immunohistochemical analysis of DLL3 and RB1 was feasible only in 13 FFPE samples with sufficient tumor content and tissue preservation. Whole-Exome Sequencing was successfully performed in 18 patients, while RNA sequencing was carried out in 37 cases with available fresh-frozen tissue.

Figure 1. CONSORT-style flow diagram of patient enrollment and sample availability for molecular analyses.



Inclusion criteria:

1. Age ≥18 years, both sexes;
2. Histologically confirmed diagnosis of neuroendocrine neoplasm (NET or NEC);
3. Availability of tumor tissue (FFPE or fresh frozen);
4. Signed informed consent.

Clinical information was collected in a dedicated electronic database and included:

- Demographic data;
- Primary tumor site and grade (G1–G3, NEC);
- Ki-67 index;

- Functional status (syndromic/non-syndromic);
- PET/CT results with ^68Ga-DOTATATE and ^18F-FDG;
- Presence or absence of metastases at diagnosis;
- Treatments received and outcomes (progression-free survival and overall survival).

All samples were anonymised and labelled with identification codes to ensure patient confidentiality.

3.3 Immunohistochemistry (IHC)

A subset of 13 FFPE tumor samples from IRST underwent immunohistochemical staining for DLL3 and RB1 to assess their potential prognostic value.

Immunostaining was performed using the *VENTANA BenchMark Ultra* system (Ventana Medical Systems Inc., Tucson, AZ, USA). The following antibodies were used: DLL3 (SP347) Ventana Assay (Ventana Medical Systems Inc.), prediluted by the supplier; RB1 (Cell Signalling Technology, Beverly, MA, USA) diluted 1:1000. All reactions were carried out for 1 hour at room temperature, and sections were counterstained with hematoxylin II (Ventana Medical Systems Inc.). Stained sections were evaluated by an experienced pathologist blinded to clinical data.

For each section, IHC staining was analysed only if a sufficient proportion of tumor cells was available for evaluation. Expression values of DLL3 and RB1 were treated as dichotomous variables (positive/negative):
- DLL3 expression was considered positive if >1% of tumor cells showed cytoplasmic or membranous staining.
- RB1 expression was considered positive if nuclear staining was present in >1% of tumor cells. Stromal cells served as internal positive controls for RB1.

3.4 DNA Extraction and Whole-Exome Sequencing (WES)

At least five unstained microtome sections (10 µm) of archived formalin-fixed paraffin-embedded (FFPE) tumor samples were collected from each patient. Whole-Exome Sequencing (WES) analysis was performed on tumor DNA.

Two sequencing platforms were employed in successive phases.

First phase:
A first set of 35 tumor samples underwent WES. Libraries were prepared using the *SureSelect XT HS Target Enrichment System* with *Human All Exon V7 probes* (Agilent), starting from 100 ng of DNA. Enzymatic DNA fragmentation was performed, followed by the preparation of individually indexed and molecularly barcoded libraries through the following steps: DNA end-repair and dA-tailing, ligation of barcoded adaptors, amplification of adaptor-ligated libraries, hybridisation with target-specific probes, and capture with streptavidin-coated beads. Captured libraries were then amplified and purified. Library quality and quantity were assessed using the *Bioanalyzer High Sensitivity DNA Assay* to verify the presence of the expected 200–400 bp peak. Libraries were denatured and diluted to a final concentration of 1.2 pM, then sequenced using the *NextSeq™ 550Dx High Output Reagent Kit v2.5 (300 cycles)* (Illumina) in paired-end 2×150 mode.

Unfortunately, due to technical limitations related to this platform, data obtained from this subset of patients did not yield significant results. Most of these samples corresponded to NEC cases, which limited the possibility of performing a direct NET vs NEC genomic comparison at this stage.

Second phase:
To overcome these limitations, a second sequencing strategy was adopted.

18 well-differentiated NETs (7 ileal and 11 pancreatic) underwent WES at the *IRCCS IRST Biosciences Laboratory*. Libraries were prepared using the *Illumina DNA Prep with Exome 2.0 Plus Enrichment Kit* and sequenced on the *NovaSeq 6000 Sequencing System*. In this case, the sequencing protocol produced good quality data and, therefore, it was possible to proceed with the bioinformatic analysis, as follows.

Variant identification and annotation were performed using a validated institutional bioinformatics pipeline. Matched normal samples (tissue) were included to distinguish somatic from germline variants. Sequencing quality was adequate, with an average coverage of >100× across samples.

Raw reads were processed through the optimised institutional pipeline of the IRST Biosciences Laboratory. Variants were classified as synonymous, missense, nonsense, frameshift, or splice-site.

3.5 RNA Extraction and RNA Sequencing (RNA-seq)

RNA-seq analysis was introduced to explore transcriptional profiles associated with tumor aggressiveness.
High-quality RNA was extracted from fresh-frozen surgical tissue and purified using the *RNeasy Mini Kit* (Qiagen). Libraries were prepared using the *Illumina Stranded Total RNA Prep Ligation with Ribo-Zero Plus Library Kit*. The quality of the resulting libraries was assessed with the *Agilent Bioanalyzer 210*, and concentrations were determined using a *Qubit Fluorimeter*. Libraries were then sequenced using the *NextSeq™ 550Dx High Output Reagent Kit v2.5 (150 cycles)*.

For clinical correlation, aggressiveness was defined by the presence or absence of metastases at diagnosis, identified as the most discriminant clinical parameter in our cohort.

A differential expression analysis was performed between the two groups, followed by *Gene Set Enrichment Analysis* (GSEA) to identify significantly enriched biological pathways.

3.6 Statistical Analysis

Overall survival (OS) was calculated from the time of diagnosis to death or last follow-up. Kaplan–Meier survival curves were compared using the log-rank test.
Correlations between molecular variables (e.g., DLL3 expression, RB1 loss) and clinical parameters were assessed using the Chi-square or Fisher's exact test, as appropriate.
All analyses were performed using *R* (v4.2).
A p-value < 0.05 was considered statistically significant.

RESULTS

A total of 104 patients with histologically confirmed neuroendocrine neoplasms (NENs) from the three participating centres (IRST, CROB, CSS) were enrolled.

Immunohistochemistry for DLL3 and RB1

Immunohistochemical analysis was performed on 13 NEN samples (4 NET G1, 3 NET G2, 6 NEC). DLL3 positivity was observed exclusively in NECs (4/6 – 66.7%), whereas nuclear RB1 expression was preserved in most NETs (9/13 – 69.2%) but frequently lost in NECs (**Table 3**).
DLL3-positive tumors showed significantly shorter overall survival (OS) compared with DLL3-negative cases (median OS: 8.5 vs 22.4 months, $p = 0.0383$) **Figure 2**.
RB1 loss did not reach statistical significance ($p = 0.0720$) **Figure 3**.

Although an association between marker expression and overall survival was observed, the limited sample size precludes definitive statistical conclusions.

Table 3. DLL3 and RB1 expression in 13 samples of NENs

Case	Grade	Ki-67 (%)	DLL3	RB1
1	NET G1	<3	–	+
2	NET G1	<3	–	+
3	NET G1	<3	–	+
4	NET G1	<3	–	+
5	NET G2	12	–	–
6	NET G2	5	–	+
7	NET G2	7	–	+
8	NEC	<20	+	–
9	NEC	40	+	–
10	NEC	70	+	–
11	NEC	35	–	+
12	NEC	35	–	+
13	NEC	80	+	+ (focal)

Figure 2. Overall Survival (OS) according to DLL3 expression (exploratory analysis).

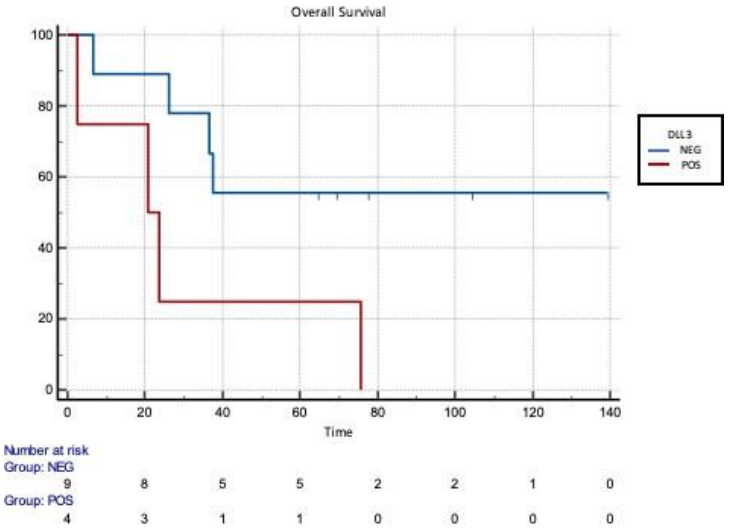
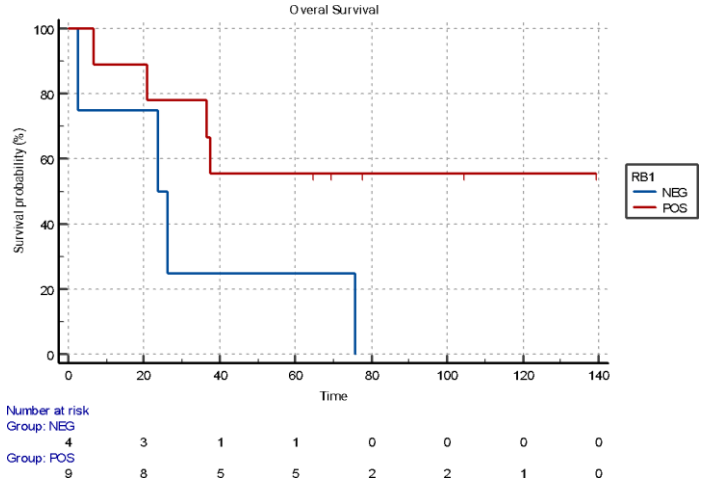


Figure 3. Overall Survival (OS) according to Rb1 expression (exploratory analysis).



Whole-Exome Sequencing (WES)

A subgroup of 18 well-differentiated NETs (11 pancreatic and 7 ileal) successfully underwent WES using the Illumina NovaSeq platform (**Table 4**).

The average target coverage exceeded 100×, with more than 95% of bases covered at ≥20×.

All samples yielded high-quality data suitable for downstream analyses.

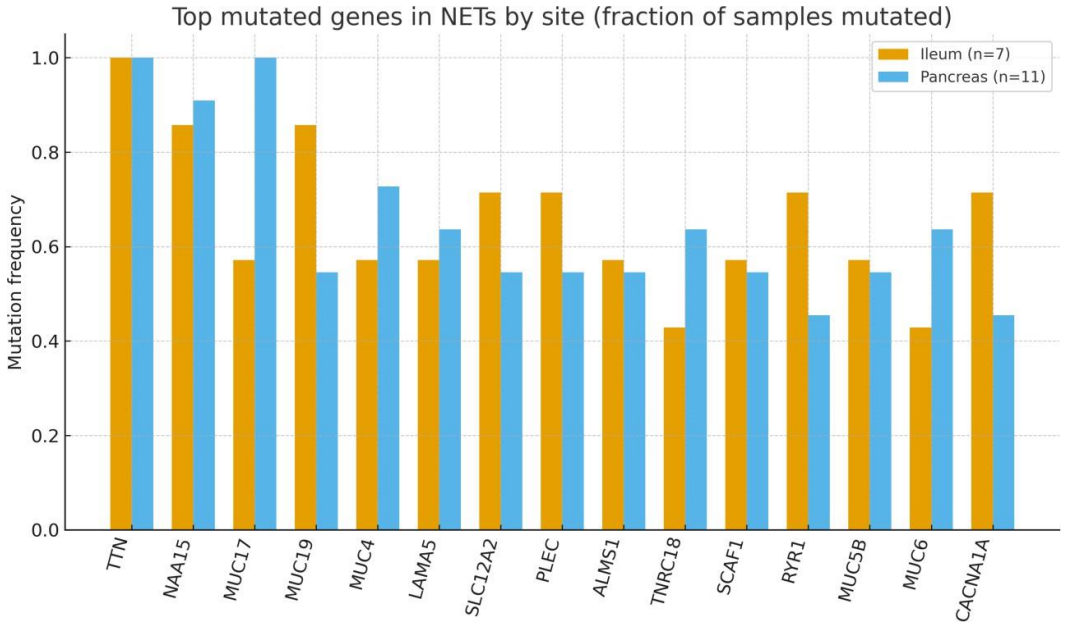
Table 4. Clinical and pathological characteristics of the 18 NET patients analyzed by WES

	Total N 18	NET pancreas (N=11)	Ileal NET (N=7)
Gender	Male 10 (55.6%), Female 8 (44.4%)	6 M/ 5F	4M/3F
Primary site	G1: 10 (55,6%) G2: 8 (44,4%)	G1:5 G2:6	G1:5 G2:2
Median Age	59 (41–79)	58	61

A total of 1,248 somatic variants were identified across the 18 tumors, including 934 missense, 88 nonsense, 104 frameshift, and 122 splice-site mutations.

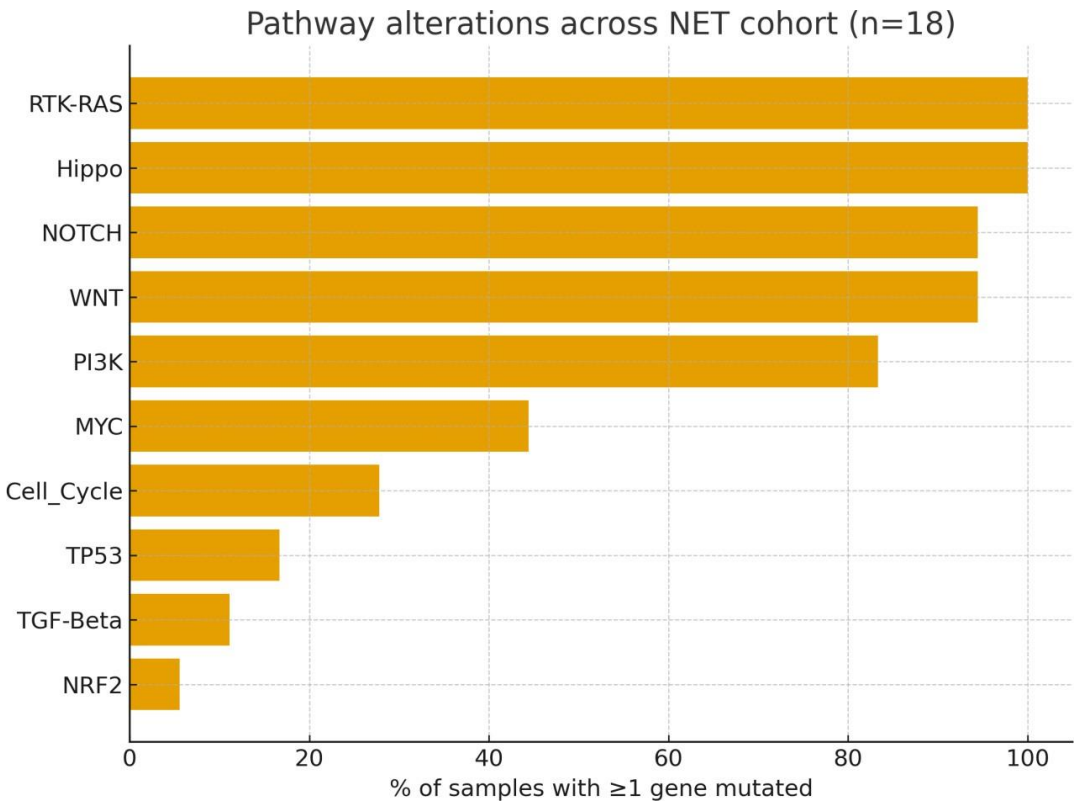
The most frequently altered genes included **TTN, MUC19, MUC17, MUC4, RYR1 (Figure 4)**.

Figure 4. Top mutated genes in NETs (ileal vs pancreatic).
Bar chart showing the frequency of mutations in the top recurrently altered genes across the NET cohort (n=18), stratified by anatomical site. Ileal NETs (n=7) and pancreatic NETs (n=11) display overlapping mutational patterns with frequent alterations in structural genes (TTN, MUC17, MUC4).



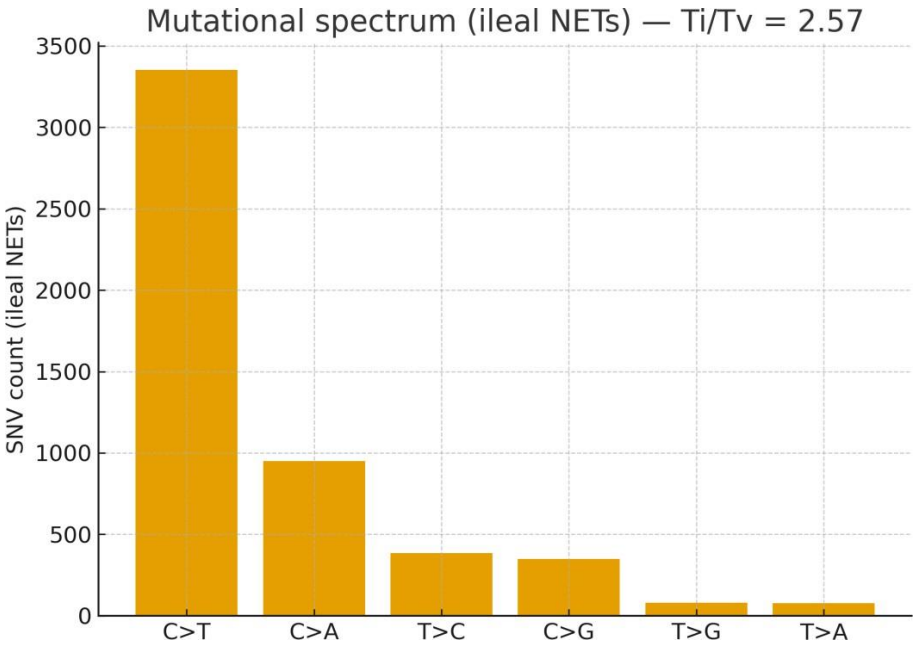
Pathway enrichment analysis revealed frequent alterations in **RTK–RAS, PI3K/mTOR, NOTCH, WNT**, and **Hippo** signalling pathways (**Figure 5**), which are involved in the regulation of cell proliferation, growth, differentiation, and angiogenesis.

Figure 5. Pathway alterations across NET cohort (n=18).
Pathway-level analysis summarizing the percentage of NET cases harboring at least one mutation in selected signalling pathways. The most frequently affected axes include RTK–RAS, PI3K/mTOR, NOTCH, WNT and Hippo signalling. These results suggest convergent pathway dysregulation despite the overall low mutational burden of NETs.



The tumor mutational burden (TMB) was low in all samples (median 1.2 mut/Mb), consistent with the indolent behaviour of NETs. Mutational signature analysis showed a predominance of **C>T transitions**, consistent with spontaneous deamination processes related to ageing and oxidative stress (**Figure 6**).

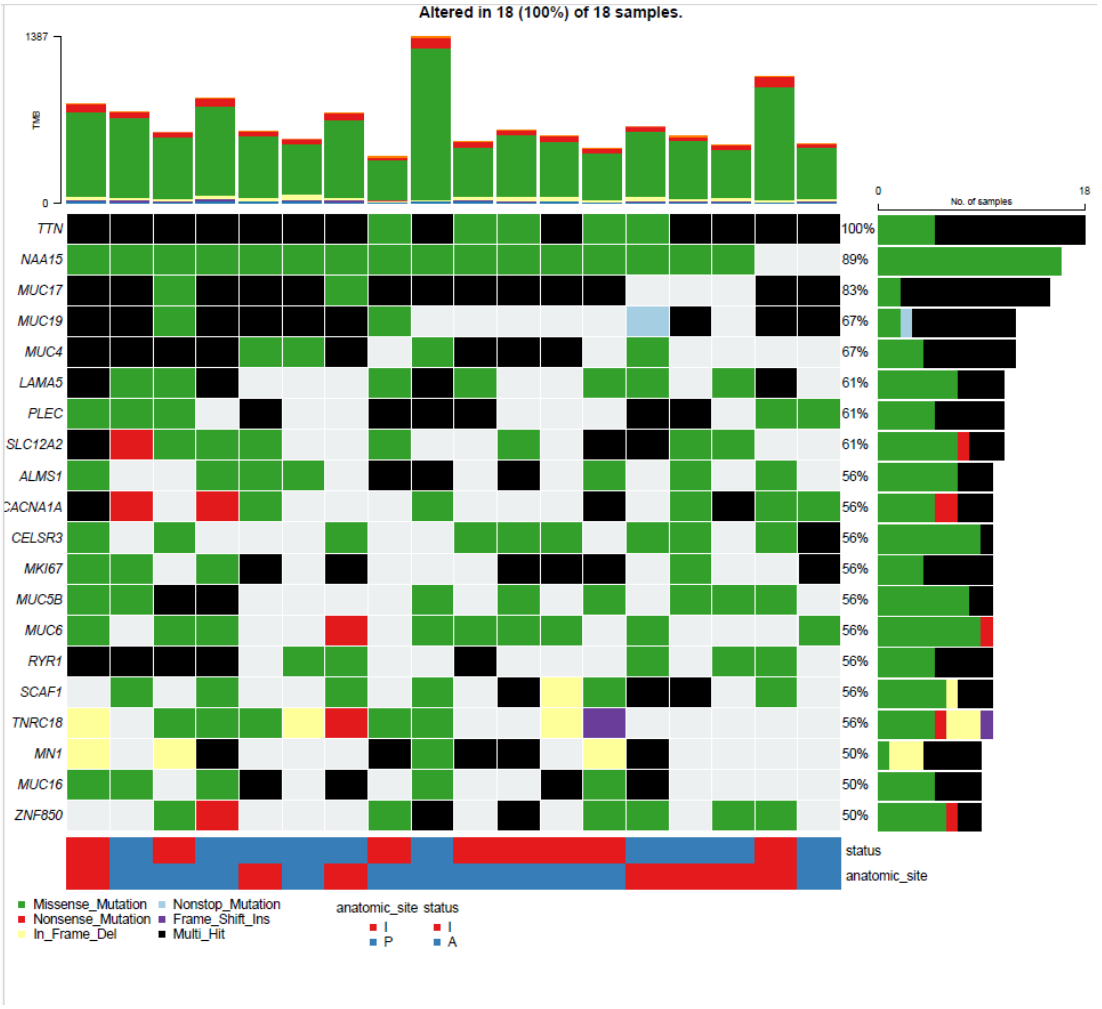
Figure 6. **Mutational spectrum (ileal NETs) with Ti/Tv ratio.**
Spectrum of single nucleotide variants (SNVs) observed in ileal NETs (n=7). The predominance of C>T transitions reflects spontaneous deamination processes commonly observed in low-TMB tumors. The calculated transition/transversion (Ti/Tv) ratio falls within the expected range for exome sequencing data, supporting the overall reliability of variant calls.



No strong mutual exclusivity among alterations was observed, although the co-occurrence of **TSC2, MKI67,** and **MUC16** mutations suggested potential cooperative oncogenic interactions (**Figure 7**).

Figure 7. Oncoprint of recurrently mutated genes in NETs.

Visualization of mutations across the 18 NET samples, stratified by anatomical site (ileal vs pancreatic). The plot highlights the distribution of missense, nonsense, frameshift, and other mutation types, showing that all cases (100%) harbor at least one genetic alteration.



Transcriptomic Analysis (RNA-seq)

RNA-seq was successfully performed on 37 fresh-frozen tissue samples, including 21 metastatic at diagnosis and 16 non-metastatic cases (**Table 5**).
Most tumors originated from the ileum (46%) and pancreas (49%).

Table 5. Clinical and pathological characteristics of patients analyzed by RNA-seq (n=37)

	Primitive Tumor	Grade	Sex M/F
Metastatic (N 21)	Ileal 16 (76%) Pancreas 5 (24%)	G1: 12 (57%) G2: 9 (43%)	10 / 11
Non metastatic (N 16)	Pancreas 13 (81%) Ileal 1, Other 2	G1: 9 (56%) G2: 7 (44%)	10 / 6

Differential expression analysis comparing tumor versus normal tissue was conducted. The volcano plot (**Figure 8**) illustrates the most significantly up- and down-regulated genes.

The heatmap (**Figure 9**) depicts hierarchical clustering of the top 100 differentially expressed genes, clearly separating tumor from normal samples.

Figure 8. Volcano plot of differentially expressed genes between tumor and normal tissue samples.

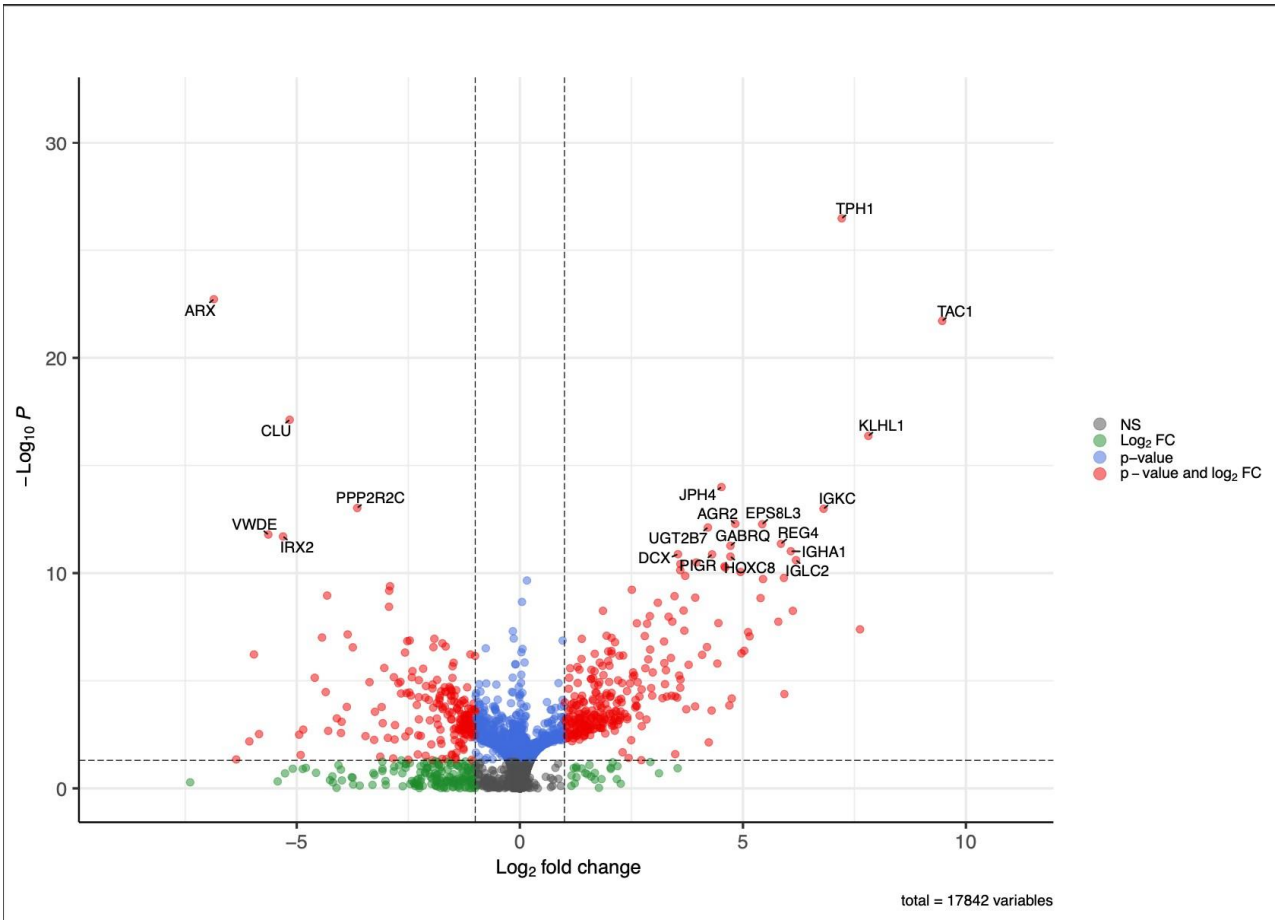
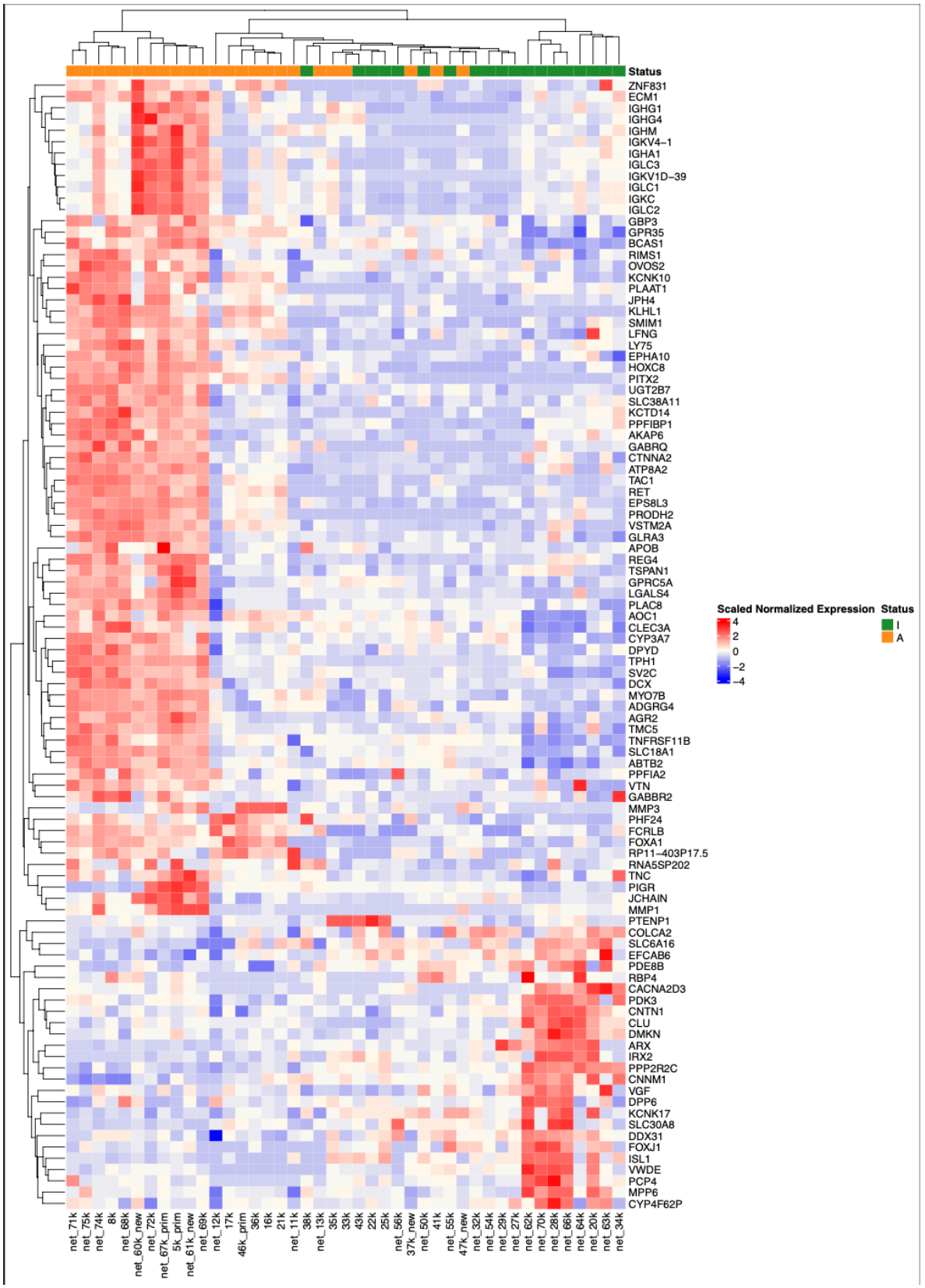


Figure 9. Heatmap of the top 100 differentially expressed genes (tumor vs normal).

Tumor samples cluster distinctly from healthy controls, highlighting transcriptional signatures associated with neuroendocrine differentiation and secretory function.

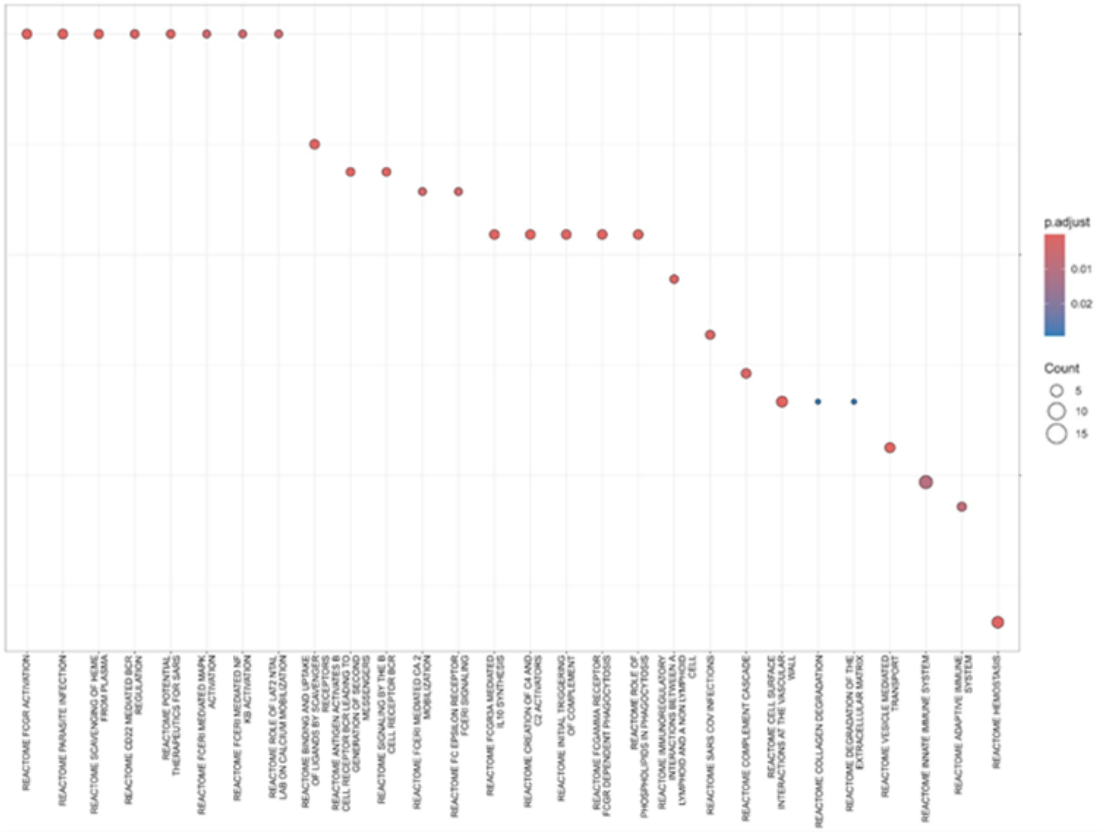


Gene Set Enrichment Analysis (GSEA) identified **28 pathways** significantly enriched in metastatic samples (**Figure 10**), mainly related to:

- activation of the immune system
- extracellular matrix degradation
- vesicle-mediated transport
- cytokine signalling and cell–cell adhesion.

Figure 10. GSEA results in aggressive vs indolent NENs.

Bubble plot showing pathways upregulated in tumors with metastasis at diagnosis (n=21) compared with non-metastatic cases (n=16). Enriched pathways mainly involve immune system processes, extracellular matrix degradation, and vesicle transport, highlighting biological mechanisms underlying tumor aggressiveness.



Integration of WES and RNA-seq data revealed convergent biological themes, including:

- activation of the mTOR pathway (TSC2 mutations and transcriptional activation of metabolic genes);
- suppression of the NOTCH pathway (consistent with DLL3 overexpression);
- modulation of the immune microenvironment in more aggressive forms.

Overall, these findings support the hypothesis that NET progression arises from the interplay between intrinsic oncogenic events and extrinsic microenvironmental factors.

DISCUSSION

This PhD project represents a comprehensive multicentric effort aimed at elucidating the genetic and transcriptomic landscape of neuroendocrine neoplasms (NENs).

Through the integration of immunohistochemical, genomic and transcriptomic approaches, the study identified candidate biomarkers and highlighted molecular mechanisms associated with tumour progression, thereby offering a refined perspective on the biological heterogeneity of these neoplasms.

DLL3 and RB1 as Prognostic Biomarkers

The immunohistochemical study confirmed that DLL3 is overexpressed in poorly differentiated neuroendocrine carcinomas (NEC) and is associated with worse clinical outcomes [44,45].
This finding is consistent with previous literature on small cell lung carcinoma and large cell neuroendocrine carcinoma, where DLL3 acts as a surrogate marker of Notch pathway inactivation and correlates with RB1 loss [44,46].

In the analysed cohort, all DLL3-positive cases belonged to the NEC subgroup and displayed significantly shorter overall survival compared with DLL3-negative tumors.
Although the limited sample size reduces the strength of statistical inference, the observed association should be considered as an observational trend, suggesting that DLL3 may serve as a prognostic biomarker and a potential therapeutic target [44,45,47].
Antibody–drug conjugates (ADC) directed against DLL3 (e.g., *rovalpituzumab tesirine*) have already been clinically evaluated in high-grade neuroendocrine carcinomas and could be tested in extra-pulmonary NECs as well [47]. Rovalpituzumab tesirine (Rova-T) is a humanized anti-DLL3 monoclonal antibody linked to a dimeric PBD toxin. In the Phase 2 TRINITY study in patients with SCLC after ≥2 prior lines, the objective response rate (ORR) was 12.4%, 14.3%, and 13.2% in all, DLL3-high, and DLL3-positive patients, respectively with grade 3–5 adverse events (AEs) in 63% of patients [48].

Tarlatamab (a bispecific T-cell inhibitor directed against DLL3) was evaluated in the DeLLphi-303 study as maintenance therapy in patients with SCLC after standard-of-care first line chemo-immunotherapy in combination with intravenous atezolizumab or durvalumab. Serious adverse events occurred in 57% of patients. The most common serious adverse events were cytokine release syndrome (24%), pyrexia (7%), immune effector cell-associated neurotoxicity syndrome (5%) and pneumonia (5%) [49].
The absence of DLL3 expression in well-differentiated NETs further confirms its specificity for aggressive phenotypes [45].

RB1, on the other hand, showed heterogeneous expression among samples.
Its loss was more frequent in NECs, supporting its role in cell-cycle deregulation and dedifferentiation, although its prognostic value appeared less evident than that of DLL3 [44,46].
This observation aligns with the hypothesis that DLL3 upregulation reflects functional inactivation of Notch signalling, which may precede or accompany RB1 loss during tumor progression [46].

Insights from Whole-Exome Sequencing (WES)

WES results provided key insights into the mutational profile of well-differentiated NETs.
Despite the overall low mutational burden, recurrent alterations were identified in structural and signalling genes.
Mutations in TSC2, particularly in ileal NETs, highlight the central role of the mTOR pathway in neuroendocrine tumor biology — a finding consistent with clinical evidence supporting the efficacy of mTOR inhibitors such as *everolimus* in NETs [50-52].
Recurrent alterations in MUC family genes (MUC19, MUC17, MUC4) and OBSCN suggest modifications in cell adhesion and mucin biology, which may influence tumor–microenvironment interactions [52].
Mutations in MKI67 (encoding the proliferation marker Ki-67) could reflect genomic instability associated with higher proliferative rates.
Pathway-level analysis revealed consistent involvement of the RTK–RAS, PI3K/mTOR, NOTCH, WNT, and Hippo signalling cascades, indicating convergence on mechanisms governing growth and differentiation.
The predominance of C>T transitions is consistent with endogenous mutagenic processes, typical of tumors with low TMB and slow growth [52].

Unfortunately, it was not possible to perform the same assessment in NECs; however, future studies aim to assess these parameters in poorly differentiated tumors to delineate a molecular signature of aggressiveness.

Transcriptomic Signatures of Aggressiveness

RNA-seq analysis of 37 frozen samples enabled exploration of transcriptional programs associated with metastatic disease at diagnosis, used here as a surrogate for aggressiveness.
Data revealed 28 pathways significantly upregulated in metastatic tumors, including:

- Immune response and cytokine signalling, suggesting possible activation of inflammatory circuits or immune evasion mechanisms;
- Extracellular matrix degradation, reflecting increased invasiveness and metastatic potential;
- Vesicle-mediated transport, linked to altered cellular trafficking and secretion of pro-tumoral factors.

These enrichment patterns align with emerging evidence that the tumor microenvironment and immune modulation are key elements in NET progression.

Although NETs are often considered immunologically “cold” [53], our data suggest that immune-related pathways remain transcriptionally active in the more aggressive forms.

Understanding the tumor-immune microenvironment is therefore crucial to improving NET therapies and assessing the feasibility of ICI treatment in this tumor setting. Data demonstrate that the tumor-immune microenvironment differs between localized and metastatic NETs. Immune cell signalling pathways are significantly activated in metastatic NETs.

In some studies, more advanced pancreatic NETs (pNETs) appear to exhibit a significantly higher degree of CD3+ PDCD1+ T cell infiltration; however, conflicting studies exist regarding the degree of TIL infiltration within pNETs and prognosis [54-55].

In addition to the activation of immune and stromal pathways, transcriptomic data revealed upregulation of vesicle-mediated transport and exosome-related genes, which may contribute to intercellular communication that promotes tumor progression. Exosomes derived from NET cells have been shown to carry specific microRNAs and proteins capable of modulating angiogenesis, immune evasion, and metastatic niche formation [56].

Overall, these findings support a model in which tumor aggressiveness is not solely determined by intrinsic proliferative potential, but also by the dynamic interaction between tumor cells and the surrounding microenvironment. Future studies integrating multi-omics data with spatial transcriptomic analyses and immune deconvolution could clarify the spatial relationships between tumor and stromal compartments, paving the way for personalized therapeutic strategies.

A comprehensive understanding of the TME and its regulatory network along with further future studies integrating spatial transcriptomics and immune profiling could validate and deepen these observations as well as guide therapeutic choices in patients with NETs.

Integration of Genomic and Transcriptomic Findings

Integration of the WES and RNA-seq datasets suggests a continuum of molecular events spanning from indolent NETs to aggressive NECs:

1. Early lesions (NET G1–G2) show low mutational burden, mTOR pathway activation, and intact Notch signalling.
2. Intermediate lesions (NET G3) may gain proliferative advantages through deregulation and partial Notch suppression.
3. Aggressive forms (including NEC) display DLL3 overexpression, RB1 loss, and transcriptional activation of inflammatory and extracellular matrix genes.

This multistep model supports a molecularly based classification of NENs rather than one purely grounded in morphology.
The results pave the way for a future diagnostic approach combining histopathology with selected molecular markers.

Study Limitations and Future Directions

The study presents several limitations:

- The number of NEC samples with high-quality WES data was limited due to the degraded nature of FFPE material, preventing a direct genomic comparison between NET and NEC to improve differential diagnosis. Therefore, the molecular differences between indolent and aggressive neuroendocrine neoplasms were investigated through an integrated approach, combining transcriptomic profiling and immunohistochemical markers;
- The multicentric retrospective design introduced variability in sample processing and storage;
- The RNA-seq cohort size was moderate.

Despite these constraints, the integrative approach adopted here represents a significant advance in understanding the molecular features of NENs.

Future studies may focus on:

- validating biomarkers in larger prospective cohorts;

- extending analyses in NEC samples to improve the differential diagnosis between well- and poorly differentiated tumors;

- integrating multi-omics to combine genomics, transcriptomics, epigenomics, and proteomics for a systems-level understanding.

Implementation of these approaches could improve prognosis, improve the molecular classification of NENs, and personalize therapy.

CONCLUSIONS

This PhD project delineated the molecular heterogeneity of neuroendocrine neoplasms through an integrated genomic and transcriptomic approach.
The main findings can be summarised as follows:

1. DLL3 expression is restricted to high-grade NECs and correlates with poor survival, confirming its role as a prognostic marker.
2. TSC2 mutations, low TMB and activation of the PI3K/mTOR pathway are recurrent in well-differentiated NETs, particularly of ileal origin.
3. Transcriptomic analysis highlights immune and extracellular matrix–related pathways as distinctive features of tumor aggressiveness.
4. The integration of clinical, genomic, and transcriptomic data provides a more nuanced molecular classification of NENs, bridging the gap between morphology and biology.

Overall, these results identify potential biomarkers and therapeutic pathways that could guide the future management of neuroendocrine tumors and improve their molecular classification.

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