
# 2022 NETRF Symposium (November 16-18)
# Oral Presentation Abstracts

## Session 1: NET Models

**Patient Derived Tumor Organoid models reveal druggable growth dependencies in Neuroendocrine Cancers**

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***Net Type:*** Multiple NET Types; ***Research Type:*** Basic; ***Keywords:*** Organoids Genomics Drug resistance Biomarkers

***Background:*** Organoid cultures are a powerful model system for the study of cell biology and human disease. We have applied the organoid culture system to study specialized hormone- and neuropeptide-producing cells, neuroendocrine (NE) cells, and the tumors derived from these cells, NE cancers. NE cancers, NE neoplasms (NENs), display a high degree of inter-tumor heterogeneity — comprising highly aggressive carcinomas (NECs) as well as low-grade well-differentiated NE tumors (NETs). Although low and intermediate grade NETs are generally associated with a favorable prognosis, 28% of these tumors present at advanced stages and are unresponsive to therapy. This statistic points to a fundamental gap in our understanding of how these tumors arise and progress, partly due to a lack of robust and faithful experimental systems.
***Methods:*** Using a completely defined growth medium, we have developed the first described patient-derived tumor organoids (PDTOs) from low-grade pulmonary NETs, including a newly defined, clinically aggressive subtype of pulmonary NET, a supra-carcinoid. We have also established PDTOs from an understudied subtype of NEC, large cell NEC (LCNEC) from multiple different tissue sites.
***Results:*** We show that NEN PDTOs maintain the intratumoral heterogeneity and major gene expression patterns of their matched tumors as well as their corresponding tumor subtypes. In a case study fashion, guided by these individual growth factor dependencies, we have performed targeted drug sensitivity analyses on NET and LCNEC PDTO lines and have uncovered therapeutic sensitivities to an inhibitor of NAD salvage biosynthesis and to an inhibitor of BCL-2. Finally, through targeted screening of growth factor components in our NEN PDTO growth media, we identified an EGF-dependency common to a subset of pulmonary NET PDTOs. These findings imply a therapeutically targetable vulnerability for this clinically identifiable subset of NETs.
***Discussion:*** More broadly, our results suggest that different NETs have distinct growth factor dependencies, and further imply trajectories to progression that are reliant on acquired independence specifically from these extrinsic growth signals. Furthermore, our work argues that PDTOs can be used to study intratumor heterogeneity and tumor evolutionary processes in vitro. Our current work is focused on identifying growth factor dependencies in other subsets of pulmonary NETs. We are also using NET PDTOs to model progression to clinically aggressive NET subtypes, including supra-carcinoids.

**Mechanisms, Models, and Treatments for Neuroendocrine Tumors**

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***Net Type:*** Multiple NET Types; ***Research Type:*** Basic; ***Keywords:*** Biomarkers Mouse model

***Background:*** Neuroendocrine tumors (NETs) occur in various forms sporadically or as the consequence of causally-linked mutations. They are generally characterized by their indolent course, debilitating symptoms, and untreatable lethality. Advances that have improved outcomes for these cancers have been limited. Following the serendipitous discovery that mechanisms that cause neurodegeneration in the central nervous system can also cause NET tumorigenesis, we have been studying various types of NETs to understand the mechanistic causes, create novel clinically accurate models, identify diagnostic biomarkers, and test new treatments.
***Methods:*** Experimental models such as human tumors, cell lines, and organ-specific inducible bitransgenic animal models were utilized for the characterization of NETs. Whole exome sequencing, bulk transcriptomics, phosphoproteomics, immunohistochemistry, and tissue microarray profiling were performed.
***Results:*** Here we will summarize some of the most notable advances made in our NET research program. We will overview the models we have created including those for medullary thyroid carcinoma (MTC), pheochromocytoma (PC), and pancreatic NETS (PNETs). We will describe some of our latest mechanistic and multi-omic studies, and present selected findings on the potential of experimental and preclinical treatment approaches.
***Discussion:*** The development of inducible NETs mouse models provides a useful preclinical tool for testing new therapies. The novel downstream effectors of Cdk5 could serve as predictive molecular signatures for the early detection of tumors in patients. The next step involves developing a clinically relevant multiplex assay system that could allow infallible quantitation of biomarker levels from the core biopsies of patient tumors. The extended current and future directions for this research will be discussed, some of which are made possible through NETRF 2022 Accelerator Award. The information, tools, results, databases, models, and drugs described in this study will be openly shared with the NET cancer research communities.

**Improving SBNET therapy by targeting serotonin metabolism**

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***Background:*** Neuroendocrine neoplasms (NENs) are rare cancers that can be well-differentiated neuroendocrine tumors (NETs) or poorly differentiated neuroendocrine carcinomas (NECs). Therapeutic options for patients with NENs are limited, due in part to the difficulty of growing well-differentiated NETs in vitro. Small bowel neuroendocrine tumors (SBNETs) represent over 50% of gastrointestinal NETs and yet few in vitro and in vivo models are available to investigators. To date, surgery remains the only cure for SBNETs since effective medical therapy remains to be identified. To facilitate drug testing, our group have developed a strategy to use patient NET and NEC samples as 3-dimensional spheroid cultures for drug testing. Our screen corroborated that SBNET spheroids are insensitive to many FDA-approved anti-cancer drugs. SBNET highly express serotonin and we recently demonstrated that serotonin inhibition by genetic and pharmacologic interventions decrease tumor formation in vivo.

***Methods:*** Surgically resected SBNETs and PNETs were established in culture as spheroids. Spheroids were embedded in Matrigel and seeded onto 96 well plates. Spheroid cultures were grown in the presence of 3 uM concentration of 175 different compounds (147 FDA-approved anti-cancer drugs, 8 lab selected drugs, and 20 structurally diverse molecules) and levels of growth inhibition measured relative to controls. Drug sensitivity profiles were compared between SBNETs, PNETs, and NECs in order to identify specific classes of inhibitors for each category of NENs. To identify new effective therapy for SBNETs, we genetically and pharmacologically target tryptophan hydroxylase 1 (TPH1), the rate limiting enzyme involved in serotonin biosynthesis using shRNAs specific against TPH1 and the TPH1 small molecule inhibitor telotristat ethyl (TE).

***Results:*** We screened 20 NENs, including 11 SBNETs, 6 PNETs, and 3 NECs. SBNET spheroids displayed the least sensitivity drugs in the panel, with 21 drugs causing >50% growth inhibition. PNETs were sensitive to 51 drugs and NECs to 60 drugs. SBNETs were most sensitive to topoisomerases, while PNETs were sensitive to mTOR inhibitors, tyrosine kinase inhibitors (TKIs), and anti-neoplastic agents. NECs were most sensitive to anti-neoplastics, TKIs, and topoisomerases. There were 21 compounds which all 3 tumor types were sensitive to. SBNET cells are highly resistant to anti-cancer therapies. We improved SBNET drug sensitivity by targeting serotonin metabolism using genetic knockdown approach and TE. In addition, we identified a set of TKI that can improve the anti-tumor effect of TE.

***Discussion:*** Screening well-differentiated NETs for drug sensitivity is possible using in vitro spheroid cultures. Several classes of drugs were identified as important for inhibiting growth of SBNETs, PNETs, and NECs, representing an important advance for drug testing in NENs. Combining metabolic perturbation of serotonin biosynthesis in SBNETs represent a new avenue of therapy for targeting this highly drug resistant cancer.

# Session 2: Genetics

**Immunophenotypic and molecular characterization of pancreatic neuroendocrine tumors producing serotonin**

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***Net Type:*** Pancreas; ***Research Type:*** Basic; ***Keywords:*** Extracellular matrix Genomics

***Background:*** Serotonin producing pancreatic neuroendocrine tumors (SP-PanNET) account for 0.58% to 1.4% of all pancreatic neuroendocrine tumors (PanNET). They may present with atypical symptoms, such as acute pancreatitis and are often radiologically characterized by main pancreatic duct dilatation. SP-PanNET are well differentiated neuroendocrine tumors (NET) distinct from classical PanNET by atypical serotonin secretion and abundant dense stroma deposition, like serotonin producing ileal NET leading in some cases to difficulties to reliably distinguish SP-PanNET from ileal NET metastases. The biology and molecular profile of SP-PanNET remain poorly characterized and the cell of origin within the pancreas is unclear.
***Methods:*** To address these questions, we analyzed a large cohort of SP-PanNET by immunohistochemistry (n=29; ATRX, DAXX, MENIN, Islet1, PAX6, PDX1, ARX, CDX2), whole genome copy number array (Oncoscan™) and a large NGS panel (NovoPM™) (n=10), FISH (n=13) and RNA sequencing (n=24) together with 21 ileal NET and 29 nonfunctioning PanNET (NF-PanNET).
***Results:*** These analyses revealed a unique genomic profile with frequent isolated loss of chromosome 1 (14 cases-61%) and few pathogenic mutations (KMT2C in 2 cases, ARID1A in 1 case). Unsupervised RNAseq-based clustering showed that SP-PanNET were closer to NF-PanNET than ileal NET with an exclusive beta cell-like signature. SP-PanNET showed TGF-ß pathway activation signatures associated with extracellular matrix remodeling and similar signature were reproduced in vitro when pancreatic stellate cells were exposed to serotonin. SP-PanNET immunohistochemical profile resemble that of

ileal NET except for PDX1 and PAX6 expression to a lesser extend suggesting that these two markers may be useful to diagnose SP-PanNET.
***Discussion:*** Taken together, this suggests that SP-PanNET are a very specific PanNET entity with a peculiar biology leading to the characteristic fibrotic aspect.

**Genomic and epigenomic analyses of multifocal ileal neuroendocrine tumors**

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***Net Type:*** Gastrointestinal; ***Research Type:*** Basic; ***Keywords:*** Genomics Epigenetics

***Background:*** Small intestinal neuroendocrine tumors (SI-NETs) are the most common tumors of the small bowel, accounting for ~40% of all small intestinal malignancies. They originate from enterochromaffin cells of the digestive tract. Most SI-NETs are located in the terminal ileum with a high incidence of multiple synchronous primary tumors. Previous high-throughput sequencing studies have reported low somatic mutation rates in SI-NETs. Loss of heterozygosity (LOH) at chromosome (chr) 18 is the most frequent genomic event identified, occurring in ~60% of tumors. The only recurrent mutations reported are CDKN1B loss-of-function mutations in ~10% of tumors. Recently, we showed that synchronous primary tumors from the same SI-NET patient display distinct somatic mutational profiles, suggesting these tumors originate independently. Our results indicate that the tumorigenesis of multifocal SI-NETs is unlikely driven by hitherto discovered genomic alterations and underscore the need of a deeper understanding of the molecular mechanisms that underlie SI-NETs.
***Methods:*** This study comprised of 119 de-identified synchronous primary tumors and metastases, as well as their matched adjacent normal ileal mucosa and/or whole blood specimens, from 14 patients with multifocal ileal NETs. We generated whole-genome sequencing (WGS), RNA-sequencing, Infinium Methylation EPIC array and reduced representation bisulfite sequencing data from these samples. WGS data were used to study germline variation and field cancerization as potential causes of multifocal SI-NETs, whereas RNA-sequencing data were used to analyze differential gene expression

between multifocal SI-NETs, metastases, and normal ileum samples. Additionally, DNA methylation profiling was used to identify aberrant methylation events in multifocal SI-NETs.
***Results:*** Although we identified 278 somatic genomic alterations in normal ileum DNA samples of SI-NET patients compared to blood DNA controls, none of them were recurrent among the patients. Differential gene expression analysis revealed 16,197 statistically significant differentially expressed genes between multifocal SI-NETs and normal ileum samples. Approximately half of these genes were upregulated and half downregulated. We did not observe clear differences in the global gene expression patterns of tumors with and without chr18 LOH, or between metastasized and non-metastasized primary tumors. We also identified a set of receptors that were overexpressed in the SI-NETs and metastases when compared to the normal ileum, suggesting that overexpression of these receptors may be required for the promotion of tumor growth. Differential methylation analysis revealed 483,743 differentially methylated positions (DMPs) between multifocal SI-NETs and normal ileum samples. The majority of DMPs (74.95%) were hypomethylated in tumor samples. DMPs were enriched at intragenic and intronic sites and global hypomethylation was observed across genomic regions. We also detected 20,482 differentially methylated regions (DMRs), of which 3,963 overlapped promoters. Interestingly, few promoter DMRs were detected in chr18 where LOH is frequent.
***Discussion:*** Our results indicate that SI-NETs are unlikely to arise from morphologically normal small intestine as a result of a mosaic genomic event leading to field cancerization. We identified various differentially expressed genes in multifocal SI-NETs compared to normal ileum. The most statistically significant differentially expressed genes were upregulated in SI-NETs. DNA methylation profiling of multifocal SI-NETs revealed that, relative to normal ileum, methylation in CpG-rich regions of SI-NETs is disrupted, most frequently through loss of methylation, which is in line with the differential gene expression analysis. Stratifying DMPs by region revealed that this pattern is maintained regardless of genomic context. Both differential gene expression and methylation analyses provide interesting candidates for in vitro studies.

**Characterising aggressive pulmonary carcinoids through integrative omics analysis within the lungNENomics project**

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***Net Type:*** Lung; ***Research Type:*** Basic; ***Keywords:*** Biomarkers Epigenetics Genomics Tumor microenvironment Organoids Single cell -omics

***Background:*** Pulmonary carcinoids are well differentiated low to intermediate grade lung neuroendocrine tumours (LNETs), that belong to the group of lung neuroendocrine neoplasms which also include highly aggressive lung neuroendocrine carcinomas (LNECs). Carcinoids are further divided into atypical and typical, based on mitotic count and presence of necrosis. Although pulmonary carcinoids show relatively good prognosis in comparison to carcinomas, metastatic disease and relapse do occur. In a previous study we introduced the concept of molecular groups of carcinoids: A (further separated into A1 and A2) and B, which, importantly, contained a mixture of the two histological types. Additionally, we identified six tumours, termed supra-carcinoids, that displayed genuine carcinoid-like morphology, but had clinical and molecular characteristics of LNECs. In comparison to carcinoid A, overall survival rates were lower for the more aggressive carcinoid B and supra-carcinoid tumours. As yet, little is known about the underlying biology or developmental origins of these two groups of aggressive carcinoids, hampering efforts to identify predictive markers and suitable therapeutic options. The focus of our work is therefore to better understand the biological and clinical characteristics of these molecular groups of carcinoids which we aim to do through integrative multi-omic analysis.
***Methods:*** In order to address this aim we have performed comprehensive multi-omic molecular, morphological and clinical characterisation of LNETs. To this end, we have designed the lungNENomics study, an international cohort of over 250 cases of pulmonary carcinoids, enriched for the very rare atypical type, with clinical data and central pathology review by six pathologists, as well as whole-genome sequencing, RNA sequencing, DNA methylation array, and digital spatial profiling data for a subset of these. These data have been combined with previously published LNET and LNEC data to perform integrative analysis using multi-omics factor analysis (MOFA), resulting in a molecular map of lung neuroendocrine neoplasms for exploration.
***Results:*** Through the integration of multi-omic data from 242 pulmonary carcinoids and 74 large cell neuroendocrine carcinoma (LCNEC) with MOFA we obtained five axes (factors) of variation. Visualising the first three factors results in a tetrahedron, with each vertex corresponding to a previously proposed group (A1, A2, B, and LCNEC). Each was characterised by specific clinical and genomic features, with enrichment for older males with MEN1 alterations and chromosome 11 loss in the more aggressive carcinoid B. Factor 2 separated the high grade LCNEC from LNET, and was strongly associated with overall survival and level of immune infiltration (measured by RNA sequencing and digital spatial profiling). Along Factor 2 are a subset of carcinoids clustered deeply within the LCNEC

group (supra-carcinoids), whilst others appeared to straddle the carcinoid/carcinoma boundary, suggesting potential progression from an indolent to a more aggressive phenotype through the acquisition of molecular alterations and changes in microenvironment.
***Discussion:*** While progress has been made in recent years in the molecular characterisation of pulmonary carcinoids, there are many clinically-relevant questions which remain. These can be addressed by investigating all molecular layers, including whole-genome sequencing, that until now has been lacking in carcinoids. The lungNENomics project aims to address these important questions, and having identified unique molecular and morphological characteristics of aggressive pulmonary carcinoids analyses, will continue to improve the biological understanding of tumour development and progression in this exceedingly rare and understudied disease.

**Developmental Lineages and Mediators of Metastasis in PNETs at Single-Cell Resolution**

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***Net Type:*** Pancreas; ***Research Type:*** Translational; ***Keywords:*** Genomics Single cell -omics Tumor microenvironment

***Background:*** Pancreatic neuroendocrine tumors (PNETs) are a diverse set of tumors that derive from the neuroendocrine cells of the pancreatic islets. Some secrete excess hormones and are termed “functional” whereas others do not and are referred to as “non-functional.” Moreover, the clinical behavior of PNETs varies widely but approximately half of cases progress to metastases and cancer-related death after surgery. A clinically-relevant molecular classification for PNETs to predict patient outcomes and guide therapeutic decision-making has been elusive, in part because prior studies have primarily looked at the entire tumor in aggregate, leading to an unknown mixture of cancer cells, immune cells, and other stromal components.
***Methods:*** To this end, we applied single-nucleus RNA-seq (snRNA-seq) to 20 frozen banked surgically-resected primary PNETs, including one neoadjuvant-treated sample, one functional glucagonoma, and one patient with multifocal metastatic well-differentiated PNET for which we profiled the primary tumor and two independent liver metastases over a 6-year time span as well as matched adjacent non-malignant tissue from the pancreas and liver. In parallel, we performed whole exome sequencing (WES) of all the specimens (primary, metastatic, adjacent non-malignant) derived from the patient with multifocal metastatic well-differentiated PNET. This collection of primary and metastatic samples from

the same patient offers a unique opportunity to identify potential somatic drivers of tumorigenesis as well as mediators of tumor dissemination in well-differentiated PNET.
***Results:*** We captured 187,461 nuclei profiles after quality control filtering and identified clusters corresponding to malignant neuroendocrine cells, cancer-associated fibroblasts, endothelial cells, myeloid cells, and lymphocytes, among others. Malignant cells were identified by a combination of expression markers and inferred copy number variations (CNVs), and we learned gene expression programs across malignant cells by performing consensus non-negative matrix factorization. Through WES, we identified 13,178 indels and 200,262 single nucleotide variations (SNVs) in the germline as well as 920 somatic indels and 1,939 somatic SNVs. Across the three malignant samples, we found a high degree of consistency in their mutational signature, including germline mutations in EXO1, MCM9, MSH3, MSH4, PARP4, and MUTYH, which all play a role in the DNA mismatch repair pathway. We found 14 exonic missense somatic mutations across three malignant samples, out of which key regulator genes such as FOXI1 and PHF2 were mutated at a high predicted impact. Furthermore, we found 2 missense exonic mutations in VCX3B and MUC5B and 7 intronic mutations exclusively in metastatic samples.
***Discussion:*** We are investigating the effects of intronic mutations on alternative splicing. We are also in the process of combining the WES and snRNA-seq datasets to examine the accumulation of CNVs during metastasis, generate hypotheses about the clonal evolution of PNETs, and draw correlations between mutational changes and downstream transcriptional states.

# Session 3: Tumor Microenvironment and Immunology

**Vascular regulation of liver metastasis in pancreatic neuroendocrine tumors**

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***Net Type:*** Pancreas; ***Research Type:*** Basic; ***Keywords:*** Tumor immunology Tumor microenvironment Tumor vasculature

***Background:*** Nearly half of pancreatic neuroendocrine tumors (PanNET) patients already possess liver metastases at the time of diagnosis, while many others develop liver metastasis after surgical resection of the primary tumor. Unfortunately, the immunosuppressive microenvironment of PanNET limits the efficacy of current immunotherapies such as immune checkpoint inhibitors for patients with metastatic disease. Thus, there is an urgent need to understand the mechanism(s) of liver metastatic progression and immune evasion that result in resistance to immunotherapies.
***Methods:*** PanNET mouse models: We used a spontaneous PanNET mouse model, RIP-Tag2 (RT2) which expresses the oncogenic SV40 large T antigen under transcriptional control of the insulin promoter and

develops PanNET after birth. We also used an experimental PanNET metastasis model using PanNET cell lines. Manipulation of ANGPT2/Tie2 signaling: For pharmacologic targeting of angiopoietin-2 (ANGPT2)/Tie2 signaling, we used selective antibodies. We also used a conditional knockout mouse for genetic deletion.
***Results:*** 1. Angiopoietin-2 (ANGPT2)-mediated vascular destabilization in human and mouse PanNET is associated with progression of PanNET liver metastases. 2. Blockade of ANGPT2 inhibition suppresses metastatic growth in the liver and improves the survival of mice with metastasis. 3. ANGPT2 inhibition promotes vascular normalization and increases CD8+ T-cell infiltration in PanNET liver metastases. 4. The antimetastatic effects following ANGPT2 inhibition are negated in immunodeficient mice and in CD8+ T-cell-depleted immunocompetent mice.
***Discussion:*** ANGPT2-mediated suppression of Tie2 signaling promotes liver metastatic progression in PanNET through vascular destabilization and consequent impaired CD8+ T-cell infiltration

**Ex vivo expansion of TILs from panNET liver metastasis: in search of novel adoptive transfer strategies for the treatment of NETs**

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***Net Type:*** Pancreas; ***Research Type:*** Translational; ***Keywords:*** Tumor immunology

***Background:*** It is currently unknown whether tumor infiltrating lymphocytes (TILs) from NETs may be expanded to the numbers needed for adoptive infusion in patients.
***Methods:*** Small fragments (median: 11 fragment-derived cultures per patient) from different regions of 10 individual panNET liver metastasis have been subjected to different protocols of TILs isolation/expansion (i.e., tissue enzymatic digestion vs in vitro culture of whole fragments; early CD3 enrichment vs late CD3 enrichment vs no CD3 enrichment; culture in the presence of low-concentration IL-2 vs high-concentration IL-2 vs IL-7/IL-15). TILs were expanded for up to 42 days and were enumerated and phenotyped weekly. At each time point, the expanded cells were analyzed to assess: (i) the immune cell composition (B cells, NK cells, total T cells and T cell subsets); (ii) the % distribution of different T cell differentiation subsets: naïve (TN), stem cell memory (TSCM), central memory (TCM), effector memory (TEM), and effectors (TE); iii) the expression of exhaustion markers including PD-1, CD39, TIGIT and TIM3. Whole-exome sequencing and RNA-sequencing of tumor

fragments is currently ongoing and will be used to define the neoantigen landscape of individual tumors to allow the identification of neoantigen-reactive T cells within the TIL cultures.
***Results:*** The culture of whole tumor fragments (with no CD3 enrichment step) in the presence of IL-2 yielded to the highest number of TILs. TILs were successfully grown from 9/10 patients, and a mean of 400x106 and 5x106 T cells were obtained at the peak of the expansion phase when fresh (n=5) and cryopreserved samples (n=5) were used respectively. There was no difference in the growth rate of TILs on the basis of the tumor grading, Ki-67, or vascular/perineural invasion, while wide differences were observed in terms of T cell yield according to the different tumor regions analyzed. Immediately after digestion, the immune cell infiltration of tumors appeared minimal (approximately 1% of total cell population, range 0.25-2.5%). T lymphocytes were the predominant population to grow in the TIL cultures at the time of cryopreservation (median CD3+: 80%), followed by NK cells (median: 2%) and NKT cells (median: 1%). Among T cells in these TIL cultures, CD4+ was the main subset (median CD4+: 80%), exceeding CD8+ T cells by a median ratio of 8:1. Treg cells represented the 5% and 10% of CD4+ and CD8+ T cells respectively at the time of cryopreservation. The differentiation of T cells changed over time in culture. In particular, we observed a drastic phenotype change in CD8+ T cells after 2 weeks of culture in the presence of IL-2 (such effect was attenuated by culture in the presence of IL-7/IL-15 instead of IL-2), with the emergence of effector memory T cell clones and the disappearance of the originally present terminally differentiated T cell clones. Among exhaustion markers, CD39 and TIGIT were mostly expressed by both CD4+ and CD8+ T cells.
***Discussion:*** Fresh tumor samples can be used to obtain the number of TILs needed for adoptive cell therapy in humans. TCR repertoire skewing may occur during ex vivo culture of T cells from panNET liver metastases.

## Session 4: Tumor Biology and Rare NETs

**Human and murine single-nucleus RNA-seq (snRNA-seq) reveals potential mechanisms of TMEM127-mediated susceptibility to pheochromocytomas**

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***Net Type:*** Pheo/Para; ***Research Type:*** Basic; ***Keywords:*** Single cell -omics

***Background:*** TMEM127 is a tumor suppressor gene that has been linked to inherited susceptibility to pheochromocytomas (pheos), rare neuroendocrine tumors arising from the adrenomedullary cells that

produce catecholamines, epinephrine and norepinephrine. Our previous studies showed that pheos with loss-of-function TMEM127- mutation show a transcriptional profile of kinase signaling activation, similar to pheos carrying mutations in genes that activate MAPK/mTOR signaling. TMEM127 is a ubiquitously expressed transmembrane protein localized to endomembranes. However, the mechanisms of tumorigenesis caused by TMEM127 mutation remain unknown. We generated single nuclei transcriptome profiles (snRNAseq) of human pheos and mouse models of TMEM127 deficiency to gain insights into its cell of origin and potential mechanisms of tumor suppression.
***Methods:*** Materials Five human pheochromocytomas (3 TMEM127 and 2 RET ), obtained after IRB-approved signed consent, and six paired adrenal glands, collected from adult Tmem127 knockout (KO) and wild type (WT) mice under an IACUC approved protocol were used. Methods Single nuclei RNA-seq (snRNA-seq) profiles were generated from the samples above using standard methods, through 10XGenomics and Illumina NovaSeq sequencing, and analyzed using the Seurat R package. Publicly available datasets of fetal and postnatal mouse and human adrenals were used to define cell cluster identity. Western blot and immunofluorescence staining were applied to validate the findings from snRNA-seq.
***Results:*** We generated libraries of 10,000 single nuclei from six adrenal glands from wild type (WT) and Tmem127 knockout (KO). We identified 19 cell clusters in adult mouse adrenals and noted a genotype-related differential proportion of cell types. KO mice had higher abundance and distinct expression signature of epinephrine-secreting chromaffin cells -mature chromaffin cells (MCCs). Single-cell based cell cycle analysis showed higher proliferative potential of KO MCCs. In addition, we prepared 35,752 single nuclei from five pheochromocytomas: 3 TMEM127-mutants and two with mutation in RET, an oncogene involved in pheochromocytoma and other cancers. snRNA-seq from human tumors identified subclusters of chromaffin cells shared by TMEM127-mutant and RET-mutant pheochromocytomas which overlapped with the KO mouse MCCs differentially expressed signature, supporting a conserved profile between mouse and human TMEM127 loss. We also used copy number inference (CNVinfer) to identify prototypical arm level chromosomal changes in TMEM127 and RET mutant pheos and define tumor-specific clusters. This approach uncovered shared chromaffin clusters with the lowest differentiation capacity consistent with putative initiating tumor cells, suggesting that TMEM127-mutant and RET-mutant pheos share a similar cell-of-origin. To find specific drivers of this process, we performed gene regulatory network analysis in mouse nuclei. Among transcription factors which were specifically activated in KO MCCs was Egr1. In support of these findings, we detected higher Egr1 protein in adrenal medulla by immunofluorescence staining, and by Western blot of WT and KO mice. These results were confirmed in two independent expression profile cohorts pheochromocytomas, and additional protein lysates, showing Egr1 upregulation in TMEM127-mutant tumors when compared to TMEM127-intact pheochromocytomas.
***Discussion:*** TMEM127 and RET-mutant pheos share a chromaffin-derived early tumor cell cluster. Tmem127 KO adrenal shows higher proliferation rate and abundance of mature chromaffin cells, similar to human pheos with TMEM127 mutations. The Egr1 transcription factor is higher in murine Tmem127 KO and human tumors carrying TMEM127 mutations. snRNAseq analysis uncovers early tumor cells of origin of pheos and suggests EGR1 as an early driver of adrenomedullary cell proliferation. These preliminary findings shed light on the cell of origin and common drivers in pheochromocytoma tumorigenesis.

**Mesenteric fibrosis in small intestinal neuroendocrine tumours**

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***Net Type:*** Gastrointestinal; ***Research Type:*** Translational; ***Keywords:*** Epigenetics Extracellular matrix Genomics Organoids Tumor microenvironment Biomarkers

***Background:*** Mesenteric fibrosis (MF) in patients with small intestinal neuroendocrine tumors (SI-NETs) causes significant morbidity and mortality and is poorly understood with no treatment option outside of surgery. MF occurs in up to 50% of SI-NET patients and is caused by a metastatic lesion circumscribed by an extensive fibrotic reaction in the mesentery. MF may lead in a significant percentage of patients to intestinal obstruction, oedema, and ischemia, which causes abdominal pain, cachexia and often necessitates surgery. We have previously demonstrated that the overall survival of patients with MF was significantly shorter compared to a control group of patients without mesenteric lymphadenopathy. The pathogenesis of MF in SI-NET is incompletely understood and there are no biomarkers or radiological criteria to predict long-term complications of SI-NET associated MF. The NETRF Accelerator Grant was awarded to the collaborative project from the UCL/Royal Free London and Erasmus MC Rotterdam, two leading groups in Europe with a research interest in this area, utilising skill sets specific to each centre to integrate and validate the understanding of MF pathogenesis. The programme is researching potential molecular markers both diagnostic and predictive of the risk of MF including tissue and blood RNA sequencing, understanding and defining the concept of the fibrosome as well as assessing epigenetic factors and their potential role in MF. The programme is further investigating the molecular pathways implicated in SI-NET carcinogenesis and fibrogenesis assessing the interaction between SI-NET cells and cancer associated fibroblasts as well as the role of sex steroids on these pathways. Understanding the pathways will also lead to development of antifibrotic therapies. To be comprehensive in understanding, predicting and diagnosing MF we are also developing a radiomics tool to determine if this could be predictive of the extent of MF and predictive of outcome. We present the ongoing work related to understanding molecular pathways and development of biomarkers.
***Methods:*** RNA sequencing. Tissue was collected from a cohort of 46 SI-NET patients, including normal SI (n=46), primary SI-NET (n=40) and mesenteric mass (n=38). Patients were classified into 4 groups according to severity of mesenteric fibrosis (none, minimal, mild, severe), based on radiological,

surgical, and histological assessments. RNA was extracted from snap frozen tissue using the RNeasy Mini kit (Qiagen) and RNA sequencing was performed on the NovaSeq instrument (Illumina, San Diego, US). A preranked gene set enrichment analysis (GSEA) was performed using GSEA software version 4.2.3. Gene sets downloaded from the Molecular Signatures database included the C2: REACTOME subset, C6: Oncogenic subset and gene sets with the keywords “small intestine”, “neuroendocrine” or the prefix “fibro-”. Creation of the preranked gene list was achieved using the sign of log2foldchange * -log10(pvalue) from the raw RNA sequencing data. A leading-edge analysis was also performed to determine genes involved in the enrichment of several reactomes. 3D model for SI-NET - fibroblast interaction. Fibroblasts (HPF) were transduced with the virus construct pLenti6.3, containing the fluorescent dsRED. Selection of fluorescent cells was performed using the antibiotic blasticidin and cell sorting. Similarly, dsRED-labeled GOT1 (SI-NET) cells were generated. 3D spheroids were formed with a combination of dsRED/fibroblasts and equal numbers of GOT1 cells. After 7 days spheroid size was measured, and cell numbers were analyzed using FACS analysis. Gene expression of NETest-fibrosome markers. RNA was extracted from human samples as described above and reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher). Q-PCR reactions were run using the TaqMan Fast Advanced Master Mix (Thermo Fisher) with Assays-on-Demand primers for CTGF, CD59, APLP2, FZD7, BNIP3L, and housekeeping genes HPRT1, ACTB, GUSB. Gene expression was normalized using the geometric mean of HPRT1, ACTB and GUSB.

***Results:*** RNA sequencing. The GSEA produced 25 different comparisons of which we focused on 18. Any comparisons involving the group of patients with no fibrosis were excluded due to low sample size (n=3). We analysed the GSEA results by highlighting enriched reactomes that are involved in the extracellular matrix or in the development and progression of fibrosis. Examples of these included Reactome: Extracellular matrix organisation, Mishra Carcinoma Associated Fibroblast Up. For this we deemed an FDR value of 0.25 and a p value 0.05 to be significant. Next, a gene list was generated using ‘core enriched’ genes – as determined by the GSEA software – from the highlighted reactomes. Only genes with a p value of < 0.05 and thus considered significant in the RNA sequencing data were included. Further analysis of these target genes is ongoing including a literature search and STRING analysis to determine important genes to take forward into future in vitro research. 3D model for SI-NET - fibroblast interaction. We obtained fluorescent (dsRED) fibroblasts and GOT-1 cells (>95% pure). Size of combined GOT1–dsRED/fibroblast spheroids was higher (increase 45±5%), compared to the sum of spheroid size of the single populations. The presence of dsRED/fibroblasts did not significantly increase the number of GOT1 cells (+37%). However, the presence of GOT1 cells significantly stimulated the number of fibroblasts by 475±49%. Preliminary results show the successful establishment of primary SI-NET spheroids. Gene expression of NETest-fibrosome markers. We have previously shown that a subset of fibrosis-related markers included in the diagnostic panel NETest – CTGF, CD59, APLP2, FZD7, BNIP3L – could detect microscopic MF with 100% accuracy. Gene expression analysis of the five transcripts showed significant upregulation of CTGF, BNIP3L and CD59 in the mesenteric mass compared to normal tissue across all fibrosis groups. Furthermore, CTGF and BNIP3L were also significantly upregulated in the mesenteric mass compared to the primary tissue in all fibrosis groups.

***Discussion:*** GSEA analysis of sequenced human samples showed changes in expression of fibrosis-related genes between normal SI, primary SI-NET and mesenteric mass across different fibrosis severity groups. Relevant gene targets will be further studied through in vitro models of SI-NET-fibroblast

interaction. Epigenetic analysis on the same cohort of patients is ongoing. The established 3D spheroid model will be used to evaluate the interaction between SI-NET derived cancer associated fibroblasts (CAFs) and SI-NET cells, as well as the secreted proteome. qPCR analysis of NETest-fibrosome markers confirmed significant upregulation of several genes in the mesenteric mass of patients across all fibrosis groups. The predictive value of radiomics for complications of the mesenteric mass and fibrosis will be assessed in the Royal Free Hospital SI-NET cohort and compared to results in the Erasmus MC SI-NET cohort.

**The role of CCL2 and IL-8 in the microenvironment of pituitary neuroendocrine tumors**

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***Net Type:*** Multiple NET Types; ***Research Type:*** Translational; ***Keywords:*** Tumor microenvironment Tumor immunology

***Background:*** Pituitary neuroendocrine tumors (PitNETs) are usually benign, but may cause significant morbidity due to mass effects and/or excessive or low hormone secretion. The interaction between tumor and non-tumoral cells in the microenvironment is mediated by cytokines, which may contribute for tumor proliferation, invasion and aggressiveness. Our previous work assessed the PitNET cytokine secretome, and identified CCL2 and IL-8 as key PitNET-derived cytokines, but their biological and diagnostic/prognostic significance remains unknown. We aimed to study the role of CCL2 and IL-8 in PitNETs, particularly in determining the clinical phenotype and outcomes of PitNET patients.
***Methods:*** CCL2 and IL8 expression was studied by RT-qPCR using RNA extracted from 96 fresh-frozen PitNET tissues derived from patients who underwent surgery at our center between 2014-2020: 67 non-functioning PitNETs (NF-PitNETs), 20 somatotropinomas, 6 corticotropinomas, 2 prolactinomas

and 1 thyrotropinoma. CCL2 and IL8 mRNA levels were then correlated with clinico-pathological, hormone and outcome data from the corresponding PitNET patients.
***Results:*** In our cohort, 46.9% were males, age at diagnosis was 55±15yr (mean±SD), and mean follow-up duration was 77±47months. CCL2 and IL8 expression levels correlated positively (rho=0.433; p<0.001), and overall, the CCL2 expression was 3-fold lower than IL8. CCL2 expression was higher in somatotropinomas than other PitNET subtypes (p=0.015). In the whole PitNET cohort, higher expression of CCL2 was seen in males, in patients who had hypopituitarism and lower serum cortisol at diagnosis, as well as in patients who required multimodal therapy and had active disease at last-follow-up. Within the 67 NF-PitNET patients, similar observations were seen, with a noteworthy trend for association between higher CCL2 expression and presence of tumor remnant on MRI within 1-year post-operatively (p=0.084). However, most significant associations were found in the somatotropinoma subgroup. Higher expression levels of CCL2 were seen in acromegaly patients who: i) had hypopituitarism at diagnosis (p=0.027); ii) had more pituitary hormone deficiencies at diagnosis (p=0.020); iii) required multimodal therapy (p=0.005); iv) received more treatments (p=0.039); v) had active disease at last follow-up (p=0.041); and vi) had higher IGF-1 levels at last follow-up (p=0.020). Moreover, acromegaly patients who required radiotherapy tended to display higher expression levels of CCL2 (p=0.093). There were no significant correlations between IL8 expression levels and clinical/outcome features in the whole PitNET cohort, neither among NF-PitNET or somatotropinoma subgroups.
***Discussion:*** Our data suggest that the expression of CCL2, but not IL8, may have a relevant biological role in PitNETs leading to more refractory and difficult-to-treat PitNET disease, particularly in patients with acromegaly.

**Defining distinct molecular subtypes of high-grade neuroendocrine carcinomas to predict therapeutic vulnerabilities.**

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***Net Type:*** Multiple NET Types; ***Research Type:*** Translational; ***Keywords:*** Single cell -omics Patient-Derived Xenografts

***Background:*** High-grade neuroendocrine carcinomas (hgNECs) are clinically aggressive high-grade carcinomas most commonly arising from the respiratory and gastrointestinal tracts, but, more rarely, from sites such as cervix, urinary bladder, prostate, and ovary. Owing to the rarity of most organ-

specific extrapulmonary hgNECs, there are few evidence-based treatment paradigms beyond those extrapolated from small cell lung cancer (SCLC). Unfortunately, inadequate access to molecular data, tumor tissue, and, most importantly models, from rare hgNECs, particularly extrapulmonary hgNECs, is a fundamental obstacle inhibiting more tumor- and patient-specific approaches to each disease. Along with several other labs, we have pioneered the use of circulating tumor cells (CTCs) from pulmonary hgNEC patients to generate patient-derived xenografts, which provide a renewable source of tissue for molecular and therapeutic analyses. We proposed a focused effort on identifying extrapulmonary hgNEC to generate novel models of these rare tumors, as well as libraries of tissue and plasma for collaborative efforts.

***Methods:*** Using an Artificial Intelligence-based approach, we identified patients with active diagnosis of extrapulmonary hgNEC. These patients were then electively consented to a series of research protocols, including those modified or created specifically for this project, which provide access to (1) patient clinical information, (2) archival tissue, (3) samples from future standard-of-care and research biopsies, and (4) serial plasma samples for CTCs and circulating tumor DNA. For each fresh blood or tissue sample, CTCs or tissue are transplanted into the flank of immunocompromised mice and mice for generation of patient-derived xenograft model. In parallel, disassociated cells are placed in media to culture immortalized cancer cell lines. Fresh tissue samples are always processed for single-cell RNAseq, while additional tissue and plasma samples are banked as frozen or formalin-fixed paraffin embedded (tissue). Blood is collected in 1) Streck tubes for plasma/buffy coat isolation, CTC analysis (Epic Sciences), and banking and 2) heparin tubes for peripheral blood mononuclear cell isolation and cryopreservation. These samples can be utilized for downstream molecular analyses, including whole exome sequencing, RNAseq, immunohistochemistry, T-cell repertoire profiling, CTC, and ctDNA analyses.

***Results:*** To date, we have identified and consented 26 patients with diagnoses of extrapulmonary hgNEC, as well as several patients with rare pulmonary hgNECs other than classical SCLC. These patients include patients with small cell carcinoma of the cervix, thymus, head/neck, and gastrointestinal tract, as well as Merkel cell carcinoma. For each, we have banked at least one blood sample. Additionally, 10 fresh tissue samples have been collected from these patients at the time of biopsies. We have already established seven xenograft models from tissue and CTCs collected from this cohort. Furthermore, in cases where freshly procured tissue was adequate, single-cell RNAseq was performed. Relative to SCLC, our single-cell RNAseq on hgNECs were notable for both their similarities – including the presence of similar subtype-defining transcription factors, presence of unique cell populations, and a range of neuroendocrine and inflammatory features – as well as their distinctions, including common expression of the transcription factor YAP1 - a feature that may underlie chemotherapy-resistant persister cells, but may also predict response to immunotherapy.

***Discussion:*** Despite their rarity, we were able to identify a large cohort of patients with extrapulmonary hgNEC at a single institution over the past ~1 year. We have established a large repository of banked tissue and plasma samples for bulk molecular analysis. This inter-tumoral information will be viewed in the context of the intra-tumoral data from our single-cell RNAseq to provide an ever-expanding molecular atlas of these tumors. More importantly, we have already generated several models, with the potential for more, which represent some of the first, if not the first patient-derived models from these tumor types. These resources will be made available throughout the NET community for collaborative efforts.

**All-Trans Retinoic Acid Radiosensitizes Neuroendocrine Tumor Cells via Peptidyl-prolyl cis-trans isomerase 1 Inhibition**

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***Presenting Author:*** Xavier M. Keutgen (Virtual)
***Net Type:*** Multiple NET Types; ***Research Type:*** Basic; ***Keywords:*** Combinatorial drug treatments Metastasis Theranostics

***Background:*** Peptide receptor radionuclide therapy (PRRT) is a promising radiation based therapy for metastatic neuroendocrine tumors (NETs) but remains palliative. Peptidyl-prolyl cis-trans isomerase (Pin1) is an evolutionally conserved enzyme that catalyzes the cis-trans isomerization of phosphorylated serine/threonine-proline motifs of its substrates and has recently been involved in DNA double strand break (DSB) repair in BRCA-proficient breast cancer cells. Here we study whether Pin1-inhibition with All-Trans Retinoic Acid (ATRA) radiosensitizes NET cells.
***Methods:*** The pancreatic and lung NET cell lines QGP1, BON1 and NCI-H727 were treated with 4Gy of radiation (IR) and either 50nM or 100nM of ATRA based on dose response curves. The poly (ADP-ribose) polymerase 1 inhibitor (PARPi) Talazoparib (10nM) was added to QGP1 cells to evaluate the additive vs. synergistic effects with ATRA and IR. Pin1 knockdown using siRNA, and BRCA1 and gH2AX western blot were used to determine mechanistic effects. Retinoic Acid Receptor (RAR)-alpha status was determined in cell lines using RT-PCR.
***Results:*** ATRA treatment alone showed a significant decrease in tumor cell viability in QGP1 ($p=0.013$), BON1 ($p=0.0001$), and NCI-H727 ($p=0.0003$). Combining ATRA + IR yielded further significant decrease in cell viability vs. IR alone (QGP1 ($p=0.0001$), BON1 ($p=0.0001$), NCI-H727 ($p=0.0003$)). ATRA synergized with Talazoparib and IR in QGP1 cells ($p<0.0001$). Pin1 knockdown with siRNA + IR further decreased cell viability in QGP1 ($p=0.0002$) and BON-1 ($p= 0.015$) cells when compared to IR alone, suggesting that ATRA radiosensitizes NET cells through Pin1 inhibition. ATRA also decreased BRCA1 mRNA levels in QGP1 cells after IR but this did not reach statistical significance ($p=0.3634$) and increased DNA double strand breaks as evidenced by increased gH2AX mRNA and protein expression after treatment. RAR alpha was highly expressed in all 3 cells lines with an average cycle threshold (CT) values of 20.42, 21.44, and 22.90 in QGP1, BON1, and NCI-H727 respectively.
***Discussion:*** ATRA radiosensitizes pancreas and lung NET cells through Pin1-inhibition and decreases BRCA1 levels. This ATRA-induced BRCA1-deficient phenotype synergizes with PARP1 inhibition and IR. Further studies will focus on validating these results in animal models.

## Session 5: Clinical and Theranostic Studies

**Digital Image Analysis in Prediction of Midgut and Pancreatic NET Outcomes**

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***Net Type:*** Multiple; ***Research Type:*** Clinical; ***Keywords:*** Clinical Studies

**Background.** Neuroendocrine tumors (NETs) have become an increasingly prevalent cancer, with a six-fold rise in incidence during the last several decades. Additionally, the prolonged survival contributes to high prevalence with recent estimates of nearly 200,000 cases in the U.S. Nearly half of NET patients present with late stage disease, of whom approximately three quarters derive from the midgut or pancreas and have substantial cancer-related morbidity and mortality. Clinical outcomes of NET patients are variable due to heterogeneous tumor biology, ranging from indolent to highly aggressive. Although tumor stage and grade are used to predict outcomes in NET patients, patients with similar stage and grade may still have variability in clinical course. For this reason, there is great need for additional prognostic markers to guide treatment decisions.
Systems pathology approaches, developed for other cancers, may further aid in predicting NET outcomes. Systems pathology involves the use of artificial intelligence (AI), advanced computer science and mathematical techniques, and state of the art light and immunofluorescence digital microscopy technology to identify novel morphologic features and protein expression profiles of clinical significance. Novel object-oriented image analyses using features such as tissue architecture, tumor cell distribution, and composition can provide information beyond the usual pathologic staging system. In this study, we applied PreciseDx image analysis algorithms that have been applied and validated in breast and prostate cancer. This program will allow us to identify and quantify histologic features (e.g., architectural patterns, tumor cell distribution), and correlate them with clinical features and outcomes. The final goal of our study is to develop and validate a comprehensive staging system which incorporates features from imaging analysis that can more accurately discriminate prognosis of patients with metastatic midgut and pancreatic NETs.
**Materials/Methods.** We applied the PreciseDx epithelium, nuclei, and mitotic figure detector neural networks to an initial 10 cases of pancreatic NETs and 10 cases of small bowel NETs with encouraging results. The neural networks were trained using over 300 breast and prostate slides that were annotated manually for nuclei, epithelium, and mitotic figures. The results are then compared against ground truth annotations performed by experienced pathologists. Performance was determined using the Dice overlap measure for pixel labels for the task of detecting epithelium and nuclei. We used object detection precision and recall for measuring the performance in the mitotic figure task. We also reviewed the charts of all midgut and pancreatic NET patients with pathological specimens at the

Mount Sinai Hospital and assessed baseline demographic characteristics. Tumor stage, grade, treatment, and outcome data were also assessed. Tumor grade was assigned as per 2019 WHO grading classification as G1, G2, and G3. Models incorporating clinical, histologic and image analysis features were created using sure independence screening (SIS).

**Results.** We identified 352 patients with midgut and pancreatic NETs with pathologic specimens available for review at MSH which were be divided into training (246) and validation (106) sets. The mean age was 59 years, with 52% female and 62% white. Median progression-free survival was 47 months. Midgut tumors represented 66%, while pancreatic NETs comprised 34%. Stage I, II, III, IV were 14%, 18%, 27%, and 41% of patients. The majority were low grade tumors, with 70% G1 , 27% G2, and 3% G3 tumors.

To test the accuracy of the PreciseDx mitotic figure, epithelial, and nuclear detectors on NETs we identified a development set of 20 cases (10 pancreatic NETs [4 G1, 4 G2, 2 G3] and 10 small bowel NET [5 G1, 5 G2]) to analyze using this neural network. The mitotic figure detector was evaluated on 20 NET slides with precision of 0.969 and recall of 0.573, yielding F1 = 0.720. Our epithelium detector was evaluated on 20 NET slides with a Dice overlap of 0.841. The nuclei detector was evaluated on 9 NET slides with Dice overlap of 0.841. Annotations derived during evaluation of these 20 cases were integrated into the neural network to further improve its efficacy in evaluation of neuroendocrine tumors and applied to the training set of tumors.

A preliminary training set model incorporating clinical, histologic and image analysis morphologic characteristics was created from an initial pool of 1048 features which was narrowed to 170 features using a concordance index (CI) cut off of 0.58. A model was created using SIS that included 7 features [5 image analysis features (3 mitotic features and 2 nuclear features) as well as tumor size and metastatic stage. A clinical model based only on patient age, tumor stage, tumor size and tumor grade was created for comparison. The novel preliminary model incorporating image analysis features (CI 0.75, sensitivity 0.71, specificity 0.77) outperformed the clinical model (CI 0.65, sensitivity 0.55, specificity 0.70) in predicting clinical outcome.

**Conclusions.** We show that the epithelial and mitotic figure detectors developed for other tumor types are applicable to pancreatic and small bowel NETs. We further optimized these algorithms for NETs and used these derived features to develop a preliminary model based on our training set of 246 patients that more accurately predicts clinical outcome compared to a model based on current practice based on tumor grade and stage. This model will be further evaluated and applied to our validation cohort.

**Transcriptomic Influences of Racial Disparities in Pancreatic Neuroendocrine Tumors**

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***Background:*** There are known disparities in outcomes between Black and White patients with pancreatic neuroendocrine tumors (pNETs). Recently, Black patients have been shown to have higher rates of lymph node metastasis in smaller tumors than White patients, indicating possible differences in tumor biology. Numerous prognostic gene expression differences between racial groups have been reported in other cancers, but no such analysis has been conducted in pNETs. This study aims to evaluate pNET transcriptomes for differential expression that may be influencing racially disparate outcomes.
***Methods:*** Quality control of archival resected pNETs and demarcation of cancer cells were performed by a board-certified pathologist. Cells were laser microdissected from formalin-fixed, paraffin-embedded specimens, and RNA isolated. Sequencing was performed on an Illumina NextSeq550 at 30 million reads/sample. Alignments to the GRCh38 transcriptome annotation were performed using Salmon and differentially expressed genes (DEG's) determined using DESeq2. Significant DEGs were determined by FDR-adjusted p-value (q-value; qv) < 0.05 and log2 fold-change (log2FC) = ±2. Gene set enrichment analysis was performed using clusterProfiler and the Gene Ontology (GO) consortium gene sets. Ingenuity Pathway Analysis (IPA) was then conducted to determine regulator effect networks.
***Results:*** RNA sequencing was conducted on 14 and 16 grade and sex-matched primary pNETs from self-identified Black and White patients, respectively. Mean age was 51 for Black and 56 for White patients. 11/16 (69%) of White and 9/14 (64%) of Black patients were female. 8 Black patients and 8 White patients had grade 1 tumors, while 6 Black patients and 8 White patients had Grade 2 tumors. Metastatic disease was present in 4 Black and 5 White patients. Overall, 372 genes were significantly differentially expressed and 179 GO gene sets were differentially enriched between groups. Notably, among the top 10 most significantly enriched biological processes were: angiogenesis/blood vessel and vasculature development (qv=1.34e-07, normalized enrichment score [NES]=1.89), positive regulation of cell migration and locomotion (qv=1.34e-07, NES=1.91), and humoral immune response (qv= 9.8e-07, NES=-2.06). Among the top 5 regulator effect networks identified by IPA were: angiogenesis of lesion/cell movement of monocytes (consistency score [CS]=19.3), activation of blood cells (CS=18.9), and activation of cells (17.9).
***Discussion:*** Numerous gene sets and pathways related to blood vessel development and cellular migration, representing key elements in the development of metastatic disease, are significantly enriched in pNETs from Black patients. Additionally, gene sets and pathways related to the immune response are downregulated in Black patients. These data indicate differences in tumor biology that may influence disparate outcomes reported in Black patients with pNETS. Sequencing of additional samples and incorporation of genetic ancestry are necessary to validate these findings.

## A Closer Look: Fluorescent Analogs of Clinical Stage PRRT Agents Reveal Specific Binding to Multipotent Bone Marrow Stem Cells

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**Introduction**: Peptide Receptor Radionuclide Therapy (PRRT) with the somatostatin receptor subtype 2 (SSTR2) antagonist $^{177}$Lu-DOTA-JR11 has shown promising results for the treatment of neuroendocrine tumors (NET)[1-3]. However, hematologic toxicity occurred when patients received a macroscopic bone marrow dose of ≥ 1.44 Gy - <2 Gy, which was considered a well-tolerated dose for the agonist $^{177}$Lu-DOTA-TATE[2]. We hypothesize that $^{177}$Lu-DOTA-JR11 binds with high affinity to a small SSTR2(+) subpopulation of hematopoietic stem cells, exerting increased hematotoxicity compared to agonist-PRRT. Here, our goal was to synthesize multimodal JR11-based analogs with comparable binding characteristics to DOTA-JR11 to enable investigations from the cellular to the whole-body level. Suitable candidates were subsequently used to investigate binding patterns to bone marrow stem cells.

**Methods:** We synthesized multimodal DOTA-JR11/TOC analogs by replacing DOTA with the azide-containing cyclen analog, multimodality chelator (MMC)[4], and conjugating DBCO-functionalized dyes using copper-free click chemistry. We conjugated 5 different dyes: AF488 and 4 variants of SulfoCy5 with either 0, 2, 3 or 4 sulfo groups (SxCy5; x=number of sulfo groups), resulting in 5 MMC(Dye)-JR11 and 5 MMC(Dye)-TOC variants that were characterized in comparison to DOTA-JR11/TOC. For *in vitro* experiments, we used HCT116-SSTR2 and -WT (SSTR2 negative) cells. We measured cellular uptake with radioligand assays after $^{177}$Lu-labeling. We also determined the binding kinetics using $K_D$ and $IC_{50}$ assays. Further characterization of compounds based on their fluorescence was done using microscopy and flow cytometry. Compound sensitivity was determined by analyzing mean fluorescence intensities (MFI). Binding of multimodal analogs (0-25 µM) was compared in cells with varying SSTR2 expression (HCT116-SSTR2 > AR42J > H446 > H69) and HCT116-WT. MMC(Dye)-JR11/TOC analogs were also added to pre-mixed cultures of SSTR2(+)/SSTR2(-) cells to identify the analog with the highest detection sensitivity for SSTR2(+) cell populations. To analyze binding to bone marrow stem cell populations, isolated peripheral blood mononuclear cells (PBMCs) were stained for CD34, CD38, CD45RA and CD90, before incubation with MMC(Dye)-JR11/TOC. All experiments were repeated at least three times.

**Results:** Following the successful synthesis of multimodal MMC(Dye)-JR11/TOC analogs, we compared their binding characteristics to DOTA-JR11/TOC. Binding affinity of the $^{177}$Lu-MMC(SxCy5)-JR11 series ($K_D$-values of 16.6 nM, 19.7 nM, 9.7 nM for x=2, 3 or 4, respectively) was similar to DOTA-JR11 ($K_D$: 9.2 nM). We confirmed via fluorescence microscopy that antagonistic (JR11) and agonistic (TOC) properties were conserved upon dye conjugation. Cellular uptake and binding specificity of our lead compound $^{177}$Lu-MMC(S2Cy5)-JR11/TOC were similar to $^{177}$Lu-DOTA-JR11/TOC. In flow cytometry, the antagonists consistently showed higher binding than the agonists MMC(S2Cy5)-TOC across a panel of cell lines. We successfully established protocols to identify 5 and 25% of SSTR2-positive cells in pre-mixed cell populations of tumor cells. Analysis of binding to human bone marrow cells revealed specific, blockable

binding to CD34+ PBMCs, but not to CD34- PBMC. Within the CD34+ population, specific binding was observed to long-term (LT-HSC) and short-term (ST-HSC) multipotent stem cells and multipotent progenitor cells, but not to lineage-committed progenitors. In all cases, the antagonist showed 2-5fold higher binding than the agonist, similar to tumor cells. LT-HSCs showed the highest binding levels, which were only 10-20% lower than AR42J cells, which are characterized as high SSTR2 expressing cell line. LT-HSC make up only 0.05-0.1% of the PBMCs, but if ablated, will lead to pancytopenia, as observed after multiple cycles of 177Lu-DOTA-JR11 despite a tolerable total red marrow dose.
**Conclusion**: We have successfully synthesized multimodal variants of clinically used PRRT agents and established protocols to investigate their binding to human bone marrow stem cell subpopulations. Our results indicate SSTR2-mediated binding to multipotent stem cells and progenitors. Similar to tumor cells, the antagonist JR11 showed 2-5-fold higher binding compared to the agonist, which would not be detectable on dosimetry. Based on our finding, we plan to fine-tune treatment protocols to block binding of PRRT agents to bone marrow stem cells without tumor treatment.
**Acknowledgements:** *We thank the Neuroendocrine Tumor Research Foundation (NETRF) and Education and Research Foundation for Nuclear Medicine and Molecular Imaging (ERF) for funding this study with a Nuclear Medicine Pilot grant. We also thank Dr. Johannes Notni for his support with the radiochemistry.*

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## The Wnt pathway protein Dvl1 targets Sstr2 for lysosome-dependent degradation

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***Background:*** Somatostatin receptor 2 (Sstr2) agonists are primary pharmacological therapies to suppress hormonal secretion and tumor cell proliferation in neuroendocrine tumors (NETs). In recent years, it has also been demonstrated that coupling somatostatin agonists to therapeutic radionuclides, known as peptide-receptor-radionuclide therapy (PRRT), is an effective approach to treat both locally

advanced and metastatic tumors in these patients. However, these therapeutic approaches are not universally successful in NET patients. Thus, novel approaches to improve the efficacy of Sstr2-directed therapies are needed and could offer exceptional clinical impact. A clear limiting factor in Sstr2 agonist therapy is the availability of receptor on the tumor cell surface. In general, Sstr2 is highly expressed in many NETs, where its expression is a defining characteristic. However, Sstr2 expression can vary considerably amongst patients. Mechanisms underlying the variability of Sstr2 expression in GEP NETs are not well understood. Sstr2 is a G protein coupled receptor (GPCR) that is rapidly internalized following agonist treatment and is largely thought to be completely recycled to the plasma membrane after stimulation. However, we have made the novel and unexpected observation that the essential Wnt pathway signaling protein Dishevelled 1 (Dvl1) interacts with Sstr2 and targets it for lysosomal degradation. As Wnt signaling is aberrantly activated in many cancers, this mechanism offers a cogent explanation for the variable expression of Sstr2 in NETs and may ultimately offer a path to enhancing receptor expression to potentiate Sstr2-directed therapies.

***Methods:*** Cells, transfections, and plasmids: HEK293, IMR32, and H69 cells were maintained in DMEM with 10% FBS in 5% CO2. Transient transfections were performed using JetPrime. siRNA transfections were performed via reverse transfection using INTERFERin. The triple HA-epitope tagged rat wild type Sstr2 plasmid was as described (PMID: 33313679). Human Dvl1, 2 and 3 were from Addgene. Immunoprecipitation and Western blotting: Cells were scraped into cold PBS with 1 mM PMSF and 100 nM okadaic acid and lysed in buffer containing 0.5% sodium deoxycholate and 0.5% C12E8 for 30 minutes at 4°C. HA-Sstr2 or endogenous Sstr2 was immunoprecipitated with anti-HA or anti-Sstr2 (PA3-109), respectively. Immunoprecipitates were washed 3 times with lysis buffer, eluted in urea sample buffer, heated at 65ºC, and resolved by SDS-PAGE. After transfer to PVDF membrane, immunoprecipitates were tested by western blotting using ECL, and visualized with X-ray film or via digital ECL detection (Azure C280). Proximity Ligation Assay: Interactions between endogenous Sstr2 and Dvl1 were analyzed by DuoLink Proximity Ligation Assay (PLA). IMR32 cells were stimulated with 100 nM SS14 for the indicated times. Coverslips were washed with cold PBS and fixed with cold methanol. PLA was performed using antibodies to Sstr2 and Dvl1, and cells were stained with 1 µg/mL DAPI. Coverslips were mounted with Prolong Gold Antifade reagent and images were captured using a Zeiss Axioskop 40 microscope equipped with a Zeiss AxioCam MRm MC100 Spot digital camera and AxioVision software. A minimum of 25 cells from 3 separate fields were quantified per experiment. Positive cells were defined as those with a minimum of 6 puncta per cell. Immunofluorescence and Microscopy: Cells were fixed with PFA, permeabilized with 0.3% Triton in PBS, and stained with antibodies to Sstr2, Dvl1, and Lamp1. Some cells were treated with NH4Cl to inhibit lysosome function prior to fixation. Cells were visualized using a Nikon A!R confocal microsope. Co-localization was assessed by Pearson's coefficient.

***Results:*** We have shown previously that the Dvl1 PDZ domain interacts with a phosphorylated peptide corresponding to the PDZ domain binding site of Sstr2 (Carr H.S. et al; PMID: 33313679). In the present work, we assessed the regulation and phenotypic consequences of this interaction. We found that both endogenous and transfected Dvl1 interacts with Sstr2 in the absence of Sstr2 agonist stimulation, and that treatment with agonist only marginally increases this interaction. Knockdown of Dvl1 does not affect receptor internalization or recycling. These data suggest that Dvl1 does not play an important role in regulating agonist-stimulated receptor trafficking. However, we did find that Dvl1 overexpression dramatically inhibits Sstr2 expression and that siRNA-mediated knockdown of Dvl1

enhances receptor expression. Dvl1-dependent downregulation of Sstr2 expression can be rescued by lysosome inhibition, and Sstr2 co-localizes with lysosomes only when lysosome function is blocked. Sstr2 is ubiquitylated in the absence of agonist and knockdown of Dvl1 inhibits Sstr2 ubiquitylation. Stimulation of cells with Wnts, which activate Dvl1, also promotes Sstr2 degradation. Notably, treatment of cells with small molecule inhibitors that block Dvl1 activation enhance transfected and endogenous Sstr2 expression.
***Discussion:*** We conclude that Dvl1 interacts with Sstr2 in the absence of receptor agonists to promote Sstr2 ubiquitylation and lysosomal degradation. Moreover, Dvl1 requires activation by signaling pathways normally activated by Wnts. Wnt signaling is often enhanced in many tumor types, particularly as they progress to more aggressive phenotypes. Importantly, small molecule inhibition of Dvl1 activation enhances Sstr2 expression in cultured cells, suggesting that Dvl1 inhibition in vivo may be a viable avenue to enhance Sstr2 expression and potentiate Sstr2-directed therapies in NET patients.

**uPAR-PET in Neuroendocrine Tumor Patients: Final Results from a prospective Phase II Trial and its Implications for uPAR-targeted Radionuclide Therapy**

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**Background**: As many neuroendocrine neoplasms (NENs) express somatostatin receptors (SSRs) as seen on somatostatin receptor imaging with PET (SRI-PET), radiotracers targeting somatostatin receptors (SSRs), SRI-PET, has proven useful peptide receptor radionuclide therapy (PRRT) targeting SSRs with 177Lu-DOTATATE is used in NEN patients. However, many patients, as evaluated on SRI do not express SSRs or the expression is too low to be eligible for SSR-targeting PRRT. Also, the response rates for SSR-PRRT are only modest.
Therefore, to offer more NEN patients the advantages of PRRT, therapy targeting a biomarker that is expressed in most NEN patients including G3/neuroendocrine carcinomas (NECs), which have no or low SSR expression, would be of paramount interest. We proposed the urokinase-type plasminogen activator receptor (uPAR) to be this new theranostic target. uPAR is involved in metastatic spread and is thus a marker of tumor aggressiveness.
**Materials and Methods**: In a prospective phase II clinical trial, we included 116 patients with NENs of all grades of which 96 subsequently had uPAR PET/CT performed. PET/CT was performed 20 min after injection of approximately 200 MBq of 68Ga-NOTA-AE105. uPAR target-to-liver ratio was used to define lesions as uPAR-positive when lesion SUVmax-to-liver SUVmean ratio was at least 2. Patients were followed for at least one year to assess progression-free (PFS) and overall survival (OS).

**Results**: Most patients had small intestinal NENs (n = 61) and metastatic disease (n = 86). uPAR-positive lesions were seen in 68% (n = 65) of all patients and in 75% (n = 18) of patients with high-grade (grade 3) NENs. During follow-up (median, 28 mo), 59 patients (62%) experienced progressive disease and 28 patients (30%) died. High uPAR expression, defined as a uPAR target-to-liver ratio above median, had a hazard ratio of 1.87 (95% CI, 1.11-3.17) and 2.64 (95% CI, 1.19-5.88) for PFS and OS, respectively ($P < 0.05$ for both).

**Conclusion/Next steps**: uPAR-PET revealed uPAR-positive lesions in most NEN patients regardless of grade and notably also in patients with high-grade NENs. uPAR expression was associated with a worse prognosis. We suggest that uPAR PET is relevant for risk stratification and that uPAR PRRT may become a valuable option for NEN patients. Our work, generously supported by the NETRF, on developing uPAR-targeted therapy for NEN patients continues.