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White paper formatted for Endocrine Oncology

NET models meeting 2024 White Paper: the current state of neuroendocrine tumour research models and our future aspirations

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Abstract (249 words, limit 250)

Current models for the study of neuroendocrine tumours (NETs) are severely limited. While *in vitro* (e.g. cell lines), *ex vivo* (e.g. organoids), and *in vivo* (e.g. mice) models all exist, each has limitations. To address these limitations and collectively identify strategies to move the NET models field forward, we held an inaugural NET Models meeting, hosted by our founding group: Dr. Lines (Oxford); Prof. Quelle (Iowa); Dr. Dayton (Barcelona); Dr. Ear (Iowa); Dr. Marinoni (Bern); and Dr. Guenter (Alabama). This 2-day meeting in Oxford (UK) was organised and supported by Bioscientifica Ltd and was solely dedicated to the discussion of NET models. The meeting was attended by ~30 international researchers (from UK, EU, Israel, USA and Canada). Plenary talks were given by Prof. Thakker who summarised NET research over the last few decades, and Dr. Schrader who described the process and pitfalls of generating new cell lines. Eight researchers also presented their work on topics ranging from human cell 3D bioprinting, to zebrafish models, and included novel ideas as well as improvements on current concepts. This was followed by an interactive workshop where discussion topics included, a summary of currently available NET models, limitations of these models, barriers to developing new models, and how we can address these issues going forward. This white paper summarises the key points raised in these discussions, as well as the future aspirations of the NET Models Consortium. The next meeting will take place in Oxford (UK) in 2025, contact contact@netcancerfoundation.com for more information.

Introduction

Neuroendocrine neoplasms (NENs) are tumours that arise in the neuroendocrine cells of the body, and commonly affect the pancreas, lung or small intestine (Chauhan*, et al.* 2020). They can occur sporadically or as part of inherited tumour syndromes such as multiple endocrine neoplasia type 1 (MEN1) (Frost*, et al.* 2018). Although considered a rare tumour type, the incidence of NENs is rising globally, with approximately 8.8 per 100,000 people diagnosed in England, and approximately 10 per 100,000 people diagnosed in the USA (Chauhan*, et al.* 2020; Das and Dasari 2021; White*, et al.* 2022). Notably, low grade NENs are particularly challenging to study and treat clinically because of their unusually slow growth, which not only makes them unresponsive to many traditional therapeutics but has impeded the development of much needed patient-derived cell systems and animal-based NEN models for research.

NENs can be classified as well differentiated neuroendocrine tumours (NETs) or poorly differentiated neuroendocrine carcinomas (NECs). The latter are highly aggressive tumours that are both biologically and clinically representative of more common cancer types such as breast or lung adenocarcinomas, as they harbour mutations in classic tumour suppressor genes including *TP53* (Rindi*, et al.* 2018). NETs, on the other hand are a more diverse and biologically distinct tumour type, that can be further subdivided into 3 different grades (G), G1, G2 and G3 (Rindi*, et al.* 2018). G1 tumours are the least aggressive whereas G3 the most aggressive with Ki-67 index and mitotic count increasing across the grades (Rindi*, et al.* 2018). Most NETs are low G1 and G2 lesions, but can still metastasize. There are >20 genes described to be mutated in NETs, with the most common being Menin 1 (*MEN1*), death-domain-associated protein (*DAXX*), and ATRX Chromatin Remodeler (*ATRX*), which occur in 40-70% of sporadic G1-3 Pancreatic NETs (PanNETs) and Cyclin-dependent kinase inhibitor 1B (*CDKN1B*) that occurs in approximately 10% of sporadic G1-3 small intestinal NETs (siNETs) (Di Domenico*, et al.* 2017; Francis*, et al.* 2013; Jiao*, et al.* 2011; Scarpa*, et al.* 2017; van Riet*, et al.* 2021). Mutations in other genes, such as Phosphatase And Tensin Homolog (*PTEN*),

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and those in the mammalian target of rapamycin (mTOR) signalling pathway have also been identified, predominately in PanNETs, but at lower frequencies (Di Domenico*, et al.* 2017; Jiao*, et al.* 2011; Maharjan*, et al.* 2021; van Riet*, et al.* 2021).

The only curative treatment for NENs of all grades is surgery, however 20-50% of patients present with unresectable tumours at diagnosis such that surgery is not an option (Buicko*, et al.* 2019; O'Dorisio*, et al.* 2020; Roeyen*, et al.* 2009). The first line medical treatment for NENs are somatostatin analogues (SSAs, either octreotide or lanreotide), which functionally engage somatostatin receptors that are often highly expressed in NENs (Caplin*, et al.* 2014; Rinke*, et al.* 2009). While SSAs stabilise disease and reduce morbidities associated with excessive hormone secretion, they do not reduce disease burden (O'Dorisio*, et al.* 2020; Zandee and de Herder 2018). Other medical therapies available to patients with NENs include the mTOR inhibitor everolimus, the tyrosine kinase inhibitor sunitinib, as well as conventional chemotherapies, although some of them show promise e.g. temozolomide shows progression free survival of ~50% in pancreatic NETs (PanNETs), many show limited and mixed efficacy (O'Dorisio*, et al.* 2020; Raymond*, et al.* 2011; Yao*, et al.* 2011; Yao*, et al.* 2016; Zandee and de Herder 2018). Radiological treatments including peptide receptor radionuclide therapy (PRRT), trans-arterial chemoembolization, trans-arterial radioemobilsation and radiofrequency ablation are also an option for NEN patients. However, similar to medical therapies, although some show promise e.g. PRRT shows progression free survival of ~40% in PanNETs, many have limited efficacy (O'Dorisio*, et al.* 2020; Singh*, et al.* 2024; Strosberg*, et al.* 2017; Zandee and de Herder 2018), although there are promising anti-tumour effects of recently developed α-emitting NEN targeted agents (Gape*, et al.* 2024; Lee*, et al.* 2024). Nonetheless, although the 5-year survival rate for patients with localised NETs is 78-93%, the 5-year survival for patients with metastatic disease is only 19-38% (Riihimäki*, et al.* 2016). Improved, and more effective targeted therapies that are developed specifically for NENs, and particularly NETs, are therefore

required. In order to develop these therapies, improved preclinical models are also required. To address this, we held the inaugural NET Models meeting in 2024, bringing together experts in the field to present their current unpublished work, and discuss the current state of NET models, the limitations of these, the barriers to improving the models, and aspirations for how models can be improved in the future. This White paper therefore summarises the presentations and discussions from this meeting.

NET Models Meeting Overview

More models of NETs are desperately needed to address the important basic and translational research questions about this disease, as those answers will ultimately help advance NET patient treatments. NETs provide a unique challenge as they are relatively rare, genetically and phenotypically diverse and nearly impossible to propagate under standard *in vitro* and *in vivo* conditions that work for common tumour types. As a consequence, generating NET models for scientific research that accurately recapitulate the patient tumours remains highly complex. This is true for *in vitro* models (predominantly cell lines), *ex vivo* models (e.g. organoids) and *in vivo* models (e.g. mice). Indeed, currently available NET research models are few in number, with many being imperfect for the molecular biology or therapeutic studies being undertaken. To address these challenges the NET Models Consortium was established in 2023. The aim of the consortium is to bring together researchers in the NET field to share expertise and experience, not only to discuss successes but also failed attempts, as well as to collaboratively develop novel models for the scientific community to use. In February 2024 the NET Models Consortium held their inaugural meeting in Oxfordshire, UK called *NET Models Meeting 2024*. There were 28 delegates attending in total, including two plenary speakers, and eight oral research presentations.

Day 1 opened with a plenary talk by Prof. Rajesh Thakker from the University of Oxford (UK). He summarised some of the most recent NET research findings and how different models, predominantly

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the cell lines BON-1 and QGP1, as well as mouse NET models have been utilised. Although this highlighted several excellent studies on genetics, epigenetics and novel treatment approaches for NETs, it also cemented the need for improved models to enable more clinically translatable research. Eight research presentations by a highly international group of speakers followed, each relating work currently being undertaken using different NEN (i.e. NET and NEC) models. Dr. Amit Tirosh (Tel Aviv, Israel) described generation of a VHL-deficient pseudohypoxic pancreatic NEN (PanNEN) cell line, Dr. Floryne Buishand (London, UK) outlined their use of canine insulinoma as a model for human malignant insulinoma, and Dr. Suganthi Chittaranjan (Vancouver, Canada) discussed their work on exploring subgroup-defining biomarkers and therapeutic vulnerabilities in PanNEN models. Dr. Anna Battistella (Milan, Italy) described their work on the development of new 3D *in vitro* models of pancreatic NETs (PanNETS), and Dr. Samira Sadowski (Bethesda, USA) outlined their work on therapeutic screening in patient derived organoids for gastroenteropancreatic NETs (GEP NETs). Three talks were focused on *in vivo* models, Dr. Ines Marques (Bern, Switzerland) presenting their work on zebrafish patient-derived xenografts (PDX) as a tool for precision medicine and establishment of an *in vivo* pipeline to evaluate NETs, Prof. Natalia Pellegata (Munich, Germany) discussed *in vivo* models of different subtypes of paragangliomas (PPGL), and Dr. Yi-Cheih Nancy Du (New York, USA) spoke about a mouse model that allows the identification of metastatic factors by somatic gene transfer.

Day 2 of the meeting began with Dr. Jörg Schrader (Husum, Germany) delivering his plenary talk on establishing innovative NET cell lines in which he described the strategies his lab has used, as well as the pitfalls. Dr. Schrader has successfully established at least two validated NET cell lines and concluded that defining the best culture conditions is of the utmost importance to stimulate growth of NET cells, particularly as they, in most cases, lack classic oncogene activation. This was followed by a workshop to discuss the next steps and aspirations for improving NET models. This workshop was subdivided into three groups, *in vitro* models (chaired by Dr. Kate Lines), *ex vivo* models (chaired

by Dr. Po Hien Ear), and *in vivo* models (chaired by Dr. Ilaria Marinoni). The outcome of these discussions is outlined in the sections below.

In vitro NET models

The term *in vitro* models refers to laboratory cultured cells, either primary cells that have a defined lifespan or immortalised cell lines that can proliferate indefinitely. Cell lines are often genetically manipulated to proliferate, or in the case of cancer may naturally harbour mutations that drive proliferation. For example, the human embryonic kidney cell line (HEK293) was generated by exposing HEK cells to adenovirus type 5, whereas HeLa cells generated from cervical cancer spontaneously proliferate in culture (Graham*, et al.* 1977; Puck and Marcus 1955). The main advantage of cell lines is that they provide a low cost, simple, high throughput model that can be used to rapidly assess tumour cell biology and test novel therapies. Cell lines also provide a model in which the role and significance of certain molecular alterations seen in patients can be efficiently studied. For example, the expression of cancer-associated genes and proteins can be easily manipulated in cultured cells and allow functional outputs, such as tumour cell proliferation and survival, to be assessed in real time.

A number of NEN cell lines have been developed and these are summarised in **Table 1**. Most cell lines commonly used were derived from human or rodent tumours. For PanNENs the most widely used cell lines are BON-1 and QGP1, which were generated over thirty years ago. BON-1 cells were originally isolated from a metastatic tumour in the pancreas, while QGP1 were derived from a human pancreatic somatostatinoma (Evers*, et al.* 1991; Kaku*, et al.* 1980). Both grow as adherent, easily maintained cultures, and harbour mutations that are commonly seen in NETS: BON-1 cells have a homozygous loss of cyclin dependent kinase 2A (*CDKN1A*) and *CDKN2B*; and QGP1 cells have a mutation in *ATRX* (Hofving*, et al.* 2018). However, whole exome sequencing has indicated that they may also harbour additional mutations that are typical of high-grade PanNETs or NECs, for example

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in *TP53* (Vandamme*, et al.* 2015). BON-1 and QGP1 cells have been used for hundreds of studies ranging from basic molecular biology of NENs to drug screening, although neither is optimal for studying hormone secretion. Therefore, for investigating effects on insulin secretion, rodent cell lines have been utilised, predominately the pancreatic beta cell derived cell lines, MIN6 and INS1 (Miyazaki*, et al.* 1990; Skelin*, et al.* 2010). More recently, cell lines that are more representative of well differentiated NETs have been described, including the human SPNE1, NT-18P and NT-3 cell lines (Benten*, et al.* 2018; Lou*, et al.* 2022; Viol*, et al.* 2022). NT-3 cells are so far the most widely used of these newer cell lines. NT-3 cells have been used, for example, to evaluate existing therapies and mutation based targeted therapies (April-Monn*, et al.* 2024; Viol*, et al.* 2022), as well as to interrogate the role of cancer-associated fibroblasts (Amin*, et al.* 2023).

Similarly to PanNETs, a few lung NETs exist including the widely used human H727 and H720 cells that represent typical and the more aggressive atypical lung NETs, respectively (Carney*, et al.* 1985). There is just one vetted human cell line for small bowel NETs, GOT1 (Kölby*, et al.* 2001). Rodent cell lines are also available for pheochromocytoma and pituitary adenomas, however although the KAT45 (pheochromocytoma), and HP75 and GX (pituitary) cell lines were developed and used historically, they have either been lost over time or have extremely limited distribution, meaning there are no widely available human cell lines (Buonassisi*, et al.* 1962; Greene and Tischler 1976; Karna*, et al.* 2024; Zhu*, et al.* 2020). The extensive use of all of these cell lines in both therapeutic and molecular biology studies, reflects their value to the NEN research community.

Limitations of existing in vitro NET models

Although NEN cell lines exist, they have many limitations. Here we will discuss the limitations that were highlighted as being the most problematic. Firstly, the existing cell lines are not representative of the genetic background in patient tumours, and particularly are unrepresentative of the genetic drivers of hereditary conditions such as MEN1. For example, there is no *MEN1* or *ATRX* knockout

cell line available for any NEN subtype seen in patients. In addition, many of the cell lines have either acquired or were selected for mutations in tumour suppressor genes such as *TP53* or oncogenes such as *KRAS*. This means the cell lines are fast growing, which is more representative of NECs rather than NETs. This limitation is exacerbated by the fact that the cell lines have been cultured by different groups over the last few decades yielding highly variant clonal populations in which novel mutations have been acquired, a phenomena highlighted by the increasing Journal requests for cell line authentication, and publications describing the characteristics and undertaking genetic analysis of the cell lines, for example (Hofving*, et al.* 2018; Luley*, et al.* 2020; Monazzam*, et al.* 2020). Such diversity in NEN cell lines studied in different labs can impact data reproducibility. One way to overcome these limitations is to develop novel cell lines. Dr. Jörg Schrader (Husum, Germany) presented his work establishing the well differentiated NT-3 insulinoma cell line, derived from a lymph node-metastasis in a PanNET patient. His team also generated NT-18P, NT-18LM and NT-36 cells PanNET cells from the primary tumour, liver metastasis and a local recurrence (12 months after initial surgery and chemotherapy treatment), respectively, from a patient with a G3 PanNET. Dr. Amit Tirosh (Tel Aviv, Israel) also summarised their work on generating a VHL-deficient pseudohypoxic pancreatic NEN cell line (Telerman*, et al.* 2023). All these cell lines, while representing an advance over previous models, are nonetheless limited by the fact they originated from high grade lesions and represent functional tumours. Most patient PanNETs are low grade (G1 or G2) and non-functional, but no cell lines representing those types of tumours have been successfully established. That remains a major gap in the NET field.

It was also highlighted that the lack of hormone secretion from some of the 'functioning' human cell lines, e.g. BON1 (Luley*, et al.* 2020), hinders the molecular biology studies that can be undertaken. This combined with the fact that there is still a lack of knowledge on the optimal culture factors and conditions required for the existing cell lines further limits the use of these cells. Murine PanNET cells are available for examining insulin secretion (for example, MIN-6, INS-1 or N134 cells),

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however, these do not recapitulate the genetics seen in humans. During this meeting, Dr. Floryne Buishand (London, UK) discussed the canine canINS cell line that was established from a canine insulinoma. As dogs more closely represent the genetics of humans than rodents, the generation of canine NET models may provide a novel and valuable platform for NET research, especially since NETs arise spontaneously in dogs in contrast to genetically induced NETs in rodents (Capodanno*, et al.* 2022).

Current barriers for the use and generation of novel NET in vitro models

We also discussed the barriers to developing new NET cell lines. It was concluded that the biggest barrier is ready access to the quantities of patient tumour tissue needed to generate cell lines. Many researchers do not have direct access to surgical theatres or any links to clinical colleagues, and therefore access to fresh samples is limited. Those researchers that do have access to samples reported a lack of interest in research from surgeons and pathologists. This can result in missed samples due to logistical planning, for example not being told about a surgery or surgeries ending late in the day such that tumour specimens could not be released to non-clinical staff. Some researchers do have good surgical links and are able to routinely access NEN tissue, however there is no defined protocol for tissue collection and storage. Thus, different laboratories will handle samples in different ways, resulting in protocols for cell line generation that are not readily transferable. Finally, it is both administratively and physically complex to share patient tissue across groups or institutes, and especially complicated and costly to share material internationally. This means that even protocols that use cryo-frozen tissue cannot be implemented by groups other than those who have directly collected the material. Even if samples are acquired and protocols for cell line establishment undertaken, cells from low grade NETs have an extremely slow growth rate. This makes both establishing and maintaining the cells extremely challenging. A major problem in this regard is the "contamination" of samples with stromal cells. As fibroblasts have an at least 10x – 100x faster growth rate than NET cells, this warrants elaborated selection strategies to achieve pure tumour cell

cultures, for example sequential trypsinization and cultivation under low-adherent conditions (Benten*, et al.* 2018). A final major barrier is the lack of obligation to deposit cell lines into a publicly available repository (for example ATCC), as well as the lack of funds for undertaking this deposit. This means that researchers commonly transfer cell lines via material transfer agreements (MTAs), which can be time consuming and/or not accepted by certain institutions, thereby restricting distribution.

Ex vivo NEN models

We define NEN *ex vivo* models as models with high levels of resemblance to surgically resected NEN samples in terms of tumour heterogeneity. This category is comprised of patient-derived tumour organoid cultures (PDTO), tumoroid or spheroid cultures, and complex multi-cell type systems. PDTO cultures can be grown and expanded in culture indefinitely and are therefore defined as long-term cultures. In contrast, most tumoroid or spheroid cultures and complex multi-cell type bioreactor systems have a limited *in vitro* lifetime in terms of growth and expansion and are thus defined as short-term cultures.

Long-term PDTO culture systems use a completely defined culture medium consisting of a cocktail of growth-factors designed to recapitulate the cellular signaling environment of the native tumor microenvironment (Clevers 2016). This growth-factor rich media enables the establishment of PDTOs from early stage and low grade tumours (Boretto*, et al.* 2019; Fujii*, et al.* 2016; Kopper*, et al.* 2019). Thus, successful derivation of PDTOs from NETs could be facilitated by prior knowledge of the growth requirements of either NETs themselves or their presumed cells of origin, neuroendocrine cells. Long-term PDTO cultures have been established for low grade lung NETs (a.k.a. pulmonary carcinoids), with a reported success rate of 37% (Dayton*, et al.* 2023). Underscoring the importance of growth factor signaling for NETs, lung NET PDTOs are largely dependent on EGF in the media for their growth. Long-term PDTO cultures have also been

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established for G3 NETs, 1 from a biliary tract NET, 1 from a PanNET, and 1 from a duodenal NET (Kawasaki, *et al.* 2020). The duodenal NET PDTO is reported to be EGF-dependent, and the pancreatic NET carries an amplification of *ERBB2*, suggesting EGF signaling may also be important for some PanNETs. A PDTO model of a G2 small intestinal NET has also been reported (D'Agosto, *et al.* 2023). NET PDTOs display an extremely slow growth rate ranging from culture expansions needed every 10 days to every 3 months (D'Agosto, *et al.* 2023; Dayton, *et al.* 2023).

Certain categories of NETs such as the gastroenteropancreatic (GEP) NETs, which include PanNETs and NETs of the gastrointestinal tract, remain challenging to culture long-term. Only 4-12% of GEP NET PDTO models have been successfully cultured beyond passage 5 or over a 6-month period, which pales in comparison to other NET and NEC PDTO models (D'Agosto, *et al.* 2023; Dayton, *et al.* 2023; Kawasaki, *et al.* 2020). As an alternative, short-term NET spheroid models have been established and demonstrated to express NET markers (April-Monn, *et al.* 2021; Ear, *et al.* 2019; Gillette, *et al.* 2021). The main advantage of short-term NET spheroid culture is the high success rate of establishment which ranges from 85-90% (April-Monn, *et al.* 2021; Gillette, *et al.* 2021). The methodology of culture is similar to long-term NET PDTO cultures where the emphasis is on the isolation of NET cells from fresh or cryo-preserved NET samples for embedding in extracellular matrix. A major difference is in the media composition (**Table 2**). Short-term cultures can be grown in either stem-cell-based media or fetal serum bovine (FBS)-containing media supplemented with additional growth factors and vitamins (EGF, FGF, PIGF, IGF-1, Insulin, or nicotinamide). FBS-containing media formulations are less expensive in comparison to stem cell media and allow the usage of short-term NET *ex-vivo* models for drug testing by many research laboratories (April-Monn, *et al.* 2024; April-Monn, *et al.* 2021; Ear, *et al.* 2019; Gillette, *et al.* 2021). Since well-differentiated NET cells are slow growing and require 7-14 days to divide, the short-term *ex vivo* model offers an advantage to bypass the long wait time for growth as it can immediately be used in drug testing experiments (April-Monn, *et al.* 2021; Ear, *et al.* 2019). Current published methods use 3,000 to 5,000

isolated NET cells per well and up to 6 drugs or drug combinations can be tested at various concentrations (April-Monn*, et al.* 2024; April-Monn*, et al.* 2021; Ear*, et al.* 2019; Gillette*, et al.* 2021). Larger collections of GEP NET short-term spheroid drug screening studies with 17 and 14-NET patient spheroids screened with different libraries of compounds are underway. With improved sensitivity of detection assays and instrumentations, several research laboratories are currently developing novel methods to use as little as 500 cells per well of GEP NETs for high-throughput drug screening for precision medicine.

In addition to NEN organoid and spheroid models which focus on 3D culture of the tumour cells, innovative 3D models with increased complexity are actively being developed. A study from colleagues at the University of Alabama successfully demonstrated the feasibility of maintaining patient PanNETs in culture in bioreactor chambers for 21 days and propagating them to secondary chambers for an additional 9 days while maintaining NET markers (Herring*, et al.* 2021). Other efforts for co-culturing NET PDTO and cancer associated fibroblasts or endothelial cells, including usage of hydrogel-based extracellular matrices, are underway. The development of tissue engineering technology based on 3D printing could also help improve current models and scale up the production for usage in drug testing experiments.

Limitations of existing ex vivo NET models

While long-term NET PDTOs can be expanded in culture indefinitely, a major limitation is their slow growth rate because they require a significant investment in terms of time and resources for expansion and long-term maintenance. This makes these NET PDTO models most suitable for mechanistic studies, targeted drug screens, or CRISPR/Cas9 experiments that can be conducted within reasonable timeframes. Nonetheless, the number of available NET PDTOs is limited. We are still in search of the optimal growth factor(s) and small molecule(s) cocktail to reliably extend the life in cultured NET cells. In contrast, short-term GEP NET spheroid models, which last 1-3 months, fit well with medium-

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scale drug screening studies. These short-term models have a significant drawback, however, in that they are not suitable for genetic studies. This is because genetic editing methods like CRISPR/Cas require long-term cultures to allow for the selection and growth of genetically altered cells.

Current barriers for the use and generation of novel NET ex vivo models

Many of the barriers faced by *ex vivo* models are similar to those mentioned for *in vitro* NET models with regards to access to tumour samples and IRB protocols that limit sharing of patient tissues. Especially in cases where different informed consent forms are used by different studies or different hospitals providing tissue for the same study, teasing apart what is allowed with samples from one hospital versus another with regards to follow up studies and sharing may be a time-consuming task that can involve multiple ethical committees and assessments.

***In vivo* NEN models**

In vivo models, or animal models, are crucial to study the impact of cancer on the entire body of a complex, living organism. Animal models enable researchers to study the mechanisms of cancer development, progression and spread to distant organs, and provide a platform for the discovery and evaluation of new therapies. *In vivo* models are particularly important for endocrine cancers as they allow the assessment of hormone secretion effects, as well as the study of rare endocrine tumour syndromes, such as MEN1, in which multiple tumours occur in different organs simultaneously.

In the field of NENs, several animal models have been developed across multiple species including mice, rats, dogs, zebrafish, and fruit flies (for example, see (Forsythe*, et al.* 2023; Karna*, et al.* 2024; Lines*, et al.* 2016; Sedlack*, et al.* 2022; Vitale*, et al.* 2014)). As those publications have already comprehensively reviewed existing NEN models, we will not revisit them all here although **Table 3** provides a brief overview. These models have been instrumental in understanding: NEN biology; mechanisms of NEN progression; systemic effects of hormone hypersecretion; and identifying novel

treatments and targets. The most widely used NEN models are genetically engineered mouse models (GEMMs), which are ideal for studying the biology of early disease progression. Many knockout (KO) GEMMs of *Men1* exist, including constitutive and conditional models and they represent a valuable model to study NEN syndromes, for example MEN1 (Bertolino*, et al.* 2003; Crabtree*, et al.* 2001; Crabtree*, et al.* 2003; Harding*, et al.* 2009; Loffler*, et al.* 2007). Mutations in *MEN1*, which encodes the menin protein, trigger NEN formation both in mice and humans and therefore the *Men1*-KO GEMMs recapitulate the NEN development observed in humans, affecting parathyroids, pancreas, and pituitary (Bertolino*, et al.* 2003; Crabtree*, et al.* 2001; Crabtree*, et al.* 2003; Harding*, et al.* 2009; Lines*, et al.* 2017; Loffler*, et al.* 2007). These models have been used to understand the role of both *MEN1* and menin, and to identify avenues for targeting dysregulated MEN1 function.

Despite not always faithfully reflecting the human tumours, existing GEMMs have been extremely valuable for studying and understanding the biological landscape of NENs. While *Men1*-knockout mice were specifically generated to target a driver mutation equivalent to a human counterpart, other animal models have been established that develop NENs through different mechanisms. For example, the RIP1-Tag2 model is a transgenic model expressing the SV40 large T antigen under the control of the rat insulin promoter where the function of the p53 and RB tumour suppressor proteins is inhibited by the T antigen in pancreatic beta cells (Hanahan 1985). RIP1-Tag2 mice develop aggressive, fast growing, and insulin-secreting pancreatic NECs (PanNECs) around 16 weeks of age (Hanahan 1985). Although the biology of these tumours does not recapitulate the slow growing nature of G1 or G2 human PanNETs, this model has been widely used to study angiogenesis, tumour progression, and to identify new potential treatments, some of which are now used clinically for patients with PanNETs (Bill*, et al.* 2015; Hanahan*, et al.* 1996).

An alternative to GEMMs are models in which a tumour develops due to spontaneously arising mutations in the animal. The MENX rat model, presented at the 2024 NET Models Meeting by Prof.

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Pellegata (Munich, Germany), carries a spontaneous mutation in the *Cdkn1b* gene (encoding the p27 tumour suppressor) and develops NENs in pituitary, adrenal, pancreas and thyroid with high penetrance within the first 8-9 months (Piotrowska*, et al.* 2004). Tumours in this model share biochemical, physiological and molecular characteristics with the cognate human NETs. Germline mutations in the human homologue *CDKN1B* cause the MEN4 syndrome (Lee*, et al.* 2013; Molatore*, et al.* 2010; Pellegata*, et al.* 2006). During the meeting, it was also highlighted that dogs spontaneously develop insulinomas and the incidence rate of insulinomas in dogs is ten times higher than in humans (Capodanno*, et al.* 2022). The advantages of working with an insulinoma model in dogs include the fact that dogs live together with humans, and are thus exposed to the same environmental factors, and may share their diet with humans. Additionally, spontaneous canine insulinomas offer intact host immunity, as well as natural tumour heterogeneity and microenvironment. When diagnosed with an insulinoma, dogs are treated as humans are, with surgery being the recommended first-line of care. Additionally, spontaneous canine insulinomas develop in different dog breeds, thus encompassing the heterogeneity observed in human patients, which certainly is a limitation of inbreed rodent strains. However, further investigation into the genetic background of these tumours to assess whether they share the same drivers as human patients are warranted, to establish the full value of this model, especially in terms of phenocopying the human disease.

Patient-derived xenotransplantation (PDX) of NENs into immunocompromised animals represents a third possible *in vivo* model. Unfortunately, NETs have a low capacity to engraft and require long time periods (up to 2 years) to grow. NEC PDX models have higher engraftment rates, and several models have been reported in recent years, for example (Tran*, et al.* 2022). These models are valuable for assessing novel treatments in the presence of a more representative microenvironment and have particularly been utilised for the study of PRRT. Although the use of zebrafish has previously been described in NENs (Vitale*, et al.* 2014), an interesting new technique was also presented at the

meeting consisting of the transplantation of SiNET and PanNETs into zebrafish embryos. This model has already been widely used for other cancer types (Fior*, et al.* 2017; Marques*, et al.* 2009). To develop a NEN PDX, tumour cells are injected into the yolk-sac of zebrafish embryos, where they are able to survive, recruit vessels, migrate to the tail vein, and even form micro-metastasis in the liver (*unpublished data*). Zebrafish embryos bearing NENs can be treated with anti-tumour drugs and the effects of the drugs on the tumour cells' behaviour can be effectively measured. In addition, zebrafish provide a model in which live imaging can be performed to follow the tumour cells.

Limitations of existing in vivo NET models

The currently available *in vivo* models of NENs have several limitations. A major limitation is the lack of models bearing some of the relevant driver mutations seen in human patients. For example, there are no animal models representing the most common mutations occurring in PanNETs: *DAXX* and *ATRX*. GEMMs bearing either of these mutations do not develop PanNETs (Sun*, et al.* 2022; Wasylishen*, et al.* 2020). A recent mouse model combining *Atrx*, *Men1*, and *Pten* deletion demonstrated development of tumours, however they were aggressive PanNECs rather than NETs (Fuentes*, et al.* 2024). Another major limitation is that the majority of available mouse models for PanNETs develop insulinomas. Only one model currently exists for non-functioning PanNETs, which represent the majority of PanNETs diagnosed in patients and have worse prognoses. Carter et al. generated an inducible, conditional mouse model of PanNETs by hyperactivating CDK5 in β cells, which promoted the development of both functional and non-functional well-differentiated tumours (Carter*, et al.* 2021).

To investigate the molecular networks that drive tumor progression and metastasis, Dr Yi-Cheih Nancy Du (New York, USA), presented their work on the development of a bitransgenic mouse model, *RIP-Tag; RIP-tva*, in which both the SV40 large T antigen and the receptor for subgroup A avian leukosis virus (*tva*) is expressed in pancreatic β cells under the control of the rat insulin

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promoter (Du*, et al.* 2007; Zhang*, et al.* 2017). As such, genetic alterations can be introduced *in vivo* into pancreatic β cells by infection with avian retroviral vectors harboring desired genetic alteration. Using this model, Dr. Du's lab has demonstrated that Bcl-xL, RHAMM^{B}, and miR-431 promotes to PanNET metastasis (Choi*, et al.* 2016; Choi*, et al.* 2019; Du*, et al.* 2007; Du*, et al.* 2011; Zhang*, et al.* 2017; Zhang*, et al.* 2020). Dr. Du also derived several insulinoma cell lines, including N134 (Du*, et al.* 2007), from PanNETs in *RIP-Tag; RIP-tva* mice for *in vitro* study. Avian retroviral vectors can infect these murine PanNETs cell lines with high efficiency to overexpress or knockdown candidate genes (Zhang*, et al.* 2017).

A major limitation to research involving GEMMs is the expense and time-consuming nature of the work, especially for slowly growing NETs that may take 1-2 years to form *in vivo*. In some cases, specialized equipment may be required to image and track tumor growth non-invasively within the pancreas, for instance, the accessibility and cost of which may be prohibitive to researchers. While a key advantage of GEMMs is the presence of an intact immune system, xenograft models suffer from the inherent lack of an immune system in the host mice. This means that interactions between tumour cells and the tumour microenvironment (including immune cells) cannot be adequately addressed using PDX mouse models and they fail to accurately mimic the human disease. Furthermore, the cell used in PDX models are generally highly mutated and therefore more representative of NECs, rather than NETs, once again failing to recapitulate the biological status of patient NETs. Finally, experimental work performed using inbred mouse strains contributes to a lack of reproducibility across different strains and even laboratories.

Current barriers for the use and generation of novel NET in vivo models

The 2024 NET Models Meeting also had discussions about the barriers to generating new *in vivo* NET models. This highlighted two main barriers, the first being that generating GEMMs or other animal models is costly, and the second that NETs have a limited capacity to proliferate *in vivo*

making the delay in NET development lengthy. Thus, the delay in tumour growth can become extremely long, wherein some PanNETs take up to two years to develop. This combined with the cost, makes it very expensive to fully characterise new models and confirm if they indeed develop the required NETs. Other animal models are also possible, for example zebrafish. These are, however, less commonly used and therefore there is a lack in the field of appropriate expertise and equipment.

Future aspirations

The main aim of the NET Models Consortium is to improve the availability and support the generation of new, more appropriate NET models. Based on the discussions at this meeting the following practical steps towards achieving this goal were proposed. Firstly, any cell lines should be deposited into a repository (for example ATCC). This would not only improve access to the cell lines but also data reproducibility as it would limit the number of sub-clones generated. It would also provide a reference genome for any authentication protocols. The limiting factor in such an effort may be accessing funds as the deposition often comes at a cost. It was therefore concluded that this should be considered when submitting grant applications. Similarly, there should be more transparency in declaring any acquired mutations or characteristics (for example that may be identified in sequencing studies or biomarker screens) in cell line sub-clones when publishing results. In addition, co-culture or 3D culture models should be considered alongside standard 2D cell line culture protocols as cells often behave differently in these conditions. Indeed, co-culture systems may more closely recapitulate the *in vivo* tumour environment, which may improve the translation of *in vitro* work into *in vivo* studies.

For all studies, it was also concluded that collaboration is key. This includes making data and protocols available, as well as sharing knowledge, reagents, and material (including cells and tissues). The aim of this meeting is to foster productive collaborations by bringing together researchers with

the necessary skills and knowledge. In addition, collaborations with researchers from other disciplines should be encouraged as that may help solve inherent challenges faced by working with NENs. The aims of these collaborations are to: standardise protocols for collection of primary material; validate findings across laboratories; generate new animal models of NETs that highly resemble disease in humans including considering expansion to other species besides the classically used rodents; share animal-related resources to reduce duplication (in line with the '3R' principle: Replace, Reduce and Refine); and apply for joint funding to further support the exchange of models/material to impact translational research of NETs and thereby ultimately improve NET patients' management and survival.

Conclusions

In conclusion, high quality *in vitro*, *ex vivo* and *in vivo* NET models that accurately mimic the patient tumours are still limited and in great demand for NET research. The NET Models Consortium has brought together international experts in this field to discuss the limitations of current models and provide tangible and deliverable aims to improve them in a collaborative manner. A framework has also been developed to continue this work at future meetings, with the next to be held in Oxford in 2025. By providing investigators an annual opportunity to present and discuss their unpublished data, including negative results, to experts in the field, it is envisaged that the NET Models Consortium meetings will foster productive collaborations required for more efficient data and resource sharing. We expect such interactions will greatly facilitate future advances in NET Model development and research.

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Tables

Table 1. Currently available cell lines for NENs

Tissue of origin	Disease	Species	Cell line name	Reference
Adrenal	Pheochromocytoma	Rat	PC-12	(Greene and Tischler 1976)
	Pheochromocytoma	Rat	PC-12 Adh	(Greene and Tischler 1976)
Lung	Typical lung NET	Human	H727	(Carney, *et al.* 1985)
	Typical lung NET	Human	H835	(Carney, *et al.* 1985)
	Atypical lung NET	Human	H720	(Carney, *et al.* 1985)
Pancreas	PanNET	Human	BON-1	(Evers, *et al.* 1991)
	PanNET	Human	QGP1	(Kaku, *et al.* 1980)
	PanNET	Human	SPNE1	(Lou, *et al.* 2022)
	PanNET	Human	NT-3	(Benten, *et al.* 2018)
	PanNET	Human	NT-18P	(Viol, *et al.* 2022)
	PanNET Liver Metastasis	Human	NT-18LM	(Viol, *et al.* 2022)
	PanNET local recurrence	Human	NT-36	(Viol, *et al.* 2022)
	PanNET	Human	HuNET	(Tillotson, *et al.* 2001)
	PanNET	Canine	canINS	(Capodanno, *et al.* 2018)
	Insulinoma	Mouse	MIN6	(Miyazaki, *et al.* 1990)
	Insulinoma	Mouse	N134	(Du, *et al.* 2007)
	Insulinoma	Rat	INS1	(Skelin, *et al.* 2010)
Pituitary		Mouse	AtT20	(Buonassisi, *et al.* 1962)
Small bowel	Ileal NET	Human	GOT1	(Kölby, *et al.* 2001)

Table 2. Summary of culture media for NEN spheroids, organoids, and tissue.

Culture Type	Manuscripts	Media Compositions
Spheroids	Ear et al. 2019	DMEM/F12, 10% FBS, 1% Penicillin/Streptomycin, 1% Glutamine, 10 mM nicotinamide, 10 µg/mL insulin.
	April-Monn et al. 2021	DMEM-F12, 5% FBS, Hepes 10 mM, 1% L-glutamine (200 mM), 1% penicillin (100 U/ mL), 1% streptomycin (0.1 mg/mL), 1% amphotericin B (0.25 mg/mL), 20 ng/mL EGF, 10 ng/mL bFGF, 100 ng/mL PlGF, 769 ng/mL IGF-1.
	Gillettes et al. 2021	DMEM/F12, 10% FBS, 1% Penicillin/Streptomycin, + 1% GlutaMAX, 10 mM HEPES + EGF.
Organoids	Kawaski et al. 2020	Advanced DMEM/F12 supplemented with penicillin/streptomycin, 10 mM HEPES, 2 mM GlutaMAX, 1 3 B27, 10 nM gastrin I, and 1 mM N-acetylcysteine. A complete medium was prepared by supplementing the basal culture medium with the following niche factors: 50 ng/ml mouse recombinant EGF, 50 ng/ml human recombinant FGF-2 , 100 ng/ml human recombinant IGF-1 (BioLegend), 100 ng/ml mouse recombinant noggin (Peprotech), 1 mg/ml recombinant human R-spondin-1 (R&D), 25% Afamin-Wnt-3A serum- free conditioned medium, and 500 nM A83-01.
	D'Agosto et al. 2023	Advanced DMEM/F12 medium supplemented with 10 mM HEPES, GlutaMAX, Primocin (1 mg/ml, InvivoGen), N-acetyl-L-cysteine (1.25 mM), Wnt3a-conditioned medium (50% v/v), R-spo1- conditioned medium (10% v/v), recombinant Noggin (100 ng/ml), Epidermal Growth Factor (EGF, 50 ng/ml), Gastrin (10 nM), Fibroblast Growth Factor 10 (FGF10, 100 ng/ml), Nicotinamide (10 mM), and A83- 01 (0.5 µM)).
	Dayton et al. 2023	Advanced DMEM/F12 supplemented with 1x GlutaMAX, 10 mM HEPES, penicillin-streptomycin, Primocin, 1% Noggin conditioned medium, 20% of RSPO1 conditioned medium (made in-house), 1x B27 supplement, 1.25 mM n-Acetylcystein, 3 µM CHIR, 1 µM Prostaglandin E2, 0.005 µg/mL FGF2, 10 µM ROCK inhibitor, 500 nM A83-01, 3 µM p38 inhibitor SB202190. All lung NET organoids and some LCNEC organoids were grown in media additionally supplemented with 0.05 µg/ml EGF.
Tissue	Herring et al. 2021	Phenol Red Free DMEM/F12 supplemented with 10% FBS, Penicillin/Streptomycin.

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Table 3. Summary of the types of existing *in vivo* NEN models

Model Type	Species					
	Drosophila	**Zebrafish**	**Mouse**	**Rat**	**Dog**	**Frog**
GEMM	Yes	Yes	Yes	Yes	No	Yes
Spontaneous	No	No	No	Yes	Yes	No
Xenograft	No	Yes	Success rate around 10%	Yes	No	No