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Germline pathogenic variants in patients with high-grade gastroenteropancreatic neuroendocrine neoplasms.

Andreas Venizelos[1,2], Halfdan Sorbye[2,3], Hege Elvebakken[4,5], Aurel Perren[6], Inger Marie B. Lothe[7], Anne Couvelard[8], Geir Olav Hjortland[9], Anna Sundlöv[10,11], Johanna Svensson[12], Harrish Garresori[13], Christian Kersten[14], Eva Hofsli[5,15], Sönke Detlefsen[16,17], Lene W. Vestermark[18], Morten Ladekarl [19,20], Elizaveta Mitkina Tabaksblat[20], Stian Knappskog[1,2]

[1]K.G. Jebsen Center for Genome-Directed Cancer Therapy, Department of Clinical Science, University of Bergen, Bergen, Norway.
[2]Department of Oncology, Haukeland University Hospital, Bergen, Norway.
[3]Department of Clinical Science, University of Bergen, Bergen, Norway.
[4]Department of Oncology, Ålesund Hospital, Møre og Romsdal Hospital Trust, Ålesund, Norway.
[5]Department of Clinical and Molecular Medicine, Faculty of Medicine and Health Sciences, Norwegian University of Science and Technology, Trondheim, Norway.
[6] Institute of Tissue medicine and Pathology, University of Bern, Bern, Switzerland.
[7]Department of Pathology, Oslo University Hospital, Oslo, Norway.
[8]Department of Pathology, Université Paris Cité and AP-HP, Bichat Hospital, F-75018 Paris, France.
[9]Department of Oncology, Oslo University Hospital, Oslo, Norway.
[10]Departmentt of Oncology, Skåne University Hospital, Lund, Sweden.
[11]Department of Medical Radiation Physics, Lund University, Lund, Sweden.
[12]Department of Oncology, Sahlgrenska University Hospital, Gothenburg, Sweden.
[13]Department of Oncology, Stavanger University Hospital, Norway.
[14]Department of Research, Hospital of Southern Norway, Kristiansand, Norway.
[15]Department of Oncology, St.Olavs Hospital, Trondheim, Norway.
[16]Department of Pathology, Odense University Hospital, Denmark.
[17]Department of Clinical Medicine, Faculty of Health Sciences, University of Southern Denmark, Odense, Denmark.
[18]Department of Oncology, Odense University Hospital, Odense, Denmark.
[19]Department of Oncology, Aarhus University Hospital, Aarhus, Denmark.
[20]Department of Oncology, Clinical Cancer Research Center, Aalborg University Hospital, Aalborg, Denmark.

Correspondence to:
Stian Knappskog, Department of Clinical Science, University of Bergen, 5021 Bergen, Norway. Tel: (+47) 55976447; E-mail: stian.knappskog@uib.no

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Abstract

High-grade gastroenteropancreatic (HG-GEP) NEN are highly aggressive cancers. The molecular etiology of these tumors remains unclear and the prevalence of pathogenic germline variants in patients with HG-GEP-NEN is unknown.

We assessed sequencing data of 360 cancer genes in normal tissue, from 240 patients with HG GEP-NEN; 198 patients with NEC and 42 with NET G3. Applying strict criteria, we identified pathogenic germline variants and compared the frequency with previously reported data from 33 different cancer types.

We found a recurrent *MYOC* variant in 3 patients and a recurrent *MUTYH* variant in 2 patients, indicating that these genes may be important underlying risk factors for HG-GEP-NEN, when mutated. Further, germline variants were found in canonical tumor suppressor genes, such as *TP53*, *RB1*, *BRIP1* and *BAP1*. Overall, we found that 4.5% of patients with NEC and 9.5% of patients with NET G3 carry germline pathogenic or highly likely pathogenic variants. Applying identical criteria for variant classification *in-silico* to mined data from 33 other cancer types, the median percentage of patients carrying pathogenic or highly likely pathogenic variants was 3.4% (range: 0-17%). The patients with NEC and pathogenic germline variants had a median overall survival of 9 months, similar to what is generally expected for metastatic GEP-NEC. A patient with NET G3 and pathogenic *MUTYH* variant had much shorter overall survival than expected.

The fraction of HG GEP-NEN with germline pathogenic variants is relatively high, but still <10%, meaning that that germline mutations cannot be the major underlying cause of HG GEP-NEN.

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Introduction

Neuroendocrine neoplasms (NEN) constitute ~2% of all malignancies and are frequently located in the gastrointestinal tract and pancreas. High-grade (HG) gastroenteropancreatic (GEP) NEN are among the most aggressive cancers; the prognosis is generally poor with frequent metastatic disease at diagnosis and median survival of less than 1 year. These neoplasms are classified based on their morphology and proliferation rate, and further sub-dived into well-differentiated neuroendocrine tumors (NET G3) or poorly differentiated neuroendocrine carcinomas (NEC) (2019). The molecular features of HG-GEP-NEN are not well characterized, but some recent reports have shed new light on the landscape of somatic mutations in these cancers (Abel et al., 2021; Tang et al., 2016a; Tang et al., 2016b; Venizelos et al., 2021a). Importantly, a pattern emerges where the molecular features of NEC differ from NET G3 (Venizelos et al., 2021a). While NET G3 resembles other well-differentiated NET, GEP-NEC frequently harbors mutations in major cancer genes, such as *TP53*, *APC*, *KRAS* and *BRAF*.

In addition to assessment of driver mutations in well-established cancer genes, efforts have been made to pinpoint the mutational processes (Alexandrov et al., 2013) moulding the molecular characteristics of GEP-NEC. Thus, Yachida and colleagues found the mutational signatures in ductal-type pancreatic NEC to be dominated by the contribution of single base substitution signature 1 (SBS1), whereas in acinar-type pancreatic NEC the dominant contribution of mutations was from SBS5 (Yachida et al., 2022). Regarding gastric NEC, signatures SBS17a and SBS17 were more frequent, indicating that these cancers undergo a distinct mutational process as compared to other NEC and therefore may have a separate molecular etiology (Yachida et al., 2022). Despite these interesting findings, the mutational signatures observed in NEC have not been linked to specific underlying

mechanisms of tumorigenesis or tumor evolution. Thus, the molecular etiology of GEP-NEC remains largely unknown.

A proportion of all cancer cases are caused by inherited pathogenic mutations or by soma-wide de novo mutations. Among the most classical examples are pathogenic mutations of *RB1, CDKN2A* and *BRCA1* underlying retinoblastoma, melanoma and breast and ovarian cancer, respectively (Benavente and Dyer, 2015; Collins and Politopoulos, 2011; Meindl et al., 2010). In addition, some genes may confer a very high risk of multiple cancer types when mutated in the germline or de novo, such as *TP53* underlying the Li-Fraumeni syndrome with a very high risk of sarcomas, breast cancers etc. (Malkin et al., 1990). Pan-cancer studies in large sample sets have enabled assessment of the contribution of germline pathogenic mutations as underlying causes of many cancer types. In a recent comprehensive study (Huang et al., 2018), the prevalence of mutations was summarized in a total of 10 389 adult cancer patients across 33 cancer types. In various specific types of adult cancers pathogenic germline variants in tumor suppressor genes, including *ATM*, *BRCA1*, *BRCA2*, *BRIP1*, and *PALB2* were reported, while mutations in genes such as *TP53*, *RB1* and *MEN1* were mainly associated with multi cancer risk syndromes.

For low-grade pancreatic neuroendocrine tumors, germline pathogenic variants in *MEN1* have been identified as a strong underlying cause, with approximately 60% of patients with such mutations developing pancreatic NET during their lifespan (Ishida and Lam, 2022; Sakurai et al., 2012). In addition, variants in several other genes have also been linked to low-grade pancreatic NET including *CDKN1B*, *VHL*, *NF1*, *TSC1/2*, *PTEN*, *GCGR, BRCA2* and *MAFA* (Ishida and Lam, 2022). For carriers of pathogenic variants in *NF1*, about 10% of these individuals develop NET specifically in the Ampulla of Vater (Noe et al., 2018) and a pathogenic variant in *MUTYH* has been linked to small intestinal NET (Dumanski et al., 2017).

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However, the prevalence of germline pathogenic mutations as a potential underlying cause of HG GEP-NEN remains unknown. Here, we assessed targeted sequencing data of 360 cancer related genes in non-neoplastic (normal) tissue, across 198 patients with NEC and 42 patients with NET G3.

Materials and methods

Study Design

The aim of this study was to assess the landscape of pathogenic germline mutations in HG GEP-NEN and thereby to provide information on the fraction of tumors that could be explained by such mutations. In total, we assessed sequencing data for 240 patients. All patients were diagnosed with HG GEP-NEN during 2013-2017 and had been prospectively included in a Nordic database. The recruiting centers are the only referral centers for HG-NEN in their respective regions, and the cohort should therefore be representative. Inclusion criteria were histopathologically confirmed high-grade neuroendocrine neoplasm (Ki-67 > 20%) with gastroenteropancreatic primary or unknown primary site (CUP) with predominantly gastrointestinal metastases (defined by radiological CT scans). At the time of protocol development (2014) and study enrollment (2014-2017), all GEP-NEN with a Ki-67 >20% were classified as neuroendocrine carcinoma, NEC G3. The current 2019 WHO pathology grading system divides HG GEP-NEN into the well-differentiated NET G3 and the poorly differentiated NEC (Nagtegaal et al., 2020; WHO, 2019). As a result of the new classification, all cases were blinded and re-evaluated digitally in 2021-2022 by three

experienced NEN pathologists (AP, AC, IMBL). Out of the 240 patients, 198 patients were reclassified as NEC and 42 patients as NET G3.

For 180 patients, somatic mutations have been reported previously (Venizelos et al., 2021a). In the previous study, sequencing data on normal tissue was only used for filtering and supporting somatic mutation calling. In that study 181 patients were included. Upon re-audit of clinical information, for the present study, one patient (11017) was excluded from analysis due to lack of proof of GI-related disease (metastases limited to axilla). In addition, for the present study, we also sequenced normal tissue from an additional 60 patients with HG GEP-NEN, reaching the total number of 240.

DNA isolation

Genomic DNA was isolated from non-neoplastic tissue (blood) using QIAamp DNA MiniKit (Qiagen, Hilden, Germany).

MSI status

MSI status was drawn from our previously published somatic data (Venizelos et al., 2021a) for the 180 patients included in that study. In brief, those analyses were performed using the Promega MSI analysis system (Version1.2, Promega). For the additional 60 patients included in the present study, only DNA from normal tissue (blood) was analysed, precluding any assessment of MSI status.

Library preparation and sequencing

Targeted massive parallel sequencing was performed on DNA from normal peripheral blood leukocyte DNA. Illumina libraries were prepared applying Kapa Hyper Prep kit (Kapa

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Biosystem) and Agilent SureSelect XT-kit (Agilent). Targeted enrichment was performed using RNA baits (SureSelect, Agilent), targeted against an in-house panel of 360 cancer related genes (Yates et al., 2015). Libraries were sequenced on a MiSeq instrument (Illumina) to an average depth of 172x (range 50x-266x).

Data processing and bioinformatics analysis

Raw sequence data was aligned to the human reference genome (Build-UCSC hg19) using BWA (Li and Durbin, 2009). Germline mutations from the vcf files were annotated using ANNOVAR and Ensembl Variant Effect Predictor (VEP) with default parameters. Furthermore, we applied CharGer (Characterization of Germline Variants) (Scott et al., 2019) which queries information from ClinVar database, to classify variants, into pathogenic, likely pathogenic, variant of uncertain significance (VUS), as well as likely benign and benign.

In addition to the CharGer software tool we manually inspected each mutation in the Integrated Genomic Analysis Viewer (IGV) to omit false positives based on coverage as well as read quality. The genome aggregation database (gnomAD) was utilized to examine the population frequency of each variant. Further, variant allele frequencies in the blood samples (and in matched tumors) were used to remove variants that were likely representing clonal hematopoiesis.

Mined data set

For comparison of the prevalence of germline variants in HG GEP-NEC to other cancer types, we mined the data available from Huang et al (Huang et al., 2018), holding germline information for 10 389 patients with 33 different cancer types. In their report, Huang et al applied a variant classification utilizing tiers for variants annotation by adopting AMP-ACMG

guidelines and levels of a score of evidence generated by the CharGer algorithm (Scott et al., 2019). This approach gave a slightly higher fraction of called likely pathogenic variants, relative to pathogenic variants. For consistency, we therefore mined the raw data from their report and reclassified according to the identical procedure as we did for our HG GEP-NEN cohort (described under ”Data processing and bioinformatics analysis”).

Ethics and consent to participate

The research protocol was approved by ethics committees in Norway (REK vest 2012/940), Sweden (REC Uppsala Dnr 2012/285) and Denmark (Region Hovedstaden H-4-2012-108). All patients signed informed written consent.

Results

Patient samples

A total of 240 patients diagnosed with HG GEP-NEN were assessed for pathogenic germline variants (198 NEC and 42 NET G3). Demographics are summarized in Table 1. Regarding the primary tumor site, the largest groups were neoplasms in the rectum and colon, followed by pancreas and esophagus.

Prevalence of pathogenic germline variants in GEP-NEC

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Applying the classification tiers from ClinVar (as described under methods) on sequencing data covering 360 cancer related genes (Yates et al., 2015), we found 9 out of 198 patients (4.5%) with GEP-NEC to carry a total of 11 germline pathogenic variants (Figure 1; Table 2). These 9 patients had pathogenic germline variants in major tumor suppressors such as *TP53* and *RB1*, but interestingly also in several genes involved in homologous recombination repair; *FANCC*, *BRIP1* and *ERCC2*.

Notably, two patients (10017 and 9054) were found to have an identical germline nonsense mutation (c.1174G>A) in the *MYOC* gene, encoding myocilin which is involved in cytoskeletal functions. Both these patients were males and had large cell NEC, one with primary tumor site in the rectum and the other in the right colon. The two patients were recruited at two different centers and there was no known familial relationship between them.

Two patients harboured more than one pathogenic variant: patient 9054 harboured both the *MYOC* variant mentioned above and a nonsense variant in *BRIP*, while patient 5021 harboured both a *TP53*- and a *CTNNB1* (β-catenin) variant (Figure 1; Table 2). The remaining pathogenic variants were found in *MUTYH* (DNA repair / polyposis related gene), *BAP1* (deubiquitinating enzyme) and *AR* (androgen receptor).

Mining our previously published data on somatic mutations in tumors (Venizelos et al., 2021b) we found that none of the 9 patients with germline pathogenic variants had somatic mutations (SNVs or indels) as a “second hit” in their tumors. However, three had somatic copy number loss indicating loss of heterozygosity (LOH) of the affected loci. This was seen for the variants in *TP53*, *RB1* and *FANCC* (Table 2).

One of the 9 cases (7032, carrying a pathogenic *ERCC2* variant), was diagnosed with a prostate cancer 10 months prior the NEN-diagnosis. None of the remaining 8 patients with

pathogenic variants had prior cancers. Neither did any of these 9 patients have any family history of NEN.

The average age at diagnosis for the 9 patients with germline pathogenic variants did not differ significantly from the remaining patients (64 vs. 66 years) but notably, one of the 9 patients (5021, carrying both a *TP53* and a *CTNNB1* pathogenic variant) was diagnosed at 39 years of age, placing this patient in the lower 5% of the cohort, with respect to age.

Among the 9 carriers of germline pathogenic variants were 6 males and 3 females. Thus, the percentage of carriers was very similar between genders (4.5% and 4.7%, respectively)

In addition to the pathogenic germline variants described above we found four additional variants that fulfilled the criteria for being germline pathogenic variants: Two variants in *DNMT3A*, one in *GNAS* and one in *U2AF1*. All these four were detected with low allele frequencies in the blood DNA and they were absent from the matched tumors samples. Thus, they were considered products of clonal hematopoiesis rather than real germline variants and excluded from the analysis.

Further to the pathogenic variants, we assessed variants of unknown significance (VUS). Among the 198 NEC patients, 84 harbored germline variants classified as VUS from the Clinvar database (Figure 1).

Prevalence of pathogenic germline variants in GEP-NEC versus other cancer types

In order to compare the fraction of GEP-NEC harbouring pathogenic germline mutations to the corresponding fraction in other cancer types, we mined the data from Huang and colleagues (Huang et al., 2018). These authors assessed the germline status of 10 389 patients across 33 cancer types. Assessing their raw data of variants, within the same 360

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gene set and classifying them according to the same criteria as we used for GEP-NEC, we found that the fraction of cases with pathogenic or likely pathogenic variants in different cancer types ranged from 0% to 17% (median 3.4% Figure 2). Thus, GEP-NEC revealed a relatively high fraction of cases harbouring germline pathogenic mutations as compared to most other cancer types.

Germline mutations in GEP-NEC originating in different tissues

Among the NEC patients with pathogenic variants, three had their primary tumors in the colon, one was rectal, one esophageal, one gastric while one tumor was in the gallbladder (the remaining two had unknown primary site; Figure 3). Comparing these fractions to the corresponding fractions of the tissue's adenocarcinoma counterparts, the fraction of GEP-NEC with germline pathogenic mutations seems high, although these data must be interpreted with great caution due to the low number of observations.

Clinical impact of germline mutations in GEP-NEC

Among the 9 patients with NEC and detected pathogenic germline variants, one patient (5017, who carried a variant in *AR*) was subject to radical surgery and therefore not comparable to the other patients with respect to clinical outcome. The other eight patients with metastatic disease given palliative chemotherapy had a median overall survival of 9 months, similar to what is generally expected for metastatic GEP-NEC. Seven of these eight patients had a partial response to first-line treatment (response rate 88%), whereas median progression-free survival was 6 months. The patients with pathogenic germline variants did not differ from the rest of the cohort with respect to any other available clinical parameters.

Prevalence of germline mutations in GEP NET G3

Among the 42 patients diagnosed with NET G3, we found 3 (7.1%) to harbour pathogenic germline variants. These variants were found in the *BLM*, *MUTYH* and the *MYOC* genes. Intriguingly, the *MYOC* variant was the same variant as detected in two of the NEC patients and the *MUTYH* variants was the same as detected in one NEC patient. This finding further substantiated the notion that variants in these genes could be particular risk factors for neuroendocrine malignancies. In addition, one patient with NET G3 (2.4%) harboured a germline variant classified as likely pathogenic. This variant was found in the *FANCA* gene, affecting homologous recombination repair. Again, this finding aligns well with the findings among patients with NEC, where several pathogenic variants were found in genes involved in homologous recombination repair, further implicating this cellular function in neuroendocrine tumorigenesis.

None of the four patients with germline pathogenic or likely pathogenic variants and NET G3 had somatic mutations as “second hit” in their tumors, but one patient, carrying a germline variant in *FANCA*, had LOH of the locus (Table 2).

Assessing the overall prevalence of pathogenic and likely pathogenic variants in NET G3, 9.5% of patients harboured such variants, placing NET G3 among those tumor types with the highest prevalence of such variants (Figure 2). However, this should be interpreted with caution since the number of analysed patients with NET G3 was low. Three of the NET G3 patients harbouring pathogenic or likely pathogenic variants had tumors with the pancreas as the primary site, while the one remaining patient had tumor with unknown primary site. Given the low absolute number of observations, no formal assessment of mutation spectrum across primary sites could be performed.

 Accepted Manuscript published as ERC-23-0057.R1. Accepted for publication: 03-Jul-2023

Overall survival for the patient with metastatic NET G3 and germline pathogenic variant in *MUTYH* was only 15 months, much shorter than the 31-42 months generally expected for metastatic NET G3.

Discussion

In the present study, we found that 4.5% of patients with NEC and 9.5% of patients with NET G3 carry germline pathogenic or likely pathogenic variants. This places the prevalence of germline pathogenic variants in HG GEP-NEN in the higher end of the spectrum of different cancer types, as shown in our present comparison.

Regarding the specific genes in which pathogenic variants were detected, we made several interesting observations. Most strikingly we found two patients with NEC and one patient with NET G3 to harbour the exact same mutation in the *MYOC* gene. This gene is known to be involved in cytoskeletal functions and germline variants in this gene have been associated with hereditary juvenile-onset open-angle glaucoma (Selvan et al., 2022). A multitude of somatic mutations in *MYOC* (including Q368*, presently detected as a germline variant) have previously been reported in several cancer types, with highest frequencies in skin cancers and endometrial cancers but also with a relatively high frequency (2.5%) in colon cancers (Forbes et al., 2017). In our previous report on somatic variants in HG GEP-NEN, we found *MYOC* mutations in 2 patients out of 152 GEP-NEC. Notably among the 29 NET G3 none of the patients harbored somatic *MYOC* mutations (Venizelos et al., 2021a). Although further studies are warranted, and the mechanism(s) underlying the potential increased risk, our data indicates a role for *MYOC* variants in the tumorigenesis of HG GEP-NEN.

Another recurrent variant was observed in *MUTYH*. This variant was found in one patient with NEC and another with NET G3. The protein product of *MUTYH* is a glycosylase involved in base excision repair and germline variants have been implicated in high somatic mutation rates and risk of colorectal cancer (Robinson et al., 2022). Interestingly, a pathogenic variant in *MUTYH* has previously been linked to small intestine NET in two different families (Dumanski et al., 2017) and different *MUTYH* variants have been detected in cases of pancreatic NETs (Scarpa et al., 2017). Although the variant as detected in our present study was a different one, taken together, these observations support a role for *MUTYH* variants in NEN development. The molecular and cellular etiology of NEC is debated and the possible potential evolution from a well-differentiated NET to a poorly-differentiated NEC is controversial (Botling et al., 2020; Pelosi et al., 2021; Tang et al., 2016b). Although our current data is no evidence for linear evolution, it is interesting to note that the recurrent pathogenic mutations both in *MYOC* and *MUTYH* are found both in NEC and NET G3 cases.

Another striking observation is that a substantial fraction of the pathogenic and likely pathogenic variants we observed was in genes related to homologous recombination repair (HRR), other than *BRCA1/2*. Such variants are well established as underlying causes of the breast, prostate and ovarian cancers (Kohlhase et al., 2014; Li et al., 2019; Ramus et al., 2015). In addition to providing insight into the underlying causes of HG GEP-NEN tumorigenesis, it should be noted that the presence of HRR deficiency could be a lead forward in the exploration of new treatment options for patients with HG GEP-NEN: although sensitivity to PARP-inhibition was originally linked to *BRCA1* mutations, it has recently become evident that such sensitivity may also be caused by defects in other genes involved in HRR (Eikesdal et al., 2021).

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However, we also found several pathogenic variants in genes not directly involved in HRR or any other kind of DNA repair. Thus, taken together, the diversity in affected genes and their functions, strongly indicate that there is no single unifying mechanistic cause underlying a majority of those cases of HG GEP-NEN caused by germline pathogenic variants.

Regarding the clinical trajectory of the GEP-NEC disease, these did not differ between patients carrying germline pathogenic mutation versus those who did not. Median survival for the 8 metastatic NEC cases was 9 months, not far from the 11-12 months generally expected for metastatic GEP-NEC (Elvebakken et al., 2021; Morizane et al., 2022; Walter et al., 2017). Although the number of patients may be too limited to draw firm conclusions, our data does not suggest that any specific adaptations within the current treatment strategies should be implemented for those patients carrying pathogenic germline variants. Instead, as mentioned above, the findings reported here should rather be used to point forward to potential exploration of alternative treatments outside of current standards. The patient with metastatic NET G3 and germline pathogenic variant in *MUTYH* had an overall survival of only 15 months. This is substantially shorter than the 31-42 months expected for metastatic NET G3 in general (Chan et al., 2021; Liu et al., 2021; Spada et al., 2021), but given the single observation, no firm conclusions can be drawn.

From a technical point of view, our approach for identification of pathogenic germline variants was somewhat conservative. We identified a slightly lower fraction of variants than e.g. Huang and colleagues (Huang et al., 2018), from whose data we mined for re-assessment. Regarding the differences, these were mainly that we detected a lower fraction of variants in the category “likely pathogenic”, while the fraction of variants in the category “pathogenic” was similar. In general, the stringency in the definition of “likely pathogenic” differs between studies and potentially preclude direct comparison of

frequencies. For future studies assessing variants in GEP-NEN, such potential technical differences in the annotation of the detected variants should be taken into account. Further, all normal tissue samples in the present study were sequenced by a targeted panel of 360 cancer related genes. Although perhaps unlikely, it may be that some patients harbor mutations in genes not included in the panel, that could contribute to the development of GEP-NEC. Further, our analysis was restricted to SNVs and indels, which may be a limitation to our data: it may be that some few patients have germline copy number alterations that could be an underlying cause of cancer. Notably, in recent reports both in prospective population-based cohorts and in hospital based cohorts, we have shown embryonic (constitutional) methylation of *BRCA1* to cause a significantly increased risk of breast and ovarian cancer later in life (Lonning et al., 2018; Lonning et al., 2022). Such epigenetic events have not been assessed for the HG GEP-NEN cohort. As such, our present data may be an underestimate of the real fraction of HG GEP-NEN that have germline variants and/ or constitutional molecular features as a lead cause of disease development.

Although our data show that the fraction of HG GEP-NEN with germline pathogenic variants is relatively high as compared to other cancer types, the fraction is still only <10%. Even with the precautions discussed above and the fact that our results may slightly underestimate the fraction, this means that the presence of such mutations cannot be the major underlying cause of HG GEP-NEN. As such, the molecular etiology of the majority of these neoplasms is still largely unknown.

Declaration of interest

AV is currently an employee at Oxford Nanopore. HS has received research support from Novartis, Amgen, Ipsen and honoraria from Novartis, Ipsen, Pfizer, Keocyt, AstraZeneca, BMS, Roche, Amgen,

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References

(2019). WHO classification of Digestive System Tumours, 5th ed (Lyon: IARC).

Abel, M.K., Melisko, M.E., Rugo, H.S., Chien, A.J., Diaz, I., Levine, J.K., Griffin, A., McGuire, J., Esserman, L.J., Borno, H.T.*, et al.* (2021). Decreased enrollment in breast cancer trials by histologic subtype: does invasive lobular carcinoma resist RECIST? Npj Breast Cancer *7*.

Alexandrov, L.B., Nik-Zainal, S., Wedge, D.C., Aparicio, S.A., Behjati, S., Biankin, A.V., Bignell, G.R., Bolli, N., Borg, A., Borresen-Dale, A.L.*, et al.* (2013). Signatures of mutational processes in human cancer. Nature *500*, 415-421.

Benavente, C.A., and Dyer, M.A. (2015). Genetics and epigenetics of human retinoblastoma. Annu Rev Pathol *10*, 547-562.

Botling, J., Lamarca, A., Bajic, D., Norlen, O., Lonngren, V., Kjaer, J., Eriksson, B., Welin, S., Hellman, P., Rindi, G.*, et al.* (2020). High-Grade Progression Confers Poor Survival in Pancreatic Neuroendocrine Tumors. Neuroendocrinology *110*, 891-898.

Chan, D.L., Bergsland, E.K., Chan, J.A., Gadgil, R., Halfdanarson, T.R., Hornbacker, K., Kelly, V., Kunz, P.L., McGarrah, P.W., Raj, N.P.*, et al.* (2021). Temozolomide in Grade 3 Gastroenteropancreatic Neuroendocrine Neoplasms: A Multicenter Retrospective Review. Oncologist *26*, 950-955.

Collins, A., and Politopoulos, I. (2011). The genetics of breast cancer: risk factors for disease. Appl Clin Genet *4*, 11-19.

Dumanski, J.P., Rasi, C., Bjorklund, P., Davies, H., Ali, A.S., Gronberg, M., Welin, S., Sorbye, H., Gronbaek, H., Cunningham, J.L.*, et al.* (2017). A MUTYH germline mutation is associated with small intestinal neuroendocrine tumors. Endocr Relat Cancer *24*, 427-443.

Eikesdal, H.P., Yndestad, S., Elzawahry, A., Llop-Guevara, A., Gilje, B., Blix, E.S., Espelid, H., Lundgren, S., Geisler, J., Vagstad, G.*, et al.* (2021). Olaparib monotherapy as primary treatment in unselected triple negative breast cancer. Ann Oncol *32*, 240-249.

Elvebakken, H., Perren, A., Scoazec, J.Y., Tang, L.H., Federspiel, B., Klimstra, D.S., Vestermark, L.W., Ali, A.S., Zlobec, I., Myklebust, T.A.*, et al.* (2021). A Consensus-Developed Morphological Re-Evaluation of 196 High-Grade Gastroenteropancreatic Neuroendocrine Neoplasms and Its Clinical Correlations. Neuroendocrinology *111*, 883-894.

Forbes, S.A., Beare, D., Boutselakis, H., Bamford, S., Bindal, N., Tate, J., Cole, C.G., Ward, S., Dawson, E., Ponting, L.*, et al.* (2017). COSMIC: somatic cancer genetics at high-resolution. Nucleic Acids Res *45*, D777-D783.

Huang, K.L., Mashl, R.J., Wu, Y., Ritter, D.I., Wang, J., Oh, C., Paczkowska, M., Reynolds, S., Wyczalkowski, M.A., Oak, N.*, et al.* (2018). Pathogenic Germline Variants in 10,389 Adult Cancers. Cell *173*, 355-370 e314.

Ishida, H., and Lam, A.K. (2022). Pancreatic neuroendocrine neoplasms: Updates on genomic changes in inherited tumour syndromes and sporadic tumours based on WHO classification. Crit Rev Oncol Hematol *172*, 103648.

Kohlhase, S., Bogdanova, N.V., Schurmann, P., Bermisheva, M., Khusnutdinova, E., Antonenkova, N., Park-Simon, T.W., Hillemanns, P., Meyer, A., Christiansen, H.*, et al.* (2014). Mutation analysis of the ERCC4/FANCQ gene in hereditary breast cancer. PLoS One *9*, e85334.

Li, A.Q., Geyer, F.C., Blecua, P., Lee, J.Y., Selenica, P., Brown, D.N., Pareja, F., Lee, S.S.K., Kumar, R., Rivera, B.*, et al.* (2019). Homologous recombination DNA repair defects in PALB2-associated breast cancers. Npj Breast Cancer *5*.

Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics *25*, 1754-1760.

 Accepted Manuscript published as ERC-23-0057.R1. Accepted for publication: 03-Jul-2023

Liu, A.J., Ueberroth, B.E., McGarrah, P.W., Buckner Petty, S.A., Kendi, A.T., Starr, J., Hobday, T.J., Halfdanarson, T.R., and Sonbol, M.B. (2021). Treatment Outcomes of Well-Differentiated High-Grade Neuroendocrine Tumors. Oncologist *26*, 383-388.

Lonning, P.E., Berge, E.O., Bjornslett, M., Minsaas, L., Chrisanthar, R., Hoberg-Vetti, H., Dulary, C., Busato, F., Bjorneklett, S., Eriksen, C.*, et al.* (2018). White Blood Cell BRCA1 Promoter Methylation Status and Ovarian Cancer Risk. Ann Intern Med *168*, 326-334.

Lonning, P.E., Nikolaienko, O., Pan, K., Kurian, A.W., Eikesdal, H.P., Pettinger, M., Anderson, G.L., Prentice, R.L., Chlebowski, R.T., and Knappskog, S. (2022). Constitutional BRCA1 Methylation and Risk of Incident Triple-Negative Breast Cancer and High-grade Serous Ovarian Cancer. JAMA Oncol.

Malkin, D., Li, F.P., Strong, L.C., Fraumeni, J.F., Jr., Nelson, C.E., Kim, D.H., Kassel, J., Gryka, M.A., Bischoff, F.Z., Tainsky, M.A.*, et al.* (1990). Germ line p53 mutations in a familial syndrome of breast cancer, sarcomas, and other neoplasms. Science *250*, 1233-1238.

Meindl, A., Hellebrand, H., Wiek, C., Erven, V., Wappenschmidt, B., Niederacher, D., Freund, M., Lichtner, P., Hartmann, L., Schaal, H.*, et al.* (2010). Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. Nat Genet *42*, 410-414.

Morizane, C., Machida, N., Honma, Y., Okusaka, T., Boku, N., Kato, K., Nomura, S., Hiraoka, N., Sekine, S., Taniguchi, H.*, et al.* (2022). Effectiveness of Etoposide and Cisplatin vs Irinotecan and Cisplatin Therapy for Patients With Advanced Neuroendocrine Carcinoma of the Digestive System: The TOPIC-NEC Phase 3 Randomized Clinical Trial. JAMA Oncol *8*, 1447-1455.

Nagtegaal, I.D., Odze, R.D., Klimstra, D., Paradis, V., Rugge, M., Schirmacher, P., Washington, K.M., Carneiro, F., Cree, I.A., and Board, W.H.O.C.o.T.E. (2020). The 2019 WHO classification of tumours of the digestive system. Histopathology *76*, 182-188.

Noe, M., Pea, A., Luchini, C., Felsenstein, M., Barbi, S., Bhaijee, F., Yonescu, R., Ning, Y., Adsay, N.V., Zamboni, G.*, et al.* (2018). Whole-exome sequencing of duodenal neuroendocrine tumors in patients with neurofibromatosis type 1. Mod Pathol *31*, 1532-1538.

Pelosi, G., Bianchi, F., Dama, E., Metovic, J., Barella, M., Sonzogni, A., Albini, A., Papotti, M., Gong, Y., and Vijayvergia, N. (2021). A Subset of Large Cell Neuroendocrine Carcinomas in the Gastroenteropancreatic Tract May Evolve from Pre-existing Well-Differentiated Neuroendocrine Tumors. Endocr Pathol *32*, 396-407.

Ramus, S.J., Song, H., Dicks, E., Tyrer, J.P., Rosenthal, A.N., Intermaggio, M.P., Fraser, L., Gentry-Maharaj, A., Hayward, J., Philpott, S.*, et al.* (2015). Germline Mutations in the BRIP1, BARD1, PALB2, and NBN Genes in Women With Ovarian Cancer. J Natl Cancer Inst *107*.

Robinson, P.S., Thomas, L.E., Abascal, F., Jung, H., Harvey, L.M.R., West, H.D., Olafsson, S., Lee, B.C.H., Coorens, T.H.H., Lee-Six, H.*, et al.* (2022). Inherited MUTYH mutations cause

elevated somatic mutation rates and distinctive mutational signatures in normal human cells. Nat Commun *13*, 3949.

Sakurai, A., Suzuki, S., Kosugi, S., Okamoto, T., Uchino, S., Miya, A., Imai, T., Kaji, H., Komoto, I., Miura, D.*, et al.* (2012). Multiple endocrine neoplasia type 1 in Japan: establishment and analysis of a multicentre database. Clin Endocrinol (Oxf) *76*, 533-539.

Scarpa, A., Chang, D.K., Nones, K., Corbo, V., Patch, A.M., Bailey, P., Lawlor, R.T., Johns, A.L., Miller, D.K., Mafficini, A.*, et al.* (2017). Whole-genome landscape of pancreatic neuroendocrine tumours. Nature *543*, 65-+.

Scott, A.D., Huang, K.L., Weerasinghe, A., Mashl, R.J., Gao, Q., Martins Rodrigues, F., Wyczalkowski, M.A., and Ding, L. (2019). CharGer: clinical Characterization of Germline variants. Bioinformatics *35*, 865-867.

Selvan, H., Gupta, S., Wiggs, J.L., and Gupta, V. (2022). Juvenile-onset open-angle glaucoma - A clinical and genetic update. Surv Ophthalmol *67*, 1099-1117.

Spada, F., Maisonneuve, P., Fumagalli, C., Marconcini, R., Gelsomino, F., Antonuzzo, L., Campana, D., Puliafito, I., Rossi, G., Faviana, P.*, et al.* (2021). Temozolomide alone or in combination with capecitabine in patients with advanced neuroendocrine neoplasms: an Italian multicenter real-world analysis. Endocrine *72*, 268-278.

Tang, L.H., Basturk, O., Sue, J.J., and Klimstra, D.S. (2016a). A Practical Approach to the Classification of WHO Grade 3 (G3) Well-differentiated Neuroendocrine Tumor (WD-NET) and Poorly Differentiated Neuroendocrine Carcinoma (PD-NEC) of the Pancreas. Am J Surg Pathol *40*, 1192-1202.

Tang, L.H., Untch, B.R., Reidy, D.L., O'Reilly, E., Dhall, D., Jih, L., Basturk, O., Allen, P.J., and Klimstra, D.S. (2016b). Well-Differentiated Neuroendocrine Tumors with a Morphologically Apparent High-Grade Component: A Pathway Distinct from Poorly Differentiated Neuroendocrine Carcinomas. Clin Cancer Res *22*, 1011-1017.

Venizelos, A., Elvebakken, H., Perren, A., Nikolaienko, O., Deng, W., Lothe, I.M.B., Couvelard, A., Hjortland, G.O., Sundlov, A., Svensson, J.*, et al.* (2021a). The molecular characteristics of high-grade gastroenteropancreatic neuroendocrine neoplasms. Endocr Relat Cancer *29*, 1-14.

Venizelos, A., Elvebakken, H., Perren, A., Nikolaienko, O., Deng, W., Lothe, I.M.B., Couvelard, A., Hjortland, G.O., Sundlov, A., Svensson, J.*, et al.* (2021b). The molecular characteristics of high-grade gastroenteropancreatic neuroendocrine neoplasms. Endocr-Relat Cancer *29*, 1-14.

Walter, T., Tougeron, D., Baudin, E., Le Malicot, K., Lecomte, T., Malka, D., Hentic, O., Manfredi, S., Bonnet, I., Guimbaud, R.*, et al.* (2017). Poorly differentiated gastro-entero-pancreatic neuroendocrine carcinomas: Are they really heterogeneous? Insights from the FFCD-GTE national cohort. Eur J Cancer *79*, 158-165.

WHO, ed. (2019). WHO classification of Digestive System Tumours, 5th ed (Lyon: IARC).

 Accepted Manuscript published as ERC-23-0057.R1. Accepted for publication: 03-Jul-2023

Yachida, S., Totoki, Y., Noe, M., Nakatani, Y., Horie, M., Kawasaki, K., Nakamura, H., Saito-Adachi, M., Suzuki, M., Takai, E.*, et al.* (2022). Comprehensive Genomic Profiling of Neuroendocrine Carcinomas of the Gastrointestinal System. Cancer discovery *12*, 692-711.

Yates, L.R., Gerstung, M., Knappskog, S., Desmedt, C., Gundem, G., Van Loo, P., Aas, T., Alexandrov, L.B., Larsimont, D., Davies, H.*, et al.* (2015). Subclonal diversification of primary breast cancer revealed by multiregion sequencing. Nat Med *21*, 751-759.

Figure legends

Figure 1
Germline variants in GEP-NEN. Oncoplot showing the germline variants detected in 240 patients (columns), ordered according to affected genes (rows). Pathogenic variants are listed in the top panel, with a framed summary line at the top. Likely pathogenic variants are listed in the middle panel, with a framed summary line at the top. Variants of uncertain significance are listed in the bottom panel, with a framed summary line at the top. Variants are coloured according to molecular variant type. "Multi_hit" indicates that more than one mutation occurs in the same gene, in the same patient. The panel under the oncoplot area is composed of 3 single row colourmaps showing in order, from top to bottom, NEN group (NEC or NET G3), primary tumor site and MSI status.

Figure 2
Germline pathogenic variants in different cancer forms. Bars indicate the fraction of patients harbouring pathogenic gemline variants (dark red) and likely pathogenic variants (bright red) in 35 different cancer forms. Data for GEP-NEC and NET G3 (bold) are original data while the remaining 33 cancer types are re-assessment of raw data from Huang et al, applying the same pathogenicity scoring as for NEC and NET G3. UVM; Uveal Melanoma, CHOL; Cholangiocarcinoma, LAML; Acute Myeloid Leukemia. UCS; Uterine Carcinosarcoma, READ; Rectum Adenocarcinoma, HNSC; Head and Neck Squamous Cell Carcinoma, LUSC; Lung Squamous Cell Carcinoma, PRAD; Prostate Adenocarcinoma, LIHC; Liver Hepatocellular Carcinoma, CESC; Cervical Squamous Cell Carcinoma & Endocervical Adenocarcinoma, TGCT; Testicular Germ Cell Tumors, THCA; Thyroid Carcinoma, KICH; Kidney Chromophobe, THYM; Thymoma, LGG; Brain Lower Grade Glioma, KIRC; Kidney Renal Clear Cell Carcinoma, SKCM; Skin Cutaneous Melanoma, MESO; Mesothelioma, ESCA; Esophageal Carcinoma, UCEC; Uterine Corpus Endometrial Carcinoma, GBM; Glioblastoma Multiforme, BLCA; Bladder Urothelial Carcinoma, LUAD; Lung Adenocarcinoma, ACC; Adrenocortical Carcinoma, BRCA; Breast Invasive Carcinoma, STAD; Stomach Adenocarcinoma, COAD; Colon Adenocarcinoma, NEC; Neuroendocrine Carcinoma, KIRP; Kidney Renal Papillary Cell Carcinoma, DLBC; Lymphoid Neoplasm Diffuse Large B-cell Lymphoma, SARC; Sarcoma, PAAD; Pancreatic Adenocarcinoma, NETG3; Neuroendocrine Tumors Grade 3, OV; Ovarian Serous Cystadenocarcinoma, PCPG; Pheochromocytoma and Paraganglioma.

Figure 3
Germline pathogenic variants in GEP-NEC and NET G3 with different primary tumour sites. Bars indicate the fraction of patients harbouring pathogenic gemline variants (dark red) and likely pathogenic variants (bright red). Numbers on y-axis indicate the number of patients in each category.

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Table1. Patient characteristics

Characteristic	**Subgroup**	***n* patients**		
		NEC	**NET G3**	**HG GEP-NEN (total)**
Total		198	42	240
Age	<60	44	16	60
	≥60	154	26	180
Gender	Male	134	19	153
	Female	64	23	87
Site	Colon right	38	3	41
	Rectum	47	0	47
	Esophagus	32	1	33
	Gastric	23	1	24
	Unknown	22	7	29
	Pancreas	18	22	40
	Colon left	11	1	12
	Gallbladder/Duct	3	0	3
	Other GI	4	7	11
Metastatic site	Liver	120	39	159
	Lung	32	5	37
	Lymph node	66	9	75
	Other	54	13	67
Cell type	Large cell	119	na	na
	Small cell	74	na	na
	Unknown	5	na	na
Ki-67	21-55%	23	35	58
	>55%	171	7	178
	>20% (exact value not specified)	4	0	4
Surgery of primary tumour	Resected (prior to sampling)	69	13	82
	Not resected	129	29	158
Disease	Non-metastatic (stage I-III)	22	0	22
	Metastatic (stage IV)	176	42	218
Smoking habit	Smoker	43	5	48
	Ex-smoker	65	12	77
	Non-smoker	77	22	99
	Unknown	13	3	16

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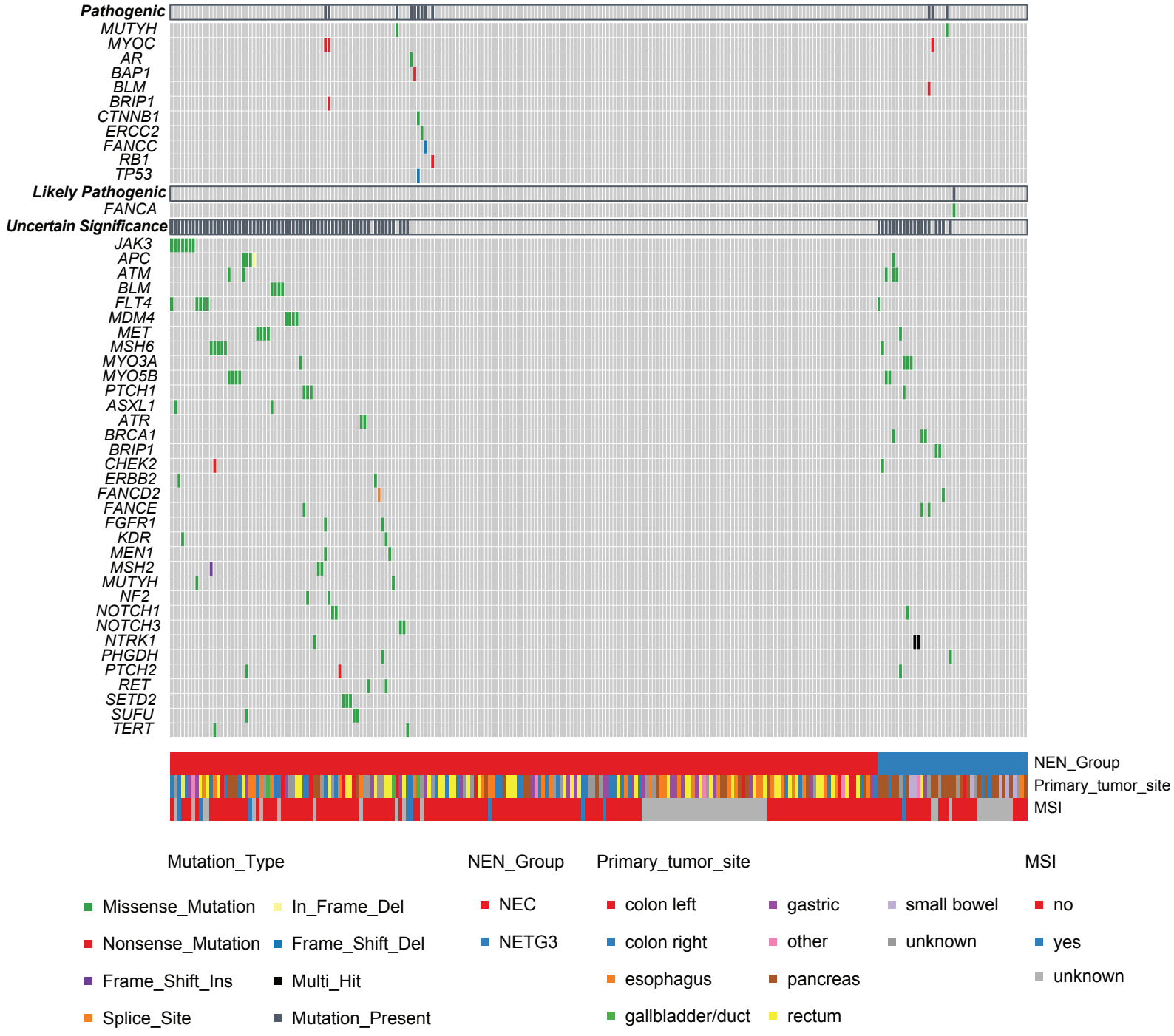
Table 2. Patients with HG-GEP-NEN and germline pathogenic variants

Study-ID	Gene	Chr.	Position	Ref.	Alt.	Variant	aa change	ClinVar Class.	LOH in tumor[3,4]	Cell type	Primary Site	Meta-stases	Gender	OS months
NEC														
5017	*AR*	chrX	66943532	C	T	missense	p.A871V	Pathogenic	No	large cell	colon right	No	female	68
5021	*TP53* *CTNNB1*	chr17 chr3	7574003 41266113	G C	- T	frameshift missense	p.R342X p.S37F	Pathogenic Pathogenic	LOH No	small cell	gallbladder /duct	Yes	male	10
7032	*ERCC2*	chr19	45856059	C	G	missense	p.R616P	Pathogenic	-	large cell	unknown	Yes	male	5
7074	*MUTYH*[1]	chr1	45798475	T	C	missense	p.Y176C	Pathogenic	-	large cell	colon left	Yes	male	9
9037	*RB1*	chr13	48955538	C	T	stop gain	p.R552X	Pathogenic	LOH	small cell	unknown	Yes	female	14
9054	*MYOC*[2] *BRIP1*	chr1 chr17	171605478 59871059	G C	A A	stop gain stop gain	p.Q368X p.E458X	Pathogenic Pathogenic	No No	large cell	rectum	Yes	male	26
9083	*FANCC*	chr9	98011507	C	-	frameshift	p.D23X	Pathogenic	LOH	large cell	gastric	Yes	female	8
10017	*MYOC*[2]	chr1	171605478	G	A	stop gain	p.Q368X	Pathogenic	No	large cell	colon right	Yes	male	8
14027	*BAP1*	chr3	52438516	A	C	stop gain	p.Y401X	Pathogenic	No	small cell	esophagus	Yes	male	10
NET G3														
5035	*FANCA*	chr16	89813023	G	A	missense	p.T1161M	Likely pathogenic	LOH		pancreas	Yes	female	26
7054	*MYOC*[2]	chr1	171605478	G	A	stop gain	p.Q368X	Pathogenic	-		pancreas	Yes	female	76
14023	*BLM*	chr15	91304245	C	T	stop gain	p.Q548X	Pathogenic	No		unknown	Yes	male	63
14028	*MUTYH*[1]	chr1	45798475	T	C	missense	p.Y176C	Pathogenic	No		pancreas	Yes	male	15

[1] Identical MUTYH variants found in one patient with NEC and one patient with NETG3
[2] Identical MYOC variants found in two patients with NEC and one patient with NETG3
[3] Copy number data for LOH assessment of tumors lacking for cases 7032, 7074 and 7054
[4] No patients had somatic mutations as “second hit” in tumor cells



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