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Whole-exome sequencing as a tool for searching for genetic background modifiers in MEN1 patients with neuroendocrine pancreatic tumours, including insulinomas

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Abstract

Introduction: Multiple endocrine neoplasia type 1 (MEN1) is a monogenic disease caused by inactivating variants in the *MEN1* gene. Although the reason for its development is well-known, disease phenotypes are unpredictable and differ even among carriers of the same pathogenic driver mutation. Genetic, epigenetic, and environmental factors may play a role in driving the individual phenotype. Those factors, however, still mostly remain unidentified. In our work, we focused on the inherited genetic background in pancreatic neuroendocrine neoplasms (pNENs) in MEN1 patients, and the pancreatic tumour subgroup with insulinoma.

Materials and methods: Whole exome sequencing was performed in MEN1 patients. The symptoms of interest were pancreatic neuroendocrine tumours in one analysis and insulinoma in the second. The study included families as well as unrelated cases. Genes with variants that are not neutral to the encoded gene product were defined in symptom-positive patients as compared to symptom-negative controls. The interpretation of the results was based on functional annotations and pathways shared between all patients with the given symptom in the course of MEN1.

Results: Whole-exome screening of family members and unrelated patients with and without pNENs revealed a number of pathways that are common for all the analysed cases with pNENs. Those included pathways crucial for morphogenesis and development, proper insulin signalling, and structural cellular organization. An additional analysis of insulinoma pNEN patients revealed additional pathways engaged in glucose and lipid homeostasis, and several non-canonical insulin-regulating mechanisms.

Conclusions: Our results show the existence of pathways that are identified in a non-literature-predefined manner, which might have a modifying function in MEN1, differentiating the specific clinical outcomes. Those results, although preliminary, provide evidence of the reasonableness of performing large-scale studies addressing the genetic background of MEN1 patients in determining their individual outcomes. **(Endokrynol Pol 2023; 74 (1): 31–46)**

Key words: *multiple endocrine neoplasia type 1; neuroendocrine tumours; pancreatic neoplasms; insulinoma; modifier; genetic background; exome sequencing*

Introduction

Multiple endocrine neoplasia type 1 (MEN1) syndrome is characterized by the simultaneous occurrence of at least 2 endocrine organ tumours in the anterior pituitary, the parathyroids, and the endocrine pancreas (making up the classical "P-triad"), with which other endocrine and non-endocrine neoplasms may co-occur [1]. The disease is inherited with high penetrance, which approaches 100% with increasing age. Almost 99% of MEN1 patients develop clinical manifestations by their early sixties, with the onset of parathyroid tumours between ages 20 and 25 years in 90% of individuals [2]. However, the expression in terms of tumour localization, age of onset, and clinical aggressiveness may vary even between affected members of the same MEN1 family and is unpredictable based on available data [3–5]. MEN1 is a dominantly inherited, monogenic disorder that develops in patients with pathogenic variants in the *MEN1* gene. All carriers of pathogenic variants in this gene will develop MEN1 syndrome throughout their lives. Tissue specificity of the tumour suppressor role of *MEN1* has been proven, which explains the general picture of the disease with the classical P-triad being a hallmark of the disease [1]. However, correlations between types of variants and clinical outcomes seem to exist only in a very limited manner. For most of the variants, no such correlations have been found despite multiple analyses that have been performed on this issue [6–8]. An association between large rearrangements in *MEN1* and earlier onset of MEN1 syndrome has been identified [9], and in some

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cases aggressiveness has been correlated with given truncating variants [10]. However, this did not seem to be the case when truncating variants were analysed all together [9]. Another study suggested an increased risk of pancreatic neuroendocrine neoplasms (pNENs) and distant metastases in patients who were carriers of *MEN1* alterations in exon 2 of the gene [11]. However, there have been multiple papers on the diversity of clinical MEN1 outcomes in different members of the same family, who are carrying the same pathogenic variant in the gene *MEN1* (for example, [12–14]). It remains elusive why some family members develop some of the symptoms while others do not, despite having the same causative *MEN1* variant.

A lot of research has been performed on the analysis of MEN1 families, and ineffective attempts to correlate the driver variant with a specific phenotype have been undertaken. Undoubtedly, the type of mutation is important because different mutant forms of the *MEN1*-encoded protein, menin, may lead to different activities and interactions of this protein; however, this kind of research has not been able to find the reason for the phenotypic variation among carriers of the same genetic variant. Our investigation proposes a different attempt that goes beyond the *MEN1* variant and targeted gene analyses, screening the patient's genome for modifiers in the genetic background. Because of the lack of clear correlations between the *MEN1* variant and clinical phenotype, also in family studies, it has been implied that other genes may act as modifiers that mask or influence the relationship between the *MEN1* gene variant and the clinical expression of the disease [15, 16]. The nature of potential genetic modifiers is a subject of great interest because further insight may enable the prediction of the course of diseases. Identifying modifier genes allows elucidation of the networks involved in the pathogenesis of disorders. Indeed, genetic modifiers can even suppress monogenic and multigenic traits in otherwise susceptible individuals [17, 18].

For other diseases, the genetic background has already been screened to indicate the impact of genetic modifiers on disease outcome complexity (e.g. [19]). There is, however, still a lack of genetic background screenings in MEN1 patients with different clinical features, which would allow for the identification of genetic modifiers that are not predefined by literature data. In this study, we focus on pancreatic tumourigenesis in MEN1. It seems highly likely that also in this case the combined effects of different genetic variants, possibly together with environmental factors, might be decisive for the individual patient's disease outcome. To address this issue at the genetic level, we performed germline exome-sequencing in relatives with MEN1 with different pancreatic tumour outcomes, as well as in unrelated patients, to identify signalling pathways with disrupted genes that are common among MEN1 patients with given clinical characteristics.

The aim of the study is to verify the existence of alterations in the genetic background of MEN1 patients, with a focus on impacted signalling cascades, which are associated with the presence of pancreatic NENs and insulinomas.

Material and methods

Patients

Two separate analyses were performed. In the first one, the presence of a pancreatic NEN in the course of MEN1 was the symptom of interest. In the second analysis, insulinoma versus non-insulinoma NEN MEN1 cases were compared. Altogether, 14 MEN1 patients were included in the study, encompassing 2 families as well as unrelated cases. Most of the patients chosen for analysis were over 40 years old, because by that age all the clinical manifestations of MEN1 syndrome are present in 95% of affected patients [1], which was particularly important in the case of subjects without the symptom ("symptom –").

Pancreatic NEN analysis

For pancreatic NEN (pNEN) analyses, 2 families were included (groups #1 and #2), as well as one group of unrelated patients (#3), all with MEN1. In family 1, 1-4 was the control without pNEN, and the remaining patients were pNEN-positive. The patients 1-1, 1-2, and 1-4 were siblings and 1-3 was their nephew, whose father passed away aged 38 years due to hepatic encephalopathy. In family 2, patient 2-1 had a pancreatic NEN, while 2-2 was the control without pNEN. The patients 2-1 and 2-2 were siblings. The unrelated MEN1 patient 3-3 without pNEN was included in analyses as a negative control, while the pNEN+ MEN1 patients 3-1 and 3-2, who were unrelated to any of the remaining study participants, served as the verification group of the obtained results. Among the families, MEN1 was determined by the same pathogenic variant in the *MEN1* gene, while in the unrelated patients different alterations in *MEN1* were responsible for the disease. For each analysis, the pNEN+ patients from a given family and all pNEN– controls were included. Characteristics of the groups are summarized in Table 1.

Insulinoma analysis

For insulinoma analyses, 3 unrelated MEN1 patients with insulinoma were included and analysed independently of each other. Unfortunately, no MEN1-positive family members with pancreatic NEN and at a suitable age to serve as controls were available. However, one of the insulinoma patients had a disease-causing *MEN1* variant that was identical to the variant found in one of the families treated in our department, despite being unrelated to this family at least 2 generations back, as was determined based on interviews with both families. For the analysis of this patient, 2 non-insulinoma pNEN patients from this family were analysed as controls (patient group #4). In the case of insulinoma patients 5-1 and 5-2, three unrelated MEN1 non-insulinoma pNEN patients, including 4-2, were included as controls. One of the insulinoma patients, 5-2, was diagnosed at a very young age. Due to the large deletion in the *MEN1* locus that was responsible for the patient's disease, the obtained results need to be interpreted carefully, because some literature data have suggested that large deletions in the *MEN1* gene might themselves be responsible for a worse disease outcome. The characteristics of the analysed patients are summarized in Table 2.

Table 1. *Clinical data of patients included in the pancreatic neuroendocrine tumours (pNEN) analysis*

Group #	Patient #	*MEN1* alteration	Age at the time of analysis	Sex	Pancreatic NEN (age at diagnosis)	Primary hyperparathyroidism	Pituitary tumour	Adrenocortical carcinoma	Other
1 (family)	1-1	c.1246_1248delGCC/p.Ala416del	Deceased (aged 46)	F	Yes (37 — disseminated)	Yes	No	No	ND
	1-2		59	F	Yes (48)	Yes	No	No	ND
	1-3		38	M	Yes (35 — locally advanced)	Yes	No	No	Suspicion of thymoma
	1-4 *		48	M	No	Yes	No	No	ND
2 (family)	2-1	c.866C>A/p.Ala289Glu	62	M	Yes (52 — locally advanced)	Yes	Yes	No	Thymoma, clear cell renal cell carcinoma
	2-2 *		59	F	No	Yes	Yes	No	Nasal cavity cancer
3 (unrelated)	3-1	c.416A>G/p.His139Arg	75	F	Yes (68)	Yes	No	Yes	ND
	3-2	c.796C>T/p.Gln266Ter	65	F	Yes (59 — locally advanced)	Yes	No	No	ND
	3-3 *	c.1256delTGC/p.Leu419del	75	M	No	Yes	Yes	Yes	ND

*patients included as pNEN-negative controls in the analysis of all patient groups, i.e. family 1, family 2, unrelated patient 3-1, and unrelated patient 3-2. *MEN1* amino acid numbering according to NP_000235; nucleotide numbering according to NM_000244; ND — no data

Table 2. *Clinical data of patients included in the insulinoma analysis*

Group #	Patient #	*MEN1* alteration	Age at the time of analysis	Sex	Pancreatic NEN type (age at diagnosis)	Primary hyperparathyroidism	Pituitary tumour	Adrenocortical carcinoma	Other
4 (unrelated)	4-1	c.945delG/p.Tyr316Profs*57	72	F	Insulinoma (47, age at surgery)	Yes	Yes	No	ND
	4-2*		59	M	Non-insulinoma (44)	Yes	No	Yes	ND
	4-3**		48	F	Non-insulinoma (43)	Yes	Yes	No	ND
5 (unrelated)	5-1	c.866C>A/p.Ala289Glu	62	M	Insulinoma (52 — locally advanced)	Yes	Yes	No	Thymoma, clear cell renal cell carcinoma
	5-2	c.(1293_1365)_(1734_?)del	24	F	Insulinoma (16)	No	No	No	ND
	5-3*	c.1246_1248delGCC/p.Ala416del	54	M	Non-insulinoma (43)	Yes	Yes	Yes	ND
	5-4*	c.796C>T/p.Gln266Ter	65	F	Non-insulinoma (59 — locally advanced)	Yes	No	No	ND

*patients included as insulinoma-negative MEN1 controls in the analysis of insulinoma-positive patients; ** additional MEN1 pNEN insulinoma-negative control, used only in the analysis of patient 4-1. *MEN1* amino acid numbering according to NP_000235; nucleotide numbering according to NM_000244; ND — no data

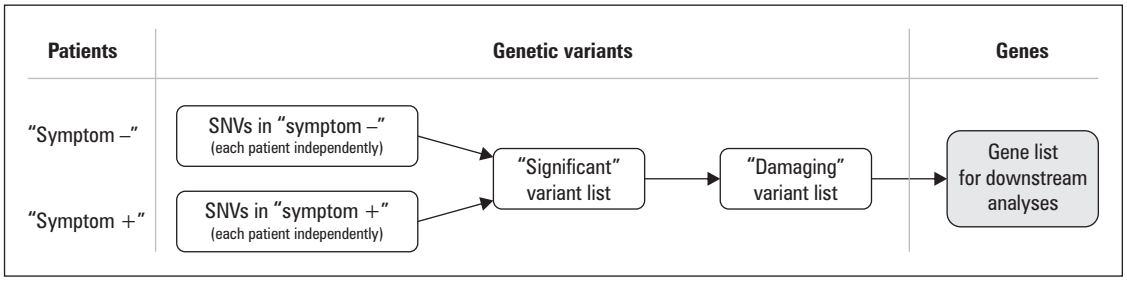

Figure 1. *Schematic representation of the analyses performed independently in each family and unrelated patients. SNV — small nucleotide variant; "symptom+" — pancreatic neuroendocrine tumour (pNEN) in pNEN analysis or insulinoma in insulinoma analysis; "symptom–" — no pancreatic NEN in pNEN analysis or non-insulinoma pNEN in insulinoma analysis; "significant" variants — alleles not present in any negative control but present in all symptom-positive patients, or alleles heterozygous in negative controls but homozygous in symptom-positive patients; "damaging" variants — variants that are not neutral to the encoded gene product, according to at least 3 sources among SIFT, LRT, MutationTaster, Polyphen2 (HumDiv or HumVar), MutationAssessor, FATHMM, and either of the meta predictors MetaSVM or MetalR*

Ethical statement

The study is in accordance with the principles set out in the Declaration of Helsinki, and the study design was approved by the Bioethics Committee of the Jagiellonian University in Krakow, Poland (Opinion No. 122.6120.267.2015). The study participants gave their informed consent for genetic analyses within the scope of the study.

DNA extraction and WES library preparation

DNA was isolated from the whole blood of the patients collected in an anticoagulation tube on a Maxwell® 16 Instrument using a Maxwell® 16 LEV Blood DNA Kit (Promega). The obtained DNA was quantified spectrophotometrically using NanoDrop and fluorometrically with the QuantiFluor dsDNA system on a Quantus instrument (Promega). The whole genomic DNA was sheared in Bioruptor (Diagenode), and sample quality was assessed using the Agilent High Sensitivity D1000 ScreenTape assay on TapeStation (Agilent). Whole-exome sequencing libraries were prepared using the Agilent Technologies SureSelect XT Reagent Kit. Exon capture was performed using the OneSeq Constitutional Research Panel from Agilent. After capture, the enriched libraries were tagged with indexes in a 10-cycle PCR reaction. The samples were multiplexed in equal molar concentrations. The pool was sequenced in an Illumina HiSeq sequencer by an external service provider (EMBLEM, Heidelberg, Germany).

NGS data analysis

Raw reads were processed with the Illumina software, generating base calls and corresponding base-call quality scores. The generated FASTQ files were fed into FastQC software (version 0.11.5) to provide quality control checks on sequenced data [20]. Reads were aligned to the human reference genome GRCh37 (hg19) using the BWA-MEM algorithm from the Burrows Wheeler Aligner (BWA, version 0.7.5) [21]. Unmapped and low mapping quality score reads were filtered out with SAMtools (version 0.1.19) [22]. Further post-alignment quality controls and annotating were performed with Strand NGS (version 4.0) (Strand Life Sciences Pvt. Ltd., Bangalore, India). Data were labelled with dbSNP151 variant indicators. Significant variants were identified, which are defined as alleles that were not present in any negative control but present in all symptom-positive patients, or that were heterozygous in negative controls but were homozygous in symptom-positive patients. Those lists were filtered for "damaging" variants, i.e. variants that were not neutral to the encoded gene product, according to at least 3 sources among SIFT, LRT, MutationTaster, Polyphen2 (HumDiv or HumVar), MutationAssessor, FATHMM, and any of the meta predictors MetaSVM or MetalR, and such that none of the variants identified in the symptom+ patient of a group is found in any other symptom+/symptom– pair in the same zygosity state, if present in this pair. The obtained region lists were translated into gene lists for downstream pathway and gene ontology (GO) analyses. The above analyses were performed independently for pancreatic NEN and for insulinoma as the analysed symptom. As an effect, we obtained one list of genes for each analysed family or unrelated patient, each of which contained genes with small nucleotide variants (SNVs) in the patients affected with the analysed symptom ("symptom+": pancreatic NEN for pNEN analysis or insulinoma for insulinoma analysis) but not the patients without the symptom ("symptom–": no pancreatic NEN for pNEN analysis or non-insulinoma pNEN for insulinoma analysis). Schematically, this is shown in Figure 1.

Downstream analysis

Gene ontology (GO) and interaction analyses were performed with the online-accessible tools Panther17.0 [23] available at geneontology.org [24, 25] (resource release 2022-03-22) and String (v11.5) [26]. An overrepresentation test for biological processes was performed using Fisher's exact test with False Discovery Rate (FDR) calculation, where appropriate. Stated minor allele frequencies were based on data from gnomAD version 2.1.1 for the European, non-Finnish population [27]. Pathway analyses for the obtained gene lists were performed in Strand NGS (WikiPathways version 20210910) and with online-accessible tools: ComPath accessible via compath.scai.fraunhofer.de [28] and NDEx v2.5.3, accessible at www.ndexbio.org [29, 30].

Results

pNEN vs. no pNEN

To investigate the genetic background of MEN1 patients with pancreatic NEN as compared to MEN1 patients without this tumour, we performed a comparison of genetic germline variants in all patient groups (family #1, family #2, patient 3-1, and patient 3-2) independently, as shown schematically in Figure 1. In this analysis, we expected to identify several variants that could render given family members susceptible or not to the development of pancreatic NEN within the course of MEN1. Therefore, in those analyses, the variants altered in all pNEN+ patients from a given group as compared to the pNEN– patients were

Table 3. *Gene ontology (GO) analysis of genes differentiating pancreatic neuroendocrine tumour (pNEN) from no-pNEN multiple endocrine neoplasia type 1 (MEN1) patients of family 2; all statistically significant [false discovery rate (FDR) p < 0.05] biological processes are listed*

GO biological process complete	Homo sapiens (REF) #	Mapped genes	Expected	Fold Enrichment	Raw p value	FDR
Positive regulation of calcium ion transport into cytosol	14	3	0.05	55.85	3.52E–05	3.45E–02
Animal organ morphogenesis	1003	17	3.85	4.42	2.36E–07	6.18E–04
Anatomical structure morphogenesis	2237	23	8.58	2.68	7.67E–06	1.34E–02
Anatomical structure development	5144	42	19.74	2.13	1.27E–07	3.99E–04
Developmental process	5677	42	21.78	1.93	1.76E–06	3.95E–03
Animal organ development	3254	35	12.49	2.80	2.17E–09	3.40E–05
Organelle assembly	803	12	3.08	3.89	5.88E–05	4.86E–02
Cellular process	15044	73	57.72	1.26	3.17E–05	3.31E–02
Homeostatic process	1424	17	5.46	3.11	2.51E–05	3.28E–02
Tissue development	1726	20	6.62	3.02	6.32E–06	1.24E–02
Nervous system development	2191	22	8.41	2.62	1.87E–05	2.67E–02
System development	3838	36	14.73	2.44	4.46E–08	1.75E–04
Multicellular organism development	4228	39	16.22	2.40	1.22E–08	9.59E–05
Behavioural response to pain	16	3	0.06	48.87	4.98E–05	4.34E–02
Multicellular organismal process	6581	50	25.25	1.98	1.59E–08	8.32E–05
Sensory perception of sound	160	6	0.61	9.77	4.20E–05	3.87E–02
Sensory perception of mechanical stimulus	181	7	0.69	10.08	7.69E–06	1.21E–02
Unclassified	2725	0	10.46	< 0.01	3.06E–05	3.69E–02

obtained and filtered for variants that were not neutral to the encoded gene product. Besides a family-derived control which allowed us to better control for the genetic background, and therefore, to exclude most of the irrelevant variants, we included also the unrelated controls from the remaining patient groups. The obtained lists of variants that are not neutral to the encoded gene product were translated into gene lists. On those lists, pathway and GO analyses were performed. Genetic modifiers may also occur commonly in untargeted populations and might themselves not result in any phenotype [31]. Therefore we have not excluded variants based on their frequency or strict disease-assessing classifiers like ACMG.

In family 1, 19 genetic variants in 14 genes were identified that were common for all pNEN-positive patients in this family. The gene ontology (GO) analysis indicated that those genes are involved in the following biological processes: cellular processes (especially cellular metabolic, cellular developmental, cellular component organization or biogenesis, cell communication, cellular response to stimulus), biological regulation, metabolic process, multicellular organismal processes, developmental processes, localization, signalling, response to stimulus, and locomotion.

In family 2, a list of 91 variants in 82 genes was obtained that were characteristic for the pNEN patient. GO biological process analysis revealed the biological processes listed in Table 3 to be significantly represented.

In the analysis of unrelated cases, 172 genes and 162 genes were identified for the unrelated patients 3-1 and 3-2, respectively. Only one gene was shared by all pNEN+ patients, i.e. *TTN*, which is, however, a large and commonly altered gene, and therefore this result needs to be interpreted with caution. The genes that were shared by 3 of the 4 pNEN+ groups (related and unrelated) are *DISC1*, *DNAH5*, *LAMA5*, and *OTOG*, which were detected in all groups except family 1, and *SYNE2*, identified in all cases but the unrelated 3-1.

GO-annotated processes that were represented in all the analysed groups, i.e. 2 families and unrelated patients, were annotations related to developmental processes but also response to stimulus and localization/transport. The pathways that are shared by all pNEN cases are presented in Figure 2.

Insulinoma vs. non-insulinoma pNEN

An analogous analysis was performed for insulinoma patients, using insulinoma as the group-differentiating symptom.

The analysis of significant genes with damaging variants (as described in Methods) in patient 4-1,

WP727 - Monoamine transport (32)			
DBH (fam.1)	*TSC2* (fam.2, pat. 3-2)	*TPH2* (pat. 3-1)	*ACHE* (pat. 3-2)

WP3925 - Amino acid metabolism (94)					
DBH (fam.1)	*ACO2* (fam.2)	*CTH* (fam.2)	*PNMT* (fam.2)	*TPO* (pat. 3-1, pat. 3-2)	*FAH* (pat. 3-1)

WP4172 - PI3K-Akt signalling pathway (340)						
PCK2 (fam.1)	*ITGB4* (fam.2)	*LAMA5* (fam.2, pat. 3-1, pat. 3-2)	*TSC2* (fam.2, pat. 3-2)	*PHLPP2* (fam.2, pat. 3-2)	*THBS4* (fam.2)	*EGF* (pat. 3-1)
NGF (pat. 3-1)	*COL4A5* (pat. 3-2)	*COL6A3* (pat. 3-2)	*LAMA2* (pat. 3-2)	*LAMA4* (pat. 3-2)	*SOS1* (pat. 3-2)	

WP4535 - Envelope proteins and their potential roles in EDMD physiopathology (46)				
SYNE2 (fam.1, fam.2, pat. 3-2)	*PLEC* (pat. 3-1, pat. 3-2)	*ADCY9* (pat. 3-1)	*TMEM43* (pat. 3-1)	*SOS1* (pat. 3-2)

WP2806 - Complement system (136)							
LRP2 (fam.1, pat. 3-2)	*LAMA5* (fam.2, pat. 3-1, pat. 3-2)	*CR1* (pat. 3-1)	*MASP2* (pat. 3-1)	*C1S* (pat. 3-2)	*C7* (pat. 3-2)	*F13A1* (pat. 3-2)	*SELE* (pat. 3-2)

WP4312 - Rett syndrome causing genes (49)		
SYNE2 (fam.1, fam.2, pat. 3-2)	*GRIN2A* (pat. 3-1)	*HTT* (pat. 3-2)

WP383 - Striated muscle contraction pathway (38)			
TTN (fam.1, fam.2, pat. 3-1 pat. 3-2)	*MYH6* (fam.1)	*ACTN2* (pat. 3-1)	*TNNT1* (pat. 3-2)

WP4298 - Acute viral myocarditis (98)					
MYH6 (fam.1)	*PARP1* (fam.2)	*ABL1* (pat. 3-1)	*LAMA2* (pat. 3-2)	*PARP1* (pat. 3-2)	*SOS1* (pat. 3-2)

Figure 2. *Pathways with altered genes in all pancreatic neuroendocrine tumour (pNEN)-positive multiple endocrine neoplasia type 1 (MEN1) patients. Pathway numbers and nomenclature according to WikiPathways, followed by brackets with the number of unique entities in the pathway. Below each pathway, the identified altered genes are presented, together with the indication about the family and/or patient in which this gene is mutated (in brackets). fam1 — family 1; fam2 — family 2; pat. — patient*

where 2 of the control patients had the same pathogenic *MEN1* variant, revealed a list of 78 genes. Gene ontology of biological processes revealed that mainly overrepresented processes were related to cellular metabolic processes but also cellular component organization or biogenesis.

For patient 5-1, the GO analysis of 159 altered genes revealed a set of significantly overrepresented annotations that were related to base-excision repair, sensory perception of mechanical stimulus, homeostatic processes, and several annotations related to anatomical structure development.

In the case of patient 5-2, among 158 significant genes, annotations were overrepresented that were related to anatomical structure development, the response to external stimulus, extracellular matrix, and organelle organization.

Thus, in our group of insulinoma patients, only functional annotations related to anatomical structure development seem to be common for all patients in the GO analysis.

Genes that were altered in all insulinoma cases are *CHAT*, *DOCK8*, *P2RX7*, *TTN*, and *TTN-AS1*.

The overview of enriched Reactome pathways revealed the following observations. For patient 4-1, several pathways related to signal transduction, developmental biology, the metabolism of proteins, and cholesterol metabolism were observed. In the case of patient 5-1, metabolism of lipids and steroids, glycogen metabolism, and extracellular matrix organization were presented repeatedly. In patient 5-2, enriched Reactome pathways clearly indicated a strong involvement of the genes in different pathways related to plasma lipoprotein metabolism, extracellular matrix organization, and metabolism of proteins.

The pathways (Wiki) shared for all 3 analysed patient groups are presented in Table 4. We took a closer look at all of them, focusing especially on pathways with a small number of genes, which are further described in the Discussion section.

Table 4. ***WikiPathways with altered genes in all insulinoma multiple endocrine neoplasia type 1 (MEN1) patients. The total numbers of nodes (genes) in the pathways are shown. Presented are all pathways in which at least one significant damaging genetic event in every insulinoma patient occurred that was not observed in non-insulinoma pancreatic neuroendocrine tumour (pNEN) patients***

Pathway identifier	Pathway name	Number of all genes in pathway
WP528	Acetylcholine synthesis	7
WP2059	Alzheimer's disease	149
WP3925	Amino acid metabolism	91
WP550	Biogenic amine synthesis	16
WP4262	Breast cancer pathway	156
WP688	Catalytic cycle of mammalian flavin-containing monooxygenases (FMOs)	5
WP558	Complement and coagulation cascades	59
WP3601	Composition of lipid particles	10
WP4016	DNA IR-damage and cellular response via ATR	80
WP3959	DNA IR-double strand breaks (DSBs) and cellular response via ATM	55
WP2858	Ectoderm differentiation	143
WP4239	EMT in colorectal cancer	162
WP474	Endochondral ossification	65
WP2853	Endoderm differentiation	145
WP5110	Familial hyperlipidaemia type 3	13
WP306	Focal adhesion	212
WP3932	Focal Adhesion-PI3K-Akt-mTOR-signalling pathway	309
WP176	Folate metabolism	69
WP5066	FOXA2 pathway	21
WP1591	Heart development	46
WP3646	Hepatitis C and hepatocellular carcinoma	51
WP3601	Lipid particles composition	10
WP702	Metapathway biotransformation phase I and II	177
WP2911	Mirna targets in ECM and membrane receptors	43
WP2064	Neural crest differentiation	102

→

Table 4. *WikiPathways with altered genes in all insulinoma multiple endocrine neoplasia type 1 (MEN1) patients. The total numbers of nodes (genes) in the pathways are shown. Presented are all pathways in which at least one significant damaging genetic event in every insulinoma patient occurred that was not observed in non-insulinoma pancreatic neuroendocrine tumour (pNEN) patients*

Pathway identifier	Pathway name	Number of all genes in pathway
WP404	Nucleotide metabolism	19
WP43	Oxidation by Cytochrome P450	63
WP4172	PI3K-Akt signalling pathway	345
WP2572	Primary focal segmental glomerulosclerosis FSGS	74
WP4900	Purinergic signalling	33
WP15	Selenium micronutrient network	88
WP430	Statin inhibition of cholesterol production	33
WP383	Striated muscle contraction	38
WP706	Sudden infant death syndrome (SIDS) susceptibility pathways	162
WP1533	Vitamin B12 metabolism	53
WP3658	Wnt/beta-catenin signalling pathway in leukaemia	24

Discussion

We expected that in different patients with pancreatic tumours in the course of MEN1, different combinations of altered genes can be identified. This is supported by the fact that the mutual exclusiveness of mutations in several genes among some pathways has already been proven in pNENs [32, 33]. Therefore, significant variants can differ between families, and in large cohorts, a heterogeneity of genetic combinations should be expected that may lead to a similar disease outcome. In our analysis, we provide a first approach to identifying such inherited genetic combinations that correlate with the development of pNENs. Patients were selected from an ethnically homogeneous group, and family analyses were performed. This allowed us to identify some of the possible genetic sets that are present in MEN1 patients with a similar outcome in means of the presence or absence of pNENs. We, therefore, verified whether common pathways might be identified in patients with pNEN in the course of MEN1 but not present in MEN1 patients without pNEN, and whether such genes would be logically associated with this symptom. Additionally, we focussed also on the group of insulinoma-positive NEN cases.

Pancreatic NENs

GO-annotations for pNEN+ MEN1 patients were related to developmental processes, response to stimulus, and localization/transport.

As a confirmation of the developmental processes recognized by GO analysis, among the pathways with genetic alterations was the monoamine transport pathway. Interestingly, in the case of the pNEN patients from our study, all of them had alterations in a narrow part of this pathway, i.e. in genes involved in tyrosine metabolism. It is well-known that neuroendocrine tumours arise from amine precursor uptake and decarboxylation (APUD) cells, which are characterized by a high uptake of amine precursors and the conversion of such precursors to amines. According to our result, alterations in tyrosine metabolism-related genes might be considered as one of the drivers associated with pNEN development in MEN1 patients.

The identified structural pathway WP4535 contains genes responsible for the structural organization of cells by interlinking elements of the cytoskeleton and anchoring organelles to the cytoskeleton, therefore playing crucial roles in maintaining cell and tissue integrity. The predominantly mutated gene in our patients in this case was the nesprin-encoding gene *SYNE2*. The role of nesprins in pancreatic tumours has been suggested but not yet defined [34].

The PI3K-Akt signalling pathway WP4172 identified in our study is very large, mainly due to the numerous factors that activate this pathway. In our analysis, the identification of this pathway was defined by several genes. Interestingly, all pNEN cases, including family 1 with only one gene recognized in this pathway, encompassed signal transduction factors that are, among others, related to insulin signalling and glucose metabolism: *PCK2* catalyses the rate-limiting step in the metabolic pathway that produces glucose from precursors [35]. *TSC2* is a tumour suppressor that functions as a negative regulator of mTOR signalling, which is a signalling network induced by several molecules including insulin [36], and *TSC2* deletion has been shown to be responsible for the hypertrophy of pancreatic beta cells

and to increase insulin secretion from pancreatic beta cells [37]. The guanine nucleotide exchange factor SOS encoded by *SOS1* links the activated insulin receptor to downstream signalling molecules [38, 39].

The complement pathway was identified due to protein-encoding genes that interact with this pathway rather than complement components themselves, because interacting genes were mutated in all the cases, as opposed to complement components. *LRP2* is a macromolecule-binding receptor expressed primarily in absorptive epithelial tissues, which is critical for the uptake of lipoproteins, sterols, vitamin-binding proteins, and hormones, including leptin, and is required in embryonic development processes [40]. The protein encoded by this *LRP2*, megalin, is an endocytic reabsorption receptor for insulin [41] and is required for the insulin-dependent internalization of the insulin receptor [42]. Its expression is supposed to be regulated by insulin [43, 44]. *LAMA5* is a laminin subunit-encoding gene. Laminins are a family of extracellular matrix (ECM) glycoproteins that mediate the attachment, migration, and organization of cells into tissues during embryonic development by interacting with other components of the ECM [40]. Extracellular matrix deposition and remodelling are known to be involved in insulin resistance, and laminin staining is increased in the biopsies from diabetic patients [45]. One of the laminins, which the *LAMA5* gene product is part of, has been shown to be a main target of antibodies in autoimmune pancreatic disease [46]. Therefore, the main functions of the genes that were indicated by this pathway are related to mechanisms other than regulation of the complement system, and they are, among others, involved in mechanisms critical for the uptake of lipoproteins and leptin, the reabsorption and internalization of the insulin receptor, and insulin resistance.

Investigations on germline variants in pancreatic tumours have been performed before. However, our data differ from previous studies in that we analysed specifically endocrine cancer-predisposed MEN1 cases with or without pNENs, in contrast to other studies, which focus on pNENs as a whole or on sporadic cases. Scarpa *et al.* have identified pathogenic germline deleterious variants in pNEN patients in DNA repair genes, including mutations in the genes *MUTYH*, *BRCA2*, and *CHEK2* [32]. Contrary to our study, they focused on apparently sporadic (i.e. non-MEN1) patients with pNEN. Also, because Scarpa *et al.* were trying to assess the incidence of hereditary syndromes in seemingly sporadic cases, the authors only looked for variants in the pNEN patient group without comparing them to a control group. We, on the contrary, analysed genes that were altered in MEN1 patients with pNEN but not in MEN1 patients without pNEN. Therefore, the results of their study must be treated as complementary to the results obtained by us.

At the somatic level, an altered expression of genes in the mTOR pathway has been found in most pNENs [47]. However, damaging variants in the mTOR pathway, including mutations in *TSC2*, *PTEN*, and *PIK3CA*, have been reported to be present only in a part of pNEN tissues [33]. In the patients from our study, germline variants in genes from this pathway are found to be a shared hallmark in all patients with pNEN that develop the tumour in the course of MEN1. The mutations most commonly identified in pNEN tissues so far are found in genes responsible for chromatin remodelling (*MEN1*, *ATRX*, and *DAXX*) [33, 48]. The predictive value of the genetic status of mutations in those genes and on the tumour's epigenetic pattern has been proposed [49, 50]. Chan *et al.* found that tumours with a somatic mutation signature in *MEN1*, *ATRX*, and *DAXX* have distinct expression clusters and methylation patterns than pNENs without this signature [48]. However, it has been shown in animal models that the loss of function of *Daxx* and *Atrx* genes, whether with or without *Men1* loss, are themselves not responsible for driving or accelerating the tumourigenesis of pNENs [51]. The authors of this latest study stress that it is the human genome context that is essential for pancreatic tumourigenesis, and this is where our study provides insight. We address the issue of the inherited genetic background of patients that do or do not develop pNENs, while being in general predisposed to endocrine tumours in the course of MEN1. Therefore, results from somatic studies must be interpreted as complementary to our study. Taken together, a whole picture of the development of pNENs and their further fate can be addressed, where inherited combined mutations in the patient's genetic background may be responsible for the occurrence of pNEN, and additional somatic events in DNA repair, intracellular signalling, and epigenetic regulation are related to the further heterogeneity among those tumours. However, those results need to be verified also specifically for the group of MEN1 patients, especially because it is known that pNENs with mutated *MEN1* and other chromatin remodelling genes in the tissue have distinct gene expression patterns compared to other pNENs [48]. To our knowledge, analyses limited to the group of patients with inherited MEN1 were not undertaken before the present analysis.

The overall picture from this part of our study is that, when compared with MEN1 patients without pancreatic NEN, in patients with MEN1-related pancreatic NENs in the course of their disease, besides disruptive *MEN1* mutations, additional damaging events

are present in genes involved in pathways crucial for neuroendocrine tumour precursor cells, structural cellular organization, and proper insulin signalling and/or lipoprotein turnover.

Insulinoma vs. non-insulinoma

The pathway analysis set many of the identified genes in a context that seems reasonable for insulinomas, including the homeostasis of glucose and lipoproteins, insulin release regulation, and immune-related interactions.

A small pathway, with only 5 entities, which was not represented in the previous pNEN analysis, was identified specifically in insulinoma patients — the mammalian flavin-containing monooxygenase (FMO) pathway (WP688). Damaging genetic variants in at least one FMO gene in each analysed patient group are responsible for its identification in our study. The link between FMOs and lipid and glucose metabolism is not new. Insulin is involved in the regulation of FMO activity [52], and the major significance of FMO3 and FMO5 in the homeostasis of glucose and lipids has been proven, where genetic knockouts of FMO3 lead to a decrease in plasma lipids, glucose, and insulin, and the inactivation of FMO5 increased glucose tolerance and insulin sensitivity [53–55]. In our patients, also deleterious mutations in FMO2 were identified. Another small pathway identified specifically in insulinoma cases is WP528, with only 7 entities. In all analysed cases, it was identified due to damaging variants in the *CHAT* gene, the product of which is responsible for catalysing the biosynthesis of acetylcholine. In pancreatic islets, there is a non-neuronal cholinergic system, in which the insulin release from beta-cells is induced by acetylcholine which is produced by adjacent *CHAT*-expressing alpha-cells [56]. Related to this network is the biogenic amine synthesis pathway (WP550), where in our study, in addition to the gene *CHAT*, also genes involved in dopamine to epinephrine conversion were identified (*DBH* and *PNMT* in patients 4-1 and 5-1, respectively). Epinephrine is a well-known direct antagonist of glucose-induced insulin action [57, 58]. It has also been shown before that the deficiency of DBH leads to the enhancement of glucose-stimulated insulin secretion, hyperinsulinaemia, and insulin resistance [59].

The FOXA2 pathway (WP5066) also consists of a small number of nodes. Here, this pathway was identified due to mutations in the genes *APOB* and *SLCO1B1*, which are located downstream of *FOXA2*, a transcriptional activator that is important in pancreas development, insulin secretion, and glucose metabolism and is suppressed by insulin [60, 61].

Those results indicate that in MEN1 insulinoma patients, additional hits in the insulin-glucose regulation occur at the germline DNA level, besides those observed in pNEN cases, and that those hits are observed in genes and pathways that are not canonical drivers of glucose homeostasis. This observation is reflected also by the conclusions of other studies. For example, in a methylome sequencing assay, it was shown that sporadic insulinomas present with abnormal methylation patterns, which affect beta-cell specification and provide alternative drivers of insulin expression. Based on their observations, the authors concluded that in insulinomas, key canonical, beta cell-specific transcription factors are hypermethylated and are therefore poorly accessible to their normal transcriptional regulatory machinery [62]. Wang *et al.* observed disturbances in the control of glucose sensing and insulin secretion, by identifying alterations in glucose transporter and *HK3* profiles [63]. They confirmed that, in their study, canonical transcription factors of beta cells (*PDX1*, *NKX6.1*, *MAFA*, etc.) were not altered in insulinomas, and their expression was not changed by mitogenic genes. In addition, the authors also observed that mutations in DNA repair genes that are characteristic for non-functioning pNENs, such as *ATRX*, *DAXX*, *BRCA2*, and mutations in genes of the PI3 kinase/mTOR pathway, were not present in insulinomas [63]. Also, this observation is confirmed by our study, although at the germline level, where a different set of pathways was identified to characterize insulinomas in contrast to pNENs as a whole.

A number of pathways related to lipid metabolism are also small and identified only in the insulinoma analysis. WP5110 — familial hyperlipidaemia type 3 was identified due to the identification of *LRP1*, *APOE*, and *SCARB1* in patients 4-1, 5-1, and 5-2, respectively. *LRP1* encodes an LDL receptor family member, which is involved in cellular processes like signalling, lipid homeostasis, and apoptotic cell clearance; *SCARB1* is a plasma membrane HDL receptor; and *APOE* is a major apoprotein of the chylomicron that is essential for the normal catabolism of triglyceride-rich lipoproteins [64, 65]. Mutations in *APOE* lead to increased plasma cholesterol and triglycerides because of impaired clearance of chylomicron and VLDL remnants. Lipid particle composition (WP3601) is on our list of identified pathways due to genetic variants in the apolipoprotein-encoding genes *APOB* and *APOE*, which are part of or bind to chylomicrons, LDL, and VLDL [64, 66, 67]. Statin inhibition of cholesterol production (WP430) is another identified example related to lipid metabolism. Among this pathway, mutations in *APOB* and *LRP1* were identified in patient 4-1, *APOA5* and *APOE* in patient 5-1, and *APOB* and *SCARB1* in patient 5-2, which means that in each patient, at least one apolipoprotein from this pathway was mutated. Folate and vitamin B12 me-

tabolism pathways were identified in our insulinoma analysis due to mutations in *APOB* — the main apolipoprotein of chylomicrons and LDL, and genes involved in the metabolism of homocysteine, *CTH* (converts cystathionine derived from methionine into cysteine) and *MTHFR* (homocysteine remethylation to methionine). The proper homeostasis of homocysteine has been shown to be significant for controlling insulin secretion and action [68, 69].

A closer look at the purinergic signalling pathway revealed that it was identified in our insulinoma patients only due to mutations in purinoreceptors, with mutations in the pro-inflammatory gene *P2RX7* being observed in all cases, and additionally other purinoreceptors in 2 of the insulinoma cases (*P2RX2* and *P2RX4* in 5-1 and *P2RY11* in 5-2). *P2RX7* is the paradigmatic cytotoxic nuclear receptor and is expressed at a high level in many malignant tumours [70].

Another identified small pathway is related to nucleotide metabolism (WP404). This network was revealed in our insulinoma analysis due to damaging variants located only in polymerase genes in all insulinoma patients: *POLA1* and *POLG* in patient 4-1, *POLG* in 5-1, and *POLB* in 5-2.

The PI3K-Akt signalling pathway has also been shown to be affected in this analysis; however, this is predominantly due to genes upstream to the pathway, i.e. involved in cellular processes that may activate this pathway. In contrast, in the pNEN analysis, downstream effectors were identified to be mutated.

Supported by their study, Wang *et al.* stress the fact that there is mutational heterogeneity of insulinoma tissues [63]. This is in accordance with the assumptions that were the basis of our study plan, which is why we focused on impacted pathways instead of looking for commonly altered genes. However, as Wang *et al.* showed, *MEN1*-related events are present in most of the investigated insulinomas, despite the absence of a germline predisposition for MEN1 in the patients [63]. It has been shown by Hamze *et al.* that the *MEN1*-encoded menin regulates the mRNA and protein levels of *MAFA*, a significant glucose-dependent transcription factor in differentiated beta-cells [71]. In our study, all patients had the *MEN1* gene mutated at the germline, and, consequently, also at the somatic level. However, despite this fact, in some of them, the pancreatic tumour was an insulinoma, while in other patients with MEN1 the tumour did not convert into insulin-secreting neoplasm. Also, the authors of the study described above came to the conclusion that *MEN1* mutations themselves cannot cause insulinoma (nor can mutations in other single genes alone) and that a bigger number of altered genes is required to drive the insulinoma phenotype [63]. However, because the genetic landscape of insulinomas was compared to normal human beta cells, the results of their study were not able to distinguish unequivocally between abnormalities that lead to the development of pancreatic tumours and those that are specifically related to insulin overproduction. This is what we addressed in our study, where we were looking for inherited predispositions among MEN1 patients with pNEN for the conversion of their tumour into insulinoma.

Henfling *et al.* found decreased expression of EGFR and mTOR pathway components, and increased expression of IGF2 in insulinoma cells [72]. Again, however, because those results were compared to normal pancreatic islets, it was not possible to determine whether this was associated with the tumour state or specifically with insulinomas. Also, in a study based on data from the GEO database, the differential expression of genes in insulinomas was described in comparison to normal pancreas tissues, leaving the same question unanswered. In this study, the results of pathway enrichment revealed the association with genes implicated in insulin secretion and pancreatic secretion [73]. Those results are confirmed by our study, with the difference, however, that in the case of our data, inherited germline incidences are presented, and secondly that we were able to ascribe those results specifically to insulinomas, as compared to non-insulinoma pNENs.

Therefore, we propose a complementary picture to the previously published conclusions according to which, in insulinomas the beta cell transcription factors do not need to be mutated for insulinoma signalling deregulation, while other, less canonical factors involved in the proper functioning of beta cells are disturbed. Altogether, the analysis of insulinoma-positive MEN1 patients as compared to MEN1 patients with non-insulinoma pancreatic tumours indicates the disturbance of pathways involved in the homeostasis of glucose and lipoproteins, and several insulin release regulation pathways and immune-related interactions. To our knowledge, we are the first to identify this relationship in MEN1 patients in a study that is not based on literature data to predefine the pathways under investigation.

Broader context of the study

The *MEN1* variant type itself is not responsible for the clinical outcome of MEN1 patients, because different symptoms are observed in relatives with the same pathogenic *MEN1* variant. This was seen also in our analysis, in which for the pNEN/no pNEN analysis, patients from families were investigated who had the same disease-causing *MEN1* alteration but different clinical symptoms. Also in our insulinoma analysis, the variant type could not be made responsible for

the observed phenotype because one patient group had the same frameshift variant but different symptoms, and 2 patients had gene-shortening events in *MEN1* but a different disease course.

If a single gene or a limited number of genes were responsible for the occurrence of pNENs in all affected patients, this gene or genes would have been previously identified by association studies. It is probable that some genes will repeatedly be mutated in different families and that different sets of mutated genes will lead to the same clinical effects in different patients. For this reason, and knowing that it is impossible to identify statistically significant genes in a small patient cohort, we hypothesized that, if the genetic background of MEN1 patients is involved in the appearance of pancreatic tumours in some of them, at least some of the genes involved in this process will be involved in pathways that are common to all identified cases in a small cohort. Hence, we did not expect to identify a single gene responsible for the observed symptoms. Instead, we hypothesized that the genetic background, i.e. several genetic variants, most probably without currently known major clinical significance, when present in a patient together with a significant disease-causing variant in *MEN1*, further regulate the patient's metabolism or other signal transduction pathways to drive the clinical outcome in a given direction. Our assumptions about the significance of the genetic background in the outcome of MEN1 patients are supported by the findings of other research groups. In a large MEN1 cohort of 797 patients from 265 families, Thevenon *et al.* demonstrated that there are strong intrafamilial correlations for MEN1 outcome, proving the existence of modifying genetic factors in MEN1 [16].

Strong premises about the genetic background playing an important role in MEN1 come also from studies in mice. Lemos *et al.* tested the characteristics of MEN1 syndrome in *Men1*-knockout mice on different backgrounds, C57BL/6 and 129S6/SvEv [74]. They demonstrated that the genetic background, and hence, likely genetic modifiers, influence the phenotype of embryonic lethality and neural tube defects in $Men^{-/-}$ mice. Some defects were found only or predominantly in C57BL/6 strains, whereas others only in strains on the 129S6/SvEv background. Their results were consistent with a role for genetic modifiers in influencing the expression of the MEN1 phenotype. Recently, Lines *et al.* have shown in heterozygous $Men1^{+/-}$ C57BL/6 and 129S6/SvEv mice that the genetic background significantly influences the risk and outcome of tumour development [75]. Mohr and Pellegata reviewed the clinical differences observed in different *Men1*-knockout mouse models. Differences in the frequency of hyperparathyroidism and the frequency and types of pituitary tumours, pancreatic tumours, and adrenal gland tumours were evident [76]. Of note, the described mouse models differed not only in the type of the introduced *Men1* mutation, but also in the genetic background of the used strains – they were usually a mixture of different background strains. Importantly, the distinct strain susceptibility to disease development, including different tumours, has been proven before [77].

In humans, it is obviously impossible to perform similar analyses on the genetic background as have been performed in mice, which is why most often the identification of modifier genes is literature-based, and selected genes are investigated for their correlation with clinical manifestations of MEN1. One of the genes investigated in this context is *CDKN1B*, which was shown to be associated with tumour development in multiple glands in MEN1 patients [78] or with tumour aggressiveness [79]. This gene has been typed out for analysis based on previous knowledge due to its causality of MEN4 syndrome. Still, there is a lack of and need to perform analyses that are not predefined by literature data.

Pancreatic NENs are among the best genetically characterized NENs. However, most of the available studies do not specifically focus on NENs that are part of MEN1 syndrome. Many studies have been performed on somatic genetic changes in those tumours [80], but it remains unclear why in some patients, pNENs occur at all, while in others not, also among families. It seems very likely that genetic, epigenetic, and environmental factors interact in driving the phenotype. Our study was an attempt to initiate the search for those factors, beginning with genetic factors, because those that undergo the least changes during a lifetime might also predispose to different responses to environmental factors.

It is known that functioning pNENs have different genetic characteristics than non-functioning pNENs, and there is also a genetic difference between malignant and benign insulinoma [80]. Similarly to the general Polish population, in which there is a more frequent occurrence of insulinoma and a lower prevalence of gastrinoma compared to other populations [81], insulinomas were relatively well represented also in our cohort. We, therefore, performed an additional analysis that compared insulinoma and non-insulinoma pNEN MEN1 cases.

In both analyses, one of the shared mutated genes was *TTN*. The significance of this gene in cancer studies has been extensively commented on in the literature because it has been repeatedly associated with many tumours, in which it is one of the most highly mutated genes. This is not surprising, due to the very large size of the gene, so the possible association with cancers is still questionable [82–84]. In our analyses, however, we

also observed shared mutations in its antisense gene, *TTN-AS1*, which were present in all insulinoma cases as well as familial pNENs. Lately, *TTN-AS1* has gained lots of attention in the context of cancer pathogenesis. It has been shown to be an oncogene in different cancers, e.g. bladder cancer [85], melanoma [86], breast cancer [87], colorectal cancer [88], and other tumours. In digestive system neoplasms, it has been shown to promote malignancy by sponging miRNAs [89]. Its expression correlates with disease stage and prognosis in some cancer types, which is why *TTN-AS1* has been suggested as a potential diagnostic and prognostic biomarker in multiple cancers [89]. However, due to insufficient data, conclusions about its role in pancreatic tumours cannot be made.

In both analyses performed in the present study, the identified genes and pathways were not predefined by literature data. The obtained genetic results were based only on clinical parameters used for defining the analysed groups — pNEN or insulinoma in the course of MEN1. However, the identified genes and pathways seemed to be logically linked to the analysed symptoms.

There are different approaches to obtaining information about genetic modifier effects in monogenic disorders. In our study, we implemented a typical workflow for genetic background analyses based on whole exome sequencing [31]. Like in other studies of this type, the last step of our analysis was based on candidate variant assessment based on databases because computational methods to identify true modifiers among candidate genes are currently not yet available [31]. Undoubtedly, this is a serious limitation of the interpretation of such results. To hold subjective prioritization of candidate genes in the interpretation as low as possible, we based our analyses on shared gene ontology areas and on shared pathways, which then indicated the genes to be discussed.

We are aware that gene ontology and pathway analyses are not perfect tools for the interpretation of genetic data because they are based strictly on our current knowledge and identified interactions. Therefore, not all actual functions and interactions of a given gene or group of genes will be encompassed, and pieces of information that have not yet been put in the context of known interactions will be missed. Yet, the results obtained in our study, which was based on gene and pathway identification that was not predefined before the study by literature data, turned out to be logically sound. Therefore, our work is the first published evidence that the future performance of large-scale studies addressing the genetic background of MEN1 patients that would focus on statistical frequencies, is reasonable.

Conclusions

The assumption that underlaid the idea of the project was that the genetic background of MEN1 patients drives the specific outcome of the disease, and that this is the reason why no correlations between causative *MEN1* variants and the patients' phenotypes could have been established so far. The presented study was not expected to give a final explanation for the heterogeneity of MEN1 syndrome outcomes. It aimed at being one of the first steps towards a broader view of the disease, suggesting genetic regions or signalling pathways of special interest in research on the disease that might explain the lack of correlation between the disease-causing *MEN1* mutation and the observed variety of MEN1 phenotypes.

From the clinical point of view, genetic studies on familial syndrome patients are expected to be a tool that can improve the quality of patient care. A better understanding of the genetic background of a disease and a broader area of available laboratory analyses can, on the one hand, aid the identification of patients at risk of developing a given disease outcome and who will need to be covered with earlier diagnostics and monitoring or even treatment at early stages of the disease. On the other hand, the possibility to type out family members in an affected family, in whom a given symptom or increased aggressiveness of the disease is less likely to occur, allows for the exclusion or less frequent clinical monitoring of those people, which helps to lower diagnostic costs and frequency in people who do not identify with the disease due to a lack of complaints and therefore do not turn up for control visits regularly.

The goal of investigating altered or disrupted genes and cellular pathways that are crucial to a given symptom is the introduction of individualized treatment. In the case of MEN1, despite many investigations that have already been undertaken, we are currently at the beginning of this journey. To address this goal in MEN1, in which the disease-causing factor is known but no predictions on the individual outcomes can be made, it seems to be of importance to investigate the patient's genomic background and evaluate the course of the disease in different family members with the same disease-causing genetic variants. In addition, it is crucial to evaluate if a given finding is true also for other patients. This was addressed in our investigation because we compared not only family members with different MEN1 symptoms but also families between each other as well as unrelated patients. The presented results were common for all the groups. Nonetheless, it is possible that in another patient group under investigation, alternative genetic regions may be identified.

This will need to be addressed in additional cohorts. Due to the rarity of the disease, it is a challenge to choose a representative number of patient groups for comparison who have the symptoms of interest, are at an appropriate age, are willing to be included in such a study, and for whom an appropriate control group is available. The ethnical origin may be an additional factor of diversity in the identified genetic regions. Hence there is a need for further extensive investigation of the modifying effect of the genetic background on MEN1 outcome in different cohorts to enable the use of genetic markers as predictors of disease outcomes and the genetic-based choice of appropriate therapies, possibly also with the consideration of environmental and lifestyle factors.

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Conflict of interest
The authors have no conflicts of interest to declare.

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