MULTIPLE ENDOCRINE NEOPLASIA TYPE 1: LATEST INSIGHTS

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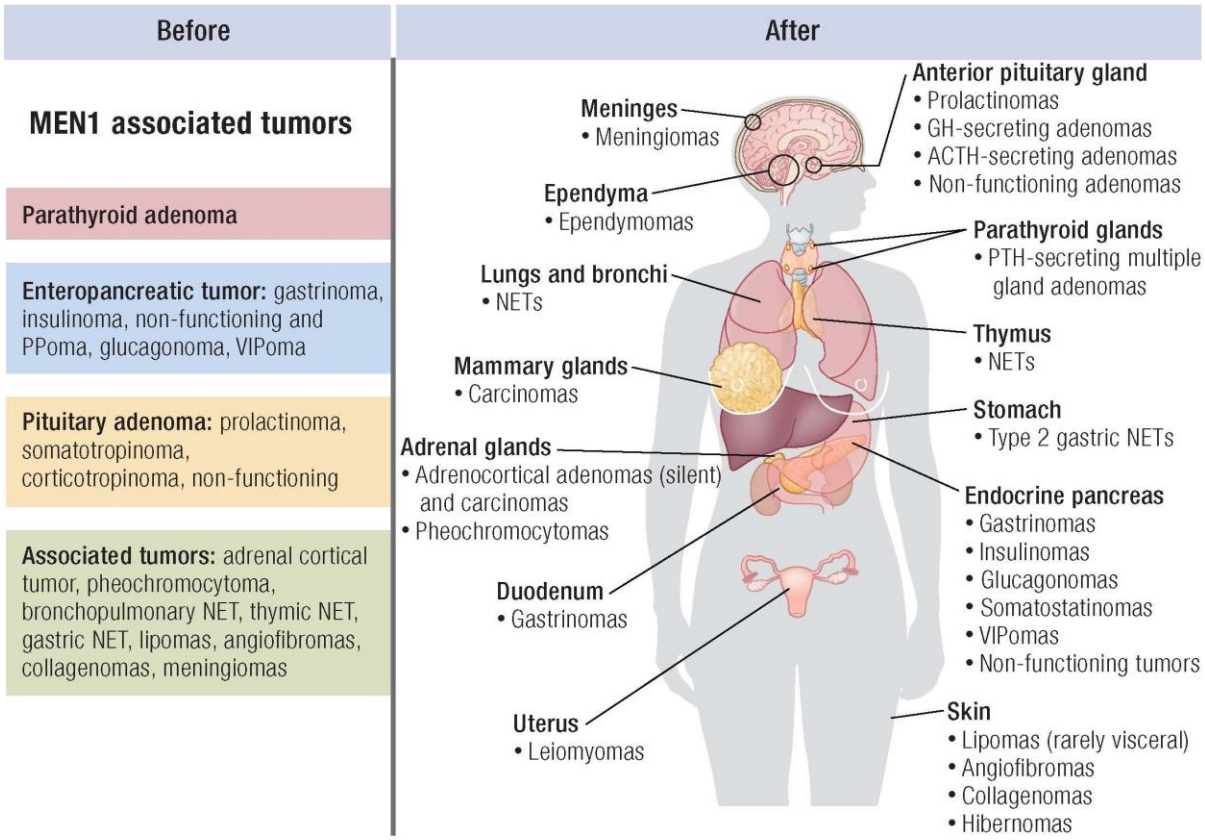
ABSTRACT

Multiple Endocrine Neoplasia Type 1 (MEN1), a rare tumor syndrome that is inherited in an autosomal dominant pattern, is continuing to raise great interest for endocrinology, gastroenterology, surgery, radiology, genetics and molecular biology specialists. There have been two major clinical practice guidance papers that were published in the past two decades, with the most recent publication 8 years ago. Since then, several new insights on the basic biology and clinical features of MEN1 have appeared in the literature and those data are discussed in this review. The genetic and molecular interactions of the *MEN1* encoded protein menin with transcription factors and chromatin modifying proteins in cell signaling pathways mediated by TGF-β/BMP, few nuclear receptors, Wnt/β-catenin and Hedgehog (Hh), and preclinical studies in mouse models have facilitated the understanding of the pathogenesis of MEN1-associated tumors and potential pharmacological interventions. The advancements in genetic diagnosis have offered a chance to recognize MEN1 related conditions in germline *MEN1* mutation negative patients. There is a rapidly accumulating knowledge about clinical presentation in children, adolescents and pregnancy that is translatable into the management of these very fragile patients. The discoveries about the genetic and molecular signatures of sporadic neuro-endocrine tumors support the development of clinical trials with novel targeted therapies, along with advancements in

diagnostic tools and surgical approaches. Finally, quality of life studies in patients affected by MEN1 and related conditions represent an effort necessary to develop a pharmacoeconomic interpretation of the problem. Because advances are being made both broadly and in focused areas, this timely review presents and discusses those studies collectively.

Keywords: Multiple Endocrine Neoplasia Type 1; MEN1; MEN1-like; Phenocopy; Menin; *MEN1* Gene Mutations; Mutation-negative; Neuroendocrine Tumors; Cell Signaling; Epigenetics; Mouse Models; Pharmacological Therapies; Surgical Approaches; Quality of Life.

Graphical abstract



INTRODUCTION

Multiple Endocrine Neoplasia Type 1 (MEN1) or Wermer's syndrome (OMIM *131100) is a rare (prevalence 3-20/100,000) highly penetrant autosomal dominant disorder caused by germline mutations in the tumor suppressor gene *MEN1,* which encodes a 610 amino acid protein, menin (1,2). The diagnosis of MEN1 in a patient has relevant implications for family members, as first-degree relatives have a 50% risk of developing the syndrome and can be identified by *MEN1* mutational analysis (3). Even though, as an autosomal dominant disorder, a gender dimorphism is not expected in MEN1, a female prevalence has been described (4), but the implications of these findings may need further validation. The age-related penetrance of MEN1 for all clinical features surpasses 50% by age 20 years and 95% by age 40 years (3). Also, instances of geographical clustering as a consequence of founder effects have been reported (5).

MEN1 is characterized by varying combinations of more than 20 endocrine and non-endocrine tumors that show loss of heterozygosity at 11q13, the location of the *MEN1* gene, resulting in biallelic loss of *MEN1* (3, 4, 6-8) (Fig. 1). Endocrine tumors become evident either by hormonal overproduction or by growth of the tumor itself. The diagnosis is clinically suspected by the combined occurrence of two or more of the following

classical endocrinopathies: primary hyperparathyroidism (PHPT) due to parathyroid glands hyperplasia, anterior pituitary tumors, and duodeno-pancreatic neuroendocrine tumors (DP-NETs). Other MEN1-associated tumors include thymus and lung NETs, type 2 gastric NETs, adrenocortical tumors, pheochromocytomas, facial angiofibromas, collagenomas, hibernomas, meningiomas, ependymomas, leiomyomas, and lipomas, and an increased risk to develop breast cancer in female patients (9, 10-12). Uncommon neoplasia associated with MEN1, such as carcinoid tumors, mammary cancer, parathyroid carcinoma (13), or adrenocortical carcinoma, are the causes of death among MEN1 patients.

MEN1 patients have a decreased life expectancy and the outcomes of treatment used in sporadic endocrine tumor counterparts are not as successful because of tumor multiplicity and aggressiveness (3). The prognosis is considerably improved by presymptomatic tumor detection and undertaking treatment specific for MEN1 tumors. This can happen only if clinical care for MEN1 patients and families is provided by a multidisciplinary MEN1-specialists' team, a true permanent task-force fully dedicated to this disorder, as suggested by the last guidance report published in 2012 (3). In the past 8 years several important discoveries have been published about the genetic diagnosis of MEN1, genetics of sporadic endocrine tumors, MEN1 related conditions, the

biological functions of menin, potential pharmacological therapies, disease pathogenesis, surgical advances, and the clinical course and prognosis of MEN1. Therefore, a review focusing on these latest findings is certainly timely and useful both for the basic science investigators and clinicians to understand the molecular basis of MEN1 and for the management of MEN1 patients.

NEW FINDINGS ABOUT THE *MEN1*-ENCODED PROTEIN MENIN AND ITS FUNCTIONAL CHARACTERIZATION

Menin, the protein product of the *MEN1* gene, consists of 610 amino acids (menin isoform 2, NCBI Reference Sequence: NM_130799.2). There is a very rare minor isoform of menin (menin isoform 1, NCBI Reference Sequence: NM_000244.3), from a potential alternative splice site 15 bp downstream of exon-2 that inserts five amino acid residues (after amino acid 148). In the gene and protein databases, the norm is to designate the longest transcript as the primary isoform; therefore, sometimes menin is described as a 615 amino acid protein.

All studies of menin and its mutants have been conducted with the 610 amino acid isoform because the rare 615 amino acid isoform has not been observed in any cell types. The 67 kDa menin is widely expressed, and at the C-

terminus contains two nuclear localization signals (NLSs) (NLS1: 479-497 and NLS2: 588-608) and one accessory NLS (aNLS: 546-572) (9, 14). Therefore, menin is detected in the nucleus as shown from experiments using Green Fluorescent Protein (GFP)-tagged menin, immunofluorescence, and western blot analysis of sub-cellular fractions (9, 14). Post-translational modifications of menin include phosphorylation at Ser394, Thr397, Thr399, Ser487, Ser543 and Ser583, SUMOylation and palmitoylation, that may enhance or suppress menin's action as a tumor suppressor in the nucleus or its potential association with the cell membrane (15-17). The functional contribution of these post-translational modifications has not been studied in MEN1-associated endocrine cell types or in a clinical context.

The three-dimensional (3D) crystal structure of human menin (Protein Data Bank No. 3U84) has been successfully deciphered after the deletion of a single internal loop region that was predicted to be unstructured (amino acid residues 460–519) (18). The 3D structure of menin resembles a 'curved left hand', with a pocket formed by the 'thumb' and the 'palm'. The structure consists of four domains: a long β-hairpin N-terminal domain, a transglutaminase-like domain ('thumb'), a helical domain that contains three tetratricopeptide motifs ('palm'), followed by a C-terminal domain ('fingers')

(Fig.2). The pocket or cavity formed by the 'thumb' and the 'palm' has been shown to facilitate protein-protein interactions (18, 19).

The tumor suppressor role of menin in MEN1 and the tissue restricted pattern of MEN1-associated tumors has been replicated in mouse models. Germline homozygous knockout of *Men1* (*Men1*$^{-/-}$) is embryonic lethal at E11.5-14.5, and germline heterozygous knockout of *Men1* (*Men1*$^{+/-}$) generates viable mice that develop (at age >12-15 months) hormone hypersecreting tumors in the pancreatic islets (mainly insulinoma), anterior pituitary (mainly prolactinoma), and the parathyroid glands (mainly hyperplasia) (20-24). Consistent with the tumor suppressor role of menin in human MEN1 tumors, a second hit to the non-targeted copy of *Men1* resulting in loss of heterozygosity (LOH) is essential for tumor formation in *Men1*$^{+/-}$ mice. The pancreatic islets of *Men1*$^{+/-}$ mice show a pre-tumor stage of hyperplasia and dysplasia prior to LOH at the *Men1* locus (25). Investigating the molecular aspects involved in these pre-tumor events can be helpful to understand tumor initiation and progression from tissue-specific menin haploinsufficiency and menin loss.

Tissue-specificity of the tumor suppressor role of menin has been shown in two conditional mouse models. First, mice with conditional loss of menin in the liver (*Men1*$^{f/f}$;ALB-Cre) do not develop tumors in the liver (26). Second, mice with conditional loss of menin in the whole pancreas (*Men1*$^{f/f}$;PDX1-Cre)

develop tumors that originate from the β-cells of endocrine pancreas (insulinoma), and not from any cells of exocrine pancreas (27). Interestingly, mice with conditional loss of menin in the glucagon-secreting pancreatic islet α-cells (*Men1*$^{f/f}$;GLU-Cre) do not develop glucagonomas, instead they predominantly develop β-cell tumors (insulinomas) (28, 29). It is possible that after menin loss, α-cells may trans-differentiate into β-cells, or paracrine signals from menin-null α-cells induce β-cell proliferation (28, 29). As expected, parathyroid-specific *Men1*-knockout mice (*Men1*$^{f/f}$;PTH-Cre) develop parathyroid hyperplasia and hypercalcemia, and pancreatic islet β-cell-specific *Men1*-knockout mice (*Men1*$^{f/f}$;RIP-Cre) develop insulinomas (25,30-32). The *Men1*$^{f/f}$;RIP-Cre mice also develop prolactinomas due to the leaky expression of the RIP-Cre transgene in pituitary lactotroph cells. Similar to human MEN1, the prolactinomas in mice (*Men1*$^{+/-}$ or*Men1*$^{f/f}$;RIP-Cre) are frequently observed in females. ~~The~~ However, the reason for the gender bias of prolactinomas remains unknown. Although the conditional *Men1*-knockout mice do not depend on a spontaneous second hit for homozygous loss of *Men1*, tumors develop 8-10 months after embryonic menin loss, and the basis for the delay in tumor formation is not known.

The functional characterization of menin has encountered various challenges due to the lack of any similarity to known proteins, lack of obvious

functional motifs/domains, lack of normal or menin-null endocrine cell lines, and lack of ex vivo models of MEN1 tumors or their counterpart normal tissues (organoids or patient derived xenograft (PDX)). Insights into how menin performs its tumor suppressor activity have been gained from the identification of interacting partners of menin in cell types unrelated to MEN1-associated tissues, followed by validation of some relevant targets in translational studies (33). Even though the sequence of menin does not reveal any functional attributes, direct or indirect interactions with more than 50 different proteins of known function have helped to provide clues about its role in various processes and pathways: cell adhesion, cell cycle progression, cell division, cell motility, cell signaling, cytoskeletal structure, DNA repair, genomic stability and transcriptional regulation (34). Highly enriched among the interacting partners of menin are transcription factors and epigenetic regulators. Menin serves as a multi-functional protein through prominent functional contributions in transcriptional regulation as a co-activator or co-repressor.

1. Functional contributions of menin in transcriptional regulation

The interactions of menin with various transcription factors and chromatin modifying proteins have shown a significant functional contribution in cell signaling pathways mediated by TGF-β/BMP, nuclear receptors, Wnt/β-catenin, and Hedgehog (Hh) (35, 36). These signaling pathways stimulate transcription factor recruitment to their cognate DNA binding sites to regulate gene expression. Menin interacts with SMAD3 or SMAD1/SMAD5 to promote their transcriptional activity, and loss of menin in these interactions inhibits TGF-β and BMP signaling pathways, respectively, thus antagonizing their proliferation-inhibitory effects. Nuclear receptors are transcription factors that are activated by binding to ligands such as steroid hormones. Menin has been shown to act as a co-activator of gene expression mediated by some nuclear receptors (AR, ERα, LXRα, PPARα, PPARϒ, RXR, and VDR), and loss of menin in these interactions predicts suppression of specific nuclear receptor signaling affecting cell growth and function. Conversely, menin interacts with β-catenin and its associated transcription factors TCF3 and TCF4 to suppress their activity, and loss of menin promotes Wnt/β-catenin signaling that is known to increase islet β-cell proliferation. Menin interacts with PRMT5 to antagonize Hh signaling by depositing a PRMT5-dependent repressive histone modification (H4R3me2s) to suppress the expression of genes in the Hh

signaling pathway, *GAS1* and *GLI1*. Loss of menin-PRMT5-mediated repressive marks in these genes would promote Hh signaling that can upregulate cell proliferation.

Among the AP1 family of JUN transcription factors (JUNB, cJUN and JUND) that regulate gene expression downstream of various stimuli, menin only interacts with JUND and suppresses its transcriptional activity (37, 38). The menin interacting region of JUND maps to its N-terminus. Synthetic mutations in this region of JUND (human JUND amino acid residues 33-36), can disrupt interaction with menin. Such mutants of JUND that lack menin interaction are oncogenic because they promote cell proliferation, consistent with a tumor suppressor effect of the menin-JUND interaction (39).

Menin interacts with DAXX (a transcriptional repressor and component of chromatin remodeling complex) and SUV39H1, a histone methyltransferase that deposits a repressive histone modification H3K9me3, to repress the transcription of specific genes associated with the regulation of cell proliferation - *MME*, *GBX2* and *IL6* (40).

H3K4me3 is a histone modification that is primarily located in promoter regions near the transcriptional start site (TSS) to activate gene transcription. Among the MLL family members that are responsible for depositing this

histone mark, menin interacts with two histone methyltransferases MLL1 (KMT2A) and MLL2 (KMT2B). MLL1 and MLL2 are part of multi-subunit protein complexes that contain ASH2L, hDPY30, HCF-2, RBBP5, and WDR5, and they also interact with the 140 kDa subunit of RNA-Pol-II (POLR2B) (41, 42). Loss of menin in this protein complex results in the transcriptional repression of specific genes due to gene-specific loss of H3K4me3 in the promoter region near the TSS (42).

The 3D structure of menin shows a central cavity that forms a binding pocket for protein interaction but no obvious DNA binding domain, indicating that to control gene expression menin does not directly bind to DNA and is dependent on its interactions with components of the transcriptional regulatory machinery (18, 19). Co-crystallization of menin with peptides from the interacting region of JUND or MLL1 have shown that their binding to the pocket region of menin is mutually exclusive (Fig.2) (18). Interestingly, the menin-binding peptides of JUND and MLL1 are almost identical at the 5 residues that are critical for binding to menin. Also, co-crystallization of menin with a peptide from the lens epithelium-derived growth factor (LEDGF) (amino acids 347-429) and MLL1 (amino acids 6-153, which contains motifs for menin-binding and LEDGF-binding) has shown that the interaction between menin and MLL1 creates an interface for binding LEDGF which is a protein that directs

the MLL-complex to chromatin (Fig.2) (18). Similar structural studies of menin with other interacting partners may help to determine how menin can interact one-on-one with individual factors or simultaneously with multiple factors to control transcriptional regulation.

2. Characterization of menin's target genes and role in cell proliferation

Genome-wide analysis of target genes using a menin antibody by techniques such as chromatin immunoprecipitation coupled with DNA microarray chips (ChIP-chip) or coupled with next-generation sequencing (ChIP-seq), or serial analysis of chromatin occupancy (SACO) have shown that menin is localized to hundreds of genes near promoter regions and other regions in the genome (35, 43, 44). Whether all or some of these genes are relevant to the role of menin as a tumor suppressor have not been determined. One target gene that was identified in the study by Scacheri et al. is *Hlxb9*/*Mnx1*, encoding an embryonic transcription factor responsible for islet β-cell differentiation (43). They compared ChIP-chip data of menin occupancy in human islets to gene expression data from islets of 15 and 25 week (wk) old mice that were wild type (WT) or menin-null (*Men1*$^{f/f}$;RIP-Cre). *Hlxb9* was one of the genes identified among the few genes that were both bound by menin and altered in expression in menin-null islets. The expression of Hlxb9 was higher in the menin-null islets. This finding supports the notion that tissue-

specificity of MEN1-associated tumors may be related to the regulation of tissue-specific target genes by menin such as *Hlxb9* in islet β-cells. Subsequent studies have shown menin-dependent regulation of a few β-cell differentiation factors (Foxa2, Nkx2.2, MafA, MafB, and Hlxb9) (45-48).

Given the association of menin in the MLL-complex for depositing H3K4me3 in chromatin, another approach to characterize menin's target genes is to analyze WT and menin-null cells for H3K4me3 profiles by ChIP-chip or ChIP-seq coupled with cDNA microarray analysis for differential gene expression. This approach in WT and menin-null mouse embryonic stem cells (mESCs) identified the lncRNA Meg3 as a menin target gene that acts as a tumor suppressor, and loss of MEG3 expression has been reported in human sporadic pituitary adenomas (49-51). Similar analysis of WT or menin-null mouse embryonic fibroblasts (MEFs), and pancreatic islet-like endocrine cells derived by *in vitro* differentiation of WT or menin-null mESCs, have shown *Hox* genes as targets of menin (42, 49). Menin loss suppresses the expression of *Hox* genes. The *Hox* genes encode essential transcription factors for embryonic development and tissue homeostasis, and their aberrant expression has been reported in various cancer types.

One of the obvious processes that could be dysregulated to promote increased cell proliferation in tumors upon loss of menin is the cell cycle.

Various proteins that are involved in the progression, maintenance and regulation of the cell cycle include cyclins, cyclin-dependent kinases (CDK), cyclin-dependent kinase inhibitors (CDKI), and tumor suppressors p53 and RB. Studies using menin-null MEFs have shown accelerated progression from G0/G1 to S phase, lower expression of two CDKI genes *Cdkn2c* (p18) and *Cdkn1b* (p27), and increased Cdk2 activity compared to WT MEFs or menin-null MEFs reconstituted with menin expression (52, 53). Similarly, lower expression of p18 and p27, and higher expression of Cdk4 has been observed in islet tumors of *Men1*$^{+/-}$ mice and in hyperplastic islets of mice with acute deletion of *Men1* (*Men1*$^{f/f}$;Cre-ER fed with tamoxifen) compared to islets from WT mice (53, 54). Further studies in mouse models with knockout of each of these cell cycle genes (*Rb1*, *Tp53*, *Cdk2*, *Cdk4*, *Cdkn2c*, and *Cdkn1b*) in the *Men1*$^{+/-}$ background have shown that p18 inactivation and Cdk4 activation may be critical for islet tumor formation upon menin loss (Table 1) (55-59).

Although the full spectrum of specific target genes of menin from interaction with various transcription factors that are relevant to MEN1-associated tumor cell proliferation remain to be determined, translational studies in mouse models have shown the importance of genetic interaction between *Men1* and *Pten*, *Kmt2a* (Mll1), *Kdm5a* (Rbp2), *Ctnnb1* (β-catenin), *Inhbb* (Activin-B) or oncogenic *Kras* in islet tumors and β-cell proliferation

(Table-1) (60-65). Combined genetic manipulation of these genes in mice with β-cell-specific menin loss (*Men1*$^{f/f}$;RIP-Cre) have shown that, loss of *Pten* or *Kmt2a* accelerates islet tumor formation and reduces survival, loss of *Ctnnb1* or a histone demethylase (*Kdm5a)* decreases islet tumor formation and prolongs survival, loss of *Inhbb* does not affect tumor formation but prolongs survival after 10 months of age, and expression of activated *Kras*(G12D) enhances rather than inhibits β-cell proliferation.

One approach that has not been explored to identify target genes of menin dysregulated upon menin loss in tumors, is single-cell RNA-seq analysis of MEN1-associated tumor cells and their counterpart normal cells.

3. Unexpected function of menin as a pro-oncogenic factor in MLL-rearranged leukemia and its potential therapeutic applications

The discovery of menin's interaction with MLL1 exposed a surprising functional contribution of menin as an oncogenic co-factor of MLL-fusion proteins that drive an aggressive form of leukemia (41). The *MLL1*/*KMT2A* gene on chromosome 11q23 is involved in chromosomal translocations in 10% of acute leukemias with the N-terminus of MLL1 fused to the C-terminus of over 80 different fusion partners. The most common among

the translocations are t(4;11)(q21;q23), t(9;11)(p21;q23), and t(11;19)(q23;p13) leading to the expression of MLL-AF4, MLL-AF9 and MLL-ENL fusion proteins (66). The MLL rearranged (MLLr) leukemias are a distinct subset of acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) that affect both children and adults.

MLL1 is required to maintain the expression of *HOX* family genes, that regulate normal hematopoietic differentiation. Oncogenic MLL-fusion proteins cause acute leukemia because they upregulate the expression of *HOX* genes, including *HOXA7*, *HOXA9* and a HOX cofactor *MEIS1*, which enhances the proliferation of hematopoietic stem cells (HSCs) and blocks hematopoietic differentiation. MLL-fusions that drive MLLr leukemia retain the menin-interacting part of MLL, and interaction of menin with the MLL-fusion protein is critical for the maintenance of the MLL-fusion driven gene expression program (41). Therefore, blocking the interaction of MLL in the central cavity/pocket of menin with small molecules suggested an important therapeutic strategy for the treatment of MLLr leukemias. Over the years, structurally optimized chemical design has resulted in the development of several small molecule inhibitors of menin-MLL interaction that have greater potency and specificity, that are orally bioavailable, and with improved pharmaceutical properties (67-70). These inhibitors have been tested in

various experimental model systems where they block HSC proliferation and promote differentiation - mouse models of MLLr leukemia, patient-derived leukemia cell lines, and PDX models of these cell lines and human primary leukemia cells. The promising results from these studies have translated into ongoing Phase I/II clinical trials for two compounds - Kura Oncology (KO)-539, a structurally related analog of MI-3454 (NCT04067336), and Syndax (SNDX)-5613, a close analog of VTP-50469 (NCT04065399). These compounds can also block the interaction of menin and WT MLL indicating a therapeutic role in non-MLLr leukemias. In a mouse model of non-MLLr leukemia where AML development is dependent on a mutation in the nucleophosmin (*NPM1*) gene (seen in 30% of AML patients), VTP-50469 elicited a cytotoxic effect in pre-leukemia AML cells suggesting the potential for preventative therapy (71).

Menin-MLL interaction inhibitors have also been tested in experimental models of solid tumors where menin has been shown to act as a pro-oncogenic co-factor, to block the interaction between menin and WT MLL. An earlier menin–MLL inhibitor, MI-2, has been shown to inhibit tumor cell growth of pediatric gliomas with a histone H3.3(p.Lys27Met) mutation (72). Antitumor effects of another menin–MLL inhibitor MI-503 has been demonstrated in castration resistant prostate cancer, Ewing sarcoma and hepatocellular carcinoma (68). The utility of menin-MLL interaction inhibitors in MEN1-

associated tumor cells are potentially irrelevant, because menin acts as a tumor suppressor in the context of MEN1 and the tumors show biallelic menin loss or inactivation. However, these inhibitors have not been tested in sporadic endocrine tumors that retain WT menin, or sporadic tumors with specific menin missense mutations that retain interaction with MLL (and without *MEN1* LOH). Availability of experimental models of such tumors could help to determine whether menin-MLL interaction inhibitors enhance or block the proliferation of endocrine tumor cells.

4. Role of menin as a tumor suppressor in non-MEN1 target tissues

Approximately, 45-50% of *BRAF* mutation positive colorectal cancers show abnormal regulation of the WNT pathway. In a recent study, a somatic inactivating hotspot mutation at codon R516 in the *MEN1* gene (R521 as per menin isoform 1) was detected in 4% of *BRAF* mutant colorectal neoplasia samples (73). These data support a role of menin in colorectal tissues as a tumor suppressor and adds another WNT pathway associated gene to the pathology of colorectal cancer, given that menin has been shown to participate in the regulation of the WNT pathway. In another recent study that investigated the germline susceptibility of patients with apparently sporadic

osteosarcoma (malignant bone tumors), a higher than expected frequency (0.5%) of pathogenic/likely-pathogenic variants of the *MEN1* gene were observed in patients from European ancestry (74). These findings have important implications for the genetic testing of osteosarcoma patients and suggest a role of menin as a tumor suppressor in bone. However, osteogenic carcinomas are not described in MEN1 patients.

EPIGENETIC REGULATION IN THE TUMORIGENESIS OF MEN1-ASSOCIATED ENDOCRINE TUMORS

DNA is wrapped around a histone octamer with two copies each of the four core histone proteins (H2A, H2B, H3 and H4), to form a nucleosome which is the basic unit of chromatin. Epigenetic modifications to DNA and histone proteins can impart a closed or open chromatin structure for controlling access to the transcriptional machinery and to control other processes such as DNA replication and repair. Various epigenetic factors form multi-protein complexes, that may also include lncRNAs, to function as enzymes or co-factors for 'writing', 'reading' or 'erasing' the modifications on DNA and histones. DNA modifications include methylation, hydroxymethylation and further oxidation. Posttranslational modifications (PTMs) of histones known as

histone 'marks' include methylation, acetylation, phosphorylation, ubiquitination and other modifications. The precise regulation of these epigenetic modifications and their control mechanisms is essential to prevent abnormal cell proliferation and function that can lead to neoplasia, and other conditions. Because epigenetic modifications can be written, read and erased, they offer a therapeutic opportunity to restore aberrant epigenetic changes to the normal state with drugs that can block or enhance the enzymatic activity or critical interactions of epigenetic regulators.

1. Epigenetic events in MEN1-associated tumors

Various epigenetic changes have been reported in MEN1-associated tumors (Fig. 3). Evidence for a role of epigenetic regulation in the tumors of MEN1 is supported by the interaction of menin with histone modifying proteins, particularly with histone lysine methyltransferases (KMTs) MLL1/KMT2A and MLL2/KMT2B in protein complexes that are responsible for writing the histone mark H3K4me3 (40,41). Histone methyltransferases methylate lysine or arginine residues in the chain of amino acids that protrude from the nucleosome (histone tail). Histone H3 can be methylated on lysine residues at positions 4, 9, 27, 36 and 79 with one, two or three methyl groups.

Activation or silencing of gene expression is regulated by the level of methylation or demethylation of specific histone H3 lysine residues. H3K4me3 is a mark of actively transcribed genes, and H3K9me3 and H3K27me3 are associated with transcriptional silencing. Specific lysine demethylases (KDMs) can erase mono-, di- or tri-methylation of H3K4 or H3K9, such as lysine-specific demethylase 1 (LSD1/KDM1A), lysine-specific demethylase 2 (LSD2/KDM1B) and Jumonji AT-rich interacting domain 1A (JARID1A/KDM5A/RBP2).

Epigenetic regulation in MEN1-associated islet tumors from H3K4me3 has been explored in mice with targeted β-cell-specific menin loss (*Men1*$^{f/f}$;RIP-Cre). Genome-wide distribution of the gene activation mark H3K4me3 and its counterpart recessive mark H3K27me3 has been examined in the pancreatic islets of 2-month old *Men1*$^{f/f}$;RIP-Cre and control RIP-Cre mice (75). Immunohistochemistry with anti-H3K4me3 showed no significant change in the global/overall level of H3K4me3 in menin-null islets compared with control islets. In menin-null islets, loss of H3K4me3 correlated with gain of H3K27me3 within a specific set of genes, and the expression of such genes was significantly decreased compared to control islets, particularly the gene encoding insulin-like growth factor 2 mRNA binding protein 2 (*Igf2bp2*). Interestingly, the altered epigenetic marks (loss of H3K4me3 and gain of H3K27me3) and lower expression of Igf2bp2 could be reversed in menin-null

islets with simultaneous deletion of the H3K4me3 demethylase Rbp2 (*Men1*$^{f/f}$;*Kdm5a*$^{f/f}$;RIP-Cre) (75). Immunohistochemistry with anti-H3K4me3 showed no significant change in the global/overall level of H3K4me3 in menin-Rbp2-null islets compared with control islets. The mice with β-cell-specific combined loss of menin and Rbp2 showed a decreased rate of islet tumor formation and prolonged survival (62). Therefore, specific epigenetic changes occurring as a consequence of menin loss could be reversed (by removing the Rbp2 histone demethylase), and the restoration of the epigenetic changes to the basal normal state could also reduce tumor formation.

The epigenetic regulation of MEN1-associated islet tumor cell proliferation has been explored for the interaction of menin with an arginine methyltransferase PRMT5 that deposits a repressive histone mark, H4R3me2s (36). In MEFs, menin together with PRMT5 was shown to repress the expression of the *Gas1* gene, which is involved in the activation of the Hh signaling pathway. GAS1 is required for binding of the Sonic Hedgehog (SHH) ligand to its receptor PTCH1 for the stimulation of ~~the~~ Hh signaling. Therefore, in tumors with menin loss, GAS1 repression would be released to activate Hh signaling. Treatment of 8-month old *Men1*$^{f/f}$;RIP-Cre mice with the Hh inhibitor GDC-0449 for 4 weeks, reduced the proliferation of islet β-cells by approximately 60%. The effect on tumor size and overall survival was not

investigated. This study showed that blocking aberrant signaling due to the loss of a menin-dependent epigenetic mark could inhibit cell proliferation.

Another histone modification that has been studied in MEN1-associated islet tumors is histone acetylation that is associated with active transcription. Acetylation marks at lysine residues in histone tails are deposited by histone acetyl transferases (HATs) and read by the bromodomain (BRD) contained in the bromodomain and extra-terminal (BET) proteins. JQ1 is a small-molecule inhibitor of bromodomain interactions with acetylated histones. Treatment of 30-wk old *Men1*$^{f/f}$;RIP-Cre mice with twice weekly injections of JQ1 for one month reduced the proliferation rate of islet β-cells in the tumors by 49-55% and significantly increased apoptosis (76). The effect on tumor size and overall survival was not assessed in this study. Although the specific epigenetic changes in histone acetylation have not been investigated in MEN1-associated tumors, this study highlights the potential of targeting histone acetyl marks.

A few studies have investigated DNA methylation in MEN1-associated tumors which is an epigenetic modification of CpG sites, particularly in gene promoter regions. DNA hypermethylation usually coincides with gene silencing. Also, H3K4me3 has been shown to protect CpG islands from DNA methylation to regulate gene transcription (77). DNA methylation can be blocked by directly inhibiting DNA methyltransferases (DNMTs)that establish,

propagate and maintain the stability of the DNA methylation mark. DNA hypermethylation was detected in a subset of MEN1 tumors. Global DNA hypermethylation was detected in parathyroid tumors and non-functioning pancreatic neuroendocrine tumors (pNETs) ~~(DP-NETs)~~ from MEN1 patients (78, 79). Also, promoter hypermethylation was observed as a frequent event in MEN1-associated advanced pNETs (80). Using a somatic gene transfer system in RIP-TVA mice, expression of DNMT1 increased β-cell proliferation, suggesting that DNMT1 could be targeted to inhibit DNA hypermethylation and β-cell proliferation (81).

MEN1-associated pNETs have been assessed for telomere length. Telomeres are specialized chromatin structures that protect chromosome ends. Alternative lengthening of telomeres (ALT) is a telomerase-independent process that is activated in cancer cells to prevent telomere shortening that accompanies normal proliferation of somatic cells. The correlation between ALT and prognosis is variable in different cancer types. Mutations in chromatin remodeling genes death domain-associated protein (*DAXX*) and α-thalassemia/mental retardation X-linked (*ATRX*) correlate with ALT activation in sporadic pNETs (82-85). In a study of non-functioning pNETs, 48% of sporadic and 25% of MEN1-associated pNETs were ALT-positive, and ALT was associated with disease relapse (85).

Non-coding RNA (ncRNA)-mediated gene silencing is another epigenetic mechanism that has been investigated in MEN1-associated tumors. There are two types of ncRNAs - the short ncRNAs (less than 30 nucleotides) and the long ncRNAs (greater than 200 nucleotides). MicroRNAs (miRNAs) are short ncRNAs that regulate gene expression at the transcriptional and post-transcriptional level. In the hyperplastic islets of 8-wk old *Men1*$^{f/f}$;RIP-Cre mice, and human parathyroid tumors, miR-24 (and its immature form miR-24-1) has been shown to target menin because increased miR-24 level correlated with decreased menin level (86-88). This mechanism of silencing menin may also contribute to the second somatic "hit" of *MEN1* inactivation in MEN1-associated tumors that do not show LOH at the *MEN1* locus (86).

2. Exploring epigenetic diagnostic and therapeutic options

Epigenetic changes are stable in tumors and thus can be used as diagnostic markers. Also, epigenetic changes are reversible and can be targeted to restore normal epigenetic states such as by blocking the enzymatic activity of epigenetic regulators, disrupting specific interactions in chromatin modifying protein complexes, interfering with the reading of epigenetic marks or targeting specific epigenetic factors for degradation.

Evidence for epigenetic changes in MEN1-associated tumors in *Men1*$^{f/f}$;RIP-Cre mice or human tumor samples indicates a potential for exploring epigenetic alterations as biomarkers for diagnostic and therapeutic options for these tumors. Among the epigenetic alterations that can occur in MEN1-associated tumors are loss of the active histone mark H3K4me3 in a sub-set of genes, gain of the repressive histone H3K27me3 in a sub-set of genes, enhanced Hh signaling due to loss of a repressive histone mark H4R3me2s in the promoter region of *Gas*, histone acetylation, DNA hypermethylation, ALT, and miRNA-mediated silencing of menin expression (Fig.3). Whether the epigenetic changes to DNA and histones, and ALT occur simultaneously in MEN1-associated tumors has not been investigated.

Experimental evidence for the reversal of changes to histone methylation in the tumors of *Men1*$^{f/f}$;RIP-Cre from combined loss of a demethylase Rbp2, indicates a therapeutic opportunity with epigenetic drugs to inhibit this demethylase in MEN1-associated tumors (62). Similarly, the effect of a BETi, JQ1, to lower cell proliferation in the tumors of *Men1*$^{f/f}$;RIP-Cre indicates that targeting histone acetylation can be further explored as a potential epigenetic therapeutic option in MEN1-associated tumors (76).

Epigenetic changes to DNA from advanced tumors can be measured in circulating cell free DNA (cfDNA) in blood and serum samples (89). Because

epigenetic changes are stable in tumors and because of the non-invasive sample acquisition for cfDNA, this potential diagnostic assay (cfDNA liquid biopsy) is being explored for various cancers but has not yet been applied in a clinical setting for any type of cancer. Therapeutic options for targeting DNA hypermethylation in tumors are DNA hypomethylating agents, decitabine and azacytidine, that target DNA methylating enzymes. These drugs have been approved by the FDA for the treatment of specific hematological malignancies and can be explored as potential therapeutic options in experimental models of MEN1-associated tumors that show DNA hypermethylation.

Telomere-specific FISH has been used to determine ALT status in human tumor samples and may serve as a diagnostic assay in tissue biopsies (85). Potential therapeutics targeting ALT have been proposed (90). Results from miR-24-mediated silencing of the WT *MEN1* allele in human parathyroid tumors without 11q13 LOH can be further investigated to develop RNA antagomir(s)-based strategies to restore the expression of menin from the non-mutant copy of the *MEN1* gene to control tumorigenesis (86).

Development and use of epigenetic-based therapeutics continue to face several issues and challenges such as cell-specific targeting in affected tissues, side effects, drug resistance and achieving constant, consistent and long-lasting effect on the target. Although several epigenetic drugs are in clinical

trials, these challenges need to be overcome for translating drug discovery to human patients (91).

NOVEL INSIGHTS ON THE GENETICS OF MEN1

The current germline or somatic *MEN1* genetic testing consists of DNA sequence analysis to screen coding exons and splice junctions for mutations, and Multiplex Ligation-dependent Probe Amplification (MLPA) for deletion/duplication (del/dup) analysis to screen for larger alterations. Since its discovery, the availability of genetic testing of the *MEN1* gene has become an essential part in the diagnosis and management of MEN1. Genetic screening in MEN1 is informative to confirm the clinical diagnosis, for carrier ascertainment and early monitoring for tumors. Also, in families with clinical and genetic MEN1, relatives with a negative *MEN1* genetic test can be excluded from the burden of life-long tumor monitoring.

Clinical practice consensus guidelines developed by a panel of experts including physicians, surgeons, geneticists and other specialists from international centers outlined recommendations for genetic testing in MEN1 (3). The current guidelines recommend that genetic counseling must be available to patients before and after genetic testing. In terms of who should

be tested, the guidelines state that the test should be offered to: 1) an index case with clinical MEN1 (presenting with two or more MEN1-associated endocrine tumors), 2) asymptomatic first-degree relatives of an individual with genetic MEN1 (known *MEN1* mutation carrier) as early as before 5 years of age, 3) symptomatic first-degree relatives of an individual with genetic MEN1, who are presenting with at least one MEN1-associated tumor, and 4) patients with multigland parathyroid disease or parathyroid adenomas before the age of 30 years, and gastrinoma or multiple pancreatic islet tumors at any age.

Sequencing and del/dup analysis can identify

heterozygous *MEN1* germline mutations in 70-90% of families with typical features of MEN1. A 2015 review of published germline mutations identified 576 unique mutations, and in 2019 the Universal Mutation Database of MEN1 mutations (UMD-MEN1 database) reported an additional 181 unique germline mutations (92, 93). These 757 unique *MEN1* germline mutations cover the entire coding region with no hot spots.

The obviously pathogenic category of germline mutations (69%) predict premature truncation of menin from nonsense mutations (14%), frame-shift mutations (42%), splice site mutations (10.5%), and large deletions (2.5%) (Fig. 4) (92). Missense mutations (25.5%) and in-frame insertion or deletion (indel)

of one or more amino acids (5.5%) do not predict obvious inactivation of menin, and whether they are benign or pathogenic needs further investigation (92). Across multiple studies and among family members, no clear genotype-phenotype correlation has emerged from an analysis of mutation types or their location with the clinical manifestations of MEN1 (94). Similarly, somatic *MEN1* mutations in sporadic tumors have not revealed any hot spots or a clear genotype-phenotype correlation with specific tumor types.

Germline *MEN1* mutations are not found in 10-30% of cases who develop clinical features consistent with MEN1. These *MEN1*-mutation negative cases may carry a germline *MEN1* mutation in regions that are not interrogated by current genetic testing methods (such as untranslated, intronic and regulatory regions), or the tumors may show somatic mosaicism (post-zygotic *MEN1* mutation), or they carry germline mutations in other genes (such as *CDKN1B* (Frederiksen et al., 2019)), or the clinical manifestation of multiple tumors is a sporadic coincidence with no underlying germline mutation. Candidate gene analysis, whole genome sequencing (WGS) or whole exome sequencing (WES) approaches have been applied to decipher the germline genetic defects in *MEN1*-mutation negative cases.

1. *In silico* analysis of *MEN1* missense mutations

One of the challenges of *MEN1* genetic testing is the interpretation of missense and in-frame indel mutations that do not predict obvious damaging effects to the protein structure or function. Given that menin is a multi-functional protein with many interacting partners, missense variants and in-frame indels could disrupt the function of menin in various ways. However, reliable functional assays are not available to establish the impact of *MEN1* missense mutations. The effect of amino acid substitutions on the structure or function of a protein without conducting functional studies can be assessed by computational (in-silico) predictive tools - SIFT (Sorting Intolerant From Tolerant), PolyPhen-2 (Polymorphism Phenotyping V-2), MutationTaster, MutationAssessor, and other similar tools. The prediction programs are based on various criteria such as sequence homology, physicochemical similarity between the alternate amino acids, evolutionary conservation, or available 3D structures. However, these tools are only for predictions and their interpretation of pathogenic consequence should be used carefully.

The structure of menin has been used to evaluate the impact of missense mutations. In one study, mapping of 159 unique *MEN1* missense mutations on the 3D structure of menin showed that 66% were located in buried residues that may destabilize the protein structure (19). The remaining 34% were

located at solvent exposed sites and might impair protein-protein interactions (19). This study did not compare differences between pathogenic and benign missense mutations. Another study performed an *in silico* thermodynamic analysis of 345 *MEN1* missense mutations using various structures of menin alone or in complex with peptides of interacting partners (MLL, JUND or MLL/LEDGF) or with small molecule inhibitors of menin-MLL interaction, from the Protein Data Bank (PDB) (95). Thermodynamic destabilization of protein structure was measured as the change in free energy ($\Delta\Delta G$) resulting from an amino acid substitution that was calculated by the FoldX program. A higher $\Delta\Delta G$ value (>4 kcal/mol) co-related with a strong destabilizing effect, thus providing an *in silico* positive predictive value to discriminate between pathogenic and benign missense variants.

In 2015, the American College of Medical Genetics and Genomics (ACMG) and the Association for Molecular Pathology (AMP) recommended a variant classification framework in their standards and guidelines (96). This framework suggested a five-tier variant classification system - pathogenic, likely pathogenic, uncertain significance, likely benign, and benign. This classification was based on the allele frequency, segregation, *de novo*, protein expression, functional studies, and other factors. For the interpretation of *MEN1* missense variants, the TENGEN network (French oncogenetics network of

neuroendocrine tumors) has proposed adjustments to the ACMG-AMP framework (97). These recommendations can be useful for the classification of *MEN1* missense variants and the genetic diagnosis of MEN1.

2. Advances in molecular genetic studies and their applications to genetic diagnosis of MEN1

One of the benefits of *MEN1* genetic testing is to confirm the diagnosis of clinical MEN1. However, *MEN1* genetic testing is negative in patients with clinical MEN1 who present with incomplete disease manifestations that represent phenocopies, or MEN1-like disease characterized by tumor in as few as one of the three main MEN1-associated endocrine tissues. The identification of susceptibility genes for endocrine tumor syndromes that have at least one overlapping feature with MEN1, has helped to extend the genetic diagnosis of *MEN1* mutation-negative cases to include those additional genes in the genetic testing approach (Fig. 5). Among the 10-30% of germline *MEN1* mutation-negative cases with or without a family history of clinical MEN1, a few may rarely test positive for germline mutations in genes for MEN1-like conditions (*CDKN1B* or other CDKI genes, *CDC73, CASR, GNA11, AP2S1, GCM2 and AIP*) (34, 98, 99). Therefore, the genetic predisposition in

clinical MEN1-like cases should be further evaluated by genomic approaches to identify the responsible mutations and genes.

One approach to find the potentially missing *MEN1* mutations in clinical MEN1 cases is to screen the non-coding regions of *MEN1* (promoter, introns and untranslated) that are not part of the current genetic testing methods. In one study, targeted next generation sequencing (tNGS) of the entire 7.2 Kb genomic region of *MEN1* was performed, and no mutation was detected in 16/76 patients. Also, none of the 76 cases had a point mutation or short indel mutation in the non-coding regions of *MEN1*, indicating that such mutations may be very rare (100).

There is only one study that has performed WGS of constitutional and tumor DNA samples from patients who were mutation-negative in prior *MEN1* genetic testing (101). Among the six patients analyzed, surprisingly, pathogenic *MEN1* germline heterozygous mutations were identified in three (two splice-site variants c.1186-2A>G and p.Arg223Arg (CGG>CGC), and a missense variant p.Pro12Leu) that was missed in the prior routine genetic testing. One patient showed a pathogenic germline heterozygous missense mutation in *CASR* (p.Ile555Val), and one patient had a germline heterozygous deletion on chromosome 1q which included *CDC73*. In the same study, WGS of tumor DNA samples from six other mutation-negative

patients did not detect any somatic variants in recurrent genes that may act as tumor suppressors (101). Therefore, the results of this WGS analysis raises the possibility of missing a germline mutation in prior routine genetic testing, perhaps due to older sequencing or variant classification methods. Thus it may be useful to repeat the *MEN1* genetic testing of cases who present with clinical MEN1 where finding an *MEN1* mutation is highly likely (tumors of parathyroid, pituitary and endocrine pancreas; or tumors of parathyroid and endocrine pancreas).

Collaborative efforts and other novel approaches can be considered to identify mutations in *MEN1* or other genes in the 10-30% of mutation-negative cases of clinical MEN1. Blood transcriptome sequencing has been used for the identification of rare-disease genes, which is RNA-seq analysis of RNA isolated from whole blood samples to detect any evidence of altered transcription as a consequence of DNA variants (102, 103). If the transcript is expressed in blood cells (e.g., *MEN1*), effect on splicing and expression level can be detected from missense, synonymous, and loss-of-function (LoF) mutations within the coding exons. LoF mutations can lead to lower transcript levels through nonsense-mediated decay. Also, blood transcriptome analysis can identify the decreased expression of one allele due to a variant in the regulatory region. When combined with WGS, the corresponding DNA variants in genes of interest can

be identified that are responsible for the altered transcription. One of the limitations of this approach is the inability to identify causal genes due to lack of expression in blood cells. Also, this approach may not successfully identify disease susceptibility genes if causal variants do not affect splicing or expression of the gene.

CLINICAL COURSE OF GENETICALLY (+) AND (-) MEN1 PATIENTS

MEN1 is mainly characterised by the occurrence of parathyroid tumors, duodeno-pancreatic neuroendocrine tumors (DP-NETs), pituitary adenomas, and adrenal tumors (3, 104-107). A diagnosis of MEN1 can be made based on clinical, familial or genetic criteria. Thus, for a clinical diagnosis patients should have 2 or more MEN1-associated tumors; for a familial diagnosis patients should have 1 MEN1-associated tumor plus a first degree relative with MEN1; and for a genetic diagnosis a germline *MEN1* mutation needs to be identified (105, 106). Across all of these diagnostic criteria, up to 90% of patients will be found to have a *MEN1* mutation. To date, however no genotype-phenotype correlations have been reported, and even within the same family the tumor types, and age of tumor onset can differ significantly (105). Therefore, long term radiological and biochemical screening should be performed, and

appropriate treatment undertaken, as it has been reported that early diagnosis of tumors with appropriate interventions can significantly improve patient survival (3, 105, 108, 109). It is important to note, however, that *MEN1* mutations can also give rise to familial isolated primary hyperparathyroidism (FIHP)(110). FIHP is characterised by the occurrence of hereditary primary hyperparathyroidism without occurrence of other MEN1-associated tumors during a follow up period of >10.4 years, and the development of primary hyperparathyroidism at 51.4±14 years of age (110). Therefore, a diagnosis of FIHP should also be considered in patients that present with primary hyperparathyroidism at an advanced age and show no further MEN1 manifestations after 10 year follow up.

In 10-30% of MEN1 patients that are diagnosed based on clinical criteria, *MEN1* mutations are not identified (106, 111). These are referred to as phenocopies, and are clinically challenging as the manifestations and familial penetrance of the disease are not defined, and therefore the risk of tumor occurrence and subsequently the most appropriate screening protocols are debatable. Furthermore, analysis of a large MEN1 family cohort consisting of 152 members, indicated that 10% of individuals within the family who were diagnosed as having MEN1 did not have the *MEN1* mutation, thereby indicating that phenocopies can also occur in MEN1 patients with a family

history of MEN1. Determining if an individual is an MEN1 is of particular importance because it has been reported that *MEN1* mutation negative patients have less aggressive disease, with the median age of first tumor manilfestation 13 years later, and median age of second manifestation 9 years later compared to *MEN1* mutation positive patients (5). Furthermore, no third manifestations were identified in mutation negative patients, compared to 76 patients in the mutation positive group, and median survival was 14 years greater in the negative *versus* positive *MEN1* mutation patient groups (87 *versus* 73 years of age) (106). The disease course for each *MEN1* mutation negative patient is however likely to differ, and sub-classification of this group based on genetic analysis will help inform the most appropriate screening and treatment (112). To date, a number of genes have been reported as causing MEN1 phenocopies. These genes include the: cyclin dependent kinase inhibitor 1B (*CDKN1B*) which encodes the cell cycle regulating tumor suppressor protein $p27^{Kip1}$; rearranged during transfection (*RET*) proto-oncogene which encodes a receptor tyrosine kinase (RTK) for members of the glial cell line-derived neurotrophic factor (GDNF) family of extracellular signaling molecules; *CDC73* which encodes parafibromin, a subunit of the polymerase II-associated factor (PAF) protein complex, which associates with the RNA polymerase II subunit POLR2A and with a histone methyltransferase complex; *CASR* which encodes a G-protein coupled receptor (GPCR); and aryl hydrocarbon receptor-interacting

protein (*AIP*), which encodes a tumor suppressor protein that interacts and colocalises with the protein kinase A (PKA) subunits R1-alpha (PRKAR1A) and C-alpha (PRKACA) (3, 99, 101, 113).

CDKN1B mutations have been observed in patients with MEN1-associated tumors. These patients are classified as having MEN4, and to date >25 cases are reported (114-116). The most common tumors to arise in MEN4 patients are parathyroid tumors, followed by pituitary adenomas and pancreatic NETs, and then occasional adrenal tumors and non-endocrine tumors such as lipomas and meningiomas (114-116). Furthermore, details of the age of onset of these tumors is limited, however primary hyperparathyroidism has been reported in a 15 year old individual, indicating that, similar to MEN1, tumors may develop in MEN4 patients during childhood, or adolescence (117). Although the clinical manifestations appear similar to MEN1, some key differences have been observed, for example there are currently no reported cases of prolactinomas in MEN4, and the prevalence of duodeno-pancreatic NETs in MEN4 patients is only approximately 25%, compared to up to 70% in MEN1 patients (114). In addition to *CDKN1B* mutations, mutations in other CDK family members including: *CDKN2B* encoding $p15^{INK4b}$; *CDKN2C* encoding $p18^{INK4c}$; and *CDKN1A* encoding $p21^{Cip1}$ have also been identified in MEN1 patients

(118). The number of reported cases for these mutations is low, and therefore accurate predictions of tumor manifestation is difficult, however parathyroid, pituitary and adrenal tumors have been reported, as well as prostate and breast tumors in these patients (118). Moreover, it is predicted that patients with *CDKN1B* mutations only account for ~3% of MEN1-like individuals, with mutations in *CDKN2B*, *CDKN2C* and *CDKN1A* estimated to account for up to 1%, 0.5% and 0.5%, respectively (114, 118). Therefore, mutations in the CDK family may only occur in a small proportion of patients defined as being MEN1 phenocopies.

Analysis of patients and families diagnosed initially to have MEN1 based on clinical criteria, has also highlighted additional genes that may represent MEN1 phenocopies including *RET*, *CDC73*, *CASR*, and *AIP* (3, 99, 102, 113). *RET* mutations are usually associated with MEN2 (previously MEN2A) and MEN2B (previously MEN3). MEN2 is characterised by the occurrence of medullary thyroid carcinoma (MTC), pheochromocytoma and parathyroid tumors; while in MEN2 parathyroid tumors are rare, and the occurrence of MTC and pheochromocytoma is found in association with a marfanoid habitus, mucosal neuromas, medullated corneal fibers, and intestinal autonomic ganglion dysfunction leading to megacolon (3). However, a patient presenting with Cushing's disease due to a corticotrophinoma at 48 years of age, who

later developed primary hyperparathyroidism and was diagnosed clinically to have MEN1, but in whom a *MEN1* mutation was not identified, was subsequently found to have MTC and pheochromocytoma at 66 years of age, and a *RET* mutation (116*RET*mutation (119), consistent with a diagnosis of MEN2. MEN1 phenocopies associated with *CDC73* mutations, a gene in which mutations usually cause hyperparathyroidism-jaw tumor (HPT-JT) syndrome that is characterised by parathyroid tumors, ossifying jaw fibromas, renal tumors and uterine neoplasms (120), have been reported in two unrelated patients who had an initial diagnosis of MEN1, but upon genetic analysis were shown to have *CDC73* mutation (99, 100); one had primary hyperparathyroidism and a prolactinoma (100), and the other had acromegaly, primary hyperparathyroidism and a pancreatic NET (100). MEN1 phenocopies associated with *CASR* mutations, which usually give rise to familial hypocalciuric hypercalcaemia type 1 (FHH1) and FIHP (121), have been reported in two unrelated patients; one had acromegaly and hypercalcaemia possibly due to primary hyperparathyroidism (99), and the other had a NET hepatic metastasis from an unknown primary tumor, and primary hyperparathyroidism (102). Thus, these patients represent MEN1 phenocopies as they have developed MEN1 tumor manifestations on a background of hereditary disorders associated with parathyroid tumors. MEN1 phenocopy

associated with an *AIP* mutation, which usually gives rise to familial isolated pituitary adenomas (FIPA), has been reported in a patient with acromegaly and primary hyperparathyroidism, thereby illustrating the occurrence of a MEN1 phenocopy in which parathyroid tumor development arose on a background of a hereditary disorder associated with pituitary adenomas (3, 113). These findings indicate that if a patient is diagnosed with MEN1 based on the combined occurrence of primary hyperparathyroidism, and a pituitary tumor then testing for *MEN1, RET*, *CDKN1B, CDKN2B*, *CDKN2C*, *CDKN1A, CDC73*, *CASR*, or *AIP* mutations would be advisable to determine if the patient has MEN1, or could potentially have MEN2, MEN4, HPT-JT, FHH, FIHP or FIPA. In addition, the prevalence of both primary hyperparathyroidism and pituitary adenomas is rapidly rising, with primary hyperparathyroidism increasing from 76 to 233 per 100,000 women, and 30 to 85 per 100,000 men over the past two decades (122), and pituitary tumors identified in over 25% of unselected autopsies and 20% of the population undergoing intracranial imaging (123). Therefore, the potential of patients to develop co-incidental parathyroid and pituitary tumors, and thus meeting the MEN1 clinical criteria is also increasing. This again highlights the need for detailed germline genetic testing, as well as familial investigation, of MEN1 patients (112).

Cohorts of patients who have MEN1 like-syndrome but tested negative for *MEN1*, *CDKN1A*, *CDKN1B*, *CDKN2B*, *CDKN2C*, *CDC73*, *CASR*, *RET* and *AIP* mutations have also been reported (100, 102). It is possible that these patients may have mutations in non-coding regions of the *MEN1* gene that affect menin expression, for example in promotor or enhance regions. Sequencing of cDNA may therefore be of benefit to identify, for example splicing changes. It is, however, also probable that additional yet unreported genes are involved in the pathogenesis of MEN1 phenocopies. To identify novel MEN causing genes will likely require genetic analysis of large cohorts of patients (102). This is likely because novel genes will be occurring in less than 20% of clinically diagnosed MEN1 patients. Thus, in summary the clinical course of patients who are *MEN1* mutation negative differs to that of *MEN1* mutation positive patients. Currently, genetic testing for genes including *CDKN1A, CDKN1B, CDKN2B, CDKN2C, CDC73, CASR, RET,* and *AIP* may highlight MEN1 phenocopies, however these still account for only ~5-10% of the *MEN1* mutation negative cases. Therefore, future studies identifying novel genes will be important for determining the treatment and screening of *MEN1* mutation negative MEN1 patients.

CLINICAL PRESENTATION OF MEN1 IN CHILDREN AND ADOLESCENTS

Approximately 12-17% of MEN1 patients are diagnosed with the disease in the first two decades of life (124-126). Clinical evident disease appears uncommon before adolescence, with consensus guidelines currently recommending phenotype screening of confirmed MEN1 carriers commencing by 5 years of age (3). Even if penetrance of *MEN1* mutations is age dependent clinical manifestations of MEN1 have occurred in some patients by the age of 5 years. Therefore, clinical guidelines suggest the performance of genetic testing in asymptomatic relatives of MEN1 mutated patients as soon as possible, certainly within the first decade of life. *MEN1* germline mutation la analysis should be recommended in individuals presenting at an early age with a single, apparently sporadic MEN1-associated tumor (3).

Early manifestation of the classical MEN1 endocrine disorders in young patients can be the first manifestation of the syndrome and thus, their recognition may help not only in the close monitoring of patient's treatment, but also direct the screening for other endocrinopathies.

The seminal paper on the role of germline *MEN1* mutation causing pituitary adenomas in children and adolescents described PRL-secreting tumors as the most frequent manifestation, with GH excess being rare and

possibly related to GHRH secreting pancreatic tumor (127). The age at diagnosis of MEN1 syndrome varies according to the clinical, familial or genetic diagnosis. Clinically a functional tumor is diagnosed much earlier than a non-functional tumor (128). The familial diagnosis allows the recognition of gene carriers at birth with the possibility to follow the natural history of the disease in a given patient. Future tests based on wide genome analysis will allow the identification of gene mutations even in sporadic asymptomatic carriers. Diagnosis in the children has to do with the presymptomatic screening of at-risk patients which allows for earlier detection and intervention, with a resultant decrease in mortality and morbidity associated with these tumors (129). Presymptomatic screening recommendations for MEN1 management have been based on the youngest age at which disease manifestations have been reported and this cannot be considered a precise optimal timing.

1. Diagnosis and Therapy

1a. Primary hyperparathyroidism

Primary hyperparathyroidism (PHPT) is the earliest laboratory and/or clinical manifestation in patients with MEN1. The youngest reported cases have been reported at the age of 4 years and with a pediatric prevalence of

75% in a study evaluating 122 patients with MEN1 aged <21 years (125). These data were confirmed in another cohort with a prevalence of 58% for (130). PHPT is mainly asymptomatic in young patients with MEN1 (125, 130), even though a higher incidence of rickets and osteomalacia was demonstrated in the pediatric population with PHPT than in adult patients (131). A single case of a severe complication of MEN1-associated PHPT has been described: a 14 years old boy with MEN1 experiencing a stroke in the absence of other recognized causes, but with only PHPT (132).

Development of hypercalcemia during surveillance is suggestive of PHPT and should be followed-up by the simultaneous measurement of calcium and intact parathyroid hormone. Diagnostic management is usually the same as for adult patients.

The therapy for PHPT in MEN1 is surgical parathyroidectomy. In adult patients this should not be postponed because bone complications are more severe in MEN1-associated PHPT than in its sporadic counterpart (133). The published data with 19 MEN1 adolescents developing PHPT before the age of 20 years and undergoing parathyroidectomy before the age of 25 years to control calcemia support the use of surgery to avoid PHPT complications, even though two patients developed post surgical hypoparathyroidism (134). Subtotal parathyroidectomy is always recommended.

Treatment with the calcimimetic cinacalcet has been demonstrated to be successful in children affected by neonatal severe hyperparathyroidism (135), but reports on the response to monotherapy with cinacalcet in children with MEN1 are not available.

1b. Pituitary tumors

Pituitary adenomas are the second in frequency in young MEN1 patients (>30%) with an age at diagnosis as early as 10 years (125, 130). MEN1-associated pituitary adenomas are known to be more frequent in females than males (124). Conversely in children macroadenomas and severity of symptoms are prevalent in young males rather than young females (124, 125, 130, 136-140). Similar to adults=prolactinomas are the most frequent MEN1-associated pituitary tumors in children (3, 125,130). Pituitary-associated Cushing's disease is more frequent than Cushing's syndrome caused by adrenal lesions in pediatric MEN1 patients (125, 137, 141). Pituitary tumors have been described in several publications as aggressive, since they are larger than in sporadic cases and may be multiple at the time of diagnosis with reduced response to standard therapy (124). A case of a boy with MEN1 ~~boy~~ who developed a TSH-secreting pituitary carcinoma at age 19 years was reported in the literature (142). The aggressive behavior of MEN1-associated pituitary tumors is an important reason for anticipating the screening at the age of 5 years (3).

The treatment of pituitary tumors in young patients should follow the standard therapy for the sporadic forms of the disease. For macroprolactinomas the presence of *MEN1* mutation was independently associated with resistance to dopamine therapy in up to 16% of patients (143).

1c. Neuroendocrine tumors

Neuroendocrine tumors (NETs) are the rarest lesion in young MEN1 patients, with the youngest patient diagnosed at 8 years of age (125, 130, 144). Interestingly, the systematic use of endoscopic ultrasound in young MEN1 patients has provided evidence for the ~~a~~ prevalence of clinically occult (non-functioning) pNETs in up to 42% of pediatric cases (145). Differently than adults, gastrinomas are rare in children with MEN1, but when present can be very aggressive (125, 130). The parents of MEN1 children are educated to recognize symptoms related to Zollinger-Ellison syndrome (ZES). Moreover, MEN1-associated insulinoma, the most frequent neuroendocrine tumors in the juvenile MEN1 French cohort, is very precocious in onset and its diagnosis is delayed (125). A possible explantation of this delay is the overlapping of symptoms related to hypoglycemia and epilepsy, a much less rare disorder in infancy. Finally, for the few young MEN1 patients affected by thymic NETs the disease was lethal (125, 146). Altogether these are important

elements for deciding when to start clinical surveillance in young MEN1 patients.

The duodeno-pancreatic disease is a major cause of mortality in adult patients with MEN1, but although rare is also present in the pediatric and young adult age groups and thus requires active surveillance. Conditions like insulinoma are cured by surgery- avoiding severe hypoglycemia and brain damage (147).

2. General Recommedations

The different studies carried out after the publication of the clinical guidelines in 2012 demonstrate that mortality is rare in children and young adults, while morbidity is not uncommon for the manifestation of PHPT, DP-NETs, and pituitary disease. MEN1-associated endocrine tumors in children and adolescents appear to have clinical manifestations that differ from those observed in the adults with increased severity. Identification of the endocrine tumors at an earlier stage in children could potentially reduce morbidity in MEN1 as observed in other hereditary tumor syndromes. It is important to remember that any decision related to an active early tumor surveillance and intervention should take into account patient preference and ascertain any financial and psychological burden. An important consideration in supporting

and managing these younger patients is a risk assessment of the likelihood of aggressive or incidental functional disease versus the burden of frequent medical surveillance.

ADVERSE FERTILITY, PREGNANCY OUTCOMES AND IMPACT OF PARENTAL MEN1

Women affected by MEN1 typically show classical endocrinopathies during their reproductive years. Some of these endocrine manifestations are known to potentially affect reproductive health. In a report describing a family genotyped by linkage analysis with two of the siblings found to be homozygotes, homozygosity did not result in a more severe phenotype, while resulting in unexplained infertility possibly at the time of conception (148). Surprisingly, until very recently there was little published research regarding the impact of MEN1 on pregnancy and a corresponding lack of data to guide antenatal management, except for a few case reports. There are, however, case reports that outline the management of PHPT and sporadic pituitary tumors and DP-NETs in pregnancy in MEN1. Data concerning the impact of MEN1 on fertility and pregnancy is based on the accumulated

experience with more common sporadic single organ dysfunction, such as isolated PHPT, pituitary tumors and NETs.

1. Adverse Fertility

Prolactinoma, that represents 70% of pituitary adenomas occurring before 21 years of age (125, 126), is recognized to reduce fertility, while PHPT does not affect fertility *per se* (149, 150). In a historical population-based analysis in a multigenerational kindred in Tasmania, named Tasman 1 MEN1 kindred, fertility and pregnancy outcomes were analyzed, with no adverse impact of MEN1 on patient fertility and stillbirth (151). The most likely explanation for this finding is the lack of symptomatology in parathyroid and pituitary tumors commonly occurring before 20 years of age in MEN1 (125). Indeed, in the Tasman population-based analysis the majority of pregnancies should have occurred in the context of subclinical pituitary or parathyroid disease. Similarly, in a Finnish MEN1 kindred the reproductive fitness was not affected by the disease (152). The analysis of the large Tasman kindred made possible also to exclude any adverse impact of the maternal MEN1 status on offspring gender or offspring MEN1 (151). In a recent retrospective Australian analysis that evaluated 96 pregnancies relating to 26 women within the Tasman 1 cohort, emergency cesarean deliveries and miscarriage rate were not significantly different in MEN1-positive women (153).

Overall the limited published data suggest no adverse impact of MEN1 on patient fertility; however pituitary disease in MEN1 could impair reproductive potential. Therefore, MEN1 patients should be informed about the need of a careful antenatal investigation of any potential problem, with target intervention only in selected patients.

2. Pregnancy outcomes

Surprisingly, there is little published research on the impact of MEN1 on pregnancy outcomes, making it difficult to guide doctors and patients on the management of the pregnant MEN1 patient. Case reports published offer a limited experience to conclude that endocrine disorders in MEN1 pregnant women are variable with a variable expression of maternofetal complications (154, 155).

Outcomes of pregnancy in MEN1 women could be weighted indirectly on the basis of ~~the~~ limited publications reporting on the impact of sporadic single organ endocrinopathies. Complications of PHPT in pregnancy is associated with a high incidence of maternal, fetal and neonatal complications directly proportionate to the degree of maternal calcium levels (156). Maternal complications include kidney stones, pancreatitis, acute hypercalcemia, hypertensive crisis, cardiac arrhythmias, pre-eclampsia, and miscarriage (153,

156). PHPT in pregnancy can cause – intrauterine growth restriction, preterm delivery, intrauterine fetal death and neonatal (low birth weight, hypocalcemia) (157). Adverse outcomes appear to relate to the degree of hypercalcemia rather than to the presence of PHPT (149, 150).

Calcemia needs to be monitored during pregnancy in MEN1 women and kept in the mild-moderate levels. Parathyroidectomy is usually avoided when possible and when necessary is timed in the second trimester. Neonates should be monitored for hypocalcemia.

Nonparathyroid MEN1-related endocrinopathies occurr less frequently during pregnancy (158). Pituitary disease was the second most common endocrinopathy in MEN1 pregnant women, with a prevalence of prolactinomas and nonfunctioning adenomas, and maternal and neonatal consequences (I.e. hypertension and low birthweight) (153, 158).

Gestational diabetes mellitus was found at a higher rate in MEN1 pregnant women (156) as a consequence of a high prevalence of hypoglycemia in infants with an MEN1-positive mother. Therefore, blood glucose should be actively tested in MEN1 pregnant women and in their offsprings.

3. Impact of parental MEN1

In a large survey conducted in the Tasman 1 cohort, parental MEN1 was shown to be associated with a high vulnerability to ~~of~~ neonates postpartum and this is not fully explained by the most common metabolic alteration, hypercalcemia (158). Main neonatal complications encompassed lower birth weight, longer stay and admission to a higher dependency nursery, hypoglycemia and more rarely hypocalcemia (158). Children with an MEN1 parent have an elevated risk of postnatal mortality and was not solely attributable to offsprings of MEN1 mothers, but was also present in offsprings of MEN1 fathers (158). Infections appear to be an important cause of mortality in these children and the potential for a role of menin in the control of the immune system has been proposed (158).

GENETIC AND CLINICAL SIGNATURES OF FUNCTIONING AND NON-FUNCTIONING DUODENO-PANCREATIC NEURO-ENDOCRINE TUMORS IN MEN1 AND THEIR IMPACTS IN DIAGNOSIS AND THERAPY OF THESE NEOPLASIA

In MEN1 patients, duodeno-pancreatic neuroendocrine tumors (DP-NETs) are highly prevalent and nowadays the major cause of premature MEN1-related death because of metastasized disease (124, 159, 160). As early as in

the first decade of life, a few MEN1 patients have been reported to develop DP-NETs (125). At the age of 50 years, approximately 50% of patients suffer or have suffered from a DP-NET rising to a prevalence of almost 90% at an age higher than 80 years (108). Within MEN1 families, there are some indications that tumors arise at an earlier age in patients within successive generations (161). During the lives of patients, multiple functioning as well as non-functioning tumors occur throughout the pancreas which further complicates the management for individual patients (162). Therefore, MEN1 carriers are intensively screened pre-symptomatically from a young age to enable timely interventions for preventing morbidity and metastasized disease (3). Insights into the clinical and genetic signatures of the different types of DP-NETs and the impact on the management of patients is very important for an adequate management of patients. Because of the complexity, and for well informed and up-to-date decision making tailored to the individual patients needs, MEN1 care within an experienced multidisciplinary team dedicated to collaborative research is of utmost importance (163).

Functioning (hormone producing) DP-NETs are often diagnosed because of elevation of plasma biochemical markers and the endocrine syndrome that is caused by the hormone produced by the NET. The management of functioning DP-NETs entails both the treatment of the functional syndrome

caused by hormonal hypersecretion as well as mitigating the oncologic risk of distant metastases, while minimizing treatment related morbidity and mortality. This is particularly challenging in gastrinoma, the most frequently occuring functioning DP-NETs in MEN1. Gastrinomas occur in approximately 30% of patients with MEN1 (110, 164-168). These tumors produce gastrin which, if unopposed, leads to acid hypersecretion of the stomach with subsequent severe peptic ulceration and gastro-intestinal bleeding, the so called ZES (169). Gastrinomas in MEN1 are often small and in most cases located in the submucosa of the duodenum (170, 171). Proton pump inhibitors are effective for the treatment of the peptic ulcer disease and therefore suggested for the treatment of the majority of patients with gastrinomas (3, 172). The prognosis of MEN1-related gastrinomas is hard to interpret because there is a wide variation among studies with regard to diagnosis and treatment (173-178). In a French cohort study, gastrinomas were associatied with an increased risk of distant metastases irrespective of tumor size. In this study metastasized gastrinomas were not significantly associated with the survival of patients (160). However, in a subgroup of MEN1 patients, gastrinomas appeared to have an aggressive course of disease with distant metastases and early death. In this study aggressive tumor behavior was associated with larger tumor size and higher gastrin levels (173). In a recent population-based study

the life expectancy of MEN1 patients with a gastrinoma was shown to be reduced. Also, in this study fasting serum gastrin levels were associated with overall survival and could therefore provide a justification for selecting those patients who might benefit from surgery (179). However, since surgery for gastrinomas is often extensive, not always curative and is associated with morbidity, controversies exist about indication and timing (3, 172, 180).

Interestingly, past Helicobacter pylori exposure was associated with increased prevalence and severity of hypergastrinemia in MEN1 patients (181). Based on these findings, routine Helicobacter pylori serotyping is suggested in all MEN1 patients, with eradication therapy for those who demonstrate evidence of active infection.

The most frequently occurring functioning pNETs in patients with MEN1 are insulinomas with an incidence of 10-15% (33, 108, 182). Insulinomas lead to symptomatic and potentially life-threatening hypoglycaemia. Patients are often young and localization of the insulinoma in the presence of multiple other pNETs is difficult. Therefore, deciding upon the type and extent of surgery is complex (183). Although only studied in six MEN1 patients with evidence for an insulinoma, the recently developed ^{68}Ga-Exendin-4 PET-CT scan appears to be of help for guiding selective pancreas surgery (184). This functional imaging technique makes use of the glucagon like peptide-1

receptor combined with Exendin-4, a synthetic GLP-1 analogue. Although different surgical strategies are followed, resection of MEN1 related insulinoma is often effective but depending on the individual patients' characteristics (185). The same dilemma applies to the even more seldom occurring functioning pNETs such as glucagonoma, VIPoma and GHRH-oma which also lead to symptoms because of overproduction of hormones and can have a poor prognosis (186).

Non-functioning pNETs (NF-pNET) are the most commonly occurring pNETs in patients with MEN1 (133, 187, 188). Therefore, the clinical practice guidelines advice screening for new tumors and monitoring of already existing tumors by plasma biochemical tumor markers combined with radiological examinations or endoscopic ultrasound (EUS) (3). Based on the recent literature, the annual use of tumor markers can no longer be recommended for diagnosing NF-pNET (189-191). The preferred radiological examination is the MRI scan, not only because of the lower risk of cumulative ionizing radiation exposure but also because of the better sensitivity compared with CT scans (189). Endoscopic ultrasound is the most sensitive imaging modality, however, it is operator dependent and invasive. Functional imaging using ^{68}Ga-DOTA PET-CT seems to be most useful for the detection of metastasis of prevalent NF-pNET(191). Up to now, there is no agreement on the optimal

follow-up scheme and the timing of surgical intervention which is the only curative option. Given the paucity of high-quality evidence, appraisal of the existing literature still leads to different opinions on the timing and extent of interventions for surgical interventions (192). Within the multidisciplinary teams taking care for patients with MEN1 the management of NF-pNETs is one of the greatest dilemmas. On the one hand, surgery for NF-pNET often leads to major short- and long-term complications which should be taken into account in the decision making before proceeding to surgery (193). On the other hand, up to now, surgery is the only curative option for NF-pNET. In one small, non-randomized and non-blinded study the somatostatin analogue lanreotide appeared to improve the progression free survival of patients with non-metastatic NF-pNET <2 cm (194). However, before this can be translated to clinical practice more robust evidence is needed.

In MEN1-related NF-pNETs, well informed decision making towards an optimal follow-up and treatment plan requires insight in natural course and prognostic factors (163). (NF-pNET was scarce and often at the risk of bias (195). This emphasizes the caution that is needed, before applying these scientific findings to clinical practice.

In the follow-up of patients, the size of pNETs is often used for deciding upon the follow-up scheme and when to proceed to surgery. From the

available studies it can be concluded that a larger tumor size is associated with a higher chance for metastases and a worse survival (195). In addition, from the studies comparing the effect of surgical resection with a watchful waiting strategy, it can be concluded that patients with tumors smaller than 2 cm have an overall low absolute risk for metastases and death (196-198). Therefore, a watchful waiting strategy for those patients, seems to be oncologically safe with surgery not lowering the risk for metastases enough when outweighing the risk for complications.

In daily clinical practice tumor growth over time is often used as a predictor for the course of the disease in individual patients. In the aforementioned review, two studies assessing the growth of NF-pNETs were identified (195, 199, 200). Overall, from the current available literature it can be concluded that NF-pNETs smaller than 2 cm have a generally stable course and a watch-and-wait strategy appears to be safe in those patients (195). However, metastasis of NF-pNETs smaller than 2 cm do seldom occur and reliable predictors for the course of disease in individual patients are urgently needed to enable decision making for individual patients (163).

Several studies were undertaken in MEN 1 patients assessing the prognostic value of tumor tissue-based markers as assessed by the pathological examination of surgically resected NF-pNETs (85, 201-203). From

research in non-MEN 1 related NETs it is clear that tumors with a higher WHO grade according to the mitotic index, and higher Ki67 labeling index are associated with a worse survival of patients (204). In only one study, tumor grade was assessed as a predictor for survival of MEN1 patients who were operated because of a NF-pNET (201). A higher tumor grade as assessed by mitotic index, in contrast to Ki 67 labeling index, was associated with a higher risk of liver metastasis. These patients were generally operated because of tumors larger than 2 cm.

Epigenetic changes contribute to tumor development. This mechanism is potentially reversible and might therefore be a new therapeutic target. Promotor hypermethylation is reported in sporadically occurring pNETs (205). In MEN1-related pNET, the association between promotor hypermethylation expressed as cumulative methylation index and clinical outcome was also studied. Patients with distant metastasis of NF-pNET appeared to have a higher cumulative methylation index (202).

A study of Cejas et al, showed that within the group of NF-pNETs a distinction can be made between alpha cell tumors expressing the transcription factor ARX and beta cell tumors expressing the transcription factor PDX1 (85). In this study, both transcription factors were assessed by immunohistochemistry. The occurrence of distant metastasis after surgical

resection of MEN1-related NF-pNETs that were often larger than 2 cm was strongly associated with the expression of ARX and not PDX1. Interestingly, distant metastasis almost exclusively occurred in cases with alternative lengthening of telomeres (ALT) within this subgroup of tumors expressing ARX positivity.

Of the currently available markers the WHO grade of tumors can be used to assess the risk for metastasis. Since grade 2 tumors are at a higher risk for metastasis, after resection of these tumors patients should be carefully followed. In addition, tissue-based prognostic factors such as the transcription factors ARX and PDX1 can be assessed by immunohistochemistry. Furthermore, ALT can be an important addition as a biomarker for predicting the risk for metastasis in individual patients with tumors expressing ARX. New biomarkers such as multi-analyte circulating transciptomas, tissue-based molecular factors and image-based markers are needed for predicting the course of tumors in individual MEN1 patients (195). In the coming decade the goals for MEN1-related DP-NETs should be to attain the ability to predict aggressive behaviour early on in the disease course and develop new therapies to prevent metastastic disease. Where patients with sporadic pNET often present with metastastic disease and research is aimed at devising novel treatment options for advanced disease and prediction of treatment response,

in MEN1 there is a window of opportunity to prevent metastatic disease. Since MEN1 is a rare condition and a high quality of research is urgently needed, patients should be treated in centers of expertise, in which multidisciplinary dedicated care is combined with collaborative research to search for new insights into more individualized care for patients (163).

NOVELTIES IN THE SURGICAL APPROACHES IN MEN1 ENDOCRINE TUMORS

Surgical management of MEN1 is complex and controversial, given the multifocal and multiglandular nature of the disease and the high risk of tumor recurrence even after surgical intervention. Establishing the diagnosis of MEN1 before making surgical decisions and referring affected individuals to a surgeon with experience in treating MEN1 can be critical in preventing unnecessary operations or inappropriate surgical approaches.

1. Treatment for parathyroid tumors

The timing and the extent of surgery for MEN1 PHPT remains controversial. Once surgical intervention is necessary, subtotal parathyroidectomy (removal of 3–3.5 glands) is often suggested as the initial treatment (206). If 3.5 or more glands are removed, the rate of persistent

disease is 5% to 6%. Preoperative imaging is not sufficiently reliable to justify unilateral exploration, with 86% of patients having enlarged contralateral parathyroid tumors that were missed. Fifty percent of the remaining patients had the largest parathyroid gland identified intraoperatively on the contralateral side (207). Reoperation is often necessary as hyperplastic parathyroid tissue can be stimulated to grow from embryologic locations along its path along the neck and mediastinum (206). Total parathyroidectomy with autotransplantation of parathyroid tissue to the forearm risks the devastating sequelae of rendering the patient hypoparathyroid (207). Concomitant transcervical thymectomy decreases the rate of recurrence and theoretically lessens the risk of thymic neuro-endocrine tumor development (which can occur in males) and is suggested at the initial operation (208). The standard cervical exploration remains the procedure of choice. At the initial operation, all 4 parathyroid glands are identified. Occasionally supernumerary parathyroid tissue will be identified. The size of the parathyroid glands can be different and gland weight can vary. Factor for choice of remnant creation include relative macroscopic normality, the accessibility of the preserved gland for subsequent re-operation should also be considered (inferior parathyroid may be more suitable lying more anteriorly, away from the recurrent laryngeal nerve) and vascular viability. The authors choose the remnant early in the exploration in

order to confirm viability before resection of the other glands. A remnant approximately twice the size of a normal gland (60mg) is ideal. Robotic and videoscopic techniques have been described but the resource allocation and value have not been proven to be better. However, for recurrent disease or disease noted within the mediastinum which can not be accessed through the neck, video-assisted thorascopic approaches are useful.

2. Treatment for DP-NETs

The timing and extent of surgery for DP-NETs are controversial and depend on many factors, including severity of symptoms, extent of disease, functional component, location and necessity of simple enucleation, subtotal or total pancreatectomy, and pancreaticoduodenectomy (Whipple procedure). Pancreatoduodenectomy has been associated with higher cure rates and improved overall survival, they also have higher rates of postoperative complications and long-term morbidity (209). The risks and benefits should be carefully considered, and surgical decisions should be made on a case-by-case basis. With regard to open or minimally-invasive (laparoscopic or robotic) approaches, minimally-invasive pancreatectomy appears to be safe and associated with a shorter length of stay and fewer complications in selected patients (210).

Minimally invasive surgery techniques have remarkably evolved over the past few decades in the field of surgical oncology, including minimally-invasive pancreatectomy. Because of the relative simplicity of distal pancreatectomy (DP), minimally-invasive DP has been widely accepted and increasingly performed with reported safety (211). Large retrospective analyses as well as recent systematic reviews have shown that minimally invasive DP resulted in less blood loss and shorter hospital stays compared with open DP, and there were no significant differences in incidence rates of short-term complications, including postoperative pancreatic fistula, or in mortality rates or complete gross tumor resection rates between the two techniques (212, 213). Alfieri et al. reported a large Italian multicenter comparative study, comparing laparoscopic and robotic DP for pNETs. The study included a total of 181 patients (96 robotic and 85 laparoscopic DP), and reported that both approaches are safe and efficacious for pNETs treatment, with similar conversion rate, postoperative morbidity, and pancreatic fistula rate between groups (214). Robotic approach was reported to have a higher spleen preservation rate and lower bolood loss, as compared with laparoscopic pancreaticoduodenectomy (PD) is technically demanding surgical procedure, associated with high mortality and morbidity rates. Techniques for minimally

invasive PD have continued to evolve (215, 216), most significantly with the emergence of the robotic surgery platform (217).

The LEOPARD-2 trial was conducted in The Netherlands. This multicenter national study was terminated prematurely after accrual of 99 patients owing to safety concerns, with reported 10% 90-day mortality in the laparoscopic group compared with 2% in the open group (218). The results of the LEOPARD-2 trial clearly demonstrate the safety concerns of laparoscopic PD. Augmented surgical dexterity, particularly the wide range of instrument articulation provided by the robotic surgery platform, may improve the safety and generalizability of robotic PD and several retrospective cohort studies have reported promising results (219). The accumulating reports support the use of robotic minimally invasive PD (Fig. 6); however, prospective randomized controlled trials are warranted to determine the safety and non-inferior oncologic outcomes of robotic PD compared with open PD for patients with pNETs.

Recent advances in interventional gastroenterology have allowed for new treatments as emerging adjuncts to standard care in patients with pNETs. Multiple applications of different techniques have been demonstrated to locally treat pNETs. The studies are small and none have had large cohorts of MEN1 patients but several deserve mention. Local therapies include ethanol

ablation using 1-2 or more cycles of ethanol lavage at various concentrations for local pNETs treatment (220, 221). In 2015, Park et al (222) published results from a series of 11 patients with fourteen lesions. Ten patients had non-functional NETs and two patients had symptomatic insulinomas (one with three lesions). Endoscopic ethanol ablation was performed with 98% ethanol over multiple sessions, patients were followed for 1 year. Post-procedure complete resolution was seen in 8/13 lesions (61.5%); both insulinoma patients were asymptomatic. Three patients had mild pancreatitis and required stent placement for PD stricture. All three patients with acute pancreatitis had >2 mL ethanol lavage in a single procedure. Endoscopic US guided brachytherapy, photodynamic therapy (223), laser ablation therapy (224), "Cyberknife" frameless radiosurgery, regarded as image guided radiotherapy have also been described as emerging technologies.

Radiofrequency ablation (RFA) is perhaps the most common endoscopic treatment considered safe. The RFA technique emits thermal energy resulting in coagulative necrosis of the surrounding tissue. Recent studies have shown promising results with RFA in patients with unresectable PC in open, laparoscopic, or percutaneous setting. EUS-guided RFA (EUS-RFA) allows real-time imaging of pancreatic tumors and may result in safe tissue ablation. In 2015, Armellini et al (225) demonstrated the safety and feasibility of EUS-RFA

in a patient with pNET who refused surgery. Post-ablation, the patient remained asymptomatic and the CT after one month showed complete radiological ablation – suggesting RFA can be a potential alternative to surgery in select cases. Lakhatakia et al. (226) and Waung et al. (227) reported that EUS-RFA used in 4 cases of insulinoma resulted in complete clinical resolution in all patients (complete morphological resolution in two patients) following EUS-RFA with 12 and 10 months follow up, respectively.

Benefits of affecting the systemic immunomodulatory response have also been studied with RFA of pancreas lesions. The initial results assessing a systemic response from radio frequency ablation (RFA) have proved feasibility for locally advanced pancreatic tumors (not specifically pNETs) with regard to adding the benefit of evidence of immunomodulation. One study group observed a general activation of adaptive response decrease of immunosuppression. The plausibility of this occurring with pNETs needs further study (228).

3. Treatment of NF-pNETs

Approximately 50% of individuals with MEN1 will develop NF-pNETs. These are often identified incidentally during assessment and exploration for functioning tumors. As with gastrinomas, the metastatic rate is correlated with

larger tumor size. Tumors smaller than 1.5 cm are not likely to have lymph node metastases (229), although the presence of metastatic disease has been associated with earlier age at death than in those without DP- NETs (127).

4. Treatment of pituitary tumors

Medical therapy to suppress hypersecretion is often the first line of therapy for MEN1-associated pituitary tumors. Surgery is often necessary for patients who are resistant to this treatment. The indications for surgical removal of pituitary tumors associated with MEN1 are similar to those for non-syndromic tumors. They include hormone hypersecretion unresponsive to medical therapy, compression of the optic nerves and/or chiasm endangering vision, and uncertainty of diagnosis requiring biopsy. Adenomas that secrete growth hormone (causing acromegaly) or ACTH (causing Cushing's disease) are typically addressed surgically, as medical strategies for those tumor types tend to palliate rather than cure. Prolactinomas are usually treated with dopamine agonists, with surgery reserved for patients in whom side effects limit their ability to take those medications consistently over the long term, or in whom they are incompletely effective in achieving the dual goals of hormone normalization and control of tumor growth. In one series of 136 patients, medical therapy was successful in approximately one-half of patients with secreting tumors (49 of 116, 42%), and successful suppression was correlated

with smaller tumor size (230). For the nonfunctional adenomas that form the majority of pituitary tumors seen in MEN1, surgery is the main method for tumor control when such control is needed. Small nonfunctional adenomas (microadenomas, < 10 mm) are usually watched, and intervention is only necessary if they grow close enough to the optic chiasm to warrant removal. The pituitary tumor should be given priority for treatment in patients with MEN1 only in patients with pituitary apoplexy (hemorrhage into an adenoma causing loss of vision and/or hormonal collapse) or who present with significant chiasmal compression and risk of blindness.

Most pituitary surgery today is done by the endonasal transsphenoidal approach, which can be done with visualization of the sphenoid sinus and sella by either microscope or endoscope. The microscope confers the advantage of binocular vision with depth of field; the endoscope provides a wider field of view. Both techniques are minimally invasive in nature, and such surgery typically requires short postoperative stays of 1-3 days in hospital. Pituitary tumors are typically soft and friable, and can be removed selectively with specially designed curettes that protect the adjacent normal gland, and allow its preservation. Although such removals are typically piecemeal, in the past decade *en bloc* resection has become possible due to the recognition that tumors > 2 mm are surrounded by a pseudocapsule that provides a plane for

the surgeon (231). For ACTH- and GH+ tumors in particular, *en bloc* resection provides higher rates of durable remission. Only 1-2% of patients will need craniotomy, which carries more risk of neurological injury and is reserved for those with tumors that extend laterally from the sella, encase suprasellar arteries, invade the skull base, or have a firm consistency that makes transsphenoidal removal too difficult and less safe.

Rates of success in surgical removal of MEN1-associated adenomas are similar to those seen in large general series of pituitary tumors in the general population. Although pituitary tumors in MEN1 patients have been reported as more invasive or aggressive than in those without MEN1, one recent surgical series showed that 82% of the tumors were confined to the sella (Knosp grades 0, 1, and 2), and that rates of remission of hormone hypersecretion and of tumor control matched those in patients without MEN1 (232). The lack of growth usually seen over time in asymptomatic un-operated microadenomas in MEN1 is congruent with those findings (233). Applying general principles of pituitary surgery to MEN1-associated adenomas yields excellent control of the majority of cases, and that modern techniques (en bloc removal, endoscopic assistance) have improved the safety and efficacy of such surgery in this group, with significant benefit to such patients. Radiation therapy is reserved for patients with incomplete surgical resection (3).

PHARMACOLOGICAL THERAPIES IN MEN1 ENDOCRINE TUMORS

MEN1 patients often have multiple, multifocal tumors that occur at a younger age and have a higher metastatic potential compared to the single sporadic counterpart tumors (104, 234). This means that surgery may not be possible, especially in the case of pNETs. In addition, with the increase in screening and subsequent earlier diagnosis, pharmacological treatments may also be administered in order to delay surgical intervention, or to control symptoms in functioning tumors (3). Selecting the optimal pharmacological therapy is however challenging as clinical trials are often not undertaken in MEN1 patients, and instead results are extrapolated from those undertaken in patients with a single endocrine tumor, or based on descriptive small cohort studies. Nevertheless, there are a number of pharmacological, or medical, treatments available for MEN1-associated tumors, which can be broadly divided into biotherapies and chemotherapies.

Biotherapies are agents that target specific tumor-associated receptors, or signaling pathways. For NETs, these predominately include agents that target somatostatin receptor (SSTR), mechanistic target of rapamycin (mTOR) and receptor tyrosine kinase (RTK) signaling (109). The first line

pharmacological treatment for MEN1-associated NETs is commonly a somatostatin analogue (SSA) (235). SSAs bind to SSTRs, a family of G protein-coupled receptors consisting of SSTR1-5, to activate their downstream signaling pathways (236). MEN1-associated NETs can express all 5 SSTRs, however they most commonly express SSTR2 and SSTR5 (235, 237). Targeting SSTR signaling, which affects anti-secretory and anti-proliferative pathways, is effective in facilitating symptom control due to hormone hypersecretion and in reducing tumor burden (104, 235, 236). Three SSAs, octreotide, lanreotide and pasireotide, have been used clinically for treating NETs. Octreotide and lanreotide, which primarily bind to SSTR2, have been demonstrated to have efficacy in pituitary and gastroenteropancreatic tumors NETs (238-243). Specific evidence for the efficacy of octreotide and lanreotide in MEN1 patients, has also been demonstrated in: a study of 5 patients with gastroenteropancreatic NETs associated with hypergastrinaemia, in whom 3 months treatment reduced gastrin hypersecretion, and reduced the size of liver metastasise (244); a retrospective study of 40 individuals with MEN1-associated DP-NETs, in whom 12-15 months octreotide treatment resulted in tumor response in 10%, and stable disease in 80% of patients (245); in a longitudinal open label study of 8 MEN1 patients with <2cm pNETs, in whom octreotide treatment resulted in a decrease in GEP hormones and stable

disease in ~80% of patients (246); and in a prospective observational study, in which lanreotide treatment in 23 MEN1 patients with <2cm pNETs significantly improved progression free survival (PFS) (204). Pasireotide, which binds to SSTRs 1-3 and 5, has been reported to be effective in treating pituitary adenomas and pNETs (247-250). Thus, pasireotide has been reported to ameliorate hypoglycaemia in insulinoma patients, and to be effective in patients who are non-responsive to octreotide and lanreotide (247, 248, 251-253). However, clinical trials have indicated that the efficacy of pasireotide is not significantly superior to octreotide and lanreotide in controlling disease progression, and that it is associated with a higher frequency of hyperglycaemia that requires medical intervention, thereby limiting its use (243, 247, 250). Furthermore, due to the lack of detailed clinical trials in MEN1 patients the evidence for the use of pasireotide for MEN1-associated tumors is currently unclear. SSAs have also been utilised in peptide receptor radionuclide therapy (PRRT), whereby they are labelled with a β-emitting radionuclide, for example ^{177}lutetium (254). One prospective randomized study indicated an increase in PFS in PRRT versus SSA alone in metastatic midgut NETs, and currently PRRT is recommended as a third line treatment for metastatic midgut and pNETs (111). Small retrospective case series have also highlighted the benefit of PRRT for metastatic MEN1-associated tumors (255, 256).

In addition to SSTR's, other receptors can be targeted for NET treatment. For example, NETs are often highly vascularised and express RTKs including vascular endothelial growth factor receptor (VEGFR), insulin-like growth factor 1 receptor (IGF1R) and platelet derived growth factor receptor (PDGFR) (104, 109). The RTK inhibitors sunitinib and pazopanib have been reported to increase PFS from ~5.5 to 11.4 months in patients with pNETs (107, 257). However, the evidence of the efficacy of these inhibitors in MEN1 patients is inconclusive, as only one study specifically examined sunitinib treatment in MEN1 patients, and no improvement in PFS was observed in the 2 patients included in the study (258). In addition to SSTR's and RTK's, pituitary tumors can also express G-protein coupled dopaminergic type 2 (D2) receptors, and dopamine agonists (for example cabergoline) are well known to lower prolactin levels and decrease tumor size in prolactinomas in non-MEN1 and MEN1 patients (259). As well as receptors, molecules targeting downstream signaling pathways have also been shown to have efficacy in NETs, including inhibitors of mTOR signaling (83). Everolimus, a mTOR inhibitor approved for the treatment of advanced NETs, has been demonstrated to increase PFS of metastatic pancreatic and lung NET patients from ~4 to ~11 months (260, 261). Evidence for everolimus for the treatment of locally advanced or metastatic pNETs in MEN1 patients has also been demonstrated,

in a retrospective study of 6 patients in which PFS was higher in individuals with *MEN1* mutations (33.1 months) compared to those with sporadic non-MEN1 disease (12.3 months) (258, 262).

Finally, surgical removal of the multiple parathyroid tumors that occur in patients with MEN1 is the definitive treatment (3). However, in patients in whom surgery has either failed or is contraindicated, drugs acting as CaSR agonists (calcimimetics, e.g. cinacalcet) may be sucessfully used to treat the hypercalcaemia and primary hyperparathyroidism (263). Thus, in 8 patients up to 30 mg twice daily cinacalcet treatment has been reported to reduce median serum calcium by 0.35 mmol/L, and decrease serum PTH by a median of 5.05 pmol/L, with no change in urinary calcium; none of the patients demonstrated recurrent stone formation (263).

Chemotherapies, including alkylating agents, anti-microtubule agents, topoisomerase inhibitors, anti-metabolites and cytotoxic antibodies, have been used to treat NETs. These are however, reserved for pancreatico, thymic and bronchopulmonary NET patients with metastatic disease, high tumor burden, or high proliferative index (3, 109). Commonly used agents include the alkylating agents streptozocin and temozolomide, the anti-microtubule agent docetaxel, the topoisomerase inhibitor doxorubicin, and the anti-metabolites capecitabine and gemcitabine, either alone or in combination (3, 109). Thus,

for high grade, poorly differentiated pNETs, it has been reported that streptozocin-based chemotherapy is effective (109), however the benefits for non-pNETs or for MEN1-associated NETs is unclear due to the lack of detailed clinical trials in these patients.

Over the last few decades the increased knowledge of menin function, and the development of robust MEN1 tumor models has led to the preclinical evaluation of a number of novel pharmacological therapies specifically targeting MEN1-associated tumor pathways. This includes gene replacement therapies, epigenetic targeting therapies, Wnt pathway inhibitors, and novel mTOR and RTK inhibitors. Furthermore, the use of these and existing pharmacological agents have also been evaluated for their efficacy in chemoprevention. These preclinical studies are discussed below and shown in Figure 7.

Menin is a tumor suppressor, and loss of menin expression or function results in NET tumor formation. Therefore, *MEN1* gene replacement therapy has the potential as a therapy for all MEN1-associated tumors. Thus, injection of a recombinant non-replicating adenoviral serotype 5 vector (rAd5) containing *Men1* cDNA under the control of a cytomegalovirus promoter, into pituitary tumors of a conventional heterozygous *Men1* knockout mouse ($Men1^{+/-}$) resulted in increased menin expression, and decreased proliferation

without inducing an immune response (264). Furthermore, systemic delivery of a hybrid adeno-associated virus and phage vector that displays active octreotide on the viral surface that allows targeted delivery of a tumor necrosis factor (*TNF*) transgene, significantly reduced the size of pNETs in a conditional *Men1* pancreatic knockout mouse model (*Men1*$^{f/f}$;RIP-Cre) (265). Despite the promise of gene therapy representing a potentially curative treatment for MEN1-assocaited NETs, its translation into clinical trials, may be complex as challenges around delivery, specificity and off target effects still need to be clarified.

One of the key functions of menin is the regulation of gene transcription through epigenetic mechanisms, including the modification of histones via methyltransferase and deacetylase complexes (described above). The use of epigenetic-targeting compounds may therefore have utility in treating MEN1-associated tumors. Preclinical studies have shown that an inhibitor, JQ1, of the BET protein family that binds to acetylated histone residues to promote gene transcription, may have efficacy in MEN1-associated pNETs as it significantly reduced proliferation and increased apoptosis of pNETs in a conditional *Men1* knockout mouse model (76). Furthermore, JQ1 has also been shown to reduce secretion of ACTH from the pituitary tumor cell line, AtT20 (266). In addition, another BET inhibitor CPI203, was able to

significantly reduce proliferation of a BON-1 xenograft model, further indicating the likely utility of BET inhibition for pNETs (267). The utility of histone deacetylases inhibitors (HDACi) has also been evaluated in preclinical NET models. The HDAC5 inhibitor, LMK-235, significantly reduced proliferation and increased apoptosis of pNET cell lines (268), and etinostat, a HDAC1/3 inhibitor could significantly inhibit the expression of master regulator proteins of metastatic gastreoenteropancreatic NETs, as well as reduce tumor growth of the midgut H-STS cell line xenograft mouse model (269). In addition, the HDACi SAHA has been reported to significantly decrease proliferation, and increase apoptosis of a growth hormone and prolactin secreting GH3 rat pituitary tumor cell line, and to decrease cell viability and ACTH secretion from AtT20 and human derived corticotroph tumor (hCtT) cells (270, 271). Similarly, the HDACi trichostatin also significantly decreased proliferation, and inhibited ACTH production in AtT20 cells (272). A number of BET and HDAC inhibitors are already in clinical trials, and thus these may offer a promising novel therapeutic approach for MEN1-associated NETs.

Menin has also been reported to promote β-catenin phosphorylation, resulting in inhibition of Wnt signaling, therefore when menin function is lost β-catenin accumulates in the nucleus and promotes gene transcription (104). The β-catenin inhibitor PRI-724 has been shown to

significantly decrease pNET cell line viability (273), and a preclinical study in conditional *Men1* knockout mice indicated that the β-catenin antagonist PKF115-584 decreased the number and size of pNETs, as well as increased overall mouse survival (63). PRI-724 is already in clinical trials for other conditions (273), and therefore this may provide a novel pharmacological agent for assessment in MEN1-NETs.

RTK and mTOR inhibitors are already in clinical use, although they may have limited efficacy and patients often stop responding to these inhibitors. Therefore, a number of preclinical studies have investigated different approaches for targeting RTK and mTOR signaling pathways. This includes targeting angiogenic pathways with an anti-VEGF (the cytokine that binds to the RTK, VEGFR) monoclonal antibody. However, studies in a RipTag2 insulinoma mouse model indicated that although these compounds reduced tumor burden, they increased invasiveness and metastasis (274). This increased invasiveness may be overcome by targeting multiple RTK pathways simultaneously. For example, concurrent inhibition of VEGF and cMET using sunitibib and crizotinib, respectively, reduced invasion and metastasis in the RipTag2 pNET model (275). Furthermore, the VEGF, PDGF and FGF targeting small kinase inhibitor nindetanib, has been reported to decrease tumor growth, and prolong survival of Rip1Tag2 insulinomas, without increasing local

invasiveness or metastatic spread (276). In addition, inhibition of nitric oxide (NO) synthase may also prove a novel angiogenic-targeting pharmacological approach, as the NO synthase inhibitor L-NAME could increase constriction of tumor supplying arterioles in pNETs of a conventional *Men1* knockout mouse model (277). Also, functionally active angiogenic peptides, including for thrompospondin 1 (TSP1) have been reported to suppress angiogenesis and tumor growth in RipTag2 pNETs, and as menin interacts with SMAD3, which is downstream of TSP1 receptor signaling, this may also provide a menin-targeted therapy for MEN1 patients (104, 278). Finally, novel mTOR inhibitors are being investigated, for example sapanisertib may provide a novel pharmacological agent for everolimus-resistant pNETs. Thus, sapanisertib caused tumor shrinkage of MEN1 mutant patient xenografts that were implanted in female athymic nude mice, and these included tumors that were non-responsive to everolimus (279).

Pharmacological agents have efficacy in reducing proliferation and hormone secretion, however they may also have utility for chemoprevention, which would be of particular importance in MEN1 patients, to prevent or delay tumor occurrence. SSAs may have a role in chemoprevention as paseriotide treatment of a conventional *Men1* knockout mouse model and a Pdx1-Cre *Men1* knockout model (*Men1*$^{f/f}$;Pdx1-Cre) significantly reduced pNET

occurrence (280, 281). Furthermore, lanreotide treatment of a conventional *Men1* knockout mouse model also significantly decreased the number of new pNETs, and pNET size compared to vehicle treated mice (282). In addition, clinical trials indicate that patients treated with octreotide have stable disease (245, 246). Therefore, the administration of SSAs to familial or genetically diagnosed MEN1 patients, may provide a novel approach for preventing or controlling tumor development.

Preclinical studies of novel agents targeting menin specific pathways that are perturbed upon menin loss in tumors may provide a wealth of pharmacological approaches for the treatment of MEN1-associated tumors. These, will however need to be carefully evaluated in prospective clinical trials, specifically recruiting MEN1 patients.

PROGNOSIS AND QUALITY OF LIFE

MEN1 is linked to a higher mortality when compared to a normal population or to non-affected members in MEN1 families, with DP-NETs and thymic NETs representing the main cause (70%) of death (3, 283). Even if uncommon, other tumors such as adrenal carcinoma and parathyroid carcinoma are fatal (13, 284). A predisposition to breast cancer, mainly of the

luminal type has been reported in female MEN1 patients at a mean age of 45 years, earlier than in the general population, thereby recommending breast cancer screening in MEN1 patients around the age of 40 years (285).

In females with MEN1, the mortality is lower than in males with MEN1, probably due to the lower incidence of thymic NETs in females. Moreover, sporadic MEN1 cases show higher mortality than familial cases. All these observations impact the surveillance strategies of MEN1 patients.

The publication of the last guidance paper (3) has certainly contributed to decrease the percentage of deaths related to MEN1 tumors, with a significant ~~a~~n improvement in the management of MEN1 patients.

The oncological nature of MEN1, the multiplicity of tumors, the aggressiveness of some of the neoplasms, the lack of solid data on prognosis and of phenotype genotype correlations increase the potential for a considerable influence of the disease in the health-related quality of life (HRQoL) of the affected patients. Recent studies support this hypothesis, showing a role of the disease and its management in the lower quality of life scores in adults with MEN1 (286-289). Important variables that influence HRQoL in MEN1 patients are the possibility to refer to a dedicated

mulridisciplinary center and to be supported by a dedicated advocacy group for ~~of~~ patients.

Unfortunately, specific questionnaires measuring HRQoL for MEN1 or other hereditary cancers have not yet been developed, contributing to an under- or over-estimation of specific traits of the syndrome

CONCLUSIONS AND FUTURE PROSPECTS

Considerable progress~~es~~ have been made in the past decade to study the natural history, diagnosis and management of MEN1, and in basic and preclinical research to understand the pathophysiology of MEN1-associated tumors. This review has outlined the major advances since the publication of the most recent MEN1 guidance paper (3). The combined efforts of basic researchers and clinicians have been instrumental to alleviate the morbidity and mortality of affected individuals. National Registries and Databases have been developed that can facilitate access to relatively large well-characterized patient populations for diagnostic and therapeutic developments. Patient Associations and Federations have been developed worldwide that can reinforce future screening or intervention programs. In parallel, centers for excellence are now being recognized and these represent quality contacts for

reliable information and clinical care for any type of patients' needs. Basic research on the characterization of menin and mouse models on *Men1* loss has revealed protein interactions and pathways affected in tumors that can be targeted for potential therapeutic options.

However, many important questions are still unanswered, and appropriate and timely recommendations must be offered to the clinicians involved in the care of MEN1 patients.

MEN1 is a complex disorder predisposing to over 20 benign and malignant endocrine and non-endocrine neoplasms. The multidisciplinary team involved in the clinical care of these patients should represent experts in endocrinology, radiology, gastroenterology, surgery, and genetics working in tight connection. This is an essential requirement for the best care of this difficult disease.

Periodic reassessment of the biomedical literature and raw genetic data by experts is encouraged that can help to identify new genes or as yet unrecognized syndromes in individuals with specific syndromic features but who lack known genetic susceptibility mutations. Further work is needed to identify epigenetic or modifying factors that may explain even rare

associations with uncommon tumors for MEN1. Biobanks of MEN1 biological materials should be established.

Preclinical and clinical evidences point to the efficacy of potential pharmacological treatments for MEN1 tumors These promising data are a clear indication for the development of multicenter clinical trials nationally and internationally. These studies can benefit from the development of liquid biopsy assays that could predict specific therapeutic options for patients and efficacy of the therapy.

Additionally, the design of a dedicated HRQoL questionnaire will represent a necessary step to increase the analytical effectiveness of any given treatment in MEN1. Advocacy Associations will be instrumental to carry on these studies.

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Table 1. Mouse models of menin loss to study functional interactions*

Double knockout with conventional *Men1*$^{+/-}$		
GENOTYPE	PHENOTYPE (Islet tumorigenesis)	*Men1* LOH
Rb1$^{+/-}$	Similar to *Men1*$^{+/-}$	Yes
Tp53$^{+/-}$	Similar to *Men1*$^{+/-}$	Yes
Cdkn2c$^{-/-}$	Accelerated	No
Cdkn1b$^{-/-}$	Similar to *Men1*$^{+/-}$	Yes
Cdk2$^{-/-}$	Similar to *Men1*$^{+/-}$	Yes
Cdk4$^{-/-}$	No tumors (islet and pituitary hypoplasia from *Cdk4*$^{-/-}$)	No

Double knockout with conditional menin loss in β-cells (*Men1*$^{f/f}$;RIP-Cre)	
GENOTYPE	PHENOTYPE (Islet tumorigenesis and survival)
Pten$^{f/f}$	Accelerated tumorigenesis and shorter lifespan
Kmt2a$^{f/f}$	Accelerated tumorigenesis and shorter lifespan
Kdm5a$^{f/f}$	Decreased tumorigenesis and prolonged survival
Ctnnb1$^{f/f}$	Decreased tumorigenesis and prolonged survival
Inhbb$^{-/-}$	No effect on tumorigenesis but prolonged survival

Oncogenic *KRas* mutant with conditional menin deficiency in β-cells (*Men1*$^{+/f}$;RIP-Cre)	
GENOTYPE	PHENOTYPE (Islets of P5 neonates)
Kras(G12D)	*Kras*(G12D) expression enhanced, rather than inhibited β-cell proliferation

*References are cited in the main text

FIGURES LEGENDS

Fig. 1 Tumors associated with MEN1

Fig. 2. 3D crystal structure of human menin alone or together with interacting partners. (A) Structure of menin showing a pocket/cavity for protein-protein interaction (PDB ID 3U84). The different domains of menin are color coded: 'N-terminus' in pale green (1-101), 'thumb' in green-cyan (102-230), 'palm' in olive green (231-386), and 'fingers' in dark green (387-end). **(B)** Structure of menin interacting with the menin-binding motif (MBM) of JUND (amino acid 27-47, in purple) (PDB ID 3U86). **(C)** Structure of menin interacting with the MBM of MLL1 (amino acid 6-13, in gold) (PDB ID 3U85). **(D)** The ternary complex of menin with interacting regions in LEDGF (amino acid 347-435, in red) and MLL1 (MBM-LEGDF binding motif (LBM), amino acid 6-153, in gold) (PDB ID 3U88).

To facilitate crystallization, the following regions were deleted (and not present in the structures shown in this Figure): an unstructured loop (amino acid 460–519) in menin, a short fragment (amino acid 40–45) in the JUND-MBM and two loop regions (amino acids 16–22 and 36–102) in the MLL1-MBM-LBM.

The structural images were generated by using PyMOL (Schrodinger, Inc) (https://pymol.org).

Fig. 3 Schematic representation of epigenetic regulation in MEN1-associated tumors. Epigenetic modification in normal cells is shown on the left. Aberrant epigenetic change observed or predicted upon menin loss in tumors is shown in the middle. Consequence of the aberrant epigenetic change is shown on the right. Green and red indicates the nature of the specific histone or DNA epigenetic modification, active or repressive mark of gene expression, respectively. Open black oval with a slanted line indicates loss of that histone mark. Alternative lengthening of telomeres (ALT) is activated in tumors and it is absent in normal cells (indicated by the blue open oval with a slanted line). Not shown is miR-24-mediated epigenetic regulation.

Fig. 4 Percent distribution of the different types

of *MEN1* mutations. Obvious inactivation of menin in predicted by nonsense, frameshift, and splice mutations, and large deletions that constitute 69% of the mutations. Missense and in-frame insertion or deletion (indel) mutations are sliced out of the pie chart to indicate the potential of variants of unknown significance among these two types of mutations.

Fig. 5 Schematic diagram for a suggested approach to germline genetic screening in MEN1 and MEN1-like disease. MEN1: a patient with two or more MEN1-associated endocrine tumors. MEN1-like: a patient with as few as any one of the three main MEN1-associated endocrine tumors. Clinical MEN1: patients with MEN1 or MEN1-like disease features. Genetic MEN1: germline *MEN1* mutation-positive. WGS: whole genome sequencing. WES: whole exome sequencing. +ve = genetic test is positive, -ve = genetic test is negative, and +ve* = genetic test is positive as per ACMG-AMP guidelines.

Fig. 6 Intraoperative view after robotic pancreaticoduodenectomy of a pNET in the head of the pancreas before reconstruction .

CHA: common hepatic atery, IVC: inferior vene cava, SMA: superior mesenteric artery, SMV: superior mesenteric vein

Fig. 7 Emerging therapies for MEN1. Menin, encoded by the MEN1 gene has roles in multiple pathways associated with cell proliferation. These can be targeted by emerging compounds, including receptor tyrosin kinase (RTKs) inhibitors, novel mTOR inhibitors, β-catenin antagonists, epigenetic modulators, and thrombospondin analogues. In addition, preclinical studies indicate MEN1 gene replacement may have efficacy in MEN1 patients, and somatostatin (SST) analogues may have chemopreventative efficacy.

Essential point

- The discovered pathways regulated by menin are opening new opportunities for novel therapeutical interventions in MEN1.

- Genetic diagnosis of MEN1 is making possible a distinct management of the genetically positive and negative patients.

- Sensitive areas, as MEN1 clinical course in the youngsters and in pregnancy, can be clinically managed treasuring the accumulated experiences.

- MEN1 pancreatic neuroendocrine tumors are better understood in their pathogenesis and in their impact in the management of the affected patients.

- Surgical and pharmacological therapies are opening to a brighter future in the clinical course of MEN1.

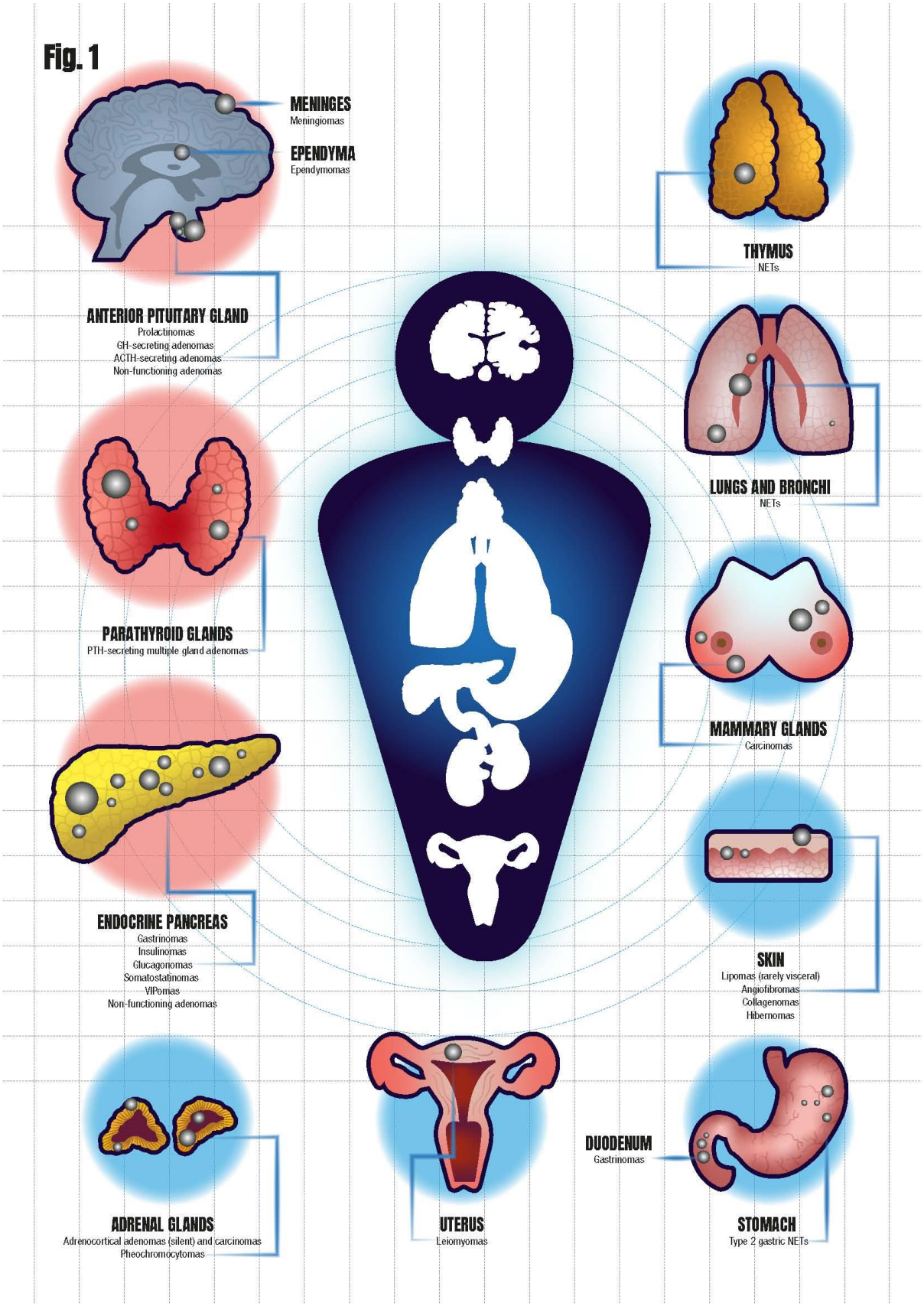

Fig. 2

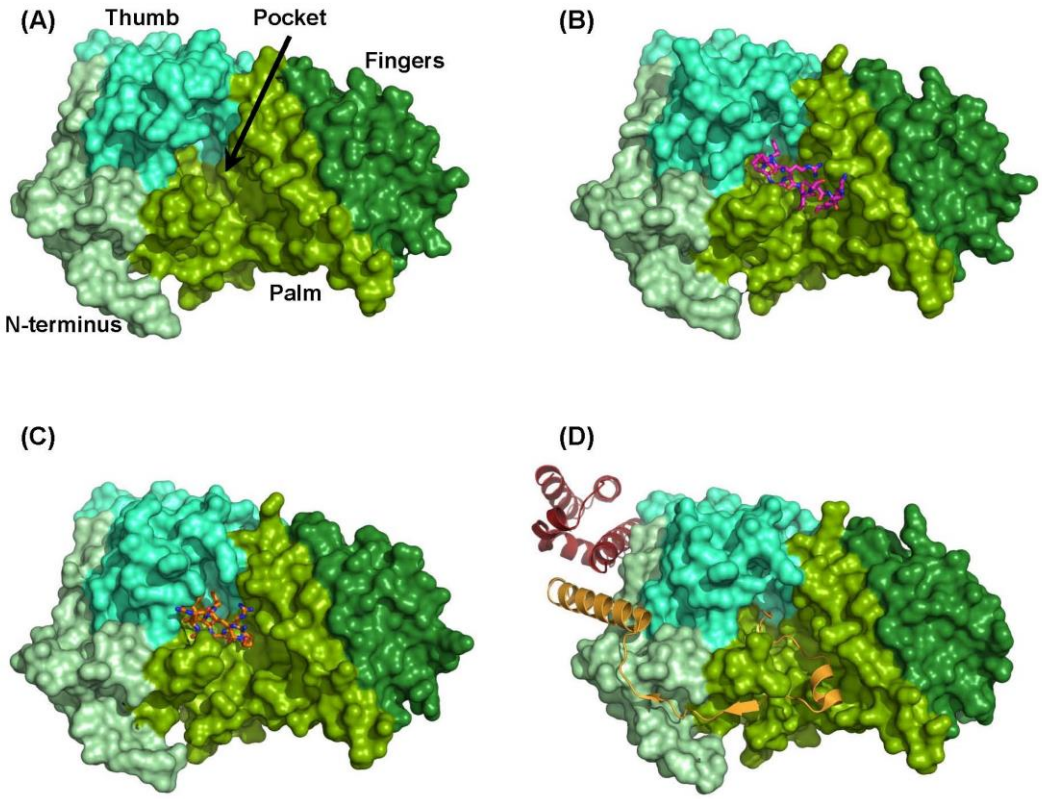

Fig. 2. 3D crystal structure of human menin alone or together with interacting partners.
(A) Structure of menin showing a pocket/cavity for protein-protein interaction (PDB ID 3U84). The different domains of menin are color coded: 'N-terminus' in pale green (1-101), 'thumb' in green-cyan (102-230), 'palm' in olive green (231-386), and 'fingers' in dark green (387-end). **(B)** Structure of menin interacting with the menin-binding motif (MBM) of JUND (amino acid 27-47, in purple) (PDB ID 3U86). **(C)** Structure of menin interacting with the MBM of MLL1 (amino acid 6-13, in gold) (PDB ID 3U85). **(D)** The ternary complex of menin with interacting regions in LEDGF (amino acid 347-435, in red) and MLL1 (MBM-LEGDF binding motif (LBM), amino acid 6-153, in gold) (PDB ID 3U88).

To facilitate crystallization, the following regions were deleted (and not present in the structures shown in this Figure): an unstructured loop (amino acid 460–519) in menin, a short fragment (amino acid 40–45) in JUND-MBM and two loop regions (amino acids 16–22 and 36–102) in the MLL1-MBM-LBM.
The structural images were generated by using PyMOL (Schrodinger, Inc) (https://pymol.org).

Fig. 3

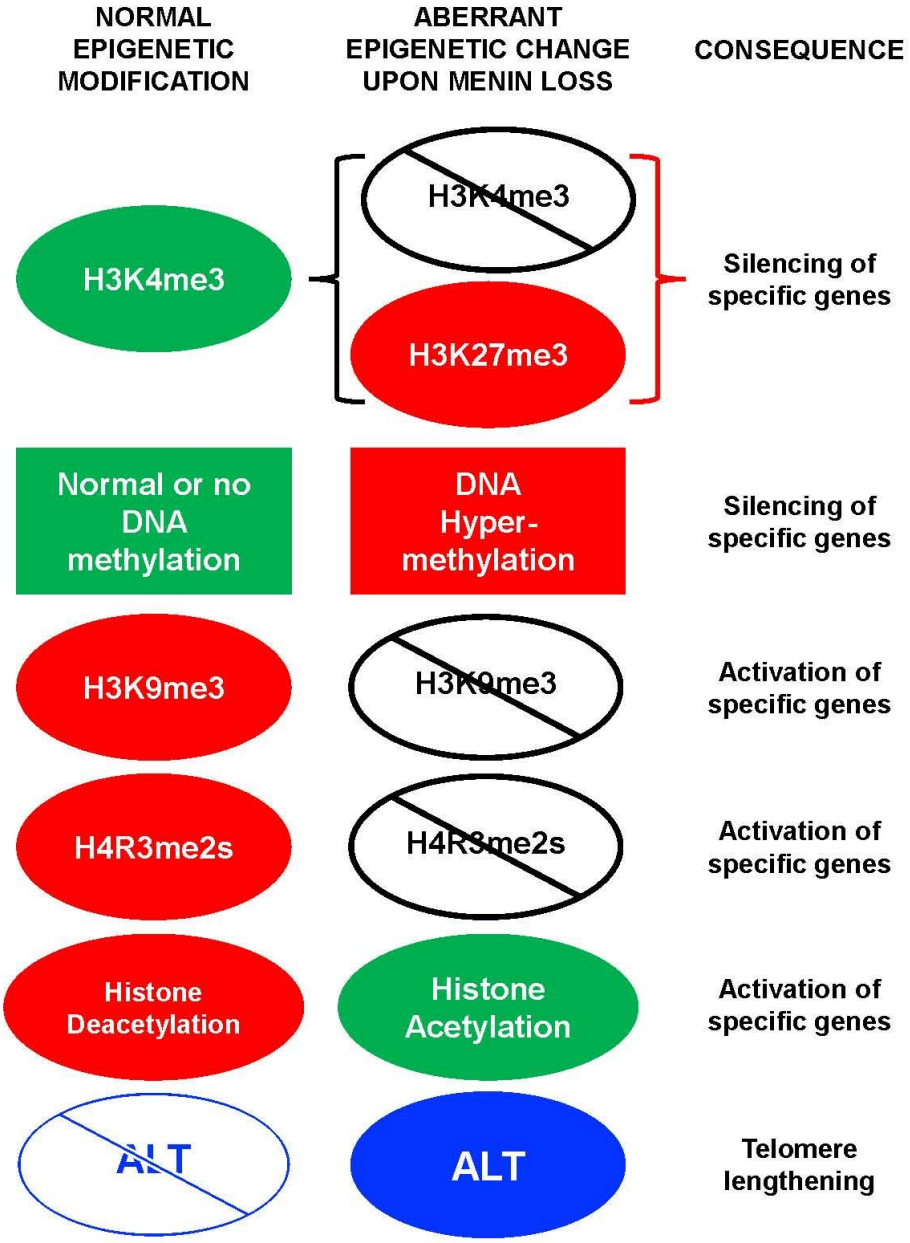

Fig. 4

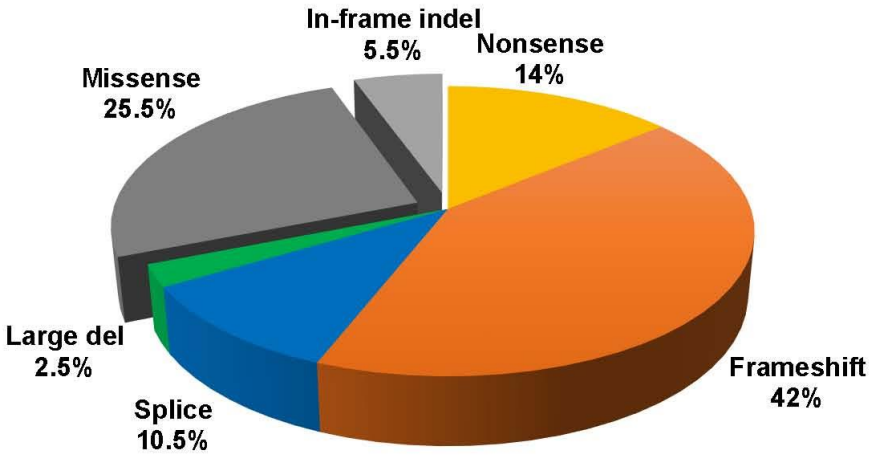

Fig. 5

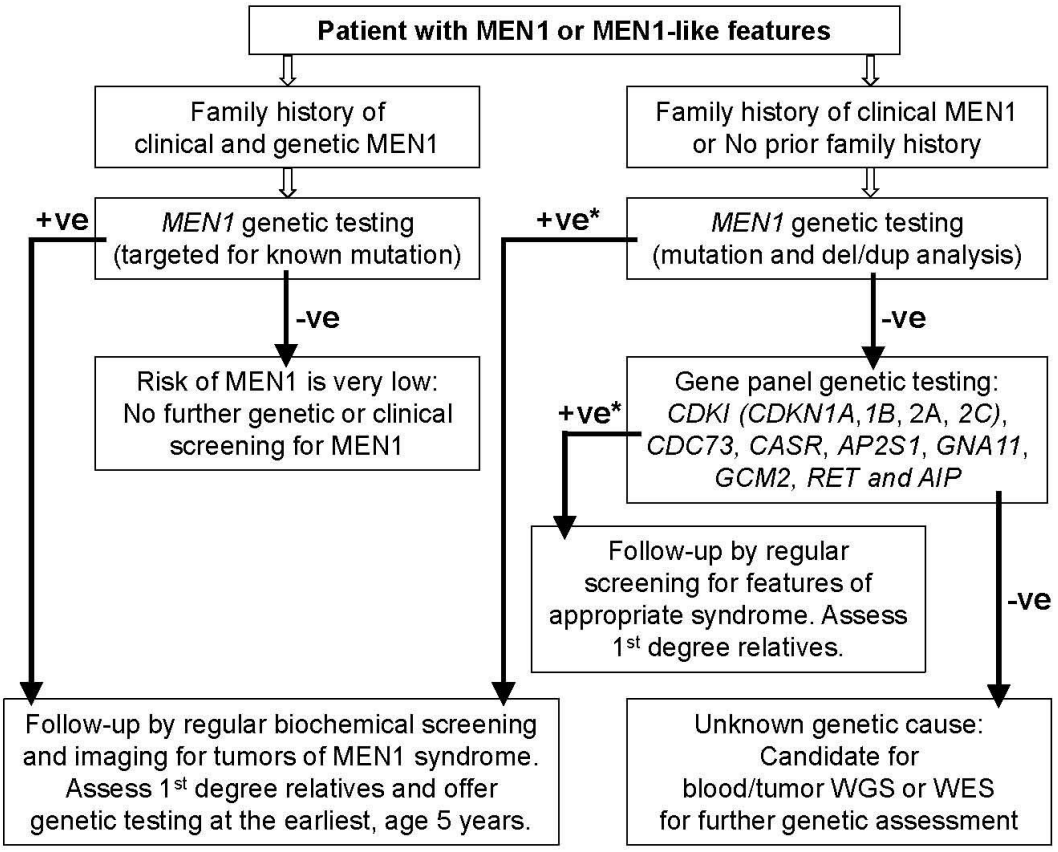

Fig. 6

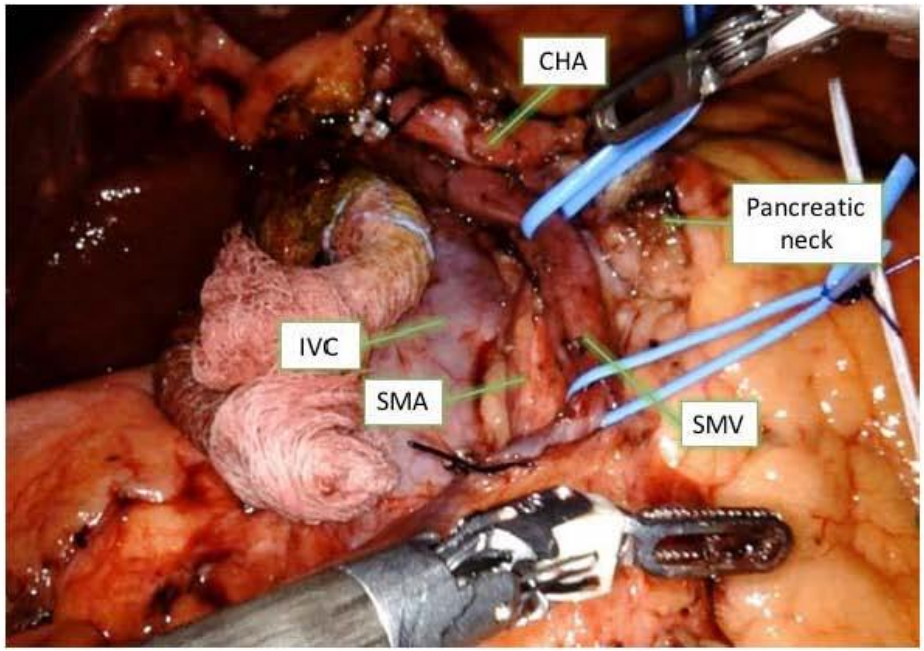

Fig. 7