Case-finding in Multiple Endocrine Neoplasia

CLUES FOR A TIMELY DIAGNOSIS

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Case-finding in Multipele Endocriene Neoplasie
aanwijzingen voor een tijdige diagnose
(met een samenvatting in het Nederlands)

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Medard Franciscus Maria van den Broek

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Promotoren:
Prof. dr. G.D. Valk
Prof. dr. M.R. Vriens

Copromotoren:
Dr. A.A. Verrijn Stuart
Dr. R.S. van Leeuwaarde

CONTENTS




CHAPTER 1

General introduction




Multiple Endocrine Neoplasia (MEN) syndromes include a group of heterogeneous disorders, characterized by a genetic predisposition for tumors in two or more endocrine glands. Over the years, four distinct MEN diseases have been identified, based on the causative genetic defect and clinical phenotype: Multiple Endocrine Neoplasia type 1 (MEN1), Multiple Endocrine Neoplasia type 2A (MEN2A), Multiple Endocrine Neoplasia type 2B (MEN2B) and Multiple Endocrine Neoplasia type 4 (MEN4).

Multiple Endocrine Neoplasia: a historical perspective

The Austrian pathologist Jakob Erdheim was the first to describe a patient with the co-occurrence of two endocrine tumors in 1903, when he identified a pituitary tumor and parathyroid adenomatosis in an acromegalic man on autopsy.[1] During the decades that followed, distinctive combinations of endocrine tumors among patients (and families) were recognized, and categorized into different MEN syndromes.[2–6] Findings in primary relatives of affected individuals led to the discovery that these entities were in fact hereditary diseases, transmitted in an autosomal dominant manner.[4] The genetic basis of these syndromes was eventually unraveled in the 1990s: activating, gain-of-function germline mutations in the *REarranged Translocation* proto-oncogene (*RET* gene) were identified to cause both MEN2 syndromes in 1993-1994, while the tumor suppressor gene *MEN1* – a gene encoding a protein called menin – was discovered as responsible gene for MEN1 in 1997.[7–9] Finally, in 2006, germline mutations in the *cyclin-dependent kinase inhibitor 1b* (*CDKN1B*) gene were detected in some patients with a MEN1-like phenotype, which led to the discovery of the latest subtype of MEN syndromes: MEN4.[10]

MEN syndromes: rare, but high-impact diseases

All MEN syndromes are characterized by their distinctive combination of endocrine tumors and non-endocrine manifestations: parathyroid, pituitary and (duodeno)pancreatic neuroendocrine tumors are considered "classic" MEN1 manifestations, although a wide variety of manifestations has been described in affected patients (see Table 1). Medullary thyroid carcinoma (MTC) and pheochromocytoma are the cardinal features in MEN2A and MEN2B. Additionally, MEN2A patients are prone to the development of parathyroid adenomas, Hirschsprung's disease and cutaneous lichen amyloidosis. In contrast to MEN2A, patients with MEN2B generally do not develop parathyroid adenomas but may suffer from numerous non-endocrine manifestations (*e.g.*, intestinal ganglioneuromatosis, mucosal neuromas, marfanoid habitus, musculoskeletal, orofacial and ocular manifestations).[11] Primary hyperparathyroidism and pituitary tumors seem common in MEN4, but the extreme rarity of this disease – with just over 40 cases reported to date – makes it hard to determine the phenotype of MEN4 with certainty.[12]

Table 1. Clinical manifestations of MEN syndromes

	MEN1	MEN2A	MEN2B	MEN4
Gene	*MEN1*	*RET*	*RET*[a]	*CDKN1B*
Inheritance pattern	autosomal dominant	autosomal dominant	autosomal dominant[b]	autosomal dominant
Endocrine manifestations[c]	pHPT (95%) dpNET (35-75%) pituitary tumor (20-65%) adrenal tumor (10-35%) foregut NET[d] (20-40%)	MTC (100%) pheochromocytoma (50%) pHPT (20-30%)	MTC (100%) pheochromocytoma (50%)	pHPT[e] pituitary tumor[e] dpNET[e] ?[f]
Non-endocrine manifestations[c]	angiofibromas (85%) collagenoma (70%) lipoma (30%) leiomyoma[e] meningioma (8%) breast cancer[e]	CLA (10-20%) Hirschsprung's disease (5-10%)	mucosal neuroma (100%) IGN (40-90%) marfanoid habitus (70%) skeletal features[g] (50%) ocular features[h] (40%)	?[f]

Abbreviations: CLA, cutaneous lichen amyloidosis; dpNET, duodenopancreatic neuroendocrine tumor; IGN, intestinal ganglioneuromatosis; MTC, medullary thyroid carcinoma; NET, neuroendocrine tumor; pHPT, primary hyperparathyroidism

a: NM_020975.6(RET):c.2753T>C (p.Met918Thr) mutation in >95% of patients.
b: *De novo* germline mutation in 75-90% of patients.
c: Lifetime penetrance is presented between parenthesis.
d: Foregut NET other dan dpNET, including bronchopulmonary (5-30%), thymic (2-8%), and gastric neuroendocrine tumors (10-30%).
e: Penetrance not clear to date.
f: Other MEN4 manifestations have not yet been established with certainty.
g: Including scoliosis, pectus excavatum and pes cavus.
h: Including alacrima, corneal hypertrophy.

Rare (cancer) diseases are often recognized late.[13] Given the estimated prevalence of 2-10 per 100.000 (MEN1), 1-3 per 100.000 (MEN2A), 0.09-0.17 per 100.000 (MEN2B) and ±40 cases of MEN4 worldwide, all MEN syndromes are considered (very) rare.[9,14–17] Nonetheless, high penetrance of disease manifestations leads to high morbidity, decreased quality of life and reduced life expectancy in patients with all MEN subtypes.[18–22] Therefore, timely case-

finding of patients with one of these syndromal hereditary diseases is both challenging and of extreme importance. Clinical guidelines have been developed by international experts to help physicians when they encounter a patient suspected of – or diagnosed with – one of these extraordinary and complex diseases.[19,23] These guidelines include recommendations for the use of DNA analysis, surveillance regimens and treatment options, and have undoubtedly improved the care for MEN patients. However, due to the uncommonness of MEN syndromes, evidence on the value of screening methods, the accuracy of surveillance intervals and efficacy of treatment modalities in these patients is scarce; only a minority of the guidelines' recommendations are substantiated by high level of evidence.[11] Therefore, in the present thesis, we aim to find evidence-based answers to some important issues concerning case-finding of MEN syndromes and case-finding within MEN patients, as outlined below.

DutchMEN study group

A large part of the research in this thesis would not be possible without the work of the DutchMEN study group (DMSG). In 2008, this initiative was founded as a national collaboration of all eight University Medical Centers (UMCs) in the Netherlands, in order to optimize care for MEN patients by conducting high-quality scientific research. The population-based design (including >90% of Dutch adult MEN1 patients), extreme long-term follow-up and standardized data collection has created a perfect setting to find answers to predefined clinically relevant research questions – drawn up in close participation with the Dutch MEN patient advocacy group (Belangengroep MEN). Recently, the retrospective data collection has developed into a prospective database with a biobank for the collection of blood samples, DNA and surgically removed tissue, and the first steps have been made to include MEN2A and MEN2B patients.[24]

Thesis outline

PART ONE

Case-finding of MEN syndromes

Timely diagnosing a MEN syndrome has been one of the major challenges since the first description of these entities. The rarity of MEN diseases has made it difficult to devise case-finding methods that are capable of early, correct identification of a MEN syndrome with sufficient yield to outweigh the (material and immaterial) costs for individual patients and health care systems. The introduction of DNA analysis and the rapid developments within the field of genetic testing technology have given physicians a powerful tool to screen for causative germline mutations in patients suspected of – or at risk for – these hereditary

diseases. Early detection of a germline *MEN1*, *RET* or *CDKN1B* mutation is extremely valuable, as it enables timely diagnosis of occult MEN-associated disease burden in other (endocrine) organs, identification of family members at risk and presymptomatic disease in probands. Therefore, current guidelines recommend genetic screening in patients with a phenotype suggestive for a MEN syndrome: *MEN1* or *RET* mutational analysis is advised in patients with two or more main MEN1-associated endocrine tumors (*i.e.*, parathyroid, pancreatic or pituitary tumors), patients with MTC or pheochromocytoma or cutaneous lichen amyloidosis, patients with a "classic phenotype of MEN2B" and "may be recommended in individuals with an atypical MEN1 phenotype".[19,23] As soon as a germline mutation is found in the index patient, first-degree relatives should be screened as well, in order to identify (presymptomatic) mutation carriers.
The category of patients with "an atypical MEN1 phenotype" leaves room for interpretation; physicians confronted with a patient with an apparently sporadic endocrine tumor may wonder whether or not to screen for a genetic cause, weighting the possible benefits of early disease identification on the one hand against the psychological burden and health care costs of unnecessary investigations on the other. In **chapter 2**, we review the current body of evidence on the clinical value of genetic screening in apparently sporadic pituitary adenoma – which can be a suggestion of MEN1 and MEN4 syndrome – and aim to formulate a tool for the use of DNA analysis in these patients in daily practice.
In addition to its benefit in patients with endocrine tumors, DNA screening can also be extremely valuable for identifying mutation carriers in asymptomatic family members of known mutation carriers. Presymptomatic *RET* mutation analysis in children of MEN2A families and subsequent prophylactic thyroidectomy in children with high risk of MTC became common practice after the discovery of the *RET* gene as the origin of MEN2 syndromes in the early 1990s. The possible effect of these developments on the incidence and outcome of pediatric MTC in the Netherlands is evaluated in **chapter 3**.

Early case-finding of MEN2B is of utmost importance, since MTC can occur already before the age of one year, can spread to adjacent lymph nodes and distant organs rapidly and can subsequently lead to disease-related death. Therefore, a preventive thyroidectomy is recommended in affected children before the age of one.[19] However, due to the syndrome's extreme rarity and frequent *de novo* presentation, diagnosing MEN2B syndrome in time can be very challenging and predicting the presence of MEN2B before the occurrence of (wide-spread) MTC appears almost impossible. Fortunately, recognition of related non-endocrine features may offer an opportunity for timely diagnosis, as non-endocrine manifestations may precede MTC in affected individuals. By meticulously studying the MEN2B population in our Dutch MEN expertise center, we describe how early non-endocrine MEN2B features can lead to timely case-finding of MEN2B patients in **chapter 4**. Additionally, we illustrate the effect of early recognition of premonitory symptoms on

prognosis.
In **chapter 5**, we focus on one particular non-endocrine MEN2B-related manifestation: "marfanoid" body habitus. This is a non-specific term which refers to a constellation of signs that are similar to the characteristics of patients with Marfan syndrome (such as tall stature, long limbs and hyperlaxity). A "marfanoid" body habitus has been reported in approximately 75% of MEN2B patients. This may lead to the assumption that patients with MEN2B have tall stature. Because literature on growth patterns and final height in MEN2B patients is very scarce, we aim to gain more knowledge on this subject by describing body proportions and longitudinal growth in our MEN2B population. Next, we try to relate growth to possible influencing parameters, including age at MEN2B diagnosis and thyroidectomy, extensiveness of MTC, body mass index (BMI), gastrointestinal manifestations and endocrine status. By augmenting knowledge on the anthropometric features in patients with MEN2B, we hope to contribute to a more timely case-finding method of MEN2B in the future.

PART TWO

Case-finding within MEN syndromes: moving towards personalized medicine

After being diagnosed with a MEN syndrome, patients are recommended to undergo regular biochemical and radiological screening to detect malignancies and other related manifestations in time. Additionally, in MEN2A and MEN2B, prophylactic thyroid surgery in early childhood is advised to prevent metastasized medullary thyroid carcinoma. In MEN2, the strong genotype-phenotype correlation has enabled the development of partially risk-stratified surveillance and therapy regimens. For example, the timing of prophylactic thyroidectomy in MEN2 patients is mainly based on the specific *RET* mutation. However, much progress has still to be made to tailor many other aspects in the care for MEN2 patients to the individual patient. In MEN1, it has not been possible to create a more personalized surveillance program to date at all, partly due to the lack of a clear genotype-phenotype association in this syndrome.[25] As a result, identification of patients with MEN1 with a deviant course of disease remains challenging; treating physicians are faced with a dilemma similar to the main issue in case-finding (diagnosing) a MEN syndrome: how to identify MEN patients suffering from aggressive and/or malignant tumors in time without disproportionately exposing the rest of the MEN population to exhaustive surveillance programs? In the second part of this thesis, we aim to generate more knowledge on the occurrence and natural course of MEN1-related tumors, thereby hoping to add to the development of more personalized care for MEN1 patients in the future.
In **chapter 6**, we discuss the possible effect of genetic anticipation in the 10 largest Dutch MEN1 families. Genetic anticipation is an unusual type of genetic inheritance characterized by a reduced age of onset and/or increased disease severity in successive generations, and

the phenomenon has been described most extensively in neuropsychiatric disorders. In diseases like Huntington's disease and myotonic dystrophy, genetic anticipation is explained by trinucleotide repeat expansions ("growing genes"): the length of the repeat is transmitted in an unstable way and can be influenced by the parental origin. Over the last two decades, examples of genetic anticipation have also been described in a few heritable cancer syndromes with "traditional" Mendelian inheritance in which the genetic defect is transmitted without alterations, such as Lynch syndrome, von Hippel-Lindau syndrome and hereditary breast and ovarian cancer syndrome.[26–28] In MEN1, literature on genetic anticipation has been limited to one report, describing a decrease in age of onset of MEN1-associated manifestations and increased frequency of metastatic disease in the youngest generations of a large (five-generation) MEN1 family.[29] In chapter 6, we search for possible clues for genetic anticipation in a much larger MEN1 population; more insight into factors influencing the age-dependent penetrance of MEN1 manifestations could help fine-tuning the recommended age to start screening in individual MEN1 patients.
In **chapter 7** and **chapter 8**, we focus on MEN1-related neuroendocrine tumors (NETs) of the lung. Previous studies suggest that MEN1-related lung NETs have an indolent course with a good prognosis.[30–34] Prior growth analysis by our own DMSG found a lung NET doubling time of 4.5 years, underlining its benign behavior.[31] However, reports of aggressive and fatal cases in other cohorts and the extraordinary malignant tumor behavior in a patient from our own cohort prompted us to re-assess tumor growth and survival of patients with MEN1-related lung NET in more depth and at long-term follow-up (chapter 7). Additionally, we compare the outcome of patients with MEN1-related lung NET to patients with sporadic and other lung NET in chapter 8.

Finally, in **chapter 9**, the main findings of the presented studies are discussed in the context of current literature. Furthermore, we discuss clinical implications for patient care and we give suggestions for future studies on MEN research.

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PART ONE

Case-finding of MEN syndromes




CHAPTER 2

Clinical relevance of genetic analysis in patients with pituitary adenomas: a systematic review

Medard F.M. van den Broek[1], Bernadette P.M. van Nesselrooij[2], Annemarie A. Verrijn Stuart[3], Rachel S. van Leeuwaarde[1], Gerlof D. Valk[1]

[1] Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Medical Genetics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Paediatrics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands

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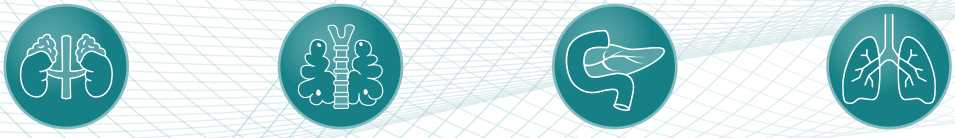

Abstract

Pituitary adenomas (PA) are amongst the most prevalent intracranial tumors, causing complications by hormonal overproduction or deficiency and tumor mass effects, with 95% of cases occurring sporadically. Associated germline mutations (*AIP*, *MEN1*, *CDKN1B*, *PRKAR1A*, *SDHx*) and *Xq26.3* microduplications are increasingly identified, but the clinical consequences in sporadic PA remain unclear. This systematic review evaluates predictors of a genetic cause of sporadic PA and the consequences for treatment outcome. We undertook a sensitive MEDLINE/Pubmed, EMBASE, and Web of Science search with critical appraisal of identified studies. Thirty-seven studies on predictors of mutations and ten studies on the influence on treatment outcome were included.

AIP and *MEN1* mutations were associated with young age of PA diagnosis. *AIP* mutations were also associated with gigantism and macroadenomas at time of diagnosis. *Xq26.3* microduplications were associated with PA below the age of five. *AIP* and *MEN1* mutation analysis is therefore recommended in young patients (≤ 30 years). *AIP* mutation analysis is specifically recommended for patients with PA-induced gigantism and macroadenoma. Screening for *Xq26*.3 microduplications is advisable in children below the age of five with increased growth velocity due to PA. There is no evidence supporting mutation analysis of other genes in sporadic PA. *MEN1*-mutation-related prolactinoma respond well to dopamine agonists while *AIP*-mutation-associated somatotroph and lactotroph adenoma are frequently resistant to medical treatment. In patients harboring an *Xq26.3* microduplication treatment is challenging, although outcome is not different from other patients with PA-induced gigantism.

Effective use of genetic analysis may lead to early disease identification, while knowledge of the impact of germline mutations on susceptibility to various treatment modalities helps to determine therapeutic strategies, possibly lowering disease morbidity.

Introduction

Pituitary adenomas (PAs) are amongst the most frequently encountered intracranial tumors with a reported prevalence for clinically relevant PAs of 68-98 per 100,000.[1–6] Pituitary adenomas are usually benign but can lead to clinical symptoms caused by hormonal overproduction or deficiency as well as by tumor mass. The majority of cases (95%) occur sporadically.[7,8] Familial clustering can be seen in the context of an inherited syndromic condition leading to an increased risk of PAs (most frequently Multiple Endocrine Neoplasia Type 1 (MEN1)) or without other (endocrine) manifestations in case of familial isolated pituitary adenoma (FIPA).

Clinical implications of identifying germline mutations in patients with PA, in terms of treatment and prognosis, have been reported by different authors.[9–12] However, to our knowledge a complete overview of literature with thorough assessment of methodological quality of studies has not been performed to date. Detection of a germline mutation enables identifying family members at risk or occult disease burden in probands. Despite the clinical need, formal guidelines defining criteria for genetic screening of patients with apparently sporadic PA are scarce. In recent years, the amount of publications concerning germline mutations in (sporadic) pituitary adenoma has increased enormously. Despite all efforts, the mechanisms underlying pituitary tumorigenesis and the role of germline mutations in PAs in a sporadic setting remain poorly understood. Still, germline mutations are often not timely identified due to *de novo* mutations, low penetrance of hereditary syndromic conditions, unclear family history or small family size.[13–15] The reported yield of genetic screening varies enormously, presumably due to a great variety of study populations, genetic screening methods and methodological quality of studies.

To provide a useful tool for daily practice in the frequently encountered dilemma whether or not to test for the presence of germline mutations in patients with apparently sporadic PA, we aim to determine the clinical value of genetic screening in apparently sporadic PA based on a rigorous systematic review and critical appraisal of the available literature.

Methods

To assess the value of genetic testing in sporadic PA without syndromic features, we formulated two clinical questions for this review that are relevant for a physician when confronted with these patients: (1) what are predictors for the presence of a genetic cause of apparently sporadically occurring pituitary adenoma? (2) What is the impact of germline mutations on course of disease and treatment outcome of PA?

Search strategy and study selection

We performed a MEDLINE/Pubmed, EMBASE, and Web of Science search in November 2018. We applied a broad search strategy using "pituitary adenoma" and "genetic analysis" with an extensive list of synonyms. The complete search string is provided in Supplemental Material 1. We included human research written in English, French, German or Dutch without restriction for year of publication. Publications using non-original data (reviews, letters to the editor, cohort duplicates) were only used for cross referencing, case-reports up to four cases were excluded.

Studies assessing predictors of a genetic cause of PA were included if (1) it was possible to retrieve data on sporadic cases separately and (2) (likely) pathogenic germline mutations of genes associated with PA were investigated. The genes of interest include the *MEN1*, *CDKN1B*, *CDKN2C*, *PRKAR1A*, *PRKACA, PRKACB, SDHx* and *AIP* genes and microduplications of *Xq26.3*. Due to insufficient evidence in literature for *GPR101* allelic variants in the tumorigenesis of PA,[15–21] studies on these variants were excluded from further review. Since the focus of this review is on patients with sporadically occurring PA, studies including patients with clear syndromic features suggestive for a certain genomic mutation were excluded.

Studies assessing the impact of a germline mutation on treatment outcome of PA were included if (1) results included information on treatment (type and number of treatments) and/or outcome (hormonal/disease control, tumor growth/reduction, complications) (2) information of the (sub)group of patients with a germline mutation was extractable and (3) at least five cases with a proven germline mutation were described.

After removal of duplications, two authors (MB and BN) independently screened all publications by title and abstract for possible relevance on the formulated questions. The full manuscript of all potentially eligible papers was then reviewed for in/exclusion by the same authors independently. In case of disagreement, consensus was reached by discussion, with the help of a third reviewer (RL). Reasons for exclusion at full-text screening were recorded (see Supplementary Material 2). All included articles, reviews and case-reports were cross-referenced for additional relevant articles.

Data extraction

Relevant data on study population (cohort origin, number of included patients, additional selection criteria, clinical subtype of adenoma, gender distribution and familial status) and investigated gene(s) (including method(s) of genetic analysis and investigation(s) of pathogenicity) were extracted. The prevalence of the investigated germline mutations was obtained. Age, gender, adenoma size and functionality were considered a potential predictor. Possible predictors of germline mutations were assessed if at least five cases with a germline

mutation were identified in the study population. All quantitative data describing determinants of treatment outcome of PA in patients with proven germline mutations were extracted. In order to determine the predictive value of determinants and the effect on treatment outcome, a combination of effect size, statistical significance, reproducibility (number of studies with comparable results) and methodological quality of studies were taken into consideration.

Critical appraisal

For the systematic evaluation of risk of bias and applicability of studies on predictors of a genetic cause of PA, we adapted the Quality Assessment of Diagnostic Accuracy Studies tool (QUADAS-2) for our review purposes.[22] For the evaluation of prognostic studies on the impact of germline mutation on treatment outcome, we customized the Quality In Prognosis Studies tool (QUIPS).[23] For more details, see Supplemental Material 3 and 4. All included studies were appraised by two authors independently (MB and BN), in case of disagreement, consensus was reached by discussion or with the help of a third reviewer (RL). The strength of recommendations was graded using the Grading of Recommendations, Assessment, Development, and Evaluation system.[24,25]

Results

Study selection

After removal of duplicates a total of 5,803 original records were identified. After systematic screening, a total of 37 studies on possible predictors of germline mutations and 10 studies on the impact of a germline mutation on treatment outcome were included. One record was included for answering both clinical questions.[26] Cross referencing did not result in additional relevant records. For further details, see Figure 1 (Flowchart).

Predictors on germline mutation status in sporadic pituitary adenoma

Studies could be categorized into three separate groups: (1) patients with a somatotroph adenoma, (2) young patients (≤ 30 years at diagnosis), and (3) other groups of patients with PA.

Sporadic somatotroph adenoma

Out of 13 studies investigating the presence of an *AIP* gene mutation, one publication identified ≥ 5 cases with a germline mutation.[27] In this study with a prevalence of an *AIP* mutation of 3.2%, predictors of the presence of a mutation were: younger age at diagnosis (mean age of *AIP* mutated patients 25 ± 10 years vs. 43 ± 14 years in wildtype, $P = 0.005$)

and gigantism (three out of five *AIP*-mutated patients suffered from gigantism compared to 17 out of 149 patients without *AIP* mutation, $P = 0.016$). This study showed a minor risk of bias and intermediate applicability (see tables 1A and 2A for more details).

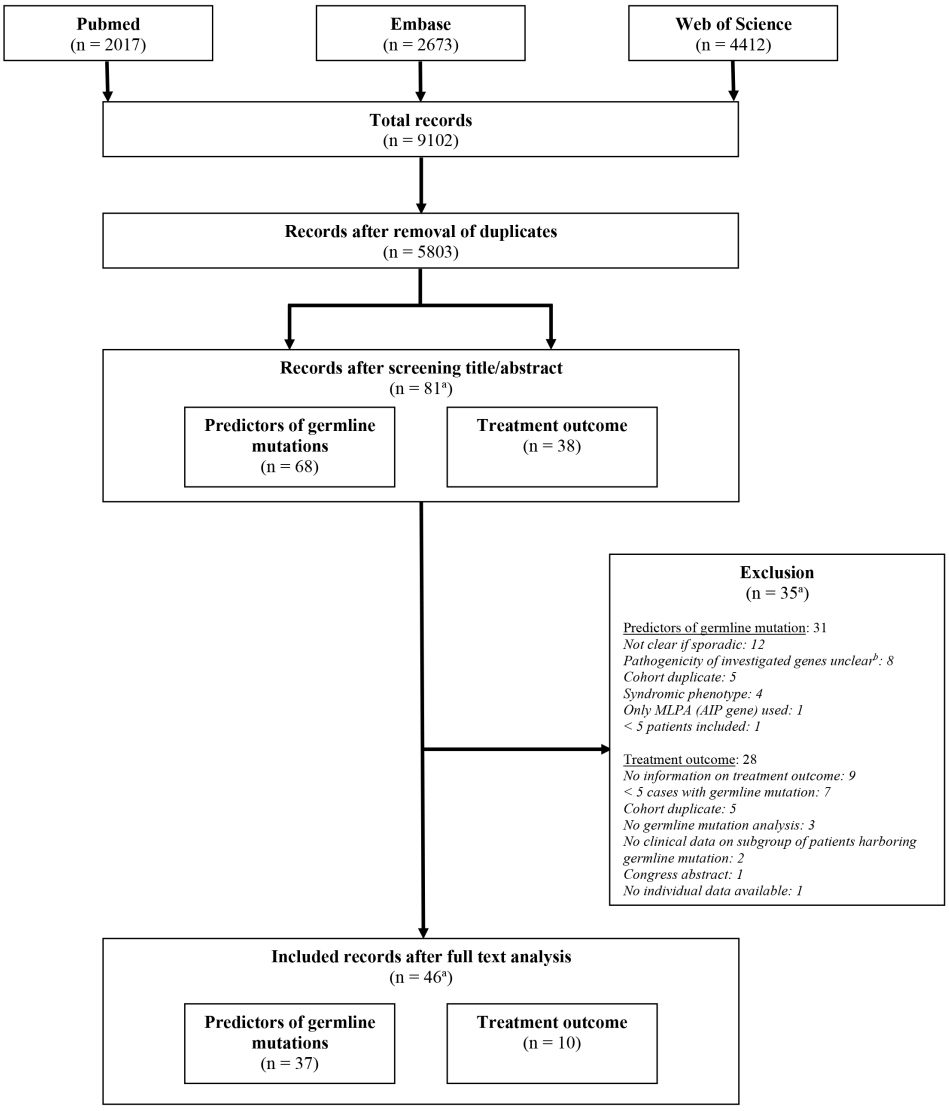

Figure 1. Flowchart
Abbreviations: MLPA, Multiplex Ligation-dependant Probe Amplification
a: Original records. Records can be included for both clinical questions (predictors of germline mutations, treatment outcome).
b: *GPR101* allelic variants, *GNAI1/2/3, CABLES1, KCNQ1/2,* genome wide association studies, SNP allele frequencies studies.

Table 1. Study characteristics of studies assessing predictors of a germline mutation

1A. Studies with sporadic somatotroph adenoma patients

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Yamasaki *et al.*[29]	Japan	GH or GH/PRL secreting PA	32	GH=30 GH/PRL=2	*PRKAR1A*	0	N/A
Vierimaa *et al.*[65]	Finland	Acromegalic patients	10[d]	GH=10	*AIP*	20.0% (2 pt)	N/A (2 cases)
Cazabat *et al.*[27]	France	GH-secreting PA Exclusion of patients with clinical features suggesting MEN1, CNC or MAS	154	GH=154	*AIP, MEN1, PRKAR1A*[e]	*AIP*: 3.2% (5 pt)[f]	Younger age Gigantism *Male gender*
Iwata *et al.*[66]	Japan	GH secreting PA	40	GH=40	*AIP*	2.5% (1 pt)	N/A (1 case)
Georgitsi *et al.*[30]	Finland	Acromegaly	50	GH=50	*CDKN1B*	0	N/A
Leontiou *et al.*[67]	International[g]	Acromegalic patients[g]	37	GH=37	*AIP*	0	N/A
Occhi *et al.*[31]	Italy	Acromegaly	131	GH=131	*AIP, CDKN1B*[h]	*AIP*: 3.1% (4 pt) *CDKN1B*: 0	N/A (4 cases)
Oriola *et al.*[68]	Spain	GH secreting PA Resistant to SSA	50	GH=50	*AIP*	4.0% (2 pt)	N/A (2 cases)
Zatelli *et al.*[69]	Italy	GH secreting	16	GH=16	*AIP*	0	N/A
Trivellin *et al.*[16]	International	*Xq26.3* duplication: gigantism	38	GH=38	*Xq26.3* duplication[i]	*Xq26.3*: 23.7% (9 pt)	N/A (insufficient data)[i]
Schöfl *et al.*[70] [j]	Germany	Acromegaly Age at diagnosis < 30 years	87	GH=87	*AIP*	2.3% (2 pt)	N/A (2 cases)
Karaca *et al.*[71]	Turkey	GH producing PA	92	GH=92	*AIP*	1.1% (1 pt)	N/A (1 case)
Ferrau *et al.*[19]	Italy	GH producing PA	210	GH=210	*AIP*	*AIP*: 1.9% (4 pt)	N/A (4 cases)
Mangupli *et al.*[28]	Venezuela	Pituitary gigantism	8	GH=8	*AIP, MEN1, Xq26.3* duplication[k]	*AIP*: 42.9% (3 pt) Xq26.3: 0 *MEN1*: ?[k]	N/A (3 cases)
Matsumoto *et al.*[72]	Japan	GH producing PA	61[l]	GH=61	*AIP*	4.9% (3 pt)	N/A (3 cases)

Table 1A. Continued (Studies with sporadic somatotroph adenoma patients)

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Ozkaya *et al.*[73]	Turkey	GH producing PA Exclusion of patients with preoperative SSA treatment or poor adherence Exclusion of MEN1, CNC, MAS	94[m]	GH=94	*AIP*	2.1% (2 pt)	N/A (2 cases)

Abbreviations: CNC, Carney complex; MAS, McCune-Albright Syndrome; MEN1, multiple endocrine neoplasia type 1; N/A, not applicable; No., number; PA, pituitary adenoma; pt, patients; SSA, somatostatin analogues.
GH, somatotroph adenoma; PRL, lactotroph adenoma; ACTH, corticotroph adenoma; TSH, thyrotroph adenoma; NFPA, non-functioning pituitary adenoma; GH/PRL, mixed somatotroph/lactotroph adenoma.

Cursive predictors: suggestive predictor but no statistical significance reached / insufficient data to calculate statistical significance.

a: Only (groups of) patients are included of which the sporadic status could be determined.
b: Mutations or variants that were considered pathogenic or likely pathogenic by the authors of each study.
c: Possible predictors are presented if a minimum of five cases of patients with a germline mutation are reported.
d: Possible founder effect (frequent occurrence of the Q14X mutation in the Finnish cohort).
e: *MEN1* and *PRKAR1A* gene analysis was performed in patients <30 years of age without *AIP* mutation (n = 28).
f: Another patient harbored a missense R304Q (c.911G>A) p.Arg304Gln mutation with conflicting interpretations of pathogenicity. Many later publications (*e.g.*, Occhi *et al.*[31], Tichomirowa *et al.*[26], Cuny *et al.*[13], Preda *et al.*[34], Tuncer *et al.*[37]) considered this a (likely) pathogenic variant.
g: Seven cases of childhood-onset gigantism (Australia, UK, Brazil, USA), 30 adult-onset acromegaly cases (cohort unknown).
h: *CDKN1B* gene analysis was performed in a subgroup of 38 patients with multiple tumors.
i: There is insufficient information provided to determine possible predictors.
j: This study is also presented in Table 1B.
k: *AIP* and *MEN1* gene analysis was performed in seven patients. The results of MEN1 gene analysis are not reported.
l: It cannot be excluded that a part of this study population is previously reported in Iwata *et al.* 2007.[66]
m: It cannot be excluded that a part of this study population is previously reported in Yarman *et al.*[41] and/or Tuncer *et al.*[37]

In only two studies on *Xq26.3* microduplication the data of apparently sporadically occurring PA could be extracted.[16,28] Both were at risk of bias and had a relatively low applicability for daily clinical practice. Trivellin *et al.* found an *Xq26.3* duplication in 9 out of 38 sporadic patients with pituitary gigantism (24%). The total group of germline-mutation-affected patients with gigantism (14 out of 43) had a female predominance (71% vs. 24%, $P = 0.007$), much earlier onset of increased growth velocity (median age 1.0 year (range 0.5-2.0) vs. 16.0 year (range 5.0-18.0), $P < 0.001$) and higher insulin-like growth factor (IGF-1) levels and more frequently elevated prolactin levels at diagnosis. Mangupli *et al.* found no cases of *Xq26.3* microduplication at all.

In the five studies investigating the presence of *MEN1*, *CDKN1B* and/or *PRKAR1A* mutations in sporadically occurring somatotroph adenoma, no predictors were identified.[27–31]

The outcomes of all included studies on sporadic somatotroph adenoma are presented in Table 1A. Methodological quality assessment of studies is presented in Table 2A. For further details on study results, see Supplemental Material 5.

Young (≤ 30 years) patients with sporadic pituitary adenoma

Three studies assessing the presence of an *AIP* mutation identified ≥ 5 cases with a germline mutation, reporting a mutation prevalence of 8.4, 8.6 and 11.7%, respectively.[13,26,32] Study characteristics of all studies are displayed in Table 1B.

In all studies, the presence of an *AIP* mutation was related with a younger age of onset or, inversely, prevalence of *AIP* mutations was higher in patients with a younger age of diagnosis (≤ 18 years). Furthermore, the two studies only including patients with macroadenoma (≥ 10 mm) reported the highest frequency of *AIP* mutations, illustrating that macroadenoma is a predictor of this specific mutation. Extrasellar extension was a frequent feature. Thirdly, *AIP* mutations were more likely identified in patients suffering from gigantism. Additionally, despite a nearly equal gender distribution in study populations, male gender was overrepresented in *AIP* mutated patients.

Data on adenoma subtype were conflicting: although Cuny *et al.* reported a higher prevalence of *AIP* mutation in non-functioning PA, results from Hernandez *et al.* showed all *AIP* mutation-related PA to be somatotroph adenomas. For further details on study results, see Supplemental Material 5.

The study of Cuny *et al.* showed only minor risk of bias and good applicability, making these results more reliable. Full quality assessment of studies can be found in Table 2B.

1B. Studies with young (≤ 30 years) sporadic pituitary adenoma patients

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Georgitsi *et al.*[74]	Italy	Age at disease onset or diagnosis < 18 years Exclusion of family history of MEN1	36[d]	GH=5 PRL=19 ACTH=3 NFPA=7[e] GH/PRL=2	*AIP*	2.8% (1 pt)	N/A (1 case)
Stratakis *et al.*[33]	USA (Bethesda)	Age at diagnosis ≤ 18 years AND (1) Cushing disease or (2) GH/PRL secreting PA	80	GH=3 PRL=3 ACTH=74	*AIP, MEN1, CDKN1B, DKN2C, PRKAR1A*	*AIP*: 3.8% (3 pt) *MEN1*: 1.3% (1 pt)	N/A (4 cases)
Tichomirowa *et al.*[26]	International[f]	Age at diagnosis < 30 years Macroadenoma (≥ 10 mm on MRI)	163	GH=83 PRL=61 ACTH=2 TSH=1 NFPA=16[g]	*AIP*	11.7% (19 pt)	Younger age *Extrasellar extension* *Male gender*
Cuny *et al.*[13]	France	Age at diagnosis < 30 years Macroadenoma (≥ 10 mm on MRI) Exclusion of patients with hypercalcemia	174[h]	GH=79 PRL=74 ACTH=8 TSH=1 NFPA=12[i]	*AIP, MEN1*	*AIP*: 8.6% (15 pt) *MEN1*: 3.4% (6 pt)	*Younger age (AIP & MEN1)* *Extrasellar extension (AIP)* *Gigantism (AIP)* *Male gender (AIP)* *NFPA (AIP)* *Prolactinoma (MEN1)*
Schöfl *et al.*[70] [j]	Germany	Acromegaly Age at diagnosis < 30 years	87	GH=87	*AIP*	2.3% (2 pt)	N/A (2 cases)

Table 1B. Continued

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Hernandez *et al.*[32]	International	Age at disease onset ≤ 30 years	404[k]	GH=290 PRL=67 ACTH=21 TSH=2 NFPA=21[l] Other=3[m]	*AIP, MEN1, CDKN1B*[n]	*AIP*: 8.4% (34 pt) *MEN1*: 0 *CDKN1B*: 0	Younger age Macroadenoma Extrasellar extension *Gigantism* *GH secreting PA*

Abbreviations: N/A, not applicable; MEN1, multiple endocrine neoplasia type 1; MRI, magnetic resonance imaging; No., number; PA, pituitary adenoma; pt, patients. GH, somatotroph adenoma; PRL, lactotroph adenoma; ACTH, corticotroph adenoma; TSH, thyrotroph adenoma; NFPA, non-functioning pituitary adenoma; GH/PRL, mixed somatotroph/lactotroph adenoma.

Cursive predictors: suggestive predictor but no statistical significance reached / insufficient data to calculate statistical significance.

a: Only (groups of) patients are included of which the sporadic status could be determined.
b: Mutations or variants that were considered pathogenic or likely pathogenic by the authors of each study.
c: Possible predictors are presented if a minimum of five cases of patients with a germline mutation are reported.
d: Three patients previously reported in Georgitsi *et al.*[43]
e: Adenoma subtype is based on "clinical diagnosis".
f: Belgium, Brazil, Bulgaria, Czech Republic, France, Germany, Italy, Lebanon and Spain.
g: Definition of NFPA is not provided. The tumor of the only NFPA patient with a germline *AIP* mutation was negative for all pituitary hormones on immunohistochemistry.
h: Fifty-nine patients previously reported in Tichomirowa *et al.*[26]
i: Definition of NFPA is not provided. The tumor of one NFPA patient with a germline *AIP* mutation had a partial (50%) immunoreactivity for GH without any pituitary hormonal hypersecretion *in vivo* (silent somatotroph adenoma). The tumors of the other three NFPA patients with a germline *AIP* or *MEN1* mutation were nonreactive on immunostaining experiments.
j: This study is also presented in Table 1A.
k: Six patients previously reported in Leontiou *et al.*[67]
l: Definition of NFPA is not provided. Immunohistochemistry results were available in 103 (out of 404) patients. All sporadic patients with a germline *AIP* mutation and available histopathology results (n = 14) had GH positive pituitary adenomas by immunohistochemistry. In the group of sporadic patients with available histopathology results but without germline *AIP* mutation (n = 89), three tumors were non-reactive (null cell PA).
m: One FSH-secreting PA, two not specified.
n: *MEN1* gene analysis is performed in 33 patients, *CDKN1B* gene analysis is performed in one patient.

1C. Studies with other groups of sporadic pituitary adenoma patients

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Zhuang *et al.*[46]	USA (Bethesda), Canada (Toronto)	Patients who had undergone full preoperative endocrine evaluation	38	GH=8 PRL=8 ACTH=14 TSH=1 Other=7[d]	*MEN1*	0	N/A
Schmidt *et al.*[47]	Germany	Exclusion of patients with a familial history of MEN1-associated tumors	61	GH=16 PRL=6 ACTH=1 TSH=1 NFPA=37[e]	*MEN1*	0	N/A
Farrell *et al.*[48]	UK	Patients previously shown to harbour allelic deletion on 11q13	23	GH=15 PRL=2 ACTH=1 NFPA=5[f]	*MEN1*	0	N/A
Yu *et al.*[38]	USA (Los Angeles)	-	63	GH=35 PRL=15 ACTH=5 NFPA=8[g]	*AIP*	0	N/A
DiGiovanni *et al.*[39]	Canada	-	66	GH=50 Other=16[h]	*AIP*	0	N/A
Barlier *et al.*[40]	France, Belgium, Italy	Exclusion of a history of MEN1 or CNC	107	GH=26 PRL=49 ACTH=2 TSH=1 NFPA=29[i]	*AIP*	0	N/A
Georgitsi *et al.*[43]	USA (Cleveland), Italy	USA (n = 113): patients undergoing PA resection Italy (n = 71): acromegaly	184	GH=84 PRL=11 ACTH=13 NFPA=76[j]	*AIP*	1.1% (2 pt)	N/A (2 cases)
Buchbinder *et al.*[44]	Germany	Exclusion of MEN1 en CNC	110	GH=10 PRL=38 ACTH=5 NFPA=55[k] Other=2[l]	*AIP*	2.7% (3 pt)[m]	N/A (3 cases)

Table 1C. Continued

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Cai *et al.*[45]	China	-	216	GH=80 PRL=39 ACTH=39 NFPA=58[n]	*AIP*	2.8% (6 pt)	Younger age GH secreting PA Male gender
Preda *et al.*[34]	UK	Adult patients with age at disease onset ≤ 40 years	127	GH=48 PRL=43 ACTH=15 TSH=1 NFPA=20[o]	*AIP*	1.6% (2 pt)	N/A (2 cases)
Yarman *et al.*[41]	Turkey	Functional PA	91	GH=47 PRL=21 ACTH=23	*AIP*	0	N/A
Lecoq *et al.*[15]	France	-	766[p]	GH=218 PRL=256 ACTH=68 TSH=14 NFPA=165[q] GH/PRL=45	*AIP*	*AIP*: 2.9% (22 pt)	N/A (insufficient data)[r]
De Sousa *et al.*[35]	Australia	Age of onset ≤ 40 years[s]	30	?	*AIP, MEN1, CDKN1B, PRKAR1A* and *SDHx*[t].	*AIP*: 13.3% (4 pt) Other genes: 0	N/A (4 cases)
Araujo *et al.*[36]	Brazil	Macroadenoma diagnosed ≤ 40 years or adenoma of any size diagnosed < 18 years of age	132	GH=74 PRL=38 ACTH=10 NFPA=10[u]	*AIP*	2.3% (3 pt)	N/A (3 cases)
Foltran *et al.*[42]	Brazil	GH producing PA or NFPA	62	GH=41 NFPA=21[v]	*AIP*	0	N/A

Table 1C. Continued (Studies with other groups of sporadic pituitary adenoma patients)

References	Population		No. of sporadic patients[a]	Subtype adenoma	Investigated genes	Prevalence of mutations[b]	Possible predictors[c]
	Cohort	*Additional selection criteria*					
Tuncer *et al.*[37]	Turkey	Functional PA No clinical suggestions of MEN1 or CNC Age at diagnosis ≤ 40 years for PRL and ACTH producing PA, age at symptom onset ≤ 40 years for GH producing PA	97[w]	GH=55 PRL=25 ACTH=17	*AIP*	2.1% (2 pt)	N/A (2 cases)

Abbreviations: CNC, Carney complex; MEN1, multiple endocrine neoplasia type 1; N/A, not applicable; No., number; PA, pituitary adenoma; pt, patients. GH, somatotroph adenoma; PRL, lactotroph adenoma; ACTH, corticotroph adenoma; TSH, thyrotroph adenoma; NFPA, non-functioning pituitary adenoma; GH/PRL, mixed somatotroph/lactotroph adenoma.

Cursive predictors: suggestive predictor but no statistical significance reached / insufficient data to calculate statistical significance.

a: Only (groups of) patients are included of which the sporadic and non-syndromic status could be determined.
b: Mutations or variants that were considered pathogenic or likely pathogenic by the authors of each study.
c: Possible predictors are presented if a minimum of five cases of patients with a germline mutation are reported.
d: Two oncocytomas, two mixed (GH/PRL) PAs, one gonadotroph PA, one glycoprotein PA, one PRL+ ACTH PA (two separate PA with independent biochemical function).
e: Subtype definition based on pre-operative hormonal status.
f: Definition of NFPA is not provided.
g: "Clinically non-functioning adenomas", without further specification.
h: No further subtype specification or subtype definitions.
i: Definition of NFPA is not provided.
j: Definition of NFPA is not provided. All patients from the USA cohort (n = 113) underwent biochemical and immunohistochemistry confirmed diagnosis.
k: An adenoma was declared as non-functioning when it was associated with levels of TSH, ACTH, PRL and GH in the normal range.
l: Two gonadotropinomas.
m: Two of these patients harbored a R16H (c.47G>A) mutation, which other authors (Georgitsi *et al.*[43], Cazabat *et al.*[27], Ferrau *et al.*[19]) considered not (likely) pathogenic.
n: Definition of NFPA is not provided.
o: Definition of NFPA is not provided.
p: This cohort includes all 443 patients reported in Cazabat *et al.*[75] (which is therefore excluded from this review).
q: Including both NFPA and gonadotropinomas. Adenoma subtype was based on clinical, biological and/or histological criteria.
r: No information on subgroup of only (likely) pathogenic mutations are presented.
s: Other subgroups of patients (family and/or personal history of endocrine neoplasia) are excluded.
t: *SDHA, SDHB, SDHC, SDHD*.
u: Definition of NFPA is not provided. Immunohistochemical staining was performed in cases who underwent surgery.
v: Definition of NFPA is not provided. Tumor samples for immunohistochemical staining were available in 45 out of 62 cases (NFPA: 18 out of 21 cases).
w: Fifty-six patients previously reported in Yarman *et al.*[41]

Regarding *MEN1* mutations, the study of Cuny *et al*. was at the lowest risk of bias and highest applicability.[13] In this series of patients younger than 30 years (prevalence of *MEN1* mutation: 3.4%), patients with a *MEN1* mutation tended to be younger: 3 out of 46 (6.5%) patients ≤ 18 years harbored a germline *MEN1* mutation vs. 3 out of 128 (2.3%) patients from 19 to 30 years at diagnosis. *MEN1* mutations did also occur more frequently in prolactinomas (5.4%) than in other PA subtypes (2.0%).

In the studies on the presence of the *CDKN1B*, *CDKN2C* and *PRKAR1A* gene mutations no germline mutations were identified.[32,33]

Other groups of patients with sporadic pituitary adenoma
Sixteen studies applied a different set of in- and exclusion criteria than somatotroph adenoma or age at diagnosis ≤ 30 years, although four publications did use age criteria.[34–37] The reported prevalence of germline mutations within these studies is relatively low, with the exception of one study reporting a prevalence of 13.3 %.[35]

The presence of *AIP* mutations was assessed in 13 studies. No *AIP* mutation was found in five of these studies,[38–42] and six studies described one to four cases with *AIP* mutation.[34–37,43,44] Lecoq *et al.* detected 22 cases, but unfortunately, there were insufficient data reported for the identification of possible predictors of *AIP* status.[15] In a publication of high methodological quality, Cai *et al*. detected six persons with *AIP* mutations (2.8%) in a group of 216 Han Chinese sporadic PA patients.[45] The prevalence of an *AIP* mutation was higher in patients with a younger age at diagnosis (patients ≥ 18 years 6.3% vs. 2.5% in patients ≥ 18 years at diagnosis) and in the subgroup of somatotroph adenoma (6.3% vs. 0.7% in non-GH producing PA). In this study, male gender also appeared to be related with a higher prevalence of *AIP* mutations (5.3% vs. 0.8%).

Four studies on predictors for *MEN1* gene mutations[35,46–48] and one study on *CDKN1B*, *PRKAR1A* and *SDHx*[35] did not reveal any mutation in the patients under study. See Table 1C for further study detail and Table 2C for all results on quality assessment.

Impact of a germline mutation on treatment outcome in pituitary adenoma
Ten studies reported on treatment outcome in patients with a germline mutation. In seven publications, treatment outcome was compared with a cohort of patients without germline mutation. Study characteristics are presented in Table 3.

Table 2. Quality assessment of studies assessing predictors of a germline mutation

2A. Studies with sporadic somatotroph adenoma patients

References	Gene(s) studied	Risk of bias			Applicability	
		Patient selection	Reference standard	Flow and timing	Patient selection	Reference standard
Yamasaki *et al.*[29]	*PRKAR1A*	-	+/-	+/-	+/-	-
Vierimaa *et al.*[65]	*AIP*	-	-	+/-	-	-
Cazabat *et al.*[27]	*AIP, MEN1, PRKAR1A*	+	+	+ /-	+/-	+/-
Iwata *et al.*[66]	*AIP*	-	-	+/-	+/-	-
Georgitsi *et al.*[30]	*CDKN1B*	-	+	+ +	-	+/-
Leontiou *et al.*[67]	*AIP*	-	+	++	+/-	+/-
Occhi *et al.*[31]	*AIP, CDKN1B*	-	+ +	+/-	+/-	+
Oriola *et al.*[68]	*AIP*	-	+ +	+ +	+/-	+
Zatelli *et al.*[69]	*AIP*	-	+	+ +	-	+/-
Trivellin *et al.*[16]	*Xq26.3* duplication	-	+ +	-	-	+
Schöfl *et al.*[70 a]	*AIP*	+ +	+ +	+ +	+/-	+
Karaca *et al.*[71]	*AIP*	-	+	+ +	+/-	+/-
Ferrau *et al.*[19]	AIP	+	- -	+ +	+/-	-
Mangupli *et al.*[28]	*AIP, MEN1, Xq26.3* duplication	+	+	- -	+/-	-
Matsumoto *et al.*[72]	*AIP*	+	+	-	+/-	+
Ozkaya *et al.*[73]	*AIP*	-	+	+ +	+/-	+/-

a: This study is also presented in Table 2B.

All seven studies on *AIP* mutations showed a potential risk of (patient) selection bias. The study of Daly *et al*. was at lowest risk of bias.[9] (see Table 4 for full reporting of quality assessment) In this study, 75 patients with an *AIP*-mutation-associated somatotroph adenoma were compared with 232 somatotropinomas without an *AIP* mutation. The proportion of patients receiving multimodal treatment was comparable (61.3% vs. 66.4%, respectively) and there was no significant difference in disease control (70.4% vs. 80.5%, respectively, $P = 0.06$). There were however some clear discrepancies in treatment characteristics and outcome: among patients with a higher cumulative treatment burden (≥ 3 distinct modalities), long-term disease control rates were significantly worse in *AIP*-mutation-associated adenoma (55.6% vs. 82.9%, $P = 0.01$). Furthermore, somatostatin analogue (SSA)-induced GH and IGF-1 reduction and tumor size reduction was significantly less in *AIP*-mutation-associated PA. In line with these data, patients harboring an *AIP*

2B. Studies with young (≤ 30 years) sporadic pituitary adenoma patients

References	Gene(s) studied	Risk of bias			Applicability	
		Patient selection	Reference standard	Flow and timing	Patient selection	Reference standard
Georgitsi *et al.*[74]	*AIP*	+	+	+ +	+/-	+/-
Stratakis *et al.*[33]	*AIP, MEN1, CDKN1B*[a], *PRKAR1A*	-	+	+ +	-	+/-
Tichomirowa *et al.*[26]	*AIP*	-	+ +	+ +	+/-	+
Cuny *et al.*[13]	*AIP, MEN1*	+	+ +	+ +	+/-	+
Schöfl *et al.*[70 b]	*AIP*	+ +	+ +	+ +	+/-	+
Hernandez *et al.*[32]	*AIP, MEN1, CDKN1B*	-	+ +	+/-	-	+

a: *CDKN2C* was also investigated.
b: This study is also presented in Table 2A.

mutation more often underwent a reoperation (21.9% vs. 5.5%). Although the prevalence of hypopituitarism in follow-up did not differ (*AIP*-mutation-associated PA 22.5% vs. controls 25.2%), patients with an *AIP* mutation had a significantly higher number of pituitary deficiencies. Other studies on *AIP*-mutation-associated somatotropinomas showed similar results.[26,49] One study focused on *AIP* mutations in patients with apparently sporadically occurring PA and not familial cases.[26] In this study, 4 out of 11 (36%) patients with *AIP* mutations underwent multiple surgical interventions, while postoperative SSA therapy achieved disease control in only one out of nine patients.

Two studies focused on patients with PA-induced gigantism. Since these patients represent a distinct group with particularly high disease severity, these results are separately displayed. In contrast, Rostomyan *et al.* reported better treatment outcomes in *AIP*-mutation-associated gigantism than in patients suffering from gigantism without genetic abnormalities.[14] Within an international cohort of 208 patients with pituitary gigantism, hormonal control was more frequently reached in *AIP*-mutation-associated PA. Multimodal treatment was seldom necessary in *AIP*-mutation-associated somatotropinoma gigantism (23.8% vs. 42.7% in controls, $P = 0.04$). Long-term control (>12 months) was reached more often in the *AIP*-mutated patients (55.3% vs. 38.4%), but this was not statistically significant ($P = 0.08$). The frequency of hypopituitarism at follow-up was similar between both groups (73% vs. 66%). In another study including 153 patients with PA-induced gigantism, no significant difference in number of treatments or in prevalence of hypopituitarism was found between 63 patients with *AIP*-mutation-associated gigantism and patients with gigantism but without genetic abnormalities.[17]

2C. Studies with other groups of sporadic pituitary adenoma patients

References	Gene(s) studied	Risk of bias			Applicability	
		Patient selection	Reference standard	Flow and timing	Patient selection	Reference standard
Zhuang *et al.*[46]	*MEN1*	-	- -	+/-	+	-
Schmidt *et al.*[47]	*MEN1*	+	- -	+ +	-	-
Farrell *et al.*[48]	*MEN1*	- -	-	++	-	-
Yu *et al.*[38]	*AIP*	-	- -	+ +	+	-
DiGiovanni *et al.*[39]	*AIP*	-	- -	+/-	-	-
Barlier *et al.*[40]	*AIP*	- -	+ +	+ +	+/-	+
Georgitsi *et al.*[43]	*AIP*	-	+	+/-	-	+/-
Buchbinder *et al.*[44]	*AIP*	-	+	+ +	+	+/-
Cai *et al.*[45]	*AIP*	+	+ +	+ +	+	+
Preda *et al.*[34]	*AIP*	+	+ +	+ +	+/-	+
Yarman *et al.*[41]	*AIP*	-	-	+ +	+/-	+/-
Lecoq *et al.*[15]	*AIP*	+	+ +	+/-	+	+
De Sousa *et al.*[35]	*AIP, MEN1, CDKN1B, PRKAR1A, SDHx*	-	+ +	+/-	-	+
Araujo *et al.*[36]	*AIP*	+	+ +	+/-	+/-	+
Foltran *et al.*[42]	*AIP*	-	+	+ +	+/-	+/-
Tuncer *et al.*[37]	*AIP*	-	+ +	+ +	-	+

In search for factors associated with response to dopamine agonists in macroprolactinoma, Salenave *et al.* found *AIP* mutations not to be a significant determinant. However, in this study only a small sample of *AIP*-mutated PA ($n = 4$) was included.[50] Failure of dopamine agonists in *AIP*-mutation-related PA has been described frequently (50% of cases) in other studies as well and multiple surgical interventions were needed regularly.[9,26] In the cohort of *AIP* mutations in apparently sporadically occurring PA, five out of seven patients (71%) underwent surgery and four out of seven patients (66.7%) had to undergo multiple surgeries,[26] which was comparable with results from another study cohort of mainly familial *AIP* cases.[9]

Table 3. Study characteristics of studies assessing the impact of a germline mutation on treatment outcome

References	Population			No. of patients with germline mutation[a]	No. of patients without germline mutation	Subtype adenoma (germline/ wildtype)	Investigated treatment outcome
	Cohort	*Gene(s)*	*Additional selection criteria*				
Verges *et al.*[52]	Belgium, France	*MEN1*	MEN1 based on clinical or genetic criteria Non-MEN1 PA were matched for age, year of diagnosis, and FU period	136	110	GH=12/15 PRL=85/68 ACTH=6/7 NFPA=20/18[b] Mixed=13/2	Normalization of hypersecretion
Daly *et al.*[9]	International[c]	*AIP*	GH producing PA	75[d]	232	GH=75/232	Treatment characteristics, controlled & active disease, hypopituitarism
Tichomirowa *et al.*[26]	International[e]	*AIP*	Sporadic Age at diagnosis < 30 years Macroadenoma (≥ 10 mm on MRI)	19	144[f]	GH=11/72 PRL=7/54 ACTH=2 TSH=1 NFPA=1/15[g]	Treatment characteristics, disease control, tumor shrinkage
Beckers *et al.*[54]	International	*Xq26.3* duplication	*Xq26.3* duplication: gigantism	18[h]	-[f]	GH=18	Treatments characteristics, hormonal control, tumor shrinkage, hypopituitarism
De Laat *et al.*[53]	The Netherlands	*MEN1*	MEN1: based on clinical or genetic criteria ≥ 16 years of age	123	-[f]	GH=8 PRL=52 ACTH=4 NFPA=52[i] Mixed=5 Other=2[j]	Tumor growth, control of excess hormonal secretion
Salenave *et al.*[50]	France	*AIP* *MEN1*	Macroprolactinoma < 20 years of age	*AIP*: 5 *MEN1*: 3	AIP: 50[k] MEN1:59[k]	PRL=59	DA resistance

Table 3. Continued (Study characteristics of studies assessing the impact of a germline mutation on treatment outcome)

References	Population			No. of patients with germline mutation[a]	No. of patients without germline mutation	Subtype adenoma (germline/ wildtype)	Investigated treatment outcome
	Cohort	*Gene(s)*	*Additional selection criteria*				
Rostomyan *et al.*[14]	International[l]	*AIP* *Xq26.3* duplication	Gigantism	*AIP*: 42 *Xq26.3*: 14	77[m]	GH=42/14/77	Multimodal treatment, GH/IGF-1 control at FU, age when control achieved, long-term control, hypopituitarism
Iacovazzo *et al.*[17]	International[n]	*AIP* *Xq26.3* duplication	Gigantism and acromegaly patients Exclusion of MEN1, CNC, MAS	*AIP*: 63 *Xq26.3*: 12	78	GH=63/12/78	Number of treatments, hypopituitarism
Nagata *et al.*[49]	Japan	*AIP* *PRKAR1A*	GH producing PA Age of diagnosis ≤ 20 years	*AIP*: 5 *PRKAR1A*: 2	18[o]	GH=5/2/18	Hormonal control
Caimari *et al.*[51]	International[p]	*AIP*	FIPA or age at disease onset ≤ 30 years or referred patients Exclusion of MEN1, MEN4, CNC, X-LAG, DICER1 syndrome	134	1271	GH=119/648[q] PRL=11/333 ACTH=0/74 NFPA=4/181[r] Other=0/11[s]	Number of treatments

Abbreviations: CNC, Carney complex; DA, dopamine agonist; FIPA, familial isolated pituitary adenoma; FU, follow-up; GH, growth hormone; IGF-1, insulin-like growth factor 1; MAS, McCune-Albright Syndrome; MEN1, multiple endocrine neoplasia type 1; MEN4, multiple endocrine neoplasia type 4; MRI, magnetic resonance imaging; No., number; PA, pituitary adenoma; X-LAG, X-linked Acrogigantism.
GH, somatotroph adenoma; PRL, lactotroph adenoma; ACTH, corticotroph adenoma; TSH, TSH secreting adenoma; NFPA, non-functioning pituitary adenoma.

a: Mutations or variants that were considered pathogenic or likely pathogenic by the authors of each study.
b: Diagnosis of adenoma subtype was made based on (increased) plasma levels of pituary hormones. Immunohistochemistry data were available in 42 cases. In 2 out of 15 cases of NFPA histologically examined, immunostaining was positive for LH and FSH.
c: Belgium, Finland, France, Italy, Spain, Germany, Bulgaria, The Netherlands, Brazil, Argentina, the United States of America, Australia, New Zealand, and Lebanon.
d: The study included 96 patients with *AIP* mutation (of which 41 reported for the first time). The clinical behavior of somatotropinoma adenoma ($n = 75$) is compared with controls.
e: Belgium, Brazil, Bulgaria, Czech Republic, France, Germany, Italy, Lebanon and Spain.
f: No comparison is made with wildtype PA.
g: Definition of NFPA is not provided. The immunohistochemical staining of the tumor of the only NFPA patient with a germline *AIP* mutation was negative.

h: Thirteen patients previously reported in Trivellin *et al.*[16]
i: Adenoma subtype classification was based on laboratory test results, no immunohistochemistry data available.
j: Two gonadotroph adenomas.
k: The study included 77 patients with macroprolactinoma. Germline mutation analysis was conducted in 50 patients (*AIP*) and 59 patients (*MEN1*).
l: Argentina, Australia, Belgium, Brazil, Bulgaria, Canada, Denmark, India, Italy, Finland, France, Germany, New Zealand, Romania, Russia, Spain, the Netherlands and the United States of America.
m: The study included 208 patients with pituitary gigantism. In 143 patients genetic analysis was performed. Seven cases of MAS, two cases of CNC and one case of MEN1 were excluded from this comparison.
n: Not further specified. it cannot be excluded that a part of this study population is previously reported. One X-LAG patient was previously described in Trivellin *et al.*[16], one X-LAG patient was previously described in Beckers *et al.*[54]
o: The study included 25 patients. Only 13 patients were tested for *AIP* mutations. Negative germline analysis and no germline analysis is reported as "no mutation" in this study.
p: Not further specified. It cannot be excluded that a part of this study population is previously reported.
q: Including PA with prolactin cosecretion.
r: Definition of NFPA is not provided.
s: Any other type of functioning pituitary tumor.

Table 4. Quality assessment of studies assessing the impact of a germline mutation on treatment outcome

References	Gene(s) studied	Risk of bias			
		Patient selection	Determination of germline status	Outcome measurement	Analysis and reporting
Verges *et al.*[52]	*MEN1*	+ +	- -	-	+ +
Daly *et al.*[9]	*AIP*	+/-	+ +	+ +	+ +
Tichomirowa *et al.*[26]	*AIP*	-	+ +	- -	+/-
Beckers *et al.*[54]	*Xq26.3* duplication	+/-	+ +	-	+/-
De Laat *et al.*[53]	*MEN1*	+ +	+/-	+ +	+/-
Salenave *et al.*[50]	*AIP, MEN1*	+/-	- -	+/-	+/-
Rostomyan *et al.*[14]	*AIP, Xq26.3* duplication	+	- -	-	+ +
Iacovazzo *et al.*[17]	*AIP, Xq26.3* duplication	-	- -	- -	+ +
Nagata *et al.*[49]	*AIP, PRKAR1A*	+/-	- -	+ +	+/-
Caimari *et al.*[51]	*AIP*	+/-	+ +	-	+ +

No comparative data have been published on treatment outcome in *AIP*-mutation-associated vs. wildtype non-functioning PA (NFPA). However, Daly *et al.* did report seven cases with *AIP*-mutation-related NFPA: six patients underwent surgery (of which one also underwent radiotherapy), long-term control of tumor size was achieved in all cases.[9]
One of the largest studies on *AIP*-mutation-associated PA (134 cases) showed a trend towards a higher number of treatments in both functioning and non-functioning *AIP*-mutation-related PA (median 2 (IQR 1-3)) compared to patients without mutation (n = 1,271, median 1 (IQR 1-2)) (P = 0.055).[51] All data are shown in Supplementary Material 5.

Treatment-related outcome of PAs in MEN1 patients was described in three studies.[50,52,53] A population-based multicenter study including 123 MEN1 patients with PA by de Laat *et al.* was at lowest risk of bias. This study showed that prolactinomas in MEN1 patients respond well to medical treatment. Furthermore, this study showed that tumor growth was very limited over time and almost always without clinical consequences. In contrast, Verges *et al.* found a significant difference in normalization of pituitary hypersecretion between MEN1 and non-MEN1 functional PA (42% vs. 90%, respectively, $P < 0.001$). Normalization of plasma prolactin was significantly less frequent in MEN1 (44%) vs. non-MEN1 patients

(90%) ($P < 0.001$). Salenave *et al*. reported the presence of a *MEN1* mutation as a significant and independent predictor of dopamine agonist resistance in a regression analysis of 77 patients with prolactinoma ($t = 3.052$, $P = 0.004$). However, in this study a low number of MEN1 patients ($n = 3$) was included.

Treatment outcome in patients with *Xq26.3* microduplications (also known as X-Linked Acrogigantism, or X-LAG) is described in three studies.[14,17,54] Since *Xq26.3* microduplications lead to an excessive growth velocity in the first years of life, X-LAG patients have a younger age at diagnosis and younger age at therapy-induced hormonal control than non-mutated counterparts.[14] Due to this distinctive phenotype, it is hard to compare these results with other (sporadic) patients with PA. The proportion of patients in which disease control was reached varied due to the use of different definitions (41.7-91.7%). Multimodal treatment was necessary in the majority of cases, and hypopituitarism occurred frequently (71-75%). Hormonal control could almost never be achieved by medical therapy (dopamine agonists or SSA) alone.[54] When comparing treatment outcome with pituitary-induced gigantism without genetic abnormalities, Rostomyan *et al*. and Iacovazzo *et al*. found no differences in number of treatment modalities or prevalence of hypopituitarism between groups. The percentage of patients with long-term disease control (>12 months) did not differ significantly (X-LAG: 41.7%, controls: 38.4%), but appropriate control of GH/IGF-1 levels at last follow-up was reached more frequently in X-LAG patients (58.0% vs. 43.0%, $P = 0.02$).[14] For more study results, see Supplementary Material 5.

No eligible studies were found on the implications of germline mutations in *PRKAR1A*, *CDKN1B* and *SDHx*.

Discussion

The prevalence of germline mutations in unselected sporadically occurring PA is low. Therefore, germline analysis is not advisable for all patients. Based on the best-available evidence, the best predictor of an *AIP* or *MEN1* mutation appears to be a younger age at diagnosis (≤ 30 years). Moreover, the prevalence of an *AIP* mutation is significantly higher in pediatric patients in comparison to young adults.[13,26,32]

Focusing on *AIP* mutations, the presence of gigantism and macroadenoma seem to be additional predictors of these mutations. The overgrowth may be attributed to the effect of GH/IGF-1 excess before full bone maturation. A male predominance in *AIP* affected individuals was found in a number studies.[13,26,45] However, since it is conceivable that men are more prone to gigantism due to later growth cessation and male predominance was not

observed in large families with an *AIP* mutation, this phenomenon might be explained by ascertainment bias.[32] Both younger age at diagnosis and macroadenoma can be an expression of a more aggressive course of *AIP*-mutation-related PAs. Data on other factors such as adenoma subtype or the extent of tumor expansion are conflicting or too limited to draw clear conclusions.

MEN1 mutation analysis is recommended in young patients (≤ 30 years). In one study it is even suggested that *MEN1* mutations are more frequently found in prolactinomas.[13] However, this is not yet confirmed in other studies.

Given the relatively high disease burden and younger age, patients suffering from pituitary-related gigantism constitute a separate category. Germline *Xq26.3* microduplications were strongly associated with an early increased growth velocity and female gender. Since all reported patients harboring *Xq26.3* microduplication experienced a start of rapid growth already below five years of age, it is reasonable to perform genetic analysis for *Xq26.3* microduplications especially in this subset of patients with sporadic pituitary gigantism.[14,16,17,54]

No cases of germline mutations in the *PRKAR1A* gene, *SDHx* genes and *CDKN1B* or *CDKN2C* gene were reported in the included articles, which can be explained by our focus on apparantly sporadically occurring PA instead of PA occurring with other syndromic manifestations. In addition, PA only very rarely occur as manifestation of these, also rare, genetic syndromes. Therefore, genetic analysis of *PRKAR1A*, *SDHx* and *CDKN1B* should only be conducted in selected cases with suggestive (syndromic) features.

AIP mutated somatotroph adenomas are more frequently resistant to SSA treatment than their non-mutated counterparts and reoperation is needed more often. Low *AIP* protein expression in tissue is correlated with worse response to SSA treatment,[55] but since *AIP* downregulation may occur regardless of *AIP* mutations, it is still uncertain which mechanisms are involved.[56] Failure of response to dopamine treatment is also described frequently in *AIP*-mutation-associated prolactinoma.[9,26] Treatment outcome seems similar when comparing study results of cohorts of sporadic and mainly familial occurring *AIP* mutation related PA patients, but data are too limited to draw clear conclusions.[9,26] Multimodal treatment is needed regularly but comparable with the treatment modalities in non-mutated controls, and difference in disease control did not reach statistical significance.[9] There are too little reliable comparative data to determine the influence of an *AIP* mutation on treatment outcome in NFPA.

Best available evidence shows that *MEN1*-mutation-associated prolactinomas respond well to medical treatment and NFPA show no to very little tumor growth in virtually all cases.[53] These findings are in contrast with earlier findings,[52] partially due to the population-based cohort studied by de Laat *et al.* and the inclusion of PA diagnosed by screening (n = 66).

The presence of *Xq26.3* microduplication is not related to a different treatment outcome compared to other cases of pituitary gigantism. Nonetheless, multiple treatment modalities are needed in most patients and complications such as hypopituitarism are frequent.[14,17,54] Due to scarcity of reported quantitative information on treatment outcome of PA associated with mutations in *PRKAR1A*, *CDKN1B* and *SDHx*, the impact of these germline mutations on therapy and outcome could not be predicted. The summary of recommendations and findings is presented in Table 5.

Table 5. Summary of recommendations and findings

Recommendations for genetic testing	**Quality of evidence[a]**	**Strength of recommendation[b]**
Genetic analysis should not be done routinely in patients with sporadic pituitary adenoma	Low	Strong
AIP mutation analysis is recommended in young (≤ 30 years at diagnosis) sporadic pituitary adenoma, especially in the presence of gigantism and macroadenoma	Low	Weak
MEN1 mutation analysis is recommended in young (≤ 30 years at diagnosis) sporadic pituitary adenoma patients (mainly prolactinoma)	Low	Weak
Genetic analysis for *Xq26.3* microduplications must be considered in sporadic pituitary gigantism with early start of rapid growth (< 5 years), especially in female	Very low	Weak
Mutation analysis of *CDKN1B*, *PRKAR1A* and *SDHx* genes is not recommended in sporadic non-syndromic pituitary adenoma	Low	Strong
Summary of findings on treatment outcome		
• *AIP* associated somatotroph adenoma are more frequently resistant to somatostatin analogue treatment than non-mutated controls. Multimodal treatment is needed frequently but comparable with non-mutated controls, difference in disease control did not reach statistical significance. • There is some evidence that treatment outcome is better in *AIP* associated gigantism, but given the considerable risk of bias and limited publications, no well-founded conclusions can be drawn for this subgroup. • Failure of dopamine agonists is described frequently in *AIP* associated prolactinoma, and multimodal treatment is necessary in the majority of cases. There are too little reliable comparative data to determine the influence of an *AIP* mutation on treatment outcome in prolactinoma. • *MEN1* associated prolactinoma respond well to dopamine agonist treatment and tumor growth of NFPA is often without clinical consequences. • Treatment is challenging in X-LAG patients given the frequent use of multiple modalities and the occurrence of hypopituitarism. No significantly difference in long-term disease control, hypopituitarism and the number of treatments is reported between X-LAG and other pituitary induced gigantism patients. • Due to scarcity of reported quantitative data on treatment outcome of pituitary adenoma in Carney Complex, MEN4 and patients with *SDHx* mutations, it turned out to be impossible to draw well-founded conclusions on the impact of these germline mutations		

Abbreviations: NFPA, non-functioning pituitary adenoma; X-LAG: X-Linked Acrogigantism.
Evidence Grading of Recommendations, Assessment, Development, and Evaluation (GRADE).
a: Quality of evidence (scale): High, Moderate, Low, Very Low.
b: Strength of recommendation (scale): Strong, Weak.

The majority of studies showed a considerable risk of bias, which can be partially explained by small study sizes inherent to the rarity of the disease. Most of the reported study populations were included in a non-random and non-consecutive manner and study cohorts were frequently selected from tertiary care centers, leading to potential patient selection bias. In some, mostly older studies, genetic analysis was not performed according to current quality standards. Furthermore, classification of genetic variants regarding the appropriate level of pathogenicity did not always take place according to the American College of Medical Genetics and Genomics and Association for Molecular Pathology (AMCG-AMP) guidelines.[57] These genetic issues introduce a risk of detection bias. The retrospective design and lack of standardized data collection in most studies further hamper the methodological quality. Moreover, it cannot be excluded that parts of included study cohorts were reported previously, introducing a possible distortion in results. Therefore, results must be interpreted with caution before drawing conclusions and especially before being used for decision-making in daily clinical practice.
Still, the aim of this review was to retrieve highly applicable best-available evidence on specific clinically relevant questions. Although we attempted to retrieve additional results, insufficient reporting of outcomes concerning our predefined topics led to exclusion of otherwise valuable records. We did exclude too small-sized studies to avoid imprecise estimations. In addition, we did not perform a meta-analysis of data because of the high heterogeneity of studies to avoid unreliable outcomes. Additionally, we used the presented results on the adenoma subtype as described in the individual papers, because immunochemistry results were not always provided. This could have resulted in slightly inaccurate results in NFPA, since immunostaining can reveal clinically silent or "whispering" adenomas with some evidence of biochemical hypersecretion. Given the distinctive clinical behavior of these subtypes, a thorough investigation of adenoma subtype according to the most recent World Health Organization guidelines would have provided us with more accurate results.[58,59] However, we provided all available data on immunohistochemistry of NFPA in the results tables. Finally, the large range of publication dates introduced a challenge in the interpretation of pathogenicity of genetic variants. By adopting the author's judgement, outdated knowledge or techniques can have resulted in inaccuracy of the results. Optimally, all historic results would have to be confirmed by the current standards of DNA analysis and interpretation. Therefore, the DNA analysis techniques and interpretation of genetic variants (*e.g.*, loss of heterozigosity studies, worldwide SNP databases, *in silico* analysis, functional studies) were evaluated thoroughly in our critical appraisal to put the results into the right perspective.

In general, our results support earlier findings and reviews on genetic analysis in PA.[60–63] Recently, Caimari *et al.* developed a user-friendly risk category system to find *AIP*-mutation-

associated PA using a large international cohort of 2,227 individuals. Young age of onset, familial status, GH excess and macroadenoma were the strongest predictors.[51] However, in contrast to these study results and earlier reviews, our recommendations are focused on apparently sporadically occurring PA in patients without other features of genetic syndromes. Furthermore, they come with the proper strength of recommendations as a result of the systematic literature search and critical appraisal of articles.

A number of unanswered questions and challenges for the future still remain. As a result of the rarity of diseases and/or PA as presenting manifestation, the clinical impact of a *CDKN1B*, *PRKAR1A* and *SDHx* mutations on treatment outcome of PA is still uncertain. Only worldwide networks of collaborating centers sharing clinical information can help unravel this issue. Secondly, the implications of an *AIP* mutation in apparently unaffected family members are unknown. To our knowledge, results from systematic follow-up of unaffected *AIP*-positive family members are not available. Therefore, surveillance guidelines in these cases await further studies. Furthermore, the number of germline variants of uncertain significance will continue to increase in the (near) future due to the increased genetic analysis modalities, further emphasizing the need for studies of functional status combined with data on clinical outcome from large worldwide databases. Lastly, despite our efforts to produce reliable recommendations, it remains difficult to predict the benefits of our recommendations when implementing them in daily practice. For example, in a recent study by Daly *et al*., no germline mutations in the *AIP* or *MEN1* gene were identified in a group of 55 PA patients, despite the use of risk criteria.[64] These results show that no risk stratification system or set of screening recommendations is flawless. By external validation and further (clinical) research these tools can be optimized in the future, but will never be all-comprehensive.

Based on the yet available literature on the value of genetic analysis of sporadic PA, we can conclude that effective use of genetic analysis can lead to early disease identification (with possibly beneficial treatment outcome) on the one hand, and can lower health care costs and psychological burden on the other hand if unnecessary investigations can be limited. Knowledge of the effect of germline mutations on treatment outcome helps to determine therapy strategy and possibly lowers disease morbidity. Now, large and unselected cohort studies are needed to further guide the indications and the consequences of mutation analysis in individual patients with PA.

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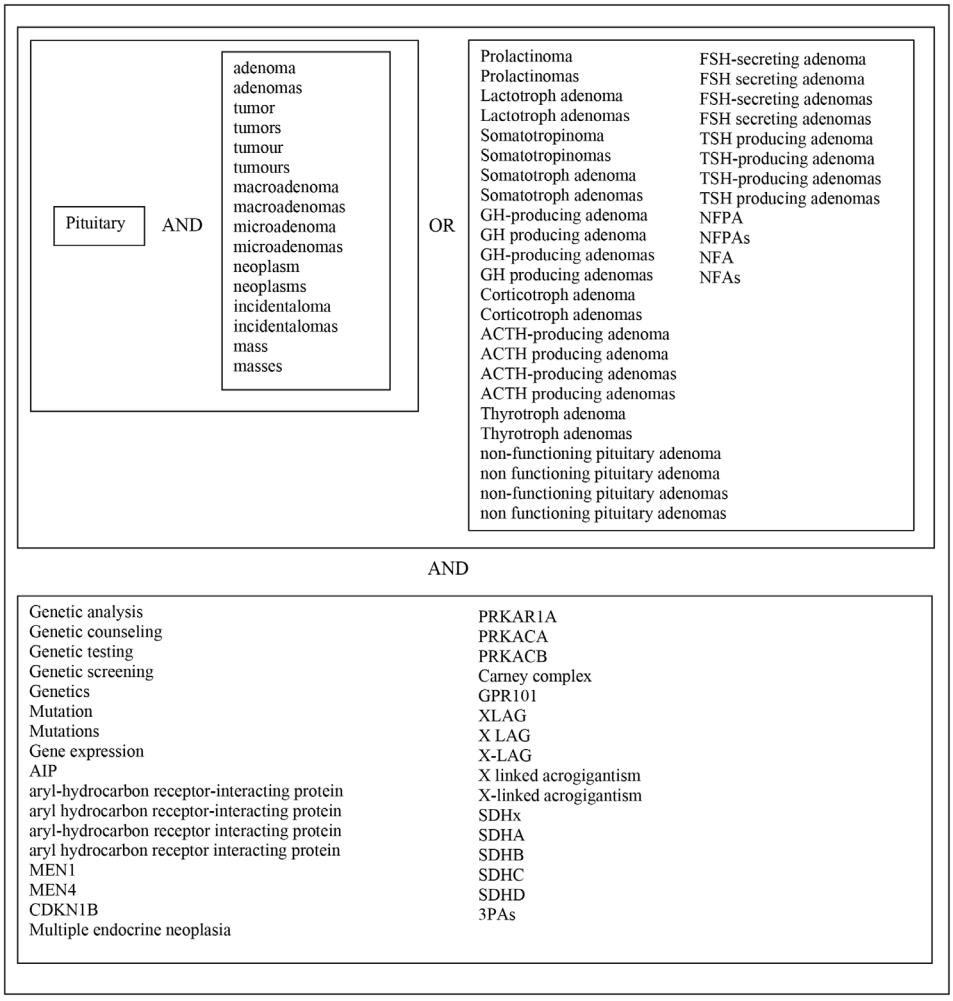
Supplemental Material 1: Search strategy

1. Graphic search overview



2. Pubmed search

#1 pituitary[Title/Abstract]

2 ((((((((((((((adenoma[Title/Abstract]) OR adenomas[Title/Abstract]) OR tumor[Title/Abstract]) OR tumors[Title/Abstract]) OR tumour[Title/Abstract]) OR tumours[Title/Abstract]) OR macroadenoma[Title/Abstract]) OR macroadenomas[Title/Abstract]) OR microadenoma[Title/Abstract]) OR microadenomas[Title/Abstract]) OR neoplasm[Title/Abstract]) OR neoplasms[Title/Abstract]) OR incidentaloma[Title/Abstract]) OR incidentalomas[Title/Abstract]) OR mass[Title/Abstract]) OR masses[Title/Abstract]

#3 #1 AND #2

#4 ((((((((((((((((((((((((((((((((prolactinoma[Title/Abstract]) OR prolactinomas[Title/Abstract]) OR "lactotroph adenoma"[Title/Abstract]) OR "lactotroph adenomas"[Title/Abstract]) OR somatotropinoma[Title/Abstract]) OR somatotropinomas[Title/Abstract]) OR "somatotroph adenoma"[Title/Abstract]) OR "somatotroph adenomas"[Title/Abstract]) OR "GH-producing adenoma"[Title/Abstract]) OR "GH-producing adenomas"[Title/Abstract]) OR "GH producing adenoma"[Title/Abstract]) OR "GH producing adenomas"[Title/Abstract]) OR "corticotroph adenoma"[Title/Abstract]) OR "corticotroph adenomas"[Title/Abstract]) OR "ACTH-producing adenoma"[Title/Abstract]) OR "ACTH-producing adenomas"[Title/Abstract]) OR "ACTH producing adenoma"[Title/Abstract]) OR "ACTH producing adenomas"[Title/Abstract]) OR "thyrotroph adenoma"[Title/Abstract]) OR "thyrotroph adenomas"[Title/Abstract]) OR "TSH producing adenoma"[Title/Abstract]) OR "TSH producing adenomas"[Title/Abstract]) OR "TSH-producing adenoma"[Title/Abstract]) OR "TSH-producing adenomas"[Title/Abstract]) OR "FSH-secreting adenoma"[Title/Abstract]) OR "FSH-secreting adenomas"[Title/Abstract]) OR "FSH secreting adenoma"[Title/Abstract]) OR "FSH secreting adenomas"[Title/Abstract]) OR NFPA[Title/Abstract]) OR NFPAs[Title/Abstract]) OR NFA[Title/Abstract]) OR NFAs[Title/Abstract]) OR "non-functioning pituitary adenoma"[Title/Abstract]) OR "non-functioning pituitary adenomas"[Title/Abstract]) OR "non functioning pituitary adenoma"[Title/Abstract]) OR "non functioning pituitary adenomas"[Title/Abstract]

#5 #3 OR #4

#6 ((((((((((((((((((((((((((((("genetic analysis"[Title/Abstract]) OR "genetic counseling"[Title/Abstract]) OR "genetic testing"[Title/Abstract]) OR "genetic screening"[Title/Abstract]) OR genetics[Title/Abstract]) OR mutation[Title/Abstract]) OR

mutations[Title/Abstract]) OR "gene expression"[Title/Abstract]) OR AIP[Title/Abstract]) OR "aryl-hydrocarbon receptor-interacting protein"[Title/Abstract]) OR "aryl-hydrocarbon receptor interacting protein"[Title/Abstract]) OR "aryl hydrocarbon receptor-interacting protein"[Title/Abstract]) OR "aryl hydrocarbon receptor interacting protein"[Title/Abstract]) OR MEN1[Title/Abstract]) OR MEN4[Title/Abstract]) OR "multiple endocrine neoplasia"[Title/Abstract]) OR CDKN1B[Title/Abstract]) OR PRKAR1A[Title/Abstract]) OR PRKACA[Title/Abstract]) OR PRKACB[Title/Abstract]) OR "Carney complex"[Title/Abstract]) OR GPR101[Title/Abstract]) OR XLAG[Title/Abstract]) OR "X LAG"[Title/Abstract]) OR "X-LAG"[Title/Abstract]) OR "X linked acrogigantism"[Title/Abstract]) OR "X-linked acrogigantism"[Title/Abstract]) OR SDHx[Title/Abstract]) OR SDHA[Title/Abstract]) OR SDHB[Title/Abstract]) OR SDHC[Title/Abstract]) OR SDHD[Title/Abstract]) OR 3PAs[Title/Abstract]

#7 #5 AND #6
Limits: Humans, Language (English; Dutch; French; German)
Search result 22th of November 2018: 2017

3. Embase search

#1 pituitary:ab,ti

#2 'adenoma':ab,ti OR 'adenomas':ab,ti OR 'tumor':ab,ti OR 'tumors':ab,ti OR 'tumour':ab,ti OR 'tumours':ab,ti OR 'macroadenoma':ab,ti OR 'macroadenomas':ab,ti OR 'microadenoma':ab,ti OR 'microadenomas':ab,ti OR 'neoplasm':ab,ti OR 'neoplasms':ab,ti OR 'incidentaloma':ab,ti OR 'incidentalomas':ab,ti OR 'mass':ab,ti OR 'masses':ab,ti

#3 #1 AND #2

#4 'prolactinoma':ab,ti OR 'prolactinomas':ab,ti OR 'lactotroph adenoma':ab,ti OR 'lactotroph adenomas':ab,ti OR 'somatotropinoma':ab,ti OR 'somatotropinomas':ab,ti OR 'somatotroph adenoma':ab,ti OR 'somatotroph adenomas':ab,ti OR 'GH-producing adenoma':ab,ti OR 'GH-producing adenomas':ab,ti OR 'GH producing adenoma':ab,ti OR 'GH producing adenomas':ab,ti OR 'corticotroph adenoma':ab,ti OR 'corticotroph adenomas':ab,ti OR 'ACTH-producing adenoma':ab,ti OR 'ACTH-producing adenomas':ab,ti OR 'ACTH producing adenoma':ab,ti OR 'ACTH producing adenomas':ab,ti OR 'thyrotroph adenoma':ab,ti OR 'thyrotroph adenomas':ab,ti OR 'TSH producing adenoma':ab,ti OR 'TSH producing adenomas':ab,ti OR 'TSH-producing adenoma':ab,ti OR 'TSH-producing adenomas':ab,ti OR 'FSH-secreting adenoma':ab,ti OR 'FSH-secreting adenomas':ab,ti OR 'FSH secreting adenoma':ab,ti OR 'FSH secreting adenomas':ab,ti OR 'NFPA':ab,ti OR 'NFPAs':ab,ti OR 'NFA':ab,ti OR 'NFAs':ab,ti OR 'non-functioning pituitary adenoma':ab,ti OR 'non-functioning pituitary adenomas':ab,ti OR 'non functioning pituitary adenoma':ab,ti OR 'non functioning pituitary adenomas':ab,ti

#5 #3 OR #4

#6 'genetic analysis':ab,ti OR 'genetic counseling':ab,ti OR 'genetic testing':ab,ti OR 'genetic screening':ab,ti OR 'genetics':ab,ti OR 'mutation':ab,ti OR 'mutations':ab,ti OR 'gene expression':ab,ti OR 'AIP':ab,ti OR 'aryl-hydrocarbon receptor-interacting protein':ab,ti OR 'aryl-hydrocarbon receptor interacting protein':ab,ti OR 'aryl hydrocarbon receptor-interacting protein':ab,ti OR 'aryl hydrocarbon receptor interacting protein':ab,ti OR 'MEN1':ab,ti OR 'MEN4':ab,ti OR 'multiple endocrine neoplasia':ab,ti OR 'CDKN1B':ab,ti OR 'PRKAR1A':ab,ti OR 'PRKACA':ab,ti OR 'PRKACB':ab,ti OR 'Carney complex':ab,ti OR 'GPR101':ab,ti OR 'XLAG':ab,ti OR 'X LAG':ab,ti OR 'X-LAG':ab,ti OR 'X linked acrogigantism':ab,ti OR 'X-linked acrogigantism':ab,ti OR 'SDHx':ab,ti OR 'SDHA':ab,ti OR 'SDHB':ab,ti OR 'SDHC':ab,ti OR 'SDHD':ab,ti OR '3PAs':ab,ti
#7 #5 AND #6

Limits: Embase, Humans, Language (English; Dutch; French; German)
Search result 22th of November 2018: 2673

4. Web of Science search

#1 TS=pituitary

#2 TS=(adenoma OR adenomas OR tumor OR tumors OR tumour OR tumours OR macroadenoma OR macroadenomas OR microadenoma OR microadenomas OR neoplasm OR neoplasms OR incidentaloma OR incidentalomas OR mass OR masses)

#3 #1 AND #2

#4 TS=(prolactinoma OR prolactinomas OR "lactotroph adenoma" OR "lactotroph adenomas" OR somatotropinoma OR somatotropinomas OR "somatotroph adenoma" OR "somatotroph adenomas" OR "GH-producing adenoma" OR "GH-producing adenomas" OR "GH producing adenoma" OR "GH producing adenomas" OR "corticotroph adenoma" OR "corticotroph adenomas" OR "ACTH-producing adenoma" OR "ACTH-producing adenomas" OR "ACTH producing adenoma" OR "ACTH producing adenomas" OR "thyrotroph adenoma" OR "thyrotroph adenomas" OR "TSH producing adenoma" OR "TSH producing adenomas" OR "TSH-producing adenoma" OR "TSH-producing adenomas" OR "FSH-secreting adenoma" OR "FSH-secreting adenomas" OR "FSH secreting adenoma" OR "FSH secreting adenomas" OR NFPA OR NFPAs OR NFA OR NFAs OR "non-functioning pituitary adenoma" OR "non-functioning pituitary adenomas" OR "non functioning pituitary adenoma" OR "non functioning pituitary adenomas")

#5 #3 OR #4

#6 TS=("genetic analysis" OR "genetic counseling" OR "genetic testing" OR "genetic screening" OR genetics OR mutation OR mutations OR "gene expression" OR AIP OR "aryl-hydrocarbon receptor-interacting protein" OR "aryl-hydrocarbon receptor interacting protein" OR "aryl hydrocarbon receptor-interacting protein" OR "aryl hydrocarbon receptor interacting protein" OR MEN1 OR MEN4 OR "multiple endocrine neoplasia" OR CDKN1B OR PRKAR1A OR PRKACA OR PRKACB OR "Carney complex" OR GPR101 OR XLAG OR "X LAG" OR "X-LAG" OR "X linked acrogigantism" OR "X-linked acrogigantism" OR SDHx OR SDHA OR SDHB OR SDHC OR SDHD OR 3PAs)

#7 #5 AND #6

Limits: Language (English; French; German)

Search result 22th of November 2018: 4412

Predictors on germline mutation status in sporadic pituitary adenoma

Author(s)	Year	Main reason of exclusion	Explanation
Corbetta *et al.*[1]	1997	Syndromic phenotype	Genetic analysis was only performed in patients with additional MEN1-related features.
Raitila *et al.*[2]	2007	Not clear if sporadic	The family history of one of the two patients with an *AIP* germline mutation has been reported as "not available".
Georgitsi *et al.*[3]	2008	Only MLPA used	This study only reported data on multiplex ligation-dependent probe amplification (MLPA) studies.
Igreja *et al.*[4]	2009	Syndromic phenotype	The study cohort consisted of patients with a clinical MEN1 syndrome, who tested negative for mutations in the *MEN1* gene.
Daly *et al.*[5]	2010	Not clear if sporadic	This study focused on the clinical and therapeutic features of patients with an *AIP* germline mutation. The study population consisted of both sporadic and familial cases. It was not possible to investigate the sporadic cases separately.
Cazabat *et al.*[6]	2011	Cohort duplicate	All patients reported in this article were included in a study by Cazabat *et al.*[7] in 2012 and Lecoq *et al.*[8] in 2016.
Nozières *et al.*[9]	2011	< 5 patients included	Only four patients with sporadic pituitary adenoma were described in this study.
Cazabat *et al.*[7]	2012	Cohort duplicate	All study participants were included in the study by Lecoq *et al.*[8] in 2016.
Belar *et al.*[10]	2012	Not clear if sporadic	Seventy-nine patients, both sporadic and familial, were included in this study. It was not possible to investigate the sporadic cases separately.
Papathomas *et al.*[11]	2014	Not clear if sporadic	The familial status of the six included patients has been reported as unknown.
Demir *et al.*[12]	2014	Pathogenicity of investigated genes unclear	This study focused on germline loss-of-function mutations in *inhibitory guanine nucleotide (GTP) binding protein alpha (GNAI)* loci. There is insufficient evidence in literature for *GNAI* allelic variants in the tumorigenesis of PA.
Nunes *et al.*[13]	2014	Not clear if sporadic	The family history of included patients was only retrieved when *MEN1* mutation analysis showed a positive result.
Beckers *et al.*[14]	2015	Cohort duplicate	All 13 sporadic patients suffering from X-LAG have been included in the study by Trivellin *et al.*[15] in 2014.
Salenave *et al.*[16]	2015	Not clear if sporadic	The familial status of the study participants has not been described.
Ye *et al.*[17]	2015	Pathogenicity of investigated genes unclear	This paper described a genome wide association study.

Supplemental Material 2. Continued (Predictors on germline mutation status in sporadic pituitary adenoma)

Author(s)	Year	Main reason of exclusion	Explanation
Denes *et al.*[18]	2015	Syndromic phenotype	The study population consisted of patients with (1) both pheochromocytoma/paraganglioma and pituitary adenoma, and (2) the occurrence of pheochromocytoma/paraganglioma and pituitary adenoma within their family.
Rostomyan *et al.*[19]	2015	Not clear if sporadic	The study population consisted of both sporadic and familial cases. It was not possible to investigate the sporadic cases separately.
Xekouki *et al.*[20]	2015	Syndromic phenotype	This study focused on the co-occurrence of pituitary adenoma and pheochromocytoma/paraganglioma.
Hu *et al.*[21]	2016	Pathogenicity of investigated genes unclear	This study focused on the possible role of single nucleotide polymorphisms (SNP) within the *AIP* gene in non-functioning pituitary adenomas.
Iacovazzo *et al.*[22]	2016	Not clear if sporadic	The study cohort of acromegaly patients included both sporadic and familial cases. It was not possible to investigate the sporadic cases separately.
Peculis *et al.*[23]	2016	Pathogenicity of investigated genes unclear	This study focused on the possible role of SNP in seven genes in pituitary adenomas.
Ramírez-Rentería *et al.*[24]	2016	Cohort duplicate	Seventy out of 71 patients have been described previously in a study by Hernández-Ramírez *et al.*[25]
Trivellin *et al.*[26]	2016	Pathogenicity of investigated genes unclear	This research focused on allelic variants of the *GPR101* gene.
Zhang *et al.*[27]	2017	Pathogenicity of investigated genes unclear	This research focuses on genetic variations in the *cadherin-related 23 (CDH23)* gene in pituitary adenoma patients. There is insufficient evidence in literature to date for its role in the tumorigenesis of PA.
Hernández-Ramírez *et al.*[28]	2017	Pathogenicity of investigated genes unclear	The authors investigated the presence of *Cdk5 And Abl Enzyme Substrate 1 (CABLES1)* mutations/copy number variations (CNVs) in pediatric cases of ACTH-producing pituitary adenoma. There is insufficient evidence in literature to date for its role in the tumorigenesis of PA.
Hernández-Ramírez *et al.*[29]	2017	Not clear if sporadic	This study reported a case of Cushing disease due to a loss-of-function mutation in *PRKAR1A* and reported a lack of mutations in 97 other pediatric Cushing patients. The familial status of these patients is unknown.

Supplemental Material 2. Continued (Predictors on germline mutation status in sporadic pituitary adenoma)

Author(s)	Year	Main reason of exclusion	Explanation
Radian *et al.*[30]	2017	Cohort duplicate	This study focused on carrier frequency of *AIP* founder mutation R304* in specific North Ireland and Ireland regions. The R304*-positive patients were previously reported in several studies (*e.g.,* Leontiou *et al.*[31] and Hernandez-Ramirez *et al.*[25])
Iivonen *et al.*[32]	2018	Pathogenicity of investigated genes unclear	This work focused on germline mutations in the *potassium voltage-gated channel subfamily Q member 1* (*KCNQ1*) and *potassium voltage-gated channel subfamily E regulatory subunit 2* (*KCNE2*) gene. There is insufficient evidence in literature to date for its role in the tumorigenesis of PA.
Caimari *et al.*[33]	2018	Not clear if sporadic	The authors presented a new risk category system for *AIP* mutations in patients with pituitary adenomas. The study population consisted of both sporadic and familial cases. It was not possible to investigate the sporadic cases separately.
Nagata *et al.*[34]	2018	Not clear if sporadic	The study cohort included both sporadic and familial cases. It was not possible to investigate the sporadic cases separately.
Makri *et al.*[35]	2018	Not clear if sporadic	This retrospective analysis of pediatric patients with Cushing disease included both sporadic and familial cases. It was not possible to investigate the sporadic cases separately.

Impact of a germline mutation on treatment outcome in pituitary adenoma

Author	Year	Main reason of exclusion	Explanation
Scheithauer *et al.*[36]	1987	No germline mutation analysis	Surgically resected pituitary adenomas in the setting of MEN1 were compared with pituitary adenomas occurring in the general population. There was no germline mutation analysis performed in this study.
Burgess *et al.*[37]	1996	No germline mutation analysis	Prolactinomas in a large kindred with MEN1 were compared with literature. There was no germline mutation analysis performed in this study.
Burgess *et al.*[38]	1996	No germline mutation analysis	Clinical, biochemical and radiological features of pituitary disease in a large MEN1 kindred were described. There was no germline mutation analysis performed in this study.
Vierimaa *et al.*[39]	2006	No information on treatment outcome	Vierimaa *et al.* described the discovery of the *AIP* gene. Age at diagnosis, gender, and size of adenoma were compared between seven patients with a germline *AIP* mutation and patients without *AIP* mutation. No information on treatment outcome was reported.
Daly *et al.*[40]	2007	No information on treatment outcome	Seventy-three FIPA families are described in this study. Age at diagnosis, gender, and size of adenoma were compared between seven patients with a germline *AIP* mutation and patients without *AIP* mutation. No information on treatment outcome was reported.
Cazabat *et al.*[41]	2007	No information on treatment outcome	Age at diagnosis, gender and patient length is compared in *AIP*-mutated and non-*AIP* mutated patients with sporadic GH secreting pituitary adenoma. Insufficient information on treatment outcome was reported.
Trouillas *et al.*[42]	2008	No information on treatment outcome	Pituitary tissue specimens of 77 MEN1 patients were compared with 2509 unselected non-MEN1 sporadic pituitary tumors. There was no information about treatment outcome reported.
Leontiou *et al.*[31]	2008	No individual data is available	Twenty-six FIPA families and 85 patients with sporadic pituitary adenoma were described. Treatment outcome of families with a germline *AIP* mutation was described, but no individual data were presented.
Igreja *et al.*[43]	2010	No information on treatment outcome	Sixty-four FIPA families were described. Age and penetrance are compared between *AIP* mutated and non-mutated patients. There was no information about treatment outcome reported.
Cain *et al.*[44]	2010	Cohort duplicate	The study cohort was identical to the study population reported by Igreja *et al.*[43] in 2010.
De Pinho *et al.*[45]	2010	Cohort duplicate	Four FIPA families were described, of which one with an *AIP* germline mutation. Three out of four families were reported previously by Igreja *et al.*[43] in 2010.

Supplemental Material 2. Continued (Impact of a germline mutation on treatment outcome in pituitary adenoma)

Author	Year	Main reason of exclusion	Explanation
Cazabat *et al.*[7]	2012	No information on treatment outcome	The authors described 443 cases with apparent sporadic pituitary adenoma, of which 16 with *AIP* germline mutation. No information on treatment outcome is reported.
Vroonen *et al.*[46]	2012	No clinical data on subgroup of patients harboring germline mutation	This study focused on 92 cabergoline resistent prolactinoma. Patients with a "genetic basis" were compared with the rest of the study population. This group included patients with a germline *MEN1* or *AIP* mutation, an *AIP* variant of uncertain significance (VUS), familial disease (FIPA) without germline mutation/variant, and one clinical MEN1 patient without germline mutation. No clinical data on the subgroup of patients harboring germline mutation were provided.
Simonds *et al.*[47]	2012	< 5 cases with germline mutation	Only four patients with a germline mutation (*MEN1*) were included in this study.
Oriola *et al.*[48]	2013	< 5 cases with germline mutation	Only two patients with *AIP* germline mutation (and two patients with a *AIP* gene VUS) were included in this study.
Cuny *et al.*[49]	2013	No information on treatment outcome	The authors described 174 patients with sporadic pituitary macroadenoma. There was no information about treatment outcome reported.
Trivellin *et al.*[15]	2014	Cohort duplicate	Clinical and biochemical characteristics of 43 patients with gigantism and GH producing pituitary adenoma were described. The discovery of *Xq26.3* genomic duplication and the related phenotype of X-LAG (14 patients) were described. Thirteen out of 14 XLAG patients have been reported in Beckers *et al.*[14] in 2015.
Preda *et al.*[50]	2014	< 5 cases with germline mutation	Only four patients with a germline mutation were included in this study.
Schöfl *et al.*[51]	2014	No clinical information on subgroup of patients harboring germline mutation	The authors described 91 young patients with acromegaly. Five patients harbored an *AIP* germline mutation. No clinical information about the subgroup of this patients are presented.
Hernandez-Ramirez *et al.*[25]	2015	No information on treatment outcome	In this study the *AIP* mutation status and clinical characteristics of 216 FIPA families and 404 sporadic pituitary adenoma patients were described. However, there was no information about the relationship between germline mutation status and treatment outcome reported.
Ramírez-Rentería *et al.*[24]	2016	Cohort duplicate	Seventy out of 71 patients were described previously in Hernandez-Ramirez *et al.*[25] in 2015.

Supplemental Material 2. Continued (Impact of a germline mutation on treatment outcome in pituitary adenoma)

Author	Year	Main reason of exclusion	Explanation
Mangupli *et al.*[52]	2016	< 5 cases with germline mutation	Only three patients with a germline mutation were included in this study.
Matsumoto *et al.*[53]	2016	< 5 cases with germline mutation	Only three patients with a germline mutation were included in this study.
Lecoq *et al.*[8]	2016	No information on treatment outcome	This study described 766 patients with sporadic pituitary adenoma. Gender, age of diagnosis and the presence of gigantism were compared between subgroups (*AIP* mutations or rare variants, *GPR101* variants, wildtype). No information about treatment outcome has been described.
Radian *et al.*[30]	2017	Cohort duplicate	This study focused on carrier frequency of *AIP* founder mutation R304* in specific North Ireland and Ireland regions. The R304*-positive patients have been reported previously in several studies (*i.a.* Leontiou *et al.*[31] in 2008, Hernandez-Ramirez *et al.*[25] in 2015).
De Sousa *et al.*[54]	2017	< 5 cases with germline mutation	Only four patients with a (likely) pathogenic germline variant were included in this study.
Ozkaya *et al.*[55]	2018	< 5 cases with germline mutation	Only two patients with a germline mutation were included in this study.
Marques *et al.*[56]	2018	Congress abstract	In this congress abstract presented at the 100th Annual Meeting of the Endocrine Society ENDO 2018, 184 pituitary adenoma patients with a germline *AIP* mutation were compared with non-mutated pituitary adenoma patients. However, the data has not been published in a full article yet.

Supplemental Material 2 References

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Supplemental Material 3: Format of Quality Assessment: Adjusted Quality Assessment of Diagnostic Accuracy Studies tool (QUADAS-2)

Risk of bias

1A1: Was a consecutive or random sample of patients with sporadic pituitary tumors enrolled?
In order to determine if enrollment took place in a consecutive or random manner – taking into account the applied in/exclusion criteria –, it was necessarily to be informed about the inclusion place(s) and inclusion period. If patient selection took place by enrollment of patients in a consecutive or random manner, the study is scored "yes". If patient selection did not take place by enrollment of patients in a consecutive or random manner, or if this could not be determined due to a lack of information, the study is scored "no".

1A2: Did the study avoid inappropriate exclusions?
Exclusion of patients based on familial pituitary tumors is scored as "yes" (= appropriate exclusion). Exclusion of sporadic patients with syndromic features suspected for a specific germline mutation is scored as "yes" (= appropriate exclusion). Exclusion of patients with a proven germline mutation (e.g. exclusion of MEN1 patients when AIP gene mutation is investigated) is scored as "yes" (= appropriate exclusion). Including only a subgroup of patients with sporadic pituitary tumors based on potential predictors of germline mutations such as age, hormonal overproduction, tumor size or patient length is scored as "yes" (= appropriate). Exclusion of patients in which certain germline mutations are already investigated but proved to be non-carriers (e.g. exclusion of patients in which MEN1 mutation carriership was investigated earlier, but showed no pathogenic mutations, when AIP gene mutation is investigated) is scored as "no" (= <u>in</u>appropriate exclusion). In/excluding a subgroup of patients based on other (additional) criteria is scored as "no". If no (additional) exclusion criteria are used or described, the study is scored "yes".

1A3: Was the patient sample population based?
If the study population was based on a population-based database/network, it is scored "yes". If not (or unclear), it is scored "no".

1A:
If all 3 questions are answered "yes", the study is scored + + on patient selection
If 2 questions are answered "yes", the study is scored + on patient selection
If 1 question is answered "yes", the study is scored - on patient selection

If no question is answered "yes", the study is scored - - on patient selection

3A1: Is the reference standard likely to correctly classify the target condition: is the proper method used to find DNA sequence variations?

Coding exome sequencing is classified as adequate reference standard. In case of investigating Xq26.3 microduplications, Copy Number Variation (CNV) analysis or comparative genomic hybridization microarray (aCGH) must be used. If this is performed correctly, the study is scored "yes". If not, it is scored "no". Single strand conformation polymorphism (SSCP) alone is not considered an appropriate method for sequencing, and scored "no".

3A2: Is the reference standard likely to correctly classify the target condition: is the proper method used to find DNA copy number variations (CNV)?

Studies investigating germline mutations and/or CNVs in AIP, MEN1 and/ or CDKN1B gene, multiplex ligation-dependent probe amplification (MLPA) must be used in addition to coding exome sequencing as adequate reference standard. If this method is used when investigating the AIP, MEN1 and/or CDKN1B gene, the study is scored "yes". If not, it is scored "no".

3A3: Is the reference standard likely to correctly classify the target condition: is the proper method used to investigate the interpretation of mutations?

The pathogenicity of a genetic variation should have been investigated with more than one of the following tools: investigating the frequency of variation in healthy controls, investigating the frequency of variations in reference databases, in silico analysis, functional studies and/ or evidence on pathogenicity reported in literature. If pathogenicity is investigation more than one mentioned methods, the study is scored "yes". If not, it is scored "no"

3A:

If all 3 questions are answered "yes", the study is scored + + on reference standard
If 2 questions are answered "yes", the study is scored + on reference standard
If 1 question is answered "yes", the study is scored - on reference standard
If no question is answered "yes", the study is scored - - on reference standard
NB: when question 3A2 is not applicable (a study is not investigating AIP, MEN1 or CDKN1B), a study is scored + + when both questions are answered "yes". If 1 question is answered "yes", the study is scored + -. If no question is answered "yes", it is scored - -.

4A1: Did all patients receive a reference standard?

It should be clear that all patients received a reference standard. In that case, a study is scored "yes". If not (e.g. due to no informed consent for genetic analysis), a study is scored "no".

4A3: Did all patients receive the same reference standard?

A low risk of biased was given when all patients underwent the same genetic analysis. The

study is scored "yes". In all other cases, it is scored "no".
4A4: Were all patients included in the analysis?
All patients should be included in the analysis. In that case, a study is scored "yes". If not, it is scored "no".

4A:
If all 3 questions are answered "yes", the study is scored + + on flow and timing
If 2 questions are answered "yes", the study is scored + - on flow and timing
If 1 question is answered "yes", the study is scored - on flow and timing
If no question is answered "yes", the study is scored - - on flow and timing

Applicability
1B: Are there concerns that the included patients do not match the review question?
This review focusses on patients with sporadic pituitary adenoma without syndromic features suggestive for a (certain) germline mutation due to the presence of additional endocrine tumors in personal or family history. This should be the study domain of the included study. In that case, the study is scored "+". If additional in/exclusion criteria are used (e.g. age criteria, hormonal tumor production, tumor size, patient length), it introduces a difference between the study population and the target population, and the study is scored "+/-". If there are not enough baseline data reported to make a fair judgement about the applicability, the study is scored "-".
The minimum required data are: gender, age (of diagnosis or start of symptoms), familial status, subtype tumor.

3B: Are there concerns that the target condition as defined by the reference standard does not match the review question?
The presence of a germline mutation must have been evaluated using whole exome sequencing and – in the case of AIP, MEN1 or CDKN1B – also by MLPA. If this is the case, the study is scored "+". In case of investigation of AIP, MEN1 or CDKN1B gene: if MLPA is not used, the study is scored as "+/-". The pathogenicity must have been investigated, only pathogenic and likely pathogenic mutations using the classification of the Association for Clinical Genetics Science (ACGS) and American College of Medical Genetics and Genomics (ACMG) guidelines are considered as clinically relevant.[1,2] *If a study does not evaluate mutations in terms of pathogenicity, the study is scored as "-". If a study does not investigate the entire coding exome by (direct) sequencing (or in case of Xq26.3 microduplication: aCGH or CNV analysis), the study is scored as "-".*

Supplemental Material 3 References

1. Wallis Y, Payne S, Mcanulty C, et al. Practice Guidelines for the Evaluation of Pathogenicity and the Reporting of Sequence Variants in Clinical Molecular Genetics. *Assoc Clin Genet Sci Dutch Soc Clin Genet Lab Spec*. Published online 2013.
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Supplemental Material 4: Format of Quality Assessment: Adjusted Quality In Prognosis Studies (QUIPS)

1. Study participation and attrition

a. Study population is adequately described
Yes: Baseline table or adequate description must be present (gender, age, sporadic/familial status, type of adenoma, tumor size (or macroadenoma yes/no), any other endocrine tumors and/or syndromic features (if applicable), genetic status (if already partly investigated).
No: no adequate description or only some characteristics addressed.

b. Inclusion and exclusion criteria are adequately described
Yes: the inclusion and exclusion criteria are adequately described, including recruitment period and place(s).
No: insufficient description.

c The data from an adequate proportion of the study population are available for outcome measurement
Yes: Number of patients lost to follow-up and the reasons for loss to follow-up and drop out are adequately described.
No: Insufficient description of participation in outcome measurement (treatment outcome) or if there is reason to believe that the selection of patients who lost to follow-up differs from the rest of the study population.

d. The study population is population based
If the study population was based on a population-based database/network, it is scored "yes". If not (or unclear), it is scored "no".

If all 4 questions are answered "yes", the study is scored + + on patient selection
If 3 questions are answered "yes", the study is scored + on patient selection
If 2 questions are answered "yes", the study is scored + - on patient selection
If 1 question is answered "yes", the study is scored - on patient selection
If no question is answered "yes", the study is scored - - on patient selection

2. Prognostic factor measurement (germline mutation)
a. a valid and reliable modality for investigating germline status was used
Yes: the combination of coding exome sequencing and – in case of investigation of germline

mutations in MEN1, AIP or CDKN1B – multiplex ligation-dependent probe amplification (MLPA) was used. In case of investigating Xq26.3 microduplications, Copy Number Variation (CNV) analysis or comparative genomic hybridization microarray (aCGH) must be used. Furthermore, more than one method must have been used to investigate the pathogenicity of a genetic variation (frequency of variation in healthy controls, frequency of variations in reference databases, in silico analysis, functional studies and/or evidence on pathogenicity reported in literature)
No: inadequate determination of germline status and/or investigation on pathogenicity of DNA variants

b. adequate participation
Yes: All study participants have been investigated for germline mutations.
No: not all study participants have been investigated for germline mutations.

If all 2 questions are answered "yes", the study is scored + + on prognostic factor measurement
If 1 question is answered "yes", the study is scored + - on prognostic factor measurement
If no question is answered "yes", the study is scored - - on prognostic factor measurement

3. Outcome measurement (treatment outcome)
a. The investigated outcome is predefined and a clear definition of outcome is provided (including duration and follow-up)
Yes: adequate description of investigated outcome (predefined). In case of follow-up: duration of follow-up and explicit description of investigations performed during that period must be described.
No: no sufficient description of outcome definition(s), follow-up measurements and/or duration.

b. The method of outcome measurement used is adequately valid and reliable
Yes: Method of outcome measurement (treatment characteristics/outcome) must be described properly. In case of follow-up :this must be adequate to assess clinical relevant variations.
No: no adequate outcome measurement.

c. The method and setting for measurement is the same for all participants
Yes: outcome measurements are the same for patients with and without germline mutation.
No: Relevant difference in outcome measurements between patients with a germline mutation vs. non-carriers.

If all 3 questions are answered "yes", the study is scored + + on outcome measurement
If 2 questions are answered "yes", the study is scored + - on outcome measurement
If 1 question is answered "yes", the study is scored - on outcome measurement
If no question is answered "yes", the study is scored - - on outcome measurement

4. Analysis and reporting
a. Sufficient presentation of data to assess the relationship between prognostic factor and outcome
Yes: sufficient description of data to assess the relationship between germline mutation and treatment outcome.
No: insufficient description.

b. There is no selective reporting of results
Yes: no selective reporting
No: selective reporting

If all 2 questions are answered "yes", the study is scored + + on analysis and reporting
If 1 question is answered "yes", the study is scored + - on outcome measurement
If no question is answered "yes", the study is scored - - on outcome measurement

Supplemental Material 5 Study results

Table 1: Predictors on germline mutation status in sporadic pituitary adenoma

1A. Sporadic somatotroph adenoma

References	Investigated gene	Predictor	Comparison of groups	Outcome	Statistical significance
Cazabat *et al.*[1]	*AIP*	Younger age	Mean age ± SD of *AIP*-mutated patients vs. mean ± SD age of wildtype	25 ± 10 years vs. 43 ± 14 years	$P = 0.005$
		Gigantism	Frequency of gigantism in *AIP*-mutated patients vs. wildtype	3/5 (60%) vs. 17/149 (11.4%)	$P = 0.016$
		Male gender	Frequency of male gender in *AIP*-mutated patients vs. wildtype	4/5 (80%) vs. 66/149 (44.3%)	$P = 0.12$
Trivellin *et al.*[2]	*Xq26.3* microduplication[a]	Female gender	Frequency of female gender in patients with *Xq26.3* microduplication vs. wildtype	10/14 (71%) vs. 7/29 (42%)	$P = 0.007$
		Onset of rapid growth	Median age (and range) at onset of rapid growth in patients with *Xq26.3* microduplication vs. wildtype	1.0 year (0.5-2.0) vs. 16.0 year (range 5.0-18.0)	$P < 0.001$
		IGF-1 level at diagnosis	Median factor increase (multiple of ULN) (and range) of IGF-1 at diagnosis in patients with *Xq26.3* microduplication vs. wildtype	4.4 (range 2.4-5.2) vs. 2.1 (range 1.4-5.3)	$P = 0.005$
		Elevated prolactin levels at diagnosis	Number of patients with elevated prolactin levels at diagnosis in patients with *Xq26.3* microduplication vs. wildtype	13/14 (93%) vs. 6/29 (21%)	$P < 0.001$

Abbreviations: IGF-1, insulin-like growth factor 1; ULN, upper limit of normal.
a: These results included nine sporadic and five familial cases of *Xq26.3* microduplication.

1B. Young (≤ 30 years) sporadic adenoma

References	Investigated gene	Predictor	Comparison of groups	Outcome	Statistical significance
Tichomirowa *et al.*[3]	*AIP*	Younger age	Prevalence of *AIP* mutation < 18 years vs. ≥ 18 years at diagnosis	8/39 (20.5%) vs. 11/124 (8.9%)	$P < 0.01$
		Macroadenoma	N/A		
		Gigantism	N/A		
		Male gender	Frequency of males in *AIP*-mutated patients vs. wildtype	16/19 (84.2%) vs. 68/144 (47.2%)	Not reported
		Adenoma subtype	Prevalence of *AIP* mutation in somatotroph adenomas vs. prolactinomas vs. NFPA	11/83 (13.3%) vs. 7/61 (11.5%) vs. 1/16 (6.3%)	Not reported
		Extrasellar extension	Frequency of extrasellar extention in *AIP*-mutated patients	14/17[a] (82.4%)	N/A
Cuny *et al.*[4]	*AIP*	Younger age	Prevalence of *AIP* mutation ≤ 18 years vs. > 18 years at diagnosis	7/46 (15.2%) vs. 8/128 (6.3%)	Not reported
		Macroadenoma	N/A		
		Gigantism	Prevalence of *AIP* mutation in giants vs. non-giants	3/6 (50%) vs. 12/168 (7.1%)	Not reported
		Male gender	Frequency of males in *AIP*-mutated patient vs. wildtype	11/15 (73.3) vs. 85/159 (53.5%)	Not reported
		Adenoma subtype	Prevalence of *AIP* mutation in NFPA vs. non-NFPA	3/12 (25%) vs. 12/162 (7.4%)	Not reported
		Extrasellar extension	Frequency of extrasellar extention in *AIP*-mutated patients	15/15 (100%)	N/A
	MEN1	Younger age	Prevalence of *MEN1* mutation ≤ 18 years vs. > 18 years at diagnosis	3/46 (6.5%) vs. 3/128 (2.3%)	Not reported
		Adenoma subtype	Prevalence of *MEN1* mutation in prolactinoma vs. non-prolactinoma	4/74 (5.4%) vs. 2/100 (2%)	Not reported

1B. Continued

References	Investigated gene	Predictor	Comparison of groups	Outcome	Statistical significance
Hernandez *et al.*[5]	*AIP*	Younger age	Median age (and IQR) at onset in *AIP*-mutated patients vs. wildtype	16 years (14.8-22.3) vs. 22 years (16-26)	$P = 0.0054$
			Percentage of pediatric patients in *AIP*-mutated patients vs. wildtype	58.8% vs. 35.9%	$P = 0.0085$
		Macroadenoma	Frequency of macroadenoma in *AIP*-mutated patients vs. wildtype	29/29 (100%) vs. 283/328 (86.3%)	Not reported
			Frequency of adenomas ≥ 40 mm in *AIP*-mutated patients vs. wildtype	Not reported	$P = 0.7859$
			Maximum diameter in *AIP*-mutated patients vs. wildtype	Not reported	$P = 0.6965$
		Gigantism	Prevalence of *AIP* mutation in sporadic patients with gigantism (no comparison)	19/75 (25.3%)	
		Male gender	Frequency of males in *AIP*-mutated patients vs. wildtype	61.8% vs. 49.2%	$P = 0.1605$
		Adenoma subtype[b]	Frequency of somatotroph adenoma in *AIP*-mutated patients vs. wildtype	100% vs. 69.2%	Not reported
		Extrasellar extension	Frequency of extrasellar extention in *AIP*-mutated patients vs. wildtype	95% vs. 58.9%	$P = 0.0011$

Abbreviations: IQR, interquartile range; N/A, not applicable; NFPA, non-functioning pituitary adenoma.
a: Seventeen out of 19 tumors from *AIP* mutated patients were investigated on extrasellar extension.
b: Proven by clinical diagnosis and immunohistochemistry.

1C. Other groups of patients with sporadic pituitary adenoma

References	Investigated gene	Predictor	Comparison of groups	Outcome	Statistical significance
Cai *et al.*[6]	*AIP*	Younger age	Prevalence of *AIP* mutation ≤ 18 years vs. > 18 years at diagnosis	1/11 (9.1%) vs. 5/205 (2.4%)	Not reported
		Adenoma subtype	Prevalence of *AIP* mutation in somatotroph adenoma vs. non-somatotroph adenoma	5/80 (6.3%) vs. 1/136 (0.7%)	Not reported
		Male gender	Prevalence of *AIP* mutation in males vs. females	5/94 (5.3%) vs. 1/122 (0.8%)	Not reported

Table 2. Impact of a germline mutation on treatment outcome in pituitary adenoma

References	Investigated gene	Comparison of groups	Treatment (outcome)	Results	Statistical significance
Daly *et al.*[7]	*AIP*	*AIP*-mutated vs. wildtype somatotroph adenomas	Proportion multimodal treatment (≥ 2 modalities)	61.3% vs. 66.4%	Not reported
			Proportion neurosurgery	87.3% vs. 80.5%	Not reported
			Proportion reoperation	21.9% vs. 5.5%	$P = 0.00069$
			Proportion radiotherapy	41.4% vs. 24.7%	$P = 0.15$
			Long-term (≥ 12 months) disease control	50/71 (70.4%) vs. 182/226 (80.5%)	$P = 0.06$
			Median SSA-induced GH reduction	40% (range 0-99%)[a] vs. 75% (range 0-99%)[b]	$P = 0.0004$
			Median SSA-induced IGF-1 reduction	47.4% (range 0-83.4%)[a] vs. 56.0% (range: 0-100%)[b]	$P = 0.028$
			Median SSA-induced tumor shrinkage	0% (range 0-90%)[a] vs. 41.4% (range 0-95%)[b]	$P < 0.000001$
			Disease control rate achieved with SSA as primary treatment	1/6 vs. 17/32	Not reported
			Disease control rate achieved with SSA as pre-operative treatment	1/6 vs. 6/16	Not reported
			Disease control rate achieved with SSA as post-operative treatment	9/26 vs. 51/84	Not reported
			Proportion disease control with pegvisomant	1/4 vs. 19/19	Not reported
			Frequency of hypopituitarism	22.5% vs. 25.2%	Not reported
			Number of deficient pituitary hormonal axes	Not reported	$P < 0.000001$
		AIP-mutated vs. wildtype somatotroph adenomas with high cumulative treatment burden (≥ 3 modalities)	Long-term (≥ 12 months) disease control	15/27 (55.6%) vs. 63/76 (82.9%)	$P = 0.01$

Table 2. Continued (Impact of a germline mutation on treatment outcome in pituitary adenoma)

References	Investigated gene	Comparison of groups	Treatment (outcome)	Results	Statistical significance
Nagata *et al.*[8]	*AIP* (continued)	*AIP*-mutated vs. wildtype somatotroph adenomas	Hormonal control at time of last follow-up	(3/5) 60% vs. (16/18) 88.9%	Not reported
			Remission by surgery alone	(2/5) 20% vs. (12/18) 66.7%	Not reported
Tichomirowa *et al.*[3]		*AIP*-mutated somatotroph adenomas (n = 11)	Proportion ≥ two surgical interventions	4/11 (36.4)	N/A
			Disease control by secondary SSA therapy	1/9 (11.1%)	N/A
			Tumoursize reduction by SSA treatment	1/6 (16.7%)	N/A
Rostomyan *et al.*[9]		*AIP*-mutated vs. wildtype pituitary giants	GH/IGF-1 control at last follow-up	61.0% vs. 43.0%	P = 0.03
			GH/IGF-1 control < 19 years	72.7% vs. 22.7%	P = 0.0001
			GH/IGF-1 control before final height	48.6% vs. 10.9%	P < 0.0001
			Median age when first control achieved	17.3 (IQR 15-20) vs. 27 (IQR 18-37) years	P < 0.0001
			Proportion multimodal treatment (≥ 3 modalities)	23.8% vs. 42.7%	P = 0.04
			Long-term control (> 12 months)	55.3% vs. 38.4%	P = 0.08
			Frequency of hypopituitarism	73% vs. 66%	P = 0.4
Iacovazzo *et al.*[10]		*AIP*-mutated vs. wildtype pituitary giants	Median number of treatments	2 (range 1-4) vs. 3 (range 1-4)	Not significant
			Frequency of hypopituitarism	13/28 (46.4%) vs. 11/19 (57.9%)	Not significant
Salenave *et al.*[11]		DA-resistant adenoma vs. DA-sensitive adenoma	Prevalence of *AIP* mutation	2/17 (11%) vs. 2/37% (5%)	Not significant
Tichomirowa *et al.*[3]		*AIP*-mutated prolactinomas (n = 7)	Disease control by DA treatment	3/6 (50%)	N/A
			Proportion neurosurgery	5/7 (71.4%)	N/A
			Proportion multiple surgeries	4/7 (66.7%)	N/A
Daly *et al.*[7]		*AIP*-mutated prolactinomas (n = 13)	Disease control by DA treatment	6/12 (50%)	N/A
			Proportion neurosurgery	7/13 (53.8%)	N/A
			Proportion multiple surgeries	4/13 (30.8%)	N/A
			Long-term hormonal control	8/13 (61.5%)	N/A

Table 2. Continued

References	Investigated gene	Comparison of groups	Treatment (outcome)	Results	Statistical significance
Daly *et al.*[7]	*AIP* (continued)	*AIP*-mutated NFPA ($n = 7$)	Proportion neurosurgery	6/7 (85.7%)	N/A
			Long-term control of tumour size	7/7 (100%)	N/A
Caimari *et al.*[12]		*AIP*-mutated PA vs. wildtype	Median number of treatments	2 (IQR 1-3) vs. 1 (IQR 1-2)	$P = 0.055$
De Laat *et al.*[13]	*MEN1*	*MEN1*-mutated prolactinomas	Hormonal control by DA as primary treatment	27/30 (90.0%)	N/A
			Hormonal control by DA as secondary treatment	8/9 (88.9%)	N/A
			Proportion neurosurgery	4/52 (7.7%)	N/A
		MEN1-mutated NPFA	Proportion neurosurgery	8/52 (15.4%)	N/A
			Proportion stable tumour size in initially untreated cases	40/45 (88.9%)	N/A
Verges *et al.*[14]		*MEN1*-mutated vs. wildtype functional PA	Proportion normalization of pituitary hypersecretion	49/116 (42%) vs. 83/110 (90%)	$P < 0.001$
		MEN1-mutated vs. wildtype prolactinoma	Proportion of normalization of plasma prolactin	37/85 (44%) vs. (90%)	$P < 0.001$
Salenave *et al.*[11]		DA-resistant adenoma vs. DA-sensitive adenoma	Prevalence of *MEN1* mutation	3/18 (16%) vs. 0/40 (0%)	$P = 0.026$[c]
Rostomyan *et al.*[9]	*Xq26.3* microduplication	Pituitary giants with *Xq26.3* microduplication vs. wildtype pituitary giants	GH/IGF-1 control at last follow-up	58.0% vs. 43.0%	$P = 0.02$
			GH/IGF-1 control < 19 years	85.7% vs. 22.7%	Not reported
			GH/IGF-1 control before final height	54.5% vs. 10.9%	$P < 0.0001$
			Median age when first control achieved	8.0 (IQR 4-13) vs. 27 (IQR 18-37)	$P = 0.0005$
			Proportion multimodal treatment (≥ 3 modalities)	46.0% vs. 42.7%	$P = 0.6$
			Long-term control (> 12 months)	41.7% vs. 38.4%	$P = 0.1$
			Frequency of hypopituitarism	75% vs. 66%	$P = 0.7$

Table 2. Continued (Impact of a germline mutation on treatment outcome in pituitary adenoma)

References	Investigated gene	Comparison of groups	Treatment (outcome)	Results	Statistical significance
Iacovazzo *et al.*[10]	*X26.3* microduplication (continued)	Pituitary giants with *Xq26.3* microduplication vs. wildtype pituitary giants	Median number of treatments	3.5 (IQR 2-4.7) vs. 3 (IQR 1-4)	Not significant
			Frequency of hypopituitarism	8/12 (66.7%) vs. 11/19 (57.9%)	Not significant
		Pituitary giants with *Xq26.3* microduplication (n = 12)	Disease control at last follow-up	11/12 (91.7%)	N/A
			Proportion multimodal (≥ 2) treatments	9/12 (75%)	N/A
			Frequency of hypopituitarism	8/12 (66.7%)	N/A
Beckers *et al.*[15]		Pituitary giants with *Xq26.3* microduplication (n = 18)	Hormonal control at last follow-up	14/18 (77.8%)	N/A
			Control of excessive growth	13/17 (76.5%)	N/A
			Proportion multimodal (≥ 2) treatments	14/18 (77.8%)	N/A
			Proportion neurosurgery	17/18 (94.4%)	N/A
			Frequency of hypopituitarism	12/17 (70.6%)	N/A
			Hormonal control by DA or SSA as primary treatment	0/9 (0%)	N/A
			Median reduction of GH/IGF-1 by SSA as primary treatment	37.5%	N/A
			Median reduction of GH/IGF-1 by SSA as secondary treatment	14.2%	N/A
			Hormonal control by surgery as primary treatment	3/9 (33.3%)	N/A

Abbreviations: DA, dopamine agonist; GH, growth hormone; IGF-1, insulin-like growth factor 1; IQR, interquartile range; N/A, not applicable; NFPA, non-functioning pituitary adenoma; PA, pituitary adenoma; SSA, somatostatin analogue.
a: Number of *AIP*-mutated patients with long-term SSA treatment: 38.
b: Number of controls with long-term SSA treatment: 164.
c: Regression analysis: the presence of a *MEN1* mutation showed to be a significant and independent predictor of dopamine agonist (t = 3.052, P = 0.004).

Supplemental Material 5 References

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CHAPTER 3

Opposite incidence trends for differentiated and medullary thyroid cancer in young Dutch patients over a 30-year time span

Chantal A. Lebbink[1,2*], Medard F.M. van den Broek[3*], Annemiek B.G. Kwast[4], Joep P.M. Derikx[5], Miranda P. Dierselhuis[2], Schelto Kruijff[6], Thera P. Links[7], A.S. Paul van Trotsenburg[8], Gerlof D. Valk[3], Menno R. Vriens[9], Annemarie A. Verrijn Stuart[1], Hanneke M. van Santen[1,2#], Henrike E. Karim-Kos[2,4#]

**contributed equally to this manuscript, #contributed equally to this manuscript*

[1] Department of Pediatric Endocrinology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands
[3] Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[4] Department of Research and Development, Netherlands Comprehensive Cancer Organisation (IKNL), Utrecht, The Netherlands
[5] Department of Pediatric Surgery, Emma Children's Hospital, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, The Netherlands
[6] Department of Surgery, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[7] Department of Endocrinology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands
[8] Department of Pediatric Endocrinology, Emma Children's Hospital, Amsterdam University Medical Centers, University of Amsterdam, Amsterdam, The Netherlands
[9] Department of Endocrine Surgical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands

Submitted




Abstract

Background

Thyroid cancer is the most common endocrine malignancy in children. A rising incidence has been reported worldwide. Possible explanations include increased use of enhanced imaging (leading to incidentalomas) and an increased prevalence of risk factors.

Aim

To evaluate the incidence and survival trends of thyroid cancer in Dutch children, adolescents and young adults (0-24 years) between 1990 and 2019.

Methods

Age-standardized incidence rates of differentiated thyroid cancer (DTC, including papillary and follicular thyroid cancer (PTC and FTC, respectively)) and medullary thyroid carcinoma (MTC), average annual percentage changes (AAPC) in incidence rates, and 10-year overall survival (OS) were calculated based on data obtained from the nationwide cancer registry (Netherlands Cancer Registry).

Results

A total of 839 patients aged 0-24 years had been diagnosed with thyroid carcinoma (PTC: 594 (71%), FTC: 128 (15%), MTC: 114 (14%)) between 1990-2019. Incidence of PTC increased significantly over time (AAPC +3.6%; 95%CI +2.3 to +4.8), the incidence rate of FTC showed a stable trend (AAPC -1.1%; 95%CI -3.4 to +1.1), while the incidence of MTC decreased significantly (AAPC: -4.4% (95%CI -7.3 to -1.5). The 10-year OS was 99.5% (1990-1999) and 98.6% (2000-2009) in patients with DTC and 92.4% (1990-1999) and 96.0% (2000-2009) in patients with MTC.

Conclusion

In this nationwide study, a rising incidence of PTC and decreasing incidence of MTC were observed. For both groups, in spite of the high proportion of patients with lymph node involvement at diagnosis for DTC and the limited treatment options for MTC, 10-year OS was high.

Introduction

Thyroid cancer accounts for 2-6% of all pediatric malignancies, making it the most common endocrine cancer in children.[1–4] Thyroid malignancy is the eighth most frequently diagnosed cancer among adolescents (15-19 years) and the second most common cancer in adolescent girls, due to a strong female predominance of differentiated thyroid carcinoma (DTC) which usually manifests during puberty.[5,6] Thyroid cancer comprises a wide spectrum of histological subtypes; most importantly, on the one hand two types of DTC originating from follicular cells (papillary and follicular thyroid cancer (PTC and FTC, respectively)), while on the other hand medullary thyroid carcinoma (MTC), deriving from parafollicular C cells. The papillary subtype represents the large majority of cases in children with thyroid cancer (83%), followed by FTC (10%) and MTC (5%).[7,8] In pediatric patients with DTC, lymph node involvement and distant metastases at time of diagnosis occur more frequently than in adults.[9–11] Nevertheless, children with thyroid cancer – particularly PTC and FTC – have an excellent prognosis.[8,12–14] Pediatric MTC is not susceptible to radio-iodine treatment, and – especially in advanced stages – is associated with a worse survival.[7,8,15] For this reason, in familiar cases such as the multiple endocrine neoplasia type 2 (MEN2) syndrome, prophylactic thyroidectomy is advised.[16]

A rising incidence in pediatric thyroid cancer – especially PTC – over the last decades has been reported in several studies and matches epidemiological findings about thyroid malignancy in adults.[2,7,11,12,14,17–20] In the USA, the incidence of thyroid cancer in patients aged 0-19 years showed a gradual but significant annual percent change (APC) of +1.1% during the period 1973-2006, while it markedly increased thereafter (APC 2006-2013: +9.6%).[14] Studies in Europe and South Korea have demonstrated comparable results.[13,17,20] There is an ongoing debate about the underlying mechanisms that may explain this phenomenon. Some authors postulated that the rising incidence of pediatric DTC is attributable to overdiagnosis, driven by the combination of the expanding usage of imaging studies, enhanced imaging techniques, in combination with the high prevalence of indolent differentiated thyroid tumors even in juvenile population.[21,22] On the contrary, others have suggested that the concurrent increased incidence of large tumors and advanced-stage disease is proof of a "true" rise in pediatric DTC.[11,14,19,23] Although studies into etiological factors have mainly focused on adult thyroid cancers, suggested explanations in children are increased obesity prevalence and radiation exposure.[24,25] In addition, part of the increased incidence may be explained by a rise in secondary DTC in a growing number of childhood cancer survivors (CCS), due to both an increased incidence of childhood cancer as well as an improved prognosis.[26,27] For example, the 5-year survival for pediatric cancer patients improved from 63% in the 1970s to 84% in the period 2010-2016 in the USA, and

uprising trends have been documented in Europe as well.[27–29] Considering the significant number of CCS who have received external radiation therapy – an established risk factor for DTC[30] – a rise in incidence of secondary DTC seems probable.

The Netherlands has a comprehensive national cancer registry (Netherlands Cancer Registry, NCR), which creates the opportunity to investigate the true incidence trends of thyroid malignancy with great accuracy. We aimed to evaluate pediatric thyroid cancer incidence and survival trends during the period 1990 to 2019, based on patient and tumor characteristics among patients aged 0-17 years in the Netherlands using the population-based data of the NCR. Young adults (18-24 yr) were also included as a (post-pubertal) comparative group, embodying the youngest patients treated in adult oncology centers.

Patients and methods

Study population

All patients below 25 years of age diagnosed with thyroid carcinoma during the period January 1990 to December 2019 in the Netherlands were selected from the Netherlands Cancer Registry (NCR).

Definitions

Thyroid carcinoma cases were classified according to the International Classification of Diseases for Oncology, Third Edition (ICD-0-3) by topography (C73) and histology: papillary (ICD-O-3 M8050, M8140, M8201, M8260, M8340-44, M8350, and M8504), follicular (ICD-O-3 M8290, M8330-32, M8335, and M8339), medullary (ICD-O-M8345, M8510-11) thyroid carcinoma with ICD-O-3 behaviour code/3 and others (ICD-O-3 M8000, M8337, and M8346).

Tumor staging was recorded according to the TNM (Tumor, Node, Metastasis) classification system of the Union for International Cancer Control (UICC). The edition applicable at time of diagnosis of thyroid carcinoma was used.[31] In case of a missing pathological TNM classification, the clinical TNM was used.

For this study, patients were divided into the following age groups: children (0-9 years and 10-14 years), adolescents (15-17 years), and young adults (18-24 years).

Patients were classified as treated in a university hospital if they received thyroidectomy and/or radio-iodine treatment in a university hospital.

The Netherlands Cancer Registry

The nationwide population-based NCR is maintained and hosted by the Netherlands Comprehensive Cancer Organisation (IKNL) and has a national coverage since 1989 with a completeness of at least 96% of all new diagnosed malignancies in the Netherlands.[32] The NCR relies on comprehensive case notification through the Nationwide Network and Registry of Histopathology and Cytopathology, and the national Registry of Hospital Discharges. Retrospectively, data were extracted on patient, tumor and treatment characteristics. Information on vital status (*i.e.*, alive, dead, or emigration) was obtained by annual linkage of the NCR with the Nationwide Population Registries Network that holds vital statistics on all residents in the Netherlands. Last linkage was at February 1st, 2021.

Statistical analyses

Characteristics of the study population were described as percentages in relation to the three periods of diagnosis: 1990-1999, 2000-2009 and 2010-2019. In addition, patient characteristics were analysed for the following age groups: 0-9, 10-14, 15-17, and 18-24 years for DTC and 0-17, 18-24 years for MTC. Differences among categorical variables were tested with the $\chi 2$ tests or the Monte Carlo estimate for the Exact test in case of small numbers.

Annual incidence rates were calculated per million person-years, using the annual mid-year population size as obtained from Statistics Netherlands. Rates were age-standardized according to the age structure of the World standard population for age ranges 0-9, 0-17, and 0-24 years.[33] Incidence rates were presented in the figures as three-year moving averages by taking the average of the rates of each given year and the rates either side of it. Changes in incidence over time were evaluated by calculating the average annual percentage change (AAPC) along with the corresponding 95% confidence intervals (CI). AAPC was derived from linear regression modelling, including the calendar year as a continuous variable.[33] Joinpoint regression program (version 4.5.0.1) was used to check for trend transitions during the study period.[34] The null hypothesis assumed that the AAPC was constant throughout the study period. The permutation test was used to determine the number of joinpoints by default set to a maximum of four.[35] For each detected joinpoint, the AAPC and corresponding 95% CIs were reported for each of the linear segments identified prior and next to the detected joinpoint.

Survival time was calculated as the time elapsed between the date of diagnosis and the date of death due to any cause (event) or censoring (*i.e.*, loss to follow-up, emigration or February 1st, 2021), whichever came first. Traditional actuarial survival analysis was used to calculate overall survival (OS) at 10 years after diagnosis. Kaplan-Meier curves and the logrank test were used for visualization and comparison of survival between DTC and MTC, respectively. Additional survival analysis to evaluate changes in survival over time was not possible due to the low number of events.

Incidence analyses were performed using SAS software (SAS system 9.4, SAS Institute, Cary, NC), whereas STATA/SE 16.1 (StataCorp LP, College Station, Texas, USA) was used for survival analyses. A *P*-value < 0.05 was considered statistically significant.

Results

A total of 839 children, adolescents and young adults, aged 0-24 years, had been diagnosed with thyroid carcinoma between 1990-2019. The most common histopathological tumor subtype was papillary thyroid carcinoma (PTC), accounting for 71% of the cases (*n* = 594). Follicular thyroid carcinoma (FTC) and medullary thyroid carcinoma (MTC) were found in 15% (*n* = 128) and 14% (*n* = 114) of the cases, respectively. Three patients had been diagnosed with a mixed/other histologic tumor type. Overall, the incidence of thyroid carcinoma increased in children/adolescents/young adults between 1990-2019 with an AAPC of +1.4% (95%CI 0.4 to 2.4) (Figure 1). Further results are described by subgroup: DTC (consisting of PTC and FTC) and MTC.

Differentiated thyroid carcinoma (DTC)

In a 30-year time span, 722 children, adolescents and young adults had been diagnosed with DTC. The incidence of DTC in children/adolescents/young adults increased significantly over time, from 3.1 per million person-years between 1990-1999 to 5.3 per million person-years between 2010-2019, with an AAPC of +2.6% (95%CI +1.6 to +3.7) (Figure 1). No joinpoints were identified, which implicates a steady increase of incidence over time. Similar shifts in incidence were found, when specifically looking into PTC; the incidence of PTC increased significantly over time (AAPC +3.6%; 95%CI +2.3 to +4.8). In contrast, the incidence rates among FTC showed a stable trend, although the number of patients diagnosed with FTC was very low. The age-specific incidence rates are presented in Figure 2A and 2B. Incidence of DTC among boys as well as girls increased significantly over time (Supplemental Material, Table S1). When focusing on age subgroups, the increasing incidence of PTC was seen in all age groups ≥ 10 years, however this was only significant in young adults (*P* < 0.001).

Of all patients with DTC, 28% (*n* = 204) were < 18 years of age (Table 1A); only 2% (*n* = 13) of the cohort was aged < 10 years at diagnosis of DTC. The age distribution of DTC did not differ over time. Girls were more often affected than boys (78% vs. 22%, respectively) regardless of age (Figure 3). DTC as second primary cancer was observed in 2% (*n* = 18, all PTC) of the patients. Most patients with DTC (42%) were found to have a T2 stage tumor. The distribution of T-stage changed significantly over time (*P* < 0.001), with a shift from T4 stage to T3 stage and from T2 stage to T1 stage. In more than 40% (*n* = 300) of

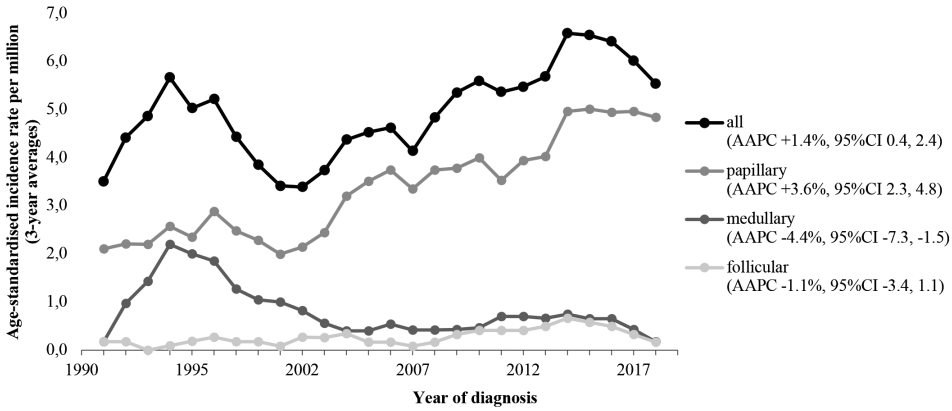

Figure 1. Time trends in incidence of patients aged 0-24 years with thyroid carcinoma in the Netherlands, 1990-2019
Three-year moving averages of the age-standardised incidence rate of thyroid carcinoma (standardised according to the World Standard Population) are shown. AAPC was estimated from a regression line, which was fitted to the natural logarithm of the rates using year of diagnosis as regressor variable.
Abbreviations: AAPC, Average Annual Percent Change; CI, Confidence Interval.

the patients with DTC, lymph node metastases were found in the pathological report. Children and adolescents were diagnosed with lymph node metastases more often compared to young adults (54% vs. 40%, $P = 0.001$). Significantly more lymph nodes metastases were reported in the period 2000-2009 (52%, $P = 0.02$). Distant metastases were reported in 3% of the patients with DTC in total, which were more frequently found in children (15% vs. 2% in older patients, $P < 0.001$). Characteristics of DTC by age group are presented in Supplemental Material, Table S2.

Over the years, patients with DTC were treated at a university hospital significantly more often (48% in 1990-1999, 67% in 2000-2009 and 73% in 2010-2019, $P < 0.001$). Especially in children and adolescents (0-17 years) this shift was noticeable (Figure S1).

The median follow-up of all patients with DTC was 12.2 years. A total of 14 patients with DTC died during follow-up (12 PTC, 2 FTC, five of them within 10 years of follow-up), from which cause of death was unknown. The 10-year overall survival was comparable between 1990-1999 and 2000-2009 (99.5% vs. 98.6%, respectively).

Medullary Thyroid Carcinoma (MTC)
A total of 114 patients had been diagnosed with MTC during the study period. The incidence of MTC significantly decreased from 1.3 per million person-years in 1990-1999 to 0.5 per million person-years in 2010-2019 (AAPC: -4.4% (95%CI -7.3 to -1.5)) (Figure 1).

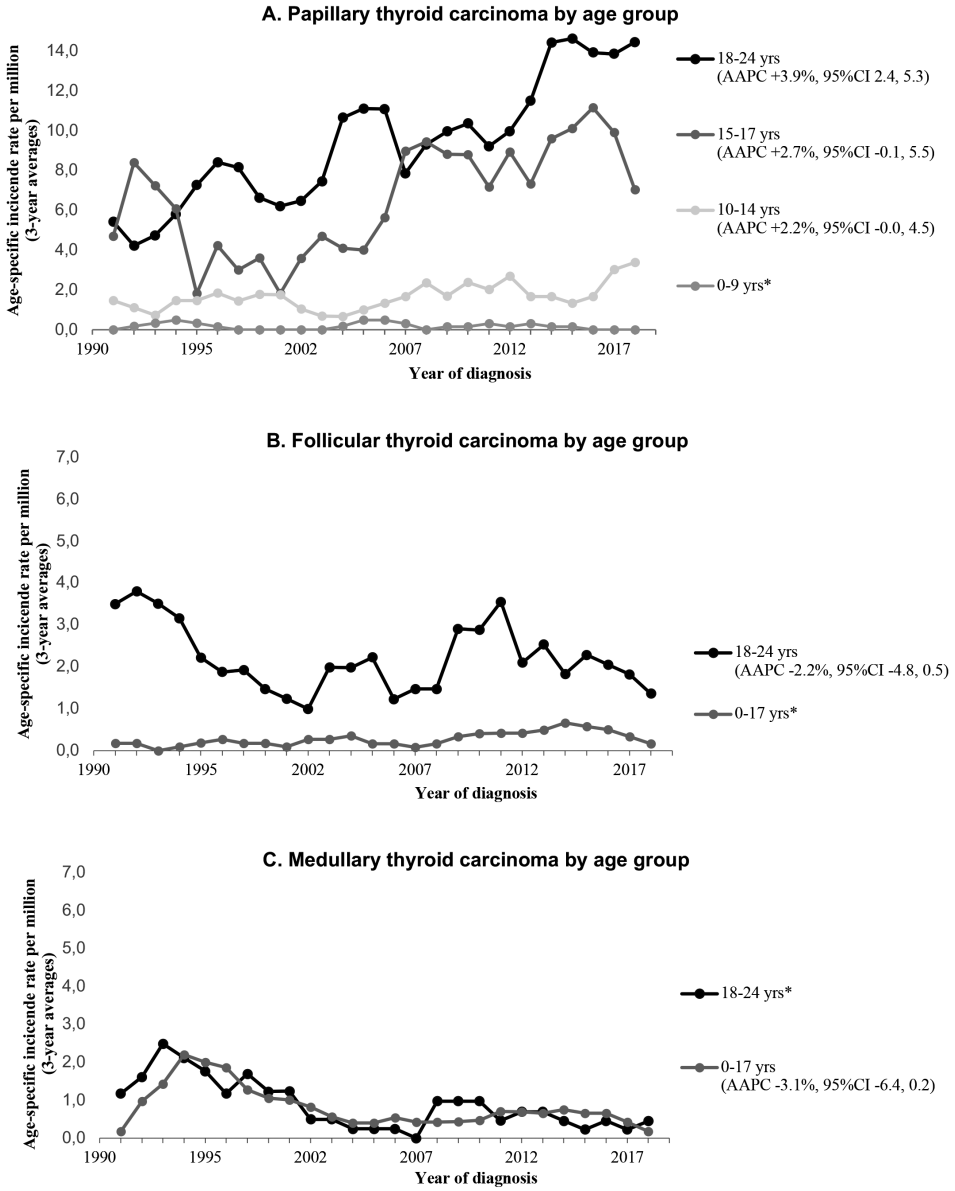

Figure 2. Time trends in incidence of patients aged 0-24 years with thyroid carcinoma by histology and age in the Netherlands, 1990-2019

Three-year moving averages of the age-specific incidence rate of thyroid carcinoma are shown. The incidence rates of the patients 0-9 and 0-17 years are age-standardised according to the World Standard Population. AAPC was estimated from a regression line, which was fitted to the natural logarithm of the rates using year of diagnosis as regressor variable.

* estimation of a reliable average annual percentage change was not possible because of $n = 0$ in >5 incidence years

Abbreviations: AAPC, Average Annual Percent Change; CI, Confidence Interval.

Table 1A. Characteristics of differentiated thyroid carcinoma patients aged 0-24 years in the Netherlands, 1990-2019

	Total		Average per year	Period of diagnosis						
				1990-1999		2000-2009		2010-2019		
	N	%	N	N	%	N	%	N	%	*P*-value
	722		24	186	26	223	31	313	43	
Age (in years)										.75
0-9	13	2	0	4	2	5	2	4	1	
10-14	61	8	2	16	9	18	8	27	9	
15-17	130	18	4	27	15	46	21	57	18	
18-24	518	72	17	139	75	154	69	225	72	
Median age (interquartile range)	20 (17-23)			21 (17-23)		20 (17-23)		20 (17-23)		.23
Sex										.98
boys	162	22	5	42	23	49	22	71	23	
girls	560	78	19	144	77	174	78	242	77	
Histology										.002
papillary carcinoma	594	82	20	138	74	185	83	271	87	
follicular carcinoma	128	18	4	48	26	38	17	42	13	
T stage[a]										<.001
1	198	28	7	25	14	64	31	109	35	
2	292	42	10	101	57	82	39	109	35	
3	145	21	5	22	13	41	20	82	26	
4	62	9	2	28	16	22	11	12	4	
unknown (3% of total)	25		1	10		14		1		
N stage[a]										.02
0	379	56	13	98	59	99	48	182	59	
1	300	44	10	68	41	108	52	124	41	
unknown (6% of total)	43		1	20		16		7		
Metastases[a]										.41
no	606	97	20	140	97	161	95	305	97	
yes	20	3	1	4	3	8	5	8	3	
unknown (13% of total)	96		3	42		54		0		
Thyroid carcinoma as second primary cancer										.65
yes	18	2	1	6	3	6	3	6	2	
no	704	98	23	180	97	217	97	307	98	

Characteristics of the study population, described as percentages in relation to the three periods of diagnosis: 1990-1999, 2000-2009 and 2010-2019. Differences among categorical variables were tested with the $\chi 2$ tests or the Monte Carlo estimate for the Exact test in case of small numbers.

Abbreviations: N, number.

a. Tumor staging was recorded according to the TNM (Tumor, Node, Metastasis) classification system of the Union for International Cancer Control (UICC). The edition applicable at time of diagnosis of thyroid carcinoma was used.

The incidence showed a downward near-significant trend for the younger age group (< 18 years) (AAPC: -3.1% (95%CI -6.4 to +0.2), whereas an estimation of a reliable AAPC was not possible for the young adults (18-24 years) due to the low incidence in this group (Figure 2C). The age at diagnosis and sex distribution did not change significantly over time. Tumor size distribution remained stable during 1990-2019, while the proportion of MTC with regional lymph node involvement showed a significant increase over time ($P = 0.045$) (Table 1B). As shown in Figures 1 and 2C, incidence rates of MTC showed a peak around 1994. Joinpoint analyses could not be performed due to the small number of events.

Table 1B. Characteristics of medullary thyroid carcinoma patients aged 0-24 years in the Netherlands, 1990-2019

	Total		Average per year	Period of diagnosis						
				1990-1999		2000-2009		2010-2019		
	N	%	N	N	%	N	%	N	%	*P*-value
	114		4	66	58	25	22	23	20	
Age (in years)										.67
0-17	78	68	3	43	65	18	72	17	74	
18-24	36	32	1	23	35	7	28	6	26	
Median age (interquartile range)	13 (6-19)			13.5 (8-19)		11 (6-18)		11 (3-18)		.32
Sex										.18
boys	55	48	2	35	53	8	32	12	52	
girls	59	52	2	31	47	17	68	11	48	
T stage[a]										.35
1	83	78	3	48	79	19	83	16	70	
2	12	11	0	7	11	1	4	4	17	
3	6	6	0	2	3	1	4	3	13	
4	6	6	0	4	7	2	9	0	0	
unknown (6% of total)	7		0	5		2		0		
N stage[a]										.045
0	67	72	2	42	82	13	59	12	60	
1	26	28	1	9	18	9	41	8	40	
unknown (18% of total)	21		1	15		3		3		
Metastases[a]										.84
Yes	5	6	0	2	5	1	6	2	9	
no	75	94	3	37	95	17	94	21	91	
unknown (30% of total)	34		1	27		7		0		

Characteristics of the study population, described as percentages in relation to the three periods of diagnosis: 1990-1999, 2000-2009 and 2010-2019. Differences among categorical variables were tested with the $\chi 2$ tests or the Monte Carlo estimate for the Exact test in case of small numbers.
Abbreviations: N, number.
a. Tumor staging was recorded according to the TNM (Tumor, Node, Metastasis) classification system of the Union for International Cancer Control (UICC). The edition applicable at time of diagnosis of thyroid carcinoma was used.

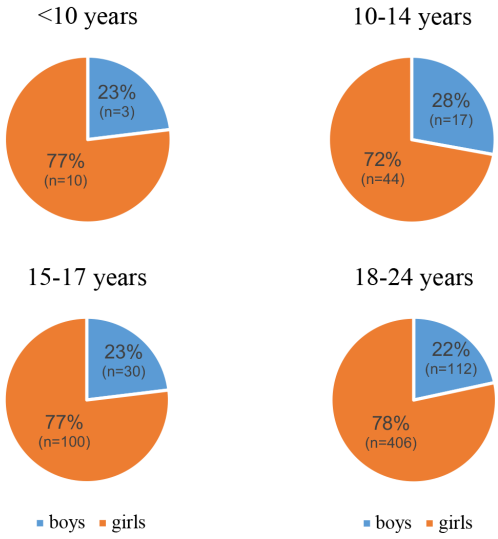

Figure 3. Sex distribution of differentiated thyroid carcinoma within different age groups in the Netherlands, 1990-2019
Sex distribution of differentiated thyroid carcinoma of the age groups < 10, 10-14 years, 15-17 years and 18-24 years. Both percentage as the absolute number of patients are shown.

Boys and girls were equally affected (48% vs. 52% of cases, respectively). In contrast to DTC, the majority of cases had been identified in childhood and adolescence (68% at age 0-17 years). Young adults (18-24 years) diagnosed with MTC suffered from more advanced disease upon diagnosis than younger patients, illustrated by the significantly higher T-stage ($P = 0.01$) and higher proportion of patients with lymph node involvement (54% in young adults vs. 17% in patients <18 years, $P < 0.001$, Table S2B). Likewise, young adults seemed to be diagnosed with metastatic disease more often than children/adolescents (13% vs. 4% respectively), but this trend did not reach statistical significance ($P = 0.14$).
Over the years, patients with MTC were treated at a university hospital significantly more often (68% in 1990-1999, 92% in 2000-2009 and 96% in 2010-2019, $P = 0.003$). This shift in MTC care was detected in both children/adolescents (0-17 year) and young adults (18-24 year) (Figure S1).
The median follow-up of all patients with MTC was 21.1 years. A total of 12 patients with MTC died during follow-up (six of them within 10 years after diagnosis), from which cause of death was unknown. The 10-year overall survival was 92.4% in patients diagnosed in the period 1990-1999 and 96.0% for patients diagnosed in 2000-2009. Patients (all ages) with MTC experienced a significantly worse survival than DTC ($P < 0.001$) (Figure 4).

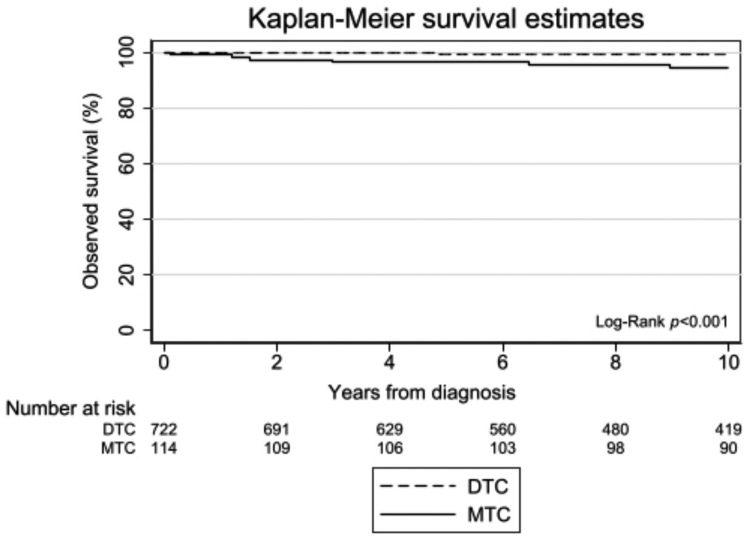

Figure 4. Observed survival of patients, aged 0-24 years with thyroid carcinoma in the Netherlands, 1990-2019
Survival time was calculated as the time elapsed between the date of diagnosis and the date of death due to any cause (event) or censoring (*i.e.*, loss to follow-up, emigration or February 1st, 2021), whichever came first. Logrank test showed a significant different 10-year survival between DTC and MTC: $P < 0.001$.
Abbreviations: DTC, differentiated thyroid carcinoma; MTC, medullary thyroid carcinoma.

Discussion

This nationwide study, spanning three decades, showed opposite incidence trends for DTC and MTC in young individuals: increasing incidence of DTC and decreasing incidence of MTC. In addition, the results of our study confirm the very good prognosis for both DTC as well as MTC in young patients (0-24 years), despite the frequent presence of advanced disease.

Differentiated thyroid carcinoma (DTC)

The incidence rate of DTC in the period 2010-2019 (5.3 per million patient-years) and AAPC of +2.6% in our cohort were comparable to previous studies.[8,11–14] In our cohort, the incidence of DTC seemed to increase over time in all age groups, which is mainly attributed to the increase in PTC over time. In accordance with two previous studies, stable incidence numbers of FTC over time were seen.[13,14] In contrast, Bernier *et al.* have reported an increasing incidence of FTC over time (APC +2.1%) in a larger cohort of children and adolescents 0-19 years with FTC ($n = 644$).[11]

In accordance with previous studies, DTC was most frequently diagnosed in patients > 18 years of age.[13] A preponderance of girls affected with DTC has been a persistent finding in previous studies.[4,8,11–14] It has been suggested that this difference may be induced by estrogens.[36,37] The fact that a predominance of affected girls was also found in patients < 10 years in our cohort was surprising and may indicate that estrogen exposure may not be expected to play a significant role in the etiology of DTC.

We found lymph nodes metastases in more than 40% of the patients with DTC. Golpanian *et al*. reported a comparable percentage of 51% lymph node metastases in a group of children and adolescents with PTC.[12] Compared to Hogan *et al.* and Golpanian *et al.*, we found relatively few patients with distant metastasis (7.8% and 7.9%, respectively, vs. 3%).[7,12] Possibly, this might be explained by a delay in diagnosis in a part of the American cohort as a result of an overall poorer insurance status compared to the Dutch cohort.[38] In line with previous studies, we found that children and adolescents were significantly more often diagnosed with more advanced disease, in terms of lymph node and distant metastases, compared to young adults.[19,39]

Over the time periods, we noted that T-stage of DTC patients at diagnosis shifted from T4 to T3 and from T2 to T1, suggesting that patients were diagnosed at earlier stages. The improved quality and increased use of diagnostic imaging tools over the last decades may have contributed to finding of DTC tumors in an earlier stage. Another explanation could be the transition in the TNM classification system editions over time. Since we based the TNM stage on the TNM edition applicable during the year of diagnosis (no adjustments to the current edition could be made), the altered T-definitions could have influenced our results. For example, the altered definition of T2 (tumor size > 1 cm to ≤ 4 cm during 1990-2002 vs. > 2 cm to ≤ 4 cm afterwards) could presumably explain the shift from T2 towards T1 tumors in recent years.

Medullary Thyroid Carcinoma (MTC)

Contrary to the trend in DTC, the incidence of MTC in children/adolescents and young adults decreased significantly during the study period. Literature on incidence trends of pediatric MTC is very limited. In 2018, Schmidt Jensen *et al*. described a group of 27 Danish children, adolescents and young adults (0-24 years) with MTC, and found no significant change in incidence over time (1980-2014).[13] A year later, Qian *et al*. reported an unchanged rate of MTC throughout the study period (1973-2013) in a cohort of children and adolescents (0-19 years) with MTC in the USA (cohort size unknown).[14] Possible explanations for the differences in incidence trends found in the Danish, the American and now in the Dutch population are the small number of patients in the other cohorts, or differences in approach to timely genetic counseling and preventive thyroidectomies at young age in children diagnosed with MEN2.

Also in contrast to DTC – but in line with previous research – MTC was diagnosed most frequently in the young age group (0-18 yr).[8] The older patients (> 18 yr) suffered from more advanced disease at the time of MTC diagnosis, which may be a reflection of a late diagnosis in non-familiar or familiar index (*de novo*) cases not yet recognized. Genetic syndromes harboring an increased risk for MTC may be difficult to recognize, contributing to the delay in diagnosis of MTC.[40]

The incidence of MTC peaked around 1994 and dropped afterwards. Synchronously, patients with MTC were found to have more advanced disease upon diagnosis in recent years. These findings can possibly be explained by the introduction of pre-symptomatic DNA screening in children from MEN2A families and prophylactic thyroidectomy in children with high risk of MTC, which became common practice after the identification of germline mutations in the *RET* gene as the origin of MEN2 syndromes in the early 90s.[41,42] In the first years after implementation of *RET* mutation screening, many children from MEN2A families were identified with local MTC, which resulted in the earlier mentioned peak incidence around 1994. After this first "wave", DNA screening in early childhood prompted to early prophylactic thyroidectomy before the onset of MTC in the majority of children with MEN2A, explaining the declined incidence of MTC in the following years. On the contrary, unfortunately, children with MEN2B are often not diagnosed until after the development of symptomatic (advanced) MTC, because *RET* mutations occur as *de novo* in 75-90% of MEN2B patients.[16,43] Therefore, implementation of DNA screening did presumably not affect the incidence of MEN2B-related MTC on a large scale. Together with the decrease in MEN2A-related MTC, this may have led to an increased proportion of (late-recognized) MTC in the context of MEN2B. This may also explain our finding of the higher percentage of MTC patients with lymph node involvement at diagnosis found in more recent years. In addition, MTC within the context of MEN2B is known to occur even earlier in life and with a more aggressive behavior when compared to MEN2A.

Site of treatment

Over the years, patients with thyroid carcinoma – both DTC and MTC – were more often treated in a university hospital, reflecting centralization of healthcare. Centralization of care is an important step in improving care for children, adolescents and young adults with rare diseases such as thyroid carcinoma in order to optimize diagnostics, management and outcomes while minimizing the long-term adverse consequences.[44] We could not detect an improvement in 10 year OS over the years. Future studies will have to evaluate whether the number of adverse effects of treatment, such as hypoparathyroidism, have decreased with increasing centralization.

Strengths and limitations

The major strengths of this study include its population-based design (national coverage) and the standardized data collection. These elements resulted in the availability of generalizable and reliable data with low risk of (information) bias. Furthermore, the long follow-up period allowed us to analyze trends in incidence and outcome over a longer period of time, including the effect of implementation of *RET* mutation analysis in MEN2A families.

Our study has several limitations. First, European and national privacy legislation prohibited the usage of data about the cause of death. Second, the NCR used clinical (including ultrasound, computed tomography imaging and functional imaging if available) and pathological data for stage registration until total thyroidectomy, which may have resulted in incorrect data about lymph node status or the presence of distant metastases found at ^{131}I- scanning post-surgery. This may have led to an underestimation of the "true" number of patients with positive lymph nodes or distant metastases. Also, for DTC specifically, as mentioned above, changes in the tumor staging system over time may have influenced the results. For MTC specifically, the low incidence and mortality numbers – despite the national coverage of this study –, prevented us from performing further analyses into factors possibly related to the incidence or survival of MTC. Finally, information about germline *RET* mutations in patients with MTC, the family history of patients and the incidence (trends) of premalignant C-cell hyperplasia would have helped to further elucidate the results of this study, but these data were not available.

In summary, the here reported outcomes of the national Dutch cohort demonstrate an increasing incidence of pediatric PTC between 1990-2019, with a shift towards smaller tumors. This may be a reflection of a true rise, or, alternatively, it may reflect increased usage and quality of diagnostics such as ultrasound of the neck. In contrast, the incidence of MTC decreased during this period, presumably explained by the implementation of pre-symptomatic DNA analysis in MEN2A families in the early 90s. Furthermore, despite more advanced disease in children and adolescents compared to adults, the overall survival rates over the last decades remain high, for both DTC and MTC in young individuals.

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Supplemental Material

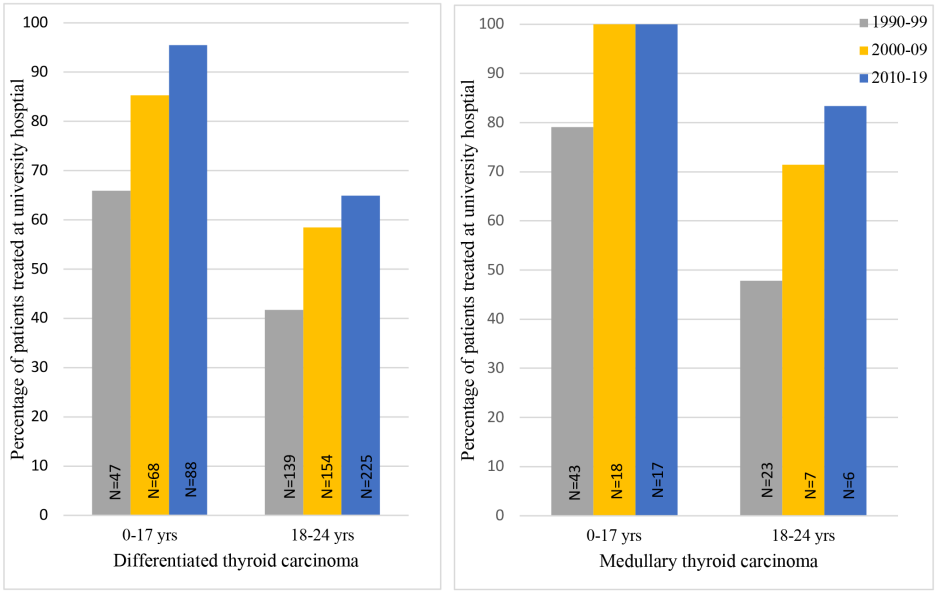

Figure S1. Proportion of patients with thyroid carcinoma aged < 18 years and aged 18-24 years, treated at a university center

Percentage of patients treated at a university hospital, by age group and time period. Patients were classified as treated in a university hospital if they received thyroidectomy and/or radio-iodine treatment in a university hospital. One patient with PTC has been excluded from this analysis, because of treatment abroad.
Abbreviations: N, number.

Table S1. Incidence of thyroid carcinoma in children, adolescents and young adults aged 0-24 years in the Netherlands, 1990-2019

		Period of diagnosis			AAPC (%)			
		1990-1999	2000-2009	2010-2019	95% CI			*P*-value
Incidence of all thyroid carcinomas								
	Average number of new cases / year	25	25	34				
	Incidence rate (per million)	4,4	4,6	5,8	**1.4**	0.4	2.4	.01
Age (in years)								
0-9	Average number of new cases / year	3	2	1				
	Incidence rate (per million)	1,3	0,7	0,8	N.A.			
10-14	Average number of new cases / year	3	2	3				
	Incidence rate (per million)	3,4	2,3	3,0	1.0	-1.6	3.6	.42
15-17	Average number of new cases / year	3	5	6				
	Incidence rate (per million)	6,1	8,4	10,1	2.7	-0.1	5.6	.06
18-24	Average number of new cases / year	16	16	23				
	Incidence rate (per million)	10,6	11,9	15,7	**1.8**	0.7	3.0	.003
Sex								
Boys	Average number of new cases / year	8	6	8				
	Incidence rate (per million)	2,7	2,0	3,0	0.9	-1.3,	3.1	.41
Girls	Average number of new cases / year	18	19	25				
	Incidence rate (per million)	6,2	7,2	8,8	**1.8**	0.7,	2.9	.002
Incidence of differentiated thyroid carcinomas								
	Average number of new cases / year	19	22	31				
	Incidence rate (per million)	3,1	4,0	5,3	**2.6**	1.6	3.7	<.001
Age (in years)								
0-9	Average number of new cases / year	0,4	0,5	0,4				
	Incidence rate (per million)	0,2	0,2	0,2	NA			
10-14	Average number of new cases / year	2	2	3				
	Incidence rate (per million)	1,7	1,8	2,7	**2.9**	0.8	5.1	.01
15-17	Average number of new cases / year	3	5	6				
	Incidence rate (per million)	4,8	7,8	9,5	**3.4**	0.4	6.5	.03
18-24	Average number of new cases / year	14	15	23				
	Incidence rate (per million)	9,1	11,3	15,3	**2.5**	1.4	3.6	<.001
Sex								
Boys	Average number of new cases / year	4	5	7				
	Incidence rate (per million)	1,4	1,7	2,4	**2.9**	1.0	4.8	.01
Girls	Average number of new cases / year	14	17	24				
	Incidence rate (per million)	4,9	6,4	8,3	**2.7**	1.4	4.0	<.001
Incidence of papillary thyroid carcinomas								
	Average number of new cases / year	14	19	27				
	Incidence rate (per million)	2,4	3,3	4,6	**3.6**	2.3	4.8	<.001
Age (in years)								
0-9	Average number of new cases / year	0,3	0,4	0,4				
	Incidence rate (per million)	0,1	0,2	0,2	NA			

Table S1. Continued

		Period of diagnosis			AAPC (%)			
		1990-1999	2000-2009	2010-2019	95% CI			*P*-value
10-14	Average number of new cases / year	1	2	2				
	Incidence rate (per million)	1,5	1,5	2,1	2.2	-0.0	4.5	.05
15-17	Average number of new cases / year	2	4	5				
	Incidence rate (per million)	4,3	6,7	8,1	2.7	-0.1	5.5	.06
18-24	Average number of new cases / year	10	13	20				
	Incidence rate (per million)	6,4	9,3	13,4	**3.9**	2.4	5.3	<.001
Sex								
Boys	Average number of new cases / year	3	4	6				
	Incidence rate (per million)	1,1	1,6	2,2	**3.4**	1.2	5.6	.004
Girls	Average number of new cases / year	10	14	21				
	Incidence rate (per million)	3,6	5,2	7,1	**4.0**	2.0	5.9	<.001
Incidence of follicular thyroid carcinomas								
	Average number of new cases / year	5	4	4				
	Incidence rate (per million)	0,8	0,7	0,7	-1.1	-3.4	1.1	.31
Age (in years)								
0-17	Average number of new cases / year	1	1	1				
	Incidence rate (per million)	0,2	0,3	0,3	NA			
18-24	Average number of new cases / year	4	3	3				
	Incidence rate (per million)	2,6	2,0	1,9	-2.2	-4.8	0.5	.10
Sex								
Boys	Average number of new cases / year	1	1	1				
	Incidence rate (per million)	0,2	0,2	0,2	NA			
Girls	Average number of new cases / year	4	3	4				
	Incidence rate (per million)	1,3	1,2	1,2	-1.3	-4.0	1.4	.32
Incidence of medullary thyroid carcinomas								
	Average number of new cases / year	7	3	2				
	Incidence rate (per million)	1,3	0,5	0,5	**-4.4**	-7.3	-1.5	.003
Age (in years)								
0-17	Average number of new cases / year	4	2	2				
	Incidence rate (per million)	1,2	0,5	0,5	-3.1	-6.4	0.2	.06
18-24	Average number of new cases / year	2	1	1				
	Incidence rate (per million)	1,5	0,5	0,4	NA			
Sex								
Boys	Average number of new cases / year	4	1	1				
	Incidence rate (per million)	1,3	0,3	0,5	NA			
Girls	Average number of new cases / year	3	2	1				
	Incidence rate (per million)	1,3	0,7	0,5	-2.9	-6.1	0.4	.08

Incidence rate is age-adjusted for the following groups: boys, girls, 0-9 years and the total group.
Statistical significant AAPCs are shown in bold.
Abbreviations: AAPC, average annual percentage change, NA, estimation of a reliable average annual percentage change was not possible because of $n = 0$ in >5 incidence years, 95% CI, 95% confidence interval.

Table S2A. Characteristics of differentiated thyroid carcinoma patients aged 0-24 years in the Netherlands by age group, 1990-2019

	Total		Age at diagnosis								
			<10 years		10-14 years		15-17 years		18-24 years		
	N	%	N	%	N	%	N	%	N	%	*P*-value
	722		**13**		**61**		**130**		**518**		
Sex											.73
boys	162	22	3	23	17	28	30	23	112	22	
girls	560	78	10	77	44	72	100	77	406	78	
Time period of diagnosis											.75
1990-99	186	26	4	31	16	26	27	21	139	27	
2000-09	223	31	5	38	18	30	46	35	154	30	
2010-19	313	43	4	31	27	44	57	44	225	43	
Histology											.48
papillary carcinoma	594	82	11	85	50	82	113	87	420	81	
follicular carcinoma	128	18	2	15	11	18	17	13	98	19	
T stage[a]											.05
1	198	28	5	45	14	24	33	27	146	29	
2	292	42	3	27	16	27	50	41	223	44	
3	145	21	2	18	20	34	26	21	97	19	
4	62	9	1	9	9	15	14	11	38	8	
unknown (3% of total)	25		2		2		7		14		
N stage[a]											.01
0	379	56	7	54	25	41	56	47	291	60	
1	300	44	6	46	36	59	63	53	195	40	
unknown (6% of total)	43		0		0		11		32		
Metastases[a]											<.001
no	606	97	10	91	45	83	117	98	434	98	
yes	20	3	1	9	9	17	3	3	7	2	
unknown (13% of total)	96		2		7		10		77		
Thyroid carcinoma as second primary cancer											.02
yes	18	2	2	15	3	5	4	3	9	2	
no	704	98	11	85	58	95	126	97	509	98	

Characteristics of the study population with differentiated thyroid carcinoma, described as percentages, by different age groups: < 10 years, 10-14 years, 15-17 years and 18-24 years. Differences among categorical variables were tested with the $\chi 2$ tests or the Monte Carlo estimate for the Exact test in case of small numbers.
Abbreviations: N, number.

a. Tumor staging was recorded according to the TNM (Tumor, Node, Metastasis) classification system of the Union for International Cancer Control (UICC). The edition applicable at time of diagnosis of thyroid carcinoma was used.

Table S2B. Characteristics of medullary thyroid carcinoma patients aged 0-24 years in the Netherlands by age group, 1990-2019

	Total		Age at diagnosis				
			<18 years		18-24 years		
	N	%	N	%	N	%	*p*-value
	114		78		36		
Sex							.51
boys	55	48	36	46	19	53	
girls	59	52	42	54	17	47	
Time period of diagnosis							.67
1990-99	66	58	43	55	23	64	
2000-09	25	22	18	23	7	19	
2010-19	23	20	17	22	6	17	
T stage[a]							.01
1	83	78	63	85	20	61	
2	12	11	7	9	5	15	
3	6	6	3	4	3	9	
4	6	6	1	1	5	15	
unknown (6% of total)	7		4		3		
N stage[a]							.001
0	67	72	54	83	13	46	
1	26	28	11	17	15	54	
unknown (18% of total)	21		13		8		
Metastases[a]							.14
no	75	94	55	96	20	87	
yes	5	6	2	4	3	13	
unknown (30% of total)	34		21		13		

Characteristics of the study population with medullary thyroid carcinoma, described as percentages, by different age groups: < 18 years and 18-24 years. Differences among categorical variables were tested with the $\chi 2$ tests or the Monte Carlo estimate for the Exact test in case of small numbers.

Abbreviations: N, number.

a. Tumor staging was recorded according to the TNM (Tumor, Node, Metastasis) classification system of the Union for International Cancer Control (UICC). The edition applicable at time of diagnosis of thyroid carcinoma was used.




CHAPTER 4

Timely diagnosis of Multiple Endocrine Neoplasia 2B by identification of intestinal ganglioneuromatosis: a case series

Medard F.M. van den Broek[1], Ester B.G. Rijks[2], Peter G.J. Nikkels[3], Victorien M. Wolters[4], Robert J.J. van Es[5], Hanneke M. van Santen[6], Bernadette P.M. van Nesselrooij[7], Menno R. Vriens[8], Rachel S. van Leeuwaarde[1], Gerlof D. Valk[1], Annemarie A. Verrijn Stuart[9]

[1] Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Netherlands School of Public & Occupational Health (NSPOH), Utrecht, The Netherlands
[3] Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
[4] Department of Pediatric Gastroenterology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[5] Department of Oral and Maxillofacial Surgery, University Medical Center Utrecht, Utrecht, The Netherlands
[6] Department of Pediatric Endocrinology, Wilhelmina Children's Hospital, University Medical Center Utrecht and Princess Maxima Center, Utrecht, The Netherlands
[7] Department of Medical Genetics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[8] Department of Endocrine Surgical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[9] Department of Pediatric Endocrinology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands

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Abstract

Background
Medullary thyroid carcinoma (MTC) in childhood is rare and has an unfavorable prognosis. To improve outcome, early diagnosis is essential. In patients with multiple endocrine neoplasia type 2B (MEN2B), MTC can occur already before the age of one year. Recognition of non-endocrine features of MEN2B may lead to timely diagnosis.

Purpose
To describe how early recognition of non-endocrine features can lead to a timely diagnosis of MEN2B as well as the effect of recognition of premonitory symptoms on prognosis.

Methods
A retrospective case series from the University Medical Center Utrecht / Wilhelmina Children's Hospital, a Dutch national expertise center for MEN patients. All eight MEN2B patients in follow-up between 1976 and 2020 were included and medical records reviewed.

Results
Intestinal ganglioneuromatosis (IGN) as the cause of gastro-intestinal (GI) symptoms was detected in seven patients. In three of them within months after birth. This led to early diagnosis of MEN2B, which allowed subsequent curative thyroid surgery. On the contrary, a MEN2B diagnosis later in childhood – in three patients (also) triggered by oral neuromas/neurofibromas – led to recurrent, persistent and/or progressive MTC in five patients.

Conclusions
Neonatal GI manifestations offer the most important window of opportunity for early detection of MEN2B. By accurate evaluation of rectal biopsies in patients with early-onset severe constipation, IGN can be timely detected, while ruling out Hirschsprung's disease. MEN2B gene analysis should follow detection of IGN and – when confirmed – should prompt possibly still curative thyroid surgery.

Introduction

Multiple endocrine neoplasia 2B (MEN2B) is an autosomal dominant inherited cancer syndrome characterized by the co-occurrence of medullary thyroid carcinoma (MTC) in nearly 100% of patients and pheochromocytoma in 50% of patients. MEN2B differs from multiple endocrine neoplasia 2A (MEN2A) in various aspects; hyperparathyroidism occurs very rarely in MEN2B, while patients do present with numerous non-endocrine manifestations. MEN2B has an estimated prevalence of 0.9-1.7 per million, making it the rarest among the MEN syndromes.[1–3] Activating, gain-of-function germline mutations in the *REarranged Translocation* proto-oncogene (*RET* gene) were identified to cause MEN2 syndromes in the early 90s.[4–6] MEN2A is usually inherited from an affected parent, while *RET* mutations occur as *de novo* in 75-90% of MEN2B patients.[7,8] The *RET* gene encodes a transmembrane tyrosine kinase receptor involved in intracellular signaling pathways of cell development required for renal organogenesis and enteric neurogenesis and is expressed in cells of the thyroid and adrenal glands, thereby explaining a part of MEN2B manifestations.[9] However, the full phenotypic spectrum of clinical manifestations associated with MEN2B has not been clarified yet.

MTC develops during the first years of life in nearly all MEN2B patients. Due to the unfavorable outcome of MTC and its early presentation, a preventive total thyroidectomy is recommended before the age of one year.[7] However, due to the syndrome's rarity and frequent *de novo* presentation, MEN2B syndrome is not frequently recognized during early childhood. As a result, many patients already suffer from locally advanced MTC or even distant metastases when symptoms are recognized and a diagnosis of MEN2B is made.[8] Pheochromocytomas are often diagnosed in the second and third decade of life.[8,10]

Several characteristic non-endocrine manifestations are associated with MEN2B, including gastro-intestinal (GI), orofacial, (musculo)skeletal and ocular manifestations.[8,11,12] It is suggested that timely identification of early MEN2B manifestations can lead to early diagnosis and prevention of (incurable) MTC, thereby improving prognosis and life expectancy.[8,12,13] Especially non-endocrine features can play a key role in early recognition, as they might occur before inoperable MTC develops.[11,12] MEN2B-associated diffuse intestinal ganglioneuromatosis (IGN) frequently leads to severe constipation, feeding intolerance and/or sometimes diarrhea in the first year of life.[11,14] Additionally, ocular symptoms, orofacial features and musculoskeletal manifestations have been reported to occur in early childhood.[11,12] Early recognition of these symptoms may lead to a timely diagnosis of MEN2B and (its associated) MTC.

By meticulously studying the MEN2B population in our Dutch MEN expertise center, we describe how early non-endocrine MEN2B features can lead to a timely recognition of MEN2B in clinical practice, and illustrate the effect of prompt detection on prognosis. Like a previous report from our institute, we aimed to increase awareness for these cardinal MEN2B-associated early symptoms.[15]

Materials and Methods

A retrospective single-center study was conducted in the University Medical Center Utrecht (UMCU), a tertiary referral and national expertise center for pediatric and adult MEN patients. Medical records of all known MEN2B patients were reviewed from first follow-up (1976) until January 2020. Information regarding endocrine and non-endocrine disease was extracted from medical records in a standardized format. Relevant physicians' notes and correspondence, laboratory results, imaging studies and results from genetic analysis were taken into account.

Age-specific reference values were used to interpret laboratory data. Tumor markers used for MTC were calcitonin and carcinoembryonic antigen. Markers for pheochromocytoma were vanillylmandelic acid (VMA) (up to 2004), urine (nor)metanephrine (from 2004 until 2013), and plasma (nor)metanephrines (2013 up to 2020). *RET* mutation analysis was performed according to standard protocols (Sanger sequencing).
Thyroidectomy was performed by an experienced thyroid surgeon together with a pediatric surgeon at the age of six months in case of neonatal diagnosis and otherwise as soon as possible after diagnosis, in line with current international guidelines.[7]
Pathological assessment of thyroid tissue was classified as normal, C-cell hyperplasia (CCH) or MTC. Rectal biopsy tissue was re-evaluated by a dedicated pathologist when the original pathology report did not provide information about the presence or absence of the MEN2B related abnormalities (IGN).

Definitions

Non-endocrine manifestations were reported descriptively, based upon the patients' medical records. Periodic structural examination at non-endocrine departments (*e.g.*, ophthalmology and oral and maxillofacial surgery) was carried out from 2007 onwards. Intestinal ganglioneuromatosis was defined by the presence of giant ganglia combined with an increase in cholinergic nerve fibers in the submucosa of gastro-intestinal tissue. Due to absence of histological diagnosis in most mucosal (oral, ocular) lesions, it was not possible to make a distinction between neuromas and neurofibromas in most cases.

Outcome of thyroid surgery regarding MTC was defined "curative" if calcitonin concentrations were undetectable postoperatively and "persistent" if still detectable post-thyroidectomy. "Recurrence of MTC" was defined as detectable calcitonin concentrations after previous curative surgery and "progressive disease" was defined as increasing calcitonin concentrations and/or evidence of metastatic disease on imaging. TNM stage was assessed using guidelines of the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, 8th edition.[16]

Diagnosis of pheochromocytoma was based on first biochemical evidence (elevated urinary VMA or urinary/plasma (nor-)metanephrines), with confirmation on imaging and pathology.

Written informed consent was obtained from parents (patients aged < 12 years), patients themselves (aged ≥ 16 years) or both (patients aged 12-16 years). The institutional review board of the UMCU approved this study.

Results

Eight MEN2B patients were identified (three males, five females), all carrying a *de novo* NM_020975.6(RET):c.2753T>C (p.Met918Thr) *RET* gene mutation. MEN2B was diagnosed at a median age of 6.3 years (range 0.1-16). Seven patients were still in follow-up at the end of the study and one had died from a metastasized pancreatic adenocarcinoma at age 54. Median clinical follow-up was 10.0 years (range 3.3-38.0). Patient characteristics are shown in Table 1.

Presenting symptoms of MEN2B

MEN2B syndrome was diagnosed solely on GI symptoms in three cases (patients 1-3) and on a combination of GI and other symptoms in two cases (patients 4-5).

The three patients diagnosed with MEN2B exclusively on GI symptoms were admitted to hospital in the first month of life for not passing stools for five days, increasing drowsiness and insufficient intake (patient 1), acute intestinal obstruction (patient 2) and abdominal distention, icterus and feeding difficulties (vomiting, insufficient intake) (patient 3). Patient 2 underwent a diagnostic laparotomy showing a cecal volvulus. Imaging studies in patient 3 revealed a colonic distention due to air retention. Pathological examination of rectal suction biopsies (patient 1 and 3, see Figure 1) and surgically removed tissue (patient 2) showed IGN. In all three cases, subsequent genetic analysis confirmed MEN2B diagnosis. Calcitonin level was 60 ng/l in patient 1 before surgery and unknown in patient 2 (at that

time under treatment elsewhere). In patient 3, the first calcitonin level (at 3 months post-thyroidectomy) was within normal range, with later values all undetectable (see Table 2). Serum calcitonin levels have been reported to be elevated in very young children, therefore we interpreted the value of 60 ng/l in patient 1 as high but not necessarily abnormal for age, based on the report of Basuyau *et al.*[17]

Table 1. Patient characteristics and presenting symptoms of MEN2B cases

Case	Sex	Age at Dx (yr)	Follow-up time (yr)	Presenting symptom(s)	Thyroid at Dx	Pheo[a], age at Dx (yr)
1	F	0.1	12.3	GI problems	CCH	No
2	F	0.3	7.6	GI problems	CCH	No
3	M	0.1	6.3	GI problems	MTC	No
4	M	11.7	13.8	GI problems, DMD, MW, oral NRs, CaL	MTC	Yes, 25
5	F	6.0	29.0	GI problems, DMD, dysmorphia, NRs	CCH[b]	Yes, 29[c]
6	F	15.8	6.0	Cheek NR, neck lump[d]	MTC	Yes, 21
7	F	6.5	3.3	DMD, MW	MTC	No
8	M	16.0	38.0	GR, marfanoid habitus	MTC	Yes, 16[e]

Abbreviations: CaL, Café au lait spot; CCH, C-cell hyperplasia; DMD, delayed motor development; Dx, diagnosis; F, female; GI, gastro-intestinal; GR, growth retardation; M, male; MTC, medullary thyroid carcinoma; MW, muscle weakness; NR, neuroma/neurofibroma; Pheo, pheochromocytoma; yr, years.
a: Anytime during follow-up. Age at first histological diagnosis of pheochromocytoma.
b: Possible MTC.
c: Second primary pheochromocytoma in contralateral adrenal gland at age 33.
d: Suspicion of MEN2B because of cheek neuromas/neurofibromas, surpassed by growing neck lump.
e: Recurrence after initial bilateral adrenalectomy at age 49.

In patient 4 and 5, severe obstipation was present since birth as well. However, rectal suction biopsy (initially) did not raise suspicion of MEN2B in these cases. Rectal biopsy of patient 4 at seven months showed no signs of Hirschsprung's disease; the original pathology report did not mention the presence or absence of IGN. Unfortunately, this tissue specimen could not be retrieved for re-evaluation. Despite extensive investigations into additional symptoms (muscle weakness, delayed motor development), no explanatory diagnosis could be made at that time. Eventually, oral neuromas/neurofibromas at the age of 11 years prompted genetic analysis and the diagnosis of MEN2B syndrome. Examination of rectal tissue of patient 5 at 11 months revealed neuronal colon dysplasia without further specification. At the age of six, the combination of ongoing constipation, delayed motor development, dysmorphic features (bumpy lips, marfanoid habitus, elongated face) and histologically proven oral neurofibromas led to the diagnosis of MEN2B. Recent re-examination of the rectal biopsy tissue did, in retrospect, show clear signs of IGN.

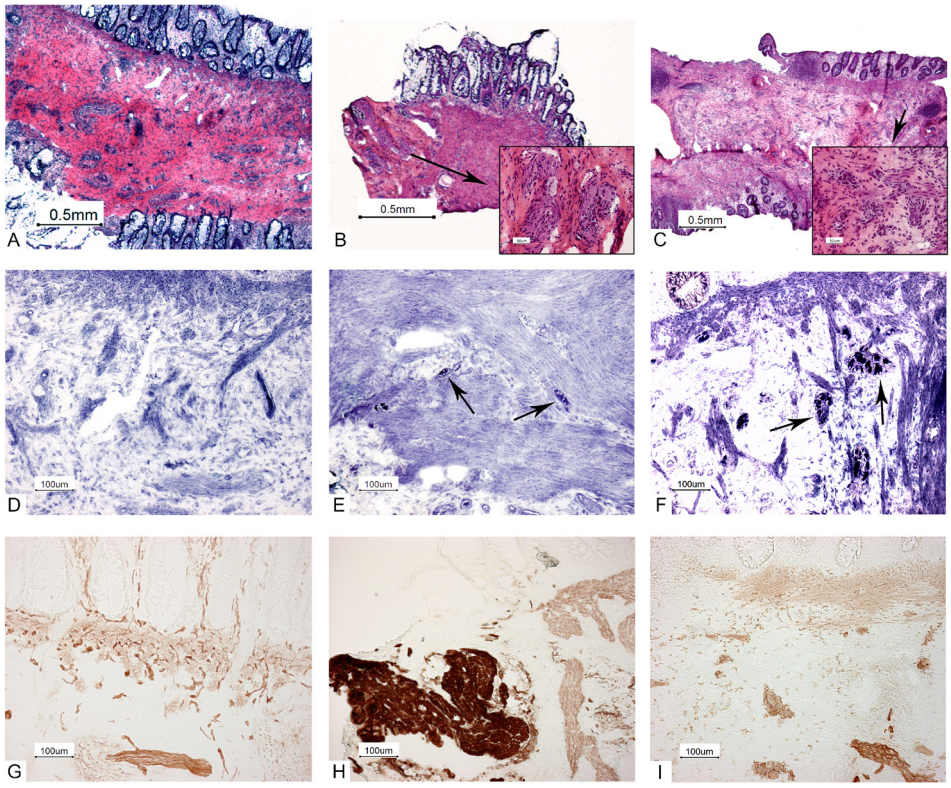

Figure 1. Three frozen rectal suction biopsies
A, **B** and **C** are stained with hematoxylin and eosin (H&E). **D**, **E** and **F** are stained with NADH enzyme stain. NADH stains the cytoplasm of ganglion cells dark blue. The round nucleus of the ganglion cells does not stain and is recognizable as a white round spot in the dark blue stained cytoplasm. **G**, **H** and **I** are stained with acetylcholinesterase without counterstain. Nerve fibres stain dark yellow and the smooth muscle cells stain very weekly positive.
Example patient (male, 2 weeks) (left column: **A**, **D** and **G**) with Hirschsprung's disease: no ganglion cells present in submucosa in NADH enzyme stain (**D**). Increase in cholinergic nerve fibres (**G**) in submusosa, muscularis mucosae and in lamina propria between the crypts (upper part of the picture), characteristic for Hirschsprung's disease.
Patient 7 (girl, 6 years) (middle column: **B**, **E** and **H**): the biopsy from this patient was very small with limited amount of submucosa and not enough for a definite diagnosis of ganglioneuromatosis, but the combination of small groups of ganglion cells (inset of **B** and arrows in **E**) and broad nerve bundles (**H**) was compatible with MEN2B.
Patient 3 (male, 1 month) (right column: **C**, **F** and **I**): biopsy showed ganglioneuromatosis with a normal lamina propria and increase in ganglion cells with giant ganglia (inset of **C** and arrows in **F**) and prominent nerve bundles in the submucosa (**I**: lower part of the picture) and not in the lamina propria (**I**: upper part of the picture).

Table 2. Thyroid disease in cases with MEN2B syndrome

Case	Age at surgery (yr)	Age at last FU (yr)	First available Ctn (ng/l)	Initial thyroid surgery	Histology	TNM (stage) at Dx[a]	Operation Curable	Disease status at last FU
1	0.6	12.4	60[b,c]	TT	CCH	T0N0M0 (n/a)	Yes	Cured
2	0.6	7.9	U[d]	TT ±LND	CCH	T0N0M0 (n/a)	Yes	Cured
3	0.5	6.4	5[e]	TT	MTC	T1aNxMx (l)	Yes	Cured
4	12.0	25.5	360[c]	TT	MTC	T1aNxMx (l)	No	Progressive
5	6.1	35.0	0,32[c,f]	TT	CCH with possible MTC	T0N0M0 or T1aNxMx (n/a or l)	Yes	Recurrence
6	16.0	21.8	8000[c]	TT + cLND + bLND	MTC, IR	T4aN1bM1 (lVc)	No	Progressive
7	6.5	9.8	3500[c]	TT + cLND + uLND	MTC, IR	T3N1bMx (lVa)	No	Persistent
8	16.0	54.0	30[c,g]	TT ±LND	MTC	TxNxMx (?)	No	Persistent

Abbreviations: bLND, bilateral lymph node dissection (LND); C, cured (no biochemical signs of thyroid disease); CCH, C-cell hyperplasia; cLND, central LND; Ctn, calcitonin; Dx, diagnosis; FU, follow-up; IR, irradical resection (tumor identified at the resection margin); ±LND, unknown if LND is performed; MTC, medullary thyroid carcinoma; n/a, not applicable; ng/l, nanogram/liter; P, persistent disease (biochemical signs); Pr, progressive disease; R, recurrent disease (biochemical signs); TNM, tumor node metastasis classification; TT, total thyroidectomy; U, undetectable; uLND, unilateral LND; yr, years; ?, unknown.

a: Staging based upon the American Joint Committee on Cancer (AJCC) Cancer Staging Manual, eight edition (Rosen *et al.*[16]).

b: Calcitonin values can be elevated in (very) young children. For reference values in children, see: Basuyau *et al.*[17]

c: Pre-operative calcitonin level.

d: Three years postoperative calcitonin level. Patient was under treatment in another country at time of surgery; calcitonin levels were not measured earlier.

e: Three months postoperative calcitonin level.

f: µg/l, basal ctn (normal range < 0.3 µg/L) – not stimulable.

g: ng/ml, basal ctn (normal range < 0.4 ng/ml). pentagastrin-stimulated ctn: 455 ng/ml (at 2 minutes), 310 ng/mL (at 5 minutes).

Oral neuromas/neurofibromas were part of the presenting phenotype in three out of eight cases and were the trigger to perform genetic analysis in two (patients 4-5). Patient 6 was referred to our hospital at the age of 15 with mucosal neuromas/neurofibromas, which had been noted for several years but had not triggered suspicion of MEN2B. Before diagnostic work-up of these lesions took place, she developed a growing neck lump caused by MTC. MEN2B diagnosis was confirmed by genetic analysis soon thereafter.

Two patients in this cohort initially presented with musculoskeletal symptoms (delayed motor development, muscle weakness) (patient 7) and the combination of growth restriction and a marfanoid habitus (patient 8).

MEN2B manifestations during follow-up

Total thyroidectomy was performed in all patients; the course of MTC is shown in Table 2. The three patients who had been diagnosed with MEN2B due to timely recognition of GI symptoms had been cured by total thyroidectomy. In total, thyroid surgery cured four out of eight patients; all were operated before the age of 6.5 years. Three other patients underwent several re-operations because of recurrent and/or progressive MTC. At last follow-up, two patients had distant metastases (patient 6: lungs and liver, patient 8: prostate). Four out of eight patients developed pheochromocytoma during follow-up (see Table 1). Complete unilateral adrenalectomy was performed in three cases (patient 4-6), while patient 8 underwent complete bilateral adrenalectomy.

Table 3 provides an overview of all non-endocrine manifestations reported in this series during follow-up. All patients experienced GI manifestations, of which chronic obstipation with varying severity was most common. Feeding problems and obstipation since neonatal period and infancy had been present in six patients. Apart from patient 2 with neonatal volvulus and rectosigmoid resection for ileus at age 3, two additional patients required GI surgery (subtotal colectomies) for therapy-resistant obstipation at a young adult age (21 and 23 years). IGN was confirmed in seven out of eight cases: by histological examination of rectal biopsies in four patients, surgical tissue in two patients and autopsy tissue in one patient.

In total, seven patients had been diagnosed with oral neuromas/neurofibromas. Other oral manifestations included thickened hypertrophic (bumpy) lips and maxillary midline diastema (space between central incisors). Ocular features were present in at least seven patients, including ocular neuromas/neurofibromas, prominent corneal nerves and alacrima (the inability to cry with tears). Joint hyperlaxity – reported in six patients – was the most common musculoskeletal manifestation.

Table 3. Non-endocrine manifestations in cases with MEN2B syndrome

Case	GI	GI therapy	IGN (method of Dx)[a]	MSK	MBH	Oral NMs	Oral	Ocular	Other manifestations
1	+	Oral laxatives Enemas CHT SD	+ (rectal biopsy)	+ HL OD	–	+	+ CD FH	+ ONR A[f] TCN	- Short stature - Transient hypogammaglobulinemia with recurrent respiratory infections - ADUS requiring meatotomy
2	+	Oral laxatives CHT Surgery	+ (surgical tissue)	?	–	–	+ CD FH	+ TCN	- Short stature - Temporarily delay of growth - Anemia due to iron deficiency - Lactose intolerance
3	+	Oral laxatives Enemas	+ (rectal biopsy)	+ HT	–	+	+ CD FH	–	- Short stature - Relapsing conjunctivitis
4	+	Oral laxatives Enemas	– (rectal biopsy)[b]	+ DMD MW HT HL	+	+	+ CD	+ ONR	- Café au lait spot cheek
5	+	Oral laxatives Enemas CHT Surgery	+ (rectal biopsy)[c]	+ DMD HL	+	+	+ CD GH FH	+ ONR TCN	- Dysfunctional voiding requiring CIC
6	+	Oral laxatives CHT Surgery	+[d] (surgical tissue)	+ HL	+	+	-	+ A	- Café au lait spots trunk
7	+	Oral laxatives Enemas	+ (rectal biopsy)[e]	+ DMD MW HT HL	–	+	+ CD FH	+ A	
8	+	Oral laxatives	+ (autopsy)	+ MW HT HL OD	+	+	+ GH	+ ONR TCN	- Temporarily delay of growth - Dysfunctional voiding requiring SCAD - Kyphoscoliosis leading to dyspnea

Non-endocrine manifestations diagnosed in MEN2B patients any time during follow-up.
Abbreviations: +, yes; –, no.
A, alacrima (inability to make tears); ADUS, anterior deflected urinary stream; CD, central diastema; CHT, colon hydrotherapy; CIC, clean intermittent catheterization; DMD, delayed motor development; Dx, diagnosis; FH, frenulum hyperplasia; GH, gingiva hypertrophy ; GI, gastro-intestinal; HL, hyperlaxity; HT, hypotonia; IGN, intestinal ganglioneuromatosis; MBH, marfanoid body habitus; MSK, musculo-skeletal; MW, muscle weakness; NMs, neuromas/neurofibromas; OD, osseous deformities; ONR, ocular neuromas/neurofibromas; SCAD, continuous suprapubic catheter; SD, manual anal internal sphincter dilatation (twice) and botulinum toxin injection into anal internal sphincter (once); TCN, thickened corneal nerves.
a: The method of acquiring intestinal tissue (rectal biopsy, intestinal surgery, autopsy) is specified between the parentheses.
b: Rectal biopsy showed no signs of Hirschsprung's disease. The original pathology report did not mention the presence or absence of IGN. This tissue specimen could not be retrieved for re-evaluation.
c: After recent re-examination of the tissue.
d: No rectal biopsy performed. Intestinal tissue from subtotal colectomy at the age of 21 showed IGN.
e: Biopsy after diagnosis of MEN2B.
f: Unilateral inability to make tears.

Discussion

Timely detection of the MEN2B syndrome is only possible if the complex of symptoms is recognized. This detailed description of MEN2B cases provides insight into the non-endocrine clinical clues for diagnosis of MEN2B, before advanced or metastatic MTC develops. Although our series is small, it firstly illustrates that prevention or curation of MTC was only reached in patients in whom IGN was recognized during diagnostic work-up and thereby led to genetic analysis confirming MEN2B. Secondly, our series underlines that all patients initially presented with non-endocrine symptoms. In retrospect, MEN2B diagnosis could have been established more timely in at least two cases by proper interpretation of gastro-intestinal and orofacial symptoms. Early MEN2B diagnosis was made in three out of eight patients after surgery or rectal suction biopsy for suspicion of Hirschsprung's disease. In two other cases, oral neuromas/neurofibromas led to genetic analysis later in childhood, making this feature of MEN2B a second key element for early diagnosis.

Over the years, reports of cohorts of MEN2B patients have shown that establishing a timely diagnosis is both challenging and critical, as diagnostic delay results in worse outcome.[13,18,19] A median age at thyroidectomy of 14 years in the largest cohort to date (including 345 MEN2B patients) reflects the typical late diagnosis, as does the relatively small fraction of patients (20 out of 338) who were operated before the recommended age of one year.[8] MEN2B has been detected relatively early in life in our case series (median age at diagnosis: 6.3 year), whereas the mean age at MEN2B diagnosis reported in literature ranges from 10.6 to 18 years.[13,18,20–22] It is important to consider the possible effect of study period on the age at MEN2B diagnosis when comparing these results, due to the lack of DNA analysis and lower awareness for MEN2B (especially non-endocrine features) in earlier years. However, the early detection of MEN2B in our case series might also be partly explained by timely referral of young children with profound GI problems to a tertiary care hospital with both a possibility to perform rectal biopsies as well as dedicated pathologists highly aware of IGN.

Although several others have described the frequent presence of neonatal and early-childhood GI symptoms in MEN2B patients [23–27], earlier studies do not focus on the clinical point we wish to make here: prevention or curation of MTC can be reached if IGN is timely recognized as the first non-endocrine manifestation of MEN2B. Severe GI symptoms in the first months of life were present in five out of eight patients in our series and IGN led to a diagnosis of MEN2B in three of them. Rectal suction biopsy is a valuable tool in diagnosing MEN2B. In this case series, IGN was reported in three out of five patients who

underwent rectal biopsies (60%), while recent re-evaluation of the biopsy from patient 5 also showed IGN. As the tissue from patient 4 could not be retrieved for re-examination, we cannot rule out that the incidence of IGN in rectal biopsies may even be 100% in our series. In a literature review on GI symptoms in MEN2B patients, IGN was found in 14 out of 25 (56%) rectal biopsies. Furthermore, IGN was detected in 32 bowel specimens when rectal and transabdominal biopsies were combined and directly led to the diagnosis of MEN2B in 27% (15 patients), which is comparable to our findings.[14]
Thus, awareness under pediatricians, pediatric gastroenterologists, pathologists and other physicians in the field of pediatrics for IGN as a distinctive early sign of MEN2B is of great importance.

The outcomes of our case series underline previous findings on premonitory symptoms of MEN2B in larger cohorts. Gastro-intestinal signs were, when reported, present in around two-thirds of the patients included in the international cohort by Castinetti *et al*., compared to 100% of patients in this case series.[8] However, differences in study setting (multicenter vs. single-center), study methods and study period make it hard to compare these results properly. In a detailed case-control study including 25 MEN2B patients, Brauckhoff *et al*. reported that constipation was the second most distinguishing early sign of MEN2B.[11] In a recent cohort study describing the age-related occurrence of physical stigmata in 24 MEN2B patients, gastro-intestinal (and musculoskeletal) symptoms preceded symptoms of MTC significantly.[12] Likewise, the onset of GI symptoms occurred in the first year of life in 29 out of 55 MEN2B patients (53%) described in the literature review by Gfroerer *et al*.[14] It was not specified whether these GI symptoms, when recognized, led to a timely (curative) thyroidectomy.

Oral neuromas/neurofibromas were the trigger to perform genetic analysis in two cases, while among the presenting symptoms in one more (out of eight cases), making it a second key element in diagnosing MEN2B. The association between mucosal neuromas/neurofibromas and MEN2B has been described earlier.[28–32] In retrospect, these manifestations had been present since childhood in most, yet unrecognized, and became more pronounced in adolescence in the majority of earlier reported cases.[11,12] In the recently published German GPOH-MET registry study, the relatively late appearance of mucosal neuromas/neurofibromas (mean age 10.1 year) did not significantly precede symptoms of MTC.[12] However, this feature should be a trigger for further diagnostic work-up and can lead to MEN2B diagnosis. [33–35] The characteristic wide maxillary midline diastema is a non-specific feature, as a midline diastema is a normal stage of dental development with a prevalence of 25-40% in children with a mixed dentition.[36] Because most children regularly visit their dentist, awareness among oral health care professionals about the typical orofacial symptoms of MEN2B should be increased.[32]

Tearless crying (alacrima) is a rare sign and though possibly a feature of multiple genetic disorders.[37] It has also been reported as a potentially promising clue for timely diagnosing MEN2B: in the earlier mentioned case-control study using questionnaires, alacrima in the first year of life was reported by 86% of parents of MEN2B patients vs. 0% of parents of healthy controls, making it the most distinguishing early sign for MEN2B in their study.[11] In other cohorts, alacrima was reported less frequently (17-40%).[8,12] This discrepancy could be explained by the different data source used in these studies (medical records), considering the potential underreporting of this symptom to the treating physicians. In our series, alacrima was present in two out of the five patients who were subjected to a structured examination at the ophthalmology department, but not part of the presenting symptoms in any of our patients. Whether alacrima has a valuable role in detecting MEN2B should be further investigated in larger prospective cohorts.

In conclusion: it is important to detect IGN in rectal biopsies even when the primary focus usually lies on the possible absence of ganglion cells, as by identification of IGN a harmful delay of diagnosis of MEN2B can be avoided. Thus, the diagnostic work-up of neonatal GI manifestations, especially severe and very early-onset constipation, may create a window of opportunity for detection of MEN2B syndrome before patients suffer from locally advanced or metastasized MTC. Oral neuromas/neurofibromas in childhood may alert oral health care professionals or treating physicians for presence of the MEN2B syndrome. Large, international, prospective studies or databases on MEN2B patients would provide further insight into the sequence of manifestations and thus may allow early identification, ameliorating the course of MTC. Education of pediatricians, pathologists, gastroenterologists, as well as medical students, dentists and medical consultation agencies upon early identification of non-endocrine manifestations – especially gastro-intestinal and oral – may help to recognize children with the MEN2B syndrome in time.

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CHAPTER 5

Children with Multiple Endocrine Neoplasia type 2B: not tall and marfanoid, but short with normal body proportions

Medard F.M. van den Broek[1], Hanneke M. van Santen[2,3],
Gerlof D. Valk[1], Annemarie A. Verrijn Stuart[2]

[1] Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands;
[2] Department of Pediatric Endocrinology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands;
[3] Princess Maxima Center for Pediatric Oncology, Utrecht, The Netherlands

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Abstract

Objective

Multiple Endocrine Neoplasia 2B (MEN2B) is characterized by early-onset medullary thyroid carcinoma (MTC), pheochromocytoma and several non-endocrine manifestations. Unfortunately, MEN2B is often diagnosed late, after the development of clinically significant MTC. Marfanoid habitus is considered an important related feature, which may lead to the assumption that patients with MEN2B have tall stature. Here, we describe the longitudinal growth and body proportions of eight MEN2B patients during childhood.

Design

Retrospective case series.

Methods

Patients were under care of a Dutch MEN expertise center. Growth patterns were assessed and interpreted in relation to body mass index (BMI), age at diagnosis and at thyroidectomy, extensiveness of disease manifestations and parental height.

Results

Seven patients were short during childhood, of whom four showed growth below target height range (THR) and three at the lowest margin of THR. Only one patient grew well within THR. All patients who attained final height ($n = 4$) ended within THR, despite short stature during childhood. Arm span/height ratio was not increased and upper segment/lower segment ratio was not reduced in any patient. Short stature in childhood in this study did not seem to be associated with age at diagnosis, age at thyroidectomy, extensiveness of MTC, endocrine deficiencies or BMI.

Conclusions

This study shows that children with MEN2B may well present with short rather than tall stature. Thereafter, final height within THR was attained in those who already reached adulthood, but none had tall stature. Finally, body proportions were normal in all children and adults in this case series, not underlining a "marfanoid" body habitus.

Introduction

Multiple Endocrine Neoplasia type 2B (MEN2B) is an autosomal dominant inherited cancer syndrome. In the majority of patients, it is caused by a *de novo* germline mutation in the *REarranged Translocation* proto-oncogene (*RET* gene) (c.2753T>C (p.Met918Thr)).[1–3] It is an extremely rare entity (estimated prevalence 0.9-1.7 per million) characterized by the combination of very early-onset medullary thyroid carcinoma (MTC) in nearly all patients, a 50% lifetime risk of pheochromocytoma as well as several non-endocrine manifestations.[4–7] Some of the latter can occur during the first years of life, thereby creating a window of opportunity for timely recognition and diagnosis of MEN2B, before the development of MTC.[8,9] More specifically, reported signs that should call for alert are intestinal ganglioneuromatosis, mucosal neuromas/neurofibromas, alacrima and a "marfanoid" body habitus.[8–10]

The frequently reported but rarely specified MEN2B-related "marfanoid" habitus refers to signs that resemble the characteristics of patients with the Marfan syndrome, such as tall stature, long limbs and hyperlaxity.[11] In the largest MEN2B cohort to date (n = 345), a "marfanoid" habitus was reported in 73% of patients.[7] Somewhat paradoxically, a recent German study suggested that short stature might be associated with the MEN2B syndrome.[9] However, data on parental height was missing and data on follow-up of patients were limited.

In this report, we aimed to describe growth patterns, body proportions and final height (FH) in children and adolescents with MEN2B syndrome in a Dutch MEN expertise center. Furthermore, we intended to relate growth patterns to age at MEN2B diagnosis and thyroidectomy, extensiveness of MTC as well as other possible growth-influencing parameters, such as body mass index (BMI), gastrointestinal manifestations and endocrine status.

Materials and Methods

A retrospective medical record review was performed of all patients diagnosed with MEN2B syndrome during the period 1976-2020 under care at the Wilhelmina Children's Hospital/ University Medical Center Utrecht (UMCU), a tertiary referral and European Reference Network (ERN) expertise center for MEN patients in the Netherlands. The primary outcomes of interest were patients' growth pattern, body proportions and FH – reported as age- and sex-related standard deviation (SD) scores (SDS)[12] – in relation to target height

(TH). Secondary, we described the association between growth and possible influencing factors. The upper segment (US)/lower segment (LS) ratio and arm span/height ratio were used to quantify anthropometric signs which have been associated with "marfanoid" habitus.[13] Written informed consent for publication was obtained from parents (for patients aged < 12 years), patients (aged ≥ 16 years) or both (patients aged 12-16 years). The study was approved by the institutional review board of the UMCU. Detailed methods are presented in the Supplemental Material.

Results

Patients; growth patterns and body proportions

A total of eight MEN2B patients were identified; the diagnosis of MEN2B had been confirmed by a *de novo* c.2753T>C (p.Met918Thr) *RET* mutation in all cases. Patient characteristics are shown in Table 1. Three patients showed growth below their target height range (THR) (Figure 1, patients 2-4). Three more patients (Figure 1, patients 1, 5 and 7) showed prepubertal growth in the lowest margin of their THR. Additionally, patient 8 had been under follow-up for (unexplained) short stature from age 4-14 for which he had been treated with testosterone preparations. Only patient 6 grew well within his THR.

During their last outpatient clinic visit, two children (patient 3,4) were prepubertal, while two others (patient 1 and 2) showed the first signs of puberty (see Table 2). Bone age was determined in six patients: all showed delayed bone maturation (calendar age minus bone age: range 0.9-4.7 years) (Figure 1, patients 1-5, 8). Data on parental pubertal development was available in four cases: menarche was reported at a normal age for Dutch females by three mothers, while a fourth reported delayed menarche. None of the fathers reported an early or delayed puberty. No data were available on the longitudinal growth patterns of the parents.

Four MEN2B patients reached FH (patient 5-8), all within THR, while all had short stature during childhood (see Table 2).

Body proportions (US/LS ratio and arm span/height ratio) were within normal range in all cases studied (n = 7). Nevertheless, four patients (patient 5-8) had been labeled "marfanoid" by the treating physician at some point during follow-up. In addition, hyperlaxity was reported in six patients (75%) (Table 2).

Possible determinants of growth

Impaired (prepubertal) growth was seen in patients diagnosed during late childhood and adolescence as well as in patients who had been diagnosed and successfully treated by thyroidectomy early in life. Details on MTC status as well as thyroid disease after surgery are reported in Table 1.

Table 1. Patient characteristics

Patient	Sex	Age at Dx (year)	Age at thyroidectomy (year)	Follow-up time (year)	Thyroid disease at Dx[a]	Thyroid disease at last FU[b]
1	F	0.1	0.6	13.3	CCH	Cured
2	F	6.5	6.5	4.0	MTC, T3N1bMx (IVa)	Persistent
3	F	0.3	0.6	8.4	CCH	Cured
4	M	0.1	0.5	7.3	MTC, T1aNxMx (I)	Cured
5	F	15.8	15.9	7.0	MTC, T4aN1bM1 (IVc)	Progressive
6	M	11.7	11.7	14.6	MTC, T1aNxMx (I)	Progressive
7	F	6.0	6.1	30.3	CCH[c]	Recurrence
8	M	16.0	16.0	38.0[d]	MTC, T1aNxMx (I)	Persistent

Abbreviations: CCH, C-cell hyperplasia; Dx, diagnosis; F, female; FU, follow-up; M, male; MTC, medullary thyroid carcinoma; ND, not determined; SD, standard deviation

a: Histological diagnosis and tumor node metastasis – classification, based upon the *American Joint Committee on Cancer (AJCC) Cancer Staging Manual*, eight edition (Rosen JE, Lloyd RV, Brierly JD, et al. Thyroid - Medullary. In: AJCC Cancer Staging Manual, 8th, Amid AB (Ed), Springer, New York 2017. p.891. Corrected at 4th printing, 2018)

b: Thyroid disease has been defined as "cured" if calcitonin levels remained undetectable after the surgery. Thyroid disease was labeled, as "recurrence" if calcitonin were initially undetectable after surgery, but became detectable afterwards. Thyroid disease was defined as "persistent" if calcitonin levels were elevated before surgery and remained elevated afterwards. Thyroid disease was labeled as "progressive" in case of increasing calcitonin concentrations and/or evidence of metastatic disease on imaging.

c: Possible MTC.

d: Patient died at age 54.0 years due to a metastasized pancreatic adenocarcinoma.

As illustrated in Figure 1, neither growth during childhood nor FH appeared to be influenced by age at MEN2B diagnosis, age at thyroidectomy or extensiveness of MTC. Late MEN2B diagnosis with progressive loco-regional or metastasized MTC did not seem to be associated with reduced FH (patients 5 and 6). Of note, we could not relate calcitonin levels to biochemical parameters of bone mineralization (calcium, phosphate and/or parathyroid hormone (PTH) levels) (data not shown).

Thyroid replacement therapy after thyroidectomy, initiated to maintain thyroid-stimulating hormone (TSH) and free thyroxine (FT4) levels within age-specific reference range levels, had been adequate in all patients. Insulin-like growth factor-1 (IGF-1) level was < 2 SD below the age- and sex-related mean in three patients (patient 2-4). Growth hormone (GH) stimulation testing in patient 3 and 4 ruled out GH deficiency as well as GH resistance (data shown in the Supplemental Material). In patient 2, GH testing had not been performed as

GH therapy was not considered advisable with simultaneous presence of persistent MTC. In five patients, BMI SDS was within the reference range (± 2) during their entire childhood (Figure 1, patient 1, 3-6). A decline in BMI in the three other patients (patient 2, 7 and 8) did not co-occur with a decrease in height growth velocity (Figure 1). Patient 3 underwent

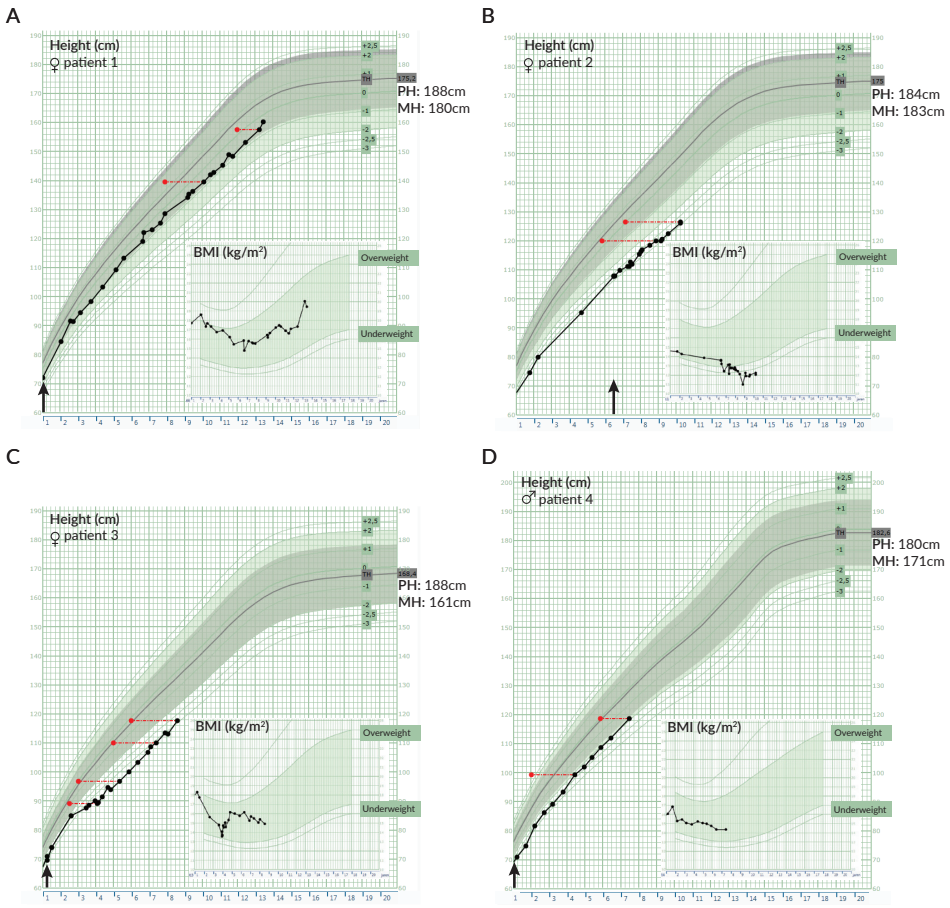
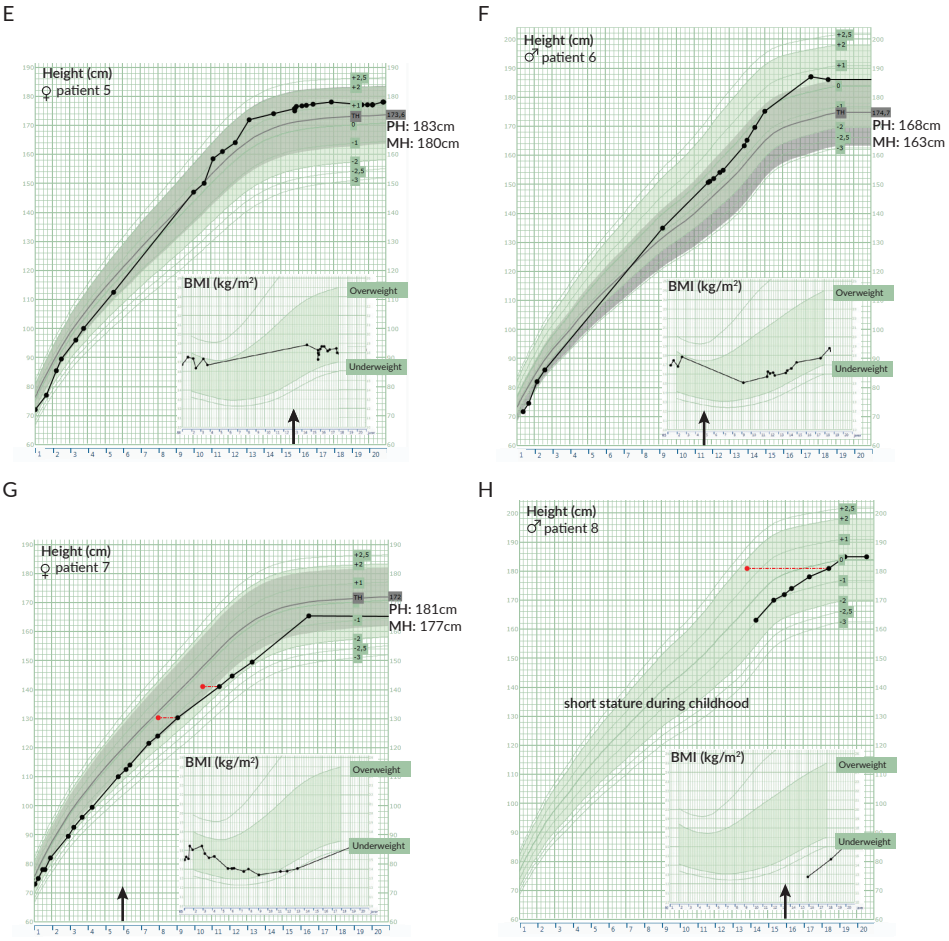


Figure 1. Growth charts
Height (cm) for age (years) charts and BMI (kg/m^2) for sex and age (years) charts of all included patients.
Age at MEN2B diagnosis is indicated by an arrow just above the *X*-axis.
Target height and parental height are reported (in cm) along the right *Y*-axis (parental data missing for patient 8).
Green color background (online version) indicates age- and sex -specific Dutch population mean ± 2 standard deviations for height and BMI (Schönbeck Y, Talma H, Van Dommelen P, et al. ▶

a partial small bowel removal after a neonatal volvulus and thereafter, at age three, a rectosigmoid resection. She suffered from ongoing mild malabsorption (iron and fat-soluble vitamin deficiencies) thereafter, and the rectosigmoid resection coincided with a temporary reduced growth velocity (Figure 1).



▶ The world's tallest nation has stopped growing taller: The height of Dutch children from 1955 to 2009. *Pediatr Res*. 2013;73(3):371-377).Grey color background (online version) marks the target height range (data missing for patient 8). Bone age is marked by a red dot (online version).
Abbreviations: BMI, body mass index; MEN2B, Multiple Endocrine Neoplasia type 2B; MH, maternal height; PH, paternal height.

Table 2. Anthropometric data and musculoskeletal features related to "marfanoid" habitus

Patient (age at last FU)	Target height[a]	Height at last FU[b]	BMI at last FU[c]	Pubertal development[d]	US/LS ratio[e]	Arm span ratio[f]	Musculoskeletal features[g]	Labeled as "marfanoid" by physician[g]
1 (13.3 yr)	175 cm +0.71 SD	-0.61 SD	+0.74 SD	Tanner stage at last FU: P2 M2	0.98	0.96	Hyperlaxity Scoliosis Pes cavus	-
2 (10.5 yr)	175 cm +0.68 SD	-2.86 SD	-3.49 SD	Tanner stage at last FU: P1 M2	1.05	0.96	Hyperlaxity Hipdysplasia Muscle weakness Hypotonia	-
3 (8.7 yr)	168 cm -0.38 SD	-2.87 SD	-0.52 SD	Tanner stage at last FU: P1 M1	1.13	0.96	-	-
4 (7.5 yr)	183 cm -0.17 SD	-1.87 SD	-0.82 SD	Tanner stage at last FU: P1 G1	1.19	0.96	Hypotonia	-
5 (22.8 yr)	174 cm +0.45 SD	178 cm[h] +1.16 SD	-1.91 SD	Normal (age at menarche: 13 yr)	1.13	0.94	Hyperlaxity	+
6 (26.3 yr)	175 cm -1.29 SD	186 cm[h] +0.39 SD	-0.87 SD	Normal	0.96	0.99	Hyperlaxity Muscle weakness Hypotonia DMD	+
7 (36.3 yr)	172 cm +0.2 SD	165 cm[h] -0.52 SD	+0.15 SD	Somewhat delayed (age at menarche: 15 yr)	1.14	1.00	Hyperlaxity DMD	+
8 (54.0 yr)	ND	186 cm[h] +0.17 SD	-2.35 SD	Delayed[i]	ND	ND	Hyperlaxity Scoliosis Pectus carinatum Pes cavus Muscle weakness Hypotonia	+

Abbreviations: DMD, delayed motor development; FU, follow-up; LS, lower segment; ND, not determined; SD, age-related sex specific standard deviation; US, upper segment; yr, year; yes, +; no, -

a: In cm and in standard deviations above or below the sex-specific population mean.

b: Standard deviations above or below the age-related sex-specific population mean. Absolute height in cm is also reported for patients who have reached their final height.

c: Standard deviations above or below the age-related sex-specific population mean.

d: Pubertal development is described by Tanner stadium at last follow-up in case of pediatric patients. In adult patients, the pubertal development was classified as 'normal', 'somewhat delayed' or 'delayed', and in female patients, age at menarche has also been reported. ▶

▶ e: Upper segment/lower segment ratio. A reduced ratio, defined as <1 for age 0-5 years, <0.95 for age 6-7, <0.9 for age 8-9 and <0.85 for age ≥10, is associated with Marfan syndrome (Loeys BL, Dietz HC, Braverman AC, et al. The revised Ghent nosology for the Marfan syndrome. *J Med Genet*. 2010;47(7):476-485)
f: Arm span-to-height ratio. An increased arm span-to-height ratio, defined as > 1.05, is associated with Marfan syndrome.
g: Based on medical record notes by treating physicians.
h: Final height.
i: According to medical correspondence, patient 8 had been treated with testosterone preparations due to (unexplained) short stature at pubertal age. Remarkably, at age 20, his height was 186 cm with a 4.7 year delay in bone maturation. Further details on his pubertal development could not be retrieved.

Discussion

In contrast to the expected "marfanoid" tall stature, 50% of the patients with MEN2B described in our report had been diagnosed with short stature. Another three patients (38%) showed growth around the lower limit of their THR – at least until puberty. Until now, growth restriction and short stature during childhood have not been fully acknowledged as associated features of MEN2B. Results from this detailed case series confirm a previous report; short stature in childhood may be considered as an associated features of MEN2B. This is an important finding because it illustrates that short stature during childhood does not decrease the chance of a diagnosis of MEN2B when other MEN2B symptoms are present.
Interestingly, none of the four patients who thus far have reached adulthood remained short. Thus, short stature in childhood in MEN2B patients is not necessarily associated with final height below predicted target height.

As the results on growth patterns were contradictory of what is generally assumed, factors that could have influenced growth were investigated. Although the small number of patients prevented us to draw firm conclusions about the effect of several clinical aspects on growth patterns, some important observations could be made: first, in our patients, age at MEN2B diagnosis or age at thyroidectomy did not seem to influence growth velocity. Second, there appeared to be no relationship between MTC status and either patients' growth or FH (both related to TH).
With regard to endocrine status: although levels of IGF-1 were low in three patients with growth restriction, GH stimulation testing ruled out GH deficiency in two cases. Delayed bone maturation may partly explain the low IGF-1 concentrations, as IGF-1 levels should be interpreted considering both bone and calendar age in children. Furthermore, since the GH-IGF-1 axis is influenced by malnutrition, low levels of IGF-1 might be partially explained by the low BMI in patient 2 and malabsorption in patient 3.[14,15] Growth charts

did not show a clear co-occurrence of reduced BMI and impaired growth, while intestinal surgery was accompanied by a temporary reduced height velocity in one patient.
Whether the abovementioned factors – and gastrointestinal manifestations in particular – may have impacted growth velocity in this case series cannot be clearly determined. Malabsorption has not been not assessed at regular intervals, investigations were based on clinical judgement of the treating physicians.
Given the delayed bone development, we considered the possibility of familial constitutional delay in growth and puberty; however, the available data overall do not suggest familial constitutional delay in growth and puberty to play a significant role in the etiology of short stature in childhood in this series.

Literature on growth patterns in MEN2B is limited to a few case-reports and cohort studies.[9,16–21] In the report of Redlich *et al*. on physical stigmata in patients with MEN2B, short stature was reported in 12 out of 24 (50%) patients.[9] Although data on parental height was missing and a limited number of measurements per patient were available, these findings also suggested an association between growth restriction and MEN2B.[9] Results described in our case series clearly show that short stature and growth beneath THR are prevalent in MEN2B children. However, as the number of patients included in our case series is small, we cannot rule out that tall stature may occur in children with MEN2B as well.

Patients with MEN2B are often mentioned to have a "marfanoid" body habitus, though this is a non-specific term without standardized criteria. On the contrary, criteria for diagnosing Marfan syndrome have been meticulously defined and include a reduced US/LS ratio and increased arm span/height ratio.[13] To the best of our knowledge, no studies have been published actually describing body proportions in MEN2B children. Somewhat surprisingly, none of the hereby reported MEN2B patients showed abnormal body proportions, whereas 50% of the patients had been labeled "marfanoid" by their treating physician. Given the lack of standardized criteria for "marfanoid" habitus, it is difficult to compare our findings with other reports.[7] We, as well as previous authors, did not use specific scoring systems for hyperlaxity, which makes it more challenging to define this symptom in patients with MEN2B. Future studies may address musculoskeletal features including hyperlaxity and bone health to understand how they develop in patients with MEN2B.

To conclude, this case series shows that short stature and height below THR is frequently present in prepubertal children with MEN2B. Next, normal body proportions as measured by arm span/height- and US/LS ratios do not support a marfanoid body habitus during

childhood in this cohort. These findings suggest that short stature should by no means rule out a possible diagnosis of MEN2B when this diagnosis is suspected due to the presence of other MEN2B-related symptoms. Short stature during childhood may even be considered one of the independent characteristics of the MEN2B syndrome. Furthermore, normal adult height within THR – but not tall stature – may be reached despite the reported short stature during childhood. Larger prospective cohort studies are needed to validate these findings on growth and body proportions.

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Supplemental Methods

A retrospective medical record review was performed of all patients diagnosed with MEN2B syndrome under care at the Wilhelmina Children's Hospital/University Medical Center Utrecht (UMCU), a tertiary referral and European Reference Network (ERN) expertise center for MEN patients in the Netherlands, during the period 1976-2020. These patients are included in our recent case series focusing on non-endocrine manifestations as presenting symptoms for a MEN2B diagnosis.[1] Medical records were reviewed from first follow-up (1976) until September 2020. Relevant physician's notes, correspondence, laboratory results and imaging studies were collected.

Outcome

The primary outcomes of interest were patients' growth pattern and final height (FH) – reported as age- and sex-related standard deviation (SD) scores (SDS)[2] – in relation to target height (TH). Sex-specific TH was based on parental height (preferably as measured at the pediatric outpatient clinic) and ethnic background.[3]

Secondary, we described the association between growth and possible influencing factors, *i.e.* age at MEN2B diagnosis as well as thyroidectomy, extensiveness of MTC, age- and sex-adjusted BMI (expressed in SDS[2]), gastrointestinal manifestations and hormonal status.

Physical examination

Anthropometric data (height and weight) were extracted from medical records in the hospital. If necessary, additional data were requested from municipal Young Health Care centers, where all newborns and children are regularly screened as part of the Dutch national public healthcare services. Furthermore, sitting height (measured in a standardized setting from the highest point of the head to the sitting surface) and arm span (measured in a standardized setting by length between the fingertips when arms raised parallel to the ground) were recorded and related to patient's body height. The upper body segment was defined as the sitting height, while the lower body segment was defined as the arithmetic difference between height and sitting height. The upper segment/lower segment (US/LS) ratio was obtained by dividing the upper segment by the lower segment. Likewise, sitting height/height ratio was calculated by dividing sitting height by patient's height.[4] The US/LS ratio and arm span/height ratio were used to quantify anthropometric signs which have been associated with "marfanoid" habitus, namely a reduced US/LS ratio (defined as < 1 for age 0-5 years, < 0.95 for age 6-7, < 0.9 for age 8-9 and < 0.85 for age ≥ 10) and an arm span/height ratio > 1.05.[5] Pubertal development was evaluated by the treating physician and described using Tanner stages.[6,7]

Laboratory investigations

Adequacy of levothyroxine replacement therapy was assessed on a regular basis (during childhood every 3-6 months), with treatment aiming at thyroid-stimulating hormone (TSH) and serum free thyroxine (FT4) levels within the age-adjusted normal ranges. Calcitonin was used as tumor marker for the presence of MTC. If available, data on growth hormone (GH) status were collected, including insulin-like growth factor 1 (IGF-1) and insulin-like growth factor binding protein-3 (IGFBP-3). In case of impaired growth or short stature, a standard set of laboratory investigations were performed, as recommended by the Dutch Pediatric Association guideline for short stature.[8] If GH deficiency was suspected, GH stimulation testing (clonidine or clonidine/arginine) was performed at the discretion of the treating physician. Age- and sex-adjusted reference ranges were used for IGF-1 and IGFBP-3.

Imaging studies

Bone age determination was based on radiographs of the left hand and wrist using the Greulich and Pyle Atlas and classified by a pediatric radiologist. BoneXpert was not used for evaluation as part of the radiographs were taken well before availability of electronic radiography systems. All available radiographs were re-evaluated by one of the authors (AVS).[9]

Ethics

Written informed consent for publication was obtained from parents (for patients aged < 12 years), patients (aged ≥ 16 years) or both (patients aged 12-16 years). The study was approved by the institutional review board of the UMCU.

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S1. Supplemental Table 1. Laboratory results growth hormone tests

Test	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8
IGF-1 (SD) (highest value)[a]	-1.12	-2.02	-0.81	-0.54	-0,52[b]	-0.58[b]	0,82[b]	ND
IGF-1 (SD) (lowest value)[a]	-1.22	-2.47	-2.79	-2.31	-0,52[b]	-0.58[b]	0,82[b]	ND
IGFBP-3 (SD) (highest value)[a]	ND	ND	-1.75	-1.93[b]	0,42[b]	-2.56[b]	0,25[b]	ND
IGFBP-3 (SD) (lowest value)[a]	ND	ND	-1.78	-1.93[b]	0,42[b]	-2.56[b]	0,25[b]	ND
Peak response in GH stimulation test			41 mIU/L[c]	120 mE/L[d]				

Abbreviations: GH, growth hormone; IGF-1, insulin-like growth factor 1; IGFBP-3, insulin-like growth factor binding protein-3; ND, not determined; SD, standard deviation.
a: These include the highest and lowest IGF-1 and IGFBP-3 values measured during entire follow-up.
b: One-time measurement; therefore this value is presented as highest and lowest value.
c: At age seven years. GH stimulation using clonidine (0.15 mg/m2 body surface area, max 0.15 mg) and arginine (0.5 g/kg, max 30 g).
d: At age four years. GH stimulation using clonidine (0.15 mg/m2 body surface area, max 0.15 mg).
c and d: Partial GH deficiency was ruled out at GH levels after stimulation of >30 mU/L, according to current Dutch guidelines. GH resistance was ruled out by the combination of (1) GH levels after stimulation of >30 mU/L and (2) IGF-1 levels not repeatedly below -2 SD, according to current Dutch guidelines.
Dutch guideline: Adviesgroep Groeihormoon van de Nederlandse Vereniging voor Kindergeneeskunde, *Richtlijn Behandeling van kleine lengte bij kinderen met groeihormoondeficiëntie*. http://www.kindergeneeskunde-mca.nl/images/stories/bart/GHDbehandeling2015.pdf Established 14-12-2012. Revision 25-9-2015.

PART TWO

Case-finding within MEN syndromes: moving towards personalized medicine




CHAPTER 6

Clues for genetic anticipation in Multiple Endocrine Neoplasia type 1

Medard F.M. van den Broek[1], Bernadette P.M. van Nesselrooij[2], Carolina R.C. Pieterman[1], Annemarie A. Verrijn Stuart[3], Annenienke C. van de Ven[4], Wouter W. de Herder[5], Olaf M. Dekkers[6], Madeleine L. Drent[7], Bas Havekes[8], Michiel N. Kerstens[9], Peter H. Bisschop[10], Gerlof D. Valk[1]

[1] Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Medical Genetics, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Pediatric Endocrinology, Wilhelmina Children's Hospital, University Medical Center Utrecht, Utrecht, The Netherlands
[4] Department of Endocrinology, Radboud University Medical Center, Nijmegen, The Netherlands
[5] Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands
[6] Departments of Endocrinology and Metabolism and Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
[7] Department of Internal Medicine, Section of Endocrinology, Amsterdam UMC, location VU University Medical Center, Amsterdam, The Netherlands
[8] Department of Internal Medicine, Division of Endocrinology, Maastricht University Medical Center, Maastricht, The Netherlands
[9] Department of Endocrinology, University Medical Center Groningen, Groningen, The Netherlands
[10] Department of Endocrinology and Metabolism, Amsterdam UMC, location Academic Medical Center, Amsterdam, The Netherlands

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Abstract

Context

Multiple Endocrine Neoplasia type 1 (MEN1) is a rare autosomal dominant hereditary disease caused by the loss of function of the *MEN1* gene, a tumor suppressor gene that encodes the protein menin. It is characterized by the occurrence of primary hyperparathyroidism (pHPT), duodenopancreatic neuroendocrine tumors (dpNET), pituitary tumors (PIT), adrenal adenomas (ADR) and bronchopulmonary (bp-NET), thymic and gastric neuroendocrine tumors. More insight into factors influencing the age-related penetrance of MEN1 manifestations could provide clues for more personalized screening programs.

Objective

To investigate whether genetic anticipation plays a role in the largest known MEN1 families in the Netherlands**.**

Methods

All Dutch MEN1 families with ≥ 10 affected members in ≥ 2 successive generations were identified. Age at detection of the different MEN1-related manifestations were compared among generations using regression analyses adjusted for competing risks. To correct for the beneficial effect of being under surveillance, manifestations occurring during surveillance were also separately compared.

Results

A total of 152 MEN1 patients from 10 families were included. A significantly decreased age at detection of pHPT, dpNET, PIT and bp-NET was found in successive generations ($P < 0.0001$). Adjusted analyses led to the same results.

Conclusions

These results suggest the presence of genetic anticipation. However, due to a risk of residual bias, the results must be interpreted with caution. After independent validation in other cohorts and further translational research investigating the molecular mechanisms explaining this phenomenon in MEN1, the results might add to future, more personalized, screening protocols and earlier screening for future generations of MEN1 patients.

Introduction

Multiple Endocrine Neoplasia type 1 (MEN1) is a rare hereditary disease caused by loss of function of the *MEN1* gene. The *MEN1* gene is a tumor suppressor gene that encodes the protein menin. It has an estimated prevalence of 2-10 per 100,000 and is inherited in an autosomal dominant pattern.[1] Although a wide variety of manifestations have been described, most MEN1 patients suffer from (1) primary hyperparathyroidism (pHPT) (90-95%), (2) duodenopancreatic neuroendocrine tumor (dpNET) (35-75%), (3) anterior pituitary tumors (PIT) (20-65%), (4) adrenal adenomas (ADR) (11-35%), and (5) bronchopulmonary (bp-NET), thymic (th-NET) and gastric neuroendocrine tumors (20-30%).[2,3] *MEN1* mutations have a high penetrance, and patients with MEN1 suffer from high morbidity and a decreased life expectancy.[4] In particular, th-NET and pancreatic NET are main causes of MEN1-related death.[4,5]

In order to detect MEN1 manifestations in an early stage, periodic screening of MEN1 patients is advised. The present clinical practice guidelines advise to start screening for a number of manifestations at the age of five in all *MEN1* mutation carriers, and to expand the screening with age.[6] Despite numerous efforts, no direct genotype-phenotype correlation has been found to date.[7] Although minor familial clustering of specific tumors has been described,[8] in general both a considerable phenotypic variability of manifestations, as well as variable age at diagnosis, have been reported.[7] More insight into factors influencing the age-related penetrance of MEN1 manifestations could provide clues for more personalized screening programs for *MEN1* mutation carriers, potentially leading a decrease in patient (and parental) burden, as well as lower health care costs.

Genetic anticipation refers to the phenomenon of decreased age of disease onset or an increased disease severity in successive generations. It is best known in neuropsychiatric diseases such as Huntington's disease and myotonic dystrophy. In these diseases, trinucleotide repeat expansions ("growing genes") are responsible for the phenotype of genetic anticipation, as the length of the repeat is transmitted in an unstable way and can be influenced by the parental origin.[9] More recently, anticipation was also described in forms of heritable cancer such as dyskeratosis congenita, Lynch Syndrome, Li-Fraumeni syndrome, von Hippel-Lindau syndrome and hereditary breast and ovarian cancer syndrome.[10–14] In these syndromes the genetic defect is transmitted without alterations. Partly due to the lack of generally accepted explanatory biological mechanism and high risk of bias in this field of research, some publications suggested this observation to be the result of different forms of bias.[15–17] To our knowledge, data about genetic anticipation within MEN1 families are limited to one study, describing a MEN1 family of five generations

with clinical expression suggestive of anticipation.[18] The aim of this nationwide study is to investigate whether genetic anticipation plays a role in the largest known MEN1 families in the Netherlands.

Methods

Patient selection

Since the discovery of the *MEN1* gene in 1997 until recently, all genetic testing for *MEN1* gene abnormalities in the Netherlands has been performed centrally at the University Medical Center Utrecht. All potential Dutch MEN1 patients and mutation carriers referred for genetic testing between January 1998 and December 2017 were identified. Pedigree information was retrieved from medical records and checked using the Dutch Municipal Resident Registration. Mutation-positive MEN1 families were selected if these families comprised at least 10 affected members in two or more successive generations.

Retrieval of clinical information

Clinical information about affected family members was obtained using the national MEN1 database of the DutchMEN1 study group (DMSG). This database contains longitudinally collected clinical information of patients ≥ 16 years of age at the end of 2017 and treated at one of the Dutch university medical centers between 1990 and 2017. The study cohort includes ≥ 90% of the total Dutch MEN1 population. Data of all patients were collected from every quarter of every available year of follow-up, from 1990 to 2017. Furthermore, data concerning the occurrence of MEN1-related manifestations before 1990 and before 16 years of age were included as well. Detailed information on the DMSG database methods have been described previously.[19]

Patients deceased before 1990, < 16 years of age on December 31, 2017, or patients whose clinical or pedigree information was lacking were excluded from this study.

Definitions of MEN1 manifestations

In order to determine the exact prevalence and time of diagnosis of a MEN1-related manifestation, the following definitions of MEN1-related manifestations were used: pHPT was defined as elevated calcium combined with a normal to elevated PTH level in two consecutive measurements; dpNET was diagnosed based on tissue examination or – if not available – gastroduodenoscopy (duodenum NET) or ≥ 1 abnormality on imaging studies in at least two successive investigations (pancreas NET); pituitary, adrenal and bronchopulmonary tumors were labeled as such based on histology or – if not available

– imaging studies suggestive of these specific tumors in at least two successive investigations. Thymic and gastric NET were diagnosed on a histological basis only. Details for reference standards of MEN1-related manifestations have been described previously.[19,20]

Statistical analysis

Patients were ranked from oldest to youngest generation, based on their position within the family pedigree. Clinical characteristics were reported as mean and standard deviation or median with range based on the distribution of data. Time-to-event methods were used to evaluate the age at detection of MEN1-related manifestations. The patients' lifetimes from birth until death, lost to follow-up or the end of follow-up (December 31, 2017) were included for analysis. The age-related penetrance of MEN1-related manifestations were analyzed using cumulative incidence functions, accounting for death as a competing risk. Generations were compared using Gray's Test. Additionally, the effect of generation on phenotype was evaluated using proportional subdistribution hazards regression models, as described by Fine and Gray.[21–23] However, these results may overestimate a possible anticipation effect, since these analyses do not take into the account the benefits of surveillance programs: with regular laboratory tests and imaging studies, tumors are more likely to be detected early in life. Since older generations may have profited less from these programs, and manifestations in patients from older generations were more frequently detected because of symptoms rather than presymptomatic screening, results may be distorted. In an attempt to reduce this bias, separate time-to-event analyses were conducted focusing on MEN1-related manifestations occurring in patients within the timeframe that they were under surveillance. In this manner we attempted to reduce the risk of detection bias, since these manifestations were detected in a comparable manner (*i.e.*, presumable early diagnosis when being under surveillance) across all generations. Statistical significance was set at a two-sided $P < 0.05$. Analyses were performed using IBM SPSS 25.0 and R version 3.4.1.

Results

A total of 10 families were included, comprising 157 MEN1 patients from the DMSG database ≥ 16 years of age at the end of 2017. Five patients were excluded due to insufficient pedigree information. The study population consisted of 80 females (52.6%) with a median age at the end of follow-up (December 31, 2017, or death) of 49 years (range 19-84 yr). Genetic analysis was performed in 134 patients (88%), and a mutation (or affected allele) was found in all of these cases. Main features of the 10 families are described in Table 1. The number of affected family members ranged from 11 to 29 per family. A total of 137 affected members (90.1%) showed one or more MEN1-related manifestations during follow-

Table 1. Characteristics of the MEN1 families

Family number	Number of MEN1 patients (N)	Number of generations (N)	Gender (N (%) female)	Age (median, range)	MEN1 manifestations							Death (N)	MEN1-related death (N)
					Primary hyper-parathyroidism (N, %)	Pancreatic and duodenal NET (N, %)	Anterior pituitary tumor (N, %)	Adrenal tumor (N, %)	Bp-NET (N, %)	Th-NET (N, %)	Gastric NET (N, %)		
1	29	3	11 (38%)	45 (19-84)	27 (93%)	20 (69%)	15 (52%)	12 (41%)	11 (38%)	0	0	3	1[a]
2	23	4	15 (65%)	50 (21-80)	23 (100%)	17 (74%)	11 (48%)	10 (43%)	5 (22%)	1 (4%)	0	7	3[b]
3	18	3	8 (44%)	51 (22-72)	15 (83%)	9 (50%)	6 (33%)	6 (33%)	6 (33%)	1 (6%)	0	4	1[c]
4	14	3	8 (57%)	52 (19-70)	12 (86%)	12 (86%)	8 (57%)	6 (43%)	3 (21%)	0	1 (7%)	1	1[d]
5	12	3	8 (67%)	51 (22-75)	10 (83%)	8 (67%)	2 (17%)	2 (17%)	5 (42%)	0	0	2	1[e]
6*	12	3	7 (58%)	52 (28-84)	3 (25%)	3 (25%)	0	1 (8%)	2 (17%)	0	0	0	-
7	11	2	9 (82%)	38 (20-63)	10 (91%)	8 (73%)	4 (36%)	3 (27%)	2 (18%)	0	2 (18%)	0	-
8	11	3	5 (45%)	48 (22-77)	9 (82%)	6 (55%)	3 (27%)	3 (27%)	4 (36%)	0	1 (9%)	2	1[f]
9	11	4	5 (45%)	49 (23-76)	11 (100%)	8 (73%)	4 (36%)	3 (27%)	3 (27%)	0	0	3	2[g]
10*	11	2	4 (36%)	48 (36-80)	1 (9%)	1 (9%)	2 (18%)	2 (18%)	2 (18%)	0	0	1	0[h]
TOTAL	152		80 (53%)	49 (19 – 84)	121 (80%)	92 (61%)	55 (36%)	48 (32%)	43 (28%)	2 (1%)	4 (3%)	23 (15%)	10 (7%)

Abbreviations: Bp-NET, bronchopulmonary NET; N, number; NET, neuroendocrine tumor; th-NET, thymic NET.

* Families with low penetrance of disease

a: MEN1-related: progressive pancreatic NET. Non-MEN1-related: irresectable myxofibrosarcoma (n = 1), end-stage heart failure (n = 1).

b: MEN1-related: progressive duodenopancreatic NET (n = 1), progressive th-NET (n = 1), complications of MEN1-related operation (n = 1). Non-MEN1-related: pneumonia (n = 1), heart failure (n = 1), pulmonary embolism (n = 1), unknown (n = 1).

c: MEN1-related: progressive th-NET (n = 1). Non-MEN1-related: progressive prostate cancer (n = 1), progressive colorectal cancer (adenocarcinoma) (n = 1), subarachnoid hemorrhage (n = 1).

d: MEN1-related: progressive pancreatic NET.

e: MEN1-related: progressive pancreatic NET (n = 1). Non-MEN1-related: progressive colorectal cancer (n = 1).

f: MEN1-related: progressive nesidioblastoma (n = 1). Non-MEN1-related: dementia (n = 1).

g: MEN1-related: progressive duodenopancreatic NET (n = 2). Non-MEN1-related: unknown (n = 1).

h: Non-MEN1-related: progressive colorectal cancer (n = 1).

up. Primary hyperparathyroidism showed the highest penetrance (121 patients, 80%), thymus NET the lowest (2 patients, 1%). Two families showed an unusually low penetrance of MEN1 manifestations: (1) family 6 with mutation c.545T>C(p.Leu182Pro) in exon 3, and (2) family 10 with mutation c.670-6C>G(p.?) in intron 3. The latter family was reported in an earlier study.[24]

Age at detection of MEN1 manifestations

A total of 42 patients (28%) were labeled as first generation. The second generation included 68 patients (45%), the third generation included 40 patients (26%), and 2 patients (1%) were identified as fourth generation family members. In all MEN1-related manifestations, the median age at detection was highest in the first generation and lowest in the last (third and fourth) generations. The difference in median age at detection between first and last generation ranged between 8 years (th-NET) and 40 years (dp-NET). The median age at detection of the first encountered manifestation was 46 (range: 21-73 yr) in the first generation, compared to 14 (range: 11-17 yr) in the youngest generation. More detailed results are displayed in Table 2.

Time-to-event analyses showed a significantly higher age-related penetrance of pHPT, dpNET, PIT and bp-NET in successive generations (see Figure 1). Additional analyses investigating the age at detection of bp-NET based on pathology results alone (n = 13) showed similar results (data not shown). Although younger generations also tend to experience adrenal tumors earlier in life, this trend did not reach statistical significance (P = 0.17). Furthermore, patients from younger generations encountered their first MEN1-related tumor significantly earlier in life. When only focusing on manifestations that occurred under surveillance, the results were the same (see Figure 2). Results from the proportional subdistribution hazards regression models demonstrated evidence of genetic anticipation in MEN1-related manifestations as well. More details are provided in Table 3. In order to investigate potential interference, additional analyses were carried out excluding the two families with a low penetrance of disease (families 6 and 10), which showed similar results (data not shown). Furthermore, supplementary analyses only comparing the second and third generations demonstrated comparable evidence of genetic anticipation as well (data not shown).

The occurrence of metastatic disease occurring in patients during the time being under surveillance – as a proxy for disease severity – was equal across generations (data not shown).

Table 2. Age at detection of MEN1 manifestations per generation

Generation		Total number of MEN1 patients (N)	Gender (N (%) female)	Age at end of follow-up (yr) (median, range)	MEN1 manifestations							Age at first manifestation (yr) (median, range)
					Primary hyper-parathyroidism	Pancreatic and duodenal NET	Anterior pituitary tumor	Adrenal tumor	Bp-NET	Th-NET	Gastric NET	
1	**Age / age at detection (yr) (median, range)**			**67 (30-84)**	**46 (21-65)**	**60 (23-73)**	**49[a] (24-60)**	**55 (41-73)**	**57[a] (43-66)**	**46**	**57 (49-69)**	**46 (21-73)**
	Year at detection (median, range)				1991 (1964- 2014)	2009 (1981-2005)	2007 (1979-2011)	2002 (1983-2012)	2008 (1983-2013)	1996	2008 (2003-2017)	
	Number of patients (%)	42 (28%)	23 (55%)		37	29	12	23	10	1	4	40
2	**Age / age at detection (yr) (median, range)**			**50[a] (20-73)**	**32[a] (15-58)**	**39 (15-61)**	**33 (14-47)**	**43 (30-64)**	**43 (20-65)**	**38**	**-**	**32[a] (14-61)**
	Year at detection (median, range)				2001 (1969-2017)	2006 (1969-2016)	2004 (1987-2015)	2006 (1996-2014)	2011 (2004-2016)	2004	-	
	Number of patients (%)	68 (45%)	34 (50%)		50	42	21	21	24	1	0	60
3	**Age / age at detection (yr) (median, range)**			**28[a] (19-50)**	**18 (10-37)**	**20 (13-38)**	**18[a] (14-39)**	**32 (30-41)**	**27 (18-43)**	**-**	**-**	**17 (10-37)**
	Year at detection (median, range)				2005 (1987-2016)	2010 (1984-2016)	2008 (1988-2014)	2012 (2003-2016)	2012 (2005-2017)	-	-	
	Number of patients (%)	40 (26%)	22 (55%)		29	21	20	4	9	0	0	35

Table 2. Continued

4	**Age / age at detection (yr) (median, range)**			**24[a] (23-24)**	**14 (11-17)**	-	**18[a] (17-18)**	-	-	-	-	**14 (11-17)**
	Year at detection (median, range)				2008 (2005-2010)	-	2011 (2010- 2013)	-	-	-	-	
	Number of patients (%)	2 (1%)	1 (50%)		2	0	2	0	0	0	0	2
TOTAL Age / age at detection (yr) (median, range)				49 (19-84)	31 (10-65)	41 (13-73)	28 (14-60)	49 (30-73)	43 (18-66)	42 (38-46)	57 (49-69)	30 (10-73)
TOTAL Number of patients (%)		152	80 (53%)		118[b]	92	55	48	43	2	4	137

Abbreviations: bp-NET, bronchopulmonary NET; N, number; NET, neuroendocrine tumor; th-NET, thymic NET; yr, year.
a: Rounded numbers.
b: The moment of diagnosis of primary hyperparathyroidism could not be retrieved in three additional cases.

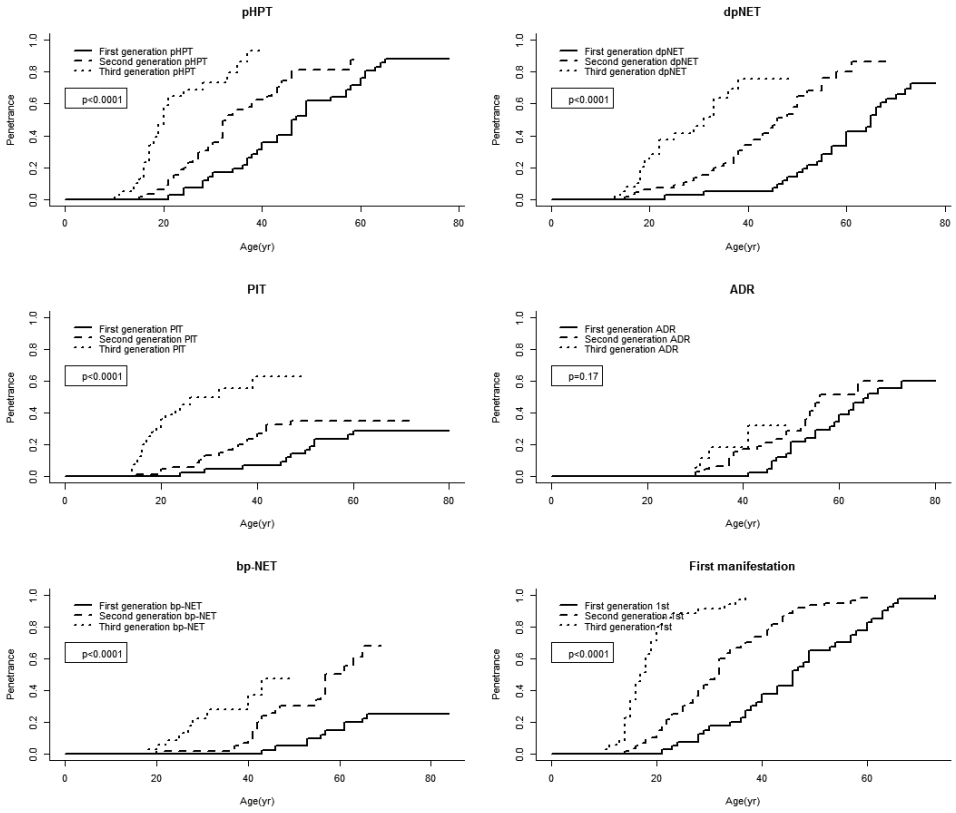

Figure 1. Age-related penetrance of MEN1 manifestations
Abbreviations: 1st, first manifestation; ADR, adrenal adenoma; bp-NET, bronchopulmonary NET; dpNET, duodenopancreatic NET; Gen 1, first generation; Gen 2, second generation; Gen 3, third generation; NET, neuroendocrine tumor; pHPT, primary hyperparathyroidism; PIT, anterior pituitary tumor.
P-value shows Gray's Test for comparison of age-related penetrance (cumulative incidence function) between generations.
Due to low penetrance of thymic and gastric neuroendocrine tumors, these manifestations were not included in the analyses.
Because of the small sample size of the fourth generation (n = 2), the age-related penetrance of MEN1 manifestations of this generation are excluded from this analyses.

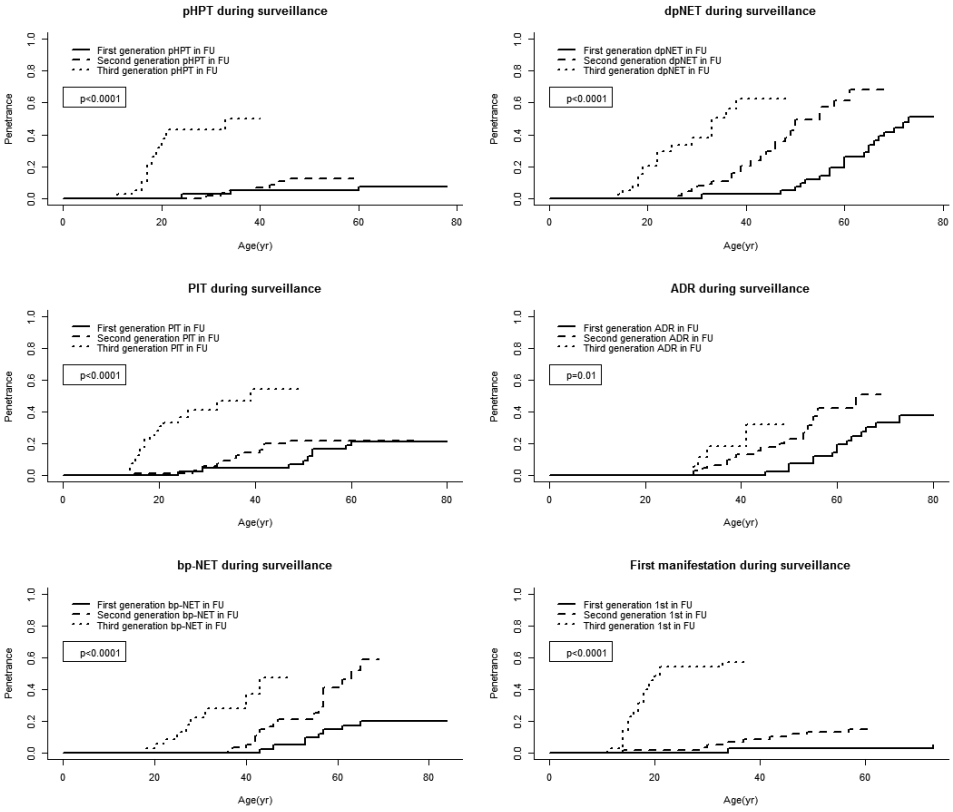

Figure 2. Age-related penetrance of MEN1 manifestations during surveillance
Abbreviations: 1st, first manifestation; ADR, adrenal adenoma; bp-NET, bronchopulmonary NET; dpNET, duodenopancreatic NET; FU, follow-up; Gen 1, first generation; Gen 2, second generation; Gen 3, third generation; NET, neuroendocrine tumor; pHPT, primary hyperparathyroidism; PIT, anterior pituitary tumor.
P-value shows Gray's Test for comparison of age-related penetrance (cumulative incidence function) between generations.
Due to low penetrance of thymic and gastric neuroendocrine tumors, these manifestations were not included in the analyses.
Because of the small sample size of the fourth generation (n = 2), the age-related penetrance of MEN1 manifestations of this generation are excluded from this analyses.

Table 3. Regression models[a]

Manifestation (during surveillance)	Generation number	Hazard Ratio (generation)[b]	Standard error	Wald *P*-value
Primary hyperparathyroidm	First	1.00[b]	-	<0.0005
	Second	1.62	0.666	
	Third	11.75	0.597	
Pancreatic- and duodenal NET	First	1.00[b]	-	<0.0005
	Second	2.07	0.249	
	Third	4.87	0.375	
Pituitary adenoma	First	1.00[b]	-	<0.0005
	Second	1.21	0.398	
	Third	6.53	0.388	
Adrenal tumor	First	1.00[b]	-	0.0076
	Second	2.14	0.340	
	Third	4.90	0.587	
Bronchopulmonary NET	First	1.00[b]	-	<0.0005
	Second	3.29	0.416	
	Third	16.00	0.533	
First manifestation	First	1.00[b]	-	<0.0005
	Second	3.38	0.754	
	Third	18.43	0.718	

Abbreviations: NET, neuroendocrine tumor

a: Proportional subdistribution hazards regression models (described in Fine and Gray[21]), assessing the effect of generation (explanatory covariate) on the occurrence of different MEN1-related manifestations diagnosed during the surveillance period (event of interest). Death and manifestations diagnosed before the start of surveillance are defined as competing risks. The occurrence of gastric NET and thymic NET are not modeled due to the low penetrance of these manifestations. Because of the small sample size of the fourth generation ($n = 2$), this generation is excluded from these analyses.

b: The subdistribution hazard of cumulative incidence function. The first generation is defined as the reference generation.

Discussion

Results from this first nationwide and multifamily study on genetic anticipation in MEN1 showed that manifestations occurred significantly earlier in the lives of patients from successive generations. Even with the adjustments for the beneficial effect of surveillance programs, our results suggested the presence of genetic anticipation in MEN1. Since metastasis occurred equally across generations, there was no indication of an increased disease severity in successive generations.

The study included a cohort of the largest Dutch MEN1 families selected from all referrals for *MEN1* mutation testing in the Netherlands, making it very unlikely to have missed any MEN1 family of relevance for answering the study questions. We expect patients from this cohort to represent the general MEN1 population, and we subsequently expect these results

to be generalizable to other MEN1 families. Clinical information was obtained using the DMSG database, in which extensive follow-up data of MEN1 patients are collected quarterly using a predefined protocol. Furthermore, possible MEN1 manifestations were interpreted using well-defined criteria. This standardization of data makes it possible to accurately investigate the natural course of MEN1-related manifestations in this population.

It should be noted, however, that studies evaluating the possibility of anticipation always suffer from a significant risk of bias. Especially in retrospective studies, one must be aware of ascertainment bias as a result of selection of families: selection of affected parents with late onset of disease, selection of affected descendants with young onset of disease and/or selection of cases with simultaneous onset in parents and offspring.[25,26] Our study used predefined inclusion criteria to analyze MEN1 families regardless of penetrance or age at detection in different generations, minimizing the risk of this type of bias.

Furthermore, bias can arise from differences in follow-up time between generations (so called "truncation bias").[27] Older generations have been under care for a longer period of time than their offspring and generally will not have been followed for the entire "at risk" period, which can introduce possible bias.

In addition, detection bias can occur in multigenerational studies as a result of a beneficial effect of surveillance programs for individuals at risk. The use of predefined surveillance protocols and well-defined criteria of MEN1 manifestations standardizes follow-up for younger generations. However, older generations have benefited less from these screening methods, introducing a possible delay in diagnosing manifestations compared to younger generations. We attempted to reduce this form of bias by conducting separate time-to-event analyses that only included manifestations detected during the period of time the patients were under surveillance for MEN1.

The effect of different observation periods (time bias) must also be taken into account. The improvement of diagnostics – such as enhanced imaging techniques with higher sensitivity – could have resulted in earlier detection of MEN1 manifestations in later generations. Also, other period-related factors (*e.g.*, improvement of medical knowledge, change of potential unknown carcinogenics, or other environmental factors) could have influenced the age at detection of different MEN1 manifestations. However, the average year at detection of dpNET, PIT and bp-NET did not differ much between generations, suggesting that time bias was not of great influence on these results. As the median year of pHPT and ADR diagnosis differed more across generations, the effect of improved diagnostics or other observation period-related factors cannot be ruled out in these cases.

Finally, the low prevalence of specific MEN1 manifestations (*e.g.*, th-NET and gastric NET) and the small sample size of fourth generation family members compromise the precision of estimations regarding the age at detection of MEN1-related manifestations and a possible effect of anticipation. With all these potential biases and limitations in mind, conclusions about the presence of genetic anticipation in MEN1 must be interpreted with caution.

In 1997, Giraud *et al*. implied the possibility of anticipation within MEN1 by describing one MEN1 family with clinical expression suggestive of this phenomenon.[18] The second and third generations of this particular family showed no clinical evidence of MEN1 to date, whereas in the fourth generation eight members were affected (including two metastatic th-NET, a case of metastatic dpNET, and a spinal ependymoma). All five fifth-generation patients showed at least one MEN1-related manifestation below the age of 22. More recently, intrafamilial correlations and heritability of MEN1 manifestations were investigated in a large French cohort of 797 patients. Thevenon *et al.* reported significant heritability of three MEN1 manifestations (PIT, ADR and th-NET). However, genetic anticipation was not a subject of the study.[8]

In order to make a valid call on the existence of genetic anticipation in MEN1, both (repeated) conclusive observations of decreased age at detection in successive generations and a commonly accepted explanatory biological mechanism are needed. However, little is known about possible molecular mechanisms that could explain anticipation in hereditary cancer syndromes like MEN1.

One potential mechanism involves progressive telomere shortening. In 2004, Vulliamy *et al*. found an association between clinical anticipation and a significant decrease in telomere length in successive generations in autosomal dominant dyskeratosis congenita, possibly owing to haploinsufficiency of the affected gene encoding the RNA component of telomerase (*TERC*).[10] This association was also reported in hereditary breast cancer syndrome, Li-Fraumeni syndrome and von Hippel Lindau disease.[14,28,29] In contrast, an association study of telomere length and single nucleotide polymorphisms (SNPs) in 43 telomere biology genes showed inverse associations between all SNPs included in the *MEN1* region and telomere length. This suggests that a loss of function would result in an increased telomere length, which is in contrast to what one would expect.[30] However, this assumption has not been investigated in affected MEN1 patients up to now.

A second hypothesis to explain anticipation has been suggested in Lynch syndrome and is based on the progressive accumulation of germline mutations prior to the loss of heterozygosity.[31] Possibly (low) levels of microsatellite instability are present in germ cells of patients with Lynch syndrome, passing on mutant alleles to their offspring. Of course, the molecular functions of mismatch repair genes associated with Lynch syndrome (*MSH2*, *MLH1*, *MSH6* and *PMS2*) are incomparable to the functions of menin, which – although not entirely unraveled yet – appear to concentrate on gene expression regulation.[32] Therefore, it is very doubtful whether this hypothesis is applicable to MEN1. To our knowledge, impairment of menin function before loss of heterozygosity has not been investigated to date.

A third mechanism for anticipation has been proposed in Li-Fraumeni syndrome. It is suggested that anticipation is caused by accumulation of DNA copy number variations in the context of *TP53* haploinsufficiency.[33] Others have proposed an alternative model in which anticipation could be explained by the inheritance of specific risk-increasing factors from the non-carrier parent.[34] Studies to explore these theories in MEN1 have not been performed yet.

In conclusion, results from this study showed a decreased age at detection of MEN1 manifestations in successive generations, suggesting the presence of genetic anticipation. However, despite our efforts, it is not possible to draw firm conclusions from these analyses due to the potential risk of residual bias. Our results require confirmation in other large population-based MEN1 cohorts with long-term follow-up to determine the true role of genetic anticipation in MEN1 syndrome. Furthermore, translational research is needed to investigate molecular mechanisms explaining this phenomenon of anticipation in MEN1. The demonstration of genetic anticipation in MEN1 would provide the opportunity of more personalized screening protocols, with the possibility of screening at a younger age in future generations of MEN1 patients.

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CHAPTER 7

The management of neuroendocrine tumors of the lung in MEN1: results from the Dutch MEN1 Study Group

Medard F.M. van den Broek[1], Joanne M. de Laat[1,2], Rachel S. van Leeuwaarde[1], Annenienke C. van de Ven[3], Wouter W. de Herder[4], Olaf M. Dekkers[5], Madeleine L. Drent[6], Michiel N. Kerstens[7], Peter H. Bisschop[8], Bas Havekes[9], Wenzel M. Hackeng[10], Lodewijk A.A. Brosens[10], Menno R. Vriens[11], Wieneke A. Buikhuisen[12], Gerlof D. Valk[1]

[1] Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[2] Department of Internal Medicine, University Medical Center Utrecht, Utrecht, The Netherlands
[3] Department of Endocrinology, Radboud University Medical Center, Nijmegen, The Netherlands
[4] Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands
[5] Departments of Endocrinology and Metabolism and Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
[6] Department of Internal Medicine, Section of Endocrinology, Amsterdam UMC, location VU University Medical Center, Amsterdam, The Netherlands
[7] Department of Endocrinology, University of Groningen, Groningen, The Netherlands
[8] Department of Endocrinology and Metabolism, Amsterdam UMC, location Academic Medical Center, Amsterdam, The Netherlands
[9] Department of Internal Medicine, Division of Endocrinology, Maastricht University Medical Center, Maastricht, The Netherlands
[10] Department of Pathology, University Medical Center Utrecht, Utrecht, The Netherlands
[11] Department of Endocrine Surgical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
[12] Department of Thoracic Oncology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

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Abstract

Introduction

Multiple Endocrine Neoplasia type 1 (MEN1)-related neuroendocrine tumors (NETs) of the lung are mostly indolent, with a good prognosis. Nevertheless, cases of aggressive lung NET do occur, and therefore the management of individual patients is challenging.

Aim

To assess tumor growth and the survival of patients with MEN1-related lung NETs at long-term follow-up.

Methods

The population-based DutchMEN1 Study Group database (n = 446) was used to identify lung NETs by histopathological and radiological examinations. Tumor diameter was assessed. Linear mixed models and the Kaplan-Meier method were used for analyzing tumor growth and survival. Molecular analyses were performed on a lung NET showing particularly aggressive behavior.

Results

In 102 patients (22.9% of the total MEN1 cohort), 164 lesions suspect of lung NETs were identified and followed for a median of 6.6 years. Tumor diameter increased 6.0% per year. The overall 15-year survival rate was 78.0% (95% confidence interval: 64.6-94.2%) without lung NET-related death. No prognostic factors for tumor growth or survival could be identified. A somatic c.3127A>G (p.Met1043Val) *PIK3CA* driver mutation was found in a case of rapid-growing lung NET after six years of indolent disease, presumably explaining the sudden change in course.

Conclusion

MEN1-related lung NETs are slow-growing and have a good prognosis. No accurate risk factors for tumor growth could be identified. Lung NET screening should therefore be based on well-informed, shared decision-making, balancing between the low absolute risk of an aggressive tumor in individuals and the potential harms of frequent thoracic imaging.

Introduction

Multiple Endocrine Neoplasia type 1 (MEN1) is a rare autosomal dominant disorder caused by loss of function of the *MEN1* gene, a tumor suppressor gene encoding the protein menin.[1] Patients with MEN1 are predisposed to the development of various endocrine tumors at a young age, with primary hyperparathyroidism due to parathyroid adenomas, neuroendocrine tumors (NETs) of the pancreas and duodenum, and pituitary adenomas being the most common, so called "major" manifestations. MEN1 patients are also at risk of adrenal tumors, lung NETs, thymic NETs, gastric NETs. Non-endocrine tumors such as angiofibromas, lipomas, leiomyomas, meningiomas and probably breast cancer are also recognized as manifestations of the syndrome.[2–5]

Lung NETs are reported in 4.7% to 31.3% of MEN1 patients, depending on whether the diagnosis was histopathologically proven or based on a combination of histopathological and radiological examinations, respectively.[6–10] Clinical practice guidelines advise annual or biannual screening for lung and thymic NET by thoracic computed tomography (CT) scan or magnetic resonance imaging (MRI) scan, although the frequency of imaging is debated.[11,12]

Outcomes of previous studies suggest that MEN1-related lung NETs are associated with a relatively indolent course and a good prognosis. Growth analysis by our group showed a 17% tumor diameter growth per year (tumor doubling time: 4.5 years), with a median patient follow-up of 3.3 years. Tumor doubling time appeared to be shorter in males compared with females (2.5 vs. 5.5 years).[7] Similar results were reported from other MEN1 cohorts, further confirming a benign natural course of disease.[8,9]

However, despite the indolent course of lung NETs in growth analyses, aggressive and fatal cases of lung NET do occur. Aggressive lung tumors, including large cell neuroendocrine carcinomas (LCNEC) and small cell neuroendocrine carcinomas (SCLC) with lethal consequences were described in seven MEN1 patients in a recent French study of the Groupe d'étude des Tumeurs Endocrines (GTE). However, given the large cohort-size of 1023 MEN1 patients, the long-term follow-up of median 48.7 years, high frequency of smokers and lack of molecular analyses, a causal relationship with MEN1 syndrome was unclear.[10]

The aggressive tumor behavior in some patients raises questions whether lung NETs truly remain indolent over the course of longer follow-up, and which factors associate with aggressive tumor biology. In this respect, of potential interest are additional somatic mutations that can drive accelerated tumor growth and smoking status, because high-grade NETs were more frequently diagnosed among smokers in the above-mentioned French GTE study.

The aims of this study were to assess growth patterns and survival of MEN1-related lung NETs during longer-term follow-up and to identify risk factors for tumor growth and survival. Moreover, we tried to elucidate the unexpected aggressive course of a lung NET in an individual patient with sudden accelerated growth and aggressive biological behavior at the molecular level.

Methods

Study design and patient selection

Patients were selected from the Dutch national MEN1 database of the DutchMEN1 Study Group (DMSG). This longitudinal database – which includes > 90% of the Dutch MEN1 population – includes all MEN1 patients ≥ 16 years of age at the end of 2017 under treatment at one of the Dutch university medical centers (UMCs) between 1990 and 2017. MEN1 diagnosis was established following current international guidelines.[11] Using a predefined protocol, clinical and demographic data were collected from 1990 to 2017 by a standardized medical record review. Detailed information on the DMSG database methods have been described previously.[13] The study protocol was approved by the medical ethical committees of all UMCs.

As previously described, patients with lung NETs or lung lesions suspect of lung NETs were identified based upon histopathological and radiological findings.[7] All pulmonary lesions on CT or MRI scan were reviewed to select potential lung NETs. Nodules were suspected of being a lung NET based on the report from a senior radiologist and confirmation in follow-up scans. In case of doubt, individual cases were discussed (MB, JL, GV). Potential lung metastases from other NETs were excluded on histological and/or radiological grounds. Contralateral lung NETs and ipsilateral recurrence of lung NETs after surgery were considered separate lung NETs for the growth analysis.

Outcome

The primary outcomes were the growth rate of lung NETs (measured in the percentage of increase of the largest tumor diameter) and all-cause mortality. The potential influence of gender, smoking status, age at lung NET diagnosis and baseline tumor size on growth rate and survival was evaluated. Previously reported genotype-phenotype associations in other cohorts were also assessed: genotype was dichotomized according to the type of mutation (missense vs. nonsense/frameshift), interacting domain (JunD, CHES1) and a combination of exon and type of mutation (nonsense and frameshift mutations in exons 2,9,10).[14–17] Furthermore, we studied the effect of tumor classification and stage – based on the Classification of Malignant Tumours (TNM) and the World Health Organization

Classification of Tumours of the Lung, Pleura, Thymus and Heart (2015) – and the effect of lung surgery on survival.[18,19] Histopathological tumor characteristics (size, mitotic index, lymph node status), type of surgery and follow-up status of histopathologically proven lung NETs were reported.

Statistical analysis

Tumor growth was studied using multilevel, linear mixed models analysis, accounting for clustering of observations within distinctive lung tumors (*e.g.*, left- and right-sided tumor) within patients. Follow-up time (years) started at the time of lung NET diagnosis. Due to the violation of model assumptions (*i.e.*, abnormal distribution of residuals), logarithmic transformed lung NET diameter was used as a dependent variable. Because current management recommendations advise surgical resection of lung NETs ≥ 20 mm upon discovery, tumors with a baseline size ≥ 20 mm in diameter were excluded from growth analysis.[20] Possible effect modification was assessed for gender, genotype, smoking status, age at lung NET diagnosis and baseline tumor size.

Survival analysis was performed using Kaplan-Meier plots. The time from diagnosis of lung NET until death, lost to follow-up or the end of follow-up was included for analysis. The effect of gender, genotype, smoking status, baseline tumor size, surgery, World Health Organization classification and lymph node involvement on survival was determined with log-rank tests.

Continuous variables are presented as mean value and standard deviation (SD) or median and interquartile range (IQR) when appropriate. Categorical variables are described as percentages. Comparisons between groups were performed using the chi-square test for categorical variables and a Student's *t* test (normal distribution) or Mann-Whitney *U* test (not normal distribution) for continuous data. Statistical significance was set at $P < 0.05$.

Investigations of the tumor showing accelerated growth

One patient with accelerated tumor growth and aggressive tumor behavior is described in more detail. Several genetic analyses were performed: next generation sequencing (NGS) was performed using Ion Ampliseq (Ion Torrent) with a custom-made panel used for analysis of lung tumors (genes specified in the Supplementary Material; all supplementary material and figures are located in a digital research materials repository).[21] Whole genome sequencing (WGS) on fresh-frozen tissue was performed at the Hartwig Medical Foundation according to all international standards (reference genome version GRCh37).[22] Copy number variation analysis was based on single nucleotide peptide (SNP) data using the Infinium CytoSNP-850K BeadChip version 1.2 array and deoxyribonucleic acid (DNA) methylation data generated by the Illumina MethylationEPIC array platform, which was analyzed with R package "Conumee".[23] By using a purity ploidy estimator on the WGS data,

the copy number profile of the tumor was assessed in more detail.[24] Additionally, ribonucleic acid (RNA) was isolated and processed to investigate possible receptor tyrosine kinase mesenchymal-epithelial transition factor *(cMET*) exon 14 skipping. The possibility of a translocation in the *REarranged Translocation* proto-oncogene (*RET* gene) was explored using fluorescence *in situ* hybridization (FISH). Furthermore, the presence of alternative lengthening of telomeres was studied by FISH, and loss of alpha-thalassemia/mental retardation syndrome X-linked protein (ATRX) and death domain-associated protein (DAXX) expression was immunohistochemically determined as previously described.[25] Likewise, menin immunohistochemistry was performed using recombinant anti-menin antibody GeneTex EPR3986.

Results

Longitudinal cohort study

A total of 446 patients (247 female, 55.4%) were included in the DMSG database by the end of 2017. The median age at MEN1 diagnosis was 37 years (range 4-82 years). The diagnosis of MEN1 was confirmed by a pathogenic *MEN1* mutation in 355 cases (79.6%) and 38 patients (8.5%) were obligate carrier of the familial occurring pathogenic *MEN1* mutation because they had at least one major MEN1-associated tumor in combination with a first-degree relative with a confirmed *MEN1* mutation. A total of 53 patients (11.9%) were diagnosed on clinical grounds (two out of the three major MEN1-associated tumors). In 51 of those patients, genetic analysis showed no pathogenic *MEN1* mutation (11.4%). A *CDKN1B* mutation was found in three of these 51 patients. In the two remaining patients diagnosed on clinical grounds, no genetic analysis was performed.

Periodic screening for lung NETs by means of interval thoracic CT scan was performed in 352 patients (78.9%). Patients who underwent CT examination did not differ from the rest of the MEN1 cohort in terms of gender, smoking status and genotype. Pulmonary nodules were detected in 177 patients (50.3% of patients who were under periodic screening). A lung NET was excluded in 75 patients based on pathology results (n = 5), radiological evidence of metastatic origin of the lesion (n = 15), radiological evidence of another (benign) origin of the lesion (n = 19), or lack of confirmation on follow-up imaging (n = 36). See Figure 1 for the full flowchart. A total of 164 lesions suspect of lung NET in 102 patients (22.9% of the entire cohort) were therefore included in the analysis.

Histopathological and clinical characteristics

Lung NETs were diagnosed based on the combination of radiological and histopathological findings in 29 patients (6.5% of the entire cohort, 28.4% of patients included in the analysis)

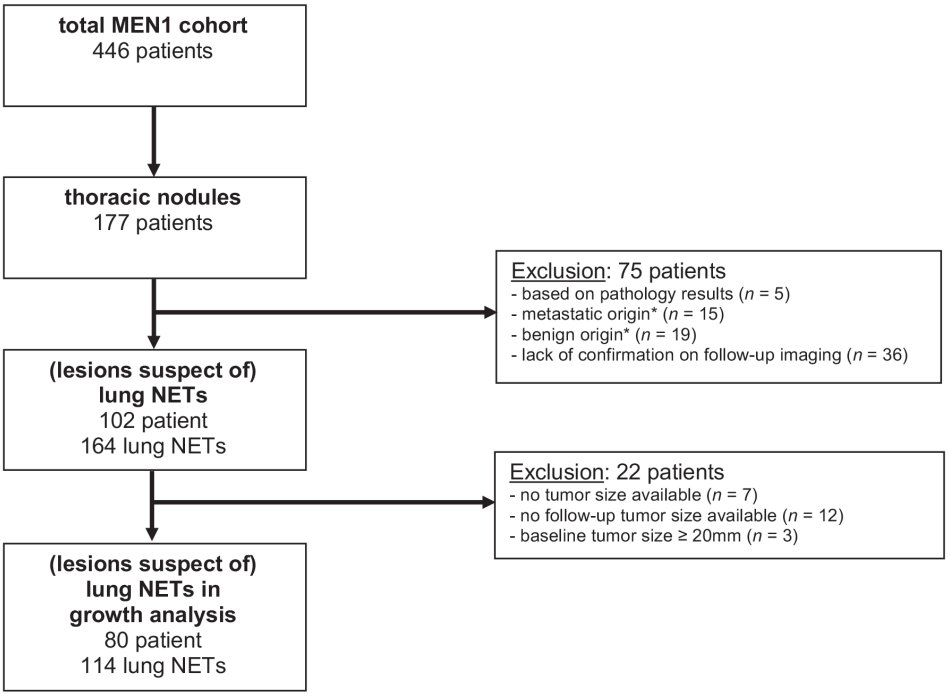

Figure 1. Flowchart of patient selection
Abbreviations: MEN1, Multiple Endocrine Neoplasia type 1; NET, neuroendocrine tumor.
* radiological evidence

and were highly suspected of lung NET solely on radiological evidence in 73 patients (71.6% of patients included in the analysis). Lung NETs were diagnosed at a median age of 43 years (IQR 38-57 years). Patients with lung NETs were more frequently female (*n* = 61, 59.8%), reflecting the overall gender distribution within the cohort. There was no significant difference in smoking status (29.0% vs. 37.3%, respectively) or genotype between patients with lung NETs and the other MEN1 patients. The prevalence of lesions suspect of lung NET was comparable between patients with a confirmed pathogenic *MEN1* mutation (25.4%) and familial cases who were an obligate carrier (23.7%). In contrast, a lesion suspect of lung NET was found in only 3 out of 53 (5.7%) clinically diagnosed MEN1 patients (two out of three major MEN1-associated tumors) without *MEN1* mutation. In this patient group, one lesion was found in one out of three patients with a *CDKNB1* mutation, one lesion in 1 out of 48 (4.8%) patients in whom genetic analysis showed neither a *MEN1* nor a *CDKN1B* mutation, and one lesion in one of the two patients in whom genetic analysis was not performed.

Median follow-up time from lung NET diagnosis until the end of follow-up (death, lost to follow-up or end of the study) was 6.6 years (IQR 3.4-9.1 years, range: 0.5-38.0 years). The clinical and histopathological characteristics of all patients with a pathological diagnosis of lung NET are shown in Table 1. Tumor size was < 15 mm without accelerating growth in only four patients who underwent surgery. Histopathological examination showed a typical carcinoid in 20 patients and an atypical carcinoid in 8 patients. The mitotic index was > 5 in only two cases. In addition, there was one case with a high-grade neuroendocrine neoplasm (patient 20), which was difficult to classify as either atypical carcinoid or LCNEC (see results, description of the case with an exceptional tumor course).
A total of 50 patients were diagnosed with one (lesion suspect of) lung NET, 43 patients were diagnosed with two, 8 patients were diagnosed with three and 1 patient was diagnosed with four (lesions suspect of) lung NETs, respectively. The baseline tumor size at diagnosis – defined as the largest nodule diameter at the first abnormal CT scan – was < 10 mm in 125 lesions and ≥ 10 mm in 27 lesions. The tumor size was not described in 12 lung NET lesions. A total of 75 lesions were identified in the left lung, compared with 89 lesions located in the right lung.

Growth analysis

Nineteen patients were excluded from the growth analysis due to the lack of sequential data. Additionally, five lung lesions were excluded because of a baseline tumor size ≥ 20 mm. Three tumors ≥ 20 mm were surgically removed. Pathology reports confirmed a lung NET in all cases. The two remaining tumors were not removed due to synchronic metastatic disease (n = 1) and apparent shrinkage in a partial cystic tumor, withholding immediate surgery (n = 1). Recurrence after surgery has occurred in one patient. Two patients with a baseline tumor of ≥ 20mm had a concurrent smaller (< 20 mm) lesion suspect of lung NET that was included in the analysis. Therefore, a total of 114 lesions suspect of lung NET in 80 patients were included in the tumor growth analysis. The median baseline tumor diameter was 5 mm (IQR: 3.0-6.3 mm, range 1-17 mm).
The increase of tumor diameter was 6.0% per year, equivalent to a doubling time of 11.8 years. The individual tumor growth is illustrated in Figure 2. Genotype, gender, smoking status, the age at diagnosis of lung NET and baseline tumor size did not significantly affect tumor growth (Table 2). Operated lung NETs were associated with a significantly higher growth rate than other lesions (P < 0.0005).

Survival analysis

Twelve patients diagnosed with one or multiple lesions suspect of lung NET died during follow-up (11.8%); their cause of death was not related to the lung NET. The overall 15-year survival rate after diagnosis of lung NET was 78.0% (95% confidence interval (CI): 64.6-

94.2%, see Figure 3); the overall 10-year survival rate was 87.8% (CI: 80.1-96.3%). The survival of operated patients was not significantly different from nonoperated patients (P = 0.18). Moreover, gender, smoking status, genotype, baseline tumor size, tumor classification and lymph node involvement did not significantly influence survival (data not shown).

Description of the case with an exceptional tumor course

A 31-year-old male MEN1 patient (patient 20) was initially diagnosed with lung NET based on thoracic imaging, which showed three small intrapulmonary nodules (5 mm) that were suspicious for NETs. For the first six years of follow-up, the nodules showed a gradual growth over the years up to a tumor diameter of 11 mm (corresponding with a doubling time of 5.1 years). However, one nodule located in the left upper lobe started to expand rapidly from 11 to 16 mm within 12 months, with new irregular tumor margins. Functional imaging (Gallium-68 DOTATATE) showed no somatostatin receptor uptake by the tumor, but a number of mediastinal lymph nodes (station 2L, 5 and 6) showed pathological uptake. Lobectomy with lymph node dissection followed soon after. Histological examination revealed a high-grade neuroendocrine neoplasm, which was difficult to classify as either atypical carcinoid or LCNEC. There was extensive vaso-invasive growth, an intralobular satellite lesion and tumor-positive mediastinal and hilar lymph nodes. The tumor was resected with free margins. An endobronchial ultrasound performed postoperatively showed six tumor-positive lymph nodes in mediastinal stations 2L and 4L. Therefore, the patient received adjuvant radiotherapy (60 Gy in 30 sessions). Follow-up CT thorax and liver showed no local recurrence for nine months postsurgery. After 12 months, new extensive liver metastases were found, which were histopathologically confirmed, showing an atypical carcinoid with a Ki67 of 15%.

To further elucidate whether this high-grade neuroendocrine neoplasm should be classified as either atypical carcinoid or LCNEC, extensive analyses were performed on the resected lung NET tissue. Histological analysis showed a tumor with a nested growth pattern composed of rather monotonous cells with round to oval nuclei and clumped chromatin (see Supplemental Material, Figure S1A).[21] Mitotic figures were frequently seen (>10 per high-power fields) and the Ki67 labelling index was 75%. By immunohistochemistry, the tumor was strongly positive for chromogranin A, synaptophysin and transcription termination factor 1 (TTF1), and it was negative for somatostatin receptor type 2a (SSTR2a). P53 immunohistochemistry revealed a wild-type expression pattern. There was no loss or ATRX or DAXX. Menin immunohistochemistry (see Supplemental Material, Figure S1B) showed loss of expression in the tumor cells.[21]

Table 1. Clinical, genetic and histopathological characteristics of patients with pathological diagnosis of lung NET

Patient	Sex	Genetic mutation	Smoking	Age at diagnosis (yr)	Type of surgery	
1	F	Frameshift exon 2: c.249_252del (p.Ile85fs)	FS	44	Wedge resection lower left lobe	
2	M	Frameshift exon 10: c.1561dup (p.Arg521fs)	FS	62	Wedge resection lower left lobe	
3	F	Frameshift exon 10: c.1430dupG (p.Glu478fs)	NS	42	Wedge resection upper left lobe	
4	F	Frameshift exon 2: c.249_252del (p.Ile85fs)	NS	42	Wedge resection upper left lobe	
5	M	Deletion exon 1 to 3: c.-110-?_669+?del (p.?)	CS	45	Lobectomy upper right lobe	
6	M	Frameshift exon 10: c.1561dup (p.Arg521fs)	FS	62	Lobectomy upper left lobe	
7	M	Frameshift exon 10: c.1561dup (p.Arg521fs)	CS	38	Wedge resection right middle lobe	
8	F	Missense exon 4: c.683T→C (p.Leu228Pro)	NS	54	Lobectomy upper right lobe	
9	M	Nonsense exon 6: c.819T→G (p.Y273X) or c.819T→A(p.Tyr273X)	NS	41	Wedge resection in upper and lower left lobe	
10	F	Frameshift exon 3: c.653_660del (p.Ala218fs)	FS	24	Segmentectomy lower left lobe	
11	F	Nonsense exon 8: c.1074C→G (p.Tyr358X)	NS	23	Bilobectomy of middle and lower right lobes	
12	M	In-frame deletion exon 2: c.358_360del (p.Lys120del)[a]	ND	37	Lobectomy right middle lobe	
13	F	Splice mutation intron 4: c.799-9G→A(p.?)	NS	38	Lobectomy upper left lobe	
14	F	Nonsense exon 2: c.377G→A (p.Trp126X)	FS	54	Lobectomy upper right lobe	
15	F	Nonsense exon 10: c.1594C→T (p.Arg532X)	NS	41	Lobectomy right middle lobe	
16	F	In-frame deletion exon 2: c.358_360del (p.Lys120del)	FS	44	Segmentectomy lower left lobe	
17	F	Nonsense exon 8: c.1192C>T (p.Gln398X)	NS	46	Wedge resection upper left lobe	
18	M	Frameshift exon 10: c.1430dupG (p.Glu478fs)	FS	43	Wedge resection lower left lobe	
19	F	Frameshift exon 2: c.249_252del (p.Ile85fs)	NS	43	Wedge resection right middle lobe	
20	M	Frameshift exon 2: c.249_252del (p.Ile85fs)	FS	38	Lobectomy upper left lobe	
21	M	Frameshift exon 2: c.249_252del (p.Ile85fs)	ND	66	Lobectomy upper left lobe	
22	M	In-frame deletion exon 2: c.358_360del (p.Lys120del)[a]	NS	56	Lobectomy lower right lobe	
23	F	In-frame deletion exon 2: c.358_360del (p.Lys120del)[a]	NS	57	Lobectomy right middle lobe	

	Lymfadenectomy (positive/total number of lymph nodes)	Tumor size on PA (mm)	Mitotic index (per 10 hpf)	WHO classification	TNM classification	Length of follow-up (yr)	Follow-up status
	N	7	1	Typical carcinoid	pT1cN0M0	9.2	Nodule ≥10mm IL
	Y (0/ND)	23	0	Typical carcinoid	pT1N0cM0	13.2	No lung lesions
	N	5	ND	Typical carcinoid	pT1c(m)N0M0	14.0	Nodule <10 mm IL, nodule ≥10mm CL[d]
	N	14	0	Typical carcinoid	pT1N0M0	8.8	Nodules <10mm IL and CL
	Y (0/11)	18	1	Typical carcinoid	pT1N0cM0	5.5	Nodules ≥10mm IL and CL; died[e]
	Y (0/11)	30	<1	Typical carcinoid	pT1N0cM0	5.0	No lung lesions; died[f]
	N	7	<2	Typical carcinoid	pT1cN0M0	5.6	No lung lesions[i]; died[g]
	Y (1/>6)	15	5	Atypical carcinoid	pT1N1cM0	23.3	Nodule <10 mm CL
	N	ND	<5	Atypical carcinoid	pT1cN0pM1	12.8	Nodule <10mm CL
	N	13	ND	Typical carcinoid	pT1cN0M0	36.2	Nodules ≥10mm CL[j]
	Y (0/6)	25	2	Atypical carcinoid	pT1N0cM0	11.3	No lung lesions
	ND	ND	ND	Typical carcinoid	ND	36.2	Nodule <10mm IL, nodule ≥10mm CL; died[h]
	Y (1/3)	20	ND	Typical carcinoid	pT1N1cM0	8.2	Nodules <10mm IL and CL
	Y (0/2)	12	<2	Typical carcinoid	pT1N0cM0	7.3	Nodule <10mm IL
	N	10	0	Typical carcinoid	pT1cN0M0	10.7	Nodules <10mm IL and CL
	Y (0/3)	35	2	Atypical carcinoid	pT2N0cM0	7.5	No lung lesions
	N	10	2	Atypical carcinoid	pT1cN0M0	0.1	ND
	N	5	1	Typical carcinoid	pT1cN0M0	6.0	No lung lesions
	Y (1/1)	6	ND	Typical carcinoid	pT1N1cM0	6.8	Nodule ≥10mm IL, nodule <10mm CL
	Y (15/16)	15	10	Atypical carcinoid[c]	pT3N2cM0	0.7	Liver metastases[k]
	Y (1/6)	15	6	Atypical carcinoid	pT1N1cM0	5.8	Nodules <10mm IL and CL
	Y (2/7)	37	4	Atypical carcinoid	pT4N2cM0	4.3	Nodule <10mm CL
	Y (2/?)	19	ND	Typical carcinoid	pT3N2cM0	2.9	ND

Table 1. Continued (Clinical, genetic and histopathological characteristics of patients with pathological diagnosis of lung NET)

Patient	Sex	Genetic mutation	Smoking	Age at diagnosis (yr)	Type of surgery	
24	F	Deletion whole gene: c.-110-?_1848+?del (p.?)	NS	64	Wedge resection lower left lobe	
25	F	Missense exon 10: c.1489C>T (p.Pro497Ser)[b]	NS	27	Partial resection upper left lobe	
26	F	Frameshift exon 10: c.1561dup (p.Arg521fs)	FS	54	Lobectomy lower right lobe	
27	F	Nonsense exon 2: c.270T>G (p.Tyr90*)	NS	44	Lobectomy upper left lobe	
28	F	Frameshift exon 10: c.1677_1684dup8 (p.Lys562fs)[a]	NS	57	CT guided biopsy upper left lobe	
29	F	In-frame deletion exon 2: c.358_360del (p.Lys120del)[a]	NS	43	Wedge resection lower left lobe	

Abbreviations: CT, computed tomography; CL, contralateral lung; CS, current smoker; F, female; FS, former smoker; hpf, high-power field; IL, ipsilateral lung; LCNEC, large cell neuroendocrine carcinoma; (m), multiple tumors; M, male; N, no; ND, not determined; NS, never smoked; PA, pathology; RTx, radiotherapy; TNM, TNM Classification of Malignant Tumors; WHO, World Health Organization; Y, yes; yr, year.

Patients 1-16 have already been reported in our earlier study on neuroendocrine tumors of thymus and lung by de Laat *et al*.[7]

a: Based on genetic analysis of family members.

b: Variant of uncertain significance.

c: High-grade tumor difficult to classify as either atypical carcinoid or LCNEC. Based on histological, immunohistochemical and molecular findings, it was concluded that this tumor was best classified as a high-grade atypical carcinoid (see the Results section).

d: The contralateral nodule was removed by a lobectomy of the middle right lobe. Histopathological examination revealed a typical carcinoid (diameter 34mm) with a mitotic index < 1 and ipsilateral positive hilar lymph nodes (TNM classification: pT2N1Mx).

e: Cause of death: adenocarcinoma of unknown origin.

f: Cause of death: prostate carcinoma.

g: Cause of death: metastatic thymic NET.

h: Cause of death: complicated surgery (not MEN1-related).

i: Patient received additional radiotherapy (unknown dose).

j: The contralateral nodules were removed by a lobectomy of the middle right lobe and segment resection of the upper right lobe. Histopathological examination showed a typical carcinoid (largest lesion: diameter 14 mm) with a mitotic index < 1 (TNM classification: pT1N0M1). Follow-up imaging afterwards revealed a nodule < 10mm in the right lung.

k: Patient received additional radiotherapy (60 Gy). After 12 months, new liver metastases were found, which were histopathologically proven, showing an atypical carcinoid with a Ki67 of 15%.

l: Patient received radiotherapy on the lesion in the upper left lobe (55 Gy) and on another – not biopsied – lesion in the lower left lobe (60 Gy).

m:The contralateral nodule was removed by a wedge resection of the lower right lobe. Histopathological examination revealed a typical carcinoid (diameter 10 mm) with a mitotic index < 1. Two lymph nodes were removed without tumor localization (TNM classification: pT1N0Mx).

	Lymfadenectomy (positive/total number of lymph nodes)	Tumor size on PA (mm)	Mitotic index (per 10 hpf)	WHO classification	TNM classification	Length of follow-up (yr)	Follow-up status
	N	9	2	Atypical carcinoid	pT1cN0M0	0.8	ND
	ND	ND	ND	Typical carcinoid	ND	38.0	Nodule <10mm IL
	Y (1/12)	12	0	Typical carcinoid	pT3N1cM0	6.0	No lung lesions
	Y (4/4)	14	<1	Typical carcinoid	pT3N2M0	2.2	Nodules <10mm IL and CL
	ND	ND	ND	Typical carcinoid	cT1N0M0	5.0	No lung lesions[l]
	ND	10	<2	Typical carcinoid	pT1cN0M0	6.3	Nodule <10 mm IL, nodule ≥10mm CL[m]

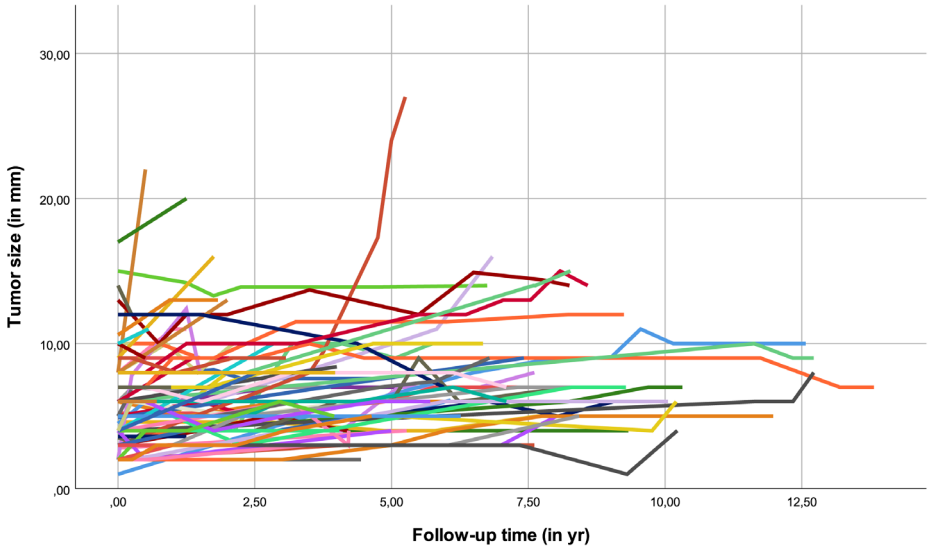

Figure 2. Individual growth of lesions suspect of lung NET
Diameter size (in mm) of lesions suspect of lung NET over time (in years). Each color represents a lesion suspect of lung NET.
Abbreviations: NET, neuroendocrine tumor; mm, millimeter; yr, years.

At initial assessment, NGS did not reveal any mutations. In addition, Reverse transcription polymerase chain reaction (RT-PCR) did not show *cMET* exon 14 skipping. FISH did not reveal a translocation of the *RET* gene or alternative lengthening of telomeres. SNP array was performed to further investigate the somatic second hit inactivation of *MEN1* and to confirm immunohistochemical menin loss, but this did not reveal loss of the *MEN1* locus. Finally, whole genome sequencing was performed, which indeed revealed a somatic inactivating c.333dupT (p.Val112fs) *MEN1* mutation of unknown clinical relevance, suggesting that this mutation – additional to the known germline frameshift c.249_252del (p.Ile85fs) mutation of the patient – was responsible for the loss of a functional *MEN1* gene in the tumor. Based on histological, immunohistochemical and molecular findings, in particular the somatic second hit inactivation of the *MEN1* gene, and lack of mutations associated with LCNEC, it was concluded that this tumor was best classified as a high-grade atypical carcinoid related to the MEN1 syndrome.
Interestingly, WGS also showed a likely pathogenic c.3127A>G (p.Met1043Val) mutation in the *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha* (*PIK3CA*) gene, associated with the PI3K-AKT-mTOR pathway. In retrospect, this mutation was also found in the NGS output with a allele frequency < 1%. Further analysis of the WGS data showed that the c.333dupT (p.Val112fs) *MEN1* mutation was unlikely to have a subclonal origin, whereas the c.3127A>G (p.Met1043Val) *PIK3CA* mutation was probably subclonal (see

Supplementary Material, Figure S2).[21] The variation in allele frequencies of the *PIK3CA* mutation between different tumor samples supports this conclusion. Although it was not possible to indisputably determine the order of events, it seems plausible to assume that the *PIK3CA* mutation occurred after the somatic *MEN1* mutation, leading to accelerating tumor growth.

Table 2. Potential determinants of tumor growth

	Tumor growth[a]
	Statistical significance and regression coefficient[a]
Overall tumor growth (β, 95% CI)	1.060 (1.038-1.083)
Effect modifiers (*P*–value for interaction)	
Gender Male, *n* = 34 (β, 95% CI) Female, *n* = 46 (β, 95% CI)	***P* = 0.437** 1.071 (1.036-1.108) 1.053 (0.975-1.138)
Age at lung NET diagnosis Reference value for age = 0 Change per year (β, 95% CI)	***P* = 0.356** 1.096 (1.019-1.178) 0.999 (0.997-1.001)
Smoking status[b] Never smoked, *n* = 39 (β, 95% CI) Former or current smoker, *n* = 19 (β, 95% CI)	***P* = 0.199** 1.065 (0.985-1.152) 1.036 (0.999-1.074)
Genotype Nonsense/frameshift exon 2,9,10 mutations, *n* = 28 (β, 95% CI) Other mutations[c], *n* = 50 (β, 95% CI)	***P* = 0.120** 1.036 (0.999-1.074) 1.074 (0.990-1.164)
Genotype JunD interacting domain mutations[d], *n* = 25 (β, 95% CI) Other mutations[d], *n* = 45 (β, 95% CI)	***P* = 0.408** 1.071 (1.033-1.109) 1.050 (0.968-1.140)
Genotype CHES1 interacting domain mutations[e], *n* = 20 (β, 95% CI) Other mutations[e], *n* = 50 (β, 95% CI)	***P* = 0.106** 1.031 (0.996-1.066) 1.068 (0.988-1.156)
Genotype Missense mutations[f], *n* = 15 (β, 95% CI) Nonsense/frameshift mutations[f], *n* = 40 (β, 95% CI)	***P* = 0.447** 1.054 (1.026-1.082) 1.076 (0.992-1.167)
Baseline tumor size Diameter < median, *n* = 55 (β, 95% CI) Diameter ≥ median, *n* = 59 (β, 95% CI)	***P* = 0.147** 1.057 (1.033-1.081) 1.071 (1.028-1.116)

β stands for the regression coefficient from the linear mixed models analysis, denoting growth as change in tumor size (factor) per year. Statistical significance is shown in bold.

Abbreviations: CHES1, checkpoint kinase 1; CI, confidence interval; NET, neuroendocrine tumor.

a: Tumor growth was assessed using multilevel linear mixed models analysis, accounting for clustering of observations within lung tumors within patients. Logarithmic-transformed lung NET diameter was used as dependent variable, follow-up time was used as main fixed effect. Potential determinants of tumor growth were treated as additional fixed (interacting) covariates.

b: Data on smoking status were available in 58 out of 80 patients included in the growth analysis (72.5%).

c: All other mutations included. Patients without genetic analysis or with a *CDKN1B* mutation were treated as missings (*n* = 2).

d: Only patients with pathogenic germline nonsense, frameshift, missense mutations and in-frame deletions included. JunD interacting domain: codons 1-40, 139-242, and 323-428.

e: Only patients with pathogenic germline nonsense, frameshift, missense mutations and in-frame deletions included. CHES1 interacting domain: codons 428-610.

f: Only patients with pathogenic germline nonsense, frameshift, and missense mutations included.

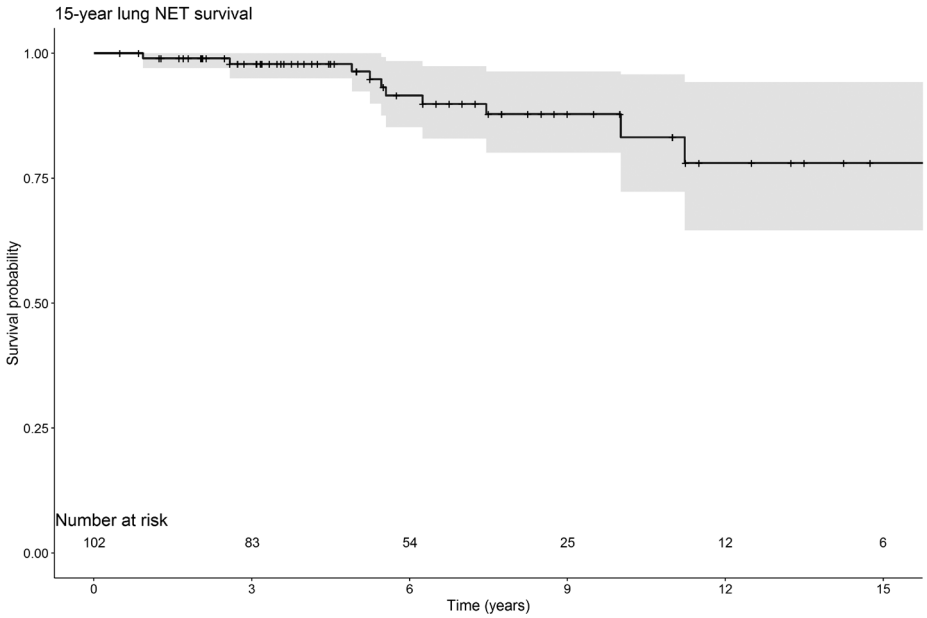

Figure 3. Fifteen-year lung NET survival rate
Abbreviations: NET, neuroendocrine tumor.
+ Censored. Grey area: 95% confidence interval.

Discussion

In the present analyses with a longer follow-up compared with most previous studies, the indolent behavior of MEN1-related lung NETs is confirmed. Approximately one in five MEN1 patients (22.9%) were diagnosed with lesions highly suspect of lung NET(s). The high overall 15-year survival rate and the absence of lung NET-related mortality in the present study emphasizes the relatively benign characteristics of MEN1-related lung NETs. Overall, tumor growth was even lower than previously reported (6.0% per year in the current study vs. 17.0% per year in our previous study). The lung lesions seemed to remain stable over longer periods of time, and growth even slowed down in some lesions, explaining the differences in outcomes of the present study when compared with our earlier results in partly the same patient cohort.[7] Further investigations of the case with a remarkable sudden growth and aggressive tumor biology revealed a somatic *PIK3CA* driver mutation, which probably led to subclonal expansion and could explain the sudden deviant course of disease.

Comparison with literature

The prevalence of histopathologically proven lung NETs in our cohort (6.5%) is comparable to earlier findings in our (4.9%) and other cohorts (4.7-6.6%)[6,8–10]. The higher prevalence of lesions radiologically suspect of lung NET in this study (22.9%) compared with the results from our previous study (13.3%) can be explained by the larger proportion of patients under regular thoracic surveillance. Similar frequencies of lung nodules found on CT scans have been described in German and Tasman cohorts (29.3% and 26.0%, respectively).[9,26] The extremely low prevalence of lung NETs in the subgroup of patients without *MEN1* or *CDKN1B* mutation (2.1%) illustrates the differences in the phenotype and clinical course between mutation-positive and mutation-negative patients, as described previously.[27]

Growth analysis showed an overall indolent course (tumor doubling time ± 12 years). Most lesions suspect of lung NET did not demonstrate significant progression, and some lesions even decreased at long-term follow-up. Through this mechanism, the longer follow-up time in this study could explain the lower overall growth rate compared with our earlier findings in 2014. Unfortunately, molecular mechanisms regulating the growth of lung NETs have not yet been revealed. Furthermore, operated lung NETs seemed to grow significantly faster than non-operated lung NETs in this study. Obviously, these results should be interpreted with caution because a larger tumor size and growth rate often are an indication for surgery. This indication bias could explain the different growth rates between these two groups rather than a difference in the type of pathology. Moreover, the fact that the mitotic index was low in most of the fast-growing and/or larger lesions necessitating surgery underlines the benign course of MEN1-related lung NETs in general. In contrast to our previous results, we were unable to confirm gender-related differences in tumor growth in the current study. Further research in other cohorts is needed to determine the true role of gender in the growth of lung NETs.

Other studies on the growth rate of pulmonary nodules in MEN1 patients showed conflicting results. In a study of 75 MEN1 patients by Bartsch *et al*., pulmonary nodules showed (slight) progression in only four MEN1 patients (18% of patients with pulmonary nodules). None grew larger than 10 mm (median follow-up 67 months).[9] In contrast, results from the Tasman cohort including 50 MEN1 patients suggested a much more aggressive course of pulmonary nodules by demonstrating tumor progression in 54% of patients with lung nodules. However, in this study, tumors were identified using fluorodeoxyglucose positron emission tomography/computed tomography (^{18}F-FDG PET/CT) scans, and tumor growth was mainly seen in FDG-avid lesions. Moreover, pulmonary metastases from other malignancies were not excluded. The more aggressive growth could therefore be a reflection of the use of different selection criteria.[26] Moreover, as the Tasman MEN1 population all share a common founder mutation ((NM_130799.2:c.446-3 C > G heterozygous), the differences in genetic background could also have contributed to the dissimilar course of disease between the two cohorts.

The excellent prognosis of lung NETs found in our study is comparable to findings in other cohorts.[6,8–10] In the largest cohort of histopathologically proven lung NETs to date ($n = 51$), overall survival was also not significantly decreased in patients with a lung NET. However, mainly poorly differentiated and aggressive lung tumors were the cause of death in seven patients. The presence of atypical carcinoid and lymph node involvement tended to be associated with higher mortality in the GTE cohort, while operated patients lived significantly longer. Furthermore, synchronous metastases were associated with shorter survival.[10] We could not reproduce these associations in our cohort, which might be explained by differences in cohort setting (population-based or not), cohort size, lung NET definition and/or selection criteria for surgery.

Extensive molecular analysis of the only high-grade neuroendocrine tumor in this cohort revealed that the somatic mutation of *PIK3CA* may have caused an aggressive course of the lung NET in patient 20. *PIK3CA* encodes the catalytic subunit of phosphatidyl 3-kinase (PI3K*)*, an intracellular central mediator of cell survival signals. *PIK3CA* mutations are associated with numerous cancer types and is most frequently found in endometrial (24-46%), breast (20-32%) and bladder cancer (20-27%).[28] *PIK3CA* mutations are also described in squamous lung cancers (5-10%), in which they possibly lead to resistance to anti-epidermal growth factor receptor therapy.[29] To our knowledge, the frequency and impact of *PIK3CA* mutations in lung NETs has not been described to date.

Strengths

Because patients were selected from the national MEN1 database, including > 90% of the Dutch MEN1 population, it is safe to assume that our study results are generalizable to the entire MEN1 population – at least in the Netherlands. Furthermore, standardized longitudinal data collection reduced the risk of information bias. Thirdly, the additional follow-up time and larger cohort size enabled us to study the natural course of MEN1-related lung NETs more accurately compared with our earlier study and previous studies in other MEN1 cohorts. Moreover, the reliability of the results has been further increased by the larger proportion of patients undergoing regular thoracic imaging (58.2% in our previous report vs. 78.9% in our current cohort).

Limitations

However, some limitations must be kept in mind when interpreting these results. First of all, the retrospective design of this study could have affected growth analyses. These analyses were dependent on data from imaging studies performed during routine patient care. Although imaging protocols and radiology reports for lung NETs have not been standardized for this clinical study, all participating UMCs have a team dedicated to NETs and have employed dedicated thoracic radiologists. Most patients had all their follow-up scans in

the same center, thereby reducing variation. Moreover, to avoid overestimation of accuracy of the outcomes, we took the aspect of longitudinal observations clustered within patients into account in the mixed models analysis.

This study included cases radiologically suspect of lung NET without pathological confirmation. This may have introduced a risk of overestimating the prevalence of lung NET by including lesions that were not truly lung NET, because the interpretation of abnormalities on imaging studies is partly subjective. Combining the interpretation of a senior radiologist, the high number of follow-up scans (including functional imaging studies) and any biopsy results largely mitigated these risks.

One might argue that the lesions found on the CT scans are diffuse idiopathic pulmonary neuroendocrine cell hyperplasia (DIPNECH). However, about half of the patients with DIPNECH complain of cough and dyspnea, often combined with signs of inflammation, bronchial obstruction and mosaic attenuation on radiological imaging.[30,31] These entities were not seen in our patient cohort. Furthermore, to our knowledge, the combination of DIPNECH and MEN1 is limited to only one patient in the literature to date.[30] Based on these considerations, we are confident that it is very unlikely that a diagnosis of DIPNECH has been missed.

Tumor growth was expressed as the change in the largest diameter of the lesions. It is important to realize that such lesions are in fact three-dimensional objects, with an estimated volume of: $\frac{4}{3} * \pi * (\text{radius})^3$ in case of spherical-shaped lesions. This means that doubling of the largest diameter of a spherical lesions is associated with a proportional eightfold increase in the volume of the lesion. The increasing availability of volumetric analysis in radiology allows for better estimation of the true tumor volume change over time – and thereby biological behavior – of lung nodules in the future.

Despite the increasing use of nuclear imaging in MEN1 patients, its exact role in the surveillance and follow-up is yet to be determined.[32–35] Although lung NETs are sporadically mentioned in some studies on nuclear imaging in MEN1 patients, none has focused on its diagnostic value in lung lesions in MEN1 patients specifically. Unfortunately, the setting and retrospective nature of our study prevented us to investigate these matters.

Clinical implications

Results from this study confirm the benign nature of MEN1-related lung NETs, reflected by low tumor growth, excellent survival and the lack of lung NET-related mortality. At long-term follow-up, tumor growth remained limited over time. From this perspective, these findings suggest justification of less frequent thoracic screening than currently advised (every one to two years).[11] This seems to be especially true for patients with clinically diagnosed MEN1 without a pathogenic *MEN1* mutation, given the very low prevalence of lung NETs in this group. The results in the subgroup of clinically diagnosed MEN1 patients

are in accordance with the recent evidence that clinically diagnosed MEN1 patients rarely develop a third MEN1-related manifestation.[27,36] However, as illustrated by one high-grade neuroendocrine tumor (atypical carcinoid), periodic screening remains essential to detect unanticipated accelerated tumor growth in time. Unfortunately, there are still no accurate clinical predictors for growth. A lower thoracic screening frequency appears to be safe at the group level, but might result in failure of timely recognition of aggressively behaving tumors in some individual cases. Nevertheless, the number needed to screen for timely identification of individual aggressive cases is high. Therefore, a personalized screening program should be discussed with individual patients, balancing between the absolute risk individual patients are willing to take and the intensity of screening and exposure to ionizing radiation.

Additionally, although uncommon in MEN1 patients, thymus NETs generally show a very aggressive course of disease and must be considered when discussing thoracic imaging in MEN1 patients.[7] In our cohort, a pathologically proven thymus NET was found in 14 MEN1 patients (3.1%). Thoracic imaging led to the diagnosis in all but one, illustrating the possible additional yield of thoracic surveillance. This must be kept in mind when reviewing the frequency of thoracic imaging with MEN1 patients.

Surgical resection is considered the first treatment of choice in MEN1-related lung NETs.[11] Tumor size and location have been suggested to be important factors when timing surgery.[20] The low growth rate and lack of beneficial effect of surgery on prognosis in this study support a watch-and-wait policy for small lung NETs. However, in case of accelerated tumor growth during follow-up, surgery should be performed without delay.

Conclusion

Overall, MEN1-related lung NETs are slow-growing and have an excellent prognosis. However, unanticipated accelerated tumor growth does occur sporadically. Because no accurate risk factors for tumor growth can be described, periodic screening programs should be based on well-informed decision-making with the individual patient, balancing between the low absolute risk of an aggressive tumor in individuals and the potential harms of frequent thoracic imaging.

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Supplementary material

Additional investigations in patient 20

1. Custom gene panel selected for next generation sequencing

Using the Ion Amliseq (Ion Torrent), DNA variations were studied in the following genes: *AKT serine/threonine kinase 1* (*AKT1*) gene, *anaplastic lymphoma kinase* (*ALK*) gene, *amelogenin Y-Linked* (*AMELY*) gene, *adenomatous polyposis coli* (*APC*) gene, *serine/threonine-protein kinase A-Raf* (*ARAF*) gene, *ataxia telangiectasia mutated* (*ATM*) gene, *B-Raf proto-oncogene, serine/threonine-protein kinase* (*BRAF*) gene, *calreticulin* (*CALR*) gene, *cadherin 1* (*CDH1*) gene, *cyclin dependent kinase inhibitor 2A* (*CDKN2A*) gene, *colony stimulating factor 1 receptor* (*CSF1R*) gene, *catenin beta 1* (*CTNNB1*) gene, *DEAD-box helicase 3 Y-linked* (*DDX3Y*) gene, *epidermal growth factor receptor* (*EGFR*) gene, *erb-b2 receptor tyrosine kinase 2* (*ERBB2*) gene, *erb-b2 receptor tyrosine kinase 4* (*ERBB4*) gene, *enhancer of zeste 2 polycomb repressive complex 2 subunit* (*EZH2*) gene, *F-box and WD repeat domain containing 7* (*FBXW7*) gene, *fibroblast growth factor receptor 1* (*FGFR1*) gene, *fibroblast growth factor receptor 2* (*FGFR2*) gene, *fibroblast growth factor receptor 3* (*FGFR3*) gene, *fms related tyrosine kinase 3* (*FLT3*) gene, *G protein subunit alpha 11* (*GNA11*) gene, *G protein subunit alpha q* (*GNAQ*) gene, *GNAS complex locus* (*GNAS*) gene, *HNF1 homeobox A* (*HNF1A*) gene, *HRas proto-oncogene, GTPase* (*HRAS*) gene, *isocitrate dehydrogenase (NADP(+)) 1* (*IDH1*) gene, *isocitrate dehydrogenase (NADP(+)) 2* (*IDH2*) gene, *Janus kinase 2* (*JAK2*) gene, *Janus kinase 3* (*JAK3*) gene, *kinase insert domain receptor* (*KDR*) gene, *KIT proto-oncogene receptor tyrosine kinase* (*KIT*) gene, *KRAS proto-oncogene*, *GTPase* (*KRAS*) gene, *MDM2 proto-oncogene* (*MDM2*) gene, *MET proto-oncogene, receptor tyrosine kinase* (*MET*) gene, *mutL homolog 1* (*MLH1*) gene, *MPL proto-oncogene, thrombopoietin receptor* (*MPL*) gene, *MYD88 innate immune signal transduction adaptor* (*MYD88*) gene, *notch receptor 1* (*NOTCH1*) gene, *nucleophosmin 1* (*NPM1*) gene, *NRAS proto-oncogene, GTPase* (*NRAS*) gene, *platelet derived growth factor receptor alpha* (*PDGFRA*) gene, *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha* (*PIK3CA*) gene, *phosphatase and tensin homolog* (*PTEN*) gene, *protein tyrosine phosphatase non-receptor type 11* (*PTPN11*) gene, *Raf-1 proto-oncogene, serine/threonine kinase* (*RAF1*) gene, *RB transcriptional corepressor 1* (*RB1*) gene, *ret proto-oncogene* (*RET*) gene, *SMAD family member 4* (*SMAD4*) gene, *SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily b, member 1* (*SMARCB1*) gene, *smoothened, frizzled class receptor* (*SMO*) gene, *SRC proto-oncogene, non-receptor tyrosine kinase* (*SRC*) gene, *Serine/Threonine Kinase 11* (*STK11*) gene, *Telomerase Reverse Transcriptase* (*TERT*) gene, *tumor protein p53* (*TP53*) gene, *von Hippel-Lindau* (*VHL*) gene.

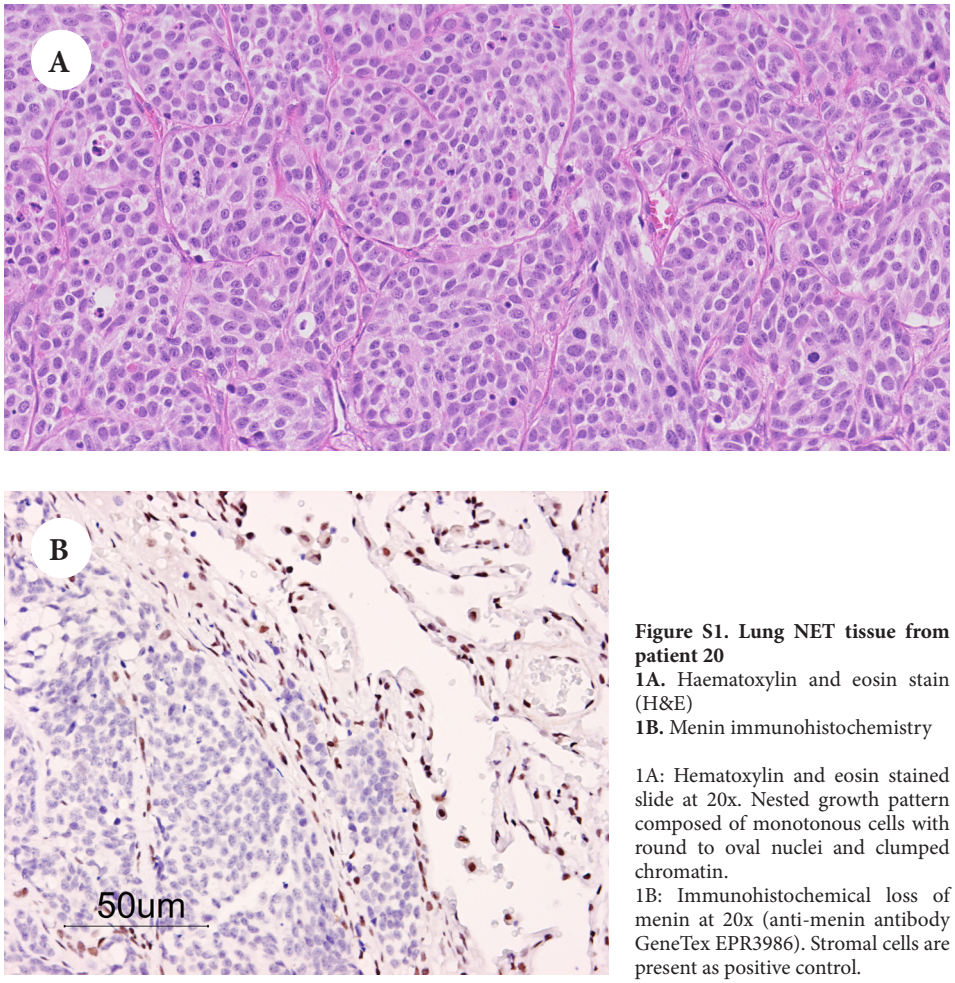

Figure S1. Lung NET tissue from patient 20
1A. Haematoxylin and eosin stain (H&E)
1B. Menin immunohistochemistry

1A: Hematoxylin and eosin stained slide at 20x. Nested growth pattern composed of monotonous cells with round to oval nuclei and clumped chromatin.
1B: Immunohistochemical loss of menin at 20x (anti-menin antibody GeneTex EPR3986). Stromal cells are present as positive control.

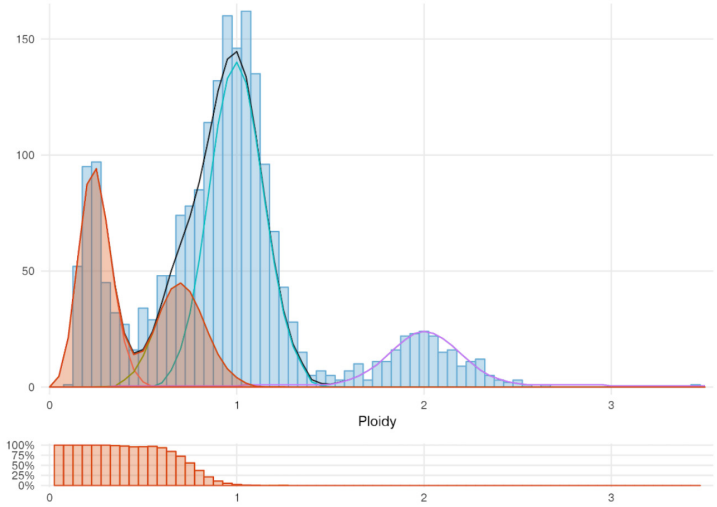

Figure S2. Clonality model lung NET patient 20
Using the whole genome sequencing data, this output of the purity ploidy estimator shows the allele frequencies of all single nucleotide variants, insertions and deletions corrected for local ploidy in blue. The black line shows the overall fitted ploidy distribution. Red filled peaks are below the 0.85 subclonal threshold. The algorithm output showed an 11% chance of the c.333dupT (p.Val112fs) *MEN1* variant being subclonal and a 56% chance of the c.3127A>G (p.Met1043Val) *PIK3CA* variant being subclonal. The full description and source code is available at https://github.com/hartwigmedical/hmftools/tree/master/purity-ploidy-estimator#10-somatic-enrichment.




CHAPTER 8

Well-differentiated bronchopulmonary neuroendocrine tumors: more than one entity

Medard F.M. van den Broek,[1*] Sonja Levy,[2*] Wieneke A. Buikhuisen,[3] Kim Dijke,[2] Koen J. Hartemink,[4] Rachel S. van Leeuwaarde,[1] Menno R. Vriens,[5] Margot E.T. Tesselaar,[2] Gerlof D. Valk[1,6]

* *Authors contributed equally*

1. Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
2. Department of Medical Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands
3. Department of Thoracic Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands
4. Department of Surgical Oncology, Netherlands Cancer Institute, Amsterdam, The Netherlands
5. Department of Endocrine Surgical Oncology, University Medical Center Utrecht, Utrecht, The Netherlands
6. On behalf of the DutchMEN Study group

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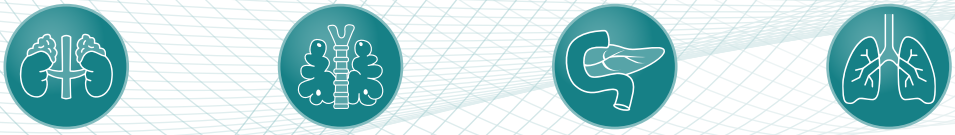

Abstract

Background

Until now, well-differentiated bronchopulmonary neuroendocrine tumors (bpNET) occurring either sporadically (sp-bpNET) or in the context of Multiple Endocrine Neoplasia Type 1 (MEN1) and Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH) are regarded as similar entities. However, in contrast to sp-bpNET: MEN1-related and DIPNECH-related bpNET rarely metastasize or lead to bpNET-related death.

Aim

To describe and compare the course of the disease of sp-bpNET, DIPNECH- and MEN1-related bpNET.

Methods

All patients with histologically confirmed MEN1-related bpNET from the DutchMEN Study Group database (1990-2017), patients with resected sp-bpNET and DIPNECH patients referred to a Dutch ENETS center between 2000-2018 were included. Fisher's exact test was used for comparison between groups. The primary endpoint was disease-specific mortality (DSM). Kaplan-Meier and logrank tests were used to compare survival. Cox regression was used to identify risk factors for DSM in the sp-bpNET subgroup.

Results

We included 112 sp-bpNET, 29 MEN1 and 27 DIPNECH patients. Tumor classification was similar across subgroups. Twenty (18%) patients with sp-bpNET died because of bpNET, compared to none in the MEN1 group and DIPNECH group. Median disease-specific survival was 12.3 (CI 6.3-18.3) years for patients with sp-bpNET, and not estimable for the other subgroups ($P < 0.001$). Differences in baseline characteristics did not explain worse survival in sp-bpNET. Tumor classification and age at diagnosis were independent risk factors for DSM in sp-bpNET.

Conclusion

Patients with sp-bpNET have a significantly higher DSM compared to MEN1- or DIPNECH-related bpNET, unexplained by differences in baseline characteristics. This implies that not all bpNET are similar entities.

Introduction

Bronchopulmonary neuroendocrine neoplasms comprise a heterogeneous group of malignancies of the lung, originating from neuroendocrine cells. These neoplasms can be classified as bronchopulmonary neuroendocrine tumors (bpNET), with a subdivision in typical carcinoid (TC) and atypical carcinoid (AC); small cell lung carcinoma (SCLC) or large cell neuroendocrine carcinoma (LCNEC). All these tumors have been grouped under "bpNET" in the most recent World Health Organization (WHO) Classification of Lung Tumors in 2015.[1] Classification is based on histopathological features, including mitotic count, the presence or absence of necrosis and a variety of cytological and morphologic features.[1] TCs and ACs – historically called "carcinoid" – account for 1-2% of all lung malignancies and are considered well-differentiated tumors with an overall favorable course.[2] Although grouped together with the poorly differentiated SCLC and LCNEC, the 2015 WHO classification recognizes the evident major clinical, epidemiological, histological and genetic differences between lung carcinoids and the high-grade SCLC and LCNEC.[1] For the purpose of this paper, we consider only the well-differentiated typical and atypical carcinoids of the lung, which we will refer to as bpNET. bpNET arise sporadically (sp-bpNET) or in the context of a hereditary predisposition, *e.g.*, Multiple Endocrine Neoplasia type 1 (MEN1). Another context in which bpNET may arise, is Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH), a proliferation of neuroendocrine cells.

The vast majority of bpNET develop sporadically. sp-bpNET are classically diagnosed in the fifth and sixth decade of life, and prognosis largely depends on histological subtype: reported 5-year survival rates are 87-94% and 44-80% for TC and AC, respectively.[3–6] Furthermore, lymph node metastases, distant metastases and higher proliferation rate have been identified as adverse prognostic factors.[5,7]

Multiple Endocrine Neoplasia type 1 is a rare hereditary disease predisposing patients to the development of several endocrine tumors. The classic manifestations of MEN1 are parathyroid hyperplasia or adenomas, neuroendocrine tumors of the pancreas and duodenum and pituitary adenomas, which are caused by inactivation of the *MEN1* gene.[8] Next to other manifestations as gastric and thymic NET, adrenal tumors and breast cancer, patients are also at risk of developing bpNET with a prevalence of 4.7-6.6% of MEN1 patients.[9–14] Clinical practice guidelines advise frequent thoracic imaging to detect and monitor these tumors. However, more recent studies have shown that MEN1-associated bpNET appear to have an indolent behavior and do not decrease overall survival in MEN1 patients, although a few aggressive cases with fatal outcome have been described.[11,12] Curative surgery is considered the first treatment of choice, but a watch-and-wait policy is suggested for small (< 2 cm) and slow-growing MEN1-related bpNET.[15,16]

Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia, an uncommon pulmonary disease characterized by proliferation of pulmonary neuroendocrine cells restricted to the bronchial and bronchiolar epithelium and presence of tumorlets, is recognized by the WHO as a pre-invasive precursor lesion for bpNET.[1] This condition typically occurs in non-smoking, middle-aged women and may cause a variety of symptoms (*e.g.*, cough, dyspnea, wheezing) for which the term "DIPNECH syndrome" has been coined.[17,18] Although the diagnosis of DIPNECH is currently not defined by stringent clinic-pathological and/or radiological criteria, Rossi *et al*. have proposed a comprehensive flow-chart for the diagnosis of either solely DIPNECH, or DIPNECH syndrome.[18] In most patients, DIPNECH is associated with a stable or slowly locally progressive disease, with only a few disease-related deaths reported to date.[19–24]

Until now, bpNET of any type are considered the same disease, which is also reflected in the recently updated international guidelines.[25,26] However, based on clinical experience and earlier reports on the natural course of sp-bpNET, MEN1-related bpNET and DIPNECH-related bpNET, the question arises whether these subtypes are in fact different entities; MEN1- and DIPNECH-related bpNET rarely metastasize or lead to bpNET-related death,[9,10,24,11–13,19–23] while the prognosis of sp-bpNET seems more heterogeneous – and perhaps worse than non-sporadic forms of bpNET.[3–7]

To our knowledge, head-to-head comparisons between sp-bpNET, MEN1-related bpNET and DIPNECH-related bpNET are lacking to date. Therefore, in this cohort study, we aimed to compare disease-specific mortality (DSM) of patients with sp-bpNET, MEN1- and DIPNECH-related bpNET. Additionally, since we describe a rather large cohort of sp-bpNET, we aimed to identify independent risk factors for DSM in patients with sp-bpNET.

Materials and methods

Study design and patients

All patients with sp-bpNET referred to the Netherlands Cancer Institute (NKI)/University Medical Center Utrecht (UMCU) European Neuroendocrine Tumor Society Center of Excellence (ENETS CoE) between 2000-2018 who had undergone surgery with curative intent were included. Similarly, all patients with histopathologically confirmed bpNET in the context of DIPNECH referred to this ENETS CoE within the same time period were included. Patients were considered to have DIPNECH or DIPNECH syndrome based on the diagnostic flowchart that has been developed by Rossi *et al*., taking into account symptoms/lung function abnormalities, compatible radiological signs and histological features.[16] Patients with bpNET in the context of DIPNECH and DIPNECH syndrome were grouped in one subgroup and further named "DIPNECH".

Patients with bpNET in the context of MEN1 were all selected from the Dutch national MEN1 database of the DutchMEN Study Group (DMSG). This database covers over 90% of the adult Dutch MEN1 population and includes all MEN1 patients ≥ 16 years of age at the end of 2017, under treatment at one of the Dutch university medical centers between 1990 and 2017. Detailed information on the DMSG database methods have been described previously.[27] To avoid misclassification of lung metastasis from NET of a different origin in patients with MEN1, only patients with histopathologically confirmed bpNET were selected for analysis.

Patient and tumor characteristics were retrieved from the longitudinal institutional neuroendocrine neoplasia database, in which all patients treated in the joint center are included, and the DMSG database. Tumor staging at time of diagnosis was based on pathological reports and derived from the 8th edition of the Tumor-, Node-, Metastasis (TNM) staging for Non-Small Cell Lung Cancer, which is also used for bpNET.[28] Since no consensus exists on TNM staging for DIPNECH, this was not performed for the DIPNECH cohort. Tumor grading in typical and atypical carcinoid was based on mitotic count and the presence of necrosis. Ki67-index was also included in the analysis. When unusually high/low mitotic count or Ki67-index were found, consensus on typical or atypical classification was reached within a multidisciplinary tumor board, based on a combination of tumor morphology and the dis-/concordance of mitotic count and Ki67-index. This study was conducted in agreement with the NKI/UMCU ethical guidelines and all patients gave consent for the use of their medical data as per institutional protocol.

Outcomes

For the three subgroups, primary outcome was disease-specific mortality. Secondary outcomes were identification of differences in patient characteristics between the subgroups that could influence survival. For patients with sp-bpNET, identification of independent risk factors for DSM was an additional outcome.

Statistics

Median with (interquartile) range was used to describe continuous variables, frequency and percentages were calculated for categorical variables. For comparison between groups Fisher's exact test was performed for categorical variables, and the Wilcoxon rank sum test for continuous variables. Disease-specific mortality (DSM) was defined as bpNET-related death. Patients who died of unknown causes were considered to have died of bpNET if recurrence or metastatic disease was present at last follow-up. Patients with no evidence of disease and death ≤ 6 months after last follow-up were considered to have died of other causes. Patients who died of other causes or were alive at end of follow-up were censored. For visualization and comparison of survival between subgroups Kaplan-Meier curves and

the logrank test was used, respectively. Cox regression was performed for uni- and multivariable analysis of risk factors for DSM. Analysis were performed using IBM SPSS Statistics software, version 25.0, and R version 3.6.2, package "survival".

Results

Patients

A total of 168 patients were included, of which 112 were patients with sp-bpNET, 29 patients had histologically proven bpNET in the context of MEN1, and 27 patients had a bpNET in the context of DIPNECH. Baseline characteristics and comparisons for all three subgroups can be found in Table 1. Since pathological characteristics are inherently associated with tumor classification, these were stratified according to typical and atypical carcinoid classification, and can be found in Table 2.

Survival

Median follow-up for all patients was 4.8 years (interquartile range (IQR) 2.2-7.5). For patients with sp-bpNET, this was 4.4 years (IQR 2.0-7.2), for patients with MEN1-related bpNET this was 6.7 years (IQR 4.9-12.0) and for patients with DIPNECH median follow-up was 2.9 years (IQR 1.3-6.7). Twenty patients (17.8%) died because of their bpNET in the sp-bpNET group. Six (5.3%) of them had an unknown cause of death but were considered to have died of bpNET due to the presence of metastatic disease at last follow-up and occurrence of death ≤ 6 months afterwards. Taking censoring of patients into account, most patients with sp-bpNET died of bpNET (50% at 10 years of follow-up, 70% at 25 years). In both the MEN1 and DIPNECH group no patients had died of bpNET. Four patients (3.6%) in the sp-bpNET group and 4 patients (13.8%) in the MEN1 group died of other causes. In the MEN1-group, only one of the patients died of a MEN1-related cancer (thymic NET), all other causes of death were non-MEN1-related cancers or the complications thereof. No deaths occurred in the DIPNECH group. Median disease-specific survival was shorter for patients with sp-bpNET, namely 12.3 years (95% confidence interval 7.4-17.1), whereas this was not estimable for patients with MEN1 or DIPNECH. The logrank test showed a significantly different survival distribution between subgroups ($P < 0.001$). Survival curves for all subgroups are shown in Figure 1.

In the sp-bpNET group, patients with AC had a significantly worse survival than patients with TC ($P = 0.003$). Survival curves for TC and AC in sp-bpNET are shown in Figure 2.

Table 1. Baseline characteristics for the three subgroups

Characteristics N (%)/median (range)	Sporadic	MEN1	Sporadic vs. MEN1 *P*-value	DIPNECH	Sporadic vs. DIPNECH *P*-value	MEN1 vs. DIPNECH *P*-value
Total	112	29		27		
Age at diagnosis	54 (18-76)	44 (23-66)	**0.008**	63 (34-85)	**0.004**	**<0.001**
Gender			0.671		**<0.001**	**0.001**
Male	46 (41.1)	10 (34.5)		0		
Female	66 (58.9)	19 (65.5)		27 (100)		
WHO PS			n/a		0.351	n/a
0	45 (40.2)			8 (29.6)		
1	45 (40.2)			16 (59.3)		
2	2 (1.8)			0		
Unknown	20 (17.9)			3 (11.1)		
Tumor classification			0.863		0.096	0.209
Typical	73 (65.2)	20 (69.0)		23 (85.2)		
Atypical	38 (33.9)	9 (31.0)		4 (14.8)		
Unknown	1 (0.9)	0		0		
T stage						
1	60 (53.6)	21 (72.4)	**0.009**		n/a	n/a
2	27 (24.1)	1 (3.4)				
3	5 (4.5)	4 (13.8)				
4	2 (1.8)	1 (3.4)				
Unknown	18 (16.1)	2 (6.9)				
N stage			0.949		n/a	n/a
N0	52 (46.4)	18 (62.1)				
N1	16 (14.3)	5 (17.2)				
N2	17 (15.2)	4 (13.8)				
Unknown	27 (24.1)	2 (6.9)				
M stage			0.206		n/a	n/a
M0	112 (100)	28 (96.6)				
M1	0	1 (3.4)				
Resection			**<0.001**		**<0.001**	**0.001**
No resection	0	1 (3.4)		9 (33.3)		
Lobectomy	64 (57.1)	14 (48.3)		4 (14.8)		
Sleeve lobectomy	7 (6.3)	0		0		
Pneumonectomy	9 (8.0)	0		0		
Wedge resection	11 (9.8)	8 (27.6)		13 (48.1)		
Segmental resection	2 (1.8)	5 (17.2)		1 (3.7)		
Bilobectomy	8 (7.1)	1 (3.4)		0		
Endobronchial approach	8 (7.1)	0		0		
Lymph node dissection	57 (50.9)	5 (17.2)	**0.001**	5 (18.5)	**0.002**	1.00

Abbreviations: bp-NET, bronchopulmonary NET; MEN1, Multiple Endocrine Neoplasia type 1; DIPNECH, Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia; WHO PS, World Health Organization Performance status; T, tumor; N, nodal; M, metastasis; n/a: not applicable.
Statistical significance is shown in bold.

Table 2. Pathological characteristics for the three subgroups, according to typical carcinoid and atypical carcinoid classification

Characteristics N (%)/median (range)	Sporadic	MEN1	Sporadic vs. MEN1 *P*-value	DIPNECH	Sporadic vs. DIPNECH *P*-value	MEN1 vs. DIPNECH *P*-value
Typical Carcinoid	73	20		23		
Ki67-index (%)	3 (0-16)	2 (1-5)	0.948	1 (0-5)	0.077	0.462
Mitotic count/2mm^2	1 (0-8)	1 (0-2)	0.623	1 (0-1)	0.231	0.253
Atypical Carcinoid	38	9		4		
Ki67-index (%)	7.5 (0-30)	10 (1-20)	0.704	2.5 (2-3)	0.089	0.250
Mitotic count/2mm^2	3 (0-27)	4 (2-10)	0.762	2 (2-2)	0.414	0.418
Necrosis[a]			**0.029**		0.104	0.119
Not present	20 (52.6)	7 (77.8)		1 (25.0)		
Present	15 (39.4)	0		1 (25.0)		
Unknown	3 (7.8)	2 (22.2)		2 (50.0)		

Abbreviations: bp-NET, bronchopulmonary NET; MEN1, Multiple Endocrine Neoplasia type 1; DIPNECH, Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia.
Statistical significance is shown in bold.
a: Since the presence of necrosis is a characteristic in the definition the tumor classification for atypical carcinoids, this was only assessed for ACs.

Comparison between subgroups

sp-bpNET with MEN1

Patients with sp-bpNET were significantly older at time of diagnosis (54 vs. 44 years in the MEN1 group). Patients with MEN1 more often had T1 (72.4% vs. 53.6%) or T3 tumors (13.8% vs. 4.5%). Histological classification (typical/atypical) and N-stage was comparable between the two groups. Tumor necrosis occurred more frequently in atypical carcinoids of patients with sp-bpNET (39.4% vs. 0%). No metastatic disease was present in patients with sp-bpNET, compared to 1 patient (3.4%) with M1 disease in the MEN1 group; this was a histologically confirmed contralateral pulmonary lesion. In patients with sp-bpNET, significantly more anatomical resections (85.7% vs. 51.7%) and more lymph node dissections (50.9% vs. 14.2%) were performed.

sp-bpNET with DIPNECH

Patients in the DIPNECH group had a significantly higher age at diagnosis (64 years vs. 54 years) and female predominance was more pronounced in this group (100% vs. 58.9% females). Also, similar to MEN1 patients, DIPNECH patients had significantly less anatomical resections (14.8% vs. 85.7%) and lymph node dissections (18.5% vs. 50.9%), compared to patients with sp-bpNET.

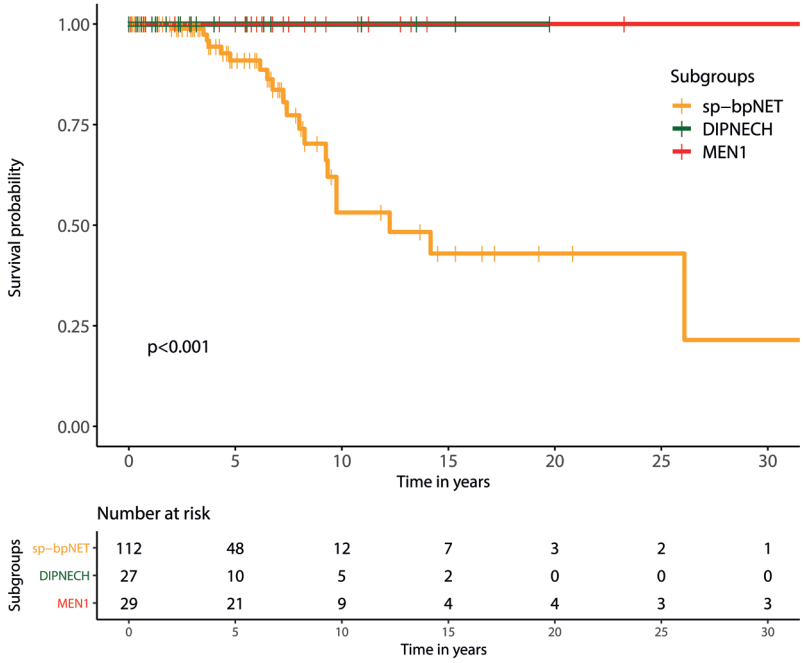

Figure 1. Kaplan-Meier curves for disease-specific survival
Abbreviations: sp-bpNET, sporadic bpNET; DIPNECH, Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia; MEN1, Multiple Endocrine Neoplasia type 1.
P-value shows logrank test for comparison between disease-specific survival.

MEN1 with DIPNECH

Patients with MEN1 were younger at time of diagnosis compared to patients with DIPNECH (44 years vs. 64 years), and more MEN1 patients were male (34.5% vs. 0%). Finally, less patients underwent resection in the DIPNECH group (66.7% vs. 96.5%).

Risk factors for disease-specific mortality in sp-bpNET (Table 3)

Univariable survival analysis for patients with sp-bpNET identified age at diagnosis (HR 1.09), atypical carcinoid (HR 4.70), Ki67-index (HR 1.17), mitotic count (HR 1.07) and lymph node dissection (HR 2.52) as risk factors for DSM. Since the number of disease-specific deaths was limited, multivariable cox regression was performed with selected variables that were deemed most contributing to DSM, according to prior clinical knowledge. Hence, age at diagnosis and tumor classification (typical vs. atypical) were included in the model. Both variables were identified as independent risk factors for DSM; a HR of 1.09 ($P = 0.001$) was found for age at diagnosis, and HR 3.61 ($P = 0.009$) for atypical carcinoids. Results of uni- and multivariable analysis can be found in Table 3.

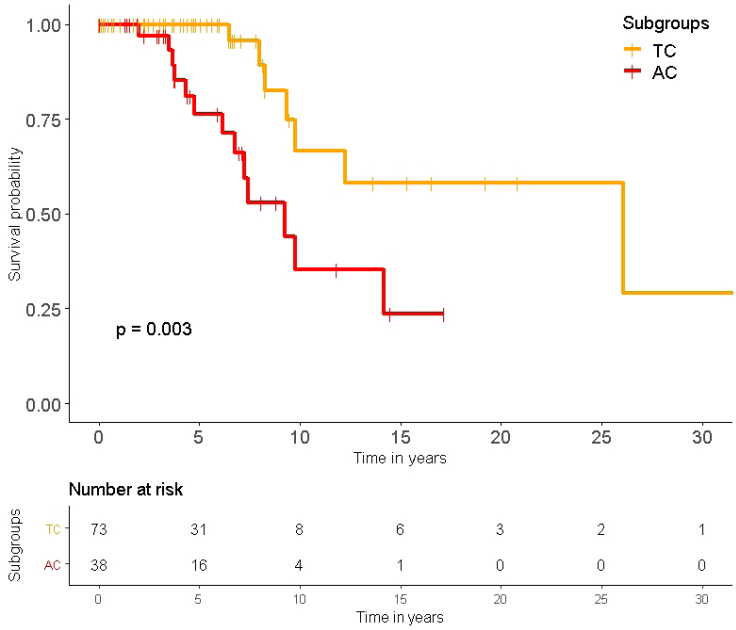

Figure 2. Kaplan-Meier curves for disease-specific survival for sp-bpNET, according to tumor classification
Abbreviations: sp-bpNET, sporadic bpNET; TC, typical carcinoid; AC, atypical carcinoid.
P-value shows logrank test for comparison between disease-specific survival.

Discussion

Results from this head-to-head comparison study showed that patients with sp-bpNET had a higher DSM than patients with MEN1-related bpNET, despite similar histological classification and a more aggressive surgical approach in patients with sp-bpNET. Furthermore, patients with DIPNECH-related and MEN1-related bpNET were found to have a similar outcome. Finally, age at diagnosis and histological classification showed to be an independent prognostic factor for survival in sp-bpNET.

The relatively good prognosis of MEN1-related bpNET in this study is in line with earlier findings in other MEN1 cohorts.[9–12] To our knowledge, only eight bpNET-related deaths in patients with MEN1 have been reported to date. In the largest cohort of histologically proven MEN1-related bpNET (n = 51), median overall survival was 20.2 years and not significantly different from the rest of the cohort.[12] Likewise, the absence of bpNET-related deaths in patients with DIPNECH in our cohort underlines the excellent prognosis of patients with DIPNECH described by others previously.[19–24] Also, the female predominance

Table 3. Univariable and multivariable analysis for disease-specific mortality in sporadic bp-NETs

	Univariable			Multivariable		
Characteristic	**HR**	**95% CI**	***P*-value**	**HR**	**95% CI**	***P*-value**
Age at diagnosis	1.09	1.04-1.14	**<0.001**	1.09	1.04-1.14	**0.001**
Gender						
Male	1					
Female	0.52	0.22-1.25	0.143			
WHO PS						
0	1					
1	2.24	0.85-5.90	0.104			
2	1.9	0.18-12.36	0.711			
Tumor classification						
Typical	1			1		
Atypical	4.70	1.81-12.18	**0.001**	3.61	1.38-9.44	**0.014**
Ki67-index (%)	1.17	1.10-1.26	**<0.001**			
Mitotic count/2mm^2	1.07	1.002-1.13	**0.044**			
T stage						
1	1					
2	0.89	0.32-2.47	0.692			
3	3.73	0.44-31.83	0.148			
N stage						
0	1					
1	1.94	0.58-6.52	0.283			
2	2.72	0.70-10.51	0.147			
Lymph node dissection	2.52	1.02-6.22	**0.045**			

Abbreviations: bp-NET, bronchopulmonary NET; HR, hazard ratio; CI, confidence interval; WHO PS, World Health Organization Performance status; T, tumor; N, nodal.
Statistical significance is shown in bold.

and high age at diagnosis (median 63 years) in our cohort of patients with DIPNECH are comparable with other cohorts.[23]

In line with previous research, patients with sporadic atypical lung carcinoid and older patients had significantly worse survival than patients with a typical carcinoid.[3–6] Others have identified additional prognostic factors associated with adverse prognosis for sp-bpNET, which – among others – were male gender, peripheral tumors and TNM stage.[5,7] Although survival was worse for patients with sp-bpNET as compared to patients with MEN1-related bpNET or DIPNECH, the number of disease-specific events was modest. This prevented us to accurately investigate additional prognostic parameters in our study.

The question arises what could explain the difference in survival between patients with sporadic and MEN1-related bpNET. Although the limited power prevents us to draw firm conclusions, the similarities in tumor classification, Ki67% count and mitotic count between both groups suggest that these histopathological prognostic factors are not responsible for the striking differences in mortality. This is also underscored by the decreasing survival in

both TC and AC in sp-bpNET, compared to MEN1-related bpNET. This shows that even the more favorable typical carcinoids behave much more aggressively in sp-bpNET, compared to MEN1-related bpNET. Interestingly, several factors could arguably have led to a better survival in patients with sp-bpNET: firstly, patients with sp-bpNET were treated more aggressively, with more anatomical resections and lymph node dissections. Secondly, the lack of lymph node involvement was based on imaging studies in 12 out of 18 (67%) MEN1 patients, while N-status in sp-bpNET was based on pathology in all cases. This could have resulted in an underestimation of the number of patients with lymph node involvement in the MEN1 group. Patients with sp-bpNET showed a significantly higher DSM nonetheless, underscoring the different course of disease between these two groups. Thirdly, indication bias could have led to the inclusion of more aggressive MEN1-related lung NET: large tumor size and high growth rate frequently are indications for surgery in MEN1 patients with thoracic nodules suspect of bpNET.[16] Nevertheless, distribution of tumor sizes was quite heterogeneous across the subgroups of MEN1 and sp-bpNET. Although patients with MEN1 had more T1 tumors compared to sp-bpNET patients, they also had a larger proportion of T3 or higher tumors, whereas patients with sp-bpNET had more intermediate (T2) tumors. This can be explained by the often multifocal occurrence of MEN1-related bpNET: the T3 classification of all MEN1-related tumors were based on the presence of a second tumor in the same lobe, while the only MEN1 patient with T4 suffered from two tumors in the same lobe and tumor spread into a major vein. Obviously, patients with sp-bpNET have to develop tumors large enough to cause symptoms before they are recognized, whilst MEN1-related bpNET are usually identified as a small asymptomatic nodule during periodic thoracic surveillance. This latter situation might prompt earlier intervention compared to the sp-bpNET group, thereby possibly explaining the difference in prognosis between groups. However, we saw no differences in N-stage between the two subgroups, which implies that the difference in T-stage did not lead to difference in metastatic disease. Taking into account the aforementioned factors, we still saw a lower DSM in patients with MEN1-related bpNET than in their sporadic counterparts, underlining the true different nature of sporadic bpNET when compared to MEN1-related bpNET.

Possibly, unidentified underlying molecular processes are responsible for the difference in outcome. This hypothesis is supported by recent data from Simbolo *et al.*[29] In their study, next-generation sequencing (NGS) in atypical carcinoids and LCNECs distinguished three transcriptional clusters; patients with a bpNET in the cluster characterized by frequent somatic *MEN1* mutations had a longer cancer-specific survival compared to a cluster with concurrent inactivation of *tumor protein p53* gene and *retinoblastoma 1* gene. However, this seems to contradict previous findings by the same research group: in a subset of 35 atypical lung carcinoids, the presence of a somatic *MEN1* mutation was associated with worse disease-specific survival ($P = 0.0045$).[30] Additionally, lung carcinoids and high-grade

neuroendocrine carcinomas with inactivation of *MEN1* had shorter survival and low *MEN1* mRNA levels correlated with distant metastasis and shorter survival.[31] Therefore, the precise role of *MEN1* mutations in the natural course and prognosis of bpNET is yet to be determined and requires further research into the molecular background of these tumors.

As for patients with DIPNECH, we showed that the clinical behavior is highly comparable with that of MEN1-related bpNET. Interestingly, although the proportion of atypical and typical carcinoids was similar across all subgroups, there seems to be a trend towards a significantly lower mitotic count and Ki-67-index range for patients with DIPNECH compared to the other two subgroups. Especially, there is a notable difference in the ranges of mitotic count and Ki-67-index, with a maximum mitotic count of 2 and a maximum Ki67-index of 5 for patients with DIPNECH. Arguably, patients who develop DIPNECH-related bpNET might be on an even more favorable end of the lung carcinoid spectrum. This suggests that the subtypes of bpNET in some ways parallel those in gastric NET; type 1 gastric NET is associated with (auto-immune) chronic atrophic gastritis and is characterized by multiple lesions but has an excellent prognosis, illustrated by a very low frequency of submucosal invasion or metastasis (like DIPNECH-related bpNET). Type 2 gastric NETs are usually detected in patients with MEN1-related gastrinomas, invade into the underlying tissue somewhat more commonly than type 1 gastric NET but still have a very good prognosis with only a small risk of disease-related death (like MEN1-related bpNET). On the contrary, type 3 gastric NETs – which arise sporadically – show a more aggressive course with frequent metastasis to lymph nodes (50-100%) and liver (22-75%), resulting in a prognosis similar to gastric adenocarcinoma (which seems to mirror characteristics of sp-bpNET).[32]

Some limitations must be considered when interpreting these results. Firstly, the retrospective nature of this study could have influenced the results due to the dependency on accurate record keeping. However, we did not encounter large issues with missing data. Data concerning WHO performance status (WHO PS) of patients with MEN1-related bpNET could not be retrieved. Although WHO PS might be associated with survival, since this parameter was already quite favorable in patients with sp-bpNET – with most patients having WHO PS 0-1 – we do not expect that differences might have contributed to a worse survival for patients with sp-bpNET. Furthermore, tumor T- and N-stage at time of diagnosis were unknown in a considerable proportion of patients with sporadic bpNET (16% and 24%, respectively), presumably due to the aspect of the NKI/UMCU functioning as a tertiary referral center: patients with sp-bpNET were often referred to our center years after initial resection, leading to missing data in some cases. However, we have no reason to believe that the distribution of T- and N- stage of sp-bpNET has been significantly affected by these missing data.

Secondly, pathological samples of MEN1-related bpNET did not undergo revision. Since DIPNECH is a novel diagnosis, it might be possible that (some) MEN1-related bpNET fall in the DIPNECH category if material were to be revised. Nevertheless, this study is the first step in acknowledgement that MEN1-related bpNET are a truly different entity than sp-bpNET, and future research should be aimed in more in-depth comparison of MEN1-related bpNET and DIPNECH-related bpNET, including revision of available MEN1-related bpNET samples.
Thirdly, despite the relatively large cohort of patients with bpNET, the number of deaths was limited. This prevented us from analyzing survival in bpNET in more detail. Ideally, we would have liked to compare DSM between groups while adjusting for prognostic factors, like age at diagnosis. However, the lack of bpNET-related death in patients with MEN1- and DIPNECH-related bpNET already underscore the true divergent nature of these entities compared to sp-bpNET. Furthermore, we were able to identify the two most important prognostic factors for DSM in sp-bpNET, *i.e.*, age at diagnosis and histological classification (typical vs. atypical carcinoid). A follow-up study with even longer follow-up and more patients might result in sufficient events to analyze prognosis in these subgroups in more detail.
Finally, the predisposition to develop multiple neuroendocrine tumors (NET) in MEN1 patients could have led to a selection of MEN1 patients included in this analysis, thereby affecting comparability between groups: among other manifestations, MEN1 patients are prone to the occurrence of duodenopancreatic NET, one of the major causes of MEN1-related death. Events like these earlier in life might have prevented the diagnosis of bpNET in a significant part of the MEN population, due to (1) MEN1-related death, or (2) a lack of histological diagnosis of bpNET due to refraining from biopsy or lung surgery due to (presumed) metastatic disease or poor WHO PS. Theoretically, this might have caused us to miss patients that would have developed bpNET later in life, and perhaps would have shown a more aggressive disease course. Nevertheless, our selection of patients – by including only those patients with histologically confirmed bpNET – was done in such a manner to ensure comparability with sp-bpNET. Also, this selection remains a true representation of clinical practice over a long time period.

To the best of our knowledge, this study is the first to directly compare the outcome of patients with bpNET in the context of MEN1, DIPNECH and the sporadic variant. Despite the rarity of these entities, we were able to include a relatively large cohort by using data from the NKI/UMCU combined ENETS CoE and the population-based DutchMEN Study Group cohort of MEN1 patients. Furthermore, all participating institutions have a team of specialists dedicated to neuroendocrine tumors, including thoracic radiologists and pathologists, which has strengthened the quality of data. Lastly, the standardized and

comprehensive data collection ensured precise and detailed information about relevant patient and tumor characteristics.

Conclusion

Sporadic and MEN1-related bpNET are currently considered the same disease, but results from this study show that there is a significant difference in survival between these groups despite similar histopathological features. Paradoxically, several factors (such as the more aggressive surgical approach in sp-bpNET, possible underestimation of proportion of MEN1-related bpNET with lymph node involvement and the probable indication bias leading to a selection of aggressive MEN1-related bpNET) arguably could have led to a better survival in patients with sp-bpNET compared to MEN1-related bpNET, underscoring the true different nature of these two entities. A possible effect of earlier detection of MEN1-related bpNET cannot be excluded entirely, although potential differences in tumor size at time of surgical resection had not resulted in a difference in locoregional or distal spread. The remarkable difference in survival suggests that these are truly distinctive entities. Furthermore, patients with MEN1- and DIPNECH-related bpNET showed similar survival, suggesting that these entities are more alike, with no bpNET-related death in our study despite the presence of atypical carcinoid in a significant part of these groups. These findings call for verification in other large cohort studies and further research into underlying explanatory (molecular) mechanisms, potentially leading to prognostic guidelines for different subgroups of bpNET.

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Appendix

Members of the DutchMEN Study Group:

- prof. dr. Gerlof Valk, Department of Endocrine Oncology, University Medical Center Utrecht, Utrecht, The Netherlands.
- prof. dr. Wouter de Herder, Department of Internal Medicine, Erasmus Medical Center, Rotterdam, The Netherlands
- prof. dr. Olaf Dekkers, Department of Endocrinology and Metabolism and Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands
- dr. Annenienke van de Ven, Department of Endocrinology, Radboud University Medical Center, Nijmegen, The Netherlands
- prof. dr. Madeleine Drent, Department of Internal Medicine, Section of Endocrinology, Amsterdam UMC, location VU University Medical Center, Amsterdam, The Netherlands
- dr. Peter Bisschop, Department of Endocrinology and Metabolism, Amsterdam UMC, location Academic Medical Center, Amsterdam, The Netherlands
- dr. Bas Havekes, Department of Internal Medicine, Divison of Endocrinology, Maastricht University Medical Center, Maastricht, The Netherlands
- dr. Wouter Zandee, Department of Endocrinology, University Medical Center Groningen, University of Groningen, Groningen, The Netherlands




CHAPTER 9

General discussion




Introduction

Since the first description of a patient with multiple endocrine neoplasia almost one hundred and twenty years ago, physicians and researchers have unraveled many aspects of the MEN syndromes: their clinical manifestations have been described, related pathological features have been discovered and causative genes have been identified. Over the last couple of decades, much progress has been made to gain knowledge about the tumorigenesis, natural course of disease and genotype-phenotype correlations, taking the first steps towards personalized medicine.

Still, more work is needed to optimize care for MEN patients. Firstly, the increase in diagnostic possibilities (including genetic analysis) has led to questions about the appropriate use of diagnostics in patients suspected of a MEN syndrome in order to identify a MEN syndrome in an individual patient in time; how to achieve the right balance between the benefits of a timely diagnosis on the one hand and the physical discomfort, psychological burden and health care costs associated with intensive screening programs on the other? Secondly, as a result of the rarity of MEN syndromes, many recommendations on the surveillance in and treatment of MEN patients are based on (relatively small) observational cohort studies from tertiary referral centers, which introduces the risk of different forms of bias and hampers the quality of scientific evidence. Related to this, MEN1 patients are still offered default surveillance programs – not customized to patients' characteristics –, due to the lack of a clear genotype-phenotype relationship and insufficient data on (factors influencing) the occurrence and natural course of associated manifestations. Although the timing of prophylactic thyroidectomy and the starting age for biochemical screening in MEN2 patients has been tailored based on genotype-related risk categories, it is not possible to personalize many other aspects in the care for MEN2 patients to date.

Of course, it is long road to the holy grail of personalized medicine. Reliable information on the natural course of MEN-related manifestations, including information on prognostic factors that can predict their occurrence and behavior, is essential to take the next steps. However, researchers and physicians have been confronted with several challenges in their attempt to gain more insight into these matters, due to the rarity of MEN syndromes: the low prevalence of disease makes it hard to enroll a sufficient number of MEN patients in a study to get reliable answers to some of the clinically most relevant questions, and randomized clinical trials are often not feasible. Moreover, the rarity of disease complicates the quest for sufficient research funding and attention from policymakers, who are often more interested in more prevalent diseases.

Fortunately, despite these challenges, several dedicated MEN research groups have made continuous efforts over the years to conduct high quality, clinically relevant research in the field of these fascinating entities. This chapter discusses the main results of our own endeavors to improve diagnosing of and screening in MEN syndromes, followed by suggestions for future directions.

Case-finding of MEN syndromes

Effective case-finding to identify a MEN syndrome is of utmost importance, because prompt recognition of the syndromal condition allows timely detection of occult MEN-associated disease in index patients as well as presymptomatic manifestations in affected relatives. For that reason, genetic analysis on the presence of germline mutations in MEN-related genes (*MEN1*, *RET* and *CDKN1B*) is recommended in patients with a clear MEN-like phenotype and all first-degree relatives of a patient with a MEN-associated mutation. Diagnostic difficulties arise when physicians are confronted with patients with apparently sporadically occurring tumors with an atypical phenotype: which patients should be screened for an underlying MEN syndrome? In MEN1, experts have attempted to specify the group of patients with an "atypical MEN1 phenotype" in which genetic testing should be undertaken: "patients with primary hyperparathyroidism before age 30; multigland parathyroid disease, gastrinoma or multiple pancreatic neuroendocrine tumors (NETs) at any age; or patients with two or more MEN1-associated tumors that are not part of the classical triad of parathyroid, pancreatic islet, and anterior pituitary tumors."[1] There concrete recommendations provide guidance to treating clinicians, but unfortunately, they are based on limited data. Data from some studies have shown a high prevalence of *MEN1* mutations in patients with sporadic endocrine tumors, and earlier findings from the DutchMEN Study Group underline the suggestion that the current guideline might be too conservative.[2–4]

As one of the main features of MEN1, pituitary adenomas are reported in 18 to 52% of MEN1 patients. More importantly in this context, these tumors are the sole, first manifestation of MEN1 in 9-23% of patients.[5–10] Thus, the occurrence of pituitary adenoma can be an important first sign of MEN1. However, with an estimated prevalence of clinically relevant tumors of 1 per 1000 in general population, pituitary adenomas are amongst the most frequently encountered intracranial tumors, and only 3% of pituitary adenomas is caused by MEN1 (and 5% is caused by all hereditary tumor syndromes together).[11,12] Therefore, since screening all patients with pituitary adenoma would be disproportionate, case-finding methods are needed to identify patients at high risk of an underlying genetic cause of disease. In **chapter 2**, we performed a rigorous systematic review and critical appraisal of the available literature for this cause.[13] Although the methodological quality varied considerably among studies, we were able to formulate a set of recommendations

for daily practice, when encountered with a patient with sporadic pituitary adenoma: *MEN1* screening must be considered in young (≤ 30 years) patients, especially in prolactinoma. Additionally, testing for mutations in the *aryl hydrocarbon receptor-interacting protein* (*AIP*) gene – which are identified in 15-20% of patients with familial isolated pituitary adenoma (FIPA) syndrome – is suggested for young (≤ 30 years) patients, and especially for patients with pituitary-induced gigantism and pituitary macroadenoma. Furthermore, screening for *Xq26.3* microduplications, the genetic basis for X-Linked Acrogigantism, or X-LAG, is advisable in children < 5 years of age with increased growth velocity due to a pituitary adenoma. There was insufficient evidence to support mutational analysis of other genes (*CDKN1B*, *PRKAR1A*, *SDHx*) in sporadic pituitary adenoma without specific syndromal features.
Our efforts will hopefully lead to early case-finding of hereditary diseases like MEN1 (with potentially beneficial treatment outcome) on the one hand, and less unnecessary investigations on the other. However, it is important to realize that the large heterogeneity of study populations and methodological quality together with the rarity of germline mutations make it difficult to predict the benefits of our recommendations when implementing them in daily practice. In a recent study, 55 patients with pituitary adenoma were screened for germline *AIP* or *MEN1* mutations, based on risk criteria partly similar to our recommendations (disease-onset ≤ 18 years of age, macroadenoma ≤ 30 years, somatotropinomas and prolactinoma resistant to medical treatment and familial cases).[14] Somewhat surprisingly, no germline mutations were identified, illustrating that no risk stratification system or set of screening recommendations is flawless. By validation in large, prospective, unselected cohorts, these tools need to be further improved in the future.

First-degree relatives of a patient with a germline *RET*, *MEN1* or *CDKN1B* mutation should be offered genetic screening, given the 50% chance of passing on the mutation from parent to child. It is particularly important to perform DNA testing in the first year(s) of life in the offspring of MEN2A (and 2B) patients, due to the high risk of early-onset medullary thyroid carcinoma (MTC) in mutation carriers. Identification of the familial *RET* mutation enables prophylactic thyroidectomy before MTC has spread to adjacent or distant organs. In **chapter 3**, we evaluated the effect of the implementation of presymptomatic *RET* mutation analysis in the Netherlands by investigating the incidence and outcome trends of pediatric MTC during 1990-2019.[15] The results showed that the incidence of MTC in patients 0-24 years of age peaked around 1994 – just after the discovery and the start of wide-spread use of *RET* mutation testing – and dropped afterwards. Furthermore, children with MTC were found to have more advanced disease upon diagnosis in recent years. We hypothesize that these findings can be explained by the introduction of *RET* mutation screening in children of MEN2A families: initially, many children from MEN2A families

with local MTC were identified through DNA screening, resulting in a spiking incidence. Afterwards, with the MEN2A families and mutational status of relatives adequately documented, prophylactic thyroidectomy before the onset of MTC was possible in the majority of children with MEN2A, explaining the declining incidence in the following years. On the contrary, children with MEN2B are usually not diagnosed until after the development of symptomatic MTC, since *RET* mutations occur as *de novo* in the majority of MEN2B patients. As a result, *RET* mutation screening has presumably led to an increased proportion of (late-recognized) MTC in the context of MEN2B, which is reflected by the stage migration towards more advanced stages of MTC over the years. The 5 and 10-year overall survival remained high throughout the study period (>95 and >92%, respectively) despite this stage migration, underlining the relatively good overall prognosis of pediatric MTC.

At first sight, these findings seem to describe a complete success story: *RET* mutation screening has enabled early detection of patients with high risk of MTC, and timely intervention has prevented the development of MTC in a large number of patients. However, these results also reveal an important challenge: MEN2B patients are often still recognized too late, illustrated by the above-mentioned stage migration towards more advanced MTC. The clinical relevance of an early MEN2B diagnosis is evident, given the fact that tumor stage at diagnosis is considered the most important prognostic factor for survival in MEN2B patients.[16] In line with this, survival of pediatric MTC in our Dutch cohort was significantly worse in patients with lymph node involvement and distant metastasis.[15] At the same time, early recognition of this extremely rare syndrome is extremely difficult: in the largest cohort of MEN2B patients to date (n = 345), the median age at thyroidectomy of 14 years and the small proportion of patients (6%) who were operated before the recommended age of one year illustrate the typical late diagnosis.[17] The question then arises: how to detect MEN2B patients in time?

Therefore, in **chapters 4 and 5**, we described case-finding methods for a timely diagnosis of MEN2B. Since the extreme rarity of MEN2B prevented the development of a prediction model, we opted for a scrupulous description of the MEN2B population in our Dutch MEN expertise center. Since previous reports had shown that non-endocrine features related to the syndrome may precede MTC in MEN2B patients, we focused on these premonitory signs of disease.[18,19] In **chapter 4**, we have described how a MEN2B diagnosis in the first months of life was made in three out of eight patients after surgery or rectal biopsy for suspicion of Hirschsprung's disease; histopathological examination showed intestinal ganglioneuromatosis (IGN) in all cases, which subsequently led to genetic analysis confirming MEN2B.[20] The early recognition of IGN indirectly led to prevention and/or curation of MTC, once again illustrating the clinical importance of an early MEN2B

diagnosis. Oral neuromas/neurofibromas were the trigger for *RET* mutation analysis in two other patients (and among the presenting symptoms in three) somewhat later in childhood, underlining the relevance of this non-endocrine feature. In retrospect, the diagnosis of MEN2B could have been made much earlier in life in at least two cases, if gastrointestinal and orofacial symptoms had been recognized as signs of the disease without delay. Even though our series enrolled only a small number of MEN2B patients, these findings clearly show that neonatal gastrointestinal manifestations offer an important window of opportunity for early detection of MEN2B, if rectal biopsies of patients with severe neonatal constipation are also screened for IGN. Awareness of non-endocrine manifestations – especially gastrointestinal and oral – appear to be the key to identify children with MEN2B in time.

The results from **chapter 5** show that short stature during childhood may be prevalent in MEN2B, as seven out of eight patients showed growth beneath or at the lowest margin of their target height range.[21] This seems to dispute the assumption that MEN2B children show a "marfanoid" body habitus, which includes tall stature. Although short before puberty, all four patients who already reached adulthood showed a final height within target height range. We could not relate impaired growth to MEN2B-related manifestations, endocrine abnormalities, age at MEN2B diagnosis or age at thyroidectomy. Normal body proportions as measured by arm span/height ratio and upper segment/lower segment ratio did also not support a "marfanoid" body habitus in this case series. Regardless of these anthropometric data, four patients (50%) had been labeled "marfanoid" by their treating physician at some point during follow-up. Apparently, the term "marfanoid" is not easily captured in quantifiable anthropometric signs; other musculoskeletal features (such as hyperlaxity, scoliosis or arachnodactyly ("spider fingers"") may also play a role in the choice to use the label "marfanoid".
What are the implications of these findings? Of course, we have to take the small cohort size (n = 8) into account when interpreting these results. Larger, population-based, preferably prospective cohort studies are needed to validate these findings on growth and body proportions, as well as to elucidate underlying mechanisms. Nevertheless, our results clearly illustrate that a short stature should by no means rule out a possible diagnosis of MEN2B, thereby overturning earlier assumptions of a "marfanoid"-associated tall stature. Short stature during childhood – but normal final height – may even be considered an independent feature of MEN2B. Awareness of anthropometric features of MEN2B might hopefully lead to an earlier diagnosis of children with MEN2B.

Case-finding within MEN syndromes: moving towards personalized medicine

Current clinical guidelines advise regular biochemical and radiological surveillance in MEN patients in order to identify patients with deviant course of disease in time, thereby enabling appropriate intervention.[1,22] Unfortunately, as pointed out earlier, a significant part of these surveillance recommendations are based on data from small cohorts (or even case-reports) of MEN patients from highly-selected populations (tertiary referral centers), which hampers the generalizability of results. Therefore, we aimed to add clinically relevant knowledge on the natural course of MEN1-related manifestations using data from the population-based DutchMEN Study Group (DMSG) database in the second part of this thesis. Hopefully, this will contribute to more effective case-finding of MEN1 patients with unfavorable course of disease in the near future.

In **chapter 6**, we focused on the potential effect of a patient's position in his/her family pedigree (generation) on the age-dependent penetrance and severity of MEN1 manifestations, in search for a potential effect of genetic anticipation (the phenomenon of decreased age of disease onset or increased disease severity in successive generations).
We compared the age at detection of different MEN1-related manifestations among subsequent generations in the 10 largest Dutch MEN1 families. We found a significantly decreased age at detection of primary hyperparathyroidism, duodenopancreatic NET, pituitary adenoma and lung NET ($P < 0.0001$) in successive generations, even after adjusting for the beneficial effect of surveillance programs.[23] However, these results must be interpreted with caution, as studies evaluating the possibility of anticipation always suffer from a significant risk of different forms of bias (*e.g.*, truncation bias, time bias). Moreover, a commonly accepted explanatory biological mechanism behind this phenotype in MEN1 (or in other hereditary cancer syndromes with Mendelian inheritance) is lacking. The results from our study should therefore primarily be considered a trigger for translational research into molecular mechanisms explaining this phenotype in MEN1, and call for validation in other (population-based) cohorts.

In **chapter 7** and **chapter 8**, we concentrated on MEN1-related neuroendocrine tumors of the lung. Pathologically proven lung NETs are diagnosed in approximately 5% of MEN1 patients, while pulmonary lesions radiologically suspected of lung NET are found in up to 30% of MEN1 patients.[24–28] Results from earlier publications – including growth analysis by our own DMSG – illustrated that MEN1-related lung NETs generally are slow-growing and do not significantly affect survival in MEN1 patients. Nonetheless, poorly differentiated and aggressive lung NETs were the cause of death in several patients in the recent French publication.[28] Furthermore, a lung NET in a MEN1 patient under care at the UMC Utrecht showed sudden accelerated tumor growth and malignant biological behavior after years of

indolent growth. These events urged us to meticulously re-evaluate the natural course of MEN1-related lung NET in our DMSG cohort and to conduct extensive investigations in the remarkably aggressive lung NET at a molecular level (**chapter 7**).[29]
A total of 164 lesions highly suspected of lung NET were found in 102 MEN1 patients (22.9% of the cohort). Linear mixed models showed that tumor diameter increased by 6.0% per year (equal to a tumor doubling time of nearly 12 years). Moreover, the overall 15-year survival rate was 78.0% (95% confidence interval 64.6-94.2%), without lung NET-related death. No prognostic factors could be distinguished for both tumor growth or survival.[29] Histological, immunohistochemical and molecular investigations in the case with exceptional tumor growth showed some very interesting results: apart from the (expected) somatic second hit inactivation of the *MEN1* gene, whole genome sequencing of the tumor also showed a likely pathogenic c.3127A > G (p.Met1043Val) mutation in the *phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha* (*PIK3CA*) gene, associated with the PI3K-AKT-mTOR pathway. Further analysis revealed that – in contrast to the second *MEN1* mutation – this driver mutation was probably subclonal, which supports the hypothesis that the somatic *PIK3CA* mutation was responsible for the sudden deviant tumor behavior. Thus, the results of our study confirm the overall benign nature of MEN1-related lung NETs, but at the same time show that unpredictable accelerated tumor growth does occur in a very limited number of patients.

In **chapter 8**, we compared disease-specific mortality between 29 patients with pathologically confirmed MEN1-related lung NET, 112 patients with resected sporadic lung NET and 27 patients with lung NET in the context of Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH).[30] Tumor progression was the cause of death in 20 (18%) patients with sporadic lung NET, while no patients with lung NET in the context of MEN1 or DIPNECH had died due to their lung NET. Histological features such as tumor classification, Ki67% count, mitotic count and presence of necrosis could not explain the remarkable difference in prognosis between sporadic and MEN1-related lung NET, as these factors were comparable between the two groups. Paradoxically, several aspects (such as the more aggressive surgical approach in sporadic lung NET and the probable indication bias resulting in a selection of large and fast-growing MEN1-related lung NET) could have contributed to a better survival in patients with sporadic lung NET compared to MEN1-related lung NET, emphasizing the true different nature of these two entities. It is feasible that the distinctive prognosis of these subtypes can be explained by unidentified molecular processes: translational studies of atypical lung carcinoids and large cell neuroendocrine carcinomas have revealed that somatic *MEN1* mutations, *MEN1* inactivation and low *MEN1* mRNA levels are associated with (cancer-specific) survival.[31–33] However, these studies have shown contradicting results on the effect (positive vs. negative) of somatic *MEN1* mutations

on survival. Therefore, further research into the molecular background of these tumors is required to determine the exact role of (somatic) *MEN1* mutations in the natural course of lung NET. As this was the first head-to-head comparison study between these subgroups of lung NET to date, verification in other cohorts is needed before these results can be implemented in prognostic guidelines for different forms of lung NET.

What do these new insights into the natural course of MEN1-related lung NET mean for clinical care in MEN1 patients? Firstly, the overall low tumor growth and lack of MEN1-related lung NET-related mortality encourage to discuss the frequency of periodic thoracic imaging as currently recommended in the clinical guidelines (every 1-2 years).[1] Given the estimated tumor doubling time of approximately 12 years and excellent prognosis, less stringent radiological screening may be justifiable, which would reduce radiation exposure, patients' physical and psychological distress and health care costs. However, the case with a somatic *PIK3CA* driver mutation has illustrated that sudden accelerated tumor growth and aggressive biological tumor behavior do take place and call for timely detection and intervention. Unfortunately, the lack of prognostic factors for tumor growth or survival prevents the development of more personally tailored screening programs to date. Thus, less frequent thoracic imaging seems defendable at a group level (given the extremely high number needed to screen for timely identification of a case of lung NET with unanticipated malignant behavior), but might lead to inoperable or metastatic disease in individuals with exceptional aggressive tumor behavior in rare individual cases. Therefore, treating physicians should discuss the benefits and disadvantages of a strict surveillance program with their individual patients, weighing the (low) chance of developing a malignant tumor against the potential harm of (frequent) thoracic imaging.
Secondly, the indolent tumor growth and lack of favorable effect of surgery on survival in our cohort indicate that a watch-and-wait policy (instead of surgical resection promptly after detection) is possible for the subset of small, slow-growing lung NETs. As suggested previously, large (> 2 cm) tumors, tumors localized close to vital structures and fast-growing lung NETs should be resected without delay.[34]

Future directions

It remains challenging to effectively identify patients with a MEN syndrome, especially patients with MEN2B and sporadic patients with an atypical MEN phenotype. More insight into the sequence of occurrence of (endocrine and non-endocrine) manifestations of MEN2B, together with education of clinicians to create more awareness for these signs, is needed to facilitate an earlier identification of MEN2B. Given the extreme rarity of disease,

this can only be accomplished by international cooperation: by connecting national (population-based) registries and creating large international databases, sufficient scientific power can be gained to accurately investigate the age-related penetrance of all MEN2B-associated features and discover all distinctive premonitory symptoms of disease. Subsequently, much effort should be made in educating pediatricians and other relevant medical specialists, as well as general practitioners and medical consultation agencies, in order to increase awareness for early signs of this syndrome; effective case-finding of rare diseases like MEN2B will only be possible if the acquired knowledge is put into practice among the full spectrum of (first-line) health care professionals.
With regard to sporadic patients with an atypical MEN phenotype: the high incidence of some MEN-related manifestations in the general population – especially compared to the low prevalence of MEN syndromes – creates difficulties for identifying patients at risk of an underlying genetic disorder. By systematical review of all available literature, we established evidence-based recommendations for genetic analysis in patients with sporadic pituitary adenoma (chapter 2). Next steps in this field of research would include conducting cost-effectiveness studies and investigating the implementation of such recommendations in daily practice: what is the yield of DNA analysis when using these tools? What are reasons to deviate from screening recommendations? How many diagnoses would be missed? The answers to these questions can help to improve current recommendations. Apart from this, the rising incidence of germline variants of uncertain significance (VUS) call for large translational studies on the functional status of these DNA variations, combined with data on clinical outcome. Furthermore, efforts should be undertaken to generate robust evidence on the use of DNA analysis in patients with other sporadically occurring MEN-related manifestations such as primary hyperparathyroidism (MEN1 and MEN2A) or duodenopancreatic NET (MEN1). Like in MEN2B research, such efforts will only be successful if research groups around the world would join forces, thereby gathering enough power to establish well-founded recommendations.

International collaboration is also of vital importance in order to fill the knowledge gaps in the occurrence and natural behavior of MEN-related tumors. Validating previous findings in other cohorts and combining data from several registries is necessary in order to determine the exact value of (presumed) prognostic factors and determine appropriate surveillance programs for individual patients. Previous teamwork between the DMSG and several international research groups has been very fruitful and the possibilities to increase such efforts should be explored.[35–37]

Special focus should be given to molecular translational research. Unraveling explanatory biological mechanisms which influence the course of MEN disease will open the door for

targeted surveillance and treatment. This includes research into epigenetics, a heritable phenotypic mechanism of DNA transcription regulation independent of DNA nucleotide sequence alterations. The latest findings – although based on limited data – look promising: epigenetic phenotypes have been associated with tumorigenesis of several neuroendocrine tissues, which suggests that this could be a target for future biomarkers and treatments for MEN-related disease.[38–41] Other encouraging results have recently been published in the field of liquid biopsies: multianalyte algorithmic analysis of the mRNA expression levels of a set of genes in blood (the so-called NETest) have shown to be suitable to detect neuroendocrine tumors, to monitor treatment efficacy, and even appears to predict disease course in gastroenteropancreatic NETs.[42,43] If this biomarker proves to be as valuable in MEN patients, the NETest will become a very important tool for tailoring surveillance and treatment in MEN patients.

Although basic and translational research is vital for elucidating tumorigenesis and the development of new lines of screening and treatment modalities in MEN disease, understanding the perspective of patients and their families should be considered equally important to improve care for MEN patients. Fortunately, a rising number of research groups have given attention to the quality of life in MEN patients in recent years.[44–52] Data from these studies have shown that MEN syndromes affect all domains related to physical, mental, emotional and social health, and that MEN patients experience a high degree of financial burden and a high fear of disease occurrence. More insight into the determinants affecting the quality of life of MEN patients and variables that can increase health-related quality of life will help improving care for MEN patients beyond the traditional medical part. Additionally, special focus should be given to quality-of-life research in children with MEN; the effects of (frequent) medical surveillance on a child's well-being are largely undiscovered to date. Well-founded information about potential harms of surveillance would add to the discussion about the ideal surveillance strategy in MEN syndromes. Finally, we owe it to MEN patients to involve them in all steps of future (clinical) research, as they are the key stakeholders, both dependable on and indispensable for improving future care.

Conclusions

How can you identify a patient with a MEN syndrome or deviant behavior of a MEN-related tumor in time without exposing (too many) other patients to harm related to extensive screening, such as physical discomfort, psychological distress, health-related risks (ionizing radiation) and health care costs? As this thesis has shown, the answer is not clear-cut: the

rarity and heterogeneity of MEN syndromes and MEN-related manifestations call for disease-specific or even manifestation-specific case-finding methods and research methodology. For example: elaborate prediction models may be used to discover factors potentially influencing tumor growth in MEN1-related lung NET (chapter 7), but are not feasible when studying premonitory signs of MEN2B in a limited number of patients (chapter 4 and 5).

This thesis has provided several clinically relevant recommendations for more effective case-finding of MEN syndromes and MEN-related tumors, thereby contributing to the development of more personalized care for MEN patients in the future. However, it has also illustrated that it is not always possible to identify prognostic factors of disease yet. Especially when faced with medical uncertainties, it is even more important to inform patients and their families about the knowns and unknowns and to involve them in the development of a surveillance strategy, adjusted to their specific needs and values. These gaps in knowledge should be considered as an invitation for future studies, paving the way towards personalized medicine.

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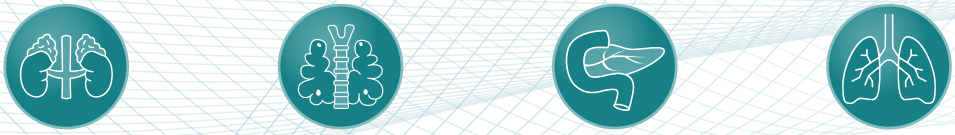
CHAPTER 10

Summary
Samenvatting (summary in Dutch)



Summary

MEN syndromes: background

MEN syndromes are part of a group of hereditary disorders, predisposing to benign and/or malignant tumors in two or more endocrine glands. Benign tumors can lead to clinical symptoms caused by hormonal overproduction or deficiency as well as by tumor mass. Malignant tumors potentially cause extra morbidity and mortality through their potential to spread to other sites. Four distinct MEN syndromes can be distinguished based on the underlying genetic defect and clinical phenotype: MEN1 is caused by loss of function of the *MEN1* gene and is primarily characterized by the co-occurrence of parathyroid, pituitary and (duodeno)pancreatic neuroendocrine tumors, although the full range of associated manifestations includes more than 20 endocrine and non-endocrine tumors. MEN2A and MEN2B – both caused by gain-of-function germline mutations in the *RET* gene – are marked by the occurrence of medullary thyroid carcinoma (MTC) in nearly all patients and pheochromocytoma in half of patients. MEN2A patients are also at risk of developing parathyroid adenomas, Hirschsprung's disease and cutaneous lichen amyloidosis. The phenotypic spectrum of MEN2B expands to a variety of non-endocrine manifestations, including intestinal ganglioneuromatosis, mucosal neuromas, marfanoid habitus, orofacial and ocular manifestations. Germline mutations in the *CDKN1B* gene are responsible for MEN4 and lead to a predisposition for the occurrence of parathyroid, pituitary tumors and possibly other tumors.

Given the estimated prevalence of 2-10 per 100,000 (MEN1), 1-3 per 100,000 (MEN2A), 0.09-0.17 per 100,000 (MEN2B) and ± 40 cases of MEN4 worldwide, all four MEN syndromes are considered (very) rare. Nonetheless, high penetrance of disease causes high morbidity, decreased quality of life and MEN-related death in a significant part of the MEN population. To improve outcome, patients with a MEN syndrome are recommended to adhere to periodic biochemical and radiological surveillance, hereby enabling detection of associated tumors in time. Moreover, patients with MEN2A and MEN2B are strongly advised to undergo prophylactic thyroid surgery in early childhood to prevent metastasized MTC.

Current thesis

The rarity of MEN syndromes and scarcity of high-level evidence on the natural course of MEN-related manifestations frequently impede efficient and timely detection of a MEN syndrome as well as prompt identification of deviant behavior of a MEN-related tumor. The first part of this thesis contains several research projects which aimed to improve early but efficient identification of a MEN syndrome. The second part focuses on the occurrence and subsequent course of MEN1-related tumors, with the goal to add to the development of personalized care for MEN1 patients in the future.

PART ONE
Case-finding of MEN syndromes

Pituitary adenomas occur in 18-52% of MEN1 patients and are the first manifestation of the syndrome in 9-23% of cases. However, pituitary adenomas are relatively common – the estimated prevalence of clinically relevant pituitary adenoma is 1 in 1,000 in general population – and only 5% of patients with a pituitary adenoma has an underlying genetic condition. Therefore, DNA screening for MEN1 (and other hereditary syndromes) in all patients with pituitary adenoma would have a very low yield. In **chapter 2**, we performed a systematic review on the clinical value of genetic screening in apparently sporadic pituitary adenoma. We focused on patients without affected family members, because DNA screening in familial cases is already considered justified. Based on the thorough assessment of 37 selected publications, we were able to formulate a set of recommendations to help physicians selecting patients for genetic screening; *MEN1* screening must be considered in young (≤ 30 years) patients, especially when patients suffer from prolactinoma. These findings will hopefully help to improve efficient case-finding of hereditary diseases like MEN1.

Patients with MEN2A have a 50% chance of passing on their germline mutation to each of their children. Therefore, clinical guidelines strongly recommend DNA screening in children of MEN2A patients in the first year(s) of life, so that *RET* mutation carriers can be identified and prophylactic thyroidectomy can be performed before aggressive MTC occurs. In **chapter 3**, we presented the incidence and outcome trends of Dutch pediatric MTC during 1990-2019. These results showed that the incidence of MTC in children, adolescents and young adults (0-24 year) peaked around 1994 – soon after the implementation of *RET* mutation testing in MEN2A families – and significantly decreased afterwards. Moreover, we noticed a trend towards more advanced disease upon diagnosis over the years. We postulate that these findings can be explained by the introduction of *RET* mutation screening in children of MEN2A patients: presumably, the implementation of prophylactic thyroidectomy has prevented the development of MTC in a significant part of children with MEN2A, thereby leading to a decreasing incidence of MTC over the years. Given the *de novo* occurrence of MEN2B in the majority of cases, presymptomatic DNA screening (based on a positive family history) is seldom possible in this subgroup of patients. Consequently, we hypothesize that the proportion of (often late-recognized) MTC in the context of MEN2B has risen due to the implementation of *RET* mutation screening, which would explain the trend towards more advanced disease upon diagnosis in recent years.

In MEN2B, timely case-finding is even more challenging than in other MEN syndromes: its extremely low prevalence and the lack of a positive family history in 75-90% of cases

often hinders an early diagnosis. Given the very early-onset of aggressive MTC in nearly all patients, many patients already suffer from advanced disease when symptoms are recognized. In **chapter 4**, we described how premonitory non-endocrine signs of MEN2B can offer clues for a timely MEN2B diagnosis in a case series of eight MEN2B patients under care at the University Medical Center Utrecht (UMCU) / Wilhelmina Children's Hospital. Intestinal ganglioneuromatosis (IGN), which was recognized during diagnostic work-up of severe neonatal gastro-intestinal symptoms in three patients, led to an early MEN2B diagnosis and (thereby) to curative thyroidectomy. This illustrates that it is important for pathologists to screen for IGN as well when evaluating rectal biopsies in young children with severe gastro-intestinal symptoms like constipation; a harmful delay of diagnosis of MEN2B can be avoided by identification of IGN. Oral neuromas/ neurofibromas were among the presenting symptoms in three other patients, and may alert (oral) health care professionals for the presence of MEN2B. Most importantly, this case series – although small – has shown that awareness of distinctive non-endocrine features is essential to identify children with MEN2B in time.

"Marfanoid" body habitus – a non-specific term referring to a constellation of features similar to characteristics of patients with Marfan syndrome, including tall stature, long limbs and hyperlaxity – is strongly linked to the MEN2B syndrome: approximately 75% of MEN2B patients has been labeled "marfanoid" by their treating physician. As a result, physicians may expect MEN2B patients to be tall, although data on longitudinal growth in MEN2B are very scarce. To test this hypothesis, we investigated growth patterns of MEN2B patients in **chapter 5**. Somewhat surprisingly, seven out of eight patients showed growth beneath or at the lowest margin of their target height range. Moreover, all four adult patients showed a "normal" final height, despite short stature during prepubertal childhood. Arm span/height ratio and upper segment/lower segment ratio were normal in all patients, which also did not support a "marfanoid" body habitus in this case series. Nonetheless, four patients had been labeled "marfanoid" by their treating physician, illustrating that – besides these anthropometric ratios – other features also play a role in labeling a patient "marfanoid". Results from chapter 5 demonstrate that short stature during childhood – but normal final height – is prevalent among MEN2B patients, and it should not hinder a possible early diagnosis of MEN2B when suspected on other grounds.

PART TWO
Case-finding within MEN syndromes: moving towards personalized medicine

After being diagnosed with a MEN syndrome, patients are recommended to undergo a comprehensive life-long surveillance program in order to identify MEN-related tumors in time. By creating more insight into the natural course of MEN-related manifestations, we aimed to contribute to more effective case-finding of MEN-related tumors with unfavorable course of disease in the future.

In **chapter 6**, we searched for clues for genetic anticipation in the largest Dutch MEN1 families. This rare type of genetic inheritance is marked by a reduced age of onset and/or increased disease severity in successive generations, and has primarily been described in neuropsychiatric diseases. In recent years, genetic anticipation has been identified in several heritable cancer syndromes with "traditional" Mendelian inheritance as well, although a widely accepted molecular explanation for this phenomenon in these diseases is lacking to date. The literature on genetic anticipation includes a case-report which reported an earlier onset of disease in younger generations in one large MEN1 family.
In order to gain insight into the potential role of genetic anticipation in MEN1, we selected all Dutch MEN1 families with ≥ 10 affected members in ≥ 2 successive generations, and compared age at detection of the most prevalent MEN1-related tumors among generations. A total of 152 MEN1 patients from 10 families were included, and regression analyses adjusted for competing risks showed a significantly decreased age at detection of primary hyperparathyroidism, duodenopancreatic NET, pituitary adenoma and lung NET in successive generations ($P < 0.0001$). Additional analyses focusing on manifestations detected during surveillance showed similar results, which suggests that these findings were not (only) the result of the beneficial effect of surveillance programs. Nonetheless, the results of this study should be interpreted with caution, keeping in mind the remaining substantial risk of various types of bias (*e.g.*, time bias) and lack of commonly accepted biological mechanism explaining this phenotype in MEN1. Only after validation of our findings in other well-designed cohorts and after elucidation of underlying biological processes, a patient's position in his/her family pedigree could be used to tailor surveillance programs for future generations of MEN1 patients.

In **chapter 7**, tumor growth and survival of MEN1-related lung NETs were investigated. These tumors are generally described as relatively benign MEN1 manifestations, but reports of metastatic and even fatal lung NETs from abroad and a recent case of a Dutch patient with a fast-growing lung NET urged us to thoroughly re-assess the clinical course in the

Dutch MEN1 cohort. A total of 164 lesions highly suspected of lung NET were detected in 102 MEN1 patients (22.9% of the cohort). Using multilevel linear mixed models, we demonstrated that tumor diameter increased by 6.0% per year on average, equal to a tumor doubling time of almost 12 years. MEN1 patients with a lung NET had an overall 15-year survival rate of 78.0% (95% confidence interval 64.6-94.2%), no deaths in the cohort were related to lung NET. No prognostic factor for tumor growth or survival could be identified. Extensive investigations in the lung NET of the Dutch patient with exceptional aggressive biological behavior demonstrated that a (probably subclonal) somatic driver mutation in the *PIK3CA* gene was most likely responsible for its malignant course.
Results from chapter 7 confirm the overall indolent course of MEN1-related lung NETs, but also illustrate that unpredictable deviant tumor growth takes place in a very small subset of patients. Thoracic imaging is currently advised annually or biannually in MEN1 patients. Based on results of this study, less frequent screening seems justified at a group level, but might result in late recognition of disease progression in a small subset of patients. Therefore, the frequency of thoracic screening should be individualized and discussed with patients, taking into account their point of view regarding the risk of aggressive tumor growth versus the potential harm of (frequent) thoracic imaging – including the psychosocial burden of frequent screening.

In **chapter 8**, we compared disease-specific mortality between 29 patients with histopathologically confirmed MEN1-related lung NET, 112 patients with resected sporadic lung NET and 27 patients with surgically removed lung NET in the context of Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia (DIPNECH). Twenty (18%) patients with sporadic lung NET died as a result of tumor progression, compared to no deaths in the other groups. This remarkable difference in prognosis between sporadic and MEN1-related lung NET could not be explained by known prognostic histological features. Furthermore, due to the more aggressive surgical approach in patients with sporadic lung NET and the presumable selection of large and fast-growing MEN1-related lung NET, one could have even expected a better rather than much worse survival rate in patients with sporadic lung NET. This underlines the true different nature of these two entities. These findings call for validation in other cohorts as well as translational studies in search for a possible underlying molecular explanation.

In **chapter 9**, we discussed the main results of the previous chapters, reflected upon clinical implications, and made suggestions for future research on the MEN syndromes.

Nederlandse samenvatting

MEN syndromen: de achtergrond

Multipele Endocriene Neoplasie (MEN) syndromen zijn zeldzame erfelijke aandoeningen, waarbij er in meerdere (multipele) hormoonvormende (endocriene) organen goedaardige en kwaadaardige tumoren (neoplasieën) kunnen ontstaan. Goedaardige tumoren kunnen door een overschot of een tekort aan hormonen én door lokale tumorgroei voor klachten zorgen. Kwaadaardige tumoren kunnen extra problemen geven omdat ze in potentie kunnen uitzaaien naar andere plekken in het lichaam. Er bestaan vier MEN types, te weten MEN1, MEN2A, MEN2B en MEN4. Ieder MEN type kenmerkt zich door de aanleg voor het ontwikkelen van tumoren in een specifieke combinatie van organen: MEN1 patiënten hebben een zeer grote kans op het ontstaan van (1) tumoren in de bijschildklieren, (2) neuroendocriene tumoren van de twaalfvingerige darm en alvleesklier, en (3) tumoren van de hypofyse, maar hebben daarnaast ook een verhoogd risico op nog circa twintig andere tumortypes. MEN2A en MEN2B patiënten daarentegen ontwikkelen bijna allemaal op jonge leeftijd een type schildklierkanker genaamd medullair schildkliercarcinoom, en hebben bovendien in de loop van hun leven ongeveer 50% kans op het optreden van een bepaalde tumor uitgaande van het bijniermerg (een feochromocytoom). Bij MEN2A komen er daarnaast bij 20% van de patiënten ook gezwellen in de bijschildklieren voor, en incidenteel een bepaalde darmziekte (de ziekte van Hirschsprung) en/of huidaandoening (cutane lichen amyloidose). MEN2B kenmerkt zich – naast het optreden van medullair schildklierkanker en feochromocytoom – door een scala aan manifestaties buiten de hormonale organen: een abnormale uitgroei van zenuwvezels in de darmen (ganglioneuromatose), onderhuidse gezwellen op de slijmvliezen van de lippen en in de mondholte (neurinomen/neurofibromen) en een lange en dunne lichaamsbouw met relatief lange ledematen en vingers (een zogenoemd 'marfanoïde' lichaamsbouw). Bij MEN4 zijn met name tumoren van de bijschildklieren en hypofyse beschreven.

De vier MEN syndromen worden ieder veroorzaakt door genetische afwijkingen (meestal veranderingen ofwel mutaties) in een specifiek deel van het DNA: veranderingen in het *MEN1* gen zijn verantwoordelijk voor het optreden van het MEN1 syndroom, mutaties in het *REarranged Translocation* (*RET*) gen veroorzaken MEN2A en MEN2B, en patiënten met MEN4 hebben een mutatie in het zogenoemde '*cyclin-dependent kinase inhibitor 1b*' (*CDKN1B*) gen.

Alle MEN syndromen zijn zeer zeldzaam: MEN1 komt voor bij ongeveer 2 tot 10 per 100.000 personen, MEN2A bij 1 tot 3 per 100.000 personen, MEN2B bij 0.9 tot 1.7 per miljoen personen, en MEN4 is wereldwijd zelfs maar in circa 40 patiënten beschreven. Omdat MEN patiënten (vaak op jonge leeftijd) te maken krijgen met verschillende tumoren

en andere MEN-gerelateerde manifestaties, gaan MEN syndromen vaak gepaard met een grote ziektelast, verminderde kwaliteit van leven en een kortere levensverwachting. Internationale experts hebben richtlijnen ontwikkeld waarin adviezen zijn opgenomen op het gebied van het opsporen, de follow-up en de behandeling van deze ziektebeelden. Zo wordt er bij alle MEN types in de vorm van een screeningsprogramma geadviseerd om regelmatig bepaalde bloedonderzoeken en scans uit te voeren om tumoren tijdig te kunnen opsporen. Bij MEN2A en MEN2B wordt daarnaast sterk aanbevolen om de schildklier op jonge leeftijd te laten verwijderen om uitgezaaide schildklierkanker te voorkomen. Deze richtlijnen hebben de zorg voor MEN patiënten enorm verbeterd, maar door de zeldzaamheid van MEN syndromen zijn helaas maar weinig van de hierin opgenomen adviezen gebaseerd op gedegen wetenschappelijk onderzoek.

Huidige proefschrift

Dit proefschrift focust zich op enkele grote uitdagingen binnen de zorg voor deze zeldzame maar tegelijkertijd zeer ernstige aandoeningen: het eerste deel richt zich op mogelijkheden om MEN syndromen tijdig te herkennen zonder een (te) grote populatie bloot te stellen aan de nadelen van diagnostische onderzoeken (zoals DNA-onderzoek). In het tweede gedeelte van dit proefschrift worden studies naar het natuurlijk beloop van MEN-gerelateerde tumoren beschreven, om in de toekomst afwijkend gedrag van deze tumoren beter te kunnen voorspellen. Op deze manier hopen we kennis te vergaren om de periodieke screeningprogramma's voor MEN patiënten beter op maat te maken.

DEEL EEN
Case-finding van MEN syndromen

Bij ongeveer 40% van de MEN1 patiënten wordt in de loop van hun leven een hypofysetumor vastgesteld. Hypofysetumoren zijn daarmee één van de 'hoofdmanifestaties' van MEN1. In 9 tot 23% van de MEN1 patiënten zijn hypofysetumoren zelfs de eerste uiting van het MEN1 syndroom. Tumoren van de hypofyse komen in de algemene bevolking echter relatief vaak voor – circa 1 op de 1000 mensen heeft een hypofysetumor die klachten veroorzaakt en/of behandeling vereist – en hebben in 95% van de gevallen géén onderliggende genetische oorzaak. Het verrichten van DNA-onderzoek bij iedereen met een hypofysetumor om MEN1 (of MEN4) op te sporen zou daarom tot veel overdiagnostiek leiden. In **hoofdstuk 2** worden de resultaten getoond van een uitgebreid literatuuronderzoek naar de waarde van DNA-diagnostiek bij patiënten met een hypofysetumor. In dit onderzoek hebben we ons specifiek gericht op patiënten zonder familieleden met een hypofysetumor (zogenoemde 'sporadische' patiënten), omdat eerder onderzoek al heeft aangetoond dat DNA-onderzoek zinvol is bij patiënten bij wie hypofysetumoren in de familie voorkomen.

Op basis van ons literatuuronderzoek hebben we een aantal aanbevelingen geformuleerd die artsen kunnen helpen om DNA-onderzoek efficiënt in te zetten: wat betreft het *MEN1* gen zou DNA-screening overwogen moeten worden bij jonge (≤ 30 jaar) patiënten, met name wanneer patiënten een prolactinoom (een tumor die prolactine produceert) hebben. Deze bevindingen dragen hopelijk bij aan een efficiëntere herkenning van erfelijke ziektes zoals MEN1.

Patiënten met het MEN2A syndroom hebben 50% kans om de mutatie in het *RET* gen door te geven aan hun kinderen. De huidige richtlijnen adviseren om bij kinderen uit MEN2A families in de eerste levensjaren DNA-onderzoek uit te voeren. Op deze manier kan bij kinderen waarbij de *RET* mutatie gevonden wordt de schildklier tijdig verwijderd worden. Zo wordt er voorkomen dat er uitgezaaid medullair schildkliercarcinoom optreedt. In **hoofdstuk 3** wordt het vóórkomen (de incidentie) en de overleving van het medullair schildkliercarcinoom bij kinderen en jongvolwassenen (0-24 jaar) gedurende de periode 1990-2019 in Nederland gepresenteerd. De resultaten laten zien dat dit type schildklierkanker relatief vaak werd vastgesteld rond 1994, net na de invoering van *RET* mutatie onderzoek in MEN2A families. In de jaren erna zagen we een significante afname van het aantal gevallen per jaar. Bovendien bleek uit de resultaten dat het aandeel patiënten waarbij de kanker al naar de lymfeklieren was uitgezaaid in de loop van de jaren is toegenomen. Wij veronderstellen dat deze trends het gevolg zijn van de invoering van *RET* mutatie screening bij kinderen van MEN2A patiënten: waarschijnlijk heeft het vroegtijdig verwijderen van de schildklier bij kinderen met een *RET* mutatie het ontstaan van schildklierkanker in een groot deel van deze kinderen voorkómen. Dit zou verklaren waarom de incidentie van medullair schildklierkanker na een piek rond de introductie van *RET* mutatie screening is afgenomen. MEN2B komt in de overgrote meerderheid van de gevallen niet binnen families voor en wordt vaak pas herkend als de schildklierkanker al is uitgezaaid; de introductie van *RET* mutatie onderzoek heeft waarschijnlijk maar weinig effect gehad op de incidentie van MEN2B-gerelateerde schildklierkanker. In combinatie met de daling in MEN2A-gerelateerde schildklierkanker zou dit geleid kunnen hebben tot een groter aandeel van MEN2B-gerelateerde medullair schildkliercarcinoom. Gezien de veelal late herkenning van MEN2B-gerelateerde schildklierkanker, zou dit een verklaring kunnen zijn voor het toegenomen aandeel van patiënten met medullair schildkliercarcinoom dat naar de lymfeklieren was uitgezaaid.

Bij 75 tot 90% van de patiënten met MEN2B treedt het syndroom '*de novo*' op, wat inhoudt dat de *RET* mutatie niet bij één van de ouders aanwezig is. Samen met de extreme zeldzaamheid van dit ziektebeeld zorgt dit ervoor dat het heel moeilijk is om het MEN2B syndroom op tijd te herkennen. Het merendeel van de MEN2B patiënten heeft dan ook al

uitgezaaid medullair schildkliercarcinoom op het moment dat de ziekte herkend wordt. In **hoofdstuk 4** wordt aan de hand van de MEN2B patiënten die onder behandeling zijn in het Universitair Medisch Centrum Utrecht (UMCU) / Wilhelmina Kinderziekenhuis (WKZ) beschreven hoe vroege tekenen van het MEN2B syndroom kunnen helpen om MEN2B op tijd op te sporen. Bij drie kinderen met ernstige maag-darm klachten in de eerste maand na de geboorte werd bij weefselonderzoek van de darm 'intestinale ganglioneuromatose' gevonden. Intestinale ganglioneuromatose is een afwijkende uitgroei van zenuwvezels in de darmen en is sterk gelinkt aan het MEN2B syndroom. Bij alle drie de kinderen kon de diagnose MEN2B met DNA-onderzoek bevestigd worden, wat vervolgens in alle drie de gevallen tot een schildklierverwijdering heeft geleid voordat er uitgezaaid medullair schildklierkanker was ontstaan. Hoewel er maar een klein aantal patiënten aan deze studie deelnam, benadrukken deze resultaten het belang van zorgvuldig weefselonderzoek bij zeer jonge kinderen met ernstige maag-darm klachten. In deze gevallen dient het zeldzame intestinale ganglioneuromatose ook uitgesloten te worden.
Drie kinderen hadden bij het eerste contact met de arts gezwellen op de slijmvliezen in de mondholte (neurinomen/neurofibromen). Deze neurinomen/neurofibromen vormen daarmee een tweede belangrijke aanwijzing voor de aanwezigheid van MEN2B. Deze resultaten kunnen gebruikt worden om artsen en andere zorgverleners bewust te maken van de kenmerkende tekenen van MEN2B die vroeg in het leven optreden, aangezien vroege herkenning van het syndroom essentieel is om uitgezaaide schildklierkanker te voorkomen.

Ongeveer 75% van alle MEN2B patiënten heeft volgens hun behandelend arts een 'marfanoïde' lichaamsbouw. Deze (niet exact-gedefinieerde) term verwijst naar kenmerken die doen denken aan het syndroom van Marfan, zoals een magere lichaamsbouw, grote lichaamslengte en relatief lange ledematen. Artsen zouden de verwachting kunnen hebben dat MEN2B patiënten een lang postuur hebben, ook al is er maar weinig bekend over de groei van kinderen met MEN2B. In **hoofdstuk 5** zijn de groeigegevens van acht MEN2B patiënten onderzocht. Enigszins verrassend bleken zeven patiënten op de onderrand van of zelfs onder hun zogenoemde 'streeflengtegebied' te groeien. Het streeflengtegebied geeft aan wat normale groei is voor een specifieke persoon, op basis van het geslacht, etniciteit en de lengte van ouders. Daarnaast bleek dat alle vier inmiddels volwassen patiënten ondanks de kleine lengte in hun vroege kinderjaren een 'normale' eindlengte bereikt hadden. Bovendien hadden géén van de patiënten afwijkende lichaamsverhoudingen die gerelateerd worden aan een 'marfanoïde' lichaamsbouw: geen enkel kind had een relatief grote armspanwijdte (t.o.v. de lichaamslengte) of een relatief groot onderste lichaamssegment (t.o.v. de bovenste lichaamssegment). Desalniettemin waren vier patiënten door hun behandelend arts als 'marfanoïde' bestempeld, wat suggereert dat andere kenmerken ook een rol spelen in het gebruik van deze term. De resultaten van dit hoofdstuk vormen een

belangrijke aanwijzing dat MEN2B patiënten op kinderleeftijd frequent een korte lengte hebben, terwijl ze op volwassen leeftijd veelal een normale (maar géén extreem grote) eindlengte bereiken. Dat kinderen met MEN2B klein kunnen zijn is een belangrijke les voor (kinder)artsen, omdat – misschien in tegenstelling tot eerdere aannames – een kleine lengte een MEN2B diagnose dus zeker niet uitsluit als daar vanwege andere kenmerken aan wordt gedacht.

DEEL TWEE
Case-finding binnen MEN syndromen: op weg naar 'personalized medicine'

Nadat een MEN syndroom is vastgesteld, worden patiënten frequent gescreend op de aanwezigheid van MEN-gerelateerde tumoren. In dit deel van het proefschrift hebben we geprobeerd meer inzicht te vergaren in het natuurlijk beloop van enkele van deze tumoren, wat kan helpen om tumoren met een ongunstig beloop in de toekomst effectiever op te sporen. Uiteindelijk kan deze kennis hopelijk gebruikt worden om de intensieve screeningprogramma's voor MEN patiënten te individualiseren.

In **hoofdstuk 6** onderzochten we aanwijzingen voor 'genetische anticipatie' binnen het MEN1 syndroom. Genetische anticipatie is een type overerving dat zich kenmerkt door het steeds vroeger en/of heftiger optreden van ziekte in opeenvolgende generaties. Dit fenomeen is met name beschreven bij enkele erfelijke neuropsychiatrische ziektebeelden, zoals bij de ziekte van Huntington. De laatste decennia zijn er echter ook aanwijzingen voor genetische anticipatie gevonden in enkele erfelijke kankersyndromen, ook al is het achterliggende moleculaire werkingsmechanisme in deze gevallen tot nu toe niet opgehelderd. Eén Frans onderzoek beschreef een MEN1 familie waarbij tumoren in de jongste twee generaties op opvallend vroege leeftijd waren opgetreden ten opzichte van de oudere generaties.
Om genetische anticipatie binnen MEN1 gedegen te kunnen onderzoeken, selecteerden we alle Nederlandse MEN1 families met ≥ 10 MEN1 patiënten in ≥ 2 generaties. Vervolgens vergeleken we de leeftijden waarop de meest voorkomende MEN1-gerelateerde tumoren ontdekt werden tussen de verschillende generaties. Op basis van de gegevens van 152 MEN1 patiënten van de 10 grootste MEN1 families bleek dat jongere generaties MEN1 patiënten significant eerder in hun leven gediagnosticeerd werden met vier van de vijf onderzochte tumorsoorten, te weten bijschildkliertumoren, alvleeskliertumoren, hypofysetumoren en longtumoren. Omdat jongere generaties ten opzichte van oudere generaties meer profijt hebben gehad van regelmatige bloedonderzoeken en scans, werden er aanvullende analyses

verricht die hier rekening mee hielden. Deze toonden vergelijkbare resultaten, wat suggereert dat er ook bij MEN1 sprake zou kunnen zijn van genetische anticipatie. Een aantal zaken zou echter alsnog voor een vertekening van de resultaten gezorgd kunnen hebben; zo zijn scans in de loop van de jaren technisch steeds beter geworden, waardoor afwijkingen steeds eerder opgespoord kunnen worden. Hierdoor zijn manifestaties bij jongere generaties MEN1 patiënten mogelijk eerder vastgesteld. Ook andere factoren die samenhangen met een bepaalde tijdsperiode (zoals bijvoorbeeld omgevingsfactoren met kankerverwekkende eigenschappen) zouden de resultaten beïnvloed kunnen hebben. Bovendien is er tot nu toe geen verklarend biologisch mechanisme voor dit fenomeen gevonden in ziektes zoals MEN1. Daarom zouden deze onderzoeksresultaten vooral geïnterpreteerd moeten worden als aanmoediging voor vervolgonderzoek om de uitkomsten te valideren en een verklarend mechanisme te ontdekken. Afhankelijk van de resultaten uit dergelijke toekomstige onderzoeken zouden de resultaten van deze studie in de richtlijnen kunnen worden verwerkt.

In **hoofdstuk 7** beschrijven we de groei en prognose van MEN1-gerelateerde neuro-endocriene tumoren van de long (long NET). Deze tumoren worden over het algemeen gekenmerkt door hun gunstige beloop. Helaas kwamen er de laatste jaren ook meldingen uit het buitenland van patiënten die overleden waren aan hun long NET, en bleek een Nederlandse patiënt een zeer agressieve tumor te hebben. Daarom besloten we het natuurlijk beloop van deze tumoren uitvoerig te onderzoeken binnen ons Nederlands cohort van MEN1 patiënten. In 102 MEN1 patiënten (23% van het cohort) werden er in totaal 164 longtumoren gevonden. Deze tumoren bleken zeer langzaam te groeien: gemiddeld duurt het bijna 12 jaar voordat de tumordiameter verdubbelt. Patiënten met een NET in de longen hadden een 15-jaars overleving van 78%, terwijl er tot nu toe geen enkele patiënt overleed als gevolg van deze tumor. Er werden geen factoren gevonden die invloed hadden op de groeisnelheid of prognose van deze longtumoren. Weefsel van een patiënt met een longtumor die plotseling uitzonderlijk snelle groei had laten zien werd uitgebreid onderzocht, en er bleek een bepaalde verandering in het DNA (een zogenoemde *PIK3CA* mutatie) te zijn die zeer waarschijnlijk de oorzaak was van het afwijkende biologische gedrag van deze tumor.
De resultaten van dit onderzoek bevestigen het gunstige beloop van MEN1-gerelateerde NET van de long, maar tonen tegelijkertijd aan dat een heel klein aantal tumoren (plotseling) agressief groeit. De huidige richtlijn adviseert om bij MEN1 patiënten iedere één tot twee jaar röntgenonderzoek van de borstholte (een CT-scan van de thorax) te verrichten. Op basis van de langzame tumorgroei en goede prognose lijkt minder frequente beeldvorming gerechtvaardigd, maar kan leiden tot een te late herkenning van agressieve ziekte in een heel klein deel van de patiënten. Daarom dient de follow-up op individueel niveau bepaald

te worden, waarbij behandelend artsen samen met de patiënt het absolute risico op een agressieve tumor moeten afwegen tegenover de potentiële schade van (frequent) röntgenonderzoek.

Alle long NET worden tot nu toe als één ziektebeeld beschouwd en op dezelfde manier behandeld, terwijl er in de praktijk aanwijzingen zijn dat de prognose van patiënten met een long NET wel degelijk beïnvloed wordt door een eventuele onderliggende ziekte. Daarom hebben we in **hoofdstuk 8** de overleving van drie groepen patiënten met een long NET met elkaar vergeleken: (1) 29 MEN1 patiënten met een long NET, (2) 112 patiënten met een long NET zonder onderliggende ziekte (zogenoemde 'sporadische' long NET), en (3) 27 patiënten met een long NET gerelateerd aan de longaandoening genaamd 'Diffuse Idiopathic Pulmonary Neuroendocrine Cell Hyperplasia' (DIPNECH). Twintig patiënten met een sporadische long NET overleden ten gevolge van de ziekte, terwijl geen enkele patiënt met MEN1 of DIPNECH overleed ten gevolge van zijn of haar long NET. Omdat de MEN1-gerelateerde en sporadische tumoren vergelijkbare weefselkenmerken hadden, moet er een andere verklaring gezocht worden voor het grote verschil in overleving tussen deze patiëntengroepen. Omdat er (vermoedelijk) met name grotere en snelgroeiende MEN1-gerelateerde longtumoren voor deze studie geselecteerd zijn, en patiënten met een sporadische tumor een uitgebreidere operatie (resectie) hebben ondergaan, zou men juist een betere in plaats van een slechtere overleving van patiënten met een sporadische long NET verwachten. De resultaten uit deze studie suggereren dat MEN1-gerelateerde long NET en sporadische long NET twee verschillende entiteiten zijn met ieder een eigen beloop. Aanvullend onderzoek is essentieel om onderliggende mechanismen te ontdekken die dit verschil in beloop kunnen verklaren.

In **hoofdstuk 9** worden de belangrijkste resultaten uit de eerdere hoofdstukken besproken, inclusief de gevolgen die deze resultaten hebben voor de dagelijkse patiëntenzorg. Ook bevat dit hoofdstuk suggesties voor toekomstig wetenschappelijk onderzoek op het gebied van MEN syndromen.

APPENDICES

Review committee
Acknowledgements (dankwoord)
List of publications
Curriculum vitae

Review committee

prof. dr. M.R. van Dijk
prof. dr. H.M. Verkooijen
prof. dr. M.G.E.M. Ausems
dr. H.M. van Santen
prof. dr. A.S.P. van Trotsenburg
dr. C.R.C. Pieterman

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te becommentariëren. Met jouw scherpe blik wist je zelfs in een 8ste versie van een document nog spelfouten eruit te pikken en verhelderende suggesties voor een tabel te doen. Het siert je dat je naast je oog voor detail op werkgebied ook veel interesse hebt voor persoonlijke zaken zoals het thuiswerken in coronatijd en de ontwikkelingen van David. Door jouw frequente toelichtingen en de uitnodiging om een ochtend mee te lopen op de polikliniek heb je mij een onmisbare inkijk gegeven in de zorg voor MEN syndromen vanuit het perspectief van de kinderendocrinologie. Bedankt voor alle moeite en energie die je in onze gezamenlijke projecten hebt gestoken, je persoonlijke aandacht en de zeer aangename samenwerking.

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* *these authors contributed equally to this work*
\# *co-senior authors*

Curriculum vitae

Medard Franciscus Maria van den Broek was born on the 3rd of August 1988 in Nijmegen, The Netherlands. In 2006, he completed secondary school at the Stedelijk Gymnasium Nijmegen *summa cum laude* (with highest honors), after which he moved to Utrecht to start his medical studies at the University of Utrecht. After obtaining his bachelor degree *cum laude* (with honors) in 2009, he took a seat in the study association board MSFU "Sams" of the Faculty of Medicine (2009-2010). During his medical master's studies, Medard participated in the extracurricular Honors Research Program of the Faculty of Medicine (2010-2012), in which he collaborated with dr. J.M. de Laat to investigate the natural course of MEN1-related neuroendocrine tumors of the lung and thymus under supervision of prof. dr. G.D. Valk. In his final year of medical training, his continuing scientific interest in MEN syndromes translated into a research internship on genetic anticipation in MEN1, under supervision of prof. dr. G.D. Valk and drs. B.P.M. van Nesselrooij. After acquiring his medical degree in 2013, he started working at the department of Internal Medicine at the St. Antonius Hospital in Nieuwegein. In May 2015, he started his residency in Internal Medicine at the same institute under the supervision of dr. A.B.M. Geers and dr. P.C. de Jong. In November 2017, he continued his residency at the University Medical Center Utrecht under supervision of prof. dr. H.A.H. Kaasjager. After completing his fourth year of residency, Medard decided to pursue his ongoing scientific fascination by starting his PhD research on MEN syndromes under supervision of prof. dr. G.D. Valk, prof. dr. M.R. Vriens, dr. A.A. Verrijn Stuart and dr. R.S. van Leeuwaarde, which led to the present thesis.

He started his endocrinology training at the University Medical Center Utrecht under supervision of dr. A.M.E. Stades in May 2021.

Medard is married to Linda Raeven. They have a son (David) and a daughter (Rosa).