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Molecular studies of endocrine tumors

Insights from genetics and epigenetics

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ACTA
UNIVERSITATIS
UPSALIENSIS
UPPSALA
2020

ISSN 1651-6206
ISBN 978-91-513-1042-8
urn:nbn:se:uu:diva-423098

Dissertation presented at Uppsala University to be publicly examined in Rosénsalen, Akademiska sjukhuset, Ingång 95, Uppsala, Thursday, 10 December 2020 at 13:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish. Faculty examiner: Associate professor Robert Bränström (Department of Molecular Medicine and Surgery, Karolinska Institute).

Abstract

Backman, S. 2020. Molecular studies of endocrine tumors. Insights from genetics and epigenetics. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1694. 59 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-1042-8.

Endocrine tumors may be benign or malignant and may occur in any of the hormone producing tissues. They share several biological characteristics, including a low mutation-burden, and may co-occur in several hereditary tumor syndromes. The aim of this thesis was to identify genetic and epigenetic aberrations in endocrine tumors.

In paper I we performed a comprehensive DNA methylation analysis of 39 pheochromocytomas/paragangliomas as well as 4 normal adrenal medullae on the HumanMethylation27 BeadChip array. We validated two previously described clusters based on DNA methylation with distinct genetic associations.

In Paper II we performed a transcriptomic analysis of 15 aldosterone producing adenomas. *CTNNB1*-mutated tumors were found to form a distinct subgroup based on gene expression and to share gene expression similarities with non-aldosterone producing adrenocortical tumors with *CTNNB1* mutations, including overexpression of *AFF3* and *ISM1*.

In paper III we used whole genome sequencing to identify germline genetic variants in 14 patients with Multiple Endocrine Neoplasia type 1 previously found to be wildtype for the *MEN1* gene on routine clinical testing. Three patients were found to carry previously undetected *MEN1* mutations. Two patients were confirmed to have phenocopies caused by variants affecting *CASR* or *CDC73*. In total 9/14 patients were not found to have a disease-causing germline variant, suggesting that the syndrome may in some cases be due to chance co-occurrence of several sporadic tumors.

In paper IV RNA-Seq and whole genome sequencing of a cohort of SI-NETs selected on the basis of unusually short or long survival was performed in order to identify disease causing genes and potential prognostic factors. We confirmed known genetic aberrations and found rare variants in known cancer driver genes. Based on gene expression two clusters that differ in prognosis were detected. Moreover, through integration of copy number variation data and gene expression, we identied novel potential disease causing genes.

Keywords: Neuroendocrine tumors, Carcinoid, Pheochromocytoma, Aldosterone, Cancer, MEN1, Multiple endocrine neoplasia

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ISSN 1651-6206
ISBN 978-91-513-1042-8
urn:nbn:se:uu:diva-423098 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-423098)

This thesis is dedicated to my family

Me? Books! And cleverness! There are more important things: friendship and bravery." – Hermione Granger

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I **Backman, S.**, Maharjan, R., Falk-Delgado, A., Crona, J., Cupisti, K., Stålberg, P., Hellman, P., Björklund, P. (2017) Global DNA methylation analysis identifies two discrete clusters of pheochromocytoma with distinct genomic and genetic alterations. *Scientific Reports* (7): 44943

II **Backman, S*.**, Åkerström, T*., Maharjan, R., Cupisti, K., Willenberg, H.S., Hellman, P#., Björklund, P.# (2019) RNA sequencing provides Novel Insights into the transcriptome of Aldosterone producing Adenomas. *Scientific Reports* (9): 6269

III **Backman, S.**, Bajic, D., Crona, J., Hellman, P., Skogseid, B., Stålberg, P. (2020) Whole genome sequencing of apparently mutation-negative MEN1 patients. *European Journal of Endocrinology*, 182(1):35–45

IV **Backman, S.**, Barazeghi, E., Norlen, O., Hellman, P., Stålberg, P. Potential prognostic markers and candidate genetic drivers in small intestine neuroendocrine tumours. *Manuscript*

*, #, denotes equal contribution

Related publications

1) Åkerström, T., Willenberg, H.S., Cupisti, K., Ip, J., **Backman, S.**, Moser, A., Maharjan, R., Robinson, B., Iwen, K.A., Dralle, H., Volpe, C.D., Bäckdahl, M., Botling, J., Stålberg, P., Westin, G., Walz, M.K., Lehnert, H., Sidhu, S., Zedenius, J., Björklund, P., Hellman, P. (2015) Novel somatic mutations and distinct molecular signature in aldosterone-producing adenomas. *Endocrine-Related Cancer*, 22(5):735-744
2) Crona, J., **Backman, S.**, Maharjan, R., Mayrhofer, M., Stålberg, P., Isaksson, A., Hellman, P., Björklund, P. (2015) Spatiotemporal heterogeneity characterizes the genetic landscape of pheochromocytoma and defines early events in tumorigenesis. *Clinical Cancer Research*, 21(19):4451-4460
3) **Backman, S.,** Norlén, O., Eriksson, B., Skogseid, B., Stålberg, P., Crona, J. (2017) Detection of somatic mutations in gastroenteropancreatic neuroendocrine tumors using targeted deep sequencing. *Anticancer research*, 37(2):705-712
4) Maharjan, R., **Backman, S.**, Åkerström, T., Hellman, P., Björklund, P. (2018) Comprehensive analysis of CTNNB1 in adrenocortical carcinomas: Identification of novel mutations and correlation to survival. *Scientific reports*, 8(1):1-10
5) Björklund, P., **Backman, S.** (2018) Epigenetics of pheochromocytoma and paraganglioma. *Molecular and Cellcular Endocrinology*, 469:92-97
6) Crona, J., **Backman, S.,** Welin, S., Taïeb, D., Hellman, P., Stålberg, P., Skogseid, B., Pacak, K. (2018) RNA-sequencing analysis of adrenocortical carcinoma, pheochromocytoma and paraganglioma from a pan-cancer perspective. *Cancers*, 10(12):518
7) Paulsson, P.O.†, **Backman, S.**†, Wang, N.†, Stenman, A., Crona, J., Thutkawkorapin, J., Ghaderi, M., Tham, E., Stålberg, P., Zedenius, J., Juhlin, C.C. (2020) Whole-genome sequencing of synchronous thyroid carcinomas identifies aberrant DNA repair in thyroid cancer dedifferentiation. *The Journal of Pathology*, 250(2):183-194
   † These authors contributed equally to this work

Contents

Abbreviations

AFF3	AF4/FMR2 family member 3
AIP	Aryl hydrocarbon receptor-interacting protein
APA	Aldosterone producing adenoma
ARR	Aldosterone-to-renin ratio
ATM	Ataxia-telangiectasia mutated
ATP1A1	Na/K-transporting ATPase subunit alpha-1
ATP2B3	Plasma membrane calcium-transporting ATPase 3
ATRX	Alpha-thalassemia/mental retardation, X-linked
CACNA1D	Calcium Voltage-Gated Channel Subunit Alpha 1D
CASR	Calcium Sensing Receptor
CCND2	Cyclin D2
CDC73	Cell Division Cycle 73
CDKN1B	Cyclin-dependent kinase inhibitor 1B
cDNA	Complementary DNA
CGH	Comparative Genome Hybridization
CGI	CpG-Island
CIMP	CpG island methylator phenotype
CSDE1	Cold Shock Domain Containing E1
CTNNB1	Catenin beta-1/ β-catenin
CYP11B1	Cytochrome P450 Family 11 Subfamily B Member 1 (11b-hydroxylase)
CYP11B2	Cytochrome P450 Family 11 Subfamily B Member 2 (aldosterone synthase)
DNA	Deoxyribonucleic acid
DNMT1/3A/3B	DNA Methyltransferase 1/3A/3B
ENaC	Epithelial sodium channel
ENC1	Ectodermal-Neural Cortex 1
EPAS1	Endothelial PAS domain-containing protein 1
FH	Fumarate hydratase/Fumarase

GAPP	Grading of Adrenal Pheochromocytoma and Paraganglioma
HIF2α	Hypoxia-Inducible Factor 2α
(p)HPT	(Primary) Hyperparathyroidism
HPT-JT	Hyperparathyroidism-Jaw Tumor syndrome
HRAS	Harvey rat sarcoma viral oncogene homolog
ISM1	Isthmin 1
KCNJ5	G protein-activated inward rectifier potassium channel 4
KMT2D	Histone-lysine N-methyltransferase 2D
LH	Luteinizing Hormone
LHCGR	Luteinizing hormone/choriogonadotropin receptor
MAML3	Mastermind like transcriptional coactivator 3
MAX	Myc-associated factor X
MDH2	Malate dehydrogenase 2
MEN1	Multiple endocrine neoplasia type 1
MEN2	Multiple endocrine neoplasia type 2
MLL	Mixed-lineage leukemia
mTOR	Mechanistic target of rapamycin
NET	Neuroendocrine tumor
NF1	Neurofibromatosis type 1
NGS	Next Generation Sequencing
NKD1	Naked cuticle 1
PA	Primary aldosteronism
PanNET	Pancreatic Neuroendocrine Tumor
PASS	Pheochromocytoma of the Adrenal gland Scaled Score
PCC	Pheochromocytoma
PCR	Polymerase chain reaction
PGL	Paraganglioma
PPGL	Pheochromocytoma/paraganglioma
PTPRM	Protein Tyrosine Phosphatase Receptor Type M
qPCR	Quantitative polymerase chain reaction
RAAS	Renin-Angiotensin-Aldosterone System
RALBP1	RalA-binding protein 1
RB1	RB Transcriptional Corepressor 1
RDBP	Negative elongation factor E
RET	Rearranged during transfection

RNA	Ribonucleic acid
SCNA	Somatic copy number aberration
SDH	Succinate dehydrogenase
SDH(A/B/C/D)	Succinate dehydrogenase subunit A/B/C/D
SDHAF2	Succinate dehydrogenase assembly factor 2
SEER	Surveillance, Epidemiology and End Results
SI-NET	Small intestine neuroendocrine tumor
SLC25A11	Solute Carrier Family 25 Member 11
SNP	Single Nucleotide Polymorphism
SOCS6	Suppressor of cytokine signaling 6
TCEB3C	Transcription elongation factor B polypeptide 3C
TCF/LEF	T-cell factor/Lymphoid enhancer-binding factor
TCGA	The Cancer Genome Atlas
TET	Ten-eleven translocation
TET1/2	Ten-eleven translocation methylcytosine dioxygenase 1/2
TMEM127	Transmembrane protein 127
VHL	von Hippel-Lindau
Wnt	Wingless homolog
5-HIAA	5-Hydroxyindoleacetic acid

Introduction

This thesis lies at the intersection of three of the most fascinating fields of medical science*: endocrinology, tumor biology, and molecular genetics. Endocrinology is concerned with the study of hormones and the diseases that relate to them. A common disease mechanism in endocrine disorders is excessive hormone production, which may occur through a variety of mechanisms, including hormone secreting tumors. Tumor biology studies mechanisms that allow cells to deviate from their normal trajectory of tightly controlled proliferation and death to form neoplastic tumors. While it is clear that this transformation of healthy tissue to a tumor requires a cascade of events, the last century has shown that the principal cause is deleterious alterations in the heritable material of the cell: DNA. The study of the sequence, structure and function of DNA is the concern of molecular genetics.

This thesis aims to identify genetic drivers and potential prognostic markers in endocrine tumors. Each of the four included papers concerns a different disease. The first two are studies of adrenal tumors, where epigenetic changes and gene expression have been studied in tumors of the medulla and the cortex, respectively. The third paper reports an investigation of inherited genetic alterations in the heritable endocrine tumor syndrome MEN1. The final paper investigates genetic variants and gene expression in neuroendocrine tumors of the small intestine. While the studied material is consequently diverse, the studies are united by a common methodological approach: the use of high-throughput molecular technologies to improve our understanding of endocrine tumor biology.

* Author's opinion

Tumor genetics

The human genome is encoded by deoxyribonucleic acid (DNA): polymers of adenine, guanine, thymine and adenosine. The DNA molecules are double stranded, with two reverse complementary strands that pair through Watson-Crick base pairing[1]. The full genome spans more than three billion bases[2] and is divided over 23 pairs of chromosomes. A total of more than 20000 protein coding genes have been located in the genome[3]. Genes are transcribed to RNA by RNA polymerases, and the RNA may then act as a template for protein synthesis (translation)[4]. Proteins, in turn, carry out a vast range of metabolic and structural functions. Only a fraction (1-2%) of the genome consists of protein-coding genes[5], although a larger fraction is known to have some function, e.g. gene regulation[6].

Each cell in a multicellular organism has a copy of the same genome (disregarding somatic mutations and certain specific cell types), yet they belong to hundreds of unique cell types with different gene expression patterns and functions. This differentiation is reached chiefly through regulation of gene expression. Epigenetic modifications, heritable alterations in gene expression that do not involve modification of the DNA sequence, play an important role in determining cellular behavior. *In vivo*, chromosomal DNA is found in complex with histone proteins, forming chromatin[7]. Histone proteins may be covalently modified to increase or reduce transcription from specific genomic regions[8]. Similarly, the DNA molecule itself may be covalently modified. The most well understood modification is methylation of cytosine residues on the 5-position, which (in vertebrates) occurs in the context of CpG dinucleotides[9]. These modifications are mitotically heritable and may be passed on to daughter cells during cell division. The methylation reaction is catalyzed by DNA Methyltransferases[10]. Mammals have three enzymatically active DNA methyltransferases: DNMT1, DNMT3A and DNMT3B. DNMT1 has a preference for hemimethylated sites and is thought to be a maintenance methyltransferase that preserves DNA methylation patterns during cell division, while DNMT3A and DNMT3B appear to be *de novo* methyltransferases that methylate unmethylated DNA[11]. DNA methylation may be reversed either passively or through the actions of TET enzymes that oxidize 5-methylcytosine[12]. Many promoter regions have clusters of CpG dinucleotides termed CpG-islands. These are usually unmethylated, and methylation of these CGIs is associated with stable silencing of gene expression[13].

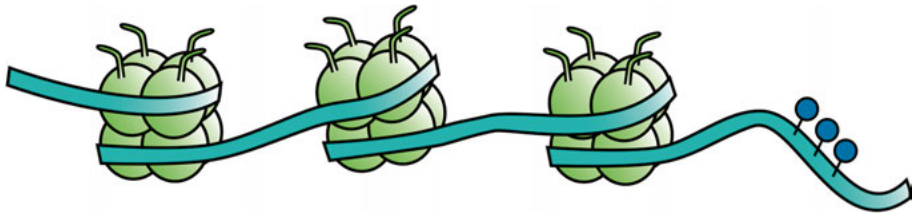

Figure 1: Chromatin. DNA is coiled around histones. Both histone proteins and the DNA molecule may be covalently modified, affecting chromatin structure and gene expression.

Tumors, benign and malignant, are caused by acquired mutations[14] that perturb the tightly controlled processes of cell survival, cell division and cell death. These mutations occur on the nucleotide level in the form of single nucleotide variants and small insertions/deletions[15] and on a larger scale as amplification, deletion and rearrangement of larger chromosomal segments[16]. Two major classes of cancer genes have been described: proto-oncogenes and tumor suppressor genes[17]. Proto-oncogenes often promote cell survival and cell division[18] and may be aberrantly activated through chromosomal rearrangements and amplifications causing increased expression, or through activating mutations that uncouple them from the physiological controls in place to regulate their activity. A mutated proto-oncogene becomes an oncogene. Tumor suppressor genes, on the contrary, physiologically suppress uncontrolled cell proliferation[18] and may be inactivated through truncating mutations or large-scale deletions. As the human genome is diploid, with two copies of each gene, two deleterious events are typically required to fully inactivate a tumor suppressor gene.

Mutations occur through several different processes[15]. In anticipation of each cell division, the entire genome is replicated to ensure that both of the resulting cells have a full genome. Several mechanisms are in place to ensure the fidelity of DNA replication, yet it has been estimated that 2-10 mutations are introduced per cell division[19]. Mutations also occur due to intrinsic metabolic processes (e.g. oxidative stress) and due to exposure to mutagens such as ultraviolet light and hydrocarbons in tobacco smoke. It is now clear that mutations in canonical cancer driver genes accumulate with age in even morphologically healthy tissue[20,21].

Tumor development occurs in a Darwinian fashion. The constant accumulation of somatic mutations provides the necessary genetic diversity, while the limitations of available space and resources, as well as the pressures of immune surveillance and medical treatment act as selective forces[22]. Cells with mutations that confer a survival advantage become more numerous, while those with mutations that have a negative effect on cell survival are weeded

out. Many mutations are neither advantageous nor deleterious and may become embedded in the tumor genome as passengers, hitch-hiking on the success of other mutations. Fully developed cancers contain a variety of clones, which may vary over time, and give rise to metastases at distant sites in the body[23].

Not all mutations that contribute to tumor development occur somatically. Certain common genetic variants cause a small but robust increase in the risk of developing a tumor[24,25]. Other rare variants, often deleterious mutations in one allele of a tumor suppressor gene, cause a complete or near complete penetrance of a specific tumor syndrome. In most cases additional somatic mutations are required for tumor development, which is nevertheless accelerated. A notable example is retinoblastoma, a childhood tumor of the eye, which occurs in a sporadic and a familial form. The sporadic form is most commonly unilateral, and is diagnosed at a somewhat higher age, while the familial form is often bilateral and is diagnosed at a younger age[26]. Both forms are due to mutations in the tumor suppressor gene *RB1*: in the sporadic form two inactivating mutations in the gene must occur in the same cell, while in the familial form a faulty copy of the gene is inherited and only one somatic mutation is required – leading to earlier tumor development and a higher risk of multiple tumors. Early studies of retinoblastoma led to the formulation of the Knudson hypothesis which states that two independent 'hits' are required to inactivate a tumor suppressor gene[27].

While most tumors are thought to be driven by changes in the coding sequence of DNA, it has become clear that aberrant epigenetic modifications also play a significant role. Large scale genome sequencing studies have revealed frequent mutations in key epigenetic regulators in both hematological[28,29] and solid malignancies[30,31]. Moreover, aberrant epigenetic marks are frequently detected in cancers. Genome-wide hypomethylation and hypermethylation of specific promoter-near CpG-islands is common[32]. For several cancers, DNA methylation patterns have been used to identify subgroups with strong prognostic value[33,34], further highlighting the biological importance of epigenetic aberrations. Finally, epigenetic aberrations that silence expression of key genes have been suggested as possible drivers of tumor development in lieu of recurrently mutated protein-coding genes[35].

A key issue in cancer research is the identification of the mutations that drive tumor development. Modern genome sequencing technologies have revolutionized this hunt: in 2004, 61 genes were known to be somatically altered in human cancers[36] while at the time of writing, the Cancer Gene Census[37] includes 576 Tier 1 cancer genes. Identifying the mutations that matter among a plethora of passengers is usually a matter of identifying recurrently mutated genes and pathways, sometimes using complex statistical models to correct

for confounding factors[38]. Elucidation of the genes that drive cancer has led to a shift in our understanding of cancer biology, and in a number of cases, to the development of novel therapies.

Pheochromocytoma and paraganglioma

The adrenal glands are located in the retroperitoneal space, cranial to the kidneys. The outer cortical layer produces steroid hormones, while the inner medulla consists of chromaffin cells which secretes catecholamines (primarily epinephrine) and is an extension of the sympathetic nervous system. Similar cells are found in the thoracic and lumbar paraganglia[39]. Epinephrine (and norepinephrine) bind and activate adrenergic receptors (α1, α2, β1-β3) which are present in a range of tissues, leading to (among other effects) increased heart rate and contractility, bronchodilation, and redistribution of blood perfusion through both vasodilation and vasoconstriction[40] – in short physiological preparation for a 'fight or flight' situation.

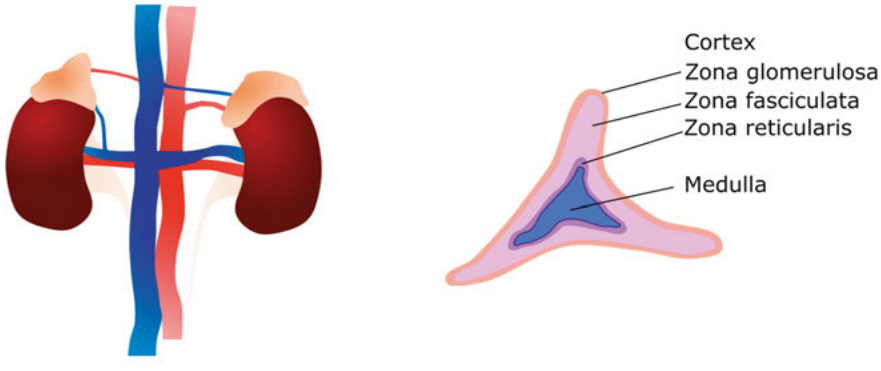

Figure 2: The adrenal glands are located cranial to the kidneys. They consist of an outer cortex which secretes steroid hormones and an inner medulla which secretes catecholamines. The cortex consists of three histological layers; zona glomerulosa, fasciculata and reticularis which produce aldosterone, cortisol and androgens, respectively.

Pheochromocytomas and paragangliomas are tumors of the adrenal medullae and the sympathetic or parasympathetic paraganglia, respectively. They are a rare entity with a reported incidence ranging from 2 to 8 cases per million inhabitants per year[41,42], with a recent nationwide study from the Netherlands showing an increasing incidence of PCC and sympathetic PGL of 5.7/1000000/year[43]. The tumors are usually benign, with only 10-17% causing distant metastases[44]. Nevertheless, untreated PPGL, due to their frequent secretion of vasoactive catecholamines (epinephrine, norepinephrine, and occasionally dopamine) into the bloodstream, may have life threatening complications[45]. The symptoms are caused by catecholamine excess and include headaches, palpitations, sweating, nausea, pallor, anxiety and hypertension. The symptoms are often paroxysmal[46].

The diagnostic work-up is based on biochemical tests and imaging. The recommended biochemical test is measurement of free metanephrines (catecholamine metabolites) in plasma, or fractionated metanephrines in urine. Imaging studies (using e.g. computed tomography) enable localization of biochemically active tumors and is the only means of identifying biochemically non-functioning tumors[47].

The treatment is predominantly surgical. In order to avoid intra- and perioperative hemodynamic instability which may occur due to an extreme release of catecholamines when the surgeon handles the tumor, or due to hypovolemia and vasodilation after removal of the tumor, the patient needs to be pretreated with alpha-blockade for a period leading up to the surgery[47].

Malignancy is established by demonstration of metastasis. Predicting metastatic behavior has proven difficult, although two scoring systems (PASS[48] and GAPP[49]) have been proposed. A recent meta-analysis concluded that these scoring systems are largely successful in ruling out malignancy in cases with a benign course but perform less well in identifying cases with high risk of metastasis[50]. Several clinical and molecular parameters have traditionally been considered to confer an elevated risk of metastatic disease. However, in a recent meta-analysis of 21 studies only the presence of an *SDHB* mutation, secretion of norepinephrine, and secretion of dopamine were associated with an elevated risk of metastasis[51]. Metastatic disease is associated with a five-year survival range around 60%[52], with a highly variable disease course[53]. Owing to the rarity of the disease randomized trials on treatment are lacking. Nevertheless, chemotherapy (cyclophosphamide, vincristine and dacarbazine[54]), as well as radiotherapy with ^{131}I-MIBG[55] and ^{177}Lu-DOTATATE[56] have been used with some success.

Genetics

A substantial fraction of PPGL cases have an underlying hereditary component. Pheochromocytomas are a key component in the Multiple endocrine neoplasia syndrome type 2, and may also occur in von Hippel-Lindau syndrome[57]. In the past two decades a number of additional PPGL genes have been identified and it is now estimated that up to 40% of cases have an underlying germline mutation[58]. Due to the high frequency of heritable causes genetic testing should be offered to all patients[47].

Mutations in the succinate dehydrogenase subunit genes *SDHA-D*[59-62] and the succinate dehydrogenase assembly factor 2 gene *SDHAF2*[63], as well as mutations in *FH*[64] cause hereditary paraganglioma. These genes all encode key enzymes in the tricarboxylic acid cycle. Rare mutations in the related genes, *MDH2*[65] and *SLC25A11*[66] have also been proposed as rare causes of hereditary

paraganglioma. Mutations in *MAX* [67,68], and *TMEM127* [69] cause hereditary pheochromocytoma. Sporadic cases often carry somatic driver mutations in any of a set of genes, including *VHL* and *RET*[70], *HRAS*[71], *NF1*[72,73], *EPAS1*[74], *ATRX*[75] and *KMT2D*[76].

Comprehensive, integrative studies incorporating somatic mutations, copy number aberrations, gene expression and DNA methylation patterns have identified several distinct subgroups[70,77,78]. The most comprehensive analysis to date, carried out by the TCGA consortium[77], identified four subgroups: Wnt-altered, Kinase-signaling, Pseudohypoxia and cortical admixture. The Wnt-altered tumors often have gene fusion events involving *MAML3* or mutations in *CSDE1*, and are characterized by expression of Wnt targets including β-catenin. The most common alterations in the kinase signaling group are mutations in *NF1*, *HRAS* and *RET*, leading to activation of the MAPK signaling pathway. The pseudohypoxia subgroup commonly carries mutations in the *SDHx*-genes, *VHL* or *EPAS1*. Mutations that inactivate the succinate dehydrogenase complex lead to intracellular accumulation of succinate which apparently inactivates α-ketoglutarate-dependent enzymes, including the prolyl hydroxylases[79] that regulate the levels of Hypoxia Inducible Factors which are key mediators of hypoxia signaling. Similarly, inactivating mutations in *VHL* prevent the VHL protein from carrying out its role in the degradation of HIFs[80]. *EPAS1* encodes HIF2α, and the mutations found in PPGLs cause a gain of function associated with expression of key hypoxia response genes[74]. Finally, the cortical admixture subgroup has a more unclear genesis and have lower tumor purity, suggestive of contamination in the sampling process.

DNA methylation

Several early studies reported analyses of DNA methylation of specific gene promoters in pheochromocytoma and paraganglioma. One of these reported a CIMP phenotype associated with extra-adrenal location, malignant behavior and young age at diagnosis[81]. Following technological advances making genome-wide DNA methylation analysis feasible, these findings were corroborated, and an association with *SDHx* mutations was established[82]. Additionally, three distinct clusters of DNA methylation were identified: A hypermethylated cluster of tumors with SDHx-mutations, an intermediate cluster of tumors with *VHL*-mutations, and a hypomethylated cluster of tumors with mutations in *NF1* or *RET*, as well as tumors which had no known mutations at the time. These results were largely validated in the TCGA study[77]. Further studies have identified potential prognostic markers based on DNA methylation, and have found hypermethylation of the *RDBP* promoter to be an independent prognostic marker for metastatic spread[83].

Aldosterone producing adenomas

Primary aldosteronism is a common cause of secondary hypertension and is characterized by inappropriate secretion of aldosterone from the adrenal glands. Aldosterone is a mineralocorticoid steroid hormone physiologically produced in the Zona glomerulosa of the adrenal cortex, which activates the mineralocorticoid receptor. The mineralocorticoid receptor is expressed in a range of tissues[84], although the effects of aldosterone are best characterized in the distal nephrons of the kidneys where aldosterone through regulation of gene expression increases the activity of apical ENaC and Na^+/K^+-ATPase at the basolateral membrane, leading to increased resorption of sodium from the primary urine[85]. The resorption of sodium leads to a simultaneous secretion of potassium and to resorption of water through osmosis. Physiologically, aldosterone secretion is regulated through the Renin-Angiotensin-Aldosterone (RAAS) system and serum potassium levels. In PA, aldosterone is instead secreted autonomously - leading to hypertension and occasionally hypokalemia.

In a majority of cases the inappropriate aldosterone secretion is due to bilateral idiopathic hyperplasia of the adrenal glands, while approximately a third of cases are due to a unilateral benign adrenal tumor. Unilateral hyperplasia and aldosterone secreting adrenocortical carcinoma underlie the syndrome in rare cases[86]. Finally, a small fraction of cases can also be attributed to inherited monogenic syndromes, often presenting with severe disease early in life[87].

Primary aldosteronism was initially considered a rare entity[88]. Since the introduction of modern screening methods it has been shown that PA is a highly prevalent cause of hypertension. In a study of 1225 newly diagnosed patients referred to hypertension clinics, 10.8% of patients could be conclusively diagnosed with PA[89]. Similarly, in a primary care setting, PA was diagnosed in 5.9% of patients with hypertension, with proportions up to 11.8% in the most severely hypertensive patients[90].

Correct differentiation between primary aldosteronism and essential hypertension is important for two main reasons. Firstly, primary aldosteronism may be curable through surgical removal of a diseased adrenal gland, or in the case of bilateral disease, guide the choice of medications. Secondly, primary aldosteronism is associated with an increased prevalence of end organ damage[91]. The recommended screening method for hypertensive patients at risk of PA is measurement of the Aldosterone/Renin ratio. Most patients with a positive ARR need to undergo confirmatory testing. Once the diagnosis has been established it is necessary to differentiate between unilateral and bilateral disease, which is accomplished through adrenal CT and/or adrenal venous sampling[92].

Unilateral cases, i.e. APAs and unilateral or asymmetrical adrenal hyperplasia should be treated surgically with unilateral adrenalectomy, if feasible. If the disease is symmetrically bilateral, or the patient is inoperable for other reasons, mineralocorticoid receptor antagonists are the recommended treatment[92]. Although biochemical success (i.e. decreased/normalized aldosterone secretion and correction of hypokalemia) is seen in 83-100% of surgically treated patients, remission of hypertension occurs only in 17-62%[93], although some benefit is seen in 84% of patients which translates to a strong rationale for treatment as a reduction in blood pressure, even in the absence of complete normalization is thought to lower the risk of adverse events.

Genetics

The advent of next generation sequencing has led to a rapid characterization of the genetics of APAs. In 2011, an exome sequencing study identified hotspot mutations in *KCNJ5*[94]. Further studies published in 2013 detected recurrent mutations in *ATP1A1*[95,96] (encoding a Na^+/K^+-ATPase subunit), *ATP2B3*[95] (encoding a Ca^{2+}-ATPase subunit) and *CACNA1D*[96,97] (encoding a calcium channel). The *KCNJ5*-mutations altered specific residues near the selectivity filter of the encoded potassium channel, causing increased sodium conductance[94]. The increased sodium conductance leads to membrane depolarization, influx of calcium, and likely aldosterone synthesis. Mutations in *ATP1A1* and *ATP2B3* have both been shown to lead to disturbed intracellular ion composition and increased cytoplasmic concentrations of calcium, similarly contributing to aldosterone synthesis[95]. Mutations in *CACNA1D* have also been shown to result in altered electrochemical properties of the resulting protein, with activation at lower membrane voltages, likely leading to increased intracellular calcium concentrations[96,97].

Mutations in *CTNNB1* in APAs were initially reported in 2015, and were reported to be associated with sensitivity of the tumor cells to circulating LH resulting in presentation of the disease during periods of high LH concentrations in the blood: pregnancy and menopause[98]. A further study found *CTNNB1* mutations in 5.1% of tumors, without any association to pregnancy or menopause[99]. Unlike *KCNJ5*, *ATP1A1*, *ATP2B3* and *CACNA1D*, *CTNNB1* is not a regulator of membrane electrophysiology. The encoded protein, β-catenin, is a proto-oncogene in the canonical Wnt signaling pathway[100]. Physiologically, in the absence of Wnt signaling, β-catenin is phosphorylated by a degradation complex which leads to its ubiquitination and degradation[101]. Mutations affecting specific residues in exon 3 of *CTNNB1* render β-catenin insensitive to destruction, leading to its accumulation in the cytoplasm and constitutive activity[102]. Cytoplasmic β-catenin is translocated to the nucleus where it mediates its activity through activation of TCF/LEF family transcription factors[101].

The different genes are mutated in a mutually exclusive manner and APAs carrying different mutations have been found to have distinct characteristics. Histologically, the cells of *KCNJ5*-mutated APAs have been reported to resemble the cortisol-producing cells of the zona fasciculata while the cells of APAs with mutations in other genes have been reported to resemble of the aldosterone producing cells of the zona glomerulosa[96,103]. However, not all studies have replicated this finding[104]. *KCNJ5*-mutated adenomas are larger, more common in females, and curiously more prevalent in Asian cohorts than in cohorts of Australian, European or North American origin[105]. Moreover, studies of gene expression have shown that *KCNJ5*-mutated APAs form a subgroup based on gene expression[96,106].

Multiple endocrine neoplasia type 1

Multiple endocrine neoplasia type 1 (MEN1) is a multi-organ tumor disorder principally presenting with tumors in the pituitary gland, parathyroid glands and the endocrine pancreas[107]. In addition to these organs, patients may develop other tumors including foregut neuroendocrine tumors, adrenal gland tumors and meningioma. While the co-occurrence of multiple endocrine tumors in single patients had been observed as a pathological rarity since the early 20th century, it was not until New York physician Paul Wermer in 1954 published a report[108] on a family with several afflicted members, that the syndrome was recognized as genetic and heritable.

In 1988, a genetic linkage study identified a region on chromosome 11 as underlying the syndrome[109]. In 1997, the MEN1 gene was cloned[110], enabling genetic testing. *MEN1* is a tumor-suppressor gene whose function is incompletely characterized. Mutations occur throughout the gene and most studies do not report a genotype-phenotype relationship[111,112]. The encoded protein, Menin, consists of 610 amino acids and is a nuclear protein[113]. While its functions are incompletely characterized, it is known to interact with several proteins, including transcription factor JunD[114] and histone methyltransferases of the MLL family[115], regulating the expression of genes including cyclin dependent kinase inhibitors[116].

Clinical presentation

The most common presentation is hyperparathyroidism, which occurs in 95% of the patients and is the most frequent initial presentation. The parathyroid glands are usually four in number, located posterior to the thyroid gland and regulate calcium metabolism[117]. Parathyroid hormone, which is inappropriately elevated in pHPT, increases serum calcium concentrations through bone resorption and increased urinary calcium re-uptake[118]. Untreated hyperparathyroidism may lead to osteoporosis, nephrolithiasis, pancreatitis, cognitive symptoms and is a risk-factor for cardiovascular disease[118]. In contrast to sporadic primary hyperparathyroidism, HPT in MEN1 is often due to variable levels of enlargement of several glands rather than a solitary adenoma[119].

The pituitary gland is located intracranially in the *sella turcica*, and comprises two parts of different embryological origin: the adenohypophysis (anterior pituitary) and the neurohypophysis (posterior pituitary)[120]. The posterior pituitary secretes vasopressin, while the anterior pituitary secretes FSH, LH, TSH, ACTH, prolactin and growth hormone. Pituitary adenomas are reported to occur in 38-42% of MEN1 patients[121,122], most commonly in the form of prolactinomas (42-63%) or non-secreting tumors (15-42%). While normally benign, pituitary tumors are associated with significant morbidity related to hormone

secretion and compression of the optic chiasm leading to visual field defects. MEN1 associated adenomas have been reported to be larger than sporadic lesions and to respond less well to treatment[121], although a more recent study (including cases detected through screening) report a high fraction of microadenomas (<10mm) that rarely progress and good response to treatment[122].

MEN1 patients have a lifetime risk in excess of 80% of developing pancreatic neuroendocrine tumors (PanNETs)[123]. PanNETs are thought to develop from the hormone producing islets of Langerhans and may be non-functioning or produce any of a range of hormones[124]. Unlike parathyroid and pituitary lesions these tumors may metastasize and are the leading cause of MEN1-related mortality[123]. The most common PanNETs in MEN1 are non-function tumors which are often small and multiple. The most common functioning PanNETs in MEN1 are gastrinomas, often located in the duodenal mucosa and frequently multiple. Gastrinomas are followed by insulinomas which are typically singular lesions and cause hypoglycemia. More rarely occurring hormone secreting PanNETs include VIPomas and glucagonomas[125].

According to contemporary guidelines, a clinical diagnosis of MEN1 can be made if two of these lesions occur (either simultaneously or subsequently) in an individual, if one lesion occurs in a first degree relative of a patient, or if a deleterious mutation in the MEN1 gene is found[107].

Clinical management

Given the propensity for developing new lesions throughout life[126], patients afflicted with MEN1 are recommended to undergo regular biochemical, clinical and radiological screening for tumors[107]. Moreover, all patients should be offered genetic testing as the detection of a mutation corroborates the diagnosis. If a mutation is detected, this could be screened for in asymptomatic relatives who then can be enrolled in surveillance programs for early detection of lesions if they have the mutation present. Genetic testing and clinical surveillance are thought to improve outcomes through the early detection of primarily the pancreatic tumors[127].

Regarding HPT, it is recommended that patients undergo annual biochemical screening with calcium and PTH measurement. As multiple glands are often affected, minimally invasive parathyroidectomy is not recommended. Instead, subtotal parathyroidectomy or total parathyroidectomy (with autotransplantation) is advised[107]. Removal of less than three glands is associated with a high rate of recurrence[128].

For PanNET, current guidelines recommend annual imaging and biochemical screening[107]. The preferred treatment depends on several factors, including tumor size and number, growth rate, and hormone production. Small gastrinomas are usually treated medically, as proton-pump inhibitors effectively ameliorate the symptoms caused by gastrin[129], and the frequent multiplicity of the tumors necessitates extensive interventions to achieve surgical cure. A majority of small (<2 cm) non-functioning tumors are stable in size during follow-up[123], and can be managed with watchful waiting[130], although the exact timing and criteria for surgery remain a matter of controversy[125]. Other symptomatic hormone producing tumors, e.g. insulinomas, as well as larger or rapidly growing non-functioning tumors and gastrinomas are often surgically removed[107]. Medical treatments include somatostatin analogues, the mTOR inhibitor everolimus, chemotherapy and peptide receptor radiotherapy[107,125].

Pituitary adenomas may be identified through biochemical assays or radiological screening at intervals. The treatment is similar to that of sporadic pituitary adenomas and is predominantly medical, using somatostatin analogues or dopamine antagonists[107].

Mutation negative MEN1

In previous studies between 5 and 25% of patients with a clinical presentation of MEN1 are not found to carry a mutation upon sequencing[107]. Among patients referred for *MEN1* mutation screening in Sweden, factors predictive for detection of a mutation were family history, multiple pancreatic tumors or parathyroid hyperplasia, and young age at diagnosis[111].

The finding of a syndrome without the mutation may be due to a patient developing multiple incidental tumors leading to fulfillment of diagnostic criteria in the absence of a heritable genetic cause, mutations in genes other than *MEN1*, mosaicism[131] or epigenetic mutations, e.g. DNA methylation silencing a gene in the absence of sequence-level variants. It has been demonstrated that patients without a mutation are less likely to develop additional disease manifestations, are diagnosed at an older age, and have a longer life expectancy which is comparable to that of the general population[132].

Several genes other than *MEN1*, when mutated, cause syndromes with features overlapping those of MEN1. *CDKN1B*-mutations have been reported in a small number of patients with multiple endocrine tumors[133], most commonly primary hyperparathyroidism and pituitary adenomas. This syndrome has been referred to as MEN4, and appears to be very rare. Mutations in *AIP* cause the Familial Isolated Pituitary Adenoma (FIPA) syndrome[134]. Mutations in *CDC73*, encoding parafibromin, underlie the Hyperparathyroidism-Jaw Tumor syndrome (HPT-JT) which is characterized by familial HPT and multiple

ossifying jaw fibromas[135]. Mutations in the calcium sensing receptor gene *CASR* may cause any of several calcium metabolism disorders, including Familial Hypocalciuric Hypercalcemia (FHH), Familial isolated hyperparathyroidism[136] and Familial hypoparathyroidism[137]. FHH, similarly to pHPT presents with increased plasma calcium levels, although in FHH hypercalcemia is typically asymptomatic and due to an abnormal homeostatic set point.

Small intestine neuroendocrine tumors

Neuroendocrine tumors of the small intestine are thought to arise from the enterochromaffin cells that are dispersed in the intestinal mucosa[138]. Enterochromaffin cells appear to be involved in the regulation of gut motility through secretion of serotonin[139]. SI-NETs are typically slow growing and are associated with a comparatively long survival even in the context of metastatic disease[140].

The primary tumors are usually small and may be multifocal (although studies of X-chromosome inactivation patterns suggest a common origin[141]). However, a majority of patients present with metastases, most commonly to the mesenteric lymph nodes and the liver[140]. Like their precursor cells, the tumors often secrete serotonin, tachykinins and other hormones. These secreted products may cause the carcinoid syndrome; diarrhea, flush, wheezing and occasionally right-sided heart disease[142]. The carcinoid syndrome is usually associated with metastases, as the blood flow from the primary tumor in the intestine will pass the liver where the culprit molecules are metabolized prior to reaching the systemic circulation. In addition to systemic hormonal symptoms, locoregional symptoms caused by obstruction of intestinal passage or mesenteric blood supply is common[143].

The most common symptoms leading to the diagnosis are pain, diarrhea and weight loss[143]. The serotonin metabolite 5-HIAA is elevated in serum[144] and urine[145] of patients with SI-NET and is used for diagnostic testing. The tumor cells express the neuroendocrine marker Chromogranin A, which is used to establish the histopathological diagnosis[146]. Additionally, as Chromogranin A is secreted and is frequently elevated in plasma from patients with the disease, it represents an additional biochemical marker[147].

Tumors are graded based on immunohistochemistry or mitotic count in accordance with the 2019 WHO classifications[148]. Grade 1 tumors have a Ki67 index less than 3%, G2 tumors 3-20%, and G3 tumors higher than 20%. Additionally, poorly differentiated grade 3 tumors are recognized as a separate entity. Staging is based on the TNM system according to a model proposed by Rindi et al.[149] Stage I corresponds to a small (<1 cm) tumor that does not invade the muscularis propria, without metastases. Stage II corresponds to a larger or more invasive primary tumor, which does not penetrate serosa or invade other organs, still without metastases. Stage III disease requires penetration of the serosa, invasion of other organs, or regional lymph node metastases. Distant metastases are present in stage IV. Both tumor grade and stage have been demonstrated to be of prognostic value[140].

Historically surgical removal of the primary tumor has been recommended regardless of disease stage. While randomized trials are lacking, a recent propensity score matched trial has challenged this approach[150], showing no survival benefit and a higher rate of reoperations in the group treated with locoregional surgery. The medical treatment arsenal includes interferon[151], somatostatin analogues [152,153] peptide receptor radiotherapy[154], the tryptophan hydroxylase inhibitor telotristat which reduces endocrine symptoms[155], as well as mTOR inhibitor everolimus[156].

SI-NETs are relatively rare, although the reported incidence has been increasing over the last decade and is reported to be 1.05/100000/year in the latest comprehensive study of SEER data[157]. Moreover, autopsy studies reveal a high prevalence of undiagnosed SI-NETs. Including both clinically known SI-NETs and those diagnosed only at autopsy, the incidence rate has been estimated to 5.3/100000/year and the prevalence to 0.58%[158].

Genetics

Early CGH array studies revealed frequent hemizygous loss of chromosome 18[159,160]. Subsequent studies have identified additional recurrent SCNAs including loss of chromosome 1, 11, and amplification of chromosomes 4, 5, 16 and 20. Exome sequencing studies have identified truncating mutations in *CDKN1B*, a tumor suppressor gene encoding $p27^{Kip1}$ in approximately 8.5% of cases[161]. These mutations do not show correlation with clinical characteristic, and may be heterogeneously detected[162], suggesting that they are likely not the tumor initiating event, but occur later. No further recurrently mutated genes are known to date.

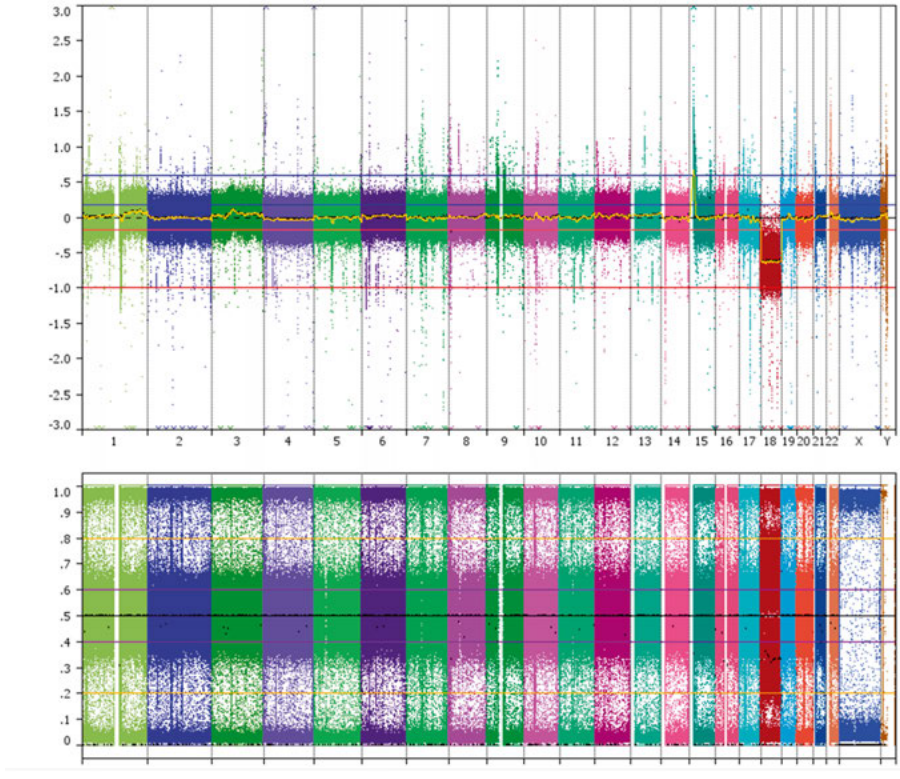

Figure 3: Hemizygous loss of chromosome 18 in a SI-NET

An integrative study combining DNA methylation, copy number, and *CDKN1B* mutation analysis found three clusters characterized by specific DNA methylation patterns and copy number aberrations[34]. These groups had prognostic impact: tumors with only loss of chromosome 18 were associated with long survival (median PFS not reached at follow-up) while those with multiple SCNAs had a median PFS of 21 months. Further evidence of epigenetic dysregulation in SI-NETs is provided by the finding that TET1 expression is abolished, and the TET2 protein excluded from the nucleus and thus presumably functionally inactivated[163].

The mechanism through which loss of chromosome 18 contributes to tumor development is not clear, although several mechanisms have been proposed. *PTPRM*, located on chromosome 18p11.2 has a growth regulatory role in SI-NETs, and may be epigenetically silenced[164]. The region also contains an imprinted gene, *TCEB3C*, which has low expression in SI-NETs. Its expression may be induced through treatment with the 5-aza-2'-deoxycytidine, and induction of *TCEB3C* expression reduces the survival of the SI-NET derived CNDT2.5 cell line[165].

Several additional molecular prognostic biomarkers have been proposed. A study of tumors from 43 patients using CGH array identified gain of chromosome 14 as a predictor of short survival[166]. Another study identified downregulation of miR-375 to be similarly associated with shorter survival[167]. Other than immunohistochemical staining for Ki67 for the purpose of grading, no molecular prognostic biomarker has reached clinical use.

A previous study has compared gene expression between primary tumors from patients with aggressive and indolent disease. No genes were found differentially expressed between the two groups, however a number of genes were found differentially expressed between primary tumors and lymph node metastases and may be involved in disease progression[168]. A subsequent study identified three clusters of tumors based on gene expression, which are associated with outcome[169].

Next generation sequencing

Three of the included studies rely heavily on next generation sequencing (NGS). Therefore, a brief overview of the technology is given. By this time, NGS might be considered a misnomer as the core technologies have been in use for more than a decade; however, the term remains in widespread use. What distinguishes NGS from traditional DNA sequencing is the sequencing of a large number of DNA fragments in parallel, leading to a radically increased throughput.

In the present studies, Illumina sequencing technology has been used. The technology is extensively described in a 2008 publication[170] and briefly reviewed here. First, the DNA sample to be sequenced is fragmented, and ligated to adapter sequences. The sequencing takes place on a flow-cell which is covered with oligonucleotides that are complementary to the adapters. The adapter-ligated DNA fragments are hybridized to these oligonucleotides and amplified in a process known as bridge-amplification, resulting in spatially concentrated clusters of identical oligonucleotides. Following cluster generation, the DNA is sequenced using sequential single-base extension with fluorophoretically labeled nucleotide bases. In each sequencing round, each cluster emits a fluorescent signal corresponding to the base added to the growing sequence. Paired-end sequencing is often performed, whereby the sequencing product is removed, a strand complementary to the sequenced strand generated, and the originally sequenced strand removed followed by sequencing from the other direction.

The generated sequencing reads are often short (50-250 bases), but numerous. The extraction of meaningful biological information requires extensive processing of the data. For a typical use case, genotyping of germline variants, the reads must first be aligned to a reference genome, followed by marking of duplicate reads which risk biasing variant calling, recalibration of base call confidences, and finally variant calling[171].

Contingent on the origin of the input DNA sample, NGS can be used for a variety of purposes. In RNA-Sequencing, cDNA is sequenced, allowing transcriptome-wide measurement of gene expression levels[172]. In exome sequencing, the coding DNA sequence has been enriched, allowing targeted sequencing of the protein coding fraction of the genome[173]. Targeted sequencing where only selected parts of the genome are sequenced allow cheap sequencing of e.g. known disease-causing genes[174]. In whole genome sequencing the entire DNA sequence can be determined.

The development of NGS technology has led to a drastic reduction in sequencing cost, paralleled by a radical increase in generated sequencing data, and has

led to the identification of a wide range of heritable and somatic disease causing genetic variants[175].

Aims

Paper I:
To investigate DNA methylation patterns in PPGL, and their relation to mutational status, and to evaluate the proposed malignancy marker *RDBP* promoter hypermethylation in our cohort.

Paper II:
To investigate the relationship between genotype and gene expression patterns in APAs.

Paper III:
To identify novel disease causing genes and phenocopies in *MEN1*-mutation negative MEN1.

Paper IV:
To identify genetic drivers and potential prognostic markers in SI-NETs.

Materials and methods

Ethics (Paper I-IV)

Ethical approval for the included studies was obtained from the regional ethical vetting board (Regionala Etikprövningsnämnden i Uppsala). All included patients provided written informed consent. Pathogenic germline mutations detected in Paper III were communicated to the treating physicians and the patients offered genetic counseling in keeping with the ethical approval for this study.

Selection of patients (Paper III-IV)

For Paper III, patients with a diagnosis of MEN1 according to standard clinical criteria treated at Uppsala University Hospital between 1984 and 2012, who had undergone routine clinical sequencing of the *MEN1* gene with negative results were identified. Patients from whom blood samples were available in local biobanks were included and all living patients provided written informed consent.

Biomaterials (Paper I-IV)

Tumor tissue was obtained from patients undergoing surgery at the Endocrine surgery unit at Uppsala University hospital as a part of routine clinical care. The tissue was snap-frozen in liquid nitrogen and stored at -70 °C.

A small number of samples studied in Papers I and II were obtained from collaborators at other centers.

Blood (Paper III) was obtained from patients undergoing treatment at either the Endocrine surgery or Endocrine Oncology units at Uppsala university hospital and stored at -20 °C.

Clinical information (Paper I-IV)

Clinical information was extracted from electronic patient records.

Nucleic acid extraction (Paper I-IV)

Extraction of DNA and RNA was performed using DNEasy Blood&Tissue, RNEasy and AllPrep extraction kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. For solid tissues, a 6 μm tissue section was cut using a cryostat, stained with Hematoxylin and Eosin, and scrutinized using light microscopy to ensure sufficient tumor cell content. If necessary and deemed possible, non-neoplastic regions were macroscopically removed using a scalpel. Subsequently, several 10 μm sections were cut and nucleic acid extracted. Concentration measurement and quality control was performed using a NanoDrop spectrophotometer (ThermoFisher Scientific, Waltham, MA).

Polymerase Chain Reaction (Paper II)

The NCBI Primer Blast tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used for primer design. PCR reactions were run on a Bio-Rad T100 thermal cycler (Bio-Rad, Hercules, CA), using Platinum Taq DNA polymerase (ThermoFisher Scientific, Waltham, MA). Reaction conditions were optimized specifically for each individual primer pair. The reaction products were analyzed using gel electrophoresis to ensure that a single product of the expected length was obtained.

DNA methylation array analysis (Paper I)

Extracted DNA was treated using the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) and fragmented. The bisulfite-treated DNA was analyzed using the Illumina Infinium HumanMethylation27 (Illumina Inc. San Diego) microarray at the Bioinformatics and Expression Analysis Core Facility at the Karolinska Institute.

Generated microarray images were imported into the Illumina GenomeStudio software where β-values were calculated. Hierachical clustering analyses were performed in R. Differential methylation analyses were performed using the GenomeStudio software with the Illumina Custom Error Model.

Quantitative Polymerase Chain Reaction (Paper I-II)

Extracted RNA was converted to cDNA using the First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Waltham, USA) according to the manufacturer's instructions. RT-qPCR reactions were run on a Bio-Rad CFX96 Real Time PCR Detection System using Bio-Rad SsoAdvanced Universal Sybr Green Supermix (Bio-Rad, Hercules, CA). All reactions were run in triplicate. *ACTB* was used as a house-keeping gene for normalization. The data was analyzed using the $2^{-\Delta\Delta Ct}$ method.

Computational analyses

Computationally intensive analyses were performed on the Bianca compute cluster at Uppsala Multidisciplinary Center for Advanced Computational Science (UPPMAX).

Whole Genome Sequencing (Paper II-IV)

Extracted DNA was subjected to whole genome sequencing on an Illumina HiSeq2500 at the SNP&Seq Platform at SciLifeLab (Uppsala branch). The target mean read depth varied between 30X and 60X. Generated reads were mapped to the reference genome (human_g1k_37) using bwa-mem[176]. Duplicate reads were marked using Picard.

In Paper II, variants were called using either MuTect 1.15[177] or HaploTypeCaller and annotated using snpEff[178].

In Paper III, germline variants were called using HaploTypeCaller, and somatic variants using FreeBayes[179]. Mutations were annotated for effect using snpEff. Somatic copy number aberrations were called using ascatNgs[180]. Germline variants were annotated using vcfanno[181] with allele counts from the SweFreq[182] dataset and filtering allele frequencies[183] form the gnomAD dataset[184]. Initially, known MEN genes were scrutinized for mutations. Subsequently, a two-hit model was constructed in which genes affected by both a germline variant with a filtering allele frequency of less than $3.6*10^{-5}$, as well as a somatic mutation were identified.

In Paper IV variants were called using FreeBayes v. 1.1.0, annotated for effect using snpEff and VEP[185], and annotated with allele frequency data from SweFreq[182] and gnomAD using vcfanno. Variants present in these databases were considered likely germline variants and were excluded from further analysis. Copy number aberrations were predicted using Control-FREEC[186].

RNA Sequencing (Papers II and IV)

Extracted RNA was subjected to DNAse treatment using Turbo-Free DNAse kit (Invitrogen, Carlsbad, CA). Libraries were generated using the RiboZero gold and TruSeq total RNA kits (Illumina). Sequencing was performed on a HiSeq2500 system using v4 chemistry in high-throughput mode. Library preparation and sequencing was performed at the SNP&Seq platform at SciLifeLab.

The generated FASTQ files were analyzed using kallisto[187] version 0.43.0 (Paper II) or 0.53.1 (Paper IV). Differential expression analysis was performed using the Wald test in sleuth[188] (v 0.29 in Paper II and 0.30 in paper IV).

For Paper II, variant calling was performed on the generated reads. Briefly, read mapping was performed using the STAR[189] 2-pass method, followed by marking of duplicates and sorting of the reads using Picard. The GATK Split'N'Trim and ReassignMappingQuality tools were run, followed by BaseQualityScoreRecalibration and variant calling using HaploTypeCaller with the default settings. Generated variants were annotated using snpEff. Variants in the genes of interest were manually extracted.

Summary of the included papers

Paper I

Pheochromocytomas and paragangliomas are caused by somatic or germline mutations in any of more than a dozen genes. Previous studies of DNA methylation have identified three clusters with hypermethylation, hypomethylation and intermediate methylation levels, respectively. These clusters correlate to mutation status, and in particular, SDHx-mutations have been shown to cause a hypermethylator phenotype[82]. Additionally, hypermethylation of the *RDBP* promoter has been suggested as a prognostic marker[83].

In the present study we studied 38 pheochromocytomas, 1 paraganglioma and 4 normal adrenal medullae using HumanMethylation27 DNA methylation arrays. Hierarchical clustering and principal components analysis revealed two distinct clusters. Cluster A (n=28) contained all the malignant tumors and showed hypomethylation compared to Cluster B and normal medullae. Cluster A had more SCNAs than cluster B, consistent with previous studies indicating that DNA hypomethylation causes chromosomal instability. The previously proposed biomarker of malignancy, *RDBP* promoter hypermethylation, was evaluated in this cohort. Two probes for the region were available, and neither differed significantly between the benign and the malignant tumors, although the studied cohort was comparatively small and these results should be interpreted with care.

In summary we validate previous findings regarding methylation clusters in PPGL, although the absence of SDHx-mutated tumors did not allow us to identify the CIMP cluster. We found no association between *RDBP* promoter methylation and malignancy.

Paper II

Aldosterone producing adenomas are known to carry mutations in *KCNJ5*, *ATP2B3*, *ATP1A1*, *CACNA1D*, and *CTNNB1* which occur in a mutually exclusive fashion and combined account for a majority of APAs. Previous transcriptomic studies have primarily identified differences between *KCNJ5*-mutated and *KCNJ5*-wildtype tumors.

In this paper, fifteen APAs (with known mutation n=13, without known mutation n=2) were subjected to RNA-Sequencing (n=15) and whole genome sequencing (n=2). The tumors without known mutations were upon resequencing found to carry mutations in two of the previously established disease genes: *CACNA1D* (p.S410L) and *ATP2B3* (p.G123R). Unsupervised hierarchical clustering separated the *CTNNB1*-mutated tumors from the rest of the cohort. Comparison of *CTNNB1*-mutated tumors with the rest of the cohort revealed 1360 differentially expressed genes, while only 106 and 75 genes were found differentially expressed in *KCNJ5*- and *ATP1A1*/*ATP2B3*-mutated tumors, respectively.

Several genes previously found overexpressed[190,191] in other (non-APA) types of CTNNB1-mutated adrenal tumors were overexpressed. also in *CTNNB1*-mutated APAs: *AFF3*, *ISM1*, *NKD1*, *ENC1* and *RALBP1*. *AFF3* and *ISM1* were selected for validation by RT-qPCR, and their overexpression confirmed.

Previous studies have reported that *CTNNB1*-mutated APAs may express ectopic hormone receptors (i.e. LHCGR) and present during periods of high plasma LH levels, such as puberty, pregnancy, or menopause[98]. Of the three *CTNNB1*-mutated tumors in the present cohort, only one had significant expression of LHCGR, although the patient did not report any association between onset of hypertension and pregnancy of puberty.

Looking at expression of hormone synthesis genes, *KCNJ5*-mutated tumors exhibited a trend towards higher expression of *CYP11B1* than *ATPase*/*CACNA1D*-mutated tumors while the opposite trend was seen for *CYP11B2*, and the *CTNNB1*-mutated tumors had greatly variable expression of these genes.

Paper III

Approximately ten percent of patients with a clinical diagnosis of MEN1 are not found to carry a *MEN1*-mutation on routine sequencing. Previous studies have shown that on a population level, MEN1 patients without a mutated *MEN1* gene have a subtly different disease course compared to those found to have a mutation, while mutations in other genes (including *CDKN1B*, *CASR*, *AIP* and *CDC73*) cause a partially overlapping disease phenotype. To investigate the genetic background of mutation-negative MEN1 patients, 13 patients fulfilling the MEN1 diagnostic criteria without mutation and one patient with a clinical suspicion of MEN1 were subjected to whole genome sequencing of constitutional DNA. Known tumor syndrome genes (*MEN1*, *CDKN1B*, *AIP*, *CASR*, *CDC73*) were investigated for mutations.

Three patients were found to carry mutations in the *MEN1*-gene (splice site/region mutations c.1186-2A>G, c.669G>C, and missense mutation p.Pro12Leu) which had not been previously detected on routine sequencing. Of note, all these patients had developed pancreatic NETs, and one of them was the only patient in the cohort with all three major MEN1 manifestations. One patient carried a p.Ile555Val missense variant in *CASR*, suggesting a diagnosis of familial hypocalciuric hypercalcemia with a coincidental neuroendocrine tumor unrelated to the patient's hyperparathyroidism. One final patient was found to carry a large-scale heterozygous deletion of part of chromosome 1 including the *CDC73* locus, indicating an alternative diagnosis of Hyperparathyroidism-Jaw Tumor syndrome. Tumor DNA from six of the patients without detected germline mutations was extracted and sequenced. Somatic and germline variants were jointly studied under a two-hit model in order to find potential novel MEN genes. Under this model, genes with a germline variant with a population frequency of less than 3.6×10^{-5} and a somatic mutation in the same patient were identified. No credible novel MEN genes were found in the cohort.

The paucity of underlying genetic lesions in 9/14 included patients, especially when viewed in an epidemiological context suggests that a fraction of patients may fulfill the diagnostic criteria for MEN1 by mere chance. Moreover, the finding of pathogenic variants in *CDC73* and *CASR* highlight the necessity of considering phenocopies.

Paper IV

Despite considerable efforts, the genetic lesions leading to development of neuroendocrine tumors of the small intestine are still largely unknown. Mutations of *CDKN1B* are found in ~9% of cases, while other recurrently mutated genes have not been found. Large scale chromosomal aberrations have been well characterized, the most common being heterozygous loss of chromosome 18q which is found in up to 80% of cases.

In the present study 30 patients with SI-NETs were included, sixteen of whom had a long survival with disease, while the remaining had short survival after diagnosis, despite similar disease characteristics. All patients were subjected to whole genome sequencing of tumor DNA, and for ten of the patients two lesions (primary tumor and metastasis) were sequenced. Additionally, ten patients from each group were included for RNA-Seq of tumor DNA.

Analysis of mutations revealed that three tumors carried *CDKN1B* mutations (frameshift n=1, nonsense n=2). Additionally, one patient carried a mutation in *NF1* in both the primary tumor and the metastasis, occurring together with

deletion of the second allele. Single tumors were also found to carry mutations in established cancer genes *MAX*, *ATRX*, and *MSH6*.

The most common copy number alteration was loss of chromosome 18, which was found in 20/40 samples. Other previously known recurrent SCNAs were also detected, including loss of chromosome 11 and gains of chromosomes 4 and 5.

Clustering based gene expression revealed two clusters, which differed in survival (p=0.004). Differential expression analysis between the two clusters identified 951 differentially expressed genes, including genes involved in angiogenesis, cell differentiation and cell migration. Direct comparison between the long-survivors and the short-survivors identified 58 differentially expressed genes which represent possible prognostic biomarkers.

Finally, we investigated the effects of known genetic aberrations on gene expression. *CDKN1B*-mutated tumors were found to have decreased expression of *CCND2* which encodes cyclin D. Loss of chromosome 11 was associated with decreased expression of tumor suppressor gene *ATM*, implicating it in SI-NET development. Loss of chromosome 18 was associated with decreased expression of 15 genes located on chromosome 18q22-qter, including putative tumor suppressor gene *SOCS6*.

Conclusions

We have validated previous findings on DNA methylation patterns in pheochromocytomas and paragangliomas, although a paucity of SDHx-mutated tumors prevented us from validating previous findings of a DNA hypermethylator phenotype in these tumors. *RDBP* promoter hypermethylation could not be validated as a marker of malignancy in our cohort.

We have identified *CTNNB1*-mutated aldosterone producing adenomas as a distinct subgroup based on gene expression signatures. These tumors shared gene expression similarities with non-aldosterone producing adrenocortical adenomas with *CTNNB1* mutations. In particular, they had overexpression of *AFF3* and *ISM1*.

While some patients presenting with a clinical MEN1 phenotype in the absence of a *MEN1* mutation detected on routine clinical genetic testing are found to carry a mutation in either *MEN1* or another endocrine tumor gene upon whole genome sequencing, a majority are not found to carry any clearly pathogenic mutation underlying the syndrome. This suggests that some of these patients have developed multiple tumors by chance.

Using RNA-Seq we identify two clusters of SI-NETs with different survival and potential prognostic markers that differ between them. Moreover, we validate previous genetic aberrations and find rare mutations in known cancer driver genes. Finally, we identify genes affected by gene dosage through recurrent chromosomal amplification or deletion, including tumor suppressor gene *ATM* which is downregulated through loss of one copy of the gene.

Future directions

Both the genomic and the epigenetic landscapes of PPGL have been thoroughly characterized in increasing detail over the last decades. Subgroups defined by driver genes, gene expression and DNA methylation have been identified. However, much work remains to be done to fully translate the biological insights into clinical benefit for the patients. The presence of clearly defined subgroups suggests that a personalized approach to management based on tumor biology may one day be possible. Studies of large cohorts, including detailed clinical and molecular data may elucidate whether 'omics' technologies are able to provide independent prognostic markers and guide treatment.

CTNNB1-mutations occur in APAs, adrenocortical carcinomas, non-functioning benign adrenocortical tumors, and benign cortisol-producing adrenocortical adenomas. Whether hormone production is dependent on the cell in which the mutation occurs, or on secondary events (such as additional mutations in other genes) remains to be elucidated. Moreover, while there is a clear mechanistic link between mutations in *KCNJ5*/*CACNA1D*/*ATP1A1*/*ATP2B3* and aldosterone synthesis through depolarization and calcium signaling, it is not clear whether these mutations are sufficient for tumor formation. Further functional studies in in model systems such as cell lines and model organisms may provide further insights on this matter.

In a majority of the apparently mutation-negative MEN1 patients we did not identify a causal genetic event in the constitutional DNA. Nevertheless, it cannot be ruled out that an alternative mechanism, e.g. DNA hypermethylation, underlies the phenotype. Such a mechanism of inactivation has been demonstrated in a case of *SDHC*-related paraganglioma[192], but is likely rare. Nevertheless, the finding that some MEN1 patients do not carry a mutation in either *MEN1* or another gene raises questions about how these patients are best managed in the clinic. Frequent follow-up with clinical controls, imaging and biochemical tests are costly and may place a psychological burden on patients. Further studies clarifying the clinical course and need for follow-up of these patients are warranted. Moreover, the risk of relatives developing disease ought to be clarified.

Our understanding of the molecular drivers of SI-NETs remains limited. In the present thesis a number of genes of potential interest have been identified,

however their biological role needs to be further investigated through e.g. knockout/overexpression studies in cell lines. A key problem for SI-NET research is the lack of available normal tissue for comparative analyses with tumors. The presumed precursor cells constitute only 1-2% of the mucosa in healthy small intestine. Recently developed technologies for single cell gene expression and epigenetic studies may provide answers to several key questions regarding the genetic pathways that are dysregulated, and the underlying mechanisms.

Acknowledgements

This work was carried out at the Department of Surgical Sciences at Uppsala University. During my time as a PhD student I've had the opportunity to work with some truly remarkable people, without whom this thesis would have never been written.

I would first like to thank my supervisors:

Per Hellman for sharing your vast scientific expertise, for your support and guidance, for plenty of good advice and for helping me develop as a scientist. Your kindness, hard work, and ability to always see new important perspectives make you an inspiration to a budding researcher like me. During most of my PhD studies you have also acted as head of the department, skillfully ensuring that it stays the great institution that it is.

Peter Stålberg for all your encouragement and guidance over the last few years, for involving me in various exciting research projects within and outside of the scope of this thesis, and for plenty of good advice (scientific and beyond) and rewarding conversations. You are a true role model for an aspiring medical doctor and researcher.

Olov Norlén for sharing your thorough knowledge of endocrine tumors and providing valuable input at various stages of my PhD studies. Your swift responses and attention to both detail and the bigger picture have been much appreciated.

I would also like to express my gratitude to the great people I've been fortunate enough to work with in the lab.

Elham Barazeghi. A brilliant researcher and a great colleague. Thank you for all the help, lunch-time discussions, and always being able to answer my questions (and for your admirable patience with my less insightful ones)!

Rajani Maharjan. It was always fun working with you. I hope that life in Scotland is treating you well!

Joakim Crona. Thank you for several successful collaborations over the years, and for your generosity with ideas (of which you always have many)!

Tobias Åkerström. I have learned a whole lot from you over the years and working with you is always both inspiring and thoroughly enjoyable!

Isabella Silins. I'm looking forward to reading your thesis down the line! Best of luck with everything!

Branislav Klimacek. I'm looking forward to continued collaboration. Best of luck with your thesis!

Birgitta Bondeson. Thank you for all the invaluable help throughout the years, and for always raising the spirits in the lab.

Gunnar Westin. Thank you for sharing your thorough knowledge of genetics and epigenetics.

All my **co-authors** for your invaluable contributions to the included studies.

All the current and former staff at Rudbecklaboratoriet R3 floor 2 for creating a great environment for science!

Christofer Juhlin, Johan Paulson, Na Wang, Adam Stenman and Jan Zedenius for nice collaborations during the last two years!

I was given the opportunity to embark on my PhD studies while in medical school, thanks to the **EDUU-programme** led by **Peter Bergsten**. When I eventually graduated, **Erik Salaneck**, **Johan Carlsson** and the rest of AT-enheten gave me the opportunity to do my Forskar-AT at Akademiska sjukhuset which allowed me to finish this thesis and continue my medical education.

Through **The National Graduate School in Medical Bioinformatics** I was able to attend first rate courses in bioinformatics and develop my skills in computational biology.

I would like to express my gratitude to **Nikola Katardjiev** for thoughtful discussions on computational complexity theory.

Quite a few times over the years I've ended up in tricky technical situations. Each time (even when I thought it impossible) the staff at **UPPMAX** have helped me out swiftly and professionally.

My friends. I am fortunate enough to have more than can reasonably be mentioned here – you know who you are! Special shoutouts to **Coola gänget** (Adam Alstermark, Adam Wolf, David "IFK-Bengan" Bengtsson, Oscar Flink, Philip Göstasson and Tomas Wigren) for all the good times in med school and beyond. The **IB squad** deserves special recognition for sticking around for the past decade.

Tove, who joined the journey somewhere between Papers I and II and has enriched every day since. I am lucky to have you in my life. I am looking forward to not understanding your thesis in a few years!

To my **family** in Finland and Sweden: Thank you for everything.

My parents Robert & Maria for always encouraging my curiosity – which eventually led me down this path - and for all your support over the years. My **brothers Oliver & Elliot** for being some of the wittiest people around, and my **sister Amelie** for never turning down a round of Mario Kart.

Finally, my sincerest apologies to those who should have been included here but were not. Compiling an exhaustive list of acknowledgments on a deadline proved nearly impossible. Thank you!

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