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New Insights in Adrenal Tumourigenesis

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Abstract
Maharjan, R. 2017. New Insights in Adrenal Tumourigenesis. *Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine* 1345. 54 pp. Uppsala: Acta Universitatis Upsaliensis. ISBN 978-91-513-0009-2.

Unilateral cortisol producing adenoma (CPA) is the most common cause of ACTH-independent Cushing's syndrome and is surgically curable. On the other hand, adrenocortical carcinomas (ACCs) are rare and aggressive tumours. Although the overall survival of the patients with ACC is very poor, the outcome can be heterogeneous and vary significantly between the patients. This thesis comprises studies showing genetic and genomic events occurring in CPAs and ACCs, their functional impact and clinical correlations.

The Wnt/β-catenin and cAMP/PKA signalling pathways are crucial in adrenal homeostasis and frequent mutations in members of these pathways (*CTNNB1, GNAS,* and *PRKACA*) are found in CPAs. Mutational analysis revealed that ~60% of the CPAs harboured mutations in either of these genes. Transcriptome signature exhibited increased expression of genes involved in steroidogenesis in *PRKACA/GNAS* mutated (Cluster1) tumours in comparison to *CTNNB1* mutated /wildtype (Cluster2) tumours. In addition we have also observed that gain of chromosome arm 9q was the most frequent arm level copy number variation (CNV) occurring in CPAs and were exclusively present in Cluster2 tumours. We also discovered novel *PRKACA* mutations occurring in ACCs, causing activation of cAMP/signalling pathway.

Comprehensive analysis of Wnt/β-catenin signalling pathway in ACCs revealed novel interstitial deletions occurring in *CTNNB1* leading to deletion of the N-terminus of β-catenin. This is a novel and yet another frequent event leading to activated Wnt/β-catenin signalling and downstream targets in ACCs. Both, mutations occurring in *CTNNB1* and nuclear expression of its protein were associated with poor overall survival. Through multiregional sampling approach we discovered intra-tumour heterogeneity in ACC tumours. Although all the multiregions within a tumour showed presence of shared basal CNVs, they encompassed private CNVs, different ploidy levels and private mutations in known driver genes. We found intra-tumour heterogeneity in *CTNNB1, PRKACA, TERT* promoter and *TP53* mutations as well as *ZNRF3* and *CDKN2A/2B* homozygous deletions.

Keywords: cortisol producing adenomas, adrenocortical carcinomas, mutation, heterogeneity, PRKACA, TERT, CTNNB1

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To my friends and family

Understanding how DNA transmits all it knows about cancer, physics, dreaming and love will keep man searching for some time
David R. Brower

List of Papers

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

I **Maharjan, R.**, Åkerström, T., Backman, S., Hellman, P., Björklund, P. Integrated Molecular Characterization of Benign Cortisol Producing Adenomas Defines Two Distinct Clusters. *Manuscript*.

II **Maharjan, R.**, Åkerström, T., Backman, S., Hellman, P., Björklund, P. Identification and Characterization of Novel *PRKACA* Mutations in Adrenocortical Tumours. *Manuscript*.

III **Maharjan, R.**, Crona, J., Åkerström, T., Backman, S., Hellman, P., Björklund, P. Intratumoural Heterogeneity of *TERT* Promoter Mutations in Adrenocortical Carcinoma. *Manuscript*.

IV **Maharjan, R.**, Backman, S., Åkerström, T., Hellman, P., Björklund, P. Comprehensive Analysis of *CTNNB1* in Adrenocortical Carcinomas: Identification of Novel Mutations and Correlation to Survival. *Manuscript*.

V **Maharjan, R.**, Backman, S., Hellman, P., Björklund, P. Multidimensional Genetic Analysis of Adrenocortical Cancers. *Manuscript*.

List of papers not included in the thesis:

I. Crona, J., A. Delgado Verdugo, R. Maharjan, P. Stalberg, D. Granberg, P. Hellman and P. Bjorklund (2013). "Somatic mutations in H-RAS in sporadic pheochromocytoma and paraganglioma identified by exome sequencing." J Clin Endocrinol Metab 98(7): E1266-1271.

II. Crona, J., R. Maharjan, A. Delgado Verdugo, P. Stalberg, D. Granberg, P. Hellman and P. Bjorklund (2014). "MAX mutations status in Swedish patients with pheochromocytoma and paraganglioma tumours." Fam Cancer 13(1): 121-125.

III. Crona, J., M. Nordling, R. Maharjan, D. Granberg, P. Stalberg, P. Hellman and P. Bjorklund (2014). "Integrative genetic characterization and phenotype correlations in pheochromocytoma and paraganglioma tumours." PLoS One 9(1): e86756.

IV. Akerstrom, T., H. S. Willenberg, K. Cupisti, J. Ip, S. Backman, A. Moser, R. Maharjan, B. Robinson, K. A. Iwen, H. Dralle, D. V. C, M. Backdahl, J. Botling, P. Stalberg, G. Westin, M. K. Walz, H. Lehnert, S. Sidhu, J. Zedenius, P. Bjorklund and P. Hellman (2015). "Novel somatic mutations and distinct molecular signature in aldosterone-producing adenomas." Endocr Relat Cancer 22(5): 735-744.

V. Crona, J., S. Backman, R. Maharjan, M. Mayrhofer, P. Stalberg, A. Isaksson, P. Hellman and P. Bjorklund (2015). "Spatiotemporal Heterogeneity Characterizes the Genetic Landscape of Pheochromocytoma and Defines Early Events in Tumorigenesis." Clin Cancer Res 21(19): 4451-4460.

VI. Delgado Verdugo, A., J. Crona, R. Maharjan, P. Hellman, G. Westin and P. Bjorklund (2015). "Exome Sequencing and CNV Analysis on Chromosome 18 in Small Intestinal Neuroendocrine Tumors: Ruling Out a Suspect?" Horm Metab Res 47(6): 452-455.

VII. Akerstrom, T., R. Maharjan, H. Sven Willenberg, K. Cupisti, J. Ip, A. Moser, P. Stalberg, B. Robinson, K. Alexander Iwen, H. Dralle, M. K. Walz, H. Lehnert, S. Sidhu, C. Gomez-Sanchez, P. Hellman and P. Bjorklund (2016). "Activating mutations in *CTNNB1* in aldosterone producing adenomas." Sci Rep 6: 19546.

VIII. Backman, S., R. Maharjan, A. Falk-Delgado, J. Crona, K. Cupisti, P. Stalberg, P. Hellman and P. Bjorklund (2017). "Global DNA Methylation Analysis Identifies Two Discrete clusters of Pheochromocytoma with Distinct Genomic and Genetic Alterations." Sci Rep 7: 44943.

Contents

Abbreviations

APA	Aldosterone Producing Adenoma
APC	Adenomatous Polyposis Coli
ACA	Adrenocortical Adenomas
ACC	Adrenocortical Carcinomas
ACTH	Adrenocorticotropic Hormone
ASCAT	Allele-Specific Copy Number Analysis of Tumours
ATRX	Alpha Thalassemia/Mental Retardation Syndrome X-Linked
BWS	Beckwith-Wiedemann Syndrome
cAMP	Cyclic Adenosine Monophosphate
CIMP	CpG island Methylator Phenotype
CDKN2A	Cyclin-Dependent Kinase Inhibitor 2A
CNA	Copy Number Aberration
CNC	Carney Complex
CNV	Copy Number Variation
CPA	Cortisol Producing Adenomas
CRH	Corticotropin-Releasing Hormone
CTNNB1	Catenin Beta 1/ Beta-Catenin
DAXX	Death-Domain Associated Protein
DHEA-S	Dehydroepiandrosterone Sulfate
ENSAT	European Network for the Study of Adrenal Tumours
FAP	Familial Adenomatous Polyposis
FFPE	Formalin Fixed Paraffin Embedment
GISTIC	Genomic Identification of Significant Targets in Cancer
GNAS	Guanine Nucleotide Binding Protein (G Protein), Alpha
IGF2	Insulin-Like Growth Factor 2
LEF	T-Cell Specific Transcription Factor 1-Alpha
LRP	Low Density Lipoprotein Receptor-Related Protein
MAS	McCune Albright Syndrome
MEN1	Multiple Endocrine Neoplasia type I
MiAH	Micronodular Adrenocortical Hyperplasia
NF1	Neurofibromin 1
PBMAH	Primary Bilateral Macronodular Adrenal Hyperplasia
PCP	Planar Cell Polarity
PCR	Polymerase Chain Reaction
PPNAD	Primary Pigmented Nodular Adrenocortical Disease
PRKACA	Protein Kinase, CAMP-Dependent, Catalytic, Alpha
PRKAR1A	Protein Kinase CAMP-Dependent Type I Regulatory Subunit Alpha
qRT-PCR	Quantitative Real Time PCR
RB1	RB transcriptional corepressor 1
SF1	Steroidogenic Factor 1

SNP	Single Nucleotide Polymorphism
SOAT1	Sterol-O-Acetyl transferase 1
STAR	Steroidogenic Acute Regulatory Protein
TCA	Tricarboxylic Acid Cycle
TCF	T-Cell Specific Transcription Factor Family
TERT	Telomerase Reverse Transcriptase
TNM	Tumour-Node-Metastasis
TP53	Tumour Protein P53
WNT	Wingless-Type MMTV Integration Site Family
WT	Wildtype
ZF	Zona Fasciculata
ZG	Zona Glomerulosa
ZNRF3	Zinc and Ring Finger 3
ZR	Zona Reticularis

Introduction

Adrenal Glands

The adrenals are endocrine glands situated superior to the kidneys. They measure roughly 3X5 cm in size and weigh approximately 10 grams together. They are sandwiched between several organs. Hence the right adrenal has a pyramidal shape and the left one has a crescent shape (Figure 1).

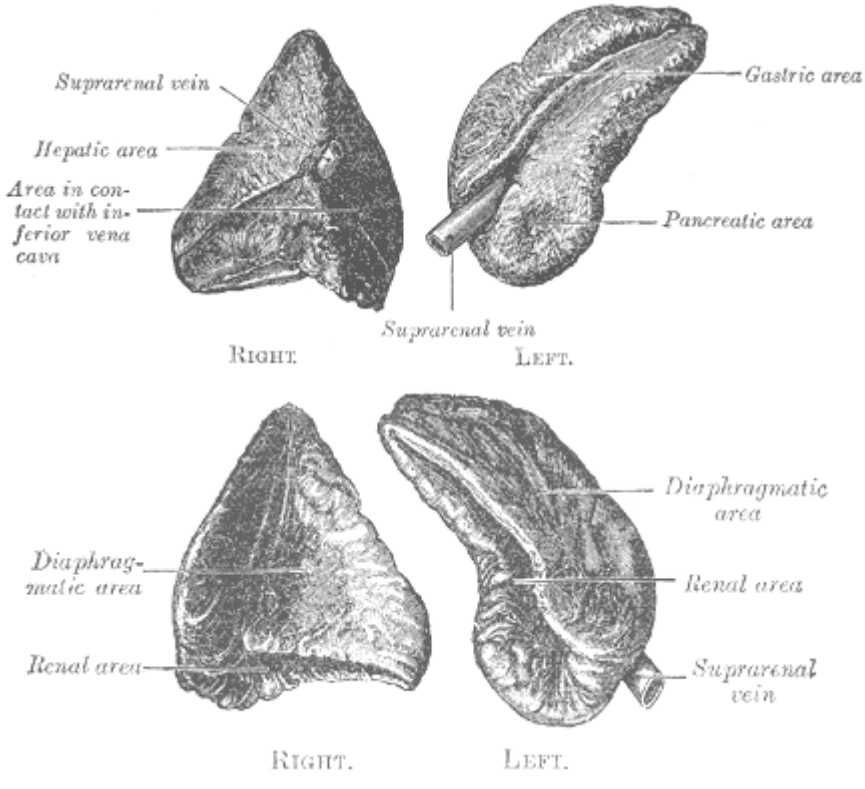

Figure 1. Front and back view of left and right adrenal gland. Figure adapted from "Anatomy of Human Body" book (20th edition)[1].

The first documented description of adrenal glands was given by Claudius Galenus of Pergamum (130-201 AD). Though not accurate, he described a connection between left adrenal gland and renal vein and referred the gland

as a 'loose flesh' projected as a renal accessory [2]. The first distinct description of adrenal glands was given by Bartholomeus Eustachius (1520-1574), Professor at the Collegio della Sapeinza, Rome in 1563 [3]. Eustachius referred adrenal glands as 'Glandulae renibus incubentis' which infers an auxiliary renal role. Along the timeline numerous scientists recognized the adrenal gland and gave them different name such as 'Renes succentuari', 'Capulae suprarenales', 'Glandulae renales'.

The two morphologically distinct layers (cortex and medulla) in the adrenal gland and the distinction between these two layers was first recognized by Baron George Cuvier (1769-1832) in 1805 [4]. However, the complete description of microscopic anatomy was first given by Albert von Kölliker (1817-1905) in 1852 along with the distinct products and functions of cortex and medulla [4].

Adrenal Zonation and function

The adrenal gland is composed of two distinct layers, cortex and medulla, derived from different embryological origin. The inner medulla is derived from neuroectoderm whereas the outer cortex is derived from mesoderm. The later accounts for major volume of the adrenal mass.

Adrenal Cortex

The adrenal cortex undergoes differentiation and develops three layers, namely zona glomerulosa, zona fasciculata and zona reticularis. Zona glomerulosa (ZG) is the outermost layer lying immediately beneath the adrenal capsule and produces mineralocorticoids in response to the plasma potassium and angiotensin II levels. Zona glomerulosa is followed by Zona fasciculata (ZF) which produces glucocorticoids in response to ACTH. Zona reticularis (ZR) is the innermost layer surrounding medulla and produces adrenal androgens.

The production of different types of corticosteroids begins with import of cholesterol into mitochondria by STAR. This is a rate limiting step in adrenocortical steroidogenesis[5]. Cholesterol undergoes a series of modification and is converted into early precursors of steroids such as pregnenolone and progesterone. Depending upon the location in the adrenal cortex, these precursors are converted into specific steroids through catalyzation by site-specific enzymes (Figure 2). Studies indicate that the non-steroidogenic capsular cells consist of progenitor cells which move centripetally from the outer cortex towards the medulla[6,7], differentiating into the corresponding type of cells as they migrate from one zona to another [8]. The evidence supporting such phenomenon was observed in studies performed as early as 1940s [9]. In this early study, regrowth of adrenal cortex from residual cells of the adrenal

capsule following adrenal cortex enucleation was observed in rat. This differentiation is regulated by Steroidogenic Factor 1 (SF1). The critical role of SF1 for ZG to ZF lineage conversion was shown by the lack of capability of SF1 deleted ZG cells to form ZF cells[8].

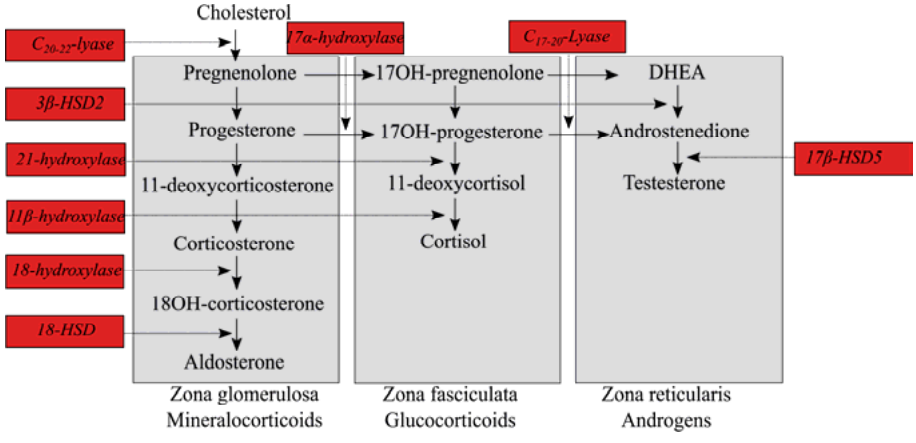

Figure 2. Schematic of adrenal steroidogenesis.

TP53 Signalling pathway

The TP53 signalling pathway is a sophisticated network of genes and signalling cascades that regulate DNA replication, chromosome segregation and cell division. Damaging signals activate different molecules that communicate with p53. Signal-specific activated molecules communicate the signal to p53 by modifications, such as phosphorylation, ubiquitination, acetylation or sumoylation of the protein.

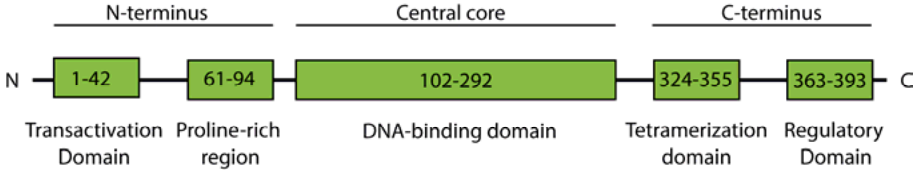

Figure 3. Summary of p53 functional domains.

The p53 structure consists of five functional domains (Figure 3)[10,11]. The N-terminus consists of two domains known to transcriptionally transactivate the p53-inducible genes. In addition, the domain is also involved in inhibit-

ing transcription of many genes involved in supressing apoptosis. The central domain consists of a sequence specific DNA binding domain. The C-terminus consists of a tetramerization domain as well as a regulatory domain. The tetramerization domain is involved in formation of dimers and tetramers of the protein, which activates transcription of its downstream targets. The plethora of signalling modalities involving p53 converges in blocking the cell cycle progression and inducing apoptosis upon a stress signal.

TP53 is one of the most frequently mutated genes in cancer[12,13] and germline mutations in *TP53* causes Li-Fraumeni syndrome[14-16]. Inactivation of the p53 results in accumulation of genetic damage and enhances tumour formation. The TP53 signalling pathway integrates and cross-talks with numerous pathways and is regulated by several negative feedback loops involving MDM-2, p73, cyclin G etc. as well as positive feedback loops involving PTEN-AKT, CDKN2A and Rb [17-19].

WNT signalling pathway

The Wnt signalling pathway is a highly conserved pathway, significant in embryogenesis as well as in the adult cell and tissue homeostasis[20,21,22]. It regulates processes such as cell proliferation, survival and differentiation and does so by three different routes; the planar cell polarity (PCP) pathway, the Wnt/Ca2+ pathway and the canonical Wnt/β-catenin pathway. In the canonical pathway, as the name suggests, Wnt and β-catenin are the key players. In the absence of Wnt ligand signalling, β-catenin is phosphorylated on its serine and threonine residues by CK1 and GSK3β in the destruction complex composed of APC, AXIN, CK1 and GSK3β. Phosphorylation starts at Ser45 by CK1 which is then followed by phosphorylation at Thr41, Ser37 and Ser33 by GSK3β[23]. Upon phosphorylation β-catenin is targeted by the E3 ubiquitin ligase β-TrCP and is subsequently subjected to proteosomal degradation (Figure 4)[24].

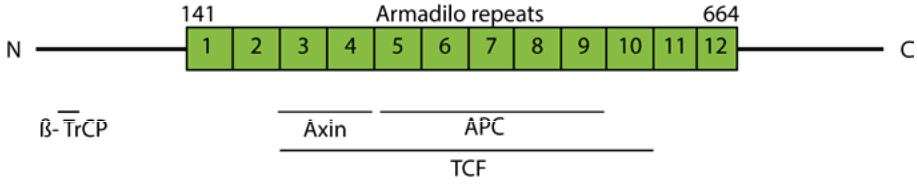

Figure 4. Summary of β-catenin functional domains. Bars indicate the regions bound by different regulators and partners.

In the presence of a Wnt ligand, it binds to its receptors Frizzled (FZD) and LRP5/6. This leads to recruitment of Axin to phosphorylated LRP causing the destruction complex to migrate to the plasma membrane leading to presence of free β-catenin. Recent studies have shown that in the presence of Wnt signalling the ubiquitination of β-catenin is blocked in the destruction complex itself. This leads to accumulation and saturation of the destruction complex with phosphorylated β-catenin leading to accumulation of newly synthesized β-catenin[25]. Cytoplasmic β-catenin then translocates to the nucleus where it acts as transcription cofactor, regulating numerous downstream genes.

The Wnt ligand family consists of 19 different evolutionarily conserved Wnt genes/proteins. In addition, there are 10 different types of FZD and 2 different types of LRP receptors known in humans to which Wnt ligand can bind. Combination of different Wnt ligands and their receptors and co-receptors regulates a diverse array of downstream targets and pathways. Newly recognized members in this signalling pathway are the E3 ubiquitin ligases ZNRF3 and RNF43. ZNRF3 and RNF43 are negative-feedback regulators involved in internalization and degradation of FZD and LRP receptors[26,27] keeping the Wnt/β-catenin signalling in check. However in the presence of R-spondin proteins, which binds to ZNRF3 and RNF43[28] prevents the degradation of the Wnt receptors leading to activation of the Wnt/β-catenin pathway[27,29].

Deregulation of this pathway affects both development and homeostasis of an organism. Knocking out CTNNB1, encoding β-catenin, leads to embryonic lethality [30]. Aberrations in molecules involved in different tiers of the Wnt signalling pathway have been observed in a wide spectrum of diseases [20]. Mutations in *FZD4* and *LRP5* are associated with familial exudative vitreoretinopathy[31,32]. Mutations and alternative splicing in *LRP5* has been associated with bone density defects [33] and hyperparathyroidism[34]. The first connection of aberrations in the Wnt signalling pathway and cancer was made in 90s with the identification of *APC* gene mutations in the familial adenomatous polyposis (FAP) syndrome [35,36]. In few cases of colon cancer without *APC* mutation, mutations in *AXIN2* or *CTNNB1* could be found instead. Mutations in *CTNNB1*, however, are not restricted to the colon cancer but have been found in different kinds of benign and malignant tumours such as melanoma, medulloblastoma, hepatocellular carcinoma, benign adrenocortical adenomas and adrenocortical carcinomas[37-40].

Mutations in *APC*, *AXIN2* and *CTNNB1* leads to inappropriate accumulation of active β-catenin that in association with transcription factors such as TCF and LEF activates the genes associated with cell proliferation. Mutations in exon 3 of the *CTNNB1* gene causing loss of serine and threonine phosphorylation sites is one of the most common cause of illicit β-catenin activation [41]. Sporadically, deletions in/of exon 3 have been observed[42].

Nuclear expression of β-catenin is observed in the subscapular region and the zona glomerulosa layer of the normal adrenal gland[43]. Conditional knockout of β-catenin in the mouse adrenal glands illuminated the role of Wnt signalling/β-catenin in adrenal development and maintenance. Complete depletion of β-catenin leads to adrenal aplasia whereas, partial depletion leads to post-partum depletion of adrenocortical cells[22]. Expression of constitutively active β-catenin on the other hand leads to hyperplasia/dysplasia of adrenal cortex in mouse models and in some even malignancy[44]. Development of malignancy in only few of them and over the time period of 17 months suggests that requirement of additional events is necessary to progress towards malignancy after gaining a *CTNNB1* mutation.

cAMP signalling pathway

The significance of the cAMP signalling pathway in steroidogenesis is well recognized [45]. In the adrenals, binding of ACTH to melanocortin receptor 2 stimulates a heterotrimeric G-protein. The G-protein consists of α, β, and γ subunits. The alpha subunit in this case is encoded by the *GNAS* gene. The activated G-protein activates adenylate cyclase which then catalyses conversion of ATP to cAMP. Subsequently cAMP binds to the PKA (protein kinase A) holoenzyme. The PKA holoenzyme is a heterotetramer consisting of two of each of the catalytic and regulatory subunits. There are four different regulatory subunits (PRKAR1A, 1B, 2A, 2B) and three different catalytic subunits (PRKACA, B, G) known in human. The catalytic subunit structure consists of a small (N) and a large (C) lobe with the active site forming a cleft in between these two lobes. The N lobe harbours the binding site for ATP whereas the C lobe consists of an activation loop which harbours a phosphorylation site. The C lobe also works as a docking surface for the substrates. In the PKA holoenzyme tetramer, the R subunit docks at an active site cleft, an activation loop site and a hydrophobic portion of catalytic subunit[46]. Binding of cAMP to the regulatory subunit leads to a conformational change releasing the active catalytic subunit. It then phosphorylates several transcription factors such as CREB, SF1, STAR[47], with subsequent activation of genes involved in steroidogenesis[48].

Mutations in the cAMP signalling pathway members are associated with several syndromes such as McCune-Albright Syndrome (MAS) and Carney Complex (CNC) characterized by development of endocrine tumours (Discussed further in familial disease section). The knowledge of familial cases has led to discovery of mutations in the cAMP signalling pathway members such as *GNAS*, *PRKACA*, *PRKAR1A*, *PDE8B* and *PDE11A* in sporadic cases of diverse adrenocortical tumours[49-54]. Deregulation of the cAMP signalling pathway and its members has been reported in various endocrine diseases[55]. *PRKAR1A* knock out cause embryonic lethality in mice [56] whereas adrenal

gland specific knock out has shown to cause ACTH independent Cushing's syndrome with autonomous expression of genes involved in steroidogenesis[57]. A mouse model study by Skalhegg et al. 2002 showed that *PRKACA* double knock out leads to immediate postnatal lethality and 27% of the mice which survive until adulthood have delayed growth[58]. However double knock out of *PRKACA* and *PRKACB* leads to embryonic lethality[59]. Adrenal specific model for *PRKACA* has not been developed yet.

Adrenocortical tumours

Adrenocortical tumours are very common and are found in up to 7% of the general population[60]. The tumours are discovered either by hormonal excess or during routine imaging. The pathophysiology of adrenocortical tumours varies from benign to malignant tumours, and with or without hormone production.

Familial syndromes

Li-Fraumeni syndrome: Germline mutations in the TP53 gene are found in 70% of patients with Li-Fraumeni syndrome, which is characterized by development of different types of cancers. *TP53* functions in DNA repair, cell cycle control and induction of apoptosis. Inactivation of this protein in different types of cancer is associated with increased germline copy number variation (CNV) [61] and chromothripsis in tumours[62,63] which highlights its potential to cause cancer. ACC occurs in about 10% of patients with the Li-Fraumeni syndrome. About 90% of paediatric ACCs are accounted by either Li-Fraumeni syndrome or a germline *TP53* mutation. Germline mutations in *TP53* have also been reported in adult ACC[64,65].

Beckwith-Wiedemann Syndrome (BWS): It is a cancer-susceptibility syndrome characterized by prenatal overgrowth, congenital malformations and susceptibility to childhood tumours, particularly adrenocortical carcinoma, hepatoblastoma, neuroblastoma and Wilms tumour. Approximately 15% of the BWS cases are inherited in an autosomal dominant manner and the rest are caused by de novo aberrations [66]. Different genetic and epigenetic modifications at the 11p15 locus deregulates imprinted genes at this locus and are found to be responsible for the manifestation of the disease in approximately 70% of the cases[67]. There are two domains in 11p15 involved, one domain with the *IGF2* and *H19* genes and another with the *CDKN1C* and *KCNQ1* genes. Alterations in the first domain are found in approximately 5%, whereas the alterations in the later are found in approximately 50% of BWS patients[68].

Multiple endocrine neoplasia type I (MEN1): The syndrome is caused due to a germline mutation in the *MEN1* gene encoding menin. MEN1 is an au-

tosomal dominant syndrome and predisposes the carrier to parathyroid, pancreatic and pituitary tumours. Adrenocortical hyperplasia and tumours are observed in 20-40% of MEN1 patients and usually occur as non-hormone producing adrenal adenomas [69]; however, cortisol and aldosterone producing cases tumours have also been observed [70,71]. Adrenocortical carcinoma is a rare manifestation (~1%) in MEN1 patients[71].

Familial adenomatous polyposis (FAP): It is an autosomal dominantly transmitted disorder with 100% penetrance and causes adenomatous polyposis in the colon and rectum. It is caused by an inactivating mutation in the adenomatous polyposis coli (*APC*) gene. The syndrome also predisposes the patients to osteomas, thyroid and adrenocortical tumours[72,73]. Adrenocortical adenoma occurs in about 7-13% of FAP patients[72,74]. While most adrenal tumours are hormonally silent, a few with cortisol or aldosterone overproduction have been reported[75,76]. Adrenocortical carcinoma occurs rarely in patients with FAP[72,73].

Carney Complex (CNC): It is a multiple tumour syndrome transmitted in an autosomal dominant manner. The syndrome is characterized by spotty skin pigmentation, cardiac myxomas, and different endocrine and non-endocrine tumours. Primary pigmented nodular adrenocortical disease (PPNAD), a subtype of Micronodular Adrenal Hyperplasia (MiAH), is observed in 60% of patients with CNC. Germline mutations in *PRKAR1A*, a regulatory subunit of PKA, are responsible for the manifestation of the disease in 40-50% of the CNC families[49,77,78]. The mutation causes inactivation of the *PRKAR1A* leading to activated cAMP signalling and hence increased cortisol production.

McCune Albright Syndrome (MAS): It is a rare syndrome and causes fibrous dysplasia of bone, café-au-lait skin pigmentation and endocrine dysfunction. Cushing's syndrome is also observed in a minority of the patients (children) with MAS. It is caused by sporadic, post-zygotic activating mutations in the *GNAS* gene[50].

Adrenocortical carcinomas are also found in other familial syndrome such as the Lynch syndrome (3%)[79], Neurofibromatosis type 1 (NF1) and carney complex[80,81] albeit rare.

Adrenocortical adenoma

One of the common manifestations of hormone-producing adrenocortical adenoma is Cushing's syndrome, occurring in patients due to glucocorticoid excess. Hypercortisolism is also observed in approximately 50% of the cases of malignant adrenocortical tumours[54,82]. Benign adrenal Cushing's syndrome generally causes due to unilateral tumour, but may be bilateral as in primary bilateral macronodular adrenal hyperplasia (PBMAH) and mi-

cronodular adrenocortical hyperplasia (MiAH); however, the latter two account for only 10% of the cases[83].

Genetics of cortisol producing adenoma

The cAMP signalling pathway is the most frequently altered pathway known in cortisol-producing adenomas (CPAs). Mutations in *PRKACA* have been found in 35-69% of CPAs[53,84-88]. In addition to recurrent mutation occurring at hotspot location p.Leu206, several other alterations activating PRKACA have been found in these tumours[87,89]. The p.Leu206 mutation alters the conserved area where the regulatory subunit PRKAR1A binds to PRKACA. Gain of this mutation enables PRKACA to achieve a constitutively active state[90]. The other member of the pathway, *GNAS*, has also been found to be mutated in adrenocortical tumours. The mutation is often found to alter p.Arg201 and p.Glu227 residues. The mutation causes inhibition of the GTPase catalytic ability of a G-protein leading to excessive production of cAMP and activation of the downstream pathway[91,92].

Wnt/β-catenin signalling is yet another frequently altered pathway in adrenocortical tumours. Hotspot mutations in β-catenin altering the serine/threonine residues and deletion of exon 3 harbouring these residues are observed in different types of adrenocortical adenomas. The mutation leads to accumulation of active β-catenin and illicit transactivation of the genes regulated by the Wnt/β-catenin signalling pathway. ACAs with *CTNNB1* mutations show nuclear and cytoplasmic accumulation of β-catenin in most cases[42]. However many tumours without *CTNNB1* mutation also exhibit such phenomenon suggesting involvement of yet unknown, other mechanisms, causing activation of this pathway [42,93].

Disease presentation, diagnosis and prognosis

Chronic exposure to excess glucocorticoids in Cushing's syndrome leads to development of severe clinical symptoms such as truncal obesity, moon face, buffalo hump, purple striae etc[94]. Hypercortisolism observed in a patient could be of adrenal (ACTH-independent) or non-adrenal (ACTH-dependent) aetiology; the latter accounts for 80% of the total[83]. ACTH-dependent hypercortisolism occur mostly due to an ACTH producing pituitary adenoma (Cushing's disease) or ectopic ACTH secretion from various neuroendocrine neoplasms. The symptoms of the syndrome such as obesity, diabetes, hypertension, depression overlaps with symptoms of many other diseases. Hence, hypercortisolism should be confirmed by biochemical testing such as 24-h urinary free cortisol, late-night salivary cortisol or an 1 mg overnight dexamethasone suppression test [95]. Management of the hypercortisolism depends on the tumour origin; therefore confirmation of hypercortisolism thereupon requires determination of the tumour origin. Combination of ACTH meas-

urements, CRH stimulation and high dose dexamethasone tests together with imaging could be used to rule out the suspects.

Molecular- clinical correlation and therapy

In general, CPA could occur at any age, though with a female predominance. Patients with tumours harbouring *PRKACA* mutations were reported to have more severe phenotype and smaller tumours[86,87] in comparison to those without *PRKACA* mutation. There are conflicting results regarding the correlation of age and *PRKACA* mutation[53,84].

The common choice for the management of adrenal Cushing's syndrome is surgical removal of the tumour in both unilateral and bilateral cases. In cases where surgical removal of the tumours is not possible or when the remission is not achieved after the surgery, palliative management by steroidogenesis inhibiting drugs such as metyrapone, ketoconazole are opted[96].

Adrenocortical Carcinoma

Adrenocortical cancer is a rare malignancy with annual incidence of 1-2 persons per million[97]. Age of diagnosis in ACC patients follow a bimodal curve with a first peak at the first decade and a second peak extending from the fifth to the seventh decades of life[64]. Patients with ACC come into attention due to symptoms caused by hormonal excess and/or abdominal mass and seldom incidentally.

Molecular signature of ACC

The knowledge of genetics of ACC tumours spawned from the knowledge of familial cases. The majority (> 90%) of childhood adrenocortical carcinoma is accounted for by the Li-Fraumeni syndrome and other *TP53* germline mutations[64,98]. Sporadic *TP53* mutations are found in approximately 20% of the adult ACCs[54,99]. Germline *TP53* mutations in adult ACCs have been reported as well[65]. High expression of IGF2 associated with alterations in chromosome band 11p15 is observed in BWS. In sporadic cases of ACCs, loss of heterozygosity at 11p15 and high expression of IGF2 are present in approximately 85% of the cases[99,100]. Mouse model studies show that IGF2 overexpression in the adrenal does not lead to formation of tumour or malignancy but has a slight promoting effect on tumour progression in β-catenin induced tumours[101,102].

Frequent activating mutations in *CTNNB1* (~15%) [54,99] and homozygous deletions of the *ZNRF3* gene makes the Wnt signalling pathway one of the most frequently altered pathway (~40%) in ACCs [54,99,103]. However, the role

of *ZNRF3* deletion in ACC tumourigenesis is yet to be explored. Although hot spot mutations in exon 3 of *CTNNB1* accounts for only 15% of ACC, nuclear accumulation of β-catenin is found in up to 40% of these tumours [93,104].

The second most frequently altered pathway in ACCs is the p53/Rb signalling pathway (~40%) with frequent mutations and alterations occurring in *TP53* and *CDKN2A*[54,99]. Mutations and deletions in *MDM-2* and *RB1* are also observed in ACCs albeit less frequently. *TP53* and *CTNNB1* mutation and nuclear accumulation of β-catenin have also been associated with poor outcome in ACC patients[93,104]. In addition, mutations leading to increased telomere length and telomerase activity have also been reported. Mutations in *ATRX* and *DAXX*, known to cause alternative lengthening of telomeres (ALT) in tumour cells[105], have been found in approximately 10% of ACCs. Recently, *TERT* promoter mutation causing increased expression of *TERT* have been reported in about 10% of ACCs[106,107].

The most frequent copy number variations (CNVs) observed in ACCs are gain of chromosomes 4, 5, 7, 8, 12, 19, 20, loss of chromosome 1p, 22 and loss of heterozygosity of chromosomes 1, 2, 3, 6, 11, 13, 17, 18 and 22[99]. The *TERT* (5p15.33) and *CDK4* (12q14) gene loci are the most frequently amplified and *CDKN2A*, *RB1*, *ZNRF3* gene loci are the most frequently homozygously deleted regions in ACCs. Frequent homozygous deletions have been observed in a few other loci containing genes such as *LINC00290/TENM3* and *LSAMP*. Frequent deletion of *LINC00290* in pan-cancer analysis and *LSAMP* in osteosarcoma has been reported before[108,109]. *LINC00290* encodes a long non-coding RNA, the importance of which is being increasingly recognized in tumourigenesis and cancer[110]. However, the role of these frequent aberrations in ACC oncogenesis is not known yet.

Disease presentation

About 60% of the patients present with clinical signs and symptoms of hormonal excess. A majority of these patients with symptomatic functional tumours present with glucocorticoids and/or androgen excess, and ~5% of them have excess production of estrogen and/or mineralocorticoids[111]. Thorough analyses of adrenal steroids have shown increased hormone production in more than 80% of the ACC patients[112]. In addition, urinary steroid profiling has revealed that almost all ACC patient present with increased excretion of steroid metabolites [113]. This emphasizes the fact that even though the patients seem to be hormonally asymptomatic, ACC is almost always accompanied by deregulated steroidogenesis. The recommended imaging and hormonal work-up for diagnosis and confirmation of ACC are listed in the table below.

Table 1. European Network for the Study of Adrenal Tumours (ENSAT) guidelines for hormonal work-up for diagnosis of adrenocortical carcinomas.

Imaging:	Hormonal	
CT/MRI abdomen CT thorax Bone scintigraphy FDG-PET (optional)	Glucocorticoid excess	- Dexamethasone suppression test - Free urinary cortisol - basal serum cortisol - basal ACTH
	Sexual steroids and steroid precursors	- DHEA-S - 17-OH-progesterone - Androstenedione - Testesterone - 17-beta-estradiol (in men or postmenopausal woman)
	Meneralocorticoid excess	- Potassium - Aldosterone/renin ratio (if with arterial hypertension and/or hypokalemia)
	Exclusion of Pheochromocytomas	- Catecholamine or metanephrine excretion - meta and normetanephrine

Staging and Prognosis

Prognosis of ACC is determined by staging of the tumour. Tumour staging by e.g. ENSAT use Tumour-Node-Metastasis (TNM) to group the tumours into different stages[114].

Table 2. Tumour staging according to ENSAT

Stage	ENSAT system
I	$T_1N_0M_0$
II	$T_2N_0M_0$
III	$T_{1-2}N_1M_0$ / $T_{3-4}N_{0-1}$ M_0
IV	$T_{1-4}N_{0-1}M_1$

T1: tumour ≤5 cm; T2: tumour >5 cm; T3: tumour infiltration into surrounding tissue; T4: tumour invasion into adjacent organs or venous tumour thrombosis; N_0: no positive lymph nodes; N_1: positive lymph node(s); M_0: no distant metastasis; M_1: distant metastasis.

Tumours in stage I-II, III and IV are termed as localized, locally advanced and advanced respectively. The overall survival for localized disease with stage I-II tumours is quite similar. Five-year survival is observed to be 60-80% for stage I-II tumours and decreases substantially from stage III to stage IV [115,116]. Radical surgery is the first choice of treatment for local and occasionally for locally advanced ACC. Resection, depending of if it is complete, incomplete microscopic, incomplete macroscopic or unknown are termed

R0, R1, R2 and Rx respectively. R1, R2 and Rx are often associated with worst overall survival. Though surgery is the only choice of curative therapy, there is a high local and distant recurrence rate (up to 80% for incomplete resection) in operated patients[112].

The risk of recurrence is determined based on various factors such as resection status, tumours size and mitotic rate. Lymphadenectomy at the time of initial resection is also shown to decrease the recurrence rate and mortality[117]. Several studies have determined Ki-67 as a promising prognostic factor for recurrence risk assessment[118]. However manual Ki-67 indexing is prone to human error. Urinary steroid profiling also seems to be a promising predicting factor for assessing recurrence risk and is also claimed to be useful in differentiating adenoma from carcinomas[113]. Patients prescribed with adjuvant therapy reportedly have better outcome[119,120]. In the cases of incomplete resection, combined treatment with mitotane and radiotherapy is also recommended[121]. Mitotane monotherapy or mitotane combined with Etoposide, Doxorubicin, and Cisplatin (EDP-M) are the first-line therapies in irresectable/advanced ACC. EDP-M is the most efficient therapy to date; with response rates of 23% and progression free survival of 5 months; however better treatment options are needed[122]. In advanced ACC, there are heterogeneous populations; some responds to the drug and have an indolent course of the disease whereas some doesn't respond and the disease progresses aggressively leading to short survival. Predicting factors for the outcome in these groups of patient are warranted. A recent study from ENSAT has proposed further classification of tumour staging[123]. According to the study, a stage III tumour with N1 status is suggested to be shifted to stage IV and the tumours in stage IV are suggested to be further classified as stage IVa, IVb, and IVc. The new tumour staging along with the GRAS (grading, R status, age and symptoms) parameters have shown to improve the prognostic classification.

Molecular and clinical correlations

The transcriptional signatures of the tumours from good and poor prognosis groups of patient are significantly different[54,99,124], where the tumours with bad prognosis shows increased expressions of genes involved in proliferation. Further, ACC tumours show a promoter CpG island hypermethylator phenotype in comparison to adenomas[125,126]. ACC tumours can be divided into three different groups based on the degree of CpG island methylation phenotype (CIMP) status: CIMP-high, CIMP-low and non-CIMP. CIMP-high and CIMP-low correlates with poor overall survival in comparison to the non-CIMP[99,126]. Similarly, different groups of ACC show different miRNA expression signatures. Studies show that tumours with poor prognosis display high expression of certain miRNAs such as miR-483-5p, miR-483-3p, miR-210 and low expressions of miR-195, miR-335 [99,127-129], whereas

tumours with good prognosis demonstrate high expression of miRNA-506-514 cluster and downregulation of the DLK1-MEG3 cluster miRNAs [99,127]. In summary, tumours with bad prognosis show different transcriptome profile, have a hypermethylation phenotype and most of them also harbour alteration in known driver genes such as *CTNNB1*, *TP53* and *ZNRF3*[99], in comparison to the group with good prognosis. In a recent TCGA study, clustering of tumours based on CNV gave three distinct clusters; silent, chromosomal and noisy. The noisy group of tumours with extensive chromosomal breaks were also found to be associated with shorter survival in this study[54].

Targeted therapy

Tyrosine kinase inhibitors such as erlotinib [130] gefitinib [131] targeting EGFR and imatinib targeting PDGFR-R [132] have been studied for their efficacy in ACC treatment. These therapies however produced minimal to no effects in the patients. Failure of these drugs is probably due to their specificity to certain kind of receptors, but there are large spectra of receptors with structural similarity activating compensatory signalling pathway [133].

Monoclonal antibodies such as bevacizumab in combination with Capecitabine [134] and the multi-kinase inhibitors sorafenib[135] and sunitinib [136] have also been used to target VEGFR. However, they also lacked any benefit. IGF2 overexpression and subsequent activation of mTOR pathway are recurrently observed in ACCs; hence several trials for targeting IGF1R and the downstream mTOR pathway have been conducted. Drugs like everolimus targeting the mTOR pathway [137], monoclonal antibodies such as cixutumumab [138] and figitumumab [139] targeting IGF1R have been studied in ACC patients but they either lacked objective response or had no effect. However, combination of cixutumumab and temsirolimus (mTOR inhibitor) treatment appeared promising in patients with metastatic ACC, and achieved stable disease in 42% of the patients for more than 6 months (range=6-21months) [140]. The latest study phase III study on listinib (IGF2R inhibitor) showed partial response in 3% of the patient with low grade ACC but no improvement in overall survival[141].

The observations made with trials of these drugs could have been confounded by the use of mitotane. Most of the patients treated with targeted therapies either had been treated with mitotane or are given therapy in conjunction to mitotane, which makes it difficult to analyse the actual potential of the tested drug. A recent study by Zhuang et al 2015 reported a patient who showed recession in tumour size after withdrawing mitotane from sunitinib plus mitotane treatment[142].

Recent studies identified sterol-O-Acetyl transferase 1 (SOAT1) as a new therapeutically targetable molecule in ACCs[143,144]. The ATR-101 molecule, capable of inhibiting SOAT1 leading to accumulation of cytotoxic free cholesterol is currently under a phase I clinical trial.

WNT/β-catenin and TP53/Rb1 signalling are the most altered pathways occurring in cancers including ACC. But they are also the most widely used pathways in human body, which makes the development of drugs targeting these pathways tricky. Several approaches have been tried for targeting mutant TP53 or restoring wild type function of TP53 but all these studies has not yet yielded any clinically approved drug[145]. In the case of the WNT/β-catenin pathway, several inhibitors targeting molecules upstream of the pathway are being tested[146-148]. However the efficacy of such inhibitors in mutated β-catenin driven tumours is doubtful. Drugs targeting downstream of this pathway such as CWP232291 enhancing degradation of β-catenin and PRI724 interrupting interaction of β-catenin and its transcriptional partners seems promising[147,149]. Adrenal specific active β-catenin and a recently developed TP53/Rb inhibited mouse model could be helpful in testing potential targeted drugs[44,150].

Materials and Methods

Patients and tumour samples (Papers I-V):

All the ACC, CPA and aldosterone producing adenoma (APA) tumours included in the study were obtained from the patients operated upon routine established diagnosis. Pieces of the tumours were either snap frozen in liquid nitrogen and stored at -70^{o}C or were formalin fixed for 24 hours and used for preparation of paraffin embedded tissues.

Patient charts were scrutinized to establish survival data. Cortisol excess was diagnosed by clinical and biochemical evaluation, including 24- hour urinary cortisol and/or midnight cortisol and early-morning plasma adrenocorticotropic hormone (ACTH), and in some cases 1 mg dexamethasone suppression test were performed. Other hormone excesses were determined based on clinical signs and appropriate blood hormone assays according to routine diagnostic procedures. Degree of aggressiveness was retrospectively evaluated by the ENSAT stage as described before[114].

Ethical approval from local ethics committees and informed consent from all the included patients (guardians if younger than 18 years old) were obtained prior to the study.

DNA/RNA extraction (Papers I-V):

Frozen tumours were cryosectioned and hematoxylin-eosin stained to evaluate the tumour cell content. Tumours were macro-dissected (when necessary and feasible) to achieve maximum content of neoplastic cells. DNA and RNA were prepared from cryosectioned tumour samples and corresponding normal tissues using Allprep DNA and RNA kit (Qiagen, Hilden, Germany). Blood DNA was prepared using DNEasy Blood and tissue kit (Qiagen, Hilden, Germany). DNA extraction from paraffin embedded tissue (Paper II) was performed using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany).The concentration and quality of obtained DNA/RNA was analysed using Nanodrop (ThermoFisher scientific, MA, USA). The integrity of RNA was analysed by running the samples on agarose gel.

PCR and Sanger sequencing (Papers I-V):

Specific primers for DNA and cDNA were used to amplify the regions of interest by PCR. The amplified products were then sequenced using automated Sanger sequencing at Beckman Coulter genomics, UK. The sequence chromatograms were analysed using Codoncode Aligner 3.7.1 (CodonCode Corporation, MA, US). Mutations were confirmed in two independent PCR reactions. Lack of mutations in heterogeneous samples in study III and V were also confirmed by sequencing two independent PCR reactions.

SNP array analysis (Papers I-V):

750ng of double stranded tumour DNA was subjected to SNP array analysis using Illumina HumanOmniExpressExome-8v (Illumina, USA) at the Science for Life core facility (Uppsala University, Uppsala, Sweden). The raw data was generated from Illumina bead studio and processed and analysed using Nexus copy number variation 7.5 software (Biodiscovery, USA) using SNP-FASST2 segmentation algorithm (Studies I-V).

ASCAT analyses were performed on the SNP array data to determine purity and ploidy of the samples in studies II-V[151]. The diploid regions were manually assigned based on ASCAT analysis. CNV calls were made based on logR ratio and B allele frequency (BAF) in studies I-V). For the analysis of focal amplifications and deletions the SNP array data was first processed using circular binary segmentation algorithm in Nexus 7.0 and subsequently analysed using GISTIC tool[152].

Expression array and cluster analysis (Paper I):

cDNA from 450ng of RNA were assayed using Illumina HumanHT-12 v4 Expression Bead Chip (Illumina, U.S.) using a direct hybridization assay at the Genomics Core Facility (University of Bergen, Bergen, Norway). Raw IDAT-files were imported into Illumina GenomeStudio V2011.1 (Illumina, San Diego, U.S.). Expression data was normalized using the cubic spline method and background removal was performed. Missing probes were excluded from the analysis. Differential expression was determined using the Illumina Custom error model which takes into account both biological variation and technical error. The False Discovery Rate (Benjamini-Hochberg) method was used to compensate for multiple hypothesis testing.

For cluster analysis, probe expression values were exported from GenomeStudio and further processed using the R statistical environment and custom Python scripts. Probes with a p-value of 0.05 or more for detection in at least 20% of samples were considered to be of poor quality and excluded

from further analysis. Probes for genes on the sex chromosomes were also excluded. Hierarchical clustering was performed in R using the Euclidean distance metric. Principal Components Analysis was performed.

Gene ontology (Paper I):

Gene Ontology enrichment analysis was performed using PANTHER Overrepresentation Test (release 20150430) using the GO Ontology database (released 2015-06-06) on differentially expressed genes. Bonferroni correction was performed and results with corrected enrichment p-values less than 0.05 were considered statistically significant.

cDNA synthesis: and qPCR (Papers I, III, IV):

Quality controlled RNA was used to prepare cDNA using RevertAid First strand cDNA Synthesis kit (ThermoFisher scientific, MA, USA). SsoAdvanced SYBR Green Supermix (Life Science, United States) was used to perform all the reactions on a CFX96 Real Time system (Bio-Rad laboratories). Beta actin was used as a reference. The samples were run in triplicates and ΔΔCT calculation was used to compare the expression[153].

Statistics (Papers I, III-V):

The Mann-Whitney U-test for comparative analysis of mRNA expression and numeric clinical data was performed in Prism. For categorical data χ^2 test was performed. Survival analysis (study IV and V) was performed using survival curve created by Kaplan-Meier estimate with Log-rank test using GraphPad Prism 7 (GraphPad Software, CA, USA). Cox proportional hazards regression method was used to determine the univariate hazard ratios of clinical criteria for overall survival. The Cox proportional hazards regression method was also used to determine the multivariate hazard ratios for the significant univariate predictors for overall survival. The analysis was performed using the SPSS software.

Mutagenesis (Paper II):

TrueORF gold expression validated cDNA clones in pCMV6-Entry vector were purchased from Origene Technologies MD, USA. Site directed mutagenesis was performed using GENEART site-directed mutagenesis system kit (Cat#A13282, Thermofisher scientific, MA, USA) according to the man-

ufacturers protocol. The specificity of the mutagenesis was verified by sequencing the entire coding region of the vectors by automated Sanger sequencing (Beckman coulter Genomics, UK).

Cell culture and transfection studies (Paper II):

Human embryonic kidney 293T (HEK293T) cells were cultured in DMEM complemented with 10% FBS. The cells were transfected for 24hr using FuGENE 6 transfection reagent in 3:1 ratio to DNA (Cat#TM350, Promega Corporation, WI, USA).

Western blot (Papers II, IV):

Samples were lysed using cytobuster (Cat#71009, Novagen) supplemented with protease inhibitor cocktail (Cat#04693124001, Roche, Basel, Switzerland). Protein lysates were denatured at 95°C with Laemmli buffer (Cat#161-0737, Bio-Rad laboratories, CA, USA) supplemented with β-mecarptoethanol for 10 mins. Samples were separated on SDS page gel and transferred to PVDF membrane. Western blotting was performed as described before [154] or by using manufacturer recommended protocols.

In silico analysis (Papers II-V):

The impact of the mutations on the presumed function were analysed using Polyphen-2[155], PROVEAN[156] and SIFT [157] in paper II, IV and V). In addition the PredictSNP[1] tool which combines six well established prediction tools (MAPP, PhD-SNP, Polyphen-1, Polyphen-2, SIFT and SNAP) was used in study II to analyse the impact of the mutations[158]. In study III in silico analysis for the putative transcription factors binding site (TFBS) was performed using PROMO[159]. Chimera software [160] was used to visualize and analyse the 3D structure of PKA RIIb tetrameric holoenzyme, 3TNP, obtained from Protein Data Bank (PDB)[161]. Clustal omega, was used in study II and IV to align and compare the *Homo sapiens* protein sequence with several other species such as *Mus musculus*, *Xenopus tropicalis*, *Danio rerio*, *Saccharomyces cerevisiae*[162,163].

Multiregional Sampling (Papers III, V):

Multiregional sampling was performed as described before[164]. Briefly, tumours obtained for research were dissected into different pieces and stored

in separate vials. The specimens were sampled from different regions of the tumours. In cases where the multiregional samplings were performed on morphologically different parts of the tumours or on a large tumour from a single initial sampling, the procedure was documented with pictures. Temporal multiregional sampling was generally performed for the cases where the recurrent tumours or metastases were available.

Immunohistochemistry (Paper IV, V):

For paraffin embedded tissue, 4um thick tissue sections were used. The following protocols were used.
The tissue section slides were
1) dried at 60^{o}C before use.
2) treated with xylene, 99.9% ethanol, 90% ethanol, 70% ethanol and dH2O in order to remove paraffin and rehydrate the tissue sections.
3) blocked for endogenous peroxidase by treating the sections with hydrogen peroxide (H_2O_2) for 15 minutes
4) heat treated using citric buffer for antigen retrieval and cooled down in room temperature for 20 minutes
5) incubated with normal horse/goat serum diluted in BSA-PBS buffer (bovine serum albumin and PBS buffer) for 30 minutes for blocking
6) incubated with primary antibody diluted in BSA-PBS buffer
7) incubated with secondary antibody diluted in BSA-PBS buffer
8) incubated with avidin/biotin complexing system
9) treated 3,3'-diaminobenzidine (DAB) substrate for chromogenic detection
10) incubated in haematoxylin for nucleus staining
(The slides were washed with PBS between each steps mentioned above)
11) dehydrated by treating them with 70% ethanol, 90%ethanol, 99.9% ethanol and xylene.
For immunohistochemistry of frozen tissue, 5um thick cryosections were generated and were
1) acetone fixed.
2) blocked for endogenous peroxidase by treating the sections with hydrogen peroxide (H_2O_2) for 15
3) blocked with avidin biotin blocking kit
Followed by steps 5, 6, 7, 8, 9 and10 as mentioned above for paraffin sections.

Phylogenetic analysis based on Copy number variation (Paper V):

In order to reduce the complexity of the copy number variation (CNV) profile due to fragmented CNV calls, the adjacent CNVs with similar logR and BAF profile located in the same chromosome arm were manually merged. CNV calls were not modified in samples with complex/chromothriptic CNV patterns; instead, they were assigned as a single event. In multiregional samples where different regions harboured different ploidy levels, whole genome copy number loss or whole genome copy number gain were considered as a separate single event. Phylogenetic analyses of the multiregional samples were performed based on CNV events in different regions. Binary tables were generated for heterogeneous samples with each event as a row and each multiregional sample as column for phylogenetic analysis as explained before[164]. These were then used for phylogenetic tree construction using MEGA7[165] employing the maximum likelihood algorithm. Obtained trees were then manually redrawn, keeping length of the trunk and branches proportional to the number of events. The frequent focal copy number aberrations (CNAs) in ACCs such as homozygous deletions in *ZNRF3*, *CDKN2A*, *CDKN2B* and somatic mutations observed in investigated genes were then mapped in the tree. Observed mutations in the multiregions were added post hoc to the tree.

Summary of included papers

The studies included in this thesis focused on analysing the genetic and genomic alterations occurring in adrenocortical tumours, especially ACCs and CPAs. The functional impact of these aberrations and possible association with clinical phenotypes and outcome were also analysed. In addition, intratumour heterogeneity occurring in ACCs was investigated.

Paper I. Integrated Molecular Characterization of Benign Cortisol Producing Adenomas Defines Two Distinct Clusters.

Mutations in *CTNNB1*, *GNAS* and *PRKACA* are observed in up to 80% of CPAs[53,84,85]. Analyses of mutations in these genes were performed in a cohort of 34 CPAs resected at the Uppsala University Hospital.

Median age at operation, tumour size and serum cortisol level of the cohort was 57.5 years, 31.5 mm and 452.5 nmol/L respectively. In addition, female predominance was observed (male: female= 1/10.33). Somatic mutations in *CTNNB1*, *GNAS* and *PRKACA* were observed in 15%, 18% and 25% of the cohort respectively. All the mutations were located at hotspot loci i.e. p.S45, p.R201 and p.L206 residues in *CTNNB1*, *GNAS* and *PRKACA* respectively. Interestingly, patients with tumours harbouring *PRKACA* mutations were significantly younger in comparison to the rest of the cohort.

Unsupervised hierarchical clustering of the tumours based on the transcriptome signature segregated tumours with *PRKACA* and *GNAS* mutation (Cluster1) from those with *CTNNB1* mutation or unknown mutation status (Cluster2). Gene ontology analysis on significantly differentially expressed genes revealed over expression of genes involved in steroidogenesis and metabolism in Cluster1 tumours. Increased expression of genes involved in steroidogenesis has been reported before in *PRKACA* mutated tumours[85]. Analysis of clinical characteristics revealed that Cluster2 tumours were significantly larger than Cluster1 tumours. No significant differences with respect to other clinical characteristics between two clusters were observed.

Copy number variation analysis showed frequent gain of chromosome arm 9q in the cohort. This event was exclusively present in Cluster2 tumours. mRNA Expression analysis revealed significantly high expression of

genes located at 9q in the group of tumours harbouring the gain. A gene dosage effect was observed for SF1, importance of which is well known in steroidogenesis and proliferation of adrenal cells. Presence of multiple copies of the gene encoding SF1 (NR5A1) has shown to cause hyperplasia and tumourigenesis in mice adrenals[166].

In conclusion, molecular classification of CPAs segregated two clusters with distinct clinical presentation where Cluster 1 tumours exhibit a hyper activated steroidogenesis pathway, which might present a therapeutical target.

Paper II. Identification and Characterization of Novel *PRKACA* Mutations in Adrenocortical Tumours.

Frequent *PRKACA* mutations in CPAs occur in residue p.L206R. Few other infrequent mutations have also been observed in CPAs without p.L206 mutation[89]. *PRKACA* mutations have not been described in other types of adrenocortical tumours[167]. In this study we have performed mutational analysis in all the coding exon of *PRKACA* in a large cohort of ACCs and a cohort of CPAs lacking p.L206R mutation.

A 15bp deletion (c.731_745del, p.Pro244_Glu249delinsGln) in one adenoma, a 3bp deletion (c.877_879del, p.Lys293del) as well as a missense mutation (c.305C>T, p.Pro102Leu) in two carcinoma samples were found. The mutations c.877_879del and c.305C>T have not been described before while c.731_745del was recently reported in a CPA[89].

In silico analysis predicted all three mutations to have damaging effect on the protein. 3D analysis revealed that all three mutation lie at or in near proximity to the critical residues of PRKACA. Molecular analysis on the tumours showed increased accumulation and phosphorylation of PRKACA and its downstream serine/threonine targets. Overexpression of mutant *PRKACA* and wildtype *PRKAR1A* in HEK293T caused increased accumulation and phosphorylation of CREB and serine/threonine targets of PRKACA, hence showing the activating impact of the mutation on *PRKACA*. Both ACC patients with tumour harbouring *PRKACA* mutation presented with excess of cortisol. One patient presented with overt Cushing's syndrome whereas the other patient presented with subclinical Cushing's syndrome. Both of the ACC patients were operated for recurrence. Mutational analysis on recurrent tumours revealed presence of the respective mutations. In summary, we found novel mutations occurring outside the hotspot locus of PRKACA in CPAs and described that activating mutations in *PRKACA* occur in ACC which has not been observed before.

Paper III. Intratumoural Heterogeneity of *TERT* Promoter Mutations in Adrenocortical Carcinoma

TERT promoter mutations leading to increased TERT expression and aberrant telomere lengthening were found in different types of malignancies including adrenocortical carcinomas (ACCs)[106,107]. In this study we have analysed TERT promoter mutation in a large cohort of adrenocortical tumours consisting of ACCs (n=61), CPAs (n=35) and APAs (n=80).

We found the previously described mutation c.124C>T (C228T) in five ACCs whereas the rest of the tumours were devoid of mutations. mRNA expression analysis showed high TERT expression in the tumours harbouring the mutation while none of the analysed benign tumours showed detectable level of expression. ACC tumours without *TERT* promoter mutations showed a wide range of TERT expression. High expression of TERT in ACC tumours without mutations has been observed before[106]. Such high expressions of TERT in ACCs could be due to frequent amplification of the *TERT* locus observed in ACCs[54,99,103].

We performed multiregional sampling (n=117) on 28 ACCs including the five ACCs harbouring the mutation. Multiregional analysis revealed intratumour heterogeneity with respect to *TERT* promoter mutations. Analysis of TERT expression in multiregional samples revealed higher expression in the multiregions harbouring the mutations in comparison to non-mutated region of the respective samples.

Paper IV. Comprehensive analysis of *CTNNB1* in adrenocortical carcinomas: Identification of novel mutations and correlation to survival.

Wnt/β-catenin signalling pathway is the most altered pathway in ACC[54,99]. The aim of this study was to analyse the mutational status of *CTNNB1* and expression of β-catenin in a large cohort of ACCs. Analysis of frequently mutated region of *CTNNB1*, exon 3 and 5, was performed. Expression analysis of β-catenin was performed using immunohistochemistry and western blot.

Missense mutations were observed in 12% of the cohort. All the mutations were located in exon 3 whereas exon 5 was devoid of any mutations. In addition to frequent missense mutation, occasional deletion of exon 3 has also been reported in adrenocortical tumours[42]. cDNA specific PCR and sequencing analysis revealed expression of truncated CTNNB1 mRNA with deletion of exon 2 and 3 in 4 ACC tumours. Utilizing whole genome sequencing we identified exact location of the deletion. Such deletions have not been reported in adrenocortical tumours; however few studies in mela-

noma tumours and cell lines have reported such deletions before[168,169]. Analyses of DNA sequences at the deletion start and end site revealed presence of microhomologous and palindromic sequence at deletion junction.

Regardless of deletion of the transcription start site, in frame expression of truncated β-catenin was observed by western blot. The tumours harbouring the deletion showed significantly higher expression of CyclinD1, AXIN2 and ZNRF3 in comparison to tumours without *CTNNB1* mutations and similar to ones harbouring *CTNNB1* hotspot mutations which corroborate that the truncated β-catenin is functional and activates downstream pathway in these tumours. The observed deletion in β-Catenin cause removal of the first 87 amino acids given that the first in frame start site in exon 4 is utilized. Such deletion of N terminal end of *CTNNB1* has shown to mimic activated Wnt/β-catenin signalling *in vitro* and in animal models[170-172].

Hotspot mutations in *CTNNB1* occur with the frequency of about 15% in ACCs. However activated Wnt/β-catenin signalling pathway inferred through nuclear accumulation of β-catenin could be observed in much higher proportion of the tumours[39]. Immunohistochemical analysis of β-catenin in a cohort of primary tumours showed nuclear accumulation of β-catenin in 51% of the cohort. All six tumours harbouring the missense mutations and three out of four tumour harbouring deletion showed nuclear accumulation of β-catenin. Accumulation of β-catenin with non-phosphorylated serine threonine residues is an indicative of an active Wnt/β-catenin signalling pathway. Analysis of expression of active β-catenin utilizing western blot showed detectable levels of the protein in 62% of the cohort. Correlative analysis of presence of nuclear and active β-catenin revealed discrepancy in 30% of the analysed tumours. Presence of *CTNNB1* mutations and expression of nuclear β-catenin were associated with significantly lower overall survival.

Paper V. Multidimensional Genetic Analysis of Adrenocortical Cancers

In this study we have investigated the mutational status of known driver genes in a large cohort of 61 ACC tumours from 52 patients. We found frequent mutations in *TP53*, *CTNNB1*, *TERT* promoter, *DAXX*, *MEN1*, *PRKACA*, APC, *CDKN2A* and *ZNRF3* in a total of 65% of the cohort. SNP array analysis revealed frequent gain of chromosome arms 5, 7, 12, 16, 19, 20 and loss of chromosome 22. GISTIC analysis revealed significant amplification at *TERT* locus and significant deletions at *ZNRF3* locus ($q < 0.01$). Survival analysis of group of tumours harbouring *CTNNB1* and *ZNRF3* alterations showed significantly lower overall survival rate. However there were no significant differences in overall survival for the patients with tumours harbouring mutations in any one of the other investigated genes. Uni-

variate cox regression analysis of age, cortisol production, ENSAT staging, gender and tumour-size for overall survival showed significant prognostic value of ENSAT staging.

We employed a multiregional sampling approach to study the possible heterogeneity occurring in ACC tumours. We performed multiregional sampling (n=68) in tumours originating from 21 patients. The multiregional samples were subjected to mutational analysis as well as SNP array analysis. Multiregional analysis of the tumours revealed heterogeneity in seven cases with respect to mutation status of driver genes *TP53, CTNNB1, TERT, PRKACA, CDKN2A*, and homozygous deletions in *ZNRF3* and *CDKN2A/2B*. The multiregions not only displayed intra-tumour heterogeneity in CNVs but also in ploidy levels. The analyses revealed that the homozygous deletions occurring in ACCs such as in *ZNRF3* and *CDKN2A* loci, occur late in tumour evolution. The heterogeneity observed in driver genes *CTNNB1* and *TP53* asserts that the mutations occurring in these genes could be a passenger event in some tumours, which raises a possible question concerning the "real" driver genes in these cases.

Single sampling of the tumours provides only a snapshot of ACC genetics and genomics. This study unveils intra-tumour heterogeneity occurring in ACCs in terms of CNVs, ploidy levels, mutational status of the driver genes and underscores the importance of multiregional sampling for better understanding of tumourigenesis and tumour progression with a possible impact on diagnosis in general.

Conclusion

We found distinct transcriptomic profile between *PRKACA/GNAS* mutant versus *CTNNB1* mutant/wildtype group of CPA tumours where *PRKACA/GNAS* mutant tumours show increased expression of genes involved in steroidogenesis and metabolism whereas the *CTNNB1*/wildtype tumour showed frequent gain of chromosome arm 9q resulting in dosage effect of genes located on this chromosome arm.

We found *PRKACA* mutations occurring outside the hotspot region in CPA. In addition, we also discovered that *PRKACA* mutations occur in ACC tumours. All three mutations lead to increased phosphorylation of downstream targets showing the detrimental impact of the mutation.

TERT promoter mutation occurred in11% of the ACC cohort. However the heterogeneous nature of *TERT* promoter mutations in ACC suggests that the mutation occurs later in tumour evolution.

Novel alterations occurring in *CTNNB1* in ACC underscores the role of the Wnt/βcatenin signalling pathway in ACC tumourigenesis and revealed that mutations in *CTNNB1* occurs more frequently in ACC than previously observed. Functional analysis shows the activating nature of these novel variants on β-catenin and the Wnt/βcatenin signalling pathway. Mutations in *CTNNB1* and nuclear expression of β-catenin were associated with significant lower overall survival.

Genetic analysis of known driver genes together with genomic analysis to study the CNVs with a multiregional approach revealed intra-tumour heterogeneity in ACC tumours. Heterogeneity with respect to mutations in known driver genes such as *TP53, CTNNB1, CDKN2A* and frequently known CNVs such as *ZNRF3* and *CDKN2A/2B* homozygous deletions was observed. Our study demonstrated that these deleterious events occur quite late during tumorigenesis and also underscores the possibility that the mutation found in the driver genes could just be a passenger event.

Future Perspective

Deregulation in the cAMP/PKA signalling accounts for the majority of CPA tumourigenesis; hence making it a good therapeutic target. Few inhibitors of PKA have been tested and were shown to decrease the PKA activity *in vitro*[85,86]. However many issues concerning specificity and drug delivery persist for these molecules[167]. Hence a mouse model to study role of PRKACA and PRKACA mutation in adrenal tumourigenesis is much warranted which certainly will be valuable for testing potential targeted drugs. Tumourigenesis in at least 20% of CPA is not accounted by currently known driver genes. Given the importance of cAMP and Wnt/β-catenin signalling pathway, perhaps evaluation of these pathways with more scrutiny might illuminate on additional driver genes or undiscovered alterations in known genes in CPA.

Most of the ACC patients presents with locally advanced or metastatic disease during diagnosis. Idealistically for these cases, development of non-invasive techniques such as use of circulating DNA for identification of tumour genotype would be desirable to opt for tailored therapy. Major fraction of resected ACC patients also suffers from recurrence. Development and use of non-invasive techniques such as steroid profile in urine, detection of biomarkers such as circulating DNA, miRNA and/or exosomes facilitating early detection are needed. Advanced techniques and research has helped understand and reveal many pathways and events occurring in ACC. However implication of laboratory findings to clinical settings needs much more knowledge about the ACC oncogenesis. Perhaps understanding and targeting currently known altered pathways would be a good choice.

Discovery of Δ(2+3) del from our studies ads on the frequency of Wnt/β-catenin alterations occurring in ACC and suggest that perhaps even other member of this pathway could account for adrenal tumourigenesis. Since β-catenin mutations occur in different types of adrenocortical tumours, it would be interesting to analyse the incidence of Δ(2+3) del in other types adrenocortical tumours such as APA and CPA as well. Comprehensive analysis of the Wnt/β-catenin signalling pathway and functional analysis of *ZNRF3* mutations/deletions in adrenal oncogenesis is much warranted.

Several CNVs such as amplification at *TERT* locus and deletions at *ZNRF3*, *LINC00290/TENM3*, *LSAMP, CDKN2A/2B* loci etc. occur in ACC. Significance of such event in ACC oncogenesis needs to be investigated as whether such deletions are significant in ACC oncogenesis or are they just a

consequential CNVs which occurs due to replication stress during excessive transcription occurring in the tumours.

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