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INSIGHTS INTO THE TRANSCRIPTOMIC PROFILING OF ADRENOCORTICAL TUMOURS

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" Our patients have the right to demand that their endocrine surgical problems are handled by specialists with deep knowledge not only on technical aspects but also of physiology and pathology of endocrine diseases... "
(Langenbecks Arch Surg 2008; 393:619)

" The rapid transformation of new experimental data to diagnosis and treatment of patients will further increase the demand of the surgeon to be updated on medical progress to avoid a future role as surgical technician, soon to be replaced by a robot. "
(Arch Surg 1998; 133:326)

Bertil Hamberger

Thesis defence

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ABSTRACT

An adrenocortical tumour (ACT) can be detected in up to 5% of the population older than 50 years of age. The frequency increases with age. More ACTs are being detected in parallel with the increased use of high-resolution imaging. When there is clinical or biochemical evidence of excess hormonal secretion from an ACT, a careful and systematic clinical evaluation is compulsory. An abnormally elevated secretion of a particular steroid such as aldosterone, cortisol or dehydroepiandrosterone is frequently associated with a benign phenotype, adrenocortical adenoma (ACA). Adrenocortical carcinoma (ACC) is a rare tumour with a relatively poor prognosis; 5-year overall survival is about 30-35%. Approximately half of ACCs are hyperfunctioning, and therefore ACC diagnosis is more often based on its imaging phenotype or by histological characteristics (Weiss Score) rather than the hormonal profile. Suspicion of malignancy or proven hormone production with clinical significance in ACTs should lead to prompt surgical resection. The overall aim of this thesis was to explore by means of transcriptomic profiling the differences between ACT regarding the malignant and hyperfunctioning phenotypes.

Paper I shows the main divergences between ACC and benign ACT at the transcriptomic level. Several genes were up regulated in ACC, including genes related to the IGF family. Transcriptional profiling through the use of cDNA arrays allows discrimination between ACC and ACA.

Paper II emphasizes the use of mRNA transcriptomics for distinguishing the hyperfunctioning phenotypes and prognosis in ACA and ACC respectively. Some specific mRNAs are closely related to certain functional phenotypes, for example aldosterone-producing adenoma (APA) with *OSBP* and *VEGFB*. Additionally, two subgroups of ACC with different survival times were distinguished based on their transcriptomic mRNA profiles.

Paper III demonstrates that the transcriptomic profile based on microRNA (miRNA) expression is also useful for discriminating ACA from ACC. Moreover, certain miRNAs were significantly associated with survival time among ACC patients. The functional role of some stochastic miRNAs determined in ACC was additionally investigated. Transcriptomic profiling of miRNAs is not limited exclusively to classification but also for predicting clinical outcome in ACC.

Paper IV also explores miRNA-based transcriptomics for classifying the most common ACA phenotypes: cortisol-producing adenoma (CPA), APA and non-hyperfunctioning adenoma (NHFA). CPA and APA clustered separately from each other, when NHFA were excluded. Moreover, NHFA were spread out in these two clusters. Certain miRNAs were specific mainly among hyperfunctioning ACA. Specific miRNAs associated with different tumour phenotypes.

Keywords: Adrenocortical tumours, adrenocortical adenoma, adrenocortical carcinoma, RNA expression, microRNA, non-hyperfunctioning, cortisol-producing adenoma, aldosterone-producing adenoma, tumour phenotype.

LIST OF PUBLICATIONS

Papers in this thesis will be referred to by their Roman numerals I-IV.

I. **Velázquez-Fernández D***, Laurell C*, Geli J, Höög A, Odeberg J, Kjellman M, Lundeberg J, Hamberger B, Nilsson P and Bäckdahl M. Expression profiling of adrenocortical neoplasms suggests a molecular signature of malignancy. *Surgery,* 138:1087-94, 2005.

II. Laurell C*, **Velázquez-Fernández D***, Lindsten K, Juhlin C, Enberg U, Geli J, Höög A, Kjellman M, Lundeberg J, Hamberger B, Larsson C, Nilsson P and Bäckdahl M. Transcriptional profiling enables molecular classification of adrenocortical tumours.
European Journal of Endocrinology, 161:141-152, 2009.

III. Özata DM, Caramuta S, **Velázquez-Fernández D**, Akcakaya P, Xie H, Höög A, Zedenius J, Bäckdahl M, Larsson C and Lui WO. The role of microRNA deregulation in the pathogenesis of adrenocortical carcinoma.
Endocrine-Related Cancer, 18:643-655, 2011.

IV. **Velázquez-Fernández D**, Caramuta S, Özata DM, Lu M, Höög A, Bäckdahl M, Larsson C, Lui WO and Zedenius J. MicroRNA expression patterns associated with hyperfunctioning and non-hyperfunctioning phenotypes in adrenocortical adenomas.
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**Authors contributed equally*

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LIST OF ABBREVIATIONS

ACA	Adrenocortical adenoma
ACC	Adrenocortical carcinoma
ACT	Adrenocortical tumour
ACTH	Adrenocorticotropic hormone
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1
APA	Aldosterone-producing adenoma
cDNA	Complementary deoxyribonucleic acid
CGH	Comparative genomic hybridization
CPA	Cortisol-producing adenoma
CT	Computed tomography
DE	Differentially expressed
DHEA	Dehydroepiandrosterone
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
FDR	False discovery rate
GO	Gene ontology
Hs	Homo sapiens
IGF2	Insulin-like growth factor II
KCNJ5	Potassium inwardly-rectifying channel subfamily J, member 5
LDL	Low density lipoprotein
miRNA	Micro ribonucleic acid
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
NHFA	Non-hyperfunctioning adenoma
OSBP	Oxysterol binding protein
PA	Primary aldosteronism
PAC	Plasma aldosterone concentration
PRC	Plasma renin concentration
PCR	Polymerase chain reaction
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNU6B	U6B small nuclear RNA 2
rRNA	Ribosomal ribonucleic acid
SAM	Significant analysis of microarrays
SD	Standard deviation
UFD1L	Ubiquitin fusion degradation 1 like
USP4	Ubiquitin specific peptidase 4
VEGFB	Vascular endothelial growth factor B
WHO	World Health Organization

1 INTRODUCTION

Bartholomeus Eustachius was the first to describe the adrenal glands in 1552. He described the location of these glands above both kidneys named them as "*glandulae renis incumbentes*" in his *Opuscula Anatomica*. Although they were described since the XIV century, many prominent physicians later on including André Vesalius failed to confirm this finding. Moreover, their function remained unknown for centuries until 1855, when Thomas Addison described for the very first time the clinical picture of 11 patients with destruction of the adrenal glands, now defined with his eponymous clinical syndrome[1].

In 1805, Georges Léopold Chrétien Cuvier describe for the very first time that the adrenal glands were composed of a peripheral cortex and central medulla. Unfortunately, Cuvier was not able to infer any functional role for these parts. Cuvier was a French naturalist with a large number of contributions, which in turn was immortalized by Gustav Eiffel by the inscription of his name among 72 other prominent French scientists, engineers and mathematicians on the sides of the tower under the fist balcony on the Eiffel Tower.

In 1856, Charles-Édouard Brown-Séquard, the one of the first to postulate the existence of substances now known as hormones, demonstrated that the removal of the adrenal glands resulted in death due to the lack of their essential hormones. Although he was very criticized and caused controversy at those times for his experimental works, he is nowadays considered the father of the modern endocrinology. He was one of the first to advocate the use of hormonal preparations obtained from tissue extracts for prolonging human life[2].

1.1 The adrenal cortex

The adrenal glands form a paired and triangle-shaped endocrine organ localized at the top of the kidneys (*Figure 1*). In human adults, the normal weight is 4-6 g and the greatest diameter 4-5 cm[3-5]. The adrenal gland is an extraordinary conjunction of two endocrine tissue types with different embryonic origin and functional properties[6-10].

The adrenal medulla originates from the neural crest and produces bioactive amines whereas the adrenal cortex arises from mesenchymal cells attached to the coelomic cavity epithelium and produces steroid-derived hormones[8-15].

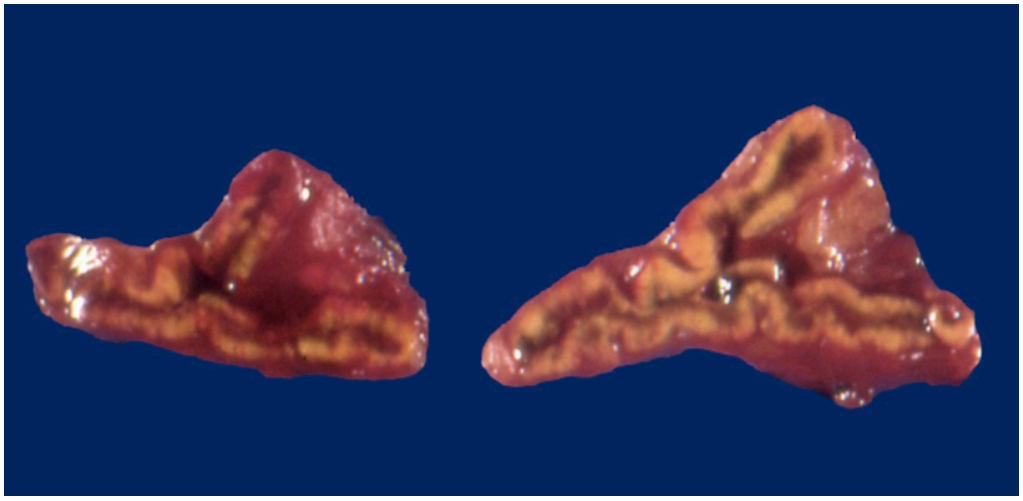


Figure 1. Macroscopic appearance of the adrenal glands. The cut surface shows a yellowish outer layer known as the cortex, and an inner layer termed the medulla.

Autonomic nerves directly stimulate the adrenal medulla and therefore the response is immediate with catecholamine secretion. In contrast, the adrenal cortex is stimulated by the adrenocorticotropic hormone (ACTH), secreted from the anterior pituitary, which in turn is also stimulated by the hypothalamus. ACTH induces synthesis and secretion of adrenal steroids in a circadian mode. In general terms, adrenocortical steroids modulate the glycaemia and electrolyte levels, mainly sodium. Compared to the rapid response of the medulla, the cortex may exert its secretion and hormonal action for longer periods of time. During foetal development until one year postpartum, two zones are evident in the adrenal glands: an inner prominent zone and an outer zone that further differentiates into the adult definitive adrenal gland[4, 10, 14]. After birth, the outer zone develops two zones; an internal one, which further differentiates into the *zona fasciculata* and an external that becomes the *zona glomerulosa*. Additionally, some growth factors such as IGF-II have been implicated in this functional development of the foetal adrenal cortex[16-22].

The adrenal cortex surrounds the medulla and constitutes a major proportion (85-90%) of the entire gland. From a functional and anatomical point of view, the adrenal cortex includes three different layers that are from the external (beneath the capsule) to the

most internal stratum the following: *zona glomerulosa, zona fasciculata* and *zona reticularis* (*Figure 2*).

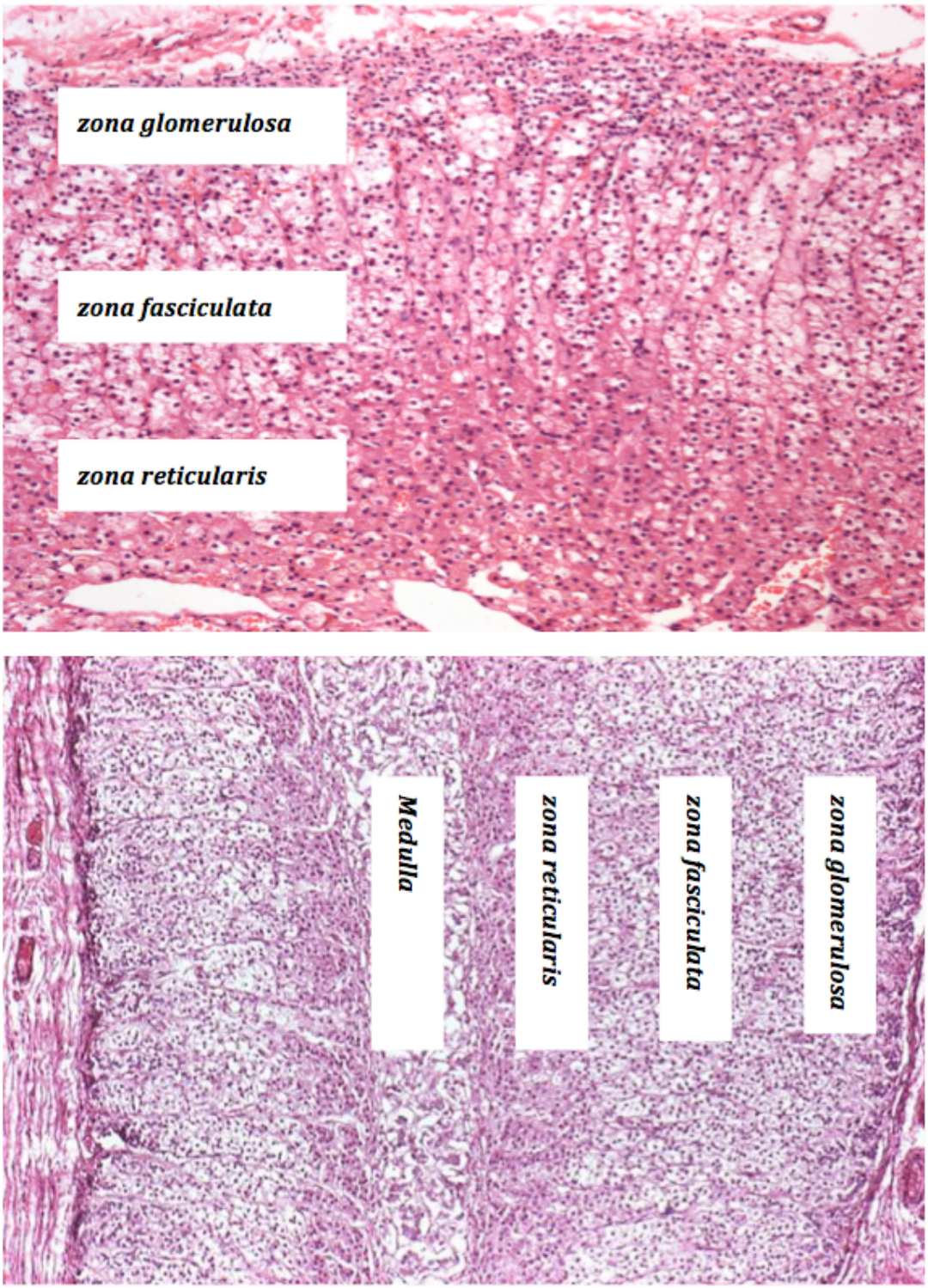
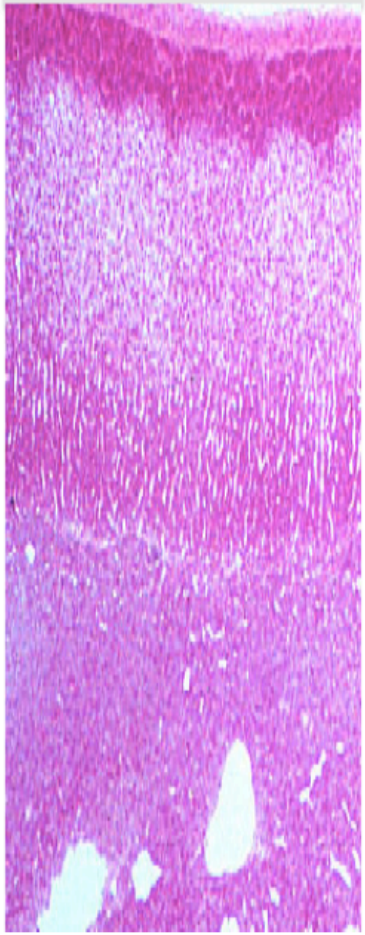


Figure 2. Microscopic features of the different anatomical and functional zones within the adrenal cortex. The *zona fasciculata* with its columnar disposition is the most abundant within the adrenal cortex. A small fraction of adrenal medulla can be seen at the centre of the image.

Traditionally it has been established that the intermediate layer (*zona fasciculata*) is the widest zone comprising 75% of the cortex and mainly produces glucocorticoids. This layer includes large and lipid-laden cells forming radial cords between the fibrovascular radial networks. Mineralocorticoids such as aldosterone are mainly produced in the outer layer (*zona glomerulosa*), while sex or gonadal steroids are produced in the innermost layer (*zona reticularis)* [23-25] (*Figure 3*). Although this conventional zonation has been recently questioned[26-30], it has long been useful for educative purposes.

However, a more complex interaction within these layers with a differential growth has been advocated by some authors especially across the period of adrenarche[24, 31-35]. The adrenarche is an endocrine developmental process occurring in humans and other non-human primates that increase cortical sex steroids such as dehydroepiandrosterone (DHEA). This phenomenon corresponds with the expansion of the *zona reticularis* of the adrenal cortex around 6 years of age[32]. The phenotypic hallmark of this adrenarche is the appearance of axillary and pubic hair, enabling the future transformation of some other steroids to active forms of sex steroids such as testosterone further until the puberty.

Steroidogenesis is a genetically guided complex biochemical and cellular process[5, 12, 36, 37] that ends up in the synthesis of steroid hormones (*Figure 4*)[37-39]. These hormones may be classified into three main types: glucocorticoids (for example cortisol and corticosterone); mineralocorticoids (aldosterone and deoxycorticosterone), and sex/gonadal steroids (such as oestrogen, testosterone and DHEA)[4, 23]. Glucocorticoids are secreted in relatively high amounts under the control of the ACTH while mineralocorticoids are secreted in lower amounts. For example while approximately 10 milligrams of cortisol are usually secreted per day, only 100 micrograms of aldosterone are secreted in the same period of time. In contrast, adrenal androgens are the most abundant steroids secreted. More than 20 milligrams of DHEA are secreted each day from an adult adrenal cortex.



PARTS	ZONES	HORMONAL PRODUCTION		ENZYME ACTIVITY	FUNCTION	REGULATION
CAPSULE						
ADRENAL CORTEX	ZONA GLOMERULOSA	Mineralo-corticoids	Aldosterone	CYP11B2	Intravascular volume and electrolytes	Angiotensin II and ACTH (MC2R)
	ZONA FASCICULATA	Glucocorticoids	Cortisol Corticosterone	*CYP17* *CYP11B1*	Regulation of glucose levels and stress response; mobilization of energy stores	Mainly regulated by ACTH (MC2R)
	ZONA RETICULARIS	Adrenal Androgens	DHEA DHEA-Sulfate	CYP17 17OH and 17,20 LYASE	Adrenarche response and sexual features	Regulated by ACTH (MC2R)/other unknown factors
ADRENAL MEDULLA	Chromaffin cells Splanchnic nerves Medullary veins	Catecholamines	Dopamine Epinephrine Norepinephrine		Inmediate response in stress response and sympathetic activity	Mainly regulated by nervous stimulii

Figure 3. Summary of the anatomical zones of the adrenal cortex and their physiological roles.

Cholesterol is the major substrate for this steroidogenesis within the adrenal cortex, which may be acquired from outer or inner sources into the circulation[40, 41]. Thereby, most steroid hormones share closely related molecular structures. Although cholesterol is the precursor of all adrenal steroids, its main source is low-density lipoproteins (LDL). These LDL can be obtained from endogenous or exogenous sources. LDL uptake is performed by specific cell surface LDL receptors located on adrenal cortex cells. Once LDL is internalized via receptor mediated endocytosis, the resulting vesicles fuse with lysozymes and free cholesterol is then produced through hydrolysis. Once cholesterol is transported into the mitochondria, a set of enzymes/proteins (mainly cytochrome P450 enzymes) participate into the further metabolic changes to obtain a specific type of steroid hormone[36, 37, 41-46].

Steroidogenic genes mainly coordinate this process, in which ACTH plays a crucial role by exerting transcriptional pressure on these genes to synthesize adrenocortical steroids[12, 36, 47] as well as the foetal adrenal development during pregnancy[16, 17, 48, 49]. Interestingly, small quantitative differences of these hormones may retain a human individual alive or paradoxically may cause an overt clinical syndrome.

Steroidogenesis is a complex series of biochemical transformations beginning with cholesterol that has a basic core composed of a cyclo-perhydro-pentano-phenanthrene structure[4, 37, 50]. All the different cortical steroids such as oestrogens, androgens, mineralocorticoids and glucocorticoids share this biochemical core. The three major steps of steroidogenesis (*Figure 4*) from cholesterol uptake to functionally active steroids are:

1. Cholesterol uptake and intracellular transportation
2. Mitochondrial inclusion of cholesterol
3. Enzymatic processes
    a) Cytochrome P450 family (oxidative enzymes)
        i. *Type 1 or mitochondrial*
        ii. *Type 2 in endoplasmatic reticulum*
    b) Hydroxysteroid dehydrogenase family
        i. *Aldo-keto reductases family*
        ii. *Short-chain dehydrogenase/reductase family*

Today, human diseases are known to be associated with all these steps. For example, in Wollman disease, adrenal insufficiency is induced due to unavailability of free cholesterol, and 21-hydroxylase deficiency is the most common cause of congenital adrenal hyperplasia[37]. The Cytochrome P450 family comprise enzymes composed with approximately 50 amino acids with a single heme group. The name reflects the fact that these enzymes are pigments and can absorb light at 450 nm in their reduced states. The genes and protein products in this family are also referred as *CYP*[4, 37]. The first conversion of cholesterol into pregnenolone is produced by P450, which is abundant in the three zones of the adrenal cortex (*glomerulosa, fasciculata and reticularis*). The participation of the other two families of enzymes determines the type of steroid to be produced and therefore its functional role. These steroidogenic enzymes also show a ”zone-specific” distribution across the adrenal cortex[3]. For example, the *zona glomerulosa* is not able to synthesize cortisol because it does not contain a 17-alpha hydroxylase, whereas this zone is able to synthesize aldosterone because it produces CYP11B2. On the first hand, classical endocrine feedback loops are in place to control the secretion of these hormones such as the corticotrophin-releasing factor (synthesized by the hypothalamus) and the ACTH (synthesized by the anterior pituitary). In addition, other stimuli may induce the synthesis of these hormones such as hyperkalaemia does for aldosterone secretion[3, 5].

On the other hand, a considerable amount of this enzymatic activity is regulated genetically and by posttranslational modifications. Moreover, discrete differences in the genetic sequences differ among this family (cluster) of steroidogenic genes. For example, *CYP11B1* and *CYP11B2* share approximately a 95% of homology, the 5 prime promoter sequences of these genes differ. This allows the specific regulation by ACTH and Angiotensin II in the final steps of glucocorticoid and mineralocorticoid biosynthesis, respectively. Rare mutations are known in the corresponding genes, and the identification of these mutations has contributed to a better understanding of the genetic basis of adrenocortical tumours (ACT)[37, 46, 51, 52].

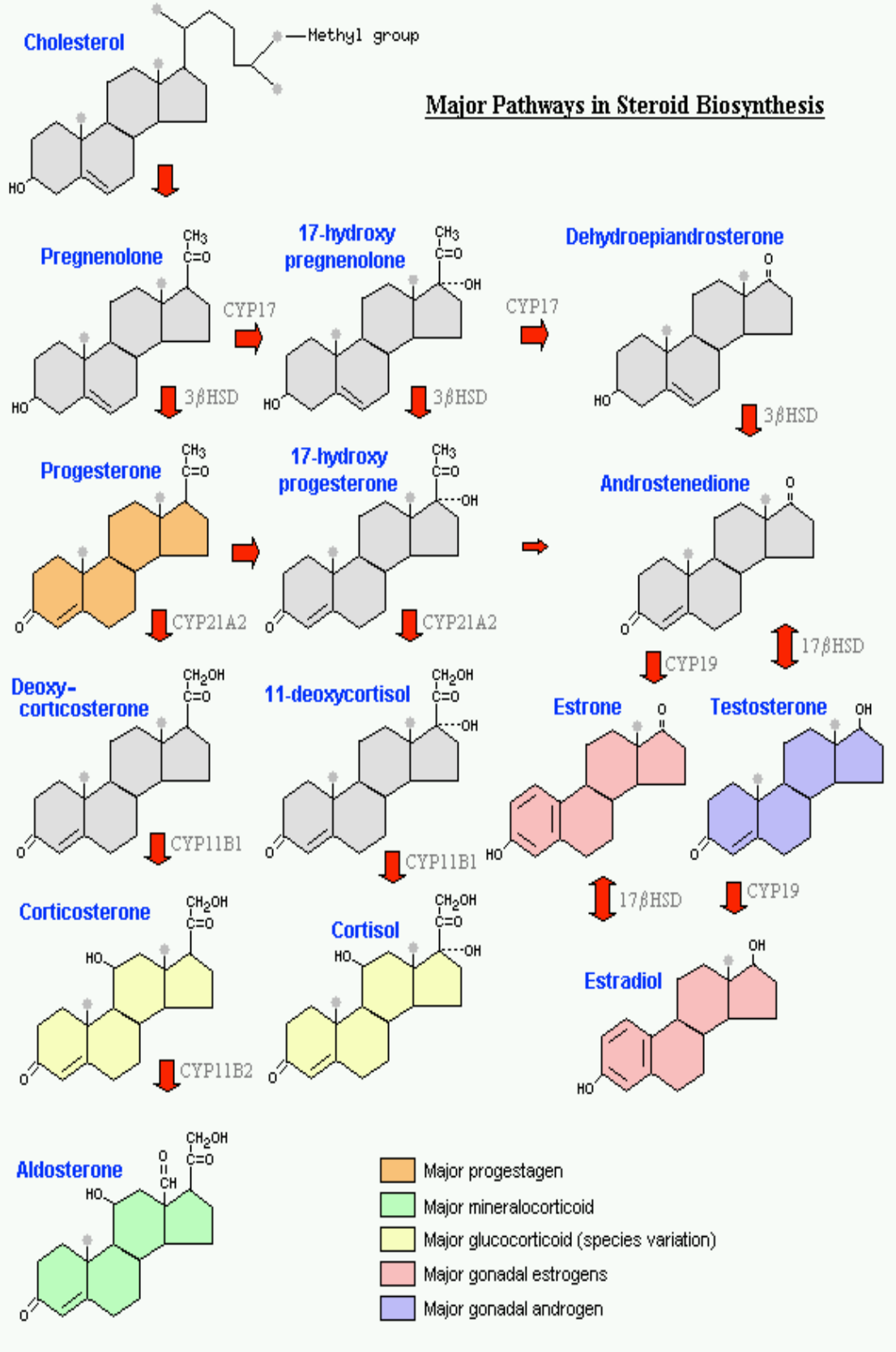


Figure 4. Schematic diagram of the human steroidogenesis (obtained from http://www.vivo.colostate.edu/hbooks/pathphys/endocrine/basics/steroidogenesis.html).

1.2 Epidemiology of adrenocortical adenomas (ACA) and carcinomas (ACC)

Adrenal tumours can be detected in up to 5% of the population older than 50 years of age[53-57]. However, adrenocortical carcinoma (ACC) is a rare malignancy, with a estimate incidence of 0.7-2 cases per million inhabitants per year based on data obtained from the National Cancer Institute[58]. ACC is also more frequent in women than in men with an estimated ratio of 1.5 times more. Although there have been a lot of speculation concerning the aetiology of ACC, this remains unclear for most of the cases.

Today, more ACTs are being detected in parallel with the increased use of high-resolution radiological imaging, especially computed tomography (CT) and magnetic resonance imaging (MRI)[59]. The general prevalence of ACT depends on the diagnostic method utilized and the patient's age. There is a correlation between age and the probability of neoplasia[60, 61]. The unsuspected discovery of an adrenal mass when a radiological examination is performed for reasons other than adrenal related symptoms is designated as adrenal incidentaloma[53, 61-68]. When there is clinical or biochemical evidence of hyperfunctioning hormonal secretion from these ACTs, a careful and systematic evaluation is compulsory[56, 69-72].

An abnormally elevated secretion of a particular steroid such as aldosterone, cortisol or DHEA is usually associated with benign growth properties. The proportion of adrenocortical adenomas (ACAs) among incidentalomas is high, while the malignant counterpart ACC is rare[73, 74]. An ACT with homogenous density, well delineated margins, without necrosis and a diameter not exceeding 4 cm are usually considered as ACA[63, 70, 75-77].

An ACA appear as a yellowish single nodule often with a microscopically well-delineated transition between normal adrenal and adenoma (*Figure 5*). Nests with high mitotic rate and eosinophilic changes without invasion may also occur as well as nuclear pleomorphism or nuclear atypia.

In contrast, the macroscopic appearance of an ACC may include necrosis and a heterogeneous pattern (*Figure 6*). Typical microscopic features are a diffuse growth pattern with capsular invasion, surrounding tissue overgrowth, vascular invasion and necrosis with haemorrhagic foci. Some of these characteristics are considered in the Weiss score system, which is commonly used as a diagnostic and prognostic tool.

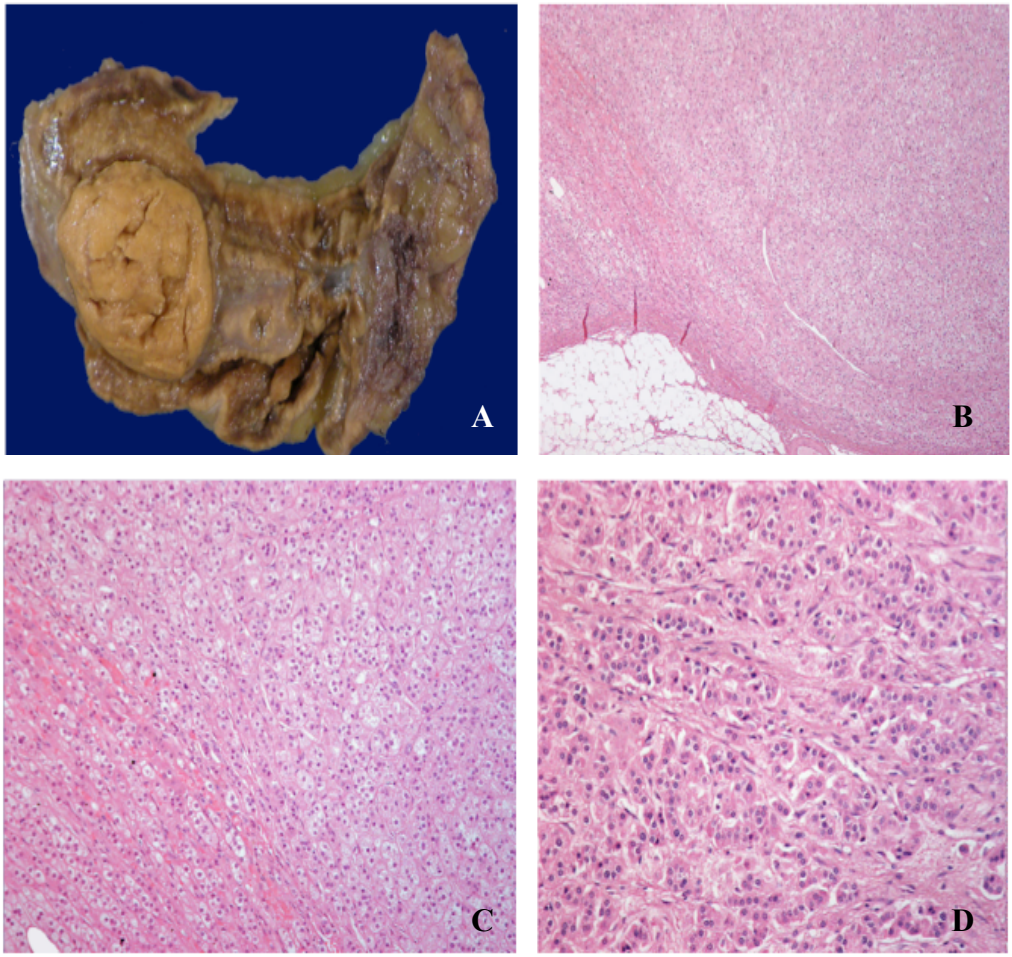


Figure 5. Aldosterone-producing adenoma (APA). (A) Macroscopic appearance. Some microscopic features are showed in B and C such as a well-delineated transition between normal adrenal and adenoma; a high mitotic rate in nests with eosinophilic changes without invasion can also occur (D).

The prognosis of ACC is poor with a 5-year overall survival of about 30-35%[54, 78, 79]. Early detection is therefore important for ACCs and ACTs that may become malignant. A considerable proportion of ACCs are hyperfunctioning, and therefore ACC diagnosis is more based on its imaging phenotype than the hormonal or biochemical profiles. Any rational suspicion of malignancy or potential malignancy

should lead to surgical resection. Therefore, large ACTs, those that grow significantly between two separate imaging procedures, and those with radiological patterns indicating malignancy (irregularity, necrosis, diffuse margins, increased attenuation, etc) should be readily excised surgically.

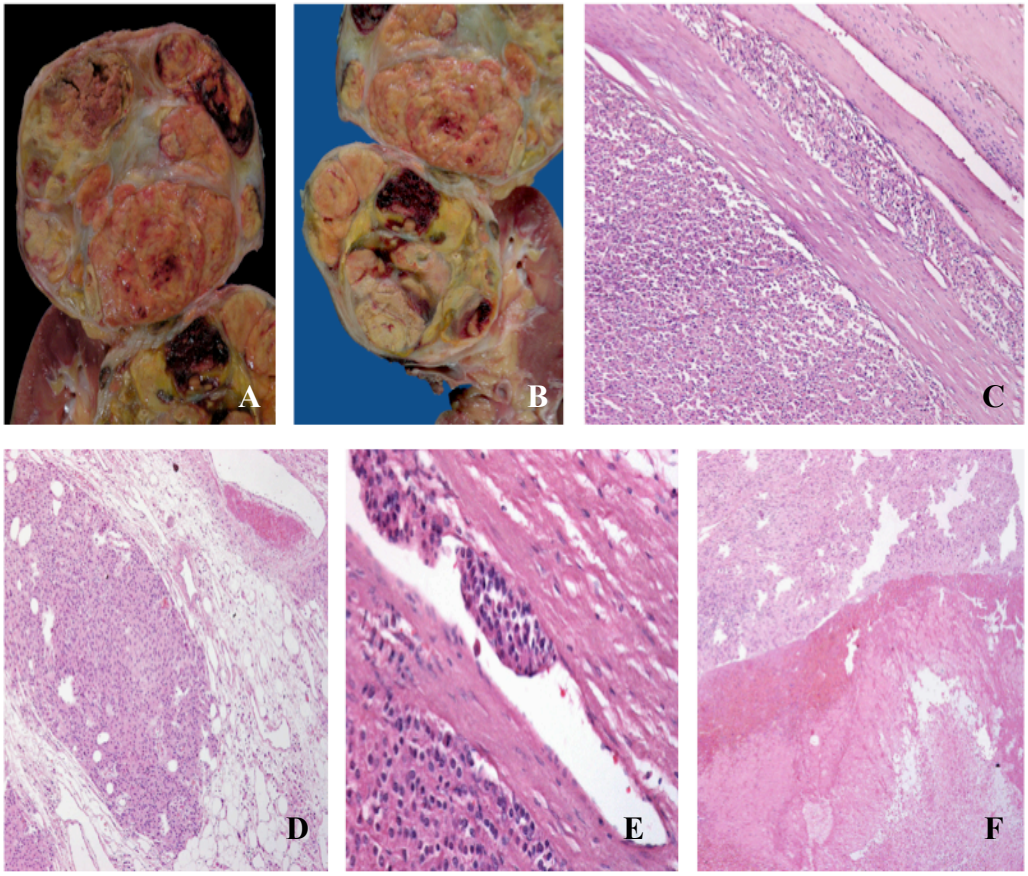


Figure 6. Adrenocortical carcinoma (ACC) Macroscopic appearance is shown in A and B. Microscopic features may include a diffuse growth pattern with capsular invasion (C), surrounding tissue overgrowth (D) vascular invasion (E) and necrosis with haemorrhagic foci (F).

1.3 Clinical aspects of ACA and ACC

In general terms, hormonally overactive ACTs are commonly benign. In ACA hormonal overproduction usually concerns one specific hormone normally produced by the adrenal cortex such as for example aldosterone or cortisol[80, 81]. However, many ACCs are also hyperfunctioning. Approximately half of ACCs produce steroids, causing clinical symptoms, often a mixture of several steroids and their derivatives,

reflecting de-differentiation of the tumour. Therefore, secretion profiling of steroids and their metabolites in urine may also serve as a tumour marker during screening, diagnosis and follow-up of ACTs[55, 82-88]. Androgen overproduction is usually restricted to the malignant phenotype of ACTs.

A genetic predisposition to ACT may also be inherited in a Mendelian fashion (*Figure 7*), which may be an important issue for genetic counselling in families with an increased risk. This is the case of a heterozygous mutation in the p53 gene located on chromosome 17p13.1, which causes the Li-Fraumeni syndrome. This syndrome is characterized by the early onset of multiple tumours such as sarcomas, osteosarcomas, breast cancer, brain tumours, brain tumours, leukaemia and ACC. This syndrome has an autosomal dominant pattern of inheritance affecting at least one first- or second relative in the same lineage at early age (before 45 years)[89-91].

Hypersecretion of aldosterone is known as primary aldosteronism (PA)[92-94]. This condition is the most frequent cause of endocrine hypertension[95] and it is commonly associated with adrenocortical entities such as hyperplasia and a single aldosterone-producing adenoma (APA)[96, 97]. The presence of a single APA is also referred in the literature to as Conn´s syndrome. Aldosterone is mainly synthesized in the *zona glomerulosa* induced by angiotensin II, hyperkalaemia or decreased intravascular volume. PA is clinically diagnosed based on resistant systemic arterial hypertension, hypokalaemia and abnormally high levels of plasma aldosterone when related to renin activity (high aldosterone:renin ratio) [98-100]. These increased levels of aldosterone are produced by the adrenal cortex constitutively without any other secondary stimulus such as angiotensin II or hyperkalaemia. It is now recognized that many patients with PA do not present with hypokalaemia[101, 102]. Thereby, proper screening must include plasma aldosterone concentration (PAC), plasma renin concentration (PRC) and the PAC/PRC ratio. Normo-kalaemia must be obtained, and some antihypertensive drugs are not allowed during biochemical work-up. A much higher prevalence of PA has been determined with this approach when compared to the previous[3].

As many other endocrine diseases, PA is more properly diagnosed through the use of a three-phase clinical approach: 1) *Detection* tests (in suspected patients), 2) *Confirmatory* tests (of PA) and 3) *Classification* tests (of the different subtypes of PA).

Classification tests are used to identify subtypes of PA. These may include CT scan and adrenal venous sampling for distinguishing between APA and bilateral hyperplasia or glucocorticoid-remediable aldosteronism. These last two should be treated medically. Adrenal venous sampling for determining the dominating side for the aldosterone over-production is today golden standard[94, 103-106]. Ideally, the adrenal without over-production should be suppressed, but a PAC/PRC ratio exceeding three or four times, is usually considered as significant, and surgery should be offered to the patient[107-111]. Most commonly the CT scan reveals an adrenal mass, but these may be very small and escape detection (referred to as microadenomas). Furthermore, localisation studies apart from CT are mandatory, as an adrenal mass in a PA patient may be a non-hyperfunctioning incidentaloma. Aldosterone-producing carcinomas are extremely rare and exhibit an atypical clinical course[112].

Hypersecretion of cortisol may induce some clinical features characterized for a wide spectrum of conditions described as Cushing's syndrome. This syndrome comprises many symptoms and signs associated with the prolonged exposure to inappropriately elevated levels of plasma glucocorticoids. These may include central obesity, facial plethora, moon facies, diabetes mellitus, arterial hypertension, gonadal dysfunction, hirsutism, bruising, red-purple striae, muscle weakness and osteoporosis among others[3, 5]. Etiological causes of Cushing`s syndrome may include exogenous iatrogenic administration, ACTH-dependent hypercortisolism and ACTH-independent which comprises the endogenous hypersecretion. Endogenous causes of Cushing`s syndrome are relatively rare and include a cortisol-producing adenoma (CPA). CPA are responsible for about 10-15% of cases with Cushing`s syndrome. Cortisol-producing carcinomas are even less frequent. In contrast, in children 65% of these cases have an adrenal origin: 15% are CPA and 50% cortisol-producing ACC[113-115]. Hypersecretion of other steroids such as androgens or mineralocorticoids is also common among these tumours.

<table>
<tr><td rowspan="6">ACT</td><td rowspan="3">Sporadic</td><td rowspan="2">Benign</td><td rowspan="2">Hyperfunctioning</td><td rowspan="2">• Aldosterone producing
• Cortisol producing
• Androgen producing</td><td>Unilateral</td><td>• APA
• CPA
• Adenomas</td></tr>
<tr><td>Bilateral</td><td>• Hyperplasia
• ACTH-independent macronodular adrenal hyperplasia (AIMAH)
• McCune-Albright Syndrome</td></tr>
<tr><td>Non-hyperfunctioning</td><td colspan="3">• Unilateral
• Bilateral</td></tr>
<tr><td rowspan="2">Malignant</td><td>Hyperfunctioning</td><td colspan="3">Most ACTs producing sex hormones (oestrogen, testosterone or DHEAS) are malignant. Many also produce cortisol.</td></tr>
<tr><td>Non-hyperfunctioning</td><td colspan="3"></td></tr>
<tr><td rowspan="4">Hereditary</td><td rowspan="2">Benign</td><td>Isolated</td><td>• AD
• AR</td><td colspan="2">• Familial adenomatous polyposis (AD)
• Hereditary ACT (CTNNB1) (AD/AR)
• Familial hyperaldosteronism (AD)</td></tr>
<tr><td>Syndromic</td><td>• AD
• AR</td><td colspan="2">• Multiple Endocrine Neoplasia 1 (AD)
• Carney complex (AD)
• Primary pigmented nodular adrenocortical disease (PPNAD) (AD)</td></tr>
<tr><td rowspan="2">Malignant</td><td>Isolated</td><td>• AD
• AR</td><td colspan="2">• Hereditary ACC (CTNNB1) (AD/AR)</td></tr>
<tr><td>Syndromic</td><td>• AD
• AR</td><td colspan="2">• Multiple Endocrine Neoplasia 1 (AD)
• Li-Fraumeni Syndrome (AD)</td></tr>
</table>

Figure 7. ACT classification based on a clinical genetics point of view. *AD*: Autosomal dominant, *AR*: Autosomal recessive.

As aforementioned for PA, Cushing`s syndrome is also more properly diagnosed through the use of a three-phase clinical approach: 1) *Detection* tests (in suspected patients), 2) *Confirmatory* tests (of Cushing´s syndrome) and 3) *Classification* tests (of the different types/causes of Cushing´s syndrome). *Detection tests* may comprehend the presence of signs or symptoms that make the suspicion high for this syndrome such as hyperglycaemia, obesity and arterial hypertension.

Confirmatory tests should comprise the measurement of 24-h plasma cortisol, secretion profiles, 24-hour urinary free cortisol excretion, and low-dose/overnight dexamethasone suppression tests. These tests have displayed heterogeneous accuracy for the diagnosis of Cushing´s syndrome. This depends on the used assays and varies from institution to institution. For example, several authors have demonstrated that a midnight salivary cortisol ≥2 ng/mL has a greater diagnostic accuracy with a 100% sensitivity and 96% of specificity for the diagnosis of Cushing`s syndrome[116-122].

Once the biochemical confirmation has been done, it is necessary to investigate what is the cause and type of Cushing´s syndrome. For this purpose, *classification* tests are necessary. These may comprise midnight (23-01 hours) plasma ACTH (normal ≤20 pg/ml), measurements of ACTH precursors (such as pro-ACTH and POMC), plasma potassium/mineralocorticoid secretion, high-dose dexamethasone suppression test, corticotropin-releasing factor test and imaging procedures. Clinical entities such as ectopic ACTH syndrome due to indolent carcinoid or subclinical Cushing may be discarded with these tests.

Bilateral adrenocortical hyperplasia is present in approximately 10-40% of patients with Cushing´s syndrome[114, 123]. This pathologic condition is associated with one or more adrenocortical nodules with a great variety of diameters and morphology. In some instances, this is associated to a chronic ACTH stimulation to the adrenal cortex. The more hyperplastic the adrenal glands become due to ACTH stimulation, the more the hypersecretion of cortisol is caused by adrenal hyperplasia. This overstimulation induces an "auto suppression" point, which may be confusing regarding this entity, as it may mimic an ACTH-independent form instead of the real ACTH-dependent origin that cause this irregular hyperplasia. A patient with CPA should have a low, if not

immeasurable, ACTH combined with an adrenal mass. Also in CPA patients, adrenal venous sampling may be mandatory. Bilateral disease, as in micronodular adrenocortical hyperplasia, is not always a contra-indication for surgery; venous sampling may allow removal of the dominant side, with sometimes a significant relief of symptoms from the cortisol over-production[124-126].

Finally, ACTs incidentally discovered by clinical imaging are commonly presenting as hormonally inactive masses or non-hyperfunctioning adenomas (NHFA)[127]. Hormonally active incidentalomas are reported to be diagnosed as CPA with or without Cushing's syndrome in approximately 9-10%, as pheochromocytomas (catecholamine-producing tumours) in 4-5% and as APA in 1-2% of the cases [54]. Approximately 60% of all diagnosed ACT but less than 10% of ACC are hormonally inactive[41, 79]. Unfortunately, a significant proportion of ACCs are initially diagnosed incidentally. Therefore, other features should also be taken in consideration for classification these tumours. For example, for tumours with a diameter less than 4 cm, there is a very low risk of malignancy, whereas this risk increases proportionally with an increasing diameter. Tumours larger than 6 cm are malignant in approximately 25% of the cases.[60, 128] Histopathological criteria included in the ***Weiss score***[129, 130] are frequently used to assess the probability of malignancy in these tumours. These criteria include:

1. High nuclear grade
2. High mitotic rate (greater than 5/50 high power fields)
3. Atypical mitoses
4. Eosinophilic tumour cell cytoplasm (>75% of tumour cells)
5. Presence of less than or equal to 25% of clear or vacuolated cells resembling the normal *zona fasciculata*
6. Diffuse architectural pattern (greater than 33% of the tumour) with broad fibrous and trabecular bands.
7. Foci of confluent necrosis
8. Venous invasion
9. Sinusoidal invasion
10. Invasion of tumour capsule

When three or more of these criteria are found in an ACT, the phenotype is considered to be malignant[129-132].

While tumour diameter and radiological features can merely suggest malignancy, surgical excision is often necessary to obtain a definitive diagnosis in most cases. Moreover, diagnosis may be unclear in some cases, even after the histopathological analysis of the surgical specimen.

Repeated radiological examinations may identify growing ACTs, and this usually leads to surgery, as malignant progression cannot be excluded. Taking the rarity of ACC into account, together with the non-negligible accumulated dose of radiation after repeated examinations, it is of importance to identify other measurable signs of malignant phenotypes in ACTs. Today the diagnostic and prognostic tools for distinguishing between benign and malignant ACTs are blunt. Therefore, a need for improved understanding of the molecular aetiologies of ACT, to develop complementary biomarkers and identify novel therapy targets, especially for ACC is of great interest[54, 133, 134].

1.4 Molecular basis of ACA and ACC

In the last decades, a multitude of genetic abnormalities have been revealed in human cancer, some with diagnostic and prognostic implications. For ACT several genetic, genomic copy-number and molecular alterations have been reported[57, 58, 128, 135], which have revealed differences between ACT and ACC[54, 58, 81, 136-138]. Many of these abnormalities have been associated to the malignant phenotype and poor prognosis such as the early presence of metastasis or local invasion in ACC (*Table 1*) [128, 139-141].

Based on comparative genomic hybridization[128, 142-144], the ACC genotype has been associated with multiple chromosomal gains and gene amplifications[144, 145]. Most commonly gains in ACC (at least 7 times more frequent than in ACA) have been observed in 5p, 5q, 12p, 12q, chromosome 19 and 4. Although similar alterations have also been detected in ACA, it seems that these gains are more prevalent in ACC. These genetic aberrations have been positively correlated to an increasing size of the

Table 1. Genetic changes and aberrations observed in adrenocortical tumours, with an emphasis on adrenocortical carcinoma.

GENE	LOCALIZATION	NAME	RELATED PHENOTYPE	GENOTYPE STATUS	POSSIBLE ROLE
IGF2A	*11p15.5*	Insulin-like growth factor 2 (Somatomedin A)	Beckwith-Wiedemann syndrome/ ACC	Most overexpressed in ACC	Cell proliferation and neoplastic transformation, regulates apoptosis
FGFR1/4	*5q35.1-qter*	Fibroblast growth factor receptors	ACC	Overexpression	Cell proliferation and tumor vascularization
PRKAR1A	*17q23-q24*	Protein Kinase A regulatory subunit	Carney complex/ACT/ACC	Somatic inactivating mutations or allelic losses	cAMP activator, can fuse to RET proto-oncogene
APC	*5q21-q22*	Adenomatous Polyposis Coli	Gardner syndrome/ACC	Activating mutations	Wnt pathway stimulation
MEN1	*11q13*	Multiple Endocrine Neoplasia Gene	MEN type 1/ACT/ACC	Inactivating mutations	Tumor suppressor and transcriptional inhibitor for JunD
RXRA	*9q34.3*	Retinoid X Receptor Alpha	ACC	Decreased expression	Oxidation and synthesis of retinoic acid
ALDH1A1	*9q21.13*	Aldehyde dehydrogenase 1 family, member A1	ACC	Decreased expression	Enzyme participating in alcohol and retinol metabolism. Determined as potential marker for cancer stem cells in human sarcoma and melanoma
CDK2	*12q13*	Cell Division protein Kinases	ACC	Overexpressed	Induces mitosis as a Ser/Thr protein kinase family
MC2R	*18p11.2*	Melanocortin 2 receptor	ACT/ACC	Decreased expression	ACTH receptor, induces steroidogenesis differentiation
TP53	*17p13.1*	Tumor protein p53	Li-Fraumeni Syndrome/ ACC	Inactivating mutation	Oncogenesis, cellular growth and division
CTNNB1	*3p21*	Catenin (Cadherin-associated protein) beta 1	ACC	Activating mutation/Overexpressed	Wnt/Beta-catenin pathway activation; associated to poor survival in ACC
CYP11B2	*8q21-q22*	Cytochrome P450, family 11, subfamily B, polypeptide 2	APA/ACC	Overexpressed/Downregulated	Stimulates steroidogenesis, aldosterone synthesis
HSD3B1	*1p13.1*	Hydroxy-delta-5-steroid-dehydrogenase, 3 beta-and steroid delta isomerase 1	CPA/ACC	Overexpressed/Downregulated	Stimulates steroidogenesis and secretion
RARRES2	*7q36.1*	Retinoic acid receptor responder	ACC	Downregulated	Initiates chemotaxis, adipokine role
SLC16A9	*10q21.2*	Solute carrier family 16, member 9	ACC	Overexpressed	Involved in cellular metabolism and communication between tissues; involved in ACC good survival
STAR	*8p11.2*	Steroidogenic acuted regulatory protein	ACC	Downregulated	Directly involved in steroidogenesis and transcriptional activation
VEGF	*6p12*	Vascular endothelial growth factor	ACC	Overexpressed	Stimulates angiogenesis
TM7SF2	*11q13*	Transmembrane 7 superfamily member 2	ACC	Overexpressed	Involved as a sterol regulatory element and MAPK activation; cholesterol synthesis
AGTR1	*3q24*	Angiotensin II receptor, type 1	APA	Overexpressed	Vasopressor activity, aldosterone secretion
PRDX5	*11q13*	Peroxiredoxin 5	ACC	Overexpressed	Antioxidant enzyme, inflammatory process
CYP17A1	*10q24.3*	Cytochrome P450, family 17, subfamily A, polypeptide 1	CPA/ACC	Overexpressed/Downregulated	Stimulates steroidogenesis produces corticoids
PINK1	*1p36*	PTEN-induced putative kinase 1	ACC	Overexpressed	Involved in ACC worst survival

tumour[144, 145]. Based on these findings, the possibility of an ontogenetic progression in these tumours has been discussed[144-147].

Many studies have consistently demonstrated that ACC specifically display an overexpression of the insulin-like growth factor 2 (*IGF-2*) and a constitutive activation of the beta-catenin as crucial events for the development of these tumours[58].

Mutations of the *TP53* gene have been reported in some ACT cases. These have been strongly associated with a higher prevalence/predisposition of ACT in some specific populations. For example in a cohort in Southern Brazil a founder germline *TP53* mutation (R337H) was detected in 95% of childhood ACT[148]. This founder mutation has conditioned in this population an unusually higher incidence (4 times more) of ACC[58]. Nevertheless, as described for many cancers, it seems that these genetic/molecular changes are quite different in tumours between the paediatric and adult population[138]. For example in studies of adult cohorts, *TP53* mutations have been established in approximately 25% of sporadic ACC[144, 149-151].

In 2011 recurrent mutations of the inwardly rectifying potassium channel gene (*KCNJ5*) were reported in approximately 36% of APA using a whole exome-capture plus Illumina sequencing approach[152]. Two recurrent mutations were identified which increase sodium conductance in the adrenal glomerulosa cells. This alteration in the channel selectivity induces aldosterone synthesis and cell proliferation. The finding has subsequently been reproduced in other populations[153] also in a cohort from our own institution[154].

Very recently, hotspot mutations were also reported in the *ATP1A1* and *ATP2B3* genes encoding an Na^+/K^+ ATPase alpha subunit and a Ca^{2+} ATPase, respectively[155]. These molecules are involved in sodium, potassium and calcium ion homeostasis. *ATP1A1* and *ATP2B3* were found mutated in 5.2% and 1.6% of APA. Patients with these somatic mutations showed autonomous aldosterone hypersecretion.

1.5 Transcriptional studies in sporadic ACA and ACC

The first step of gene expression is coding RNA or mRNA from a DNA template (a genetic process named *transcription*). This molecular process conduces regularly but

not always, to protein synthesis in a mechanism called *translation*. Gene expression has been associated to certain cellular features such as growth control, cellular death and differentiation. Therefore, it has been documented that in some pathological cellular states such as neoplasia certain gene expression levels could be changed or abnormally distorted, often showing a specific profile or pattern. In fact, cancer is usually considered as a heterogeneous group of diseases that all arise from discernible genetic changes. Changes at DNA or RNA level have been well documented in many cancers. Applied used of the whole or a subset of transcripts in medical genomics is frequently referred to as *transcriptomics*. The transcriptome constitute mainly of different forms of RNA, and it is directly related to the functional elements of the entire genome and may contribute to the better understanding of human disease[156]. Furthermore, mRNA expression profiling has unravelled some molecular markers between benign tumours and carcinoma, leading to novel diagnostic/prognostic tools such as the Mamma Print for breast and other cancers[157-159]. Moreover, this microarray-based diagnostic/prognostic tool has been demonstrated to be superior to its pathological counterpart, mainly for assessing the risk of metastases. Additionally, other molecular signature tools seem useful for improving the diagnostic classification among tumours, predominantly cancer and its subtypes[160-163]. These molecular signatures (specially a featured group of deregulated *mRNA* levels) have also proven to be reliable as predictors of malignancy, providing valuable independent information in addition to the clinical, biochemical and pathological assessment of cancer[138, 164].

Based on this appraisal, over-expression of some specific genes (*Table 1*) has been associated to certain ACT phenotypes such as *CYP11A1* and *HSD3B1* for CPA[51, 165]; *CYP11B2* for APA[166-170] and *CYP21A2* for androgen secreting ACT[170, 171]. Since many of these genes participate actively in steroidogenesis, it seems very probable that an abnormal expression of these genes may address the overproduction of specific steroid hormones in ACT and therefore a characteristic phenotype[37, 46, 138, 166, 167, 169-172]. These studies have contributed to a better understanding of the molecular pathogenesis of ACT and additionally to improve the diagnosis of certain clinical subtypes of hyperfunctioning ACT such as APA and CPA[169, 171-173]. However, due to the heterogeneous gene expression, its routine use for clinical diagnosis is still a matter of debate[54, 173]. In contrast, some specific genes have been related to ACC, such as *IGF2* and *FGF12*[81, 138, 144, 145]. In ACC, also other genes have been suggested to be

deregulated such as *WNT*, *ALDH1A1*, *DLG7* and *PINK1*[81, 138, 149, 151, 174]. In total, it seems that the malignant genetic signature is the more relevant, or useful, transcriptomic feature among ACT[81, 138, 149]. A large part of the human transcriptome consists of non-coding RNAs. Recently, it was demonstrated that these RNAs play an important role in the regulation of gene expression[175-177]. MicroRNAs (miRNAs) represent a family of small non-coding RNA molecules and probably represent the largest proportion of regulatory RNAs. These molecules may affect the functional properties of almost 30% of the protein-coding genes[176]. Although the functional roles of many miRNA molecules are still unknown, they are involved in diverse physiological and pathological processes such as tissue development, cell differentiation, apoptosis, immune response and particularly tumourigenesis[177-179]. By microarray platforms it has been demonstrated that miRNA are differentially expressed between normal and some types of tumour tissues[177, 180]. However, the link between miRNA expression and tumourigenesis is still far from being completely understood.

Moreover, few examples of the implication of these molecules in ACT have been reported[181-183]. Two separate groups[184, 185] have demonstrated the up-regulation of *miR-483* in ACC as a potential biomarker. Additionally, the role of miRNA in the regulation of steroidogenesis in ACT has been documented, with a possible function on cell proliferation[186]. Although many of these studies have demonstrated the effect of some miRNA in steroidogenesis in ovarian granulosa cells[187-189], most probably the effect of these miRNAs are similar in other steroidogenic tissues such as adrenal gland, placenta and brain. Among these miRNAs, *miR-483*, one of the most investigated miRNA in ACC, has been also associated to progesterone concentrations[190]. Other miRNAs such as *miR-184, miR-195, miR-7, miR-503, miR-511* and *miR-335* have also been documented as deregulated in ACC[184, 185, 191-193]. Furthermore, in one study[193], two miRNAs (*miR-503* and *miR-511*) have been proposed as a tool for the differentiation of ACA and ACC tumours with 100% sensitivity and 93% specificity. On the other hand, *miR-483* has demonstrated a 100% of positive predictive value and 92% of negative predictive value for the ACC diagnosis[191]. Although these findings should be validated in larger cohorts, these results are promising for a better understanding of ACC development.

2 AIMS

I. General Aims

1. To explore by means of transcriptomic profiling (*mRNA and miRNA*) the differences between ACT regarding the malignant and benign phenotypes.
2. To explore by means of transcriptomic profiling (*mRNA and miRNA*) the differences between ACT regarding the hyperfunctioning and non-hyperfunctioning phenotypes.

II. Specific Aims

1. *Paper I.* - To determine if the expression profiling based on mRNA from ACT could provide for a distinction between ACA and ACC.
2. *Paper II.* - To characterize mRNA expression profile in relation to the different subtypes of hormone producing and non-producing ACA.
3. *Paper III.* - To determine if the expression profiling based on miRNA from ACT could provide for a distinction between ACA and ACC.
4. *Paper IV.* - To characterize miRNA expression profile in relation to the different subtypes of hormone producing and non-producing ACA.

3 MATERIAL AND METHODS

3.1 Patients and Tumour Material

This thesis is based on transcriptional analysis of snap-frozen adrenocortical tissues obtained from a cohort of patients diagnosed and managed at the Department of Surgery, Karolinska University Hospital. The cohort includes more than 300 patients operated at this medical institution from 1986 to 2011. All included patients were biochemically screened and followed up for at least 1.5 years in their postoperative period. Every included sample was verified by histopathology according to the WHO classification[194]. A cellular representation equal or above 70% of tumour cells within each sample was required as an inclusion criterion. All APA cases included were confirmed with immunohistochemistry as described elsewhere[96]. Aldosterone-secreting hyperplasia or non-solitary APAs were discarded. Diagnostic criteria for ACC were vascular invasion, invasion of surrounding tissues or structures and distant metastases. Sample sizes were variable across papers:

Paper	Normal Cortices	Benign ACT	ACC
I	0	13 (5 ACA, 4 APA and 4 NHFA)	7
II	4	17 (5 ACA, 4 APA and 8 NHFA)	11
III	10	43 (13 ACA, 16 APA and 14 NHFA)	25
IV	10	43 (13 ACA, 16 APA and 14 NHFA)	0

Table 2. General description of the number of samples and their functional phenotypes included in the published papers considered for this thesis.

General clinical and pathological features of the included cohort of patients/samples are included in the corresponding papers. For the miRNA inhibition and overexpression assays a commercially available cell line of ACC (NCI-H295R) from the American Type Culture Collection (ATCC# CRL-2128; LGC Standards, Middlesex, UK) was used. These cells were maintained in a DMEM:F12 medium, containing 2.5% of Nu-Serum serum replacements (BD Biosciences, Bedford, MA), 1% of a mixture of penicillin and streptomycin and 1% sterile-filtered ITS+1 liquid media supplement (100X) at 37^{o}C with a 5% of CO_2. The ITS+1 liquid media (Sigma-

Aldrich Logistik GmbH, Schnelldorf, Germany) contains 1.0 mg/ml of recombinant human insulin, 0.55 mg/ml of human transferrin and 0.5 μg/ml of sodium selenite. The authentication of this cell line was also evaluated and verified by Bio-Synthesis, Inc. (Lewisville, TX). For this purpose a genotyping of 15 short tandem repeat loci and the amelogenin gene (*AMEL*) were used. Comparisons were realized to the genotype information from the ATCC.

3.2 Methods for mRNA studies

3.2.1. mRNA isolation and purification

Total RNA was isolated from mechanically homogenized adrenocortical frozen tissues (at least 30 mg of fresh or frozen tissue) by means of organic extraction using Trizol and purification by Qiagen Columns (RNeasy mini kits, www.qiagen.com) using standardised protocols[195, 196]. This kit provides a fast purification of high-quality RNA from tissue human cells by means of a silica-membrane included into spin columns with a binding capacity of at least 100 micrograms of total RNA. Additionally, this kit allows an efficient purification of total RNA from relatively small amounts of starting material (0.5 mg of tissue). The method simplifies the RNA isolation combining the stringency of guanidine-isothiocyanate lysis with the speed and purity of silica-membrane isolation. Once samples are lysed with a commercial buffer (lysis buffer or RLT), 70% pure (molecular grade) ethanol is added to the lysate in order to provide an ideal binding condition. This lysate plus ethanol is then loaded onto the RNeasy spin columns and centrifuged by about 3 min at full speed in a microfuge. Supernatant was removed by pipetting and then transferred to a new micro centrifuge tube (Eppendorf tube). This lysate produces a RNA-silica binding in up to 100 micrograms capacity. Subsequently several washes are repeated in order to eliminate the contaminants and salts by means of commercial buffers (RW1 and RPE). After this decontamination a technically pure RNA bind into the silica is eluted through the use of 30-100 microliters of RNase-free sterile water or TE buffer. A ND-1000 spectrophotometer (Nanodrop, San Francisco, CA, USA) and a 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA, USA) were used for assessing the quality and relative quantity of total RNA obtained in these extractions. A260/280 ratios from 1.7 to 2.1 are ideal for further applications of this total RNA.

3.2.2. Complementary DNA (cDNA) microarray

Complementary DNA oligoarrays produced by KTH were used for mRNA expression profiling, following their general standard protocols (more details can be found at www.ktharray.se). These produced microarrays consisted of 29 760 cDNA fragments that were spotted onto an ultra GAPS slides (Corning, Corning, NY) with a QArray (Genetix, New Milton, UK). These cDNA microarrays contained approximately 29 760 cDNA fragments representing 18 953 unique human UniGene IDs. Complementary DNA clones were taken from the sequence verified human clone collection, 97001.V (Research Genetics, Inc., Huntsville, AL, USA). Microarray was elaborated completely at the KTH by spotting onto Ultra GAPS crystal slides (Corning, Lowell, MA, USA) with a Qarray2 instrument with 48 K2805 pins (Genetix, Hampshire, UK) in a 25 x 25 pattern within each of the 48 blocks and with a feature centre-to-centre distance of 170 µm. The average size of the spots is approximately 110 µm.

This array also includes 23 different controls with 8 copies of each one. Complete versions of these protocols are available at www.ktharray.se and at Array Express. Quality of spotted slides was assessed by Syto61 staining (Molecular probes, Eugene, OR, USA) and random nonamer hybridization. The slides were UV cross-linked at 250 mJ/cm^2 followed by baking at 75°C for 2 hours. A complete gene list can be found at www.biotech.kth.se/molbio/microarray.

First strand cDNA target was generated by reverse transcription of 10 µg of total RNA in a 30 µl reaction using 10 µg anchored oligo dT primer (dT20VN, MWG Biotech AG), first-strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 0.01 mM DTT, 400 units Superscript II (Invitrogen AB), 2 mM d(A/G/C)TPs, 1.6 mM dTTP (Amersham Biosciences) and 0.4 aminoallyl-dUTP (Sigma-Aldrich). Secondary structures of RNA and primers were removed at 70 °C during 10 minutes followed by 2 minutes of incubation on ice for primer annealing before addition of the other reaction components. Complementary DNA synthesis was performed during 2 hours at 42 °C. This reaction was terminated by addition of EDTA. The RNA was then hydrolysed in NaOH during 15 minutes incubation at 70°C followed by neutralization with HCl (final concentrations 20 mM, 150 mM

and 150 mM, respectively). The cDNA was purified further using a MinElute spin columns (Qiagen GmbH) with the provided was buffer replaced by 80% ethanol molecular biology grade and the elution buffer by 100 mM $NaHCO_3$, pH 9.0. Monofunctional NHS-ester Cy3 or Cy5 flurophores (Amersham Biosciences) were coupled to the amino-allyl groups during 30 minutes incubation at room temperature (22°C) after which the unincorporated ester groups were inactivated through the use of hydroxylamine (final concentration 730 mM). Tumour samples were labelled with Cy5 and Cy3 as reference. The labelling reactions were purified using MinElute spin columns. The complete protocol is available at www.biotech.kth.se/molbio/microarray/index.html. Extraction and labelling quality and quantity of the total RNA and cDNA were evaluated using a nanophotometer and an Agilent bioanalyzer 2100.

Microarray slides were pre-hybridized in 5X SSC, 0.1% SDS and 1% bovine serum albumin (Sigma Aldrich) for 30 minutes at 42°C in a closed wet chamber. Hybridization buffer used for all these experiments were constituted with 25% formamide, 5X SSC, 0.1% SDS, 5X Dehardts, 20 µg poly (A) (Sigma Aldrich), 10 µg COT-1 DNA (Invitrogen), 5 µg tRNA (Sigma-Aldrich). Hybridizations were carried out in hybridization closed wet chambers (Cat number 2551, Corning) for 18 hours at 42 °C. Post-hybridization washing was necessary and carried out according to Corning's recommendations. All these hybridizations were performed in technical duplicates for decreasing the variance. All hybridizations were repeated at least twice (biological replicates) in an alternate way (benign and malign cases) for achieving an adequate statistical power.

Arrays were scanned at 532 and 635 nm with 10µm resolution through the use of a G2565bBA DNA microarray scanner (Agilent Technologies). Image analysis was performed by means of GenePix 5.1 software (Axon instruments). Defective spots were considered whenever less than 70% of the foreground pixels were below the background intensity plus 2 standard deviations in both channels or if signal to noise ratio were below 3 in both channels or if the difference between ratio of all medians and regression ratio exceeded the 20% in one of the channels. After the removal of these defective spots the remaining intensities were print tip LOWESS

normalized within R environment for statistical computing with the aroma package (www.maths.lth.se/publications).

Levels of gene expression were analysed in separate samples, tumour group (cluster of samples) and in comparison with the common RNA reference. Statistical analysis of these values (expressed as continuous dimensional data) was performed using the Aroma package for normalization and the LIMMA package for statistical testing of significance. Bayesian analysis and B-test for ranking on differentially expressed genes was also performed on the average of technical replicates on genes represented in all samples. This was performed using the Limma package. Clustering was performed with the stats and class packages (www.R-project.org). Unsupervised hierarchical clustering of all included genes for visualization of expression patterns was performed in MeV (www.tigr.org). Functional gene annotation was determined based on Gene Ontology (www.geneontology.org/GO.doc.html). Fisher's exact test or square chi test were used to evaluate the significant over-representation of GO terms and chromosomal location for differentially expressed genes among samples.

3.2.3. Validation by quantitative RT-PCR (qRT-PCR)

In order to validate the array findings for differentially expressed genes validation with quantitative real time polymerase chain reaction (qRT-PCR) was performed. A two-step TaqMan qRT-PCR was used for testing genes such as *ALDH1A1* (hs 00167445_m1), *IGF2* (hs 00171254_m1) *USP4* (hs 00234300_m1) and *UFD1L* (hs 00799945_s1). Synthesis of cDNA from total RNA was obtained using the high-capacity cDNA archive kit (Applied Biosystems). Approximately 0.5 to 1 μg of RNA was used from every included sample. An enzyme cocktail was added containing 10X reverse transcription buffer, 25X dNTP, 10X random primers, nuclease-free water and a MultiScribe reverse transcriptase (50 U/μl) in a final total volume of 50 μl. This reaction was load into 96-well plates into a GeneAMp PCR System 9700 thermal cycler (Applied Biosystems). The following conditions were used for the RTPCR: an initial hold phase of 25 °C for 10 minutes for incubation, 2 hold phases with 37°C for 60 minutes for reverse transcription, a fourth phase with 37°C for 7 minutes and a final phase with 4°C for 30 seconds. The plate was

immediately removed after completing cycling and store at -15 to 25°C for later reading and use. A 96-well plate was prepared also for the TaqMan gene expression assays (Applied Biosystems). This is a 5 prime fluorogenic nuclease assay, which quantifies the relative abundance of specific RNA transcripts. Every well approximately contained 12.5 µl of TaqMan universal master mix with 1.25 µl of primers plus probmix (Assays on Demand from Applied Biosystems), 6.25 µl of RNase-free water and 5 µl of cDNA or previously prepared dilutions (for the reference curve). Dilutions were prepared for 1:10, 1:100, and 1:10 000. Every sample and control well was done in triplicates and the housekeeping genes in duplicates according to manufacturer's recommendations. A non-template control (NTC) was included in every run. Plates were sealed with microAmp optical caps for avoiding leakage. A brief centrifugation (a couple of minutes) was done to spin down the contents and eliminate any air bubbles from the solutions contained in very well within the plate. Plates were then loaded into the ABI PRISM 7700 sequence detection system (Applied Biosystems). In order to select a housekeeping gene as a reference for the relative quantification, the TaqMan human endogenous control plate was used. With this plate, the expression of 11 selected housekeeping genes was evaluated, using a two-step RTPCR. The plate also features a unique internal positive control (IPC) designed to detect the presence of PCR inhibitor among test samples. Analysis of the following housekeeping genes was performed: an internal positive control, 18S rRNA, acidic ribosomal protein, beta-actin, cyclophilin, glyceraldehydes-3phosphate dehydrogenase, phosphoglycerokinase, beta2-microglobulin, beta-glucoronidase, hypoxanthine ribosyl transferase, transcription factor IID, TATA binding protein and the transferrin receptor.

3.3 Western blot analysis

For the mRNA based studies, fresh frozen tissue samples were initially grounded into powder by mortaring on dry ice and subsequently dissolved by sonication in a buffer containing 50 mM Tris (pH 7.4), 5 mM $MgCl_2$, 250 mM sucrose, 1 mM DTT and 1 mM ATP. Total protein extractions were then clarified by centrifugation and the lysates were snap frozen in liquid nitrogen. These aliquots were preserved in ultra-freezer until further use. For the Western blot analysis approximately 20 µg

of the protein aliquot preserved previously was used. This sample was fractionated on SDS-PAGE 4-12% gradient gels (Novex©, Invitrogen, Life Technologies), and blotted on PVDF membranes, blocked in a PBS buffer supplemented with 5% skim milk and 0.1% Tween® detergent. Then an aliquot of this sample was probed with an anti-*Usp4* antibody (C-terminal; Abgent Inc., San Diego, CA, USA) with a dilution of 1:500. Several buffer washings was followed by incubation with HRP-conjugated goat anti-rabbit serum (Amersham Biosciences). A positive control for the *Usp4* antibody was transfected human embryonic kidney 293T cells with a plasmid expressing *USP4* in fusion with green fluorescent protein and harvested these cells for western blot analysis after 2 days of transfection. All protein blots were developed by enhanced chemiluminescence method (ECL™ Western Blotting Detection System; GE Healthcare (Uppsala, Sweden). Solutions for the development were equilibrated to at room temperature and mixed in equal volumes directly before their use. Fast exposure to film was required for optimal signal detection.

For the microRNA (miRNA) based studies, total proteins were extracted from whole cell lysates obtained from adrenal references and ACT samples through homogenization within a NP40 Cell Lysis buffer (Invitrogen, Life Technologies), with the previous addition of a protease inhibitor mix (Complete protease inhibitor cocktail; Roche Diagnostics Corporation, Indianapolis, IN) and 1 mM of phenylmethanesulfonyl fluoride (Sigma-Aldrich). Protein quantification was performed by a colorimetric assay (Bio-Rad Protein Assay Kit; Bio-Rad© Laboratories). Approximately 60 µg of the lysate was separated using the Novex® 10% Tricine gels (Invitrogen, Life Technologies, Carlsbad, CA) and transferred into nitrocellulose membranes (Invitrogen). The Novex® Tricine gel system is a modification of the Tris-glycine gel system and allows a better resolution of the low molecular weight proteins. Filters were blocked with 5% skim milk diluted in TBS buffer with 0.1% Tween 20 Then they were incubated with anti-PUMA antibodies (Cell Signalling Technology) at a 1:1000 dilution, followed by an anti-rabbit IgG-HRP at a 1:3000 dilution (Bio-Rad© Laboratories, Hercules, CA) used as secondary antibody. Subsequent incubation of the filters with a GAPDH antibody (Santa Cruz Biotechnology Inc.) diluted at 1:5000 was performed for normalization purposes as a control. Blot detection was carried out with Novex ECL HRP chemiluminescent

substrate reagent (Invitrogen). The Novex Sharp Pre-stained protein standards (Invitrogen) and a Magic Mark™ XP (Invitrogen) western blot protein standard were used. This standard allows the direct visualization of different protein standard bands on a blot without the need for protein modification or special detection reagents. The range of this standard is from 20 to 200 kDa proteins. The protein levels were quantified on X-ray films from immunoblots using the Image J software (http://rsb.info.nih.gov/ij/).

3.4 Methods for microRNA (miRNA) based studies

3.4.1. MiRNA isolation and purification

For studies of miRNA expression, experimental and analysis procedures previously established in our group were used[197]. Total RNA was isolated using standard *mir*Vana™ miRNA isolation kit (by Ambion) following the protocol recommended by the manufacturer. This kit utilises a combination of an organic with a solid-phase extraction of RNA with high yield, ultra-purity and high quality results. Fresh or frozen tissue samples were slightly unfrozen and mechanically homogenized. At least 30 mg of adrenocortical tissue (either normal or pathological) were used in every extraction. A representative tumour cell population was assessed by histopathology every time RNA or DNA was extracted and purified. Tumour tissue homogenization was performed with the fragment immersed in denaturing lysis buffer. Before any extraction was done, the bench, pipettors and all surfaces were cleaned with RNaseZap© Solution (Ambion, Life Technologies) and 70% ethanol for decontaminating RNase or other contaminating molecules by simply spraying and wiping.

After disrupting the sample, this was subjected to acid-phenol chloroform extraction for improving the purification of the RNA and cleansing off the genomic DNA. For this, several aliquots were obtained with approximately 10 volumes per tissue mass of lysis-binding buffer into the homogenization tube (included in the kit). During all the disruption process, the tubes were kept submerged on crushed ice for keeping the sample cold. Disruption was continued until no clumps or tissue fragments were visible. The mixture was transferred to a new and sterile Eppendorf

tube and added 1/10 volume of miRNA homogenate additive (included in the kit) mixing well by vortexing by approximately 2-3 minutes. The sample was left on ice for 10 minutes and then the acid-phenol chloroform was added in an equal volume to the total lysate volume obtained in previous phases. Samples were vortexed for 30 to 60 seconds in order to homogenize the contents and then centrifuged for 5 minutes at maximum speed (10 000 x g) at room temperature. After centrifugation two phases were required to be noted: aqueous and organic phases, in the case these were not clear, the centrifugation was repeated. The upper aqueous phase was carefully removed with a pipette without disturbing the lower organic phase for avoiding contamination. Aqueous phase was then transferred to a new sterile Eppendorf tube and the organic phase was discarded. Then 100% ethanol (molecular grade and at room temperature) was added 1/3 volume to the aqueous phase recovered from the organic extraction. Sample was mixed thoroughly by vortexing for a couple of minutes. This lysate plus ethanol mixture was pipetted onto the filter cartridge (included also in the kit) up to 500 µl. The tube was centrifuged at 10 000 rpm for approximately 15 seconds in order to pass the mixture through the filter. The filtrate was collected and room temperature 100% ethanol (molecular grade) was added 2/3-volume. This mixture was pipetted and applied to the filter cartridge. This was centrifuged at 10 000 rpm for approximately 15 seconds. The flow-through was discarded and applied 500 µl of miRNA wash solution 1 to the filter cartridge and centrifuged for approximately 10 seconds. Approximately 500 µl of wash solution 2/3 was applied two times and repeated the centrifugation. After discarding the flow-through from the washes, the filter cartridge was transferred into a fresh collection Eppendorf tube and applied 100 µl of pre-heated (at 95 °C) elution solution to the centre of the filter carefully with the pipette and close the cap. Sample was centrifuged at 10 000 rpm for approximately 20-30 seconds in order to recover the enriched RNA. Samples were stored at least at -20°C until their use in further steps.

RNA was always assessed through the use of a spectrophotometer. A ND-1000 spectrophotometer (Nanodrop, San Francisco, CA, USA) was used for assessing the quality and relative quantity of total RNA obtained in these extractions. A260/280 ratios from 1.7 to 2.1 are ideal for further applications of this total RNA.

3.4.2. MiRNA global profiling microarray

Subsequently, samples were labelled with Cyanine 3-pCp and hybridized at 55°C for 22 hours to previously prepared Agilent oligoarrays for miRNA global profiling following a standardized protocol. This microarray contained 903 human miRNA obtained from the Mir Base release 14. After a washing phase, the arrays were scanned immediately into an Agilent scanner G2565BA. Processing of images was done using Feature Extraction Software v10.7.3.1 (Agilent). Through the use of Cluster 3.0, raw data was normalized and centred the raw data based on medians. Once miRNA were normalised, miRNA with a limited proportion (for example <50%) of missing calls were included in subsequent statistical comparisons between ACT subgroups (functioning or malignant phenotype). Significance Analysis of Microarrays (SAM) included in the multi-experiment viewer software (TM4 MeV version 4.8) was used for contrasting phenotypes. In order to reach adequate stringency for the statistical inference, a false discovery rate equal or less than 5% or 0.05 was set up.

3.4.3. Validation by quantitative RT-PCR (qRT-PCR)

Finally selected miRNA were subsequently further validated using commercially available TaqMan® (Applied Biosystems) qRT-PCR microRNA Assays (www.appliedbiosystems.com). All 43 ACAs and 10 adrenal references were included for this qRT-PCR validation. The expression levels of 14 selected mature miRNAs were measured: *miR-21* (ID 000397), *miR-186* (ID 002105), *miR-497* (ID 001043), *miR-210* (ID 000512), *miR-10b* (ID 002218), *miR-320b* (ID 002844), *miR-320c* (ID 241053_mat), *miR-30e* (ID 000422), *let-7f* (ID 000382), *miR-139-5p* (ID 002289), *miR-195* (ID 000494), *miR-1274b* (ID 002884), *miR-34a* (ID 000426) and *miR-520d-3p* (ID 002743).

The levels of the expression of 4 miRNAs (*miR-21*, *miR-210*, *miR-195* and *miR-497)* were also quantified and compared across the same ACT samples in *paper III*. *RNU6B* (ID 001093) was used as an endogenous control for normalization of the miRNA expression level. The relative expression quantification was performed by the ΔC_T method and calculated as $2^{-\Delta CT}$. All reactions were run in triplicates. Statistical analysis was performed through the use of IBM® SPSS® Statistics software version 20.0 (IBM Corp., Chicago, IL) and Excel 2011of Microsoft

Office. Unpaired Student t-test for independent samples and one-way ANOVA were performed to compare differences in miRNA expression levels between studied groups. Other variables were analysed according to their scaling using chi-square test for categorical variables and t-test for dimensional variables. All hypotheses were two-sided tests, considering any p value ≤ 0.05 or 5% as statistically significant.

3.4.4. MiRNA inhibition and overexpression assays

For this purpose a commercial line of ACC cells (NCI-H295R) was used. These cells were transfected using the Amaxa Nucleofector® technology (Amaxa Biosystems, Gaithersburg, MD). This is a commercially available and highly efficient non-viral transfection method. Approximately $3x10^6$ cells were suspended in 100 µl of the Nucleofector solution and mixed with 100 picomoles of miRNA inhibitors (anti-miR-483-3p or anti-miR-483-5p; Applied Biosystems/Ambion) or miRNA precursors (pre-miR-195 or pre-miR-497; Applied Biosystems/Ambion). An anti-miR negative control#1 or pre-miR Negative control#1 (Applied Biosystems/Ambion) containing a non-targeting sequence molecule were used as a negative control. Cells were then electroporated using the program T-20, allowed to recover in DMEM: F12 cell culture media for 15 min at 37°C and seeded in T-25 flasks with 5 ml DMEM: F12 cell culture media. Culture media was replaced after 24 hours and cells were cultured for additional 72 hours. The transfection efficiency was determined by measuring the endogenous *miR-483-3p*, *miR-483-5p*, *miR-195* or *miR-497* expression levels by qRT-PCR. All transfection experiments were performed in triplicates.

3.4.5. Cell proliferation assay

A colorimetric assay using the cell proliferation reagent WST-1 (Roche Applied Science, Mannheim, Germany) was used in order to determine the inhibitory or proliferative effects of some miRNA on cell proliferation. This assay allows the non-radioactive, spectrophotometric quantification of cell proliferation and growth of cell lines. The proliferative effect of *miR-483-3p* and *miR-483-5p* and the inhibitory effect of *miR-195* and *miR-497* on cell lines were separately assessed. This assay was performed using 96-well plates, with approximately 20 000 cells

containing in each well. After 72 hours of transfection, 20 µl of the WST-1 solution were added to the culture medium and incubated for 2.5 hours at 37°C. The absorbance was subsequently determined using a VersaMax™ microplate reader (Molecular Devices, Sunnyvale, CA). All raw data were further collected and analysed with SoftMax® Pro Version 5 software (Molecular Devices) applying the wavelengths 450 nm for measurements and 650 nm as reference. All experiments were conducted in 8 wells for each condition, and replicated at least three times. Cell proliferation was calculated by comparing the absorbance values between the samples after background subtraction. The fraction of surviving cells was calculated by defining the anti-miR (or pre-miR)-Negative control #1-treated cells as 1.

The WST-1 cell viability assay measures the metabolic activity in cell populations such as the MTT and the XTT methods. The WST-1 solution is added to the cells cultured, followed by incubation. During the incubation lapse, the WST-1 is converted into a coloured, soluble formazan salt (a water-insoluble coloured azo compound formed by reduction of a tretazolium salt) by the metabolic activity of viable cells. Then, the quantity of formazan obtained is quantified by an ELISA method by a microplate reader at 420-480 nm. This method does not require a cell transfer, is entirely performed in a single microplate with a ready-to-use solution. The main limitation of the WST-1 assay is that this method is not lineal over a broad logarithmic cell proliferation range due to the ELISA plate reader.

3.4.6. Apoptosis assay

The Caspase-3 colorimetric assay kit (Genscript, Piscataway, NJ) was used to evaluate the effects of *miR-483-3p* and *miR-483-5p* inhibition or *miR-195* and *miR-497* overexpression on cell apoptosis. This method allows the activity of caspase-3 through the spectrophotometric detection of the chromophore pnitroanilide (pNA) after its cleavage from the labelled substrate DEVD-pNA. The pNA is quantified in a microtiter plate at 400 or 405 nm. Comparing the measured absorbance of pNA between an apoptotic sample or non-apoptotic control the apoptotic (caspase-3) activity is relatively quantified. This kit includes 15 ml of lysis buffer, 5 ml of a 2X reaction buffer, 500 µl of a caspase-3 substrate and 150 µl of DTT.

The assay was performed with approximately $3x10^6$ cells into T-25 flasks. After 72 hours of transfection, proteins were isolated and quantified by Bio-Rad© Protein Assay (Bio-Rad© Laboratories). About 50 µl of lysate containing 50 µg of protein was mixed with 50 µl of 2X Reaction Buffer and 5 µl of caspase-3 substrate. This mixture was then incubated at 37°C for 4 hours. The absorbance was subsequently determined using a VersaMax™ microplate reader (Molecular Devices, Sunnyvale, CA) and analysed also with SoftMax® Pro Version 5 software (Molecular Devices) applying the wavelengths 405 nm for measurement. Apoptosis was calculated by comparing the absorbance values of the anti-miR/ pre-miR treated cells with the respective negative control treated cells. All the experiments were performed in triplicates.

3.5 Statistical Methods

In general terms, mRNA and miRNA expression levels are referred to as a quantitative dimensional scale (represented as mean ± standard deviation), even though medians may be used for normalisation. Proper statistical tests for contrasting two or more means are the unpaired t-test for independent samples, ANOVA and the non-parametric version of this: the Kruskal-Wallis test. In an experiment of multiple hypotheses testing, an adjustment for the regular p value is compulsory. Transcriptomic profiles include hundreds or thousands of mRNA/miRNA expression levels at the same time. Significance analysis of microarrays (SAM) is a statistical technique developed for discerning differentially expressed genes among samples[198, 199]. This allowed the possibility for contrasting many hundreds or thousands mRNA/miRNA by multiple t-tests, measuring the strength of the relationship between mRNA/miRNA expression and the contrasting phenotypes (regarding functionality or malignancy). Repeated permutations are frequently used in this technique in order to investigate the different mRNA/miRNA combinations regarding the phenotypes. The use of this method allows looking for correlations among mRNA/miRNA avoiding parametric assumptions (such as a Gaussian distribution) of separate mRNA or miRNA. SAM uses a false discovery rate (FDR) and fold change for establishing differences between mRNA/miRNA expression levels regarding the tumour phenotype. Once a

FDR cut-off value is set up, a list of significant or differentially expressed mRNA/miRNA can be applied. All statistical analysis was performed through the use of IBM® SPSS® Statistics software version 20.0 (IBM Corp., Chicago, IL) and Excel 2011of Microsoft Office, this last mainly for data representation and graphics. Linear correlation was also used by means of Pearson or Spearman tests. Other variables were analysed according to their scaling using chi-square test for categorical variables and t-test for dimensional variables. Any p value equal or less than 0.5 or 5% was considered significant for a two-tied hypothesis testing.

4 RESULTS AND DISCUSSION

4.1 mRNA Expression Profiles of ACC and ACA (*Paper I*). -

Expression profiling of adrenocortical tumours suggests a molecular signature of malignancy. The spotted cDNA oligo-array aforementioned by competitive hybridization was used in this study. This microarray was made and processed at the KTH which explored more than 25 000 cDNA fragments obtained from a verified and sequenced human clone collection (97001 V). In this very first approach, transcriptomic expression profiles between 7 ACC and 13 ACT were compared. Several issues were taken into account, such as histopathological representative proportion of tumour cells, hybridization duplicates and sufficient clinical follow-up in all patients.

Transcriptional profiling among the benign ACTs was more homogenous than among ACCs. Moreover, a set of stochastic expression levels for genes made it possible to categorise benign ACT from ACC clearly (Figure 1 in paper 1). In this heat map dendogram, the genes were clustered with the use of Euclidean distances and average linkage to provide an overview of the general pattern. The expression profiles of adenomas were observed homogenous while the ACC profile was heterogeneous. When clustering these samples, adenomas and ACC were clustered separately.

Additionally, several genes were significantly up regulated in ACC such as *USP4*, *IFD1L* and *IGF2*. Interestingly, several genes related to the IGF family were up regulated such as *IGF2*, *IGF2R*, *IGFBP3* and *IGFBP6*. This group of genes were good candidates for a better clinical classification between ACC and benign ACT (Figure 2 in Paper 1).

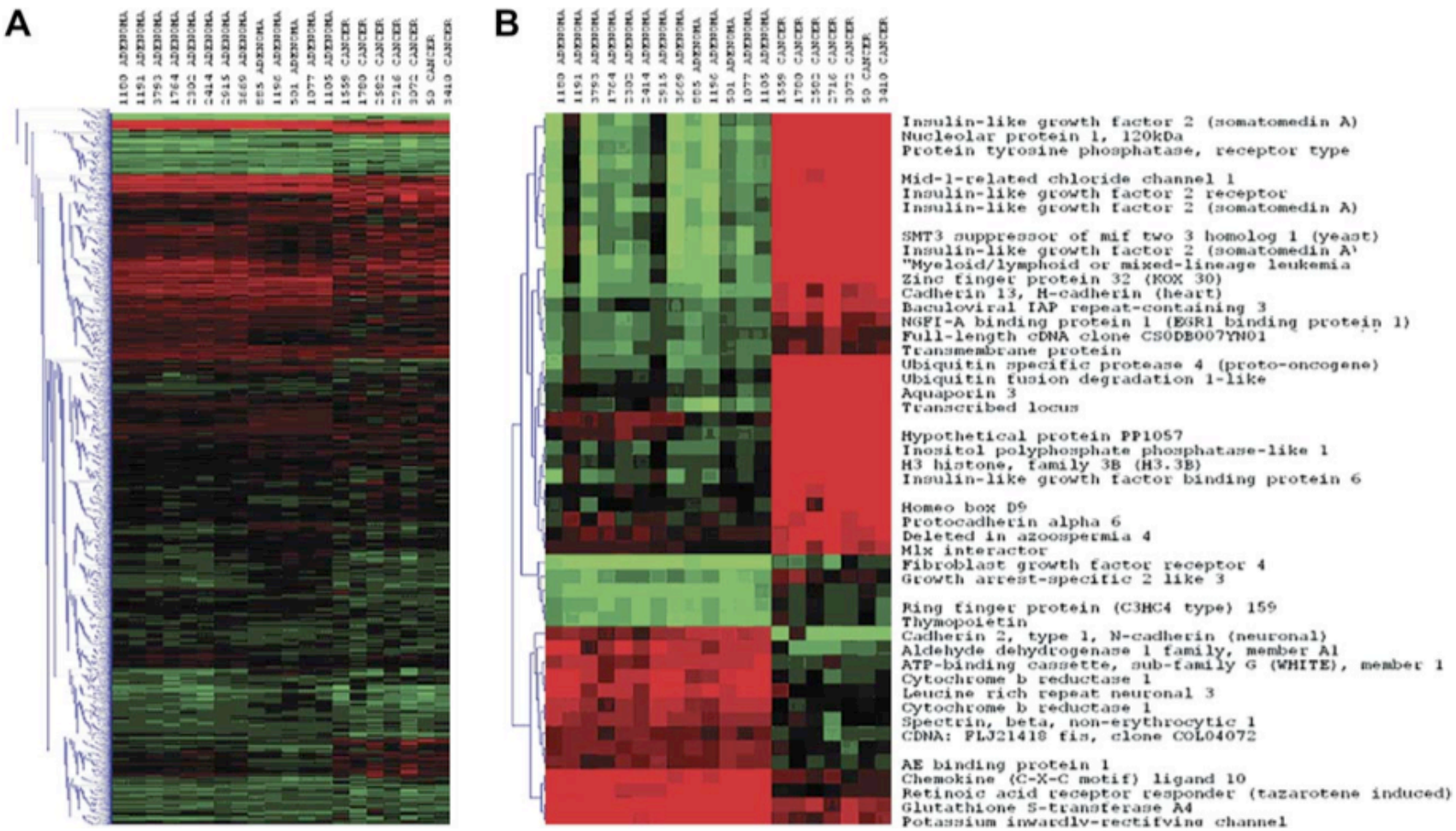


Figure 8. Heat maps representing the entire set of genes that clustered hierarchically among ACT. A, whole microarray between ACA and ACC. B, Top 50 differentially expressed genes between ACA and ACC.

Moreover, many of the 571 deregulated genes found in ACC were functionally localized in the nucleus according to GO tools. Chromosomal location was also performed through the use of Locus Link. With this tool a significant over-representation in chromosomes 5 and 12 were determined for up regulated genes mainly in ACC.

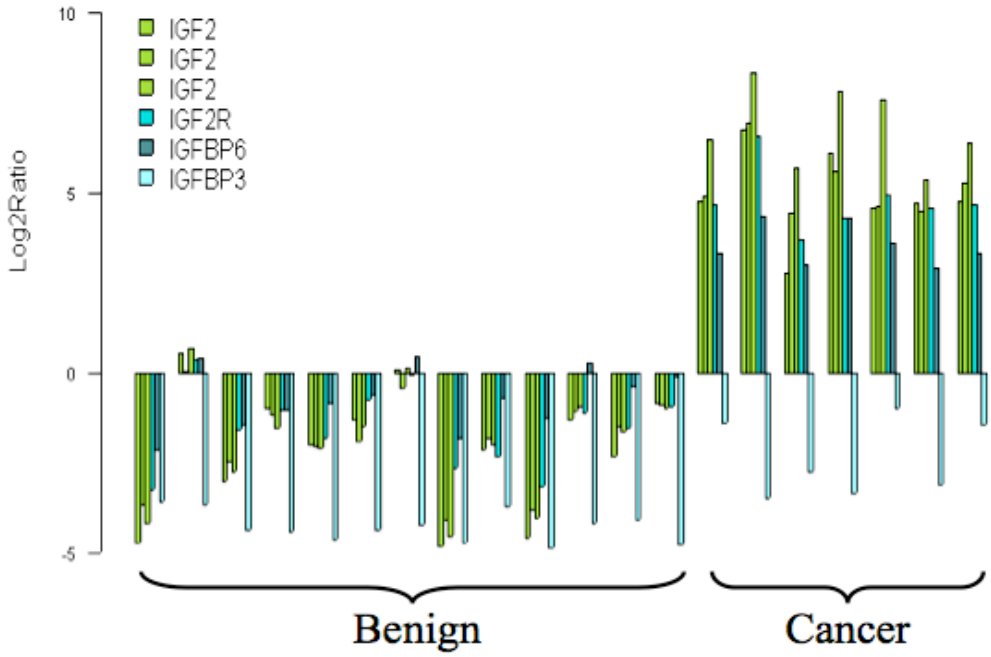


Figure 9. Histograms representing the differentially expressed levels of IGF-related transcripts between benign ACT and ACC.

Our results at this point showed that the transcriptomic profile was different between ACC and ACA. Some specific mRNA such as USP4 showed to be up regulated at least 40 times more in ACC than ACA. An important group of these up regulated genes included members of the IGF family. One of these, IGF2 was the most consistently found up regulated in ACC. It seemed evident that through the use of transcriptomic profiling ACA can be confidently discerned from ACC. Additionally, a molecular signature was feasible to construct.

In conclusion, this first paper showed the main differences between ACC and benign ACT at their intrinsic transcriptomic level. The transcriptional profiling allowed the discrimination of ACC from other ACT, even from a limited amount (the 50 most significantly) of differentially expressed genes. Despite the fact that cDNA arrays may be expensive, a simple qRT-PCR of a molecular signature composed by a limited amount of genes is not only feasible but also germane into the clinical scenario.

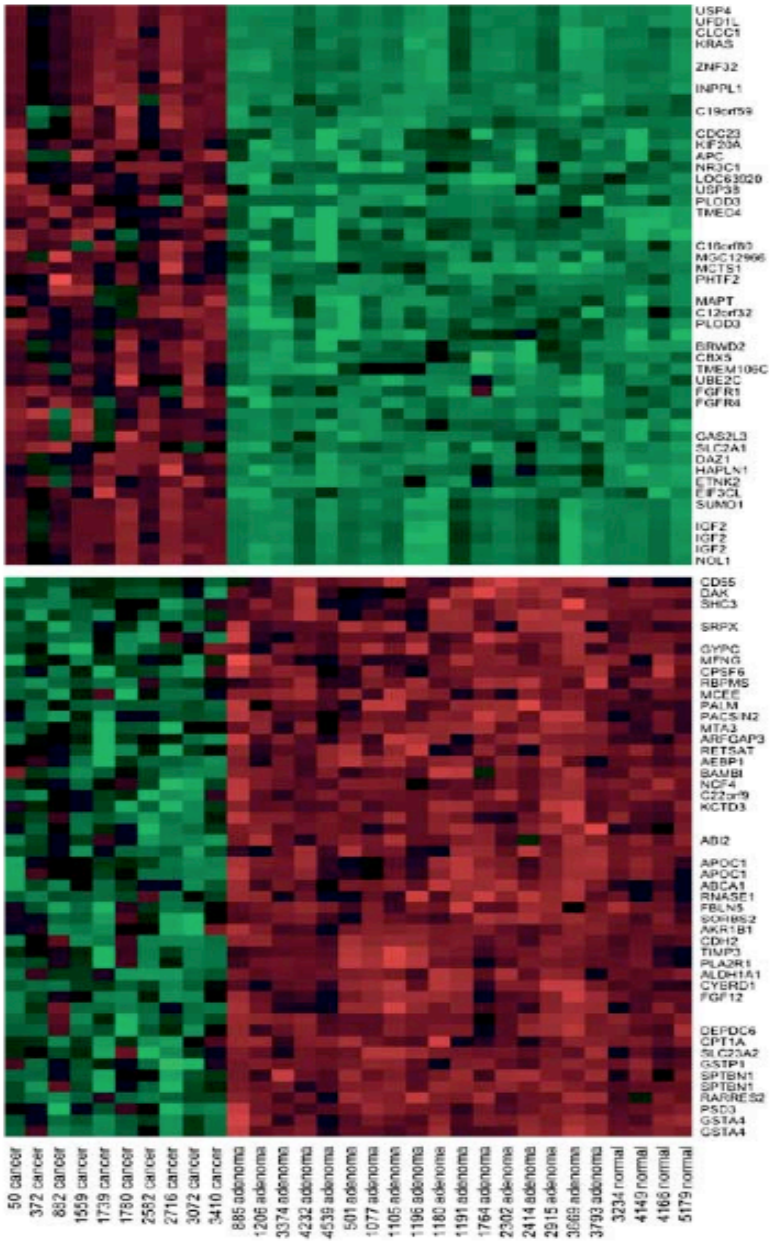


Figure 10. Heat maps representing the expression profiles of the 50 top most up- (red) and down-regulated (green) genes between ACA and ACC.

4.2 mRNA Expression Profiles of ACT Subgroups (*Paper II*). -

Transcriptional profiling enables molecular classification of adrenocortical tumours. The primary aim of this study was to better classify ACC and ACT based on their hyperfunctioning phenotype and to explore mRNA expression's implication on survival. Using the same technology (transcriptomic-based microarrays) the transcriptional profile of 17 ACA, 11 ACC and 4 normal adrenal cortices were determined. Findings were validated by RT-PCR and western blot analysis. A molecular signature comprising 50 genes was sufficient for dichotomising ACC from benign ACT and normal adrenal cortices (Figure 2 in paper II).

The discrepancy based on the transcriptomic profiled between ACC was once again striking; moreover subgroups within the ACA phenotype were noted. NHFA, APA and CPA clustered separately, even though normal references clustered with CPA. Some differentially expressed (DE) genes such as *VEGFB* and *OSBP* were related exclusively to APA and not with CPA, normal references or NHFA.

Two groups of genes especially up regulated in ACC were further validated; growth factor signalling and ubiquitin-proteasome pathway related genes. Furthermore, two subgroups of ACC with different survival outcomes based on their transcriptional profiles could be discriminated. Certain specific genes were up regulated in some specific phenotypes among ACT, such as *OSBP* and *VEGFB* in APA.

Additionally, principal component analysis and unsupervised clustering of expression profiles made it possible to discriminate between benign and malignant phenotypes in ACT (Figure 1 in Paper II). Normal references clustered together, in a similar manner as other ACTs. The normal cases clustered with benign adenomas, mainly CPAs or NHFAs.

In addition, two subgroups of ACCs were determined based on their transcriptomic profile. These subgroups behaved differently regarding their clinical survival: a short-survival group (equal or less than 9 months) and another with long-survival (more than 67 months). This fact conducted the hypothesis that this transcriptomic profile (Figure 5A in paper II) might be used for prognostic purposes among these patients (Figure 5B

in paper II). Currently, there are very limited features related to ACC, which are able to have a predictive value on the survival time in these patients.

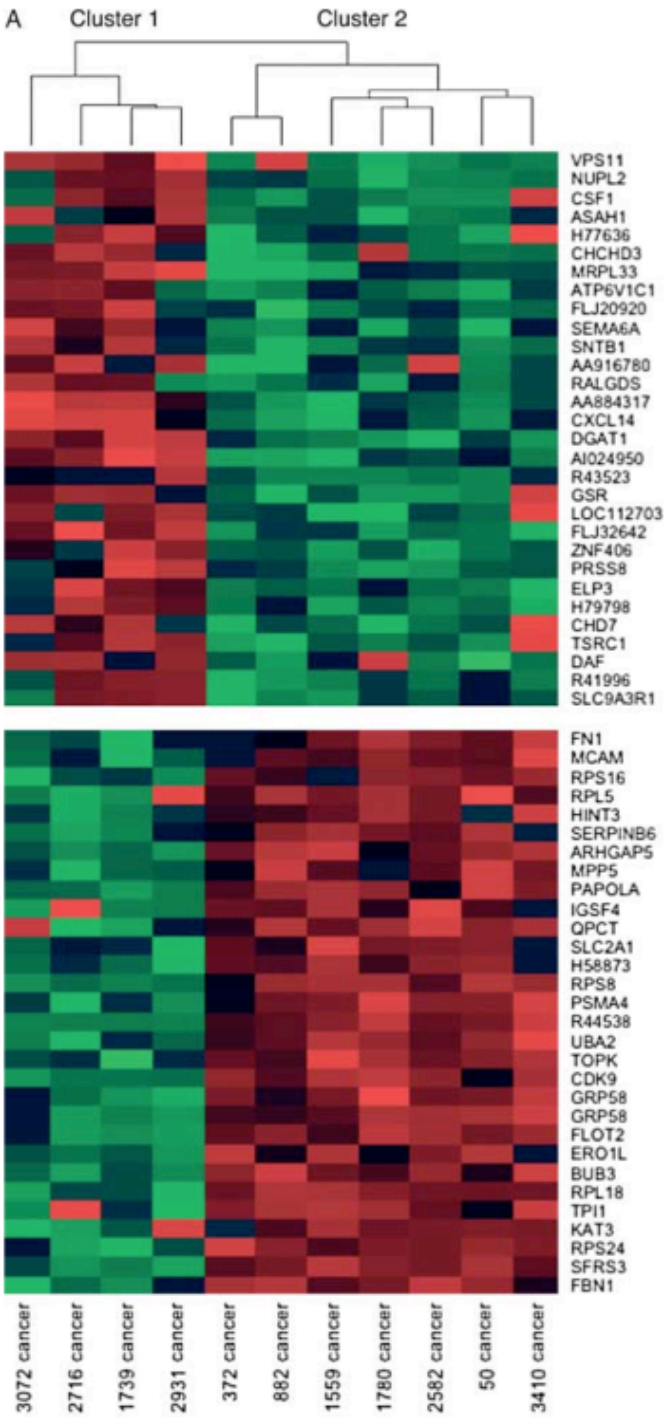


Figure 11. Heat maps representing the expression profiles of the most up- (red) and down-regulated (green) genes between two subsets of ACC.

In conclusion, paper II focused on the hyperfunctioning phenotype and prognostic discrimination in ACA and ACC respectively. This goal was achieved by the use of the transcriptomic profile and some specific genes such as *OSBP* and *VEGFB* for APA.

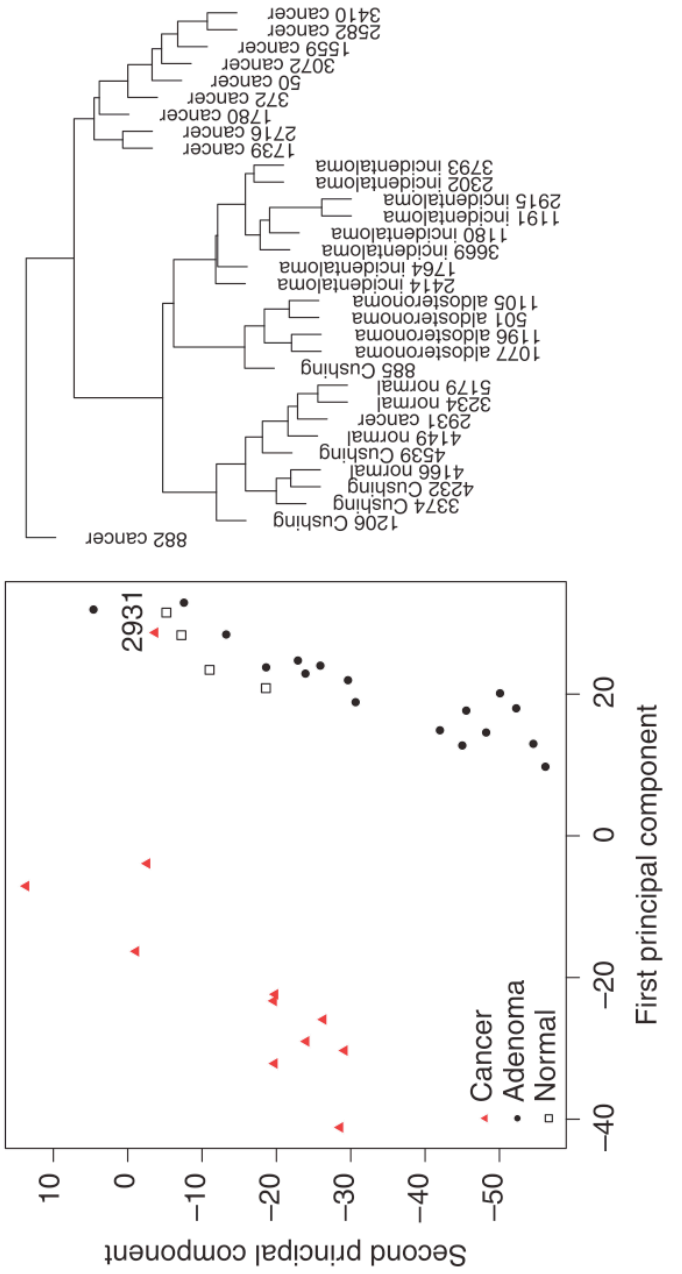


Figure 12. Unsupervised hierarchical clustering based on the differential gene expression profiles with principal component analysis demonstrating separate clustering between ACA and ACC.

Interestingly, two subgroups of ACC with different survival times were also identified based on this profile. Although microarrays may be expensive and impractical for

everyday clinical use, a PCR based assay using a limited number of genes could prove to be a useful prognostic tool in ACC patients.

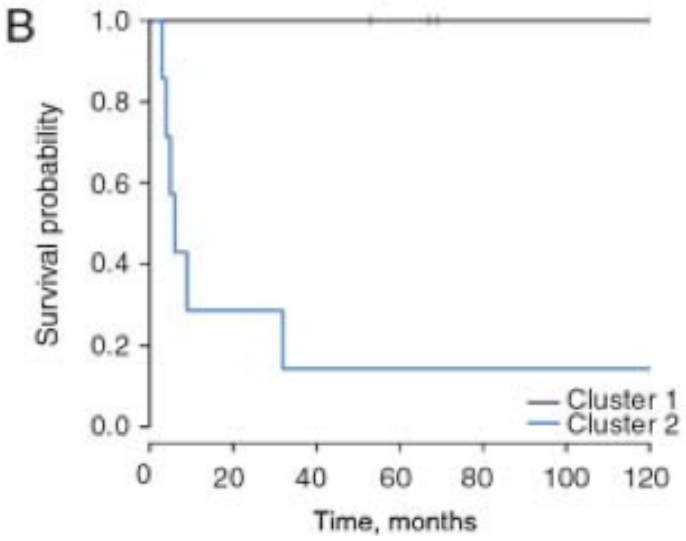


Figure 13. Kaplan-Meier survival curve showing the longer survival observed for cluster 1 of ACC regarding cluster 2 of ACC.

4.3 miRNA Expression Profiles of ACC and ACA (*Paper III*). -

The role of miRNA deregulation in the pathogenesis of ACC. Contrasting the two previous papers, miRNA expression profiling as an organization tool for discriminating ACC from ACA was used. In addition, the predictive value of miRNA expression regarding the clinical prognosis of ACC patients was evaluated. Transcriptomic profiles based on miRNA expression levels were quantified in a series of 68 ACTs (13 ACA, 16 APA, 14 NHFA and 25 ACC) and 10 normal reference adrenal cortices.

The transcriptomic profile made it possible to discriminate benign ACT from ACC. The signature for this classification included 72 deregulated miRNA such as *miR-483-3p*, *miR-210*, *miR-21*, *miR-1974* and *miR-195* among others. When survival was used as an outcome, three clusters within the ACC group were revealed by SAM analysis. Signature associated to differential survival included 9 miRNA (*miR-503, miR-379, miR-1915, miR-381, miR-638, miR-886-3p, miR-671-5p, miR-23a,* and *miR-27a*).

Relative high levels of expression of the first seven and low expression of the last two were associated with a shorter survival in patients with ACC (Figure 5 Paper III).

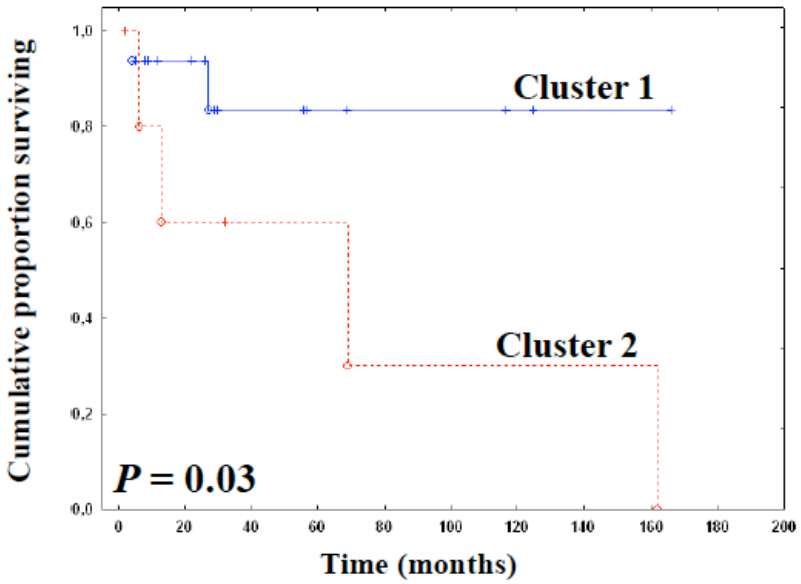


Figure 14. Cumulative survival plots for cluster 1 and 2 of ACC based on differentially expressed miRNA profiles.

The transcriptomic profile, this time based on miRNA, was again capable of discriminating ACC from ACA. For a second time, the consistency of these findings was supported by qRT-PCR validation. Moreover, cell proliferation and apoptotic assays were used in this paper in order to elucidate the functional role of some DE miRNA. This was the case of *miR-483-3p*, which showed a proliferative, but not apoptotic effect in NCI-H295R cells. Interestingly, this miRNA has been located within the second intron of the *IGF2* locus[200]. This study reported that *miR-483-3p* and *IGF2* might cooperate or even act independently as oncogenes. Hence, demonstrating two transcripts with a strong value for discriminating between ACC from ACA was very interesting.

4.4 miRNA Expression Profiles of ACA Subgroups (*Paper IV*). -

MiRNA expression patterns allow categorisation between hyperfunctioning and non-hyperfunctioning phenotypes in adrenocortical adenomas.

Distinctive miRNA profiles regarding the functional phenotype among ACTs were analysed and characterized. Using the same transcriptomic platform and 68 ACT as in Paper III, the hypothesis that these may discriminate the functional phenotype was tested. Clustering based on this transcriptomic tool allowed the discrimination between the three most common ACT phenotypes: CPA, APA and NHFA. Altogether, NHFA classified within either of the hyperfunctioning phenotypes (CPA or APA). When NHFA was discarded, CPA and APA classified separately (Fig 1a and 1B paper IV).

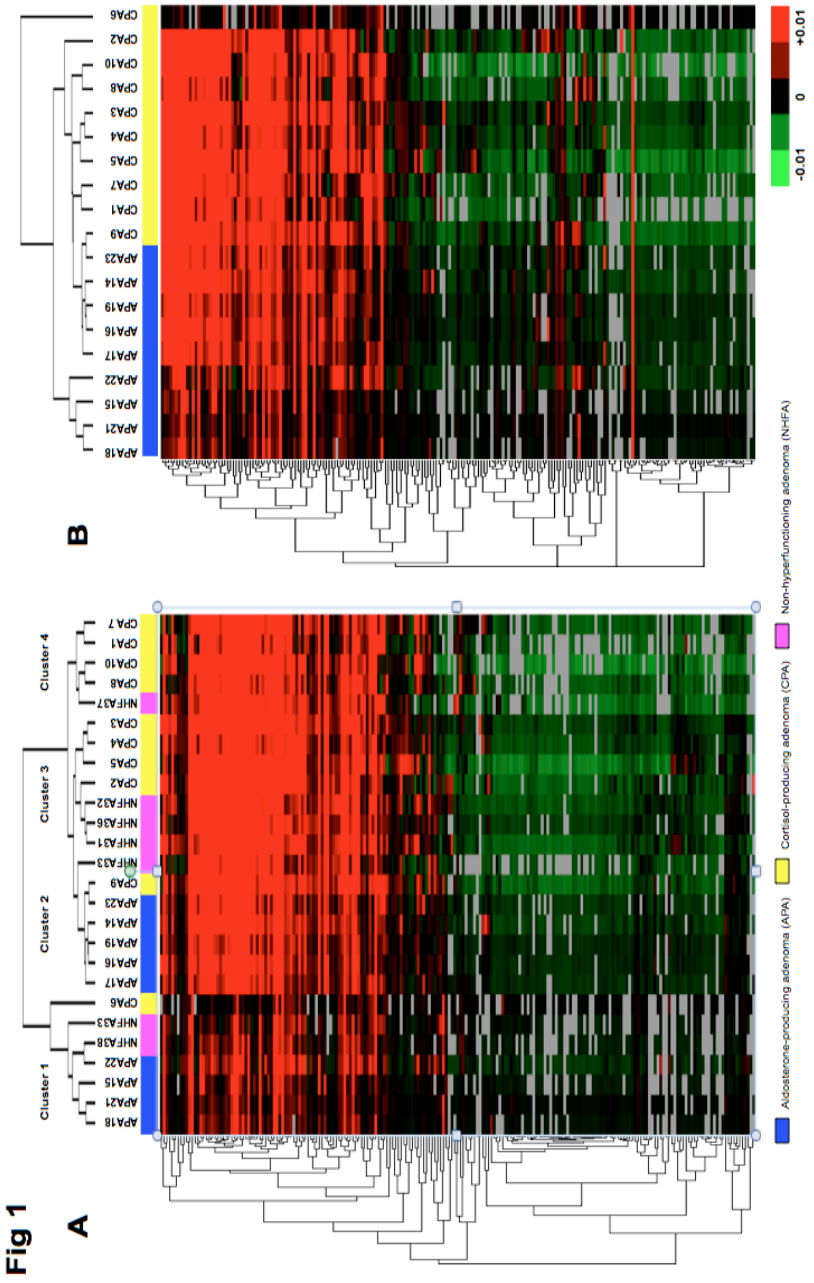


Figure 15. Heat maps based on differentially expressed miRNA between NHFA and CPA/APA (A).Heat maps showing these differentially expressed profiles based on miRNA but exclusively between APA and CPA.

Interestingly, most of the deregulated miRNAs were up regulated in hyperfunctioning phenotypes. Moreover, at least 10 miRNAs were differentially expressed in ACA when compared to normal cortices. Some of these differentially expressed miRNA were “private” or uniquely deregulated in functional ACT such as APA. Additionally, two APA clusters were observed, although the presence of *KCNJ5* mutation was not decisive for the clustering of these two subgroups.

As conclusion in this paper, transcriptional profiling based on miRNA may also contribute to a better phenotype classification of ACT regarding their hormonal over secretion. The reiterated finding that the transcriptome is categorical between ACA and ACC might elucidate stochastic differences at the biological and cellular level of ACT. In addition, it seems that some oncogenes may have an important role in ACC as well as in ACA. For example *miR-21* has been demonstrated to harbour dual functions such as promoting cell proliferation and steroidogenesis among hyperfunctioning phenotypes. Differences at the transcriptomic level are not only a reflection of the prevailing cell functional capacities but also of its future comportment.

5 CONCLUDING REMARKS

The *primary general aim* was to characterize the transcriptional patterns for mRNA and miRNA among ACTs, especially regarding the malignant phenotype. In papers I and III it was possible to correctly classify the ACTs into two different phenotypes: malignant (ACC) or benign tumours (ACA). Throughout these studies, these profiles were discrete and categorical between ACC and benign ACT phenotypes, although the transcriptomic pattern in ACC showed to be somewhat heterogeneous. Additionally, some of these transcriptional elements showed a predictive value associated with survival in patients with ACC. In paper II, mRNA expression patterns were able to identify two groups of ACC with contrasting survival times, whereas in paper III some specific miRNAs lead to the identification of ACC subgroups with varying prognosis.

The *secondary general aim* was accomplished in paper II and IV by demonstrating that it is possible to discriminate between the hyperfunctioning ACAs (especially APA and CPA) through the use of transcriptomic profiling. Additionally, NHFA clustered in either APA or CPA nearly indistinctly but differently from normal cortices and ACC. As expected, particular transcript signatures between functioning/non-functioning phenotypes, elucidating some of the molecular mechanisms involved in these phenotypes. Certain mRNA (such as *OSBP* and *VEGFB*) and miRNA (such as *miR-130a and miR-185*) were "private" or uniquely deregulated in APA.

It is hereby documented that the transcriptional profiling is a useful tool for distinguishing specific phenotypes with clinical interest such as malignant or hyperfunctioning ACTs. Additionally, this knowledge could contribute to a better understanding of the molecular mechanisms underlying the development of these tumours.

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