# Possible role of epithelial to mesenchymal transition and its associated FGF/FGFR pathway in adrenocortical carcinoma

# Mögliche Rolle des epithelial-mesenchymalen Transition und des damit verbundenen FGF/FGFR-Signalwegs beim Nebennierenrindenkarzinom

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I hereby confirm that my thesis entitled 'Possible role of epithelial to mesenchymal transition and its associated FGF/FGFR pathway in adrenocortical carcinoma' is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Hiermit erkläre ich an Eides statt, die Dissertation „Mögliche Rolle des epithelial-mesenchymalen Transition und des damit verbundenen FGF/FGFR-Signalwegs beim Nebennierenrindenkarzinom“ eigenständig, d.h. insbesondere selbständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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# Summary:

Recent studies have hinted to an involvement of epithelial to mesenchymal transition, a mechanism often associated with metastasis in epithelial cancers, in adrenocortical carcinoma. In addition, the knowledge about the FGF/FGFR pathway in pathogenesis of the adrenal gland, a pathway often associated with the epithelial to mesenchymal transition, is sparse and fragmented.

We assessed, in a large number of normal, benign and malignant adrenocortical tissues (a total of 181 different samples), the expression of canonical and novel epithelial and mesenchymal markers and compared it with their expression in typical epithelial and mesenchymal tissues. In addition, we also quantified the expression of most members of the FGF/FGFR pathway in adrenocortical tissues and compared it against well-studied epithelial and mesenchymal tissues as well as between malignant and not malignant adrenocortical tissues, in order to assess the possible connection to epithelial to mesenchymal transition and find possible drug targets. Surprisingly, both normal and neoplastic adrenocortical tissues lacked expression of epithelial markers (e.g. E-Cadhering or EpCAM) but strongly expressed mesenchymal markers (e.g. N-Cadherin or SLUG), suggesting a higher similarity of adrenocortical tissues to mesenchymal compared to epithelial tissues, reminiscent of the adrenocortical origin from the intermediate mesoderm. Despite their ubiquitous expression in all adrenocortical tissues, mesenchymal markers had a variable expression in adrenocortical carcinoma, associating either directly or inversely with different clinical markers of tumor aggressiveness. Lymph node infiltration was associated with high expression of SLUG ($p = 0.04$), and at the same time low expression of N-cadherin ($p = 0.001$), and the same pattern was observed for venous infiltration of tumoral tissue, Weiss score of tumor malignancy or Ki67 proliferation marker. In malignant compared to benign adrenal tumors, we found significant differences in the expression of 16 out of the 94 studied FGF receptor pathway related genes. Genes involved in tissue differentiation and metastatic spread through

epithelial to mesenchymal transition were most strongly altered. The therapeutically targetable FGF receptors 1 and 4 were upregulated 4.6- and 6-fold, respectively, in malignant compared to benign adrenocortical tumors, which was confirmed by using two different quantification methods in both frozen and paraffin embedded tissue material. High expression of FGFR1 and 4 was significantly associated with worse patient prognosis (High FGFR1 expression was associated with a shorter overall patient survival of 84 vs 148 months (HR=1.8, 95% CI: 1.01-3.25) as well as a shorter resection free survival of 25 vs 75 months ((HR=2.93, 95% CI: 1.25-6.84), while high FGFR4 was associated with a much shorter overall survival of 50 vs 155 months (HR=2.44, 95% CI: 1.41-4.22).

In conclusion, epithelial to mesenchymal transition does not seem to play a role in adrenocortical carcinoma tumor progression, and the FGF/FGFR pathway, even if it is probably not related to EMT, is nonetheless associated with tumor aggressiveness. Furthermore, quantification of FGF receptors may enable a stratification of adrenocortical carcinoma for the use of FGFR inhibitors in future clinical trials.

# Zusammenfassung:

Jüngste Studien weisen auf eine Beteiligung der epithelial-mesenchymalen Transition, ein Mechanismus der oft mit Metastasen bei Epithelkarzinomen assoziiert ist, beim Nebennierenrindenkarzinom hin. Darüber hinaus gibt es kaum Kenntnisse über die Rolle des FGF/FGFR-Signalweges in der Pathogenese der Nebenniere, ein Signalweg, der oft mit der epithelial-mesenchymalen Transition in Verbindung gebracht wird.

Wir haben hier an einer großen Anzahl von normalen, gutartigen und bösartigen Nebennierenrindengewebeproben (insgesamt 181 Proben) die Expression von kanonischen und anderen epithelialen und mesenchymalen Markern untersucht und mit ihrer Expression in typischen epithelialen und mesenchymalen Geweben verglichen. Darüber hinaus, haben wir auch die Expression der meisten Mitglieder des FGF/FGFR-Signalwegs in Nebennierenrindengeweben quantifiziert und mit gut definierten epithelialen und mesenchymalen Geweben verglichen sowie zwischen bösartigen und nicht bösartigen Nebennierenrindengeweben, um die mögliche Verbindung zu epithelialer-mesenchymaler Transition zu finden und mögliche therapeutische Ziele zu identifizieren. Überraschenderweise konnte weder in normalem noch in neoplastischem Nebennierenrindengewebe die Expression von epithelialen Markern (z. B. E-Cadherin oder EpCAM) nachgewiesen werden. In beiden Geweben wurde aber eine starke Expression mesenchymaler Marker (z. B. N-Cadherin oder SLUG) gefunden, was auf eine größere Ähnlichkeit von Nebennierenrindengeweben zu mesenchymalen im Vergleich zu epithelialen Geweben hindeutet. Dies könnte mit der Entwicklung des Nebennierenrinden-gewebes aus dem intermediären Mesoderm erklärt werden. Trotz ihrer ubiquitären Expression in allen Nebennierenrindengeweben, hatten mesenchymale Marker eine variable Expression in Nebennierenrindenkarzinomen, die entweder direkt oder indirekt mit verschiedenen klinischen Markern der Tumoraggressivität assoziiert waren. Die Lymphknoteninfiltration war mit einer hohen Expression von SLUG ($p = 0,04$) und einer niedrigen Expression von N-Cadherin ($p = 0,001$) verbunden. Das gleiche Muster wurde für die venöse Infiltration von Tumorgewebe, dem Weiss-Score oder dem Ki67-Proliferationsmarker beobachtet. Signifikante

Unterschiede in der Expression von 16 der 94 untersuchten Gene, die mit dem FGF-Rezeptorsignalweg in Verbindung stehen, wurden beim Vergleich von bösartigen und gutartigen Nebennierentumoren gefunden. Gene, die an der Gewebedifferenzierung und Metastasierung durch epithelial-mesenchymale Transition beteiligt sind, waren dabei am stärksten verändert. Die therapeutisch relevante FGF-Rezeptoren 1 und 4 waren bei malignen im Vergleich zu gutartigen Nebennierenrindentumoren 4,6- bzw. 6,0-fach hochreguliert. Dies wurde durch Verwendung zweier unabhängiger Quantifizierungsmethoden sowohl in gefrorenem als auch in paraffineingebettetem Gewebematerial bestätigt. Eine hohe Expression von FGFR1 und 4 war signifikant mit einer schlechteren Prognose verbunden. Eine hohe FGFR1-Expression war mit einem kürzeren Gesamtüberleben der Patienten von 84 vs. 148 Monaten (HR = 1,8; 95%CI: 1,01-3,25) sowie einem kürzeren resektions-freien Überleben von 25 vs. 75 Monaten (HR = 2,93; 95%CI: 1,25-6,84) verbunden, während eine höhere FGFR4-Expression mit einem viel kürzeren Gesamtüberleben von 50 vs. 155 Monaten assoziiert war (HR = 2,44; 95%CI: 1,41-4.22)).

Zusammenfassend lässt sich sagen, dass der Mechanismus der epithelial-mesenchymalen Transition keine Rolle bei der Tumorprogression des Nebennierenrindenkarzinoms zu spielen schein. Es konnte außerdem gezeigt werden, dass der FGF/FGFR-Signalweg, auch wenn er wahrscheinlich nicht mit der EMT zusammenhängt, mit der Aggressivität der Tumoren assoziiert. Die Untersuchung der Expression der FGF-Rezeptoren könnte für die Stratifizierung des Nebennierenrindenkarzinoms, zwecks Verwendung von FGFR-Inhibitoren in zukünftigen klinischen Studien, benutzt werden.

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# Chapter 1

# Introduction

## 1.1 The adrenal gland

The adrenal glands are small but important endocrine (hormone secreting) organs located on top of both kidneys. Structurally, they consist of an outer layer (i.e., the cortex) and an inner layer (i.e., the medulla). The medulla is derived from neural crest cells, while the cortex develops from the intermediary mesoderm. Our focus in this study was on the adrenocortical carcinoma (ACC) which is a disease of the adrenal cortex. The human adult adrenal cortex consists of three compartments with distinct morphological and functional characteristics: the zona glomerulosa, which is the outermost layer, composed of ovoid cells forming rosettes similar to glomeruli, then the zona fasciculata with its cells in radial cords that constitutes the major part of the gland and the zona reticularis, which is the inner layer bordering the medulla and is formed of cords of cells scattered in different directions. The cortex is surrounded on the outside by a thick capsule consisting of connective tissue [Lotfi et al. 2018, Kim et al. 2009] (Figure 1).

The fetal adrenal cortex has a completely different structure than the adult cortex, the human fetal adrenal is one of the largest organs at term (nearly the size of the kidney). The fetal zone is composed of large steroidogenic cells that produce large amounts of the sex hormone precursor dehydroepiandrosterone (DHEA) which is then converted by the placenta to estrogens for the maintenance of normal pregnancy. By the $8^{th}$ week of gestation, new formation adrenocortical cells emerge between the capsule and fetal zone, creating the definitive zone, formed of smaller cells that continue to differentiate and later develop into the adult cortex [Xing et al. 2015].

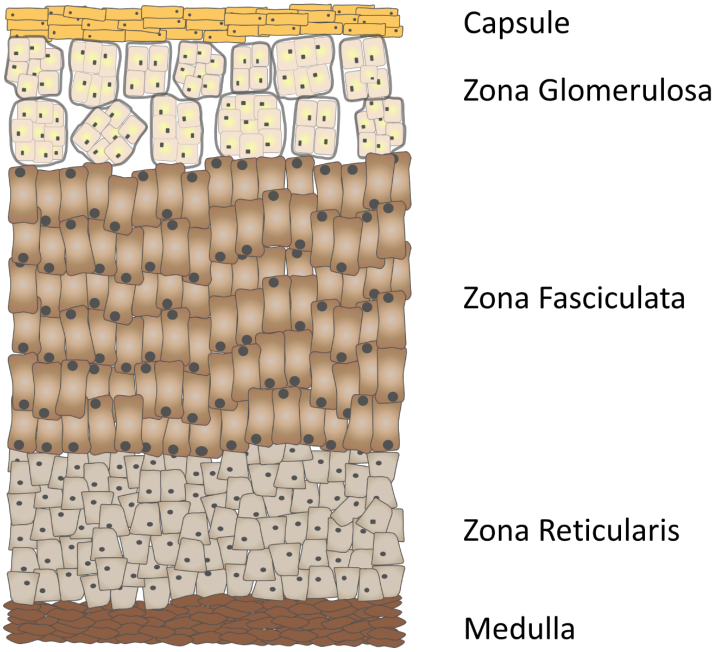



**Figure 1. Schematic of the structure and zonation of the adrenal gland.**

The function of the adrenal cortex is steroid hormone production. Three main types of hormones are produced: glucocorticoids (cortisol and corticosterone, produced in zona fasciculata), mineralocorticoids (aldosterone, produced in the zona glomerulosa, and the aldosterone precursor deoxycorticosterone produced in the zona fasciculata) as well as sex steroids produced in the zona reticularis, like DHEA. Cholesterol is the precursor of all adrenal steroid hormones, the principal source being low-density lipoprotein (LDL) cholesterol uptake [Gwynne and Strauss 1982, Miller 1988]. The normal activity of the adrenal cortex is maintained by the adrenocorticotropic hormone (ACTH) secreted by the pituitary gland [Gallo-Payet et al. 2017]. Regarding the role of the hormones produced by the adrenal cortex, the mineralocorticoids regulate blood pressure and sodium–potassium exchange in the kidney while glucocorticoids have an anti-inflammatory response and produce diverse physiological effects on energy metabolism and glucose homeostasis [Tataranni et al. 1996, Segawa et al. 2021, Jaisser and Farman 2016, Keegan and Hammer 2002].

## *1.2 The tumors of the adrenal cortex*

### 1.2.1 Tumor types

There are three types of tumors (abnormal growth) of the adrenal cortex. First is hyperplasia, which is an abnormal increase in the number of cells in a tissue, but those cells are arranged in normal fashion and respond to several physiological stimuli. Adrenal cortical hyperplasia can be divided into three broad categories: ACTH-dependent, ACTH-independent, and congenital adrenal hyperplasia (CAH) [Michelle et al. 2017]. ACTH-dependent adrenal hyperplasia is most commonly due to too much ACTH secreted either by the pituitary gland or ectopically or rarely also by over secretion of corticotropic releasing hormone. [Morani et al. 2020]. ACTH-independent adrenal hyperplasia consists of two main type: ACTH-independent macronodular adrenal hyperplasia (AIMAH), which is characterized by multiple bilateral adrenocortical nodules that cause a striking enlargement of the adrenal glands, and primary pigmented adrenocortical disease (PPNAD). Congenital adrenal hyperplasia is any of several hereditary genetic conditions caused by decreased activity of one of the enzymes involved in the synthesis of cortisol and other adrenal steroids, resulting in overstimulation of the growth of the adrenal cortex [El-Maouche et al. 2017].

In terms of neoplasia (growth of new cells that is mostly autonomous and does not respond to all physiological stimuli), we have first the adenoma, which is the benign tumor of epithelial tissues of glands, organs and other internal structures. Benign tumors grow in their primary location without invading other sites in the body, they grow slowly and have distinct and relatively smooth borders with cells that resemble quite closely in size and shape with the ones of the normal tissue. Adrenocortical adenomas (ACA) are by far the most prevalent tumor of the adrenal cortex, and several of them produce one or more of the hormones associated with this tissue, which can lead to various syndromes caused by the hypersecretion of steroid hormones [Pinto and Barletta 2015]. Non-secreting adrenal adenomas, or those that secrete low levels of hormones, are often asymptomatic and discovered incidentally on

imaging done for other issues (these are also called adrenal incidentalomas) [Fassnacht et al. 2016]. The glucocorticoid producing ACAs present the symptoms of the Cushing syndrome which includes obesity, hypertension, hyperglycemia, fatigue, depression, striae, fractures, and osteopenia. Autonomous secretion of cortisol from an adrenal adenoma accounts for 9–22% of patients with Cushing's syndrome which affects mostly women and is ACTH independent. To distinguish the adrenal Cushing syndrome from the ACTH-dependent version, which is mostly caused by corticotroph pituitary adenoma, the diagnosis relies on the demonstration of a blunted diurnal rhythm of cortisol secretion and failure of overnight suppression by administration of low doses of dexamethasone [Putignano et al. 2003, Beuschlein and Reincke 2006]. Primary aldosteronism, which is the condition caused by aldosterone-secreting ACAs may present with a hypertension that is frequently resistant to antihypertensive medications. Other symptoms can include hypokalemia, muscle weakness, hypomagnesemia, or hypernatremia [Mahmood and Anastasopoulou 2021]. The aldosterone/renin ratio (renin is an enzyme secreted by the kidney that is part of the physiological system that regulates blood pressure) is currently the most recommended screening test for primary aldosteronism, even under antihypertensive medication [Beuschlein and Reincke 2006].

The second neoplastic tumor is the malignant adrenocortical carcinoma (ACC). A malignant tumor (also known as cancer), as opposed to a benign tumor, has cells that grow uncontrollably and spread locally and/or to distant sites. A carcinoma is the version of a malignant tumor that originates in epithelial tissues, such as the skin, lungs, breasts, and other organs and glands. The cells of a carcinoma are usually larger, the edges of growth more irregular and the heterogeneity much more pronounced compared to an adenoma. ACCs can still produce steroid hormones just like ACAs, cortisol being the more common one. ACCs are usually heavy tumors and metastases formation is also common, with lung, liver, and bone being the most frequent metastatic sites [Pinto and Barletta 2015, Beuschlein and Reincke 2006].

The adrenocortical carcinoma is a rare tumor and its pathogenesis is still poorly understood. Complete surgical resection is the treatment of choice in localized ACC and is virtually the only option to achieve a cure. As recurrence is frequent, adjuvant therapy is recommended in most patients [Fassnacht et al. 2018, Jasim and Habra 2019, Fassnacht et al. 2020, Else et al. 2014]. Several genomic studies have been performed in adrenocortical tumors with the goal to better understand the mechanisms that lead to tumorigenesis, hormone excess and malignancy [Assie et al. 2014, Giordano et al. 2009, Zheng et al. 2016, Mohan et al. 2018]. Using clustering of genome wide data, these studies consistently identified a subgroup of highly malignant tumors characterized by enhanced genomic variability and altered gene expression [Jouinot and Bertherat 2018, Crona and Beuschlein 2019].

In irresectable and metastatic disease, cytotoxic chemotherapy is the standard treatment. The first and largest randomized phase III study in advanced ACC established etoposide, doxorubicin, cisplatin plus mitotane (EDP-M) as the cytotoxic chemotherapy of first choice in metastatic ACC [Fassnacht et al. 2012]. With a median progression-free survival of only 5.0 months and an overall survival of only 14.8 months in the group receiving EDP-M, the prognosis is still poor. In the meantime, several other therapeutic approaches have been investigated [Cosentini et al. 2019, Megerle et al. 2019, Henning et al. 2017, Fassnacht et al. 2015], but a clinically meaningful breakthrough has not yet been achieved, so there has been a growing interest in targeted therapies for the treatment of ACC. Overall, advanced disease still remains a major therapeutic challenge in patients with ACC [Altieri et al. 2020].

### 1.2.2 Tumorigenesis

Regulation of adrenocortical growth and differentiation is a complex process that requires a diverse array of specific transcription factors and signaling cascades. Clonality studies of adrenocortical tumors using X-chromosome inactivation analysis indicate monoclonal expansion of a single cell as the origin of ACC [Beuschlein et al. 1994]. These findings are in agreement with those in other forms of cancer, which are believed to originate

from a single transformed cell clone in response to a series of genetic alterations that result in the disruption of growth regulation. In contrast, ACAs can present as monoclonal or polyclonal tumor entities, suggesting that, in addition to tumorigenic mutations, mitogenic extra-adrenal stimuli might contribute to cellular proliferation in this benign tumor [Beuschlein and Reincke 2006, Gicquel et al. 1994].

The cAMP-PKA pathway is fundamental in regulating adrenocortical cell development, proliferation, and function. This pathway is activated by the binding of ACTH to the ACTH receptor, a G-protein coupled receptor on the adrenocortical cell membrane [Bossis and Stratakis 2004]. Aberrant cyclic adenosine monophosphate (cAMP) - protein kinase A (PKA) signaling has been shown to play a key role in the development of most benign cortisol-producing adrenocortical tumors. Mutations in the coding for the C$\alpha$ subunit of PKA (PRKACA) are mostly responsible for this aberrant signaling [Beuschlein et al. 2014, Calebiro et al. 2015], but also genetic alterations in GNAS, PRKAR1A, PRKACB, PDE11A, and PDE8B [Kamilaris et al. 2020].

Somatic mutations in genes encoding ion channels or transporters are found in more than half of aldosterone producing adenomas. Such mutations include those in KCNJ5 and CACNA1D, as well as in the ATPases ATP1A1 and ATP2B3. These genes have roles in balancing the amounts of positively charged ions of sodium, potassium, and calcium in cells [Kamilaris et al. 2020].

Most ACC cases are sporadic, however 5-10% of ACCs have been associated with hereditary germline mutations. ACC has been described in several hereditary cancer syndromes including Li-Fraumeni syndrome, Beckwith-Wiedemann syndrome, Multiple Endocrine Neoplasia Type 1 (MEN-1), Lynch syndrome and Carney complex [Else et al. 2014, Petr and Else 2018, Kamilaris et al. 2020]. Li-Fraumeni syndrome is an autosomal dominant cancer predisposition that results from a germline mutation in the TP53 gene, a tumor suppressor protein which has an important role in preventing genome mutation. ACC develops in only a few patients with this syndrome [Soon et al. 2008]. Beckwith–Wiedemann syndrome is congenital overgrowth disorder

characterized by a high risk of development of various childhood tumors, and also has a high incidence of ACC. The disease has been mapped to the human chromosome band 11p15.5, which harbors the IGF-2 gene among others, a factor that is widely associated with ACC [Beuschlein and Reincke 2006]. MEN-1 is another autosomal-dominant disorder due to an inactivating germline mutation in the menin gene, which has roles in genome stability, that often leads to various types of tumors and in some rare cases also to ACC [Fonseca et al. 2012]. Carney complex is an autosomal dominant disorder that results from inactivating mutations in the protein kinase A regulatory subunity (PRKAR1A) gene, that encodes the type 1$\alpha$ regulatory subunit of cAMP dependent PKA. Patients with Carney complex develop primary pigmented adrenocortical disease with abnormal skin pigmentation, hypercortisolism, cardiac myxomas, and tumors among which ACA and sporadically ACC [Bertherat 2012]. Lynch syndrome is another autosomal dominant cancer predisposition syndrome, that is attributed to germline mutations in one of several DNA-mismatch repair genes resulting in genomic instability. Lynch syndrome is characterized by an increased risk for colorectal cancer and cancers of the endometrium, but an increased prevalence of ACC has also been associated with it [Raymond et al. 2013, Kamilaris et al. 2020]. Investigation of the genetic and molecular characteristics of these hereditary syndromes has allowed for some elucidation of the signaling pathways involved in tumorigenesis that can also lead to the development of sporadic ACC.

In terms of the occurrence of sporadic ACC, somatic genetic alterations leading to changes in p53 signaling, Wnt-β-catenin signaling, IGF2 overexpression, and to a certain degree cAMP-PKA signaling, are some of the most often cited reasons [Kamilaris et al. 2020]. A study published in 2014 based on the molecular analysis of 122 ACCs identified nine pathogenic genetic alterations that were present in at least 5% of ACCs. The most frequently altered pathway was the Wnt-β-catenin pathway with 39% of the genetic defects affecting this pathway. Signaling by the Wnt family of glycolipoproteins is one of the fundamental mechanisms that direct cell proliferation and cell fate determination during embryonic development and in adult organs, with β-

catenin being the key effector responsible for transduction of the signal to the nucleus [MacDonald et al. 2009, Valenta et al. 2012]. Among this pathway, the most common genetic abnormality identified in 21% of tumors was that of the ZNRF3 (zing finger and ring finger protein 3) gene, a potential tumor suppressor gene related to the β-catenin. The p53-Rb pathway, which is the system that is paramount in the control of cellular responses to potentially oncogenic stimuli, was the second most frequently affected pathway according to this study, with alterations identified in 33% of ACCs [Assie et al. 2014]. A subsequent study published in 2016, based on the genomic characterization of 91 ACCs, confirmed the importance of these pathways and expanded the list of known ACC driver genes [Zheng et al. 2016]. Both studies, as well as numerous other previous studies have found that IGF-2 overexpression happens in a majority of ACC cases [Beuschlein and Reincke 2006, Fonseca et al. 2012], also IGF-2 can be used in combination with proliferation marker Ki-67 with great accuracy to differentiate ACC from ACA [Soon et al. 2009]. Several other growth factors have also been shown to be overexpressed in ACC, including TGFβ-1 and 2 [de Fraipont et al. 2005, Fassnacht et al. 2011], insulin like growth factor-related genes and VEGF [Fassnacht et al. 2011, Velazquez-Fernandez et al. 2005, Kolomecki et al. 2001]. It has also been hypothesized that some sporadic ACCs could be a multistep process with possible progression from a benign to a malignant adrenocortical tumor [Bernard et al. 2003, Kamilaris et al. 2020]. However, the low incidence of ACC and the very high prevalence of ACA argues against this as a frequent scenario.

## *1.3 Epithelial to mesenchymal transition*

### 1.3.1 Overview of the epithelial to mesenchymal transition

An epithelial-mesenchymal transition (EMT) is a biologic process that allows an epithelial cell, which normally interacts with basement membrane via its basal surface, to undergo multiple biochemical changes that enable it to assume a mesenchymal cell phenotype, which includes enhanced migratory capacity, invasiveness, elevated resistance to apoptosis, and greatly increased

production of extra cellular matrix components [Kalluri and Weinberg 2009, Kalluri and Neilson 2003]. The completion of an EMT is signaled by the degradation of underlying basement membrane and the formation of a mesenchymal cell that can migrate away from the epithelial layer in which it originated.

Historically, epithelial and mesenchymal cells have been identified based on their unique visual appearance and the morphology of the structures they create [Shook and Keller 2003]. A typical epithelium is a sheet of cells, sometimes one cell thick, with individual epithelial cells bordering each other in a uniform array. Regularly spaced cell–cell junctions and adhesions between neighboring epithelial cells hold them tightly together and inhibit the movement of individual cells away from the layer. This adhesiveness allows an epithelial sheet to enclose a three-dimensional space and provide it with structural definition and mechanical rigidity. The epithelial sheet itself is polarized, meaning that the apical and basal surfaces are likely to be visually different, adhere to different substrates, or have different functions [Lee et al. 2006, Hay 2005]. Mesenchymal cells, on the other hand, generally exhibit neither organized structure nor tight intercellular adhesion. Mesenchymal cells form structures that are irregular in shape and not uniform in composition or density. Adhesions between mesenchymal cells are less strong than in their epithelial counterparts, allowing for increased migratory capacity. They also have a more extended and elongated shape, relative to epithelial cells. In addition, mesenchymal migration is mechanically different from epithelial movement. Epithelial cells move as a sheet, whereas mesenchymal migration is considerably more dynamic, more individual [Lee et al. 2006, Hay 2005, Kalluri and Weinberg 2009].

EMT is a process that was first recognized as a feature of embryogenesis, the cells in the body can assume various phenotypic states during development, a process called differentiation. During specific steps of embryogenesis and organ development, the cells within certain epithelial tissues appear to be plastic and thus able to move back and forth between epithelial and mesenchymal states via the processes of EMT as well as its reverse process MET

(mesenchymal-epithelial transition). Upon completion of the development of epithelial tissues, the epithelial cells typically exert tissue-specific function, while the mesenchymal cells in such tissue play a supporting role [Lee et al. 2006, Thiery et al. 2009, Acloque et al. 2009, Hay 2005]. Most importantly, the mechanism of EMT, which allows the epithelial tumor cells to acquire a motile mesenchymal phenotype [Puisieux et al. 2014], is diverted by several types of cancer to promote metastasis and resistance to treatment [Kalluri and Weinberg 2009]. This process has been considered to be implicated in metastatic spread of such a large variety of human cancers like breast, prostate, lung etc. [Karlsson et al. 2017, Navas et al. 2020, Wang and Zhou 2011] (Figure 2).

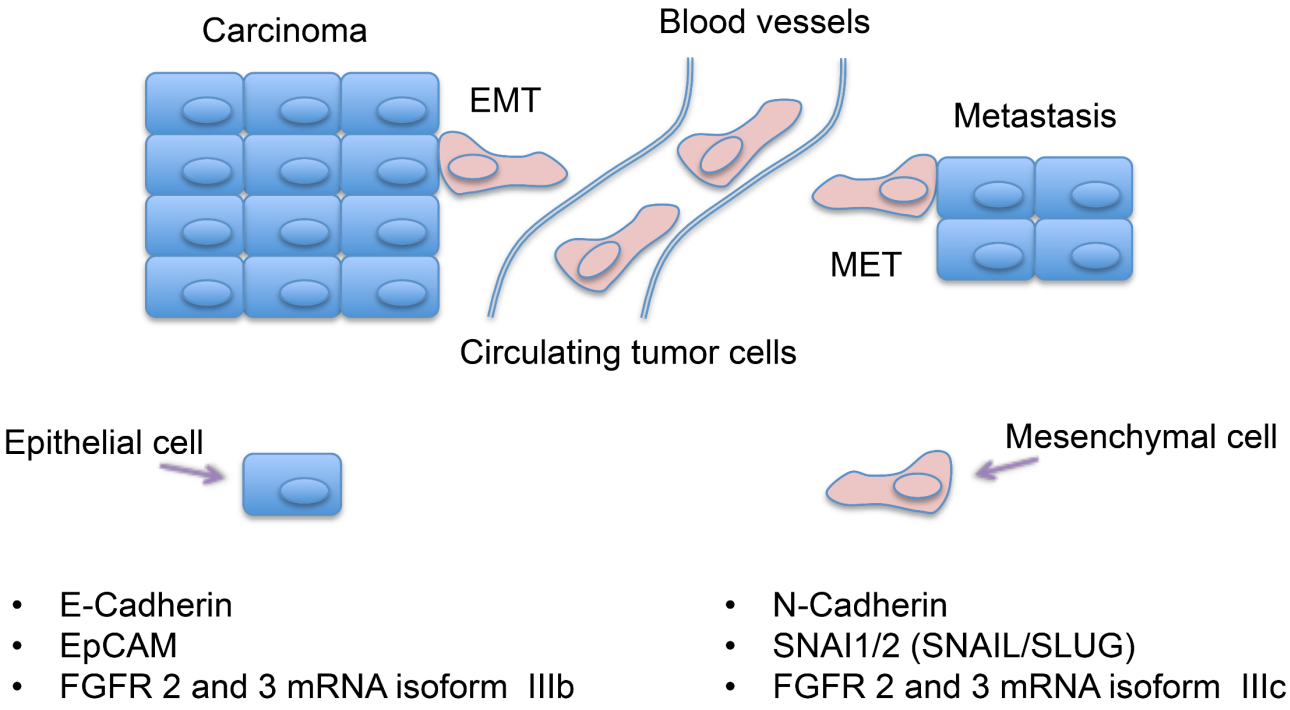



**Figure 2. Classical EMT in cancer cells.** Upper panel: EMT (Epithelial to Mesenchymal Transition) and MET (Mesenchymal to Epithelial Transition) processes in metastatic spread. Lower panel: canonical markers of epithelial (left) and mesenchymal (right) cells.

### 1.3.2 The organogenesis of the adrenal cortex

Early morphological studies on the organogenesis of the adrenal cortex placed its origins either in the dorsal coelomic epithelium [Pankratz 1931, Uotila 1940] or in the intermediate mesoderm mesenchyme that is surrounded by it [Gruenwald 1946, Roos 1967], or even both [Gruenwald 1942]. These conflicting views were partly due to the very close origin of the gonads and the adrenal cortex, which made the distinction difficult with just imaging equipment. With the use of steroidogenic factor 1 (SF-1) marker proteins, it could be later shown that this so called adreno-gonadal primordia separated in two separate populations, and the one on the coelomic epithelium side is the one that evolves into the gonads and the population towards the dorsal aorta into the adrenal cortex [Hatano et al. 1996, Morohashi 1997] but this study does not fully elucidate the initial origin of this primordia. Morphologically, Gruenwald describes the early fetal adrenal cortex as hard to distinguish from the surrounding mesenchyme, and that only when the peripheral layer that will later become the zona glomerulosa starts forming, one can talk about epithelial phenotype. Additionally, he characterizes zona glomerulosa as being the only true epithelial structure, even in the adult cortex. [Gruenwald 1946]. The more recent publications seem to agree that the mesoderm splits and undergoes epithelialization to become the coelomic epithelium (CE), and a subsequent thickening of this forms the specialized region known as the urogenital ridge, and that is where the adrenal cortex originates from [Keegan and Hammer 2002, Mesiano and Jaffe 1997, Yates et al. 2013, Pihlajoki et al. 2015, Xing et al. 2015]. The coelomic epithelium has recently been shown to be formed from two layers of immature epithelial-like cells, the outer and inner CE, and furthermore, the adrenal cortex derives from the inner CE while the gonads from the outer CE, so in case a common precursors exist, they could reside in the mesoderm [Saito et al. 2017]. In conclusion, even though the adrenal cortex is considered an epithelial tissue, its origin and development are still being studied.

### 1.3.3 Processes involved in EMT

A number of distinct molecular processes participate in EMT, like the activation of transcription factors, expression of specific cell-surface proteins, reorganization and expression of cytoskeletal proteins etc. In many cases, the factors involved are also used as biomarkers to demonstrate that a specific cell is undergoing EMT [Kalluri and Weinberg 2009]. For example, E-cadherin and epithelial cell adhesion molecule are considered as classical epithelial markers while N-cadherin, SNAIL and SLUG are considered mesenchymal markers (Figure 3) [Zeisberg and Neilson 2009, Larue and Bellacosa 2005].

Epithelial cell adhesion molecule (EpCAM) is a transmembrane glycoprotein mediating Ca2+-independent homotypic cell-cell adhesion in epithelia [Litvinov et al. 1994] associating with the actin cytoskeleton via an intermediate molecule [Balzar et al. 1998]. It has a limited expression in mesenchymal cells [Momburg et al. 1987, Brown et al. 2021]. Epithelial tumors are often characterized by strong expression of EpCAM while its expression is downregulated during EMT but then upregulated once the metastasis reaches its future tumor site, where the MET process is supposed to take place [van der Gun et al. 2010].

E-cadherin (epithelial cadherin) and N-cadherin (neural cadherin) are classical cadherins (calcium-dependent adhesion proteins) and share similar structures. They form a cadherin-catenin complex where the cytoplasmic domain consists of EC repeats that bind with catenins to moderate the cytoskeletal filament containing actin. The structural difference between E-cadherin and N-cadherin is that E-cadherin binds with the shorter isoform of p120 catenin while N-cadherin binds with the longer isoform. The switch from E-cadherin expression to N-cadherin, which mediates weaker cell-cell interactions, is classically used as a mesenchymal marker to define EMT [Loh et al. 2019]. N-cadherin is also present in few epithelial tissues such as hepatocytes but only together with a much stronger E-cadherin expression [Zeisberg and Neilson 2009].

Snail and Slug (SNAI1 and SNAI 2), are two transcription factors that suppress E-cadherin and lead to a decrease in cell-to-cell adhesion and are also commonly used to detect EMT [Nieto 2002, Davidson and Sukumar 2005]. Knockout models for both SNAIL and SLUG showed significant reduction in cancer invasiveness [Olmeda et al. 2007, Emadi Baygi et al. 2010].

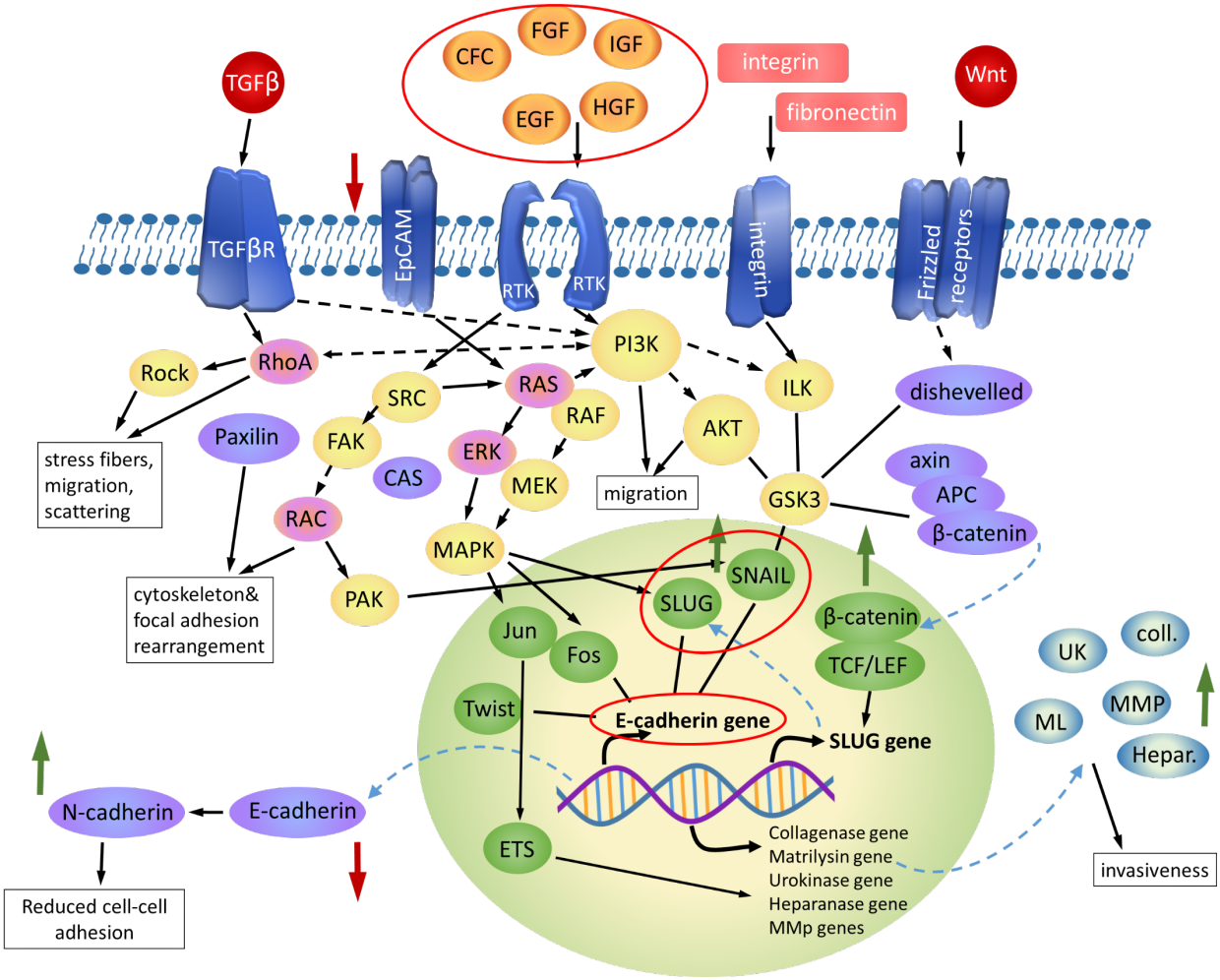



**Figure 3. Signaling pathways and downstream genes involved in epithelial to mesenchymal transition.** Green arrows show groups involved in the transition to a more mesenchymal phenotype, while red arrows show the factors that confer a resistance to this transition. Adapted from [Larue and Bellacosa 2005, Gao et al. 2015, van der Gun et al. 2010].

There are several signaling pathways, including those originating from TGF-β (transforming growth factor beta), FGF (fibroblast growth factors), EGF (epithelial growth factors), HGF (hepatocyte growth factors) and their

receptors, Wnt/beta-catenin, that can induce a mesenchymal phenotype (Figure 3) [Skromne and Stern 2001, Larue and Bellacosa 2005, Ciruna and Rossant 2001, Collignon et al. 1996, Kim et al. 2009]. These influences are mainly coordinated by growth factors and their receptors on tumor cell membranes [Alexander and Friedl 2012]. All these pathways as a common feature activate the zinc-finger transcription factors SNAIL and SLUG. Out of these signaling pathways our focus was on the FGF pathway for two reasons: the first is that, by reanalyzing microarray data from Giordano [Giordano et al. 2009], we saw a disturbance in the expression levels of some FGF family members in ACC compared to non-malignant adrenocortical tissues (Figure 4); the second reason being that it also provides an additional direct marker of transition: at mRNA level, the expression of the epithelial (IIIb) and mesenchymal (IIIc) isoforms of FGFR 2 and 3 can be used to characterize EMT as well [Turner and Grose 2010].

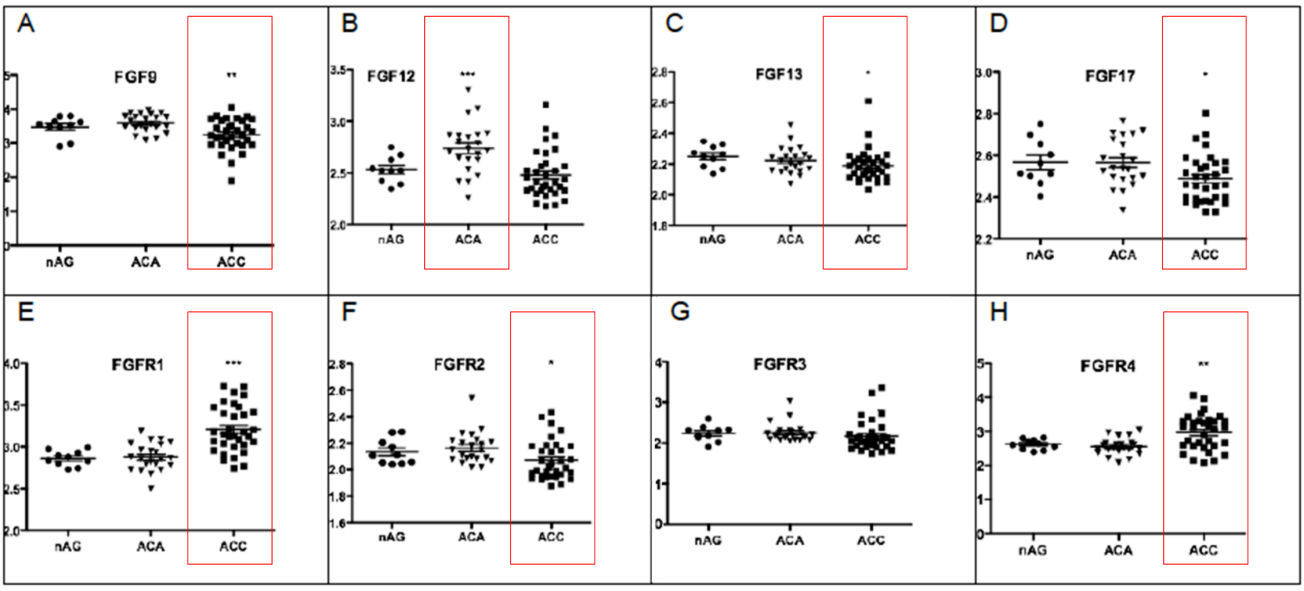



**Figure 4. Reanalysis of microarray data from Giordano et al. (2009) shows a variance in the expression of FGF family members in adrenocortical tissues.**

## *1.4 The fibroblast growth factor family and signaling pathway*

At mRNA level, the fibroblast growth factors receptors (FGFR) isoform switching is another model that can be used as a marker for EMT. Fibroblast growth factor receptors (FGFRs) are a family of receptor tyrosine kinases expressed on the cell membrane that play crucial roles in both developmental

and adult cells. The fibroblast growth factor receptor family has four members, FGFR1, FGFR2, FGFR3, and FGFR4 [Dai et al. 2019]. The FGFRs consist of three extracellular immunoglobulin-type domains (D1-D3), a single-span trans-membrane domain and an intracellular split tyrosine kinase domain. FGFs interact with the D2 and D3 domains, with the D3 interactions primarily responsible for ligand-binding specificity [Dai et al. 2019]. Thfe receptors 1 to 3 have the unique feature of having two isoforms due to alternative splicing of the D3 domain which changes the specificity (Table 1, Figure 5) [Holzmann et al. 2012]. Choice of splice site for FGFR-IIIb and IIIc variants is strictly regulated and is mutually exclusive [Gilbert et al. 1993]. For the FGFRs 2 and 3 it has been shown that the isoform IIIb is mainly present in epithelial cells while the isoform IIIc is mostly mesenchymal [Turner and Grose 2010, Ishiwata 2018] (Figure 5). This isoform switch is subject to regulation by EMT related pathways like TGF-beta, WNT/beta catenin and activation of other RTK for example by HGF or other FGF members [Thiery et al. 2009]. Thus, EMT and the FGFR isoform switch are closely interconnected tumoral processes.

| Receptor | Ligands with high affinity | |
|---|---|---|
| | Isoform IIIb | Isoform IIIc |
| FGFR1 | FGF1, FGF2, FGF3, FGF10, FGF22 | FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21, FGF23 |
| FGFR2 | FGF1, FGF3, FGF7, FGF10, FGF22 | FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21, FGF23 |
| FGFR3 | FGF1, FGF9, FGF16 | FGF1, FGF2, FGF4, FGF5, FGF6, FGF8, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21 |

**Table 1. Ligand binding spectrum of different splicing variants of FGFR1-3.**

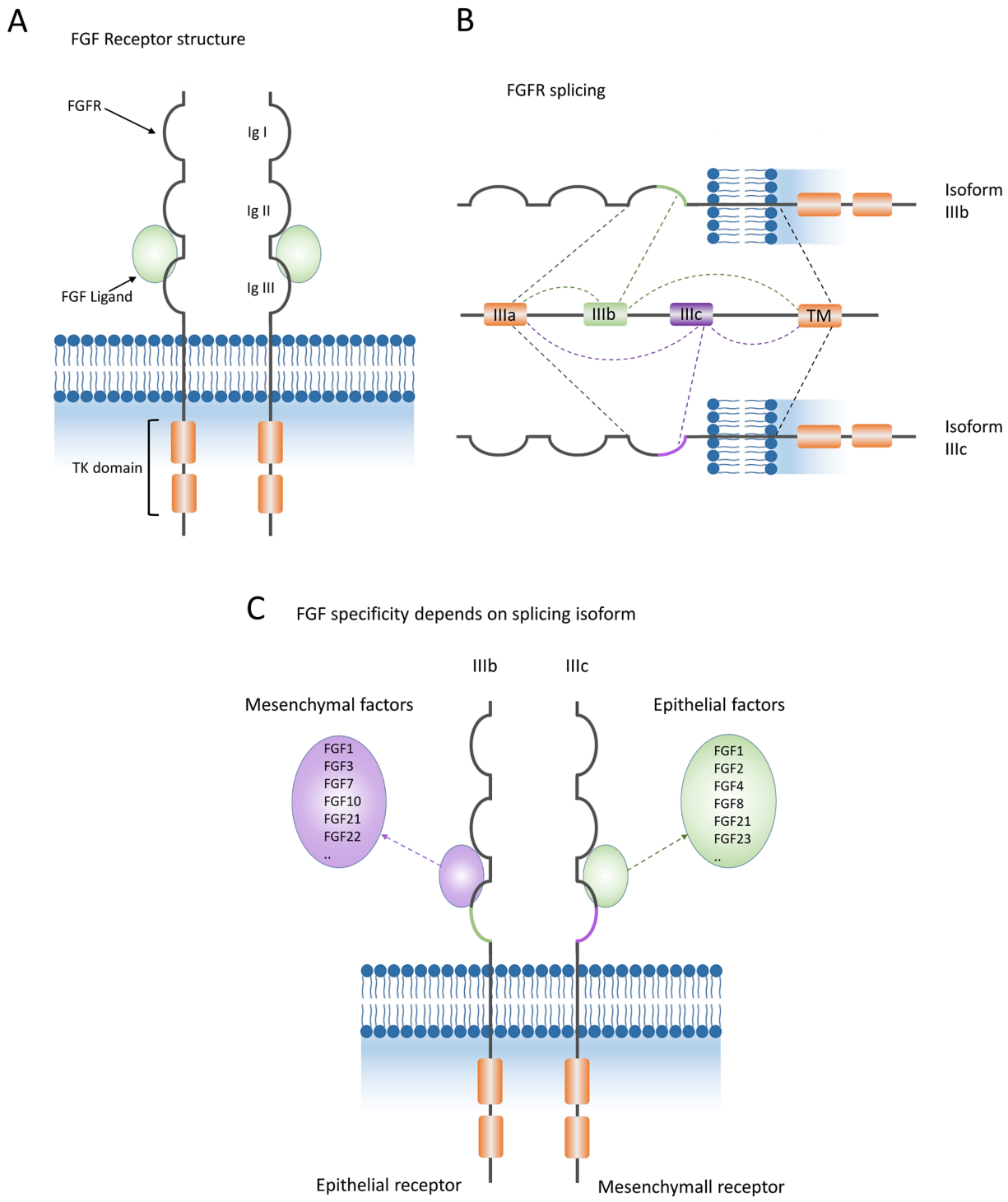



**Figure 5. FGFR alternative splicing for receptors 1, 2 and 3.** (A) shows the general structure of an FGF receptor, (B) the alternative splicing occuring at the thrid immunoglobulin repeat while (C) shows the preferred specificity of the FGFs to the different isoforms. Adapted from [Turner and Grose 2010] and [Matsuda et al. 2012].

Regarding the pathways involved in EMT (Figure 3), insulin-like growth factor (IGF) 2 is the single most overexpressed molecule in the majority of ACC, as previously discussed [Soon et al. 2009], and clinical investigation of inhibitors of the IGF pathway yielded high expectations [Altieri et al. 2019]. However, this first industry-sponsored randomized phase III clinical trial in ACC with the IGF1R-inhibitor linsitinib (OSI-906) did not significantly prolong progression free survival in the vast majority of patients although some remarkable responses were observed [Fassnacht et al. 2015]. High expression of vascular endothelial growth factor (VEGF) and its receptor VEGF-R2 in many ACC specimens [Xu et al. 2011] led to several studies targeting the tumor vasculature in ACC with bevacizumab, an anti-VEGF monoclonal antibody, and sorafenib, a multi-tyrosine kinase inhibitor in combination with paclitaxel, which however failed to demonstrate clinical efficacy [Berruti et al. 2012]. Previous *in vitro* data in ACC cell lines [Kroiss et al. 2011] led to the conception of a phase II clinical trial of the receptor tyrosine kinase (RTK) inhibitor sunitinib targeting VEGFR2, and PDGFRβ among others. 29 patients were evaluated in the study (SIRAC), all patients had progressed despite prior cytotoxic chemotherapy and suffered from significant tumor burden, however, sunitinib showed modest antitumor effects [Kroiss et al. 2012]. Despite these setbacks, tyrosine kinase inhibitors have still potential in the treatment of ACC, as demonstrated by a small study using the multi-tyrosine kinase inhibitor cabozantinib in 16 patients with progressive ACC that showed prolonged disease control in half of the patients [Kroiss et al. 2020].

In humans, the family of fibroblast growths factors (FGFs) comprises 23 different genes that encode proteins binding with high affinity to receptor tyrosine kinases (FGFRs) [Lieu et al. 2011]. Each factor has more or less affinity to each of the four receptors, also depending on the splicing variant in the case of the first three receptors (Table 1.). Members of the FGF family function in the earliest stages of embryonic development and during organogenesis to maintain progenitor cells and mediate their growth, differentiation, survival and paterning [Itoh and Ornitz 2011, Ornitz and Itoh 2015]. Four of the five known FGF receptors (FGFR1-4) are highly conserved membrane bound RTK.

After ligand binding, dimerization of the receptor causes phosphorylation of intracellular tyrosine residues that subsequently activate several crucial intracellular signaling pathways [Itoh 2007] like Phospholipase-C (PLC), Protein Kinase C (PKC) and Ras/Mitogen-activated protein kinase (MAPK). Activation of FGFR signaling may lead to carcinogenesis in several types of tissues [Thisse and Thisse 2005, Haugsten et al. 2010]. All FGFs have a high affinity for the glycosaminoglycan heparin and for cell surface heparan sulfate proteoglycans. It has been shown that heparan sulfate is essential for FGF and that tissue-specific differences in the structure of heparan may modulate the activity of FGF [Lin et al. 1999, Ornitz 2000]. Aberrant expression of some of the FGFs has been implicated in the development and progression of different tumor types [Turner and Grose 2010, Korc and Friesel 2009]. Although FGF signaling can drive tumorigenesis, it has also been shown to mediate tumor protective functions [Turner and Grose 2010]. Importantly, the association of FGFRs with tumorigenesis led to the development of tyrosine kinase inhibitors (TKI) with FGFR specificity [Capozzi et al. 2019, Tan et al. 2019], with high response rates in the first clinical studies in other types of cancer [Bedrose et al. 2020].

## *1.5 Objective*

Around 90% of all malignancies originate from epithelial tissue [Wang et al. 2012]. The adrenocortical tissue is also classically categorized as an epithelial tissue. Accordingly, adrenal tumors are also classified as carcinomas (tumors of an epithelial tissue) [Rogalla and Contag 2015] as opposed to sarcomas (tumors of a mesenchymal tissue) [Mohseny and Hogendoorn 2011]. The existing genomic studies in adrenocortical tumors identified a subgroup of highly malignant tumors characterized by enhanced genomic variability and altered gene expression [Jouinot and Bertherat 2018, Crona and Beuschlein 2019]. Our first hypothesis was, therefore, that EMT could be an explanation for the subgroup of quickly metastasizing, highly aggressive ACCs. Two studies have already provided a first indication that adrenocortical tissues are expressing

some mesenchymal markers [Bulzico et al. 2017, Rubin et al. 2016]. However, the number of adrenocortical carcinoma tissues analyzed in these studies was low (24 cases in each study) and a correlation between EMT marker expression and clinicopathological markers indicative of tumor aggressiveness was not possible.

In addition, the knowledge about the FGF/FGFR pathway in the adrenal gland is sparse and fragmented. Already since 1975, Gospodarowicz et al. demonstrated that some fibroblast growth factors increased proliferation in the mouse Y1-adrenocortical tumor cell line [Gospodarowicz and Handley 1975] and in bovine and human fetal adrenal cells [Gospodarowicz et al. 1977]. Later, FGF1 and 2 were identified as growth-stimulating factors in adrenocortical cells and adrenocortical tumors [Boulle et al. 2000, Haase et al. 2007, Feige and Baird 1991] indicating a dual role of the FGF/FGFR system in both organogenesis and tumorigenesis in the adrenal system.

There were three main objectives in this study:

1. Our first hypothesis was that, adrenocortical tissues being classically categorized as epithelial tissues, EMT could be an explanation for the subgroup of quickly metastasizing, highly aggressive ACCs. Following our first hypothesis, one would expect a decrease in epithelial markers as well as an increase in mesenchymal markers in ACC, especially advanced stages of ACC, in comparison to ACA or normal adrenals. To this end, we aimed at the investigation of the expression of diverse epithelial as well as mesenchymal markers as a way to detect EMT in a large cohort of adrenocortical tissue samples and correlated them with clinical features and patient outcome.

2. Since until now, no single study had been published that focused on both EMT and the FGF/FGFR pathway as a central mechanism that can potentially be targeted therapeutically, the second and third goal of our study aims at closing this gap. In order to achieve this, we first planned to analyze the gene expression of most members of the pathway in a cohort of malign and benign adrenocortical tissue samples and compared it against well-studied

epithelial and mesenchymal tissues to see if we can find a connection between the FGF pathway and EMT.

3. The third goal is a continuation of the second one, using its results as well as new results from a larger cohort of samples focusing on the FGF receptors, we wanted to find possible therapeutic targets from this pathway in ACC, as well as generally gathering more information about upregulated or downregulated genes in malignant versus benign adrenocortical tissues.

# Chapter 2
# Materials and Methods

## *2.1 Patient material*

We used, in total, patient material from three normal adrenal glands (NAG), 29 adrenocortical adenomas (ACA) and 149 adrenocortical carcinomas (ACC). The material was either frozen, as used for the RT-qPCR experiments, or as formalin fixed, paraffin embedded tissue blocks (FFPE), as was used for the immunohistochemistry and RNAScope experiments. The study was approved by the ethical committee of the University of Würzburg (88/11) and all patients gave informed consent. An overview of key clinical characteristics of all the patients can be found in Tables 2 and 3. Cohort I contains all the samples that were used for experiments requiring FFPE material, while cohort II all those requiring frozen material. Cohort II is mostly a subcohort of cohort I.

| | Normal adrenal | ACA | ACC |
|---|---|---|---|
| *n* | 3 | 29 | 142 |
| Sex (male/female) | 1/2 | 11/18 | 52/90 |
| Age [yr (sd)] | 49 (11) | 51 (14) | 49 (14) |
| Size of the tumor [cm (sd)] | | 3.3 (1.2) | 9.8 (4.7) |
| Hormone secretion | | | |
| Cortisol – *n* (%) | | 11 (38%) | 52 (37%) |
| Androgen – *n* (%) | | 0 (0%) | 10 (7%) |
| Aldosterone – *n* (%) | | 7 (24%) | 6 (4%) |
| Inactive – *n* (%) | | 11 (38%) | 16 (11%) |
| Unknown – *n* (%) | | 0 (0%) | 58 (41%) |
| Tumor localization – *n* (%) | | | |
| Primary - ENSAT stage I+II | | | 47 (33%) |
| Primary - ENSAT stage III | | | 36 (25%) |
| Primary - ENSAT stage IV | | | 26 (18%) |
| Local recurrences | | | 22 (16%) |
| Distant metastases | | | 11 (8%) |
| Ki67 index [median (range)] | | | 10 (1-70) |
| Weiss Score [median (range)] | | 0 (0-1) | 5 (2-9) |

**Table 2. Baseline characteristics of patients and tumors used in cohort I (FFPE material only).**

| | Normal adrenal | ACA | ACC |
|---|---|---|---|
| *n* | 3 | 15 | 21 |
| Sex (male/female) | 1/2 | 7/8 | 12/9 |
| Age [yr (sd)] | 49 (11) | 46 (12) | 51 (13) |
| Size of the tumor [cm (sd)] | | 3.2 (1.5) | 10 (4.9) |
| Hormone secretion | | | |
| Cortisol – *n* (%) | | 8 (53%) | 10 (47%) |
| Androgen – *n* (%) | | 0 (0%) | 3 (14%) |
| Aldosterone – *n* (%) | | 3 (20%) | 1 (5%) |
| Inactive – *n* (%) | | 4 (27%) | 0 (0%) |
| Unknown – *n* (%) | | 0 (0%) | 7 (34%) |
| Tumor localization – *n* (%) | | | |
| Primary - ENSAT stage I+II | | | 8 (38%) |
| Primary - ENSAT stage III | | | 8 (38%) |
| Primary - ENSAT stage IV | | | 5 (24%) |
| Local recurrences | | | 0 (0%) |
| Distant metastases | | | 0 (0%) |
| Ki67 index [median (range)] | | | 20 (3-70) |
| Weiss Score [median (range)] | | | 7 (3-9) |

**Table 3. Baseline characteristics of patients and tumors used in cohort II (frozen material only).**

In addition, the following samples served for comparison, depending on the experiment: 1 normal thyroid, 1 normal colon, 3x colon carcinoma, 1 thyroid carcinoma, 1 ovarian carcinoma, 2x osteosarcoma, 2x liposarcoma, 1x synovial sarcoma, 1x rhabdomyosarcoma.

## *2.2 Immunohistochemistry*

### 2.2.1 Materials for immunohistochemistry

| **Reagents/Kits** | **Manufacturer** |
|---|---|
| Xylene, Ethanol, Methanol | Sigma-Aldrich, VWR |
| Human AB serum | Sigma-Aldrich |
| Hydrogen peroxide | Merck |
| HiDef Detection HRP Polymer System | Cell Marque |
| DAB substrate kit | Cell Marque |
| Meyer's Hematoxylin | Carl Roth |
| Entellan | Merck |

| **Equipment** | **Manufacturer** |
|---|---|
| 'Slee Cut 5062' Microtome | Slee Medical |
| 'C35 Feather' Microtome blades | pfm medical |
| 'SuperFrost' glass slides | Langenbrick |
| Borosilicate glass coverslips | A. Hartenstein |
| 'Aperio Versa' scanning brightfield microscope | Leica Microsystems |
| Pressure cooker | Silit |

| Buffers | Manufacturer of main ingredient |
|---|---|
| Phosphate-buffer saline | Sigma-Aldrich |
| Citrate buffer, 10 mM, pH 6.5 | Sigma-Aldrich |

| Primary antibodies | Clone | Dilution | Manufacturer |
|---|---|---|---|
| E-Cadherin (CDH1) | CL1172 | 1:2250 | Sigma-Aldrich |
| EpCAM | polyclonal | 1:20000 | abcam (ref. ab71916) |
| N-Cadherin (CDH2) | D-4 | 1:125 | Santa Cruz Biotechnology |
| SLUG (SNAI2) | OTI1A6 | 1:300 | Novus Biologicals |
| SNAIL (SNAI1) | clone EC3, subclone EC11 | 1:50 | University Pompeu Fabra, Barcelona (custom antibody [Franci et al. 2006], gift) |

### 2.2.2 Immunohistochemistry

The formalin-fixed, paraffin-embedded tissues or tissue microarrays were cut manually with the help of a microtome to a thickness of ~2µm, laid shortly in warm water (55°C) to straighten the tissue slice and subsequently mounted on SuperFrost glass slides. After a minimum of one day in order to dry out, the tissues were deparaffinized twice in xylene for 10 minutes, then rehydrated in a series of ethanol in water dilutions for 5 minutes each (100%, 90%, 80% and 70% ethanol). Antigen retrieval was achieved by boiling in 10mM citrate buffer corrected to a pH of 6.5, inside a pressure cooker set at the highest setting for 13 minutes and then slowly cooled to room temperature in the buffer for 20 minutes. Endogenous peroxidase was blocked with a 3% solution of hydrogen peroxide in methanol for 10 minutes, followed by the blocking of

non-specific binding for another 10 minutes with the help of a 20% solution of human AB serum in PBS. Incubation time for all of the primary antibodies was 1 hour at room temperature in PBS. Signal amplification was done with the "HiDef Detection HRP Polymer System" from Cell Marque, which is a two-step process that uses an indirect method that universally detects mouse and rabbit primary antibodies. The first step consists of a 10 minute incubation with the Amplifier followed by two 2 minute washes in PBS, the second step is another 10 minute incubation with the HRP Polymer Detector followed by two 2 minute washes. Finally, the signal was developed using a mixture of the DAB chromogen and DAB buffer substrate reagents included in the 'DAB substrate kit' for 10 minutes. Counterstaining of nuclei was performed using Meyer's Hematoxylin for 2 minutes, followed by washing in running tap water for 5 minutes. After dehydration (2 minutes each in the following solutions in order: 70% ethanol, 100% ethanol, 100% ethanol, 100% xylene), slides were mounted using Entellan and borosilicate glass coverslips.

Stained tissue slides were imaged with the Leica 'Aperio Versa' brightfield scanning microscope at a magnification of 20x and evaluated using a semi-quantitative H-Score that estimated the intensity of the staining (scored as negative (0), low (1), medium (2), and high (3)) and the percentage of positive cells (scored as 0, 0.1, 0.5, or 1 if 0%, 1–9%, 10–49%, or ≥50% of the cells were positive, respectively). The slides were subjectively evaluated by two independent investigators, myself and the technical assistant working on the project, both blinded to clinical data. Low expression was defined as H-score <2 and high score as H-score ≥2.

## *2.3 RNAScope*

### 2.3.1 Materials for RNAScope

| **Reagents/Kits** | **Manufacturer** |
|---|---|
| Xylene, Ethanol | Sigma-Aldrich, VWR |
| Hydrogen peroxide solution | ACDbio ref. 322335 |
| Protease plus | ACDbio ref. 322331 |
| Meyer's Hematoxylin | Carl Roth |
| Entellan | Merck |
| Amplifier 1 | ACDbio ref. 322311 |
| Amplifier 2 | ACDbio ref. 322312 |
| Amplifier 3 | ACDbio ref. 322313 |
| Amplifier 4 | ACDbio ref. 322314 |
| Amplifier 5 | ACDbio ref. 322315 |
| Amplifier 6 | ACDbio ref. 322316 |
| DAB-A | ACDbio ref. 320052 |
| DAB-B | ACDbio ref. 320053 |

| **Probes** | **Ref. number (ACDbio)** |
|---|---|
| FGFR1 | 310071 |
| FGFR2 | 311171 |
| FGFR4 | 412301 |

| Equipment | Manufacturer |
|---|---|
| 'HybEZ' Oven | ACDbio |
| 'Slee Cut 5062' Microtome | Slee Medical |
| 'C35 Feather' Microtome blades | pfm medical |
| 'SuperFrost' glass slides | Langenbrinck |
| Borosilicate glass coverslips | A. Hartenstein |
| 'Aperio Versa' scanning brightfield microscope | Leica Microsystems |
| Pressure cooker | Silit |

| Buffers | Manufacturer |
|---|---|
| Target retrieval reagent 10X + dd$H_2O$ | ACDbio ref. 322000 |
| Wash buffer 60X + dd$H_2O$ | ACDbio ref. 31009 |

### 2.3.2 RNAScope in-situ hybridization

RNAScope is a custom RNA in-situ hybridization solution from Advanced Cell Diagnostics, USA. Version 2.5 of the kit was utilized for our experiments following the manufacturer's instructions. In short, the FFPE tissue sections of ~2μm thickness were prepared in the same way as the immunohistochemistry method, then deparaffinized in xylene twice for 8 minutes. Afterwards they were washed twice with 100% ethanol for 5 minutes, followed by endogenous enzyme blocking in hydrogen peroxide solution after becoming completely dry, at room temperature for 10 min. Permeabilization was performed by boiling in a pressure cooker for 13 minutes in target retrieval reagent, followed by a quick cooling down and washing in water. Afterwards,

protein digestion was achieved with the help of Protease Plus for 20 min at 40°C. All steps at 40°C were performed in a HybEZ Oven that ensures quick heating up of the samples. After protein digestion, the probes (in our case FGFR 1,2 and 4) were hybridized at 40°C for 2h. Starting with this step and until final DAB detection, the slides were washed in wash buffer instead of water after each step, twice for 2 minutes. Afterwards, the slides were treated, in order, with Amplifier solution 1 to 6. Amplifier 1 and Amplifier 3 at 40°C for 40 min, Amplifier 2 and Amplifier 4 at 40°C for 20 min, while Amplifier 5 for 50min and Amplifier 6 for 20 min, both at room temperature and in a reduced light environment. Then equal amounts of DAB-A and DAB-B were mixed and applied to the slides for 10 minutes at RT in order to develop the amplified signal. Counterstaining of nuclei was performed using Meyer's Hematoxylin for 2 minutes, followed by washing in running tap water for 5 minutes. After dehydration (2 minutes each in the following solutions: 70% ethanol 100% ethanol, 100% ethanol, 100% xylene), the slides were mounted using Entellan and borosilicate glass coverslips.

The incubation times listed for the amplifiers were generally increased in comparison to the times recommended by the manufacturer for a better batch to batch signal detection consistency.

Three pictures of representative areas of each slide were taken with the Leica 'Aperio Versa' brightfield scanning microscope at 40x magnification. Scoring the RNAScope slides was done with the help of Aperio ImageScope software (version 12.x, Leica, Germany) on the entirety of the pictures using the optional image analysis algorithm 'RNA ISH v1'. This algorithm automatically detects and counts the number of RNA molecules (each brown stained spot is one molecule of RNA) and the number of cells (by detecting the hematoxylin-stained nuclei) in a defined area. Thresholds for the detection were manually adjusted for a high fidelity assessment of the signal. In Figure 6, there is an example of a selected area for detection from a high scoring sample. We used the ratio of RNA spots per number of cells for each slide to quantify the target gene expression.

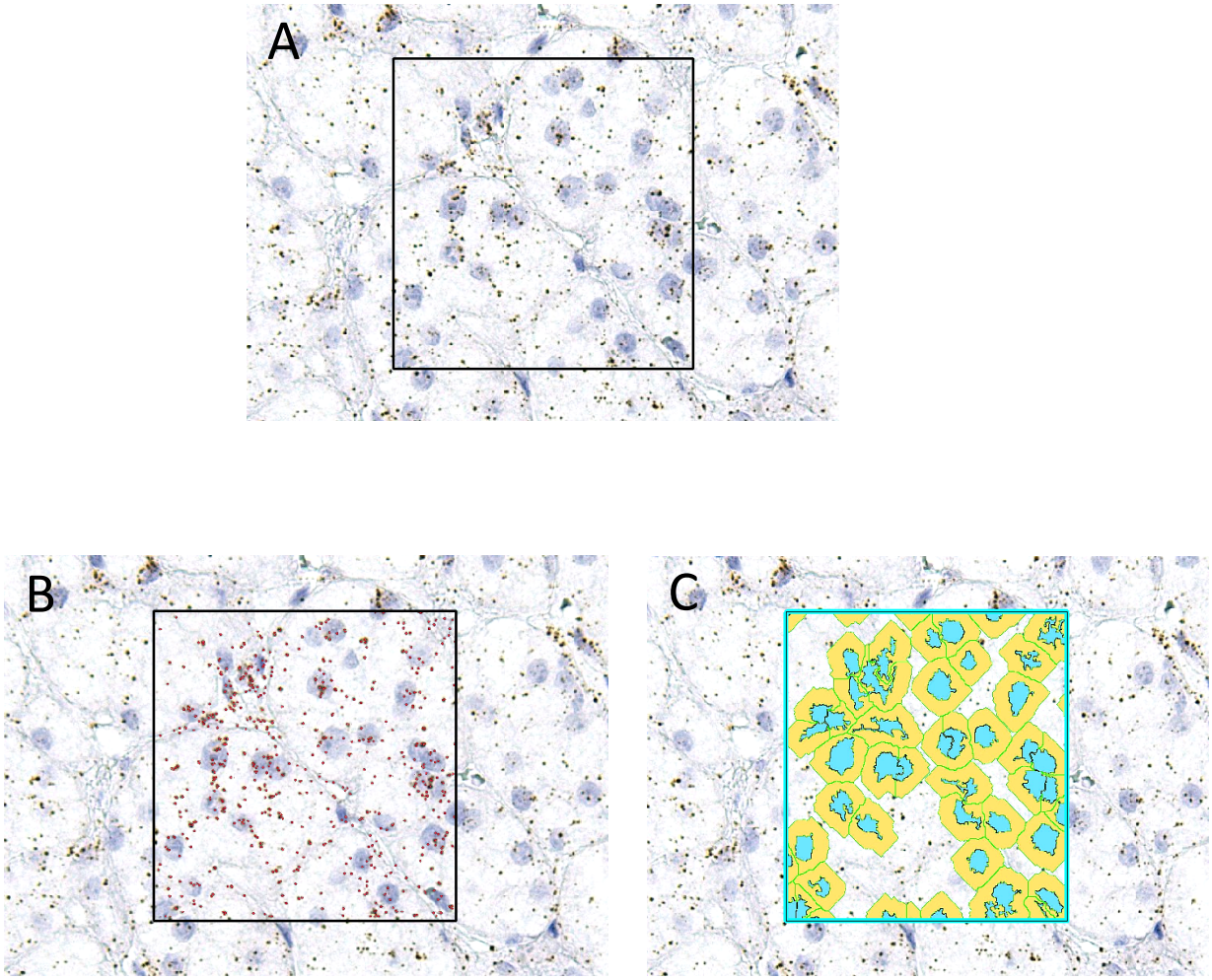



**Figure 6. An example of RNAScope signal detection using the ImageScope software.** The first image (A) is the original image; the square is the selected area for detection. (B) The same image with the detected mRNA molecules marked in red by the software, while (C) is the same image with the detected cells marked in blue (nuclei) and yellow (cytoplasm).

## *2.4 Quantification of mRNA expression*

### 2.4.1 Materials needed for all the steps of the quantification

| **Reagents/Kits** | **Manufacturer** |
|---|---|
| Xylene, Ethanol | Sigma-Aldrich, VWR |
| 'RNeasy Lipid Tissue Kit' | Qiagen |
| 'RNeasy Mini Kit' | Qiagen |
| 'TaqMan Gene Expression Master Mix' | Applied Biosystems |
| 'High-Capacity cDNA Reverse Transcription Kit' | Applied Biosystems |

| **Equipment** | **Manufacturer** |
|---|---|
| 'TissueLyser II' | Qiagen |
| 5mm stainless steel beads | Qiagen |
| 'NanoDrop 2000c' | Thermo Fisher Scientific |
| 'Mastercycler personal' | Eppendorf |
| 'CFX96' thermal cycler | Bio-Rad |
| 'StepOnePlus Real-Time PCR System' | Thermo Fisher Scientific |

| Probes | Ref. number (Thermo Fisher Scientific) |
|---|---|
| Beta-actin | Hs99999903_m1 |
| 18S | Hs99999901_s1 |
| FGFR3 IIIB | Hs01005396_m1 |
| FGFR3 IIIC | Hs00997397_m1 |
| FGFR4 | Hs01106908_m1 |
| Human FGF pathway PCR plate with 96 probes | 4418781 |

Diagram of the probes contained on the Human FGF Pathway 96-well PCR plate, the genes marked with green background are the house-keeping gene controls, and with red font the members of the FGF family:

| Gene Symbol | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 18S | GAPDH | HPRT1 | GUSB | ATF2 | CDC42 | ELK1 | FGF1 | FGF10 | FGF12 | FGF13 | FGF14 |
| B | FGF17 | FGF19 | FGF2 | FGF20 | FGF21 | FGF23 | FGF3 | FGF4 | FGF5 | FGF7 | FGF8 | FGF9 |
| C | FGFR1 | FGFR2 | FGFR3 | FRS2 | GPLD1 | GRB2 | HRAS | ITPR1 | ITPR2 | ITPR3 | KRAS | MAP2K1 |
| D | MAP2K2 | MAP2K3 | MAP2K4 | MAP2K5 | MAP2K6 | MAP3K1 | MAP3K2 | MAP3K3 | MAP3K4 | MAP3K5 | MAPK1 | MAPK10 |
| E | MAPK11 | MAPK12 | MAPK13 | MAPK14 | MAPK3 | MAPK8 | MAPK9 | MRAS | NRAS | PIK3C2A | PIK3C2B | PIK3C2G |
| F | PIK3C3 | PIK3CA | PIK3CB | PIK3CD | PIK3R1 | PIK3R2 | PIK3R3 | PIK3R4 | PIK3R5 | PLCG1 | PLCG2 | PLD1 |
| G | PLD2 | PLD3 | PRKCA | PRKCB | PRKCD | PRKCE | PRKCG | PRKCH | PRKCI | PRKCQ | PRKCZ | PRKD1 |
| H | PRKD3 | RAC1 | RAF1 | RALA | RALB | RALBP1 | RALGDS | RASA1 | RRAS | RRAS2 | SOS1 | SOS2 |

In addition to these gene probes, due to commercial unavailability, the following custom probes were used:

FGFR2 IIIB — fw: 5′-GGCTCTGTTCAATGTGACCGA-3′; rev: 5′-GTTGGCCTGCCCTATATAATTGGA-3′; TaqMan probe: 5′-TTTCCCCAGCATCCGCC-3

FGFR2 IIIC — up: 5′-CACGGACAAAGAGATTGAGGTTCT-3′; low: 5′-CCGCCAAGCACGTATATTCC-3′; TaqMan probe: 5′-CCAGCGTCCTCAAAAG-3

### 2.4.2 Methods employed for mRNA quantification

For quantification of mRNA expression, real-time quantitative RT-PCR was performed. To arrive from organic material to the mRNA expression of the gene of interest, three separate steps need to be performed in order: first RNA extraction from cryo-preserved tissues or from cell lines, this RNA was then transcribed to cDNA (complementary DNA) via a reverse transcriptase, this cDNA was subsequently used for the real-time RT-PCR.

#### 2.4.2.1 RNA extraction

For the RNA extraction from cryo-preserved tissues, the RNeasy Lipid Tissue Kit from Qiagen was used. The tissues were selected from the -80°C freezer and placed in liquid nitrogen to avoid degradation, and then 30-50mg of each tissue was broken off and immediately added to 1ml of QIAzol Lysis reagent (part of the kit, in order to inhibit the degradation of RNA) in a 2 ml reaction tube together with a stainless steel bead. The tubes with all the samples were then placed in the special reaction tube holders of the TissueLyser machine (pre-cooled at -20°C). Tissue homogenization was achieved after shaking the samples in the tube together with the beads in the TissueLyser set at 30hz for 2 minutes. This homogenate was then used as the starting step for the extraction using the reagents and equipment included in the 'RNeasy Lipid Tissue Kit' according to the manufacturer's instructions. The end result was

50µl of total RNA solution in RNase-free water, that can be stored at -80 °C. To determine the concentration of this solution for each sample as well as the quality of extracted RNA, 2µl of the solution was measured in the NanoDrop machine using RNase-free water as background signal.

#### 2.4.2.2 cDNA reverse transcription

Following RNA extraction, a quantity of 1µg of RNA was used for each sample, diluted in RNAse-free water to a total volume of 10µl, in order to transform it into cDNA. The amount of initial RNA solution used will vary therefore depending on its concentration. An additional 10µl of a mixture of reagents included in the 'High-Capacity cDNA Reverse Transcription Kit' according to manufacturer's instructions was then added and the total amount of 20µl for each sample was placed in a 0.2ml reaction tube, and then placed in the Eppendorf 'Mastercycler personal' machine. The following thermal cycling was performed in order to obtain the cDNA solution:

25 °C for 10 min
37 °C for 120 min
85 °C for 5 min
4 °C hold

The cDNA can be stored at 4°C for a short-term period or -20°C long-term. The final yield of cDNA is considered to be equivalent to the quantity of RNA used, so in our case 1 µg per sample.

#### 2.4.2.3 Quantitative real-time RT-PCR amplification

In order to get the quantitative expression of mRNA for our genes of interest (relative to the housekeeping genes), probe primers containing a fluorescent marker that will specifically attach to the cDNA corresponding to out mRNA of interest were used in this final amplification step. For each probe that was part of the 96-well FGF pathway plate, a total of 10ng of cDNA per sample was used. In the case of the probes that were not part of the FGF pathway plate, 100ng of cDNA were used per sample. The TaqMan Gene

Expression Master Mix was mixed with nuclease-free water, the probe primer (which targets our specific genes of interest) and the cDNA solution, according to the manufacturer's instructions. For each measurement, besides the samples that contained the target probe primer, there were samples with the controls consisting of 18S ribosomal RNA, beta-actin and more in the case of the FGF pathway plate. In the case of the probes that were part of the FGF pathway, the samples were run through the 'StepOnePlus Real-Time PCR System' thermal cycler from Thermo Fisher for which these plates were designed for, with the following conditions:

50 °C for 2min

95 °C for 10 min

95 °C for 15sec
60 °C for 1min
} 44 cycles

For the probes that were not part of the FGF pathway plate, the samples were run through the Bio-Rad 'CFX96 C1000' thermal cycler with the same conditions.

The raw result of the real-time PCR is the cycle number at which the fluorescence of the reporter dye contained by the probe, which is being amplified after each cycle, has reached a certain threshold (cycle threshold - CT). Then this raw data was quantified in Microsoft Excel with the ΛCT method by normalizing to the average of the house-keeping genes expression.

## *2.5 Cell culture*

### 2.5.1 Cell culture materials

**Cell lines**

NCI-H295R – adrenocortical carcinoma cells, from ATCC [Gazdar et al. 1990]

CU-ACC1 and CU-ACC2 – adrenocortical carcinoma cells, obtained from Katja Kiseljak-Vassiliades [Kiseljak-Vassiliades et al. 2018]

MUC-1 – adrenocortical carcinoma cells, obtained from Constanze Hantel [Hantel et al. 2016]

Hep G2 – liver carcinoma cells, obtained from ATCC [Knowles et al. 1980]

HEK-293 – embryonic kidney cells, obtained from ATCC [Graham et al. 1977]

**Cell culture media**

- Cell culture medium for NCI-H295R cells
  DMEM/F12 (1:1) + L-Glutamine + 15mM HEPES (gibco 11330032)
  supplemented with
  - 3% Nu-Serum 3% (Corning)
  - 1% Insulin-Transferrin-Selenium (gibco)
- Cell culture medium for CU-ACC1 and CU-ACC2 cells
  75% of F12 + L-Glutamine (gibco 21765029) and 25% of DMEM + D-Glucose + L-Glutamine + Sodium Pyruvate (gibco 41966029)
  supplemented with
  - 10% Fetal bovine serum (gibco)
  - 0.7% Hydrocortisone 50µg/ml (Sigma-Aldrich)
  - 0.09% Insulin 5mg/ml (Sigma-Aldrich)
  - 0.007 Cholera Toxin 0.1 mg/ml (Sigma-Aldrich)
  - 0.1% Adenine 20mg/ml (Sigma-Aldrich)
  - 0.009 Epidermal Growth Factor 0.1mg/ml (gibco)

- Cell culture medium for MUC-1 cells
  Advanced DMEM/F12 (1:1) + Non-essential Aminoacids + Sodium Pyruvate (gibco 12634010) supplemented with
  - 10% Fetal bovine serum (gibco)
  - 1% Penicillin-Streptomycin 10.000 U/ml (gibco)
- Cell culture medium for HepG2 cells
  DMEM/F12 (1:1) + L-Glutamine (gibco 11330032) supplemented with
  - 10% Fetal Bovine Serum (gibco)
- Cell culture medium for HEK-293 cells
  DMEM + D-Glucose (gibco 11960044) supplemented with
  - 10% Fetal bovine serum (gibco)

| **Reagents/Kits** | **Manufacturer** |
|---|---|
| Dulbecco's Phosphate Buffered Saline (DPBS) | Sigma-Aldrich |
| 'T4049' Trypsine-EDTA solution | Sigma-Aldrich |
| Trypan blue 0.4% solution | Sigma-Aldrich |
| Cell Proliferation Reagent WST-1 | Roche Applied Science |
| Dimethyl sulfoxide (DMSO) | Sigma-Aldrich |

| Equipment | Manufacturer |
|---|---|
| 'Cellstar' Cell Culture Flasks | Greiner Bio-One |
| 96-well Microplates | Greiner Bio-One |
| 'Countess 2 FL' cell counter | Invitrogen |
| 'C150' CO2 Incubator | Binder |
| 'MSC-Advantage' Class II Biological Safety Cabinet | Thermo Fisher Scientific |
| 'Victor³' plate reader | PerkinElmer |

### 2.5.2 Different cell culture methods

Cell culture work was performed at a sterile workbench to prevent contamination (MSC-Advantage Class II Biological Safety Cabinet). The cells were cultivated in appropriate culture flasks for each cell line, with a volume of 250 or 550 ml, and kept in incubators set at 37 °C, 95% relative humidity and 5% $CO_2$.

#### 2.5.2.1 Passaging of cells

Cells that were cultured in flasks were passaged prior of becoming confluent to avoid inhibition of growth. The following protocol was used in the case of the smaller 250ml flasks, and scaled accordingly for other sizes. The medium was removed and cells were washed with 10 ml DPBS. Around 1 ml Trypsin-EDTA was added and incubated for 2 minutes to initiate the detaching of cells. The Trypsin-EDTA was then removed and the cells were incubated for another couple of minutes until detached. The cells were then resuspended in 8 ml of fresh medium. Depending on the cell line and confluence, the cells were split with a ratio of one part of resuspended cells to 3 to 5 parts of fresh medium up to a total of 12 milliliter per flask. For experiments that required an exact

amount of cells, the cells were counted in the 'Countess 2 FL' automated cell counter after mixing 10 µl of resuspended cells and 10 µl of Trypan Blue (for the detection of dead cells) and adding it to the cell counting chamber slides.

#### 2.5.2.2 WST-1 assay for cell proliferation

In order to assess the cell viability and proliferation after treatment with various inhibitors, the WST-1 assay has been used. First, cell passaging has been performed and cells counted. These cells were then cultured in a 96-well microplate, each well containing $2.5x10^4$ cells in a volume of 100 µl medium. The plates were incubated until the cells were fully attached to the bottom (around 24 hours), then the medium removed with a pipette. A volume of 100 µl of new medium containing various concentration of the inhibitor or the control (consisting of medium with a DMSO concentration equal to the one in the wells containing the inhibitor) was then added, the wells on each column containing the same concentration of the inhibitor in order to make an average of results. In addition, a triplicate of these 96-well plates has been used for each inhibitor test for better accuracy. Inhibitors are mostly directly soluble in DMSO (Dimethyl sulfoxide), but since this compound is toxic to cells, the DMSO concentration has been kept under 0.3% and constant for all the inhibitor concentrations on each plate in order to not influence the results. Afterwards, the plates were incubated for a few days, and then 10 µl of WST-1 reagent was added to each well, and left to incubate for 3 hours, after which the plates were taken and the absorbance at 450 nm measured for 1 second for each well in the Victor[3] plate reader. The result consists of a table with the absorbance values for each well, a higher value signifies more metabolically active cells. This data was further analyzed in Microsoft Excel and the concentration/absorbance curves fitted in GraphPad Prism 7 to obtain the $EC_{50}$ value, the inhibitor concentration at which half-maximal response is obtained.

#### 2.5.2.3 Cell RNA extraction

After passaging of cells and cell counting, the growing medium containing a sufficient number of cells (1x $10^7$) has been placed in a tube and centrifuged at 1500rpm, then the medium discarded and the cell pellet placed in Buffer RLT (part of the RNEasy Minit Kit) in a reaction tube together with a steel bead for homogenization, the further steps were the same as the extraction of RNA from frozen tissues. The resulting RNA solution was then measured in the NanoDrop machine to assess the yield and quality of the extracted RNA, and then stored at -80 °C for further processing.

## *2.6 Bioinformatic and statistical analyses*

For non-parametrical comparisons between two groups of data, for example when comparing protein expression quantified by immunohistochemistry, the two-tailed Mann-Whitney test was used. A p-value $<0.05$ was considered statistically significant. P-values between 0.05 and 0.1 were considered indicative of a statistical trend. The statistical analyses were performed with Graph Pad Prism 7 for Windows.

For ACC patients, the Kaplan-Meier method was used to estimate overall survival (OS, in all patients with primary tumors) and recurrence-free survival (RFS, in patients with complete resection of the primary tumor. This analysis follows the occurrence of set termination events in time (patient death in the case of OS and tumor recurrence in the case of RFS), starting with the primary tumor removal. The Cox proportional hazards model was used to calculate the hazard ratio (HR, how big is the likelihood of one or more variables to affect the survival either positively or negatively) and the confidence interval (CI) for univariate as well as multivariate analyses. All survival analyses were done using IBM SPSS v 26 for Windows. For the graphs, samples were split in two categories, of low and high expression, depending on the thresholds set for each gene of interest being compared (i.e. SLUG, FGFR1,

etc), the termination events set accordingly, the HR and p-value included represent the survival data of high expression samples compared using the Cox model against the survival data of low expression. Points marked as 'censored' on the survival curve represent samples for which the patient was lost to follow-up during the study or did not experience a termination event until the cut-off time value.

In the case of the inhibitor analyses, the concentration/absorbance curves were fitted in GraphPad Prism 7 to calculate the $EC_{50}$ value, the inhibitor concentration at which half-maximal response is obtained.

For the FGF pathway gene analysis, the normalized expression data were Log2 transformed and loaded into the Gene-E software v. 3.0.215 from Broad Institute. The unsupervised cluster analysis of all the samples was performed using the column distance/similarity matrix algorithm and the average linkage method with Pearson correlation. Hierarchical clustering of the gene expression between different phenotype clusters was performed using the marker selection algorithm, using a two-sided test with 1000 permutations. The most significantly differential expressed genes were considered those where the false discovery rate (FDR) values were $<0.05$.

# Chapter 3
# Results

## *3.1 Expression of epithelial markers in adrenocortical tissues*

The expression of E-cadherin was absent in all the adrenal tissues analyzed (n=170), both normal and tumoral (Figures 7 B-E), while this marker showed a normal, membrane expression in 14 different epithelial tissues analyzed (Figures 7 A and E). Similar results were also observed for EpCAM, which was also completely missing in all adrenocortical tissues analyzed (Figures 7 G-J) while strong expression of this marker was observed in classical epithelial tissues that were used as positive controls (Figures 7 F and J).

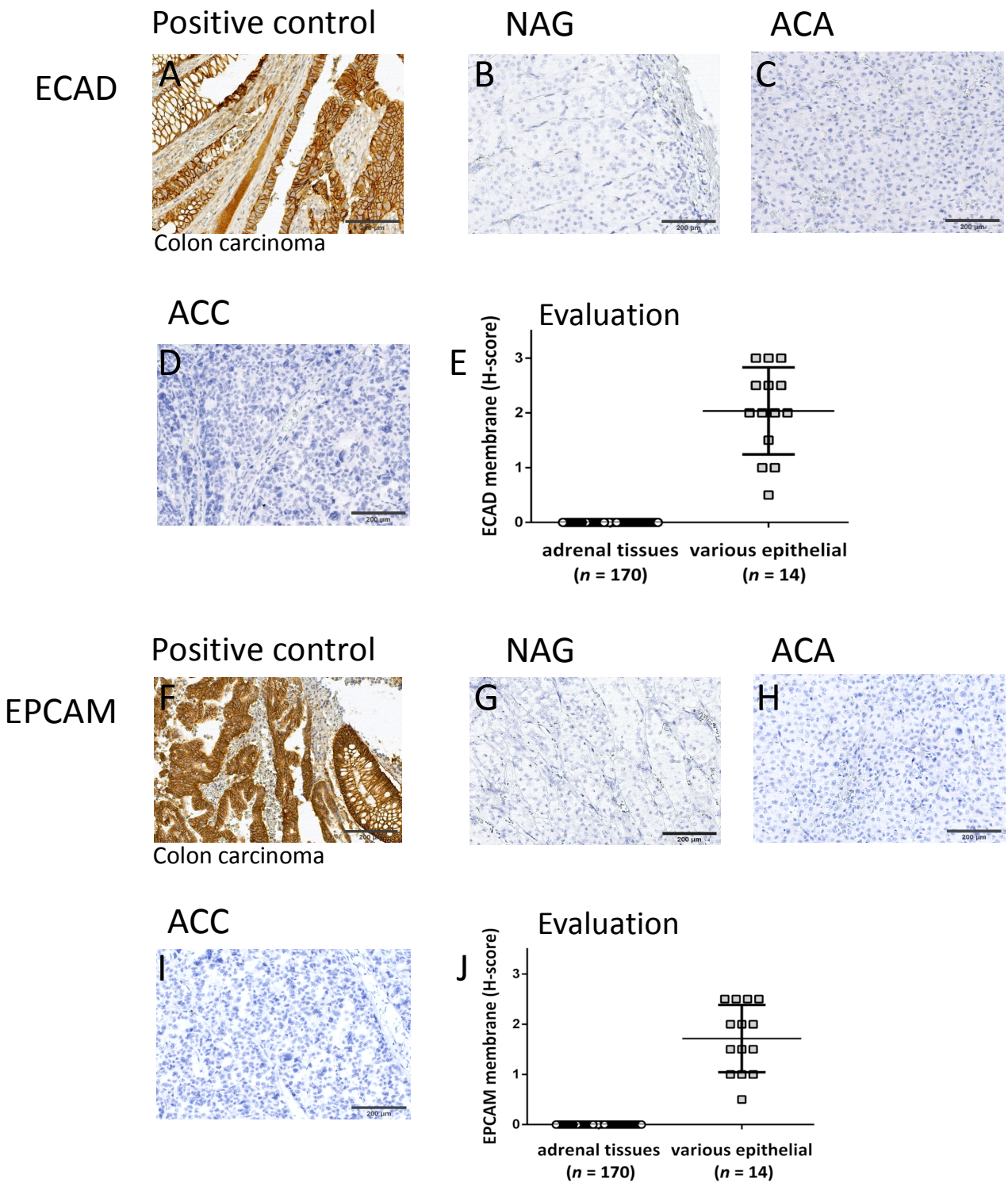
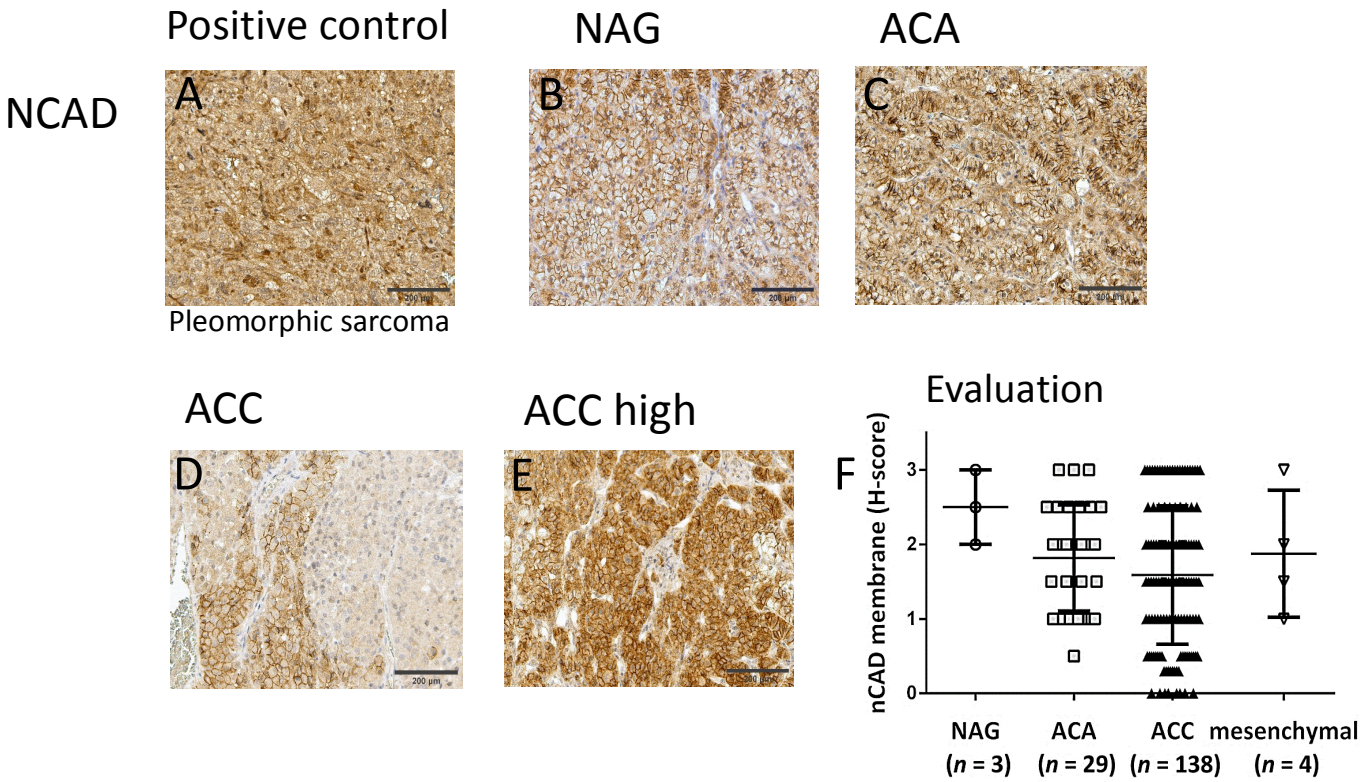



**Figure 7. Expression of canonical immunohistochemical epithelial markers in adrenocortical tissues.** Staining of epithelial markers E-cadherin (A-E) and Epithelial Cell Adhesion Molecule (EpCAM) (F-J) protein in classical epithelial tissues (A and F) vs normal adrenal glands (NAG, n=3; B and G) vs adrenocortical adenomas (ACA, n=29; C and H) vs adrenocortical carcinomas (ACC, n=138; D and I). Scale bar = 200µm. Quantitative evaluation in E and J, respectively.

## *3.2 Expression of mesenchymal markers*

Membrane N-cadherin expression was present in normal adrenocortical tissues at high levels (H-score 2.5 ± 0.5, Figure 8 B, F). The expression was distributed rather equally between the three functional zones, with slightly lower expression in the zona fasciculata (Figure 9). Most adrenocortical adenomas and carcinomas demonstrated moderate to high expression (ACA mean H-score 1.8 ± 0.7; Figures 8 C, F, ACC 1.6 ± 0.9; Figures 8 D-F) similar to the mesenchymal sarcomas (1.9 ± 0.8; Figures 8 A, F). There were no significant differences between the different adrenocortical tissues, only a trend (NAG vs ACA: p = 0.14, NAG vs ACC: p = 0.09 and ACA vs ACC: p = 0.20), however, the variability of expression of N-cadherin increased from ACA to ACC (Figure 8F) as shown by increasing coefficients of variation (ACA 39.25% and ACC 58.53%).



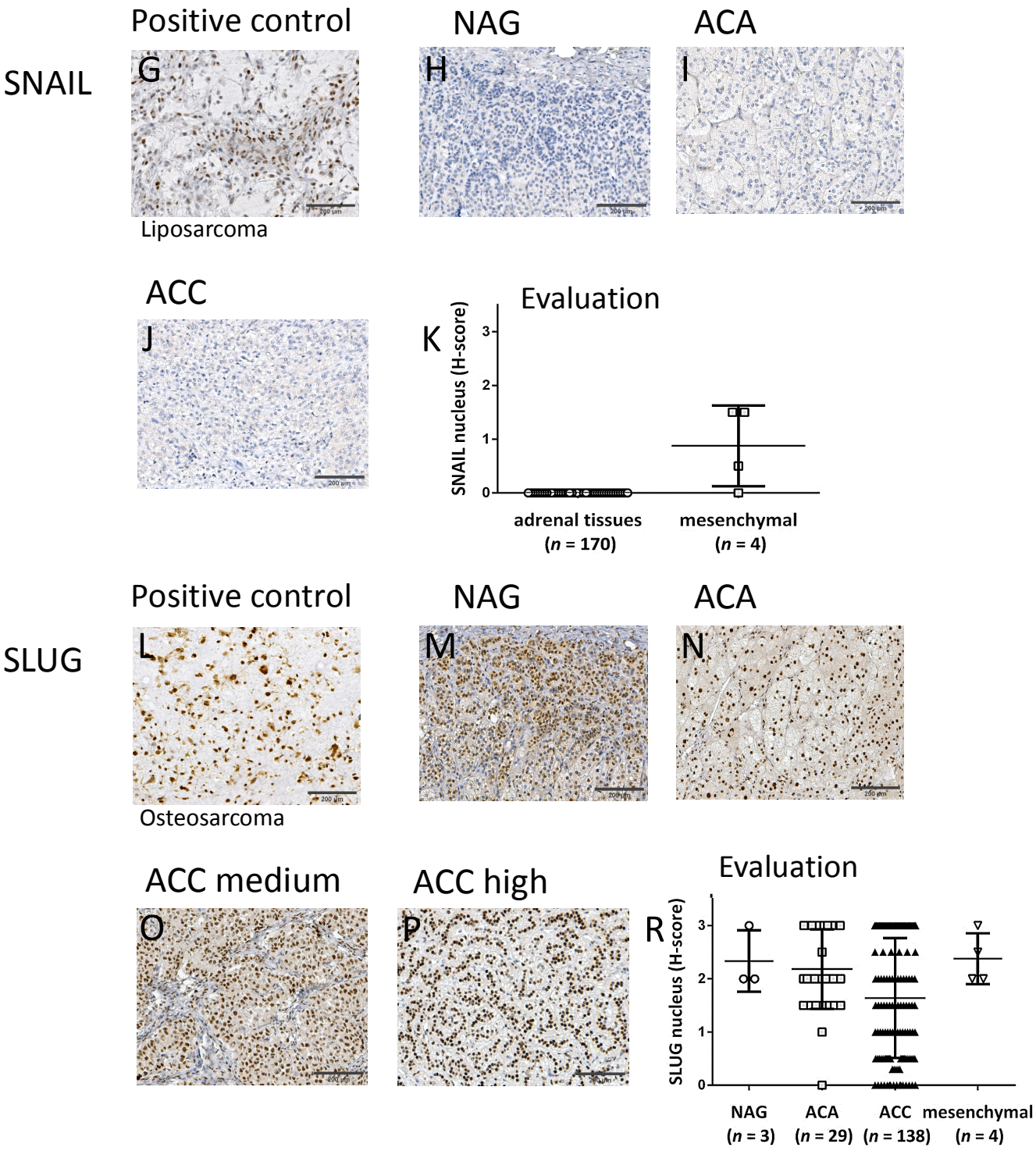



**Figure 8.** Expression of canonical immunohistochemical mesenchymal markers in adrenocortical tissues. Staining of mesenchymal markers N-cadherin (A-F), Zinc finger protein SNAI1 (SNAIL) (G-K) and Zinc finger protein SNAI2 (SLUG) (L-R) in classical mesenchymal cancers (A, G and L) vs normal adrenal glands (NAG, n=3; B, H and M) vs adrenocortical adenomas (ACA, n=29; C, I and N) and vs adrenocortical carcinomas (ACC, n=138; (D, E, J, O, and P). Scale bar = 200µm. Quantitative evaluation in (F, K and R), respectively.

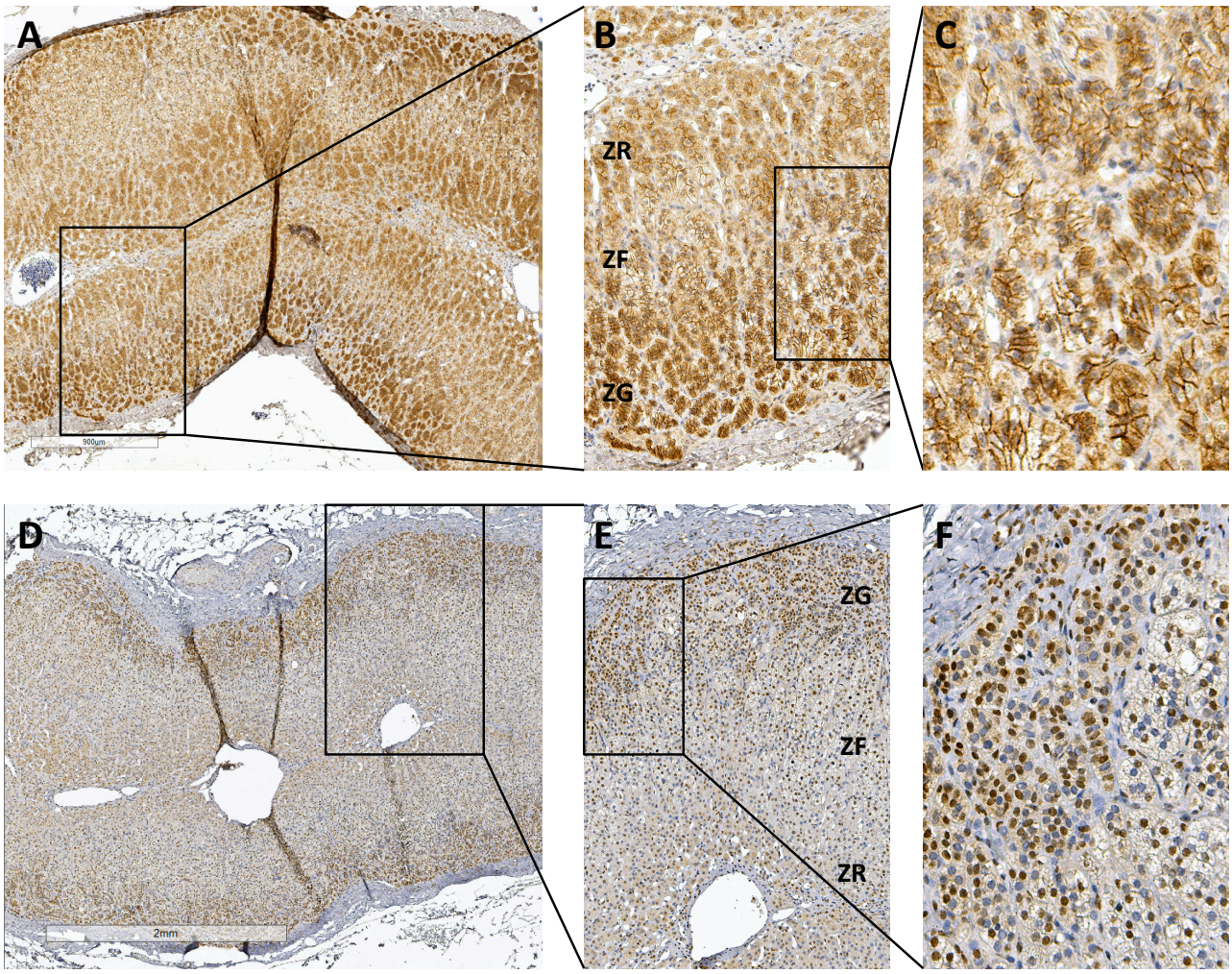



**Figure 9. Expression of canonical immunohistochemical mesenchymal markers in normal adrenal gland.** Staining of mesenchymal markers N-cadherin (A, B and C) and SLUG (D, E and F) in normal adrenal gland. Overview of the middle section of a normal adrenal gland (A and D) with zoom in a representative region (B and E) containing all three functional zones of the adrenal gland (ZG = zona glomerulosa, ZF = zona fasciculata and ZR = zona reticularis) and further zoom to visualize staining subcellular localization in C and F, respectively.

While Snail nuclear expression was found in most mesenchymal tissues tested (Figures 8 G and K), detectable expression was not observed in any of the adrenocortical tissues (Figures 8 H-J and evaluation in K). In contrast, a strong expression of Slug was found in both mesenchymal tissues (mean H-score 2.3±0.5; Figures 8 L and R) and normal and benign adrenal tissues without statistically significant differences among groups (NAG mean H-score 2.3±0.5, ACA 2.2±0.7; Figures 8 M-N and R) but variable expression in ACC (mean H-

score 1.6±1.1; Figures 8 O-P and R). Only the expression in ACC was significantly different compared to the other two adrenocortical sample sets (NAG vs ACA: p = 0.79, NAG vs ACC: p = 0.02 and ACA vs ACC: p = 0.01*) but, as with N-cadherin, the variability of expression of SLUG increased gradually from NAG to ACA and then to ACC (Figure 8R) as shown by increasing coefficients of variation (NAG 24.74%, ACA 34.29% and ACC 68.77%; mesenchymal 20.16%). In the normal adrenal gland tissue the most nuclei stained positive were localized in the subcapsular region, in the zona glomerulosa (Figure 9).

## *3.3 FGFR2-3 isotype expression shows a pattern similar to mesenchymal tissues*

In order to further elucidate the epithelial vs. mesenchymal phenotype of adrenocortical tumors, we used the ratio between the predominantly mesenchymal IIIC and the predominantly epithelial IIIB isotypes of FGFR 2-3 in a subgroup of fresh frozen adrenocortical tissue samples and cell lines. Isoform IIIC of FGFR 2 was expressed on average 4.6 times higher than IIIB in all adrenocortical tissues studied (Figure 10A) (ratio IIIC/IIIB: 5.1±2.6 for the normal adrenal glands and adrenocortical adenomas vs. 4.2±2 for the ACC samples vs. 4.8±1.2 for ACC cell-lines) similar to the mesenchymal sarcomas (2.8±0.8), but in contrast to the epithelial samples where the IIIB isoform was higher expressed than the IIIC isoform, as expected (ratio IIIC/IIIB: 0.4±0.3). For FGFR 3 the IIIC/IIIB ratios were even higher (Figure 10B) (12.2±5.5 for the normal adrenal glands and adrenocortical adenomas vs. 11.9±7.7 for the ACC samples vs. 11.7±3.2 for ACC cell-lines) similar again to the mesenchymal sarcomas (10.7±6.1). The epithelial control tissues showed again, as expected, higher IIIB than IIIC expression (ratio IIIC/IIIB: 0.06 ± 0.04).

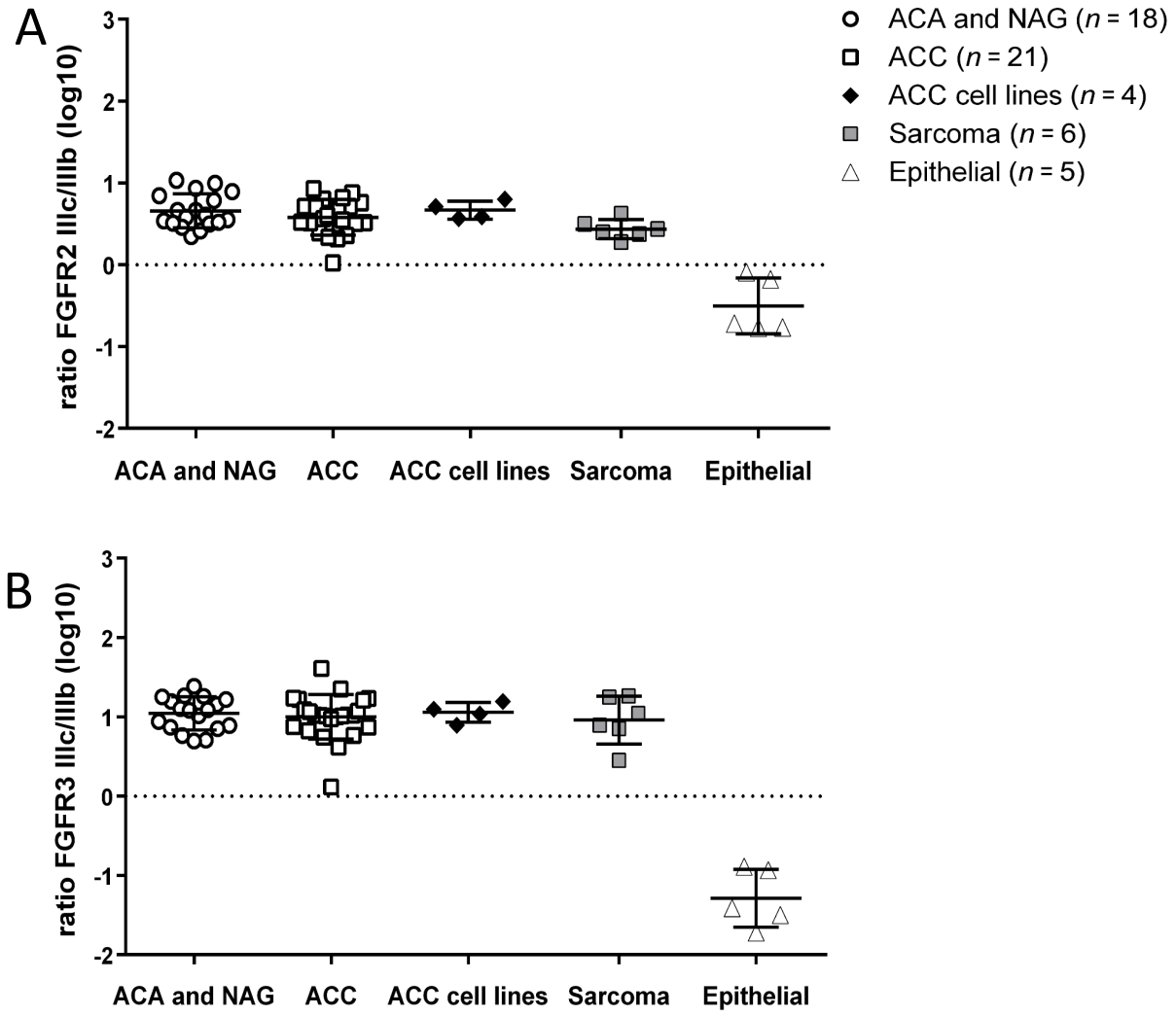



**Figure 10.** Differential expression of FGFR splice variants mRNA in adrenocortical tissues. Analysis of the ratios between the "mesenchymal" (IIIC) and "epithelial" (IIIB) splice variants for FGFR-2 (A) and 3 (B), in normal adrenal glands (NAG), adrenocortical adenomas (ACA) and carcinomas (ACC) as compared to the mesenchymal sarcomas and canonical epithelial tissues. For better visualization of the results and of the isoform switch, results are represented in log10 base.

## *3.4 SLUG and N-Cadherin are associated in an opposite manner with pathoclinical tumor aggressiveness parameters*

Since expression of NCAD and SLUG showed an increase in variability from normal, to benign, to malignant adrenocortical tissues, this suggested a modulation of these factors during the tumorigenesis and tumor progression. Therefore, we looked for possible associations between different expression levels of NCAD and SLUG and indicators of tumoral metastatic potential. The presence of venous infiltration of tumoral tissue was associated with high (H-score ≥2) vs. low (H-score <2) expression of SLUG (31 vs 44%, $\chi 2=3.6$, $p=0.05$) (Figure 11A), but with lower expression of N-cadherin (28 vs 46%, $\chi 2=6.9$, $p=0.008$) (Figure 11B). Similarly, lymph node infiltration was significantly more often present in tumors with high SLUG expression (23% vs 12%, $\chi 2=4.2$, $p=0.04$) (Figure 11C) and with low N-cadherin expression (26% vs. 9%, $\chi 2=10.0$, $p=0.001$) (Figure 11D). In addition, the mixed pathomorphological diagnostic Weiss score, an indicator for tumor malignancy, was significantly higher for samples with low NCAD expression (6.0±1.5 vs. 4.7±1.6, $p<0.001$) (Figure 11F), and for samples with high SLUG expression (6.0±1.9 vs. 5.1±1.5, $p=0.04$) (Figure 11E). A Mann-Whitney test of the distribution of N-cadherin and SLUG expression in tumors with low and high expression of the proliferation marker Ki67, the best-defined prognostic marker for the ACC [Beuschlein et al. 2015], confirmed this association. In tumors with high Ki67 expression the SLUG expression was significantly higher (2.2±0.9 vs 1.5±1.1, $p=0.03$) (Figure 11G) while the expression of N-cadherin was significantly lower (1.1±0.6 vs. 1.5±0.9, $p=0.04$) (Figure 11H).

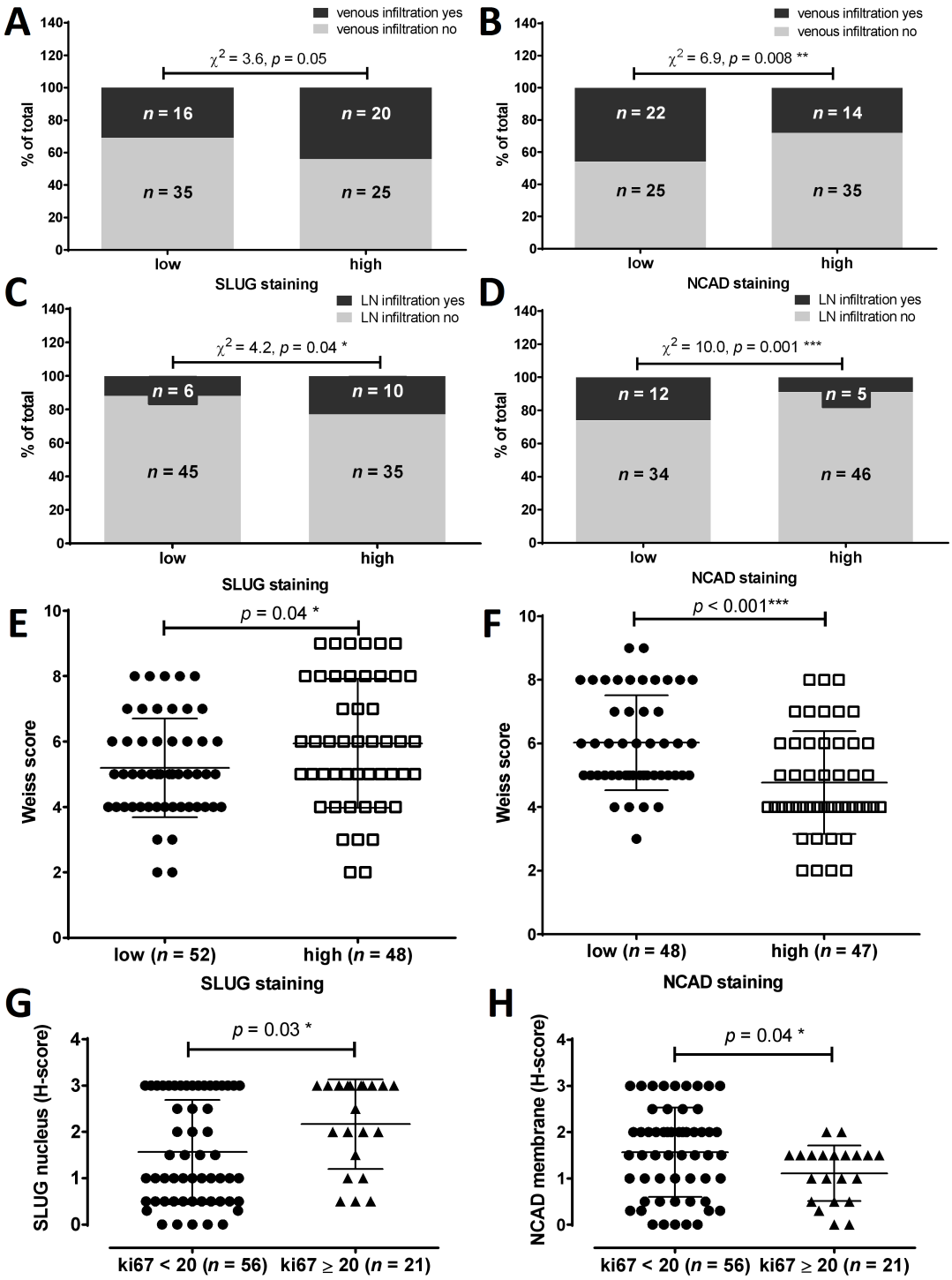



**Figure 11. Comparison between relevant clinicopathological data and expression levels of mesenchymal markers SLUG and N-Cadherin.** (A,B) venous infiltration of tumoral tissue, (C,D) lymph node infiltration of tumoral tissue, (E,F) Weiss score distribution and (G,H) proliferation marker Ki67. "n" numbers represent the absolute number of cases in each subgroup. Pearson's Chi-Square ($\chi 2$) analyses have been performed in IBM SPSS between proportions (%) in each staining intensity group while a Mann-Whitney test was used to compare the IHC scores. * $p < 0.05$,** $p < 0.01$,*** $p < 0.001$

## *3.5 SLUG and N-cadherin expression have a divergent association with ACC patients' progression-free survival*

We next investigated a potential association of SLUG and N-cadherin expression with patient outcome and found no difference on OS (low vs. high SLUG expression: median survival time 64.20 ± 10.27 vs 68.82 ± 9.14 months, HR = 1.15, 95% CI: 0.5–1.5, p = 0.79 and low vs high N-cadherin expression: median survival time 65.11 ± 9.49 vs 71.22 ± 10.66 months, HR = 0.81, 95% CI: 0.48–1.37, p = 0.44) (Figures 12 A,B) and only a trend that high SLUG expression correlated with a less favorable RFS in ACC patients after complete resection (high vs. low SLUG expression: median survival time 25.96 ± 5.40 vs 49.82 ± 10.12 months, HR = 2.15, 95% CI: 0.96–4.83, p = 0.056) (Figure 12C). For N-cadherin the situation was opposite, while again not statistically significant, there was a trend that high N-cadherin expression correlated with a better progression-free survival (mean survival time 40.12 ± 8.35 vs 21.32 ± 6.6 months, HR = 0.65, 95% CI: 0.34–1.11, p = 0.14) (Figure 12D).

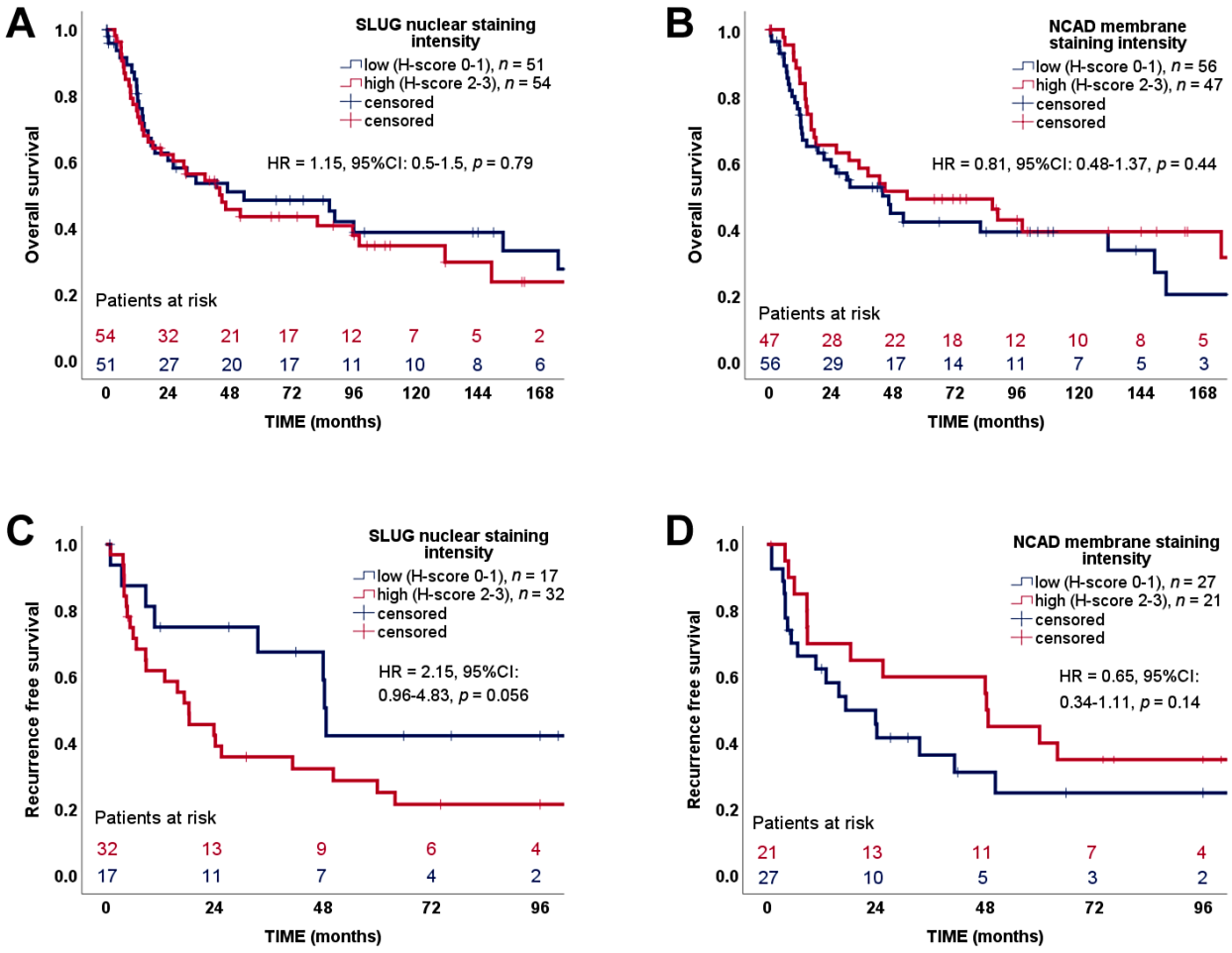



**Figure 12**. Correlation of patient survival with expression of mesenchymal markers SLUG and N-Cadherin. (A-B) overall survival (C- D) recurrence-free survival. The Kaplan-Meier method was used to build the survival curves and the Cox proportional hazards model was used to calculate the hazard ratio (HR), the confidence interval (CI) and significance values in IBM SPSS.

## *3.6 FGF pathway mRNA expression*

A similarity matrix clustering analysis of the real-time RT-PCR assessment comprising 93 genes from the FGF pathway showed a distinctive phenotype of adrenocortical tissue compared to all the other tissue samples (Figure 13). A separate cluster that contained all three NAG and most of the ACA had a distinct expression pattern compared to the majority of ACC. At

variance, typical epithelial (from thyroid and colon) and mesenchymal tissues (from sarcomas) and all cell lines showed divergent expression patterns and clustered in several small groups separately from the adrenocortical tissues. Notably, colon tissues, whether normal or malignant clustered together as did most of the sarcomas. The different cell lines, whether of adrenocortical origin or not, did not cluster with their tissue counterparts (Figure 13).

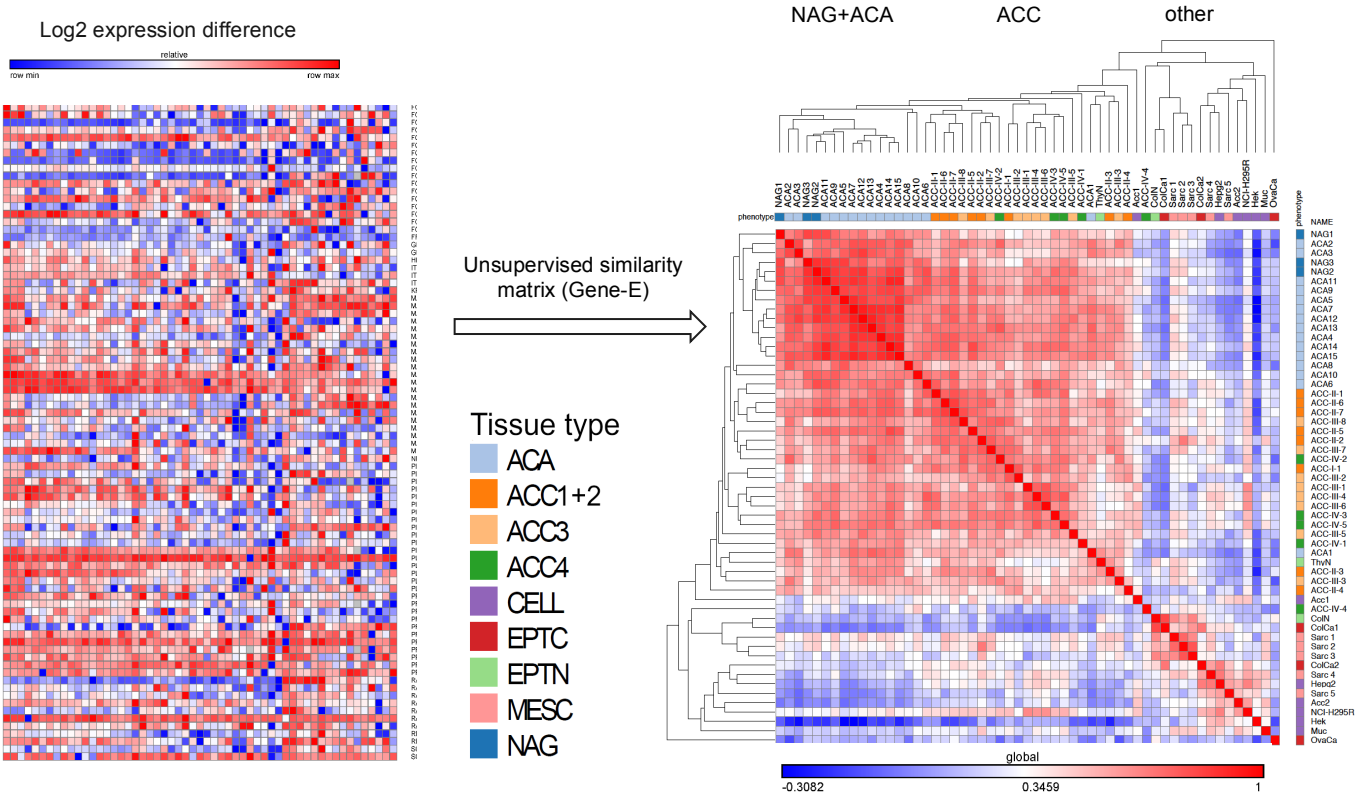



**Figure 13. Unsupervised similarity matrix based on RT-PCR FGF pathway expression, performed in Gene-E software using the column distance/similarity matrix algorithm and the average linkage method with Pearson correlation.** On the left side is a graphical representation of the Log2 transformed normalized expression data arranged vertically by tissue name and horizontally by gene name in the order that they were arranged on the PCR plate. To the right side is the data rearranged through the unsupervised similarity matrix algorithm. The colored bar under the tissue names is encoding the different types of tissues analyzed: NAG = normal adrenal glands, ACA = adrenocortical adenomas, ACC1+2 = ACC in ENSAT stages I and II, ACC3 = ACC in ENSAT stage III, ACC4 = ACC in ENSAT stage IV, EPTN = normal (non-neoplastic) classical epithelial tissues, EPTC = malignant tumors of classical epithelial tissues/carcinomas, MESC = malignant tumors of classical mesenchymal tissues/sarcomas and CELL = cell-lines.

Several genes of the FGF pathway were significantly differentially expressed among the various clusters, including 16 genes differentially expressed between ACA and ACC (software clustering shown in Figure 14 and values detailed in Table 4 and Figure 15). Among the 11 genes expressed at lower levels in ACC compared to ACA, there were the genes encoding for FGFs and their receptors like the FGF12, FGF14, and FGFR2, for phospholipases like Phospholipase D1, Phosphatidylcholine-Specific (PLD1) and Glycosylphosphatidylinositol Specific Phospholipase D1 (GPLD1), Ras-Related Protein R-Ras 1 (RRAS), 2 (RRAS2) and 3 (MRAS) and the Mitogen-Activated Protein Kinases 10 (MAPK10) and 5 (MAP3K5) as well as Phosphatidylinositol-4-Phosphate 3-Kinase Catalytic Subunit Type 2 Gamma (PIK3C2G). The five genes significantly upregulated in ACCs vs ACAs encoded for the FGFR1, FGFR4, FGF8, and FGF19, and the Neuroblastoma RAS Viral Oncogene Homolog (NRAS).

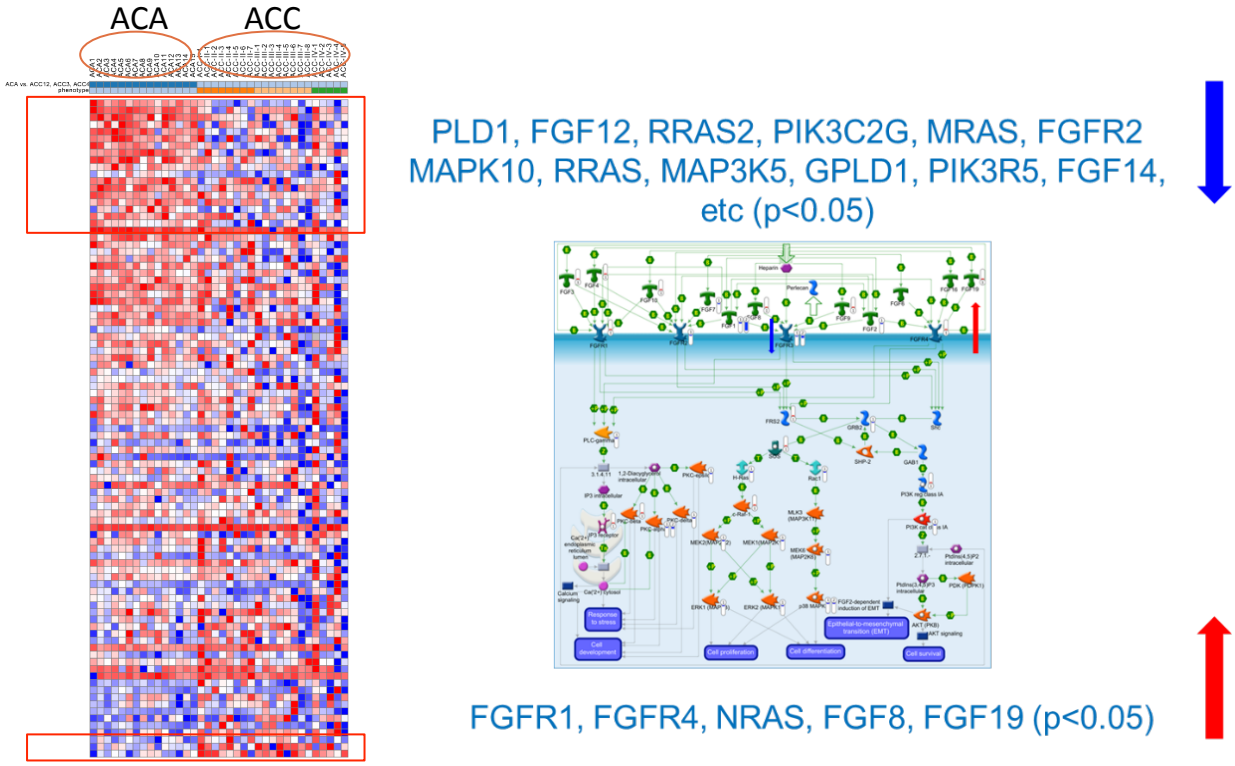



**Figure 14. Hierarchical clustering of adrenocortical adenoma vs versus adrenocortical carcinoma, using the marker selection algorithm with a two sided test in Gene-E software.** The upper region contains the genes that are downregulated in ACC and the lower part those that are upregulated. To the right a representation of the FGF pathway and some examples of genes that are down or upregulated. In the background a representation of the FGF pathway generated in MetaCore.

| Tissue | ACA (n=15) | ACC (n=21) | | |
|---|---|---|---|---|
| Gene symbol | relative expression (average±SD) | relative expression (average±SD) | ACC vs ACA fold-change (95% CI) | *p* -value |
| *PLD1* | 0.027 ± 0.0124 | 0.008 ± 0.004 | -3.4 (-2.4 to -4.4) | <0.0001 |
| *FGF12* | 0.056 ± 0.041 | 0.015 ± 0.024 | -3.7 (-1.5 to -7.7) | <0.0001 |
| *RRAS2* | 0.002 ± 0.0008 | 0.0009 ± 0.0007 | -2.1 (-1.2 to -3.1) | 0.0002 |
| *PIK3C2G* | $8.3x10^{-5} \pm 1x10^{-5}$ | $1.2x10^{-5} \pm 1.6x10^{-5}$ | -6.7 (-1.7 to -11.8) | 0.021 |
| *MRAS* | 0.025 ± 0.011 | 0.011 ± 0.008 | -2.2 (-1.3 to -3.1) | 0.0003 |
| ***FGFR2*** | 0.021 ± 0.013 | 0.013 ± 0.017 | -1.6 (-1 to -2.2) | 0.014 |
| *MAPK10* | 0.043 ± 0.028 | 0.021 ± 0.032 | -2 (-1 to -2.9) | 0.001 |
| *RRAS* | 0.005 ± 0.001 | 0.003 ± 0.002 | -1.6 (-1 to -2.1) | 0.006 |
| *MAP3K5* | 0.029 ± 0.018 | 0.011 ± 0.009 | -2.5 (-1.4 to -3.6) | 0.001 |
| *GPLD1* | $2.1x10^{-4} \pm 1.3x10^{-4}$ | $1.2x10^{-4} \pm 7.8x10^{-5}$ | -1.7 (-1.1 to -2.3) | 0.009 |
| *FGF14* | $1.1x10^{-4} \pm 1.2x10^{-4}$ | $6.6x10^{-5} \pm 9.8x10^{-5}$ | -1.7 (-1 to -2.3) | 0.04 |
| ***FGFR1*** | 0.005 ± 0.003 | 0.023 ± 0.015 | 4,1 (2.5 to 5.6) | <0.0001 |
| ***FGFR4*** | $3.3x10^{-5} \pm 2.4x10^{-5}$ | $2.0x10^{-4} \pm 1.8x10^{-4}$ | 6.1 (3.1 to 9.1) | 0.007 |
| *NRAS* | 0.012 ± 0.003 | 0.020 ± 0.009 | 1.4 (1 to 1.8) | 0.049 |
| *FGF8* | $3.4x10^{-6} \pm 4.7x10^{-6}$ | $2.8x10^{-5} \pm 5.9x10^{-5}$ | 8.32 (1.2 to 15.3) | 0.010 |
| *FGF19* | $1.7x10^{-7} \pm 1.02x10^{-7}$ | $1.71x10^{-6} \pm 3.0x10^{-6}$ | 9.81 (1.1 to 18.5) | 0.047 |

| Tissue | ACC 1+2 (n=8) | ACC 3+4 (n=13) | | |
|---|---|---|---|---|
| Gene symbol | relative expression (average±SD) | relative expression (average±SD) | ACC3+4 vs ACC1+2 fold-change (95% CI) | *p* –value |
| *RALA* | 0.016 ± 0.012 | 0.006 ± 0.002 | -2.6 (-2 to -3.3) | 0.001 |
| *PRKCA* | 0.047 ± 0.042 | 0.006 ± 0.006 | -7.8 (-3.3 to -12.3) | 0.004 |
| *MAPK9* | 0.031 ± 0.017 | 0.013 ± 0.008 | -2.7 (-1.5 to -2.9) | 0.012 |
| *MAP3K2* | 0.013 ± 0.005 | 0.007 ± 0.005 | -1.6 (-1 to -2.2) | 0.024 |
| *PIK3R1* | 0.081 ± 0.102 | 0.021 ± 0.019 | -3.8 (-1.9 to -5.7) | 0.024 |
| *RAF1* | 0.007 ± 0.003 | 0.004 ± 0.002 | -1.6 (-1.1 to -2.1) | 0.036 |
| *MAP3K1* | 0.008 ± 0.003 | 0.005 ± 0.002 | -1.5 (-1.1 to -1.9) | 0.036 |
| *FGF21* | $5.1x10^{-7}$± $5.0x10^{-7}$ | $8.8x10^{-6}$± $7.6x10^{-6}$ | 17.3 (9.3 to 25) | 0.007 |

**Table 4. Statistically significant differential mRNA expression between different groups of adrenocortical tissues.** ACA: adrenocortical adenoma, ACC: adrenocortical carcinoma, ACC 1+2: adrenocortical carcinoma in ENSAT stages I and II, ACC 3+4: adrenocortical carcinoma in ENSAT stages III and IV. The higher values between two subgroups are squared in black. The significantly differentially expressed FGF-receptors are in bold type. (Detailed view of the highlighted values, with $p < 0.05$, from Figure 14. The significance was determined in Gene-E software by using a two-sided test within the marker selection algorithm).

The differences between ACCs with localized, ENSAT I/II tumors compared to stage III/IV ACCs were less prominent with only 8 genes with statistically significant differential expression between the two groups (Table 4 and Figure 16). Most of these genes were expressed lower in advanced ACC: RAS Like Proto-Oncogene A (RALA), Raf-1 Proto-Oncogene (RAF1) and the kinases Protein Kinase C Alpha (PRKCA), Mitogen-Activated Protein Kinase 9 (MAPK9), Mitogen-Activated Protein Kinase Kinase Kinase 1 (MAP3K1) and 2 (MAP3K2), and Phosphoinositide-3-Kinase Regulatory Subunit 1 (PIK3R1). Fibroblast growth factor 21 (FGF21) was the only one of the analyzed genes that was expressed at significantly higher levels in advanced ACC.

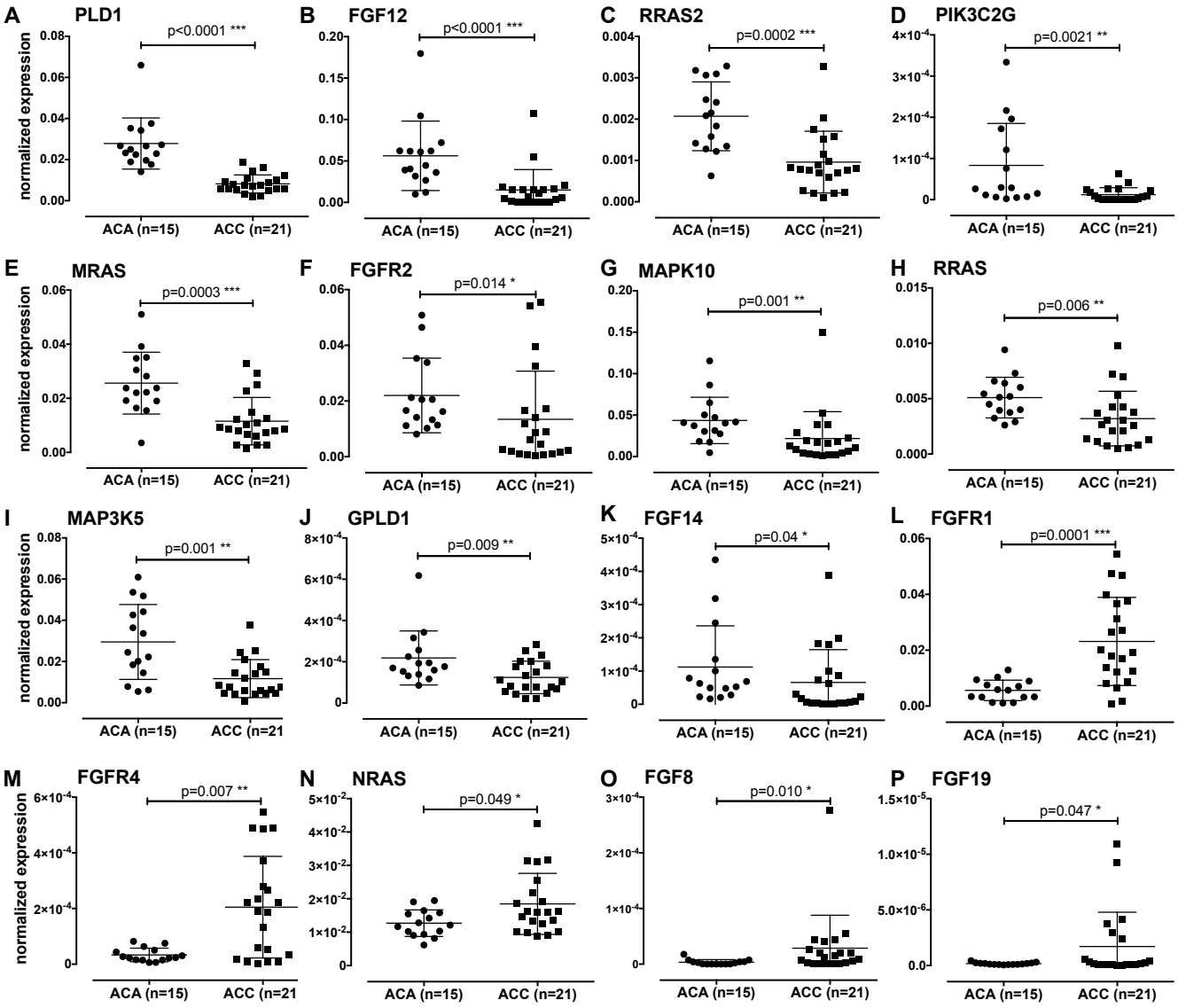



**Figure 15. Significantly differentially expressed genes in adrenocortical adenomas (ACA) compared to adrenocortical carcinomas (ACC).** Graphical representation of the significantly underexpressed (A-K) and overexpressed (L-P) genes in ACC vs ACA. Statistical significance determined with a two-sided test in Gene-E: *p<0.05, **p<0.01, ***p<0.001.

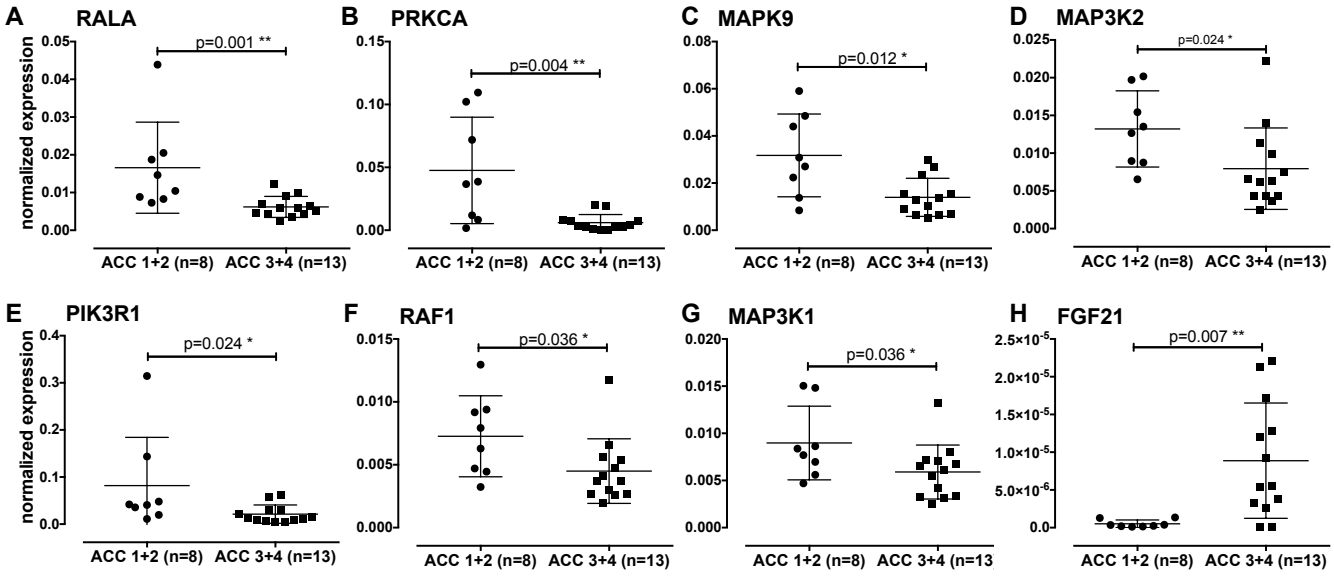



**Figure 16. Significantly differentially expressed genes in adrenocortical carcinomas with ENSAT I and II (ACC 1+2) compared to adrenocortical carcinomas with ENSAT III and IV (ACC 3+4).** Graphical representation of the significantly underexpressed (A-G) and overexpressed (H) genes in ACC 3+4 vs ACC 1+2. Statistical significance determined with a two-sided test in Gene-E: *p<0.05, **p<0.01.

## *3.6 RNAScope in situ RNA hybridization of FGF Receptors*

To assess the tissue distribution of the FGF receptors FGFR1, 2 and 4 as potential treatment targets, and to confirm real-time PCR results in a larger sample set (n=166), we applied RNAScope in situ RNA hybridization. FGFR3 has not been included in the this bigger cohort due to the results from the RT-PCR analysis, that did not show any significance. RNAScope staining with FGFR3 in small subcohort also showed a low and constant expression among the samples.

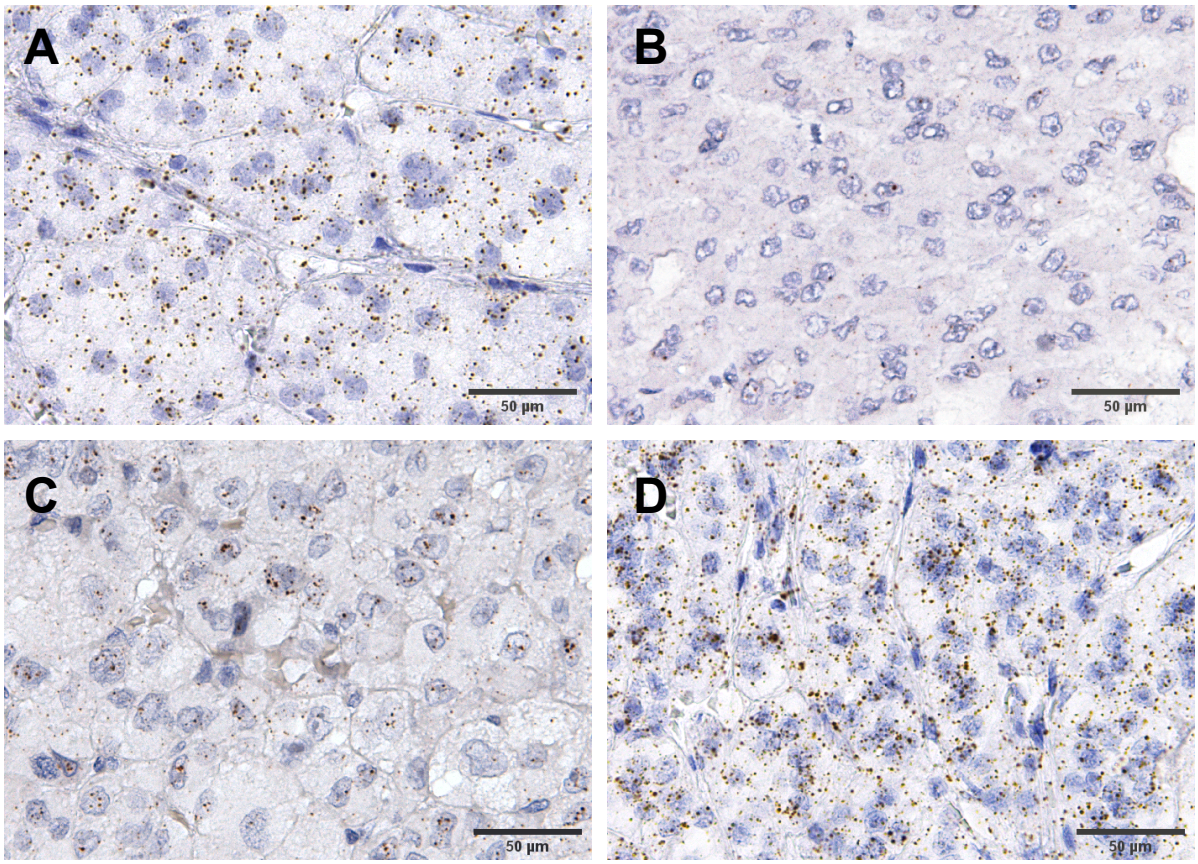



**Figure 17. Examples of expression RNAScope staining in ACC.** (A) an example of house keeping gene PPIB staining, while (B) to (D) show various levels of FGFR4 expression, from low to high.

While the housekeeping gene PPIB (Peptidylprolyl Isomerase B) showed a relatively constant average-high expression in all ACC samples analyzed (Figure 17A), expression of FGFRs was variable between the samples but quite homogenously distributed within most samples (Figures 17 B-D). FGFR expression was nearly exclusively in tumoral cells.

Significantly more FGFR1 and FGFR4 mRNAs were detected in ACC compared to ACA (Figure 18) (5.1±4.3 mRNA molecules/cell vs 1.7±1.4 mRNA molecules/cell, p=0.03 in the case of FGFR1 and 5.5±4.9 mRNA molecules/cell vs 2.1±1.4 mRNA molecules/cell, p=0.002 in the case of FGFR4). In contrast, FGFR2 was significantly higher expressed in ACA (5.2±2.7 mRNA molecules/cell for ACA vs 2.5±2.5 mRNA molecules/cell for ACC, p=0.0001) confirming our real-time RT-PCR results in frozen tissues.

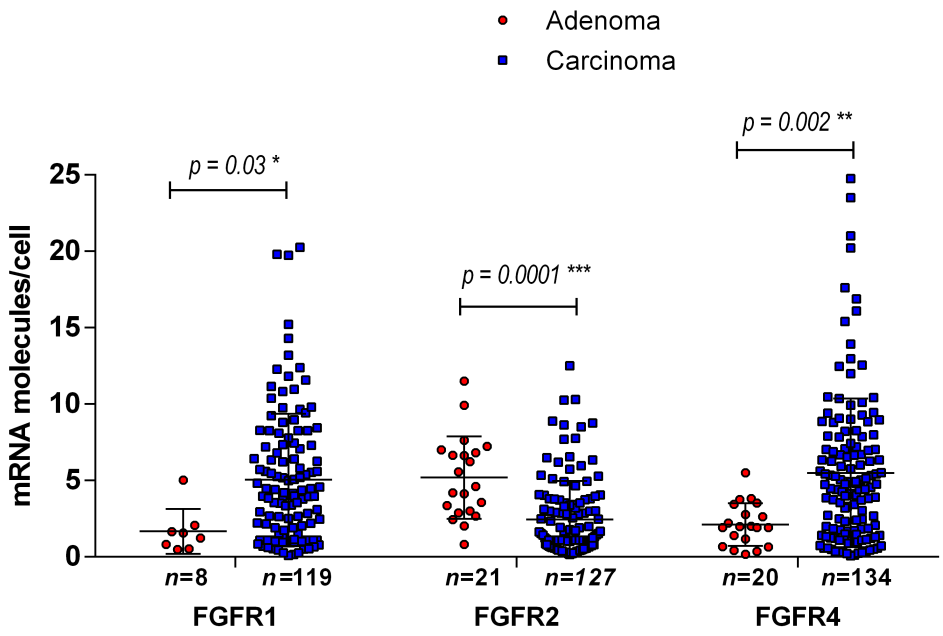



**Figure 18. Expression of FGF receptors 1, 2 and 4 in adrenocortical tissues as assessed by RNAScope.** Expression levels in ACA vs ACC. Statistical significance determined with a Mann-Whitney test in Graphpad Prism: *p<0.05, **p<0.01, ***p<0.001. (FGFR1 has a much less samples for ACA due to the FFPE blocks being sent to another center for another study and did not arrive in time to be included).

Next, we compared expression between tumors in early and advanced stages (Figure 19A) and found significantly higher expression only of FGFR4 in ENSAT stage 3 + 4 (6.2±5.2 mRNA molecules/cell) compared to ENSAT 1 + 2 (4.1±3.7 mRNA molecules/cell, p=0.02) ACC. Similarly, we found significantly higher expression FGFR4 in recurrences/metastasis compared to primary tumors (Figure 19B) (8.8±6.6 mRNA molecules/cell for recurrences vs 4.7±4.1 mRNA molecules/cell for primary tumors).

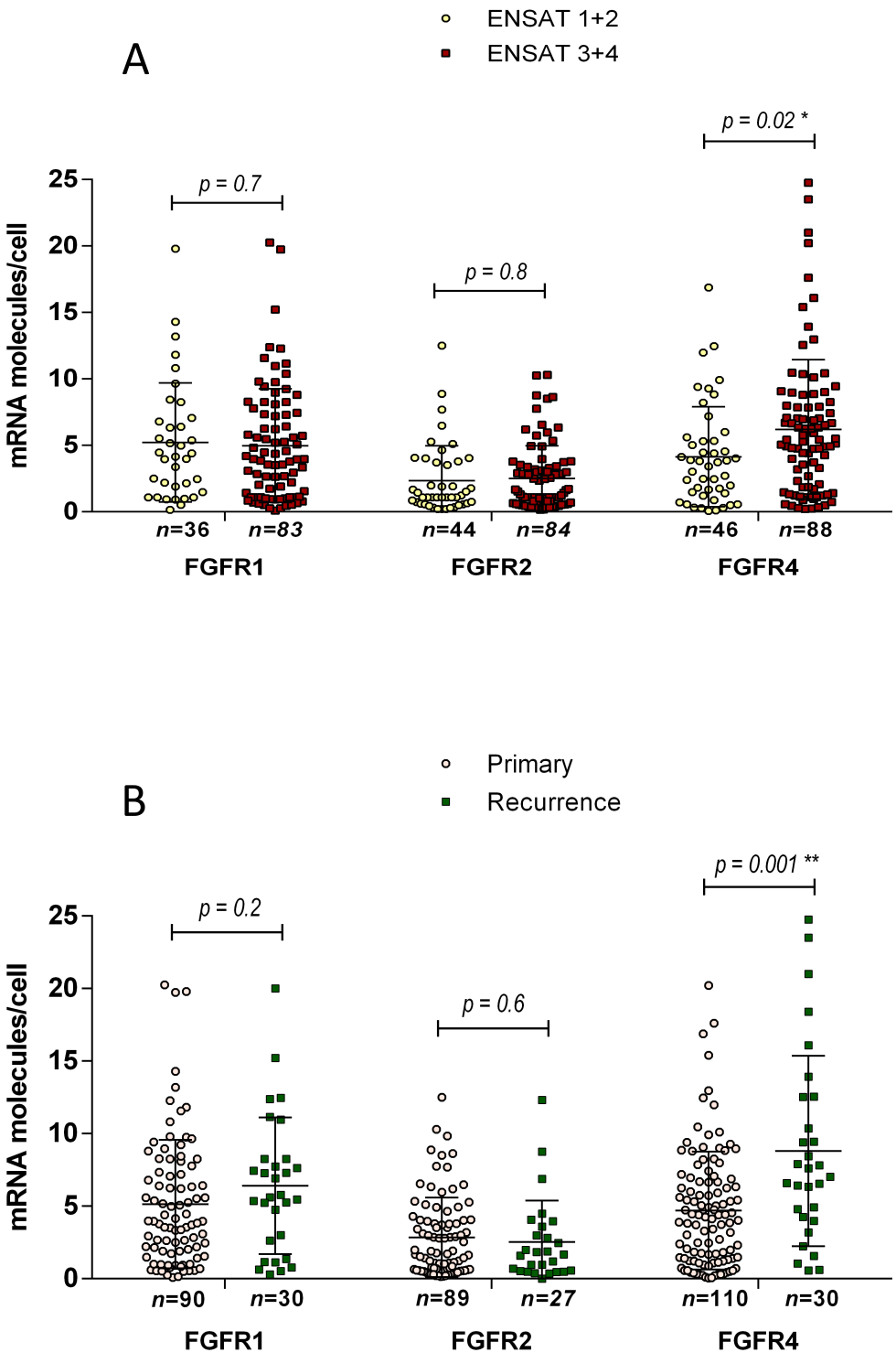



**Figure 19. Expression of FGF receptors 1, 2 and 4 in adrenocortical tissues as assessed by RNAScope.** Expression levels in ACC ENSAT stages 1+2 vs 3+4 (A) and in ACC primary tumor samples vs local or distant recurrences (B). Statistical significance determined with a Mann-Whitney test in Graphpad Prism: *p<0.05, **p<0.01, ***p<0.001

## *3.7 Influence of FGF Receptors' expression on patient survival*

In a next step, we assessed the influence of the FGFR 1, 2 and 4 on patient survival in the RNAScope ACC patient cohort. The median expression value for each receptor was chosen as cut-off between low and high expression and was 3.9 spots per cell for FGFR1, 1.9 for FGFR2 and 4.4 for FGFR4.

High FGFR1 expression was associated with both a shorter OS of 84.12±16.75 vs 147.98±23.20 months (HR=1.8, 95% CI: 1.01-3.25, p=0.047) (Figure 20A) and a shorter RFS of 24.84±6.71 vs 74.71±15.06 months (HR=2.93, 95% CI: 1.25-6.84, p=0.013) (Figure 20B), whereas FGFR2 was not associated with either OS and RFS (Figures 20 C - D) (Table 5). FGFR4, while significantly associated with a shorter OS of 50.52±7.59 vs 154.60±19.64 months (HR=2.44, 95% CI: 1.41-4.22, p=0.001) (Figure 20E), was not associated with RFS (Figure 20F) (Table 5).

In a multivariate analysis, including ENSAT tumor stage and proliferation index Ki-67, two well established prognostic factors for ACC [Fassnacht et al. 2009, Beuschlein et al. 2015], the association between FGFR1 expression and the recurrence-free survival (HR=6.10, 95% CI: 1.78 – 20.86, p=0.004) and between FGFR4 expression and the overall survival (HR=3.23, 95% CI: 1.52 – 6.88, p=0.002) remained significant (Table 5).

| overall survival | | | | | | | |
|---|---|---|---|---|---|---|---|
| | univariate analysis | | | | multivariate analysis | | |
| **variables** | **time** | **HR** | **95% CI** | ***p*** | **HR** | **95% CI** | ***p*** |
| Tumor stage | | | | | | | |
| I+II (n=46) | 171.68 ± 22.82 | | | | | | |
| III (n=33) | 90.17 ± 17.74 | 2.11 | 1.13 – 3.94 | **0.018** | 5.23 | 1.95 – 14.01 | **0.001** |
| IV (n=25) | 36.94 ± 11.28 | 4.60 | 2.35 – 8.98 | **< 0.001** | 5.64 | 1.43 – 22.18 | **0.013** |
| Ki67 | | | | | | | |
| low (<20) (n=53) | 143.21 ± 17.61 | | | | | | |
| high (≥20) (n=19) | 30.11 ± 6.77 | 4.31 | 2.12 – 8.78 | **< 0.001** | 17.4 | 5.83 – 52.20 | **< 0.001** |
| FGFR1 | | | | | | | |
| low (<median) | 147.9 ± 23.20 | | | | | | |
| high (>median) | 84.12 ± 16.75 | 1.80 | 1.01 – 3.25 | **0.047** | 2.11 | 0.91 – 4.89 | 0.07 |
| FGFR2 | | | | | | | |
| low (<median) | 103.09 ± 15.94 | | | | | | |
| high (>median) | 117.81 ± 23.76 | 1.09 | 0.60 – 1.98 | 0.75 | 1.19 | 0.50 – 2.83 | 0.68 |
| FGFR4 | | | | | | | |
| low (<median) | 154.60 ± 19.64 | | | | | | |
| high (>median) | 50.52 ± 7.59 | 2.44 | 1.41 – 4.22 | **0.001** | 3.23 | 1.52 – 6.88 | **0.002** |
| **recurrence-free survival** | | | | | | | |
| | **univariate analysis** | | | | **multivariate analysis** | | |
| **variables** | **time** | **HR** | **95% CI** | ***p*** | **HR** | **95% CI** | ***p*** |
| Tumor stage | | | | | | | |
| I+II (n=35) | 63.35 ± 11.58 | | | | | | |
| III (n=14) | 43.65 ± 14.25 | 1.36 | 0.66 – 2.81 | 0.40 | 1.74 | 0.67 – 4.51 | 0.25 |
| Ki67 | | | | | | | |
| low (<20) (n=29) | 71.22 ± 11.85 | | | | | | |
| high (≥20) (n=10) | 13.89 ± 5.57 | 4.34 | 1.87 – 10.09 | **0.001** | 8.66 | 2.64 – 28.44 | **< 0.001** |
| FGFR1 | | | | | | | |
| low (<median) | 74.71 ± 15.06 | | | | | | |
| high (>median) | 24.84 ± 6.71 | 2.9 | 1.25 – 6.84 | **0.009** | 6.10 | 1.78 – 20.86 | **0.004** |
| FGFR2 | | | | | | | |
| low (<median) | 59.56 ± 16.40 | | | | | | |
| high (>median) | 53.44 ± 12.71 | 0.99 | 0.45 – 2.18 | 0.99 | 0.87 | 0.32 – 2.39 | 0.79 |
| FGFR4 | | | | | | | |
| low (<median) | 62.94 ± 13.21 | | | | | | |
| high (>median) | 52.62 ± 13.07 | 1.06 | 0.52 – 2.18 | 0.86 | 0.77 | 0.33 – 1.80 | 0.55 |

**Table 5. Influence of FGFR - 1, 2 and 4 expression on overall and recurrence-free survival of ACC patients in univariate and multivariate analyses including Ki-67 and ENSAT stage.** The Cox model in IBM SPSS was used to determine the HR, CI and p-values. Statistical significance: *p<0.05, **p<0.01, ***p<0.001 (values highlighted in bold).

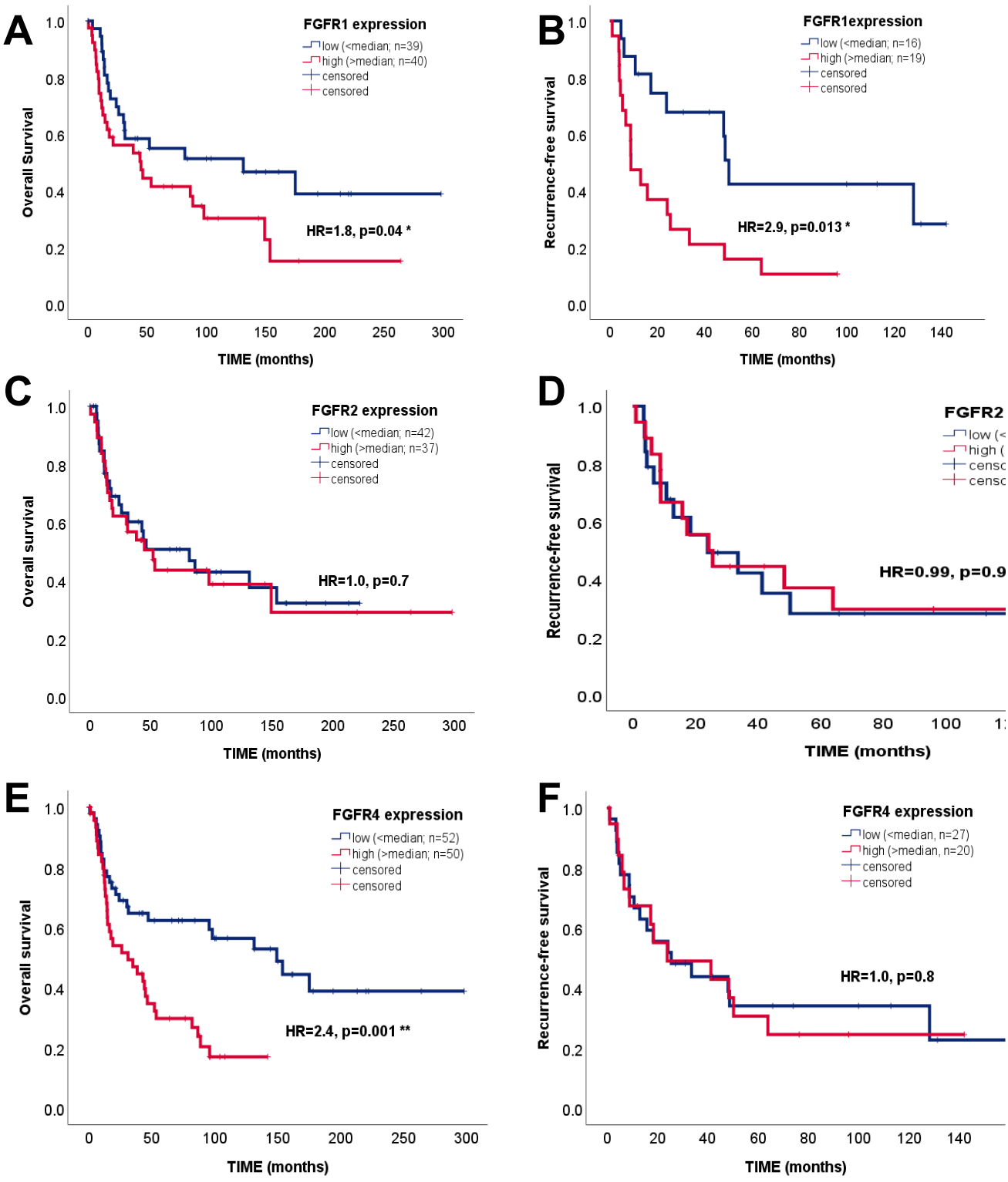



**Figure 20. Association between expression of FGF receptors 1, 2 and 4 with patient survival.** (A, C and E) overall survival (B, D and F) recurrence-free survival. The Kaplan-Meier method was used to build the survival curves and the Cox proportional hazards model was used to calculate the hazard ratios (HR) and significance values in IBM SPSS.

# Chapter 4

# Discussion

## *4.1 Adrenocortical tissues do not express typical epithelial adhesion markers but are instead characterized by relatively high expression of mesenchymal markers*

We investigated a series of both classical epithelial and mesenchymal markers in a large cohort of normal, benign and malignant adrenocortical tissues, and compared the expression of these markers with that in epithelial and mesenchymal control tissues. Against our hypothesis, our analysis revealed that EMT in adrenocortical tumors does not appear to play a role in tumor progression as suggested before in smaller studies [Bulzico et al. 2017, Zeisberg and Neilson 2009]. In contrast to our expectations, mesenchymal markers were not higher expressed in malignant than in benign neoplastic tissue. Even non-malignant adrenocortical tissues do not express established epithelial markers like E-cadherin and EpCAM but instead express a series of "classical" mesenchymal markers like Slug and N-cadherin at similar levels as mesenchymal tissues.

To validate this surprising finding, we explored a complementary method. By using the more recently discovered marker of alternative mRNA splicing of the FGFR2 and 3 [Ishiwata 2018, Shimizu et al. 2002, Paur et al. 2015, Zhao et al. 2013], we confirmed that adrenocortical tissues are more similar to mesenchymal than to epithelial tissues. This may be due to the special case of the adrenocortical tissue as it originates during embryogenesis from the intermediate mesoderm, but is considered to undergo MET to result in an epithelial tissue [Xing et al. 2015, Keegan and Hammer 2002]. It seems that this epithelial transformation is incomplete and the adrenal cortex keeps most of its mesenchymal characteristics at a molecular level.

Even though mesenchymal markers are expressed in all adrenocortical tissues, mesenchymal differentiation may still have a role in tumor aggressiveness. While higher SLUG expression is associated with more aggressive behavior of the tumors as indicated by its association with markers for lymphatic and hematogenic metastasizing and high cell proliferation, N-cadherin appears to play the role of major cell-cell adhesion molecule in the adrenocortical tissue and thus is a counterplayer of SLUG. There are also other tissues where N-cadherin is the prevalent constituent of adherens junctions such as neural tissues [Miyamoto et al. 2015]. It is hence likely that adherens junctions in adrenocortical tissue are predominantly mediated by N-cadherin instead of the E-cadherin, which is more commonly found in epithelial adherens junctions. However, only future studies at a deeper molecular level on the cell-to-cell interactions in the adrenal will be able to definitely answer this question.

Interestingly, SLUG nuclear expression in the normal adrenal gland was highest in the subcapsular area of the zona glomerulosa. This zone accommodates a subset of cells that have been reported to centripetally migrate towards the center of the gland and are responsible for the permanent renewal of the adrenocortical tissue [Vinson 2016]. The idea of a progenitor cell population that gives rise to all differentiated cell types within the adrenal cortex is old [Arnold 1866, Gottschau 1883], but while its subcapsular localization has been clarified using animal models [Chang et al. 2013, Vidal et al. 2016], there is not yet an universally accepted immunohistochemical marker that can be used to identify this population. It has been shown that Wnt, β-catenin and Shh all play an important role in this process [Walczak et al. 2014, King et al. 2009, Hammer and Basham 2021], however, their expression in the adult adrenal cortex did not coincide with cell proliferation markers [Lerario et al. 2017] so they cannot be used to identify the progenitor cell population. The best candidate to date is the Notch atypical ligand Delta-like homologue 1 (DLK1) [Hadjidemetriou et al. 2019].

It would have been especially interesting to correlate in more detail the expression of SLUG and N-cadherin also in metastases with the corresponding clinicopathological characteristics, especially Ki67. Nevertheless, these data have been inconsistently retrieved in the past, and combined with the rarity of ACC, renders the collection of such a large series of clinically well-annotated cases prospectively quite challenging. Another limitation is the perceived limited choice of both epithelial and mesenchymal markers analyzed. While the list of possible specific markers is very long [Kalluri and Weinberg 2009, Gibbons and Creighton 2018, Aiello and Kang 2019, Santamaria et al. 2017], we have focused on some of the best defined and used markers in each category, without limiting ourselves to immunohistological staining, but also investigating markers defined at mRNA level, thus covering a broader selection of pathways involved in cell adhesion, migration and response to external stimuli. The results of all these analyses corroborated with each other to give a synchronized picture on the role played by these markers in the adrenal tissues.

Based on these results, it appears that adrenocortical tissues, whether normal, benign or malignant, are characterized by a lack of expression of classical epithelial tissues and are closer to mesenchymal tissues through high expression of classical mesenchymal markers like N-cadherin and SLUG. These new factors also seem to play a role in cancer progression in ACC: while N-cadherin appears to have a positive role in the tissue structure sustainability and against metastatic spread, SLUG seems to promote this. It seems unlikely, given these results as well as previous studies, that EMT has a role in the case of adrenocortical carcinoma.

## *4.2 Adrenocortical tissues showed divergent expression patterns regarding the FGF pathway compared to other tissues*

The analysis of pan-FGF/FGFR pathway expression data showed that there are significant differences in the expression pattern of constituents of this pathway between the different subtypes of adrenocortical tissues but also between these and normal and neoplastic tissues of other organs. The normal and benign adrenocortical tissues clustered close together in unsupervised analyses but separately from the malignant adrenocortical carcinomas. This indicates that the different members of the pathway have similar expression patterns between the normal and benign adrenocortical tissues but different from ACC. Interestingly, the expression in all adrenocortical tissues clustered again separately from the expression in other normal and neoplastic tissues of both epithelial and mesenchymal origin indicating that adrenocortical tissues represent a particular tissue type, at least in terms of the components of the FGF pathway. A defining property of FGFs and their receptors is that they bind to heparin and heparan sulfate and are therefore intimately connected with the extracellular matrix of tissues [Burgess and Maciag 1989, Lin et al. 1999, Ornitz 2000] where they play an important role during epithelial morphogenesis [Li et al. 2001]. As the loss of connectivity with the extracellular matrix is an important process necessary in the establishment of 2D cell-lines [Li et al. 2001, Kapalczynska et al. 2018], it was not surprising that all cell-lines, including those of adrenocortical origin, had completely divergent FGF/FGFR pathway gene expression pattern from the corresponding tissues. Hence, these cell-lines may not be regarded as a reliable research model for FGF signaling in adrenocortical tissues and future studies addressing the therapeutic potential of modulating these pathways will need to use more physiological models such as patient-derived tumor xenograft or spontaneous adrenocortical carcinoma mouse models.

## *4.3 FGFR 1 and 4 are potential therapeutic targets in ACC*

A quantitative analysis of the genes significantly differentially expressed between the benign ACA and ACC revealed quite a high number of genes (16/93) with altered expression in ACC. A qualitative analysis of these genes showed that several of the genes that were expressed at lower levels in ACC are associated with patterns of expression indicative of tissues differentiation. Thus, a downregulation of these genes would lead to less differentiated, more disorganized tissues. For example, in the adrenal, PLD1 and MRAS are associated with hormonal secretion patterns [Tsai et al. 2014, Rabano et al. 2004, Romero et al. 2010], FGFR2 is known to regulate the differentiation [Hafner et al. 2015, Guasti et al. 2013] and the spatial organization of the adrenal gland [Leng et al. 2020] while PIK3C2G is downregulated in the adrenal glands of Gata6 knockout mice, a transcription factor with a role in adrenal development [Pihlajoki et al. 2013, Tevosian et al. 2015]. Importantly, FGFR1 and FGFR4 were considerably higher expressed in ACC [Laurell et al. 2009, De Martino et al. 2013, Brito et al. 2012]. The concordant up-regulation of their ligands FGF8 and FGF19 suggests an autocrine/paracrine growth promoting loop [Mattila and Harkonen 2007, Kuro 2019], FGF19 being also used as a biomarker for patient selection in the case of hepatocellular carcinoma [Kim et al. 2019].

The differences between the localized (ENSAT I and II) and more aggressive (ENSAT III and IV) ACCs were more subtle as can be observed also by the lower number of genes that had significantly different expression levels between the two subgroups. Most of the genes were associated with metastatic processes, such as what was classically defined as epithelial to mesenchymal transition, suggesting its involvement in the adrenocortical cancer progression. Starting with the genes downregulated in advanced ACC, RalA plays an important role during embryogenesis and regulation of epithelial-mesenchymal interaction in tissues of mesenchymal origin including the fetal adrenal [Zhao and Rivkees 2000] and it has been shown that the expression of constitutively active RalA inhibited migration in human cancer lines [Oxford et al. 2005]. The

same is true for MAPK9/JNK2, the phosphorylation status of which is controlling metastatic processes by promoting the switch between mesenchymal-epithelial transition to epithelial-mesenchymal transition [Hu et al. 2017] and its inactivation was identified as a carcinogenic factor in other types of cancer [Lessel et al. 2017, Cantrell et al. 2015]. PIK3R1 was also reported to negatively regulate the epithelial-mesenchymal transition and stem-like phenotype of renal cancer [Lin et al. 2015], as well as having a tumor suppression function [Vallejo-Diaz et al. 2019], so its downregulation would lead to the mobilization of cells and would support the establishment of metastases. RAF1, although has been associated with tumorigenesis, mostly due to frequent mutations [Nandan and Yang 2011, Maurer et al. 2011], has been reported to have a tumor suppressing effect in cancers like hepatocellular carcinoma and medullary thyroid cancer [Jeric et al. 2016, Kunnimalaiyaan et al. 2007]. Interestingly, FGF21, the only gene we found significantly upregulated in advanced vs localized ACC, is a secreted endocrine factor that functions as a major metabolic regulator stimulating the uptake of glucose, and as such, has been associated with aggressiveness in several types of cancer, like thyroid cancer, melanoma, hepatocellular carcinoma, and other solid tumors [Kang et al. 2019, Kim et al. 2019, Zheng et al. 2020, Osawa et al. 2009] but also with outcome in other types of diseases [Ebrahimi et al. 2019, Fisher and Maratos-Flier 2016]. All these findings and especially the upregulation of the secreted factor FGF21 in advanced ACC are important discoveries for ACC and should be addressed in more detail in further studies.

Importantly, from the therapeutic potential point of view, the three FGFRs that we could show to be differentially expressed between benign and malignant adrenocortical tumors, FGFR1, 2 and 4, were also confirmed in the larger cohort of FFPE tissues. The partial cross reactivity of FGFR antibodies due to the sequence similarity of the FGFR family rendered immunohistochemistry unreliable as a validation method. That is why most studies assessing FGFR expression as a prognostic marker for selective FGFR inhibitors use an in situ RNA detection method instead of

immunohistochemistry [Sanchez-Guixe et al. 2021, Schuler et al. 2019, Grunewald et al. 2019]. We opted for the in-situ hybridization technique RNAScope that allowed us to both quantify and determine the tissues distribution of the mRNA of interest, a major advantage when compared to the bulk measurement used in the targeted screening. RNAscope confirmed our previous finding that FGFR1 and 4 are overexpressed in ACC when compared to ACA while FGFR2 is higher expressed in the latter. Not surprisingly, FGFR1 and 4 expression was significantly negatively associated with patient survival endpoints while their individual role in recurrence and metastasis remains unclear from these clinical analyses.

We could show that FGF/FGFR pathways are expressed in adrenocortical tissues and that their expression pattern is different from other tissues. Expression changes in different member molecules of this pathway are associated with tumor progression (FGFR1 and 4) and loss of tissue differentiation, and aggressiveness. These include factors that are generally associated with the epithelial to mesenchymal transition including cell mobilization and metastatic spread. However, considering our findings that the adrenal cortex shares less similarity with epithelial compared to mesenchymal tissues, it is hard to say if the FGF/FGFR pathway has any connection to EMT in the case of ACC as we initially hypothesized. Nonetheless, FGFR1 and 4 being associated with patient prognosis in a relatively large cohort of ACC patients, is raising hopes that specific FGFR inhibitors will show a therapeutic potential of these novel targets in the treatment of refractory ACC.

## *4.4 Limitations of the presented study*

In terms of limitations of the study, starting with the EMT aspect, we analyzed the most typical markers and a few others, but certainly did not exhaust the list of markers that have been proposed in the literature. In addition, we have not yet identified a strong adhesion molecule that could be responsible for the epithelial phenotype, which is why we speculate that this

may be a role taken by N-Cadherin, however, this is not generally considered a strong adhesion molecule. Regarding the FGFR pathway, all the experiments performed quantified mRNA expression, in the case of both RT-PCR as well as RNAscope. This is a problem especially in the case of the FGF receptors, where protein expression would have shown more clearly the level of presence of these proposed drug targets, considering that it has been shown that mRNA expression doesn't necessarily correlate with the corresponding protein's expression [de Sousa Abreu et al. 2009]. The absence of suitable antibodies due to partial cross reactivity of FGFR made immunohistochemistry unreliable at this time however [Sanchez-Guixe et al. 2021].

The work also has fundamental limitations that apply to other retrospective studies in this rare disease. First, of course, there are all the limitations that are explained by the retrospective design (such as survival data not being rigorously followed up or patient data having gaps that might have been relevant to the study). On the other hand, the group size studied is formally a weakness of the work, since a significantly higher number of cases would certainly have allowed more reliable answers. However, it should be noted that the analyzed cohort is extremely large for the methodology used and especially for the rarity of the disease, and only very few research groups in the world are able to investigate such case numbers.

## *4.5 Clinical perspectives*

In terms of druggable targets from the EMT part of the study, SLUG is an interesting candidate that could be inhibited, for example, with the help of small inhibitor RNA (siRNA). Promising preliminary results have been achieved in breast carcinoma studies, where inhibition of SLUG reduced cell motility and the growth of cells resistant to treatment [Alves et al. 2018]. On the other hand, results from hepatocellular studies, where SLUG is also commonly overexpressed in metastases, showed that despite expectations, SLUG inhibition enhanced multidrug resistance [Zhao et al. 2016, Zhao et al. 2015].

More interestingly, the high expression of the FGFR1 and 4 in ACCs is a first indication that FGFR inhibitors could be a promising concept for the treatment of ACC. Inhibitors like Ponatinib (pan-FGFR, PDGFR, SRC, RET, KIT and FLT1 inhibitor) [Gozgit et al. 2012, Tan et al. 2019, Ahn et al. 2022], Lenvatinib (VEGFR, pan-FGFR, PDGFR$\alpha$, KIT and RET inhibitor) [Capozzi et al. 2019], Rogaratinib (selective FGFR inhibitor) [Schuler et al. 2019, Grunewald et al. 2019], Fisogatinib (previously known as Blu-554, a selective FGFR4 inhibitor) [Hatlen et al. 2019], Erdafitinib [Roskoski 2020, Tabernero et al. 2015], Futibatinib [Sootome et al. 2020] may, therefore have better therapeutic efficacy than the other RTK inhibitors that have been tested until now for the treatment of ACC. However, currently, no inhibitor with high selectivity for just both FGFR 1 and 4 exists.

Some of the listed inhibitors as well as others are already being used in trials to assess their efficacy in the treatment of various cancers. The FGFR4 inhibitor Fisogatinib is in two trials for the treatment of hepatocellular carcinoma [Kim et al. 2019, ClinicalTrials-NCT02508467 2015, ClinicalTrials-NCT04194801 2019]. Rogaratinib also is being tested in carcinomas as well as sarcomas [ClinicalTrials-NCT03473756 2018, ClinicalTrials-NCT03410693 2018, ClinicalTrials-NCT04595747 2020, ClinicalTrials-NCT04483505 2020]. Futibatinib, a promising pan-FGFR inhibitor, is being trialed against various solid tumors [ClinicalTrials-NCT04189445 2019, ClinicalTrials-NCT04601857 2020]. Ponatinib is a more wide-spectrum inhibitor including FGFR1 that is being mostly trialed in myeloid leukemia [Cortes et al. 2021], including leukemia associated with FGFR1 [Lee et al. 2013], but also in solid tumors [ClinicalTrials-NCT02272998 2014]. Another wider spectrum inhibitor with a focus on FGFR that has good potential is Erdafitinib [ClinicalTrials-NCT03210714 2017, ClinicalTrials-NCT04083976 2019]. LY2874455, a pan-FGFR and VEGFR2 inhibitor is also being trialed in advanced cancer [Michael et al. 2017], as well as the FGFR1-3 inhibitors Pemigatinib [Merz et al. 2020, ClinicalTrials-NCT04003623 2019, ClinicalTrials-NCT05202236 2022], Infigratinib (BGJ398) [ClinicalTrials-NCT04233567 2020, ] and AZD4547 [Chae

et al. 2020]. For use in ACC, a first assessment of these inhibitors would need to be performed on the existing cell culture models for ACC (cell lines like NCI-H295R, MUC-1, JIL-2266) and it should be examined how strong the inhibition effect is across a range of concentrations, based on cell viability assays. Since, as mentioned, the FGF receptors bind to heparin and heparan sulfate and are therefore connected with the extracellular matrix, and since the loss of this connection is a process necessary in the establishment of 2D cell-lines, it is however possible that the results of these assays might not show the expected effect. One possible way around this limitation would be the use of 3D culture models.

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# Author's list of publications

## First author publications:

**Sbiera, I.**, S. Kircher, B. Altieri, M. Fassnacht, M. Kroiss and S. Sbiera (2021). "*Epithelial and Mesenchymal Markers in Adrenocortical Tissues: How Mesenchymal Are Adrenocortical Tissues?*" Cancers **13**(7).

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