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Evolution and progression of adrenocortical carcinoma: the potential role of the adipose microenvironment and the isolation and characterization of circulating tumor cells

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TABLE OF CONTENTS

INTRODUCTION

1. The adrenal glands: structure, development and function

The human adrenal glands are multifunctional endocrine organs producing a variety of hormones involved in main physiological processes, like the regulation of glucose blood levels, the protein turnover, the maintenance of the hydroelectrolytic equilibrium as well as the cardiovascular tone, the tissue response to damages and infections, the adaptation to stress conditions. They are paired organs located in the retroperitoneum at the upper poles of the kidneys, from which they are separated by a weak wall of connective tissue, and directly below the diaphragm, to which they are attached by the renal fascia. Both the adrenal glands have an irregular pyramidal shape, which becomes semilunar and somewhat larger in the left gland. They measure approximately 3 cm in width, 5 cm in length and up to 1 cm in thickness, with an overall weight ranging from 7 to 10 grams, although this parameters can vary depending on age, sex and physiological conditions. Each adrenal gland is enclosed within a capsule of fibroblasts and myofibroblasts, surrounded by adipose tissue, and is constituted by two distinct parts, each with a unique function: the outer adrenal cortex, representing up to 90% of the entire gland and synthesizing glucocorticoids, mineralocorticoids and androgens; and the inner medulla, producing catecholamines, adrenalin and noradrenalin, in response to a direct sympathetic stimulation. The regulation of the hormone synthesis depends on both the gland architecture and the enzymatic pool of single cells, as well as on the blood supply. This is assured by a rich network of blood capillaries that, departing from the superior, the middle and the inferior suprarenal arteries, penetrate the capsule and spread from the external cortex to the central medulla. Thus allows to strictly control the delivery of steroid hormones to the blood and therefore to regulate the activity of the enzymes involved in their synthesis. Venous blood leaves the adrenal glands by the suprarenal veins, usually one for each gland: the right suprarenal vein, draining into the inferior vena cava, and the left suprarenal vein, draining into the left renal vein (Fig. 1). There are also subcapsular and medullary lymphatic plexus that drain to the lumbar and para-aortic lymph nodes (Dobbie & Symington, 1966).

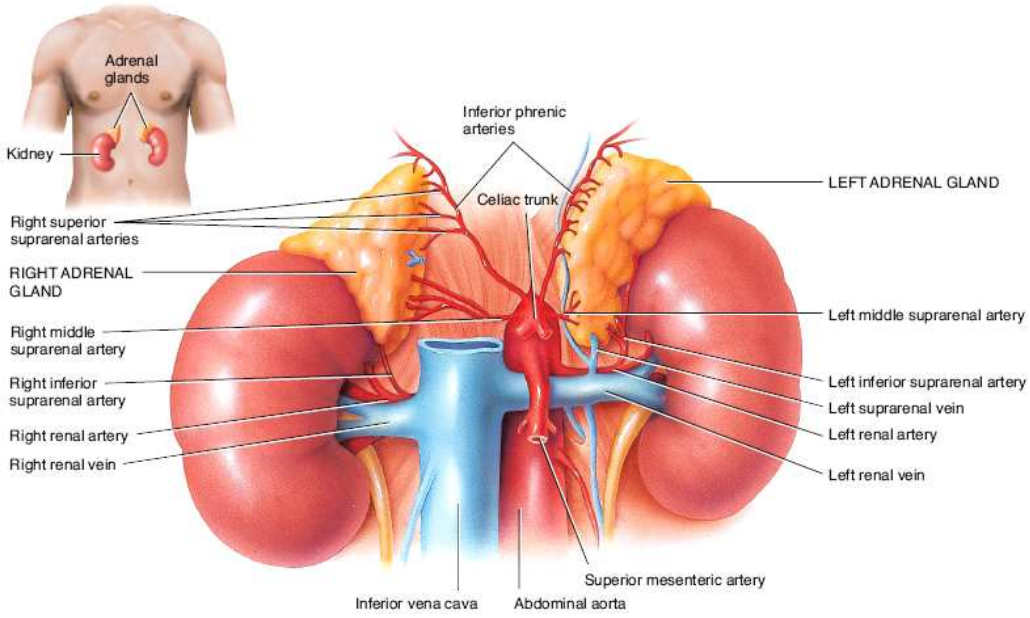


Figure 1. Representation of the anatomical location and blood supply of the adrenal gland.

A rich innervation is also present, with the majority of nerve plexus located in the capsular region: the fibers mainly originate from the splanchnic nerve and form nerve bundles that branch into an extensive subcapsular network to penetrate both the cortex and the medulla, mostly in association with blood vessels (Parker *et al*, 1993; Engeland, 1998).

The developmental process of the adrenal glands is really unique in the human organism and distinct from those of other species, since it involves different embryonic tissues and a series of maturational changes taking place during the embryonic, fetal and post-natal life, thus allowing to acquire the definitive structure and function in the adult organ (Fig. 2). The adrenal cortex derives from the mesoderm and develops around the fourth week post-conception as a thickening of the celomic epithelium between the primitive urogenital ridge and the dorsal mesentery (Parker *et al*, 2002; Else & Hammer, 2005). This primordial structure is defined as adrenogonadal primordium (AGP) and contains both adrenocortical and somatic gonadal progenitor cells specifically expressing the transcriptional factor Steroidogenic Factor-1 (SF-1) (Luo *et al*, 1994; Hatano *et al*, 1996). These cells are able to delaminate and to invade the underlying mesenchyme to form dorsolaterally the gonadal primordium (GP) and dorsomedially the adrenal primordium (AP), in which two discrete zones can be distinguished: the inner fetal zone (FZ), composed by large cells with lipid-filled vacuoles, and the smaller outer definitive zone (DZ), presenting small and tightly packed basophilic cells (Mesiano & Jaffe, 1997).

Meanwhile, cells coming from the neural crest (neuroectoderm) enter the adrenal anlage, acquiring the phenotype of chromaffin cells during their migration (Ehrhart-Bornstein *et al*, 1997; Lumb & Schwarz, 2015) and starting to form the adrenal medulla. By the ninth week of gestation, the periphery of the gland becomes heavily vascularized and the newly formed organ appears encapsulated. The fetal adrenal keeps growing until the third semester, during which a third transitional zone (TZ) appears between the DF and FZ, which has the supposed role of cortisol producing starting from 24–28 weeks of pregnancy (Mesiano *et al*, 1993). Soon after birth and during the post-natal life, the fetal adrenal undergoes a complex process of differentiation and reorganization to finally acquire the definitive structure and the hormonal secreting function: the FZ progressively regresses by an apoptotic process and the DZ and the TZ give rise to the definitive adult adrenal cortex (Ishimoto & Jaffe, 2011), while the islands of chromaffin cells coalesce to form a contiguous medulla (Wilburn *et al*, 1986). Notably, the development and maintenance of the two distinct neuroendocrine and steroid producing components are strictly interrelated: in fact, while the adrenal cortex produces factors influencing the medullary cell survival and functions, such as glucocorticoids (Parlato *et al*, 2009), growth factors (Beaujean*et al*, 2003) and androgens (Sicard *et al*, 2007; Ziegler *et al*, 2008), the catecholamines produced by the medulla control the steroid release from the cortex.

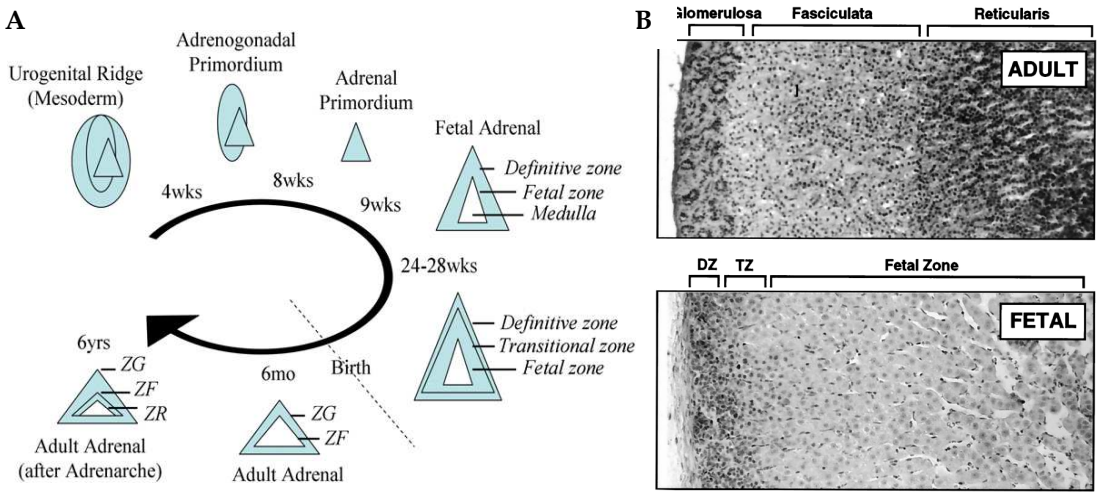


Figure 2. Schematic representation of the embryological adrenal development (**A;** from Kempná & Flück, 2008) and of adult and fetal adrenal cortex morphology of (**B;** Mesiano & Jaffe, 1997).

1.1. The adrenal cortex

The human adult adrenal cortex is composed of three distinct zones, namely zona glomerulosa (ZG), fasciculata (ZF) and reticularis(ZR), going from the outer to the inner part, that differ each other for both cell morphology and functionality (Fig. 3). The ZG constitutes up to 15% of the adrenocortex mass and it is composed of a thin region of columnar cells arranged in an arched or arcuate pattern, showing abundant endoplasmic reticulum and mitochondria with typical lamelliform ultrastructural appearance of their cristae. They produce the principal mineralcorticoid aldosterone, which is responsible for sodium reabsorption and potassium excretion by the kidneys within the signalization of the renin-angiotensin system, thus regulating the hydrosaline homeostasis and, indirectly, the extracellular fluid volume. The ZF is the thickest zone: it occupies about the 75% of the adrenal cortex and it is composed of columns of polyhedral secretory cells rich in lipid droplets, presenting mitochondria with tubulovesicular cristae and producing glucocorticoids, cortisol and corticosterone, which have both a hyperglycaemic and anti-inflammatory action. Cortisol secretion is under the direct control of the pituitary Adrenocorticotropic Hormone (ACTH), which is in turn regulated by the Corticotropin Releasing Hormone (CRH) released from the hypothalamus. A mechanism of negative feedback by cortisol itself eventually controls the entire axis. The last inner zone, the ZR, presents cells typically arranged as round nests or clumps and producing, under the ACTH and Luteinizing Hormone (LH) control, sex steroids, specifically the adrenal androgens DHEA (dehydroepiandrosterone) and DHEAS (dehydroepiandrosterone sulfate) (Balboni *et al*, 1990).

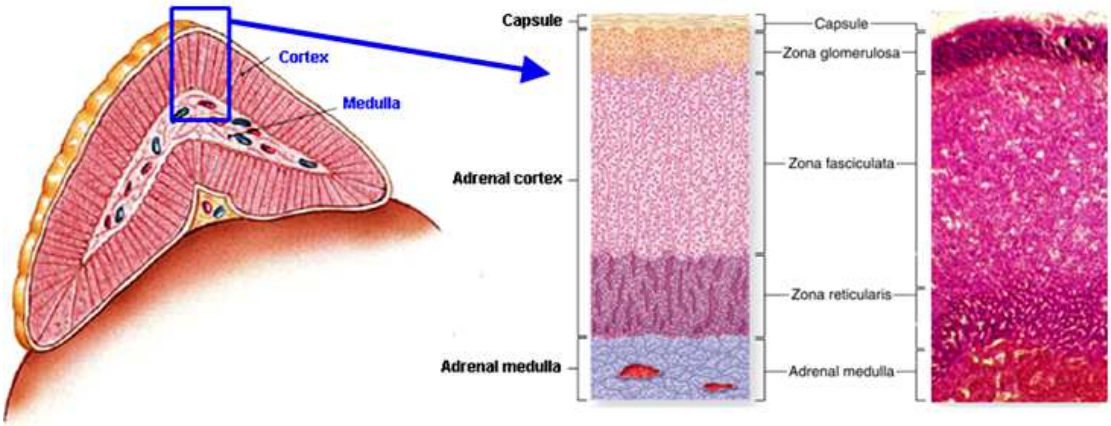


Figure3. Representation of an adrenal gland section and its histological structure.

Cholesterol is the precursor for all the steroid hormones synthesized by the adrenal cortex: it derives from a *de novo* synthesis by cortical cells or from the intracellular steroidogenic depots, and it is released thanks to the action of the cholesterol esterase. Once transferred into the mitochondria by the StAR (Steroid Acute Regulatory) protein, cholesterol is converted into pregnenolone by theP-450 (CYP) 11A1 (20,22 R-hydroxylasecholesterol side-chain cleavage), thus representing the rate-limiting step for the hormone biosynthesis and the starting point for the further enzymatic chain reactions (Payne & Hales, 2004; Hu *et al*, 2010). In fact, pregnenolone transported to the smooth endoplasmic reticulum undergoes different conversions depending on the specific zone: in ZG, pregnenolone is converted into progesterone which in turns becomes the precursor for the synthesis of corticosterone and, eventually, of aldosterone; instead, in ZF pregnenolone is transformed in 17α-hydroxypregnenolone and, further, in 17α-hydroxyprogesterone (which can also directly derive from progesterone) to obtain cortisol. Finally, in ZR the previously synthesized 17α-hydroxypregnenolone and 17α-hydroxyprogesterone are used as intermediates for the production of DHEA/DHEA-S and androstenedione, respectively (Fig. 4).

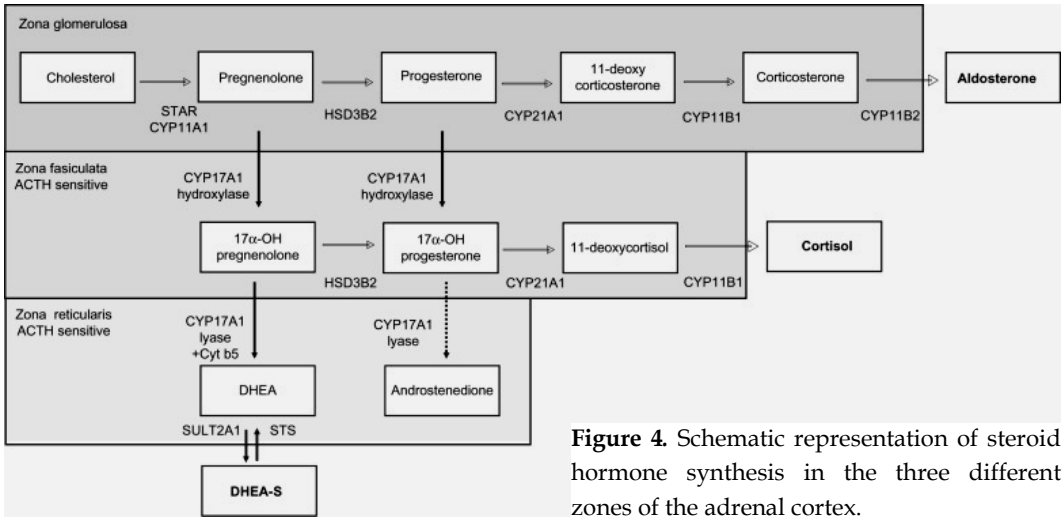


Figure 4. Schematic representation of steroid hormone synthesis in the three different zones of the adrenal cortex.

1.2. The adrenal medulla

The central portion of the adrenal gland, the adrenal medulla, is constituted by the so called chromaffin cells, which are arranged in a net pattern and are responsible for the synthesis and secretion of catecholamines (adrenalin and noradrenalin) into the blood in response to stress stimuli, through the action of

acetylcholine on the adrenergic receptors. This so called "fight or flight" response, it induces many physiological effect requiring energy (such as increased heart rate, blood pressure and blood glucose levels, with a general reaction of the sympathetic nervous system), which is supplied by substrates derived from glycogen- and lipolysis induced by catecholamines themselves in liver, muscle and adipose tissue.

The first step for catecholamines synthesis is the conversion of tyrosine to L-dihydroxyphenylalanine (L-DOPA) by the tyrosine hydroxylase, which is regulated by a negative noradrenalin feedback. L-DOPA is further decarboxylated to dopamine which enters the chromaffin granules, where it is converted to noradrenalin by the phenylethanolamine-*N*-methyltransferase (PNMT). Noradrenalin is then released into the cytoplasm by exocytosis and it is converted to adrenalin, which enters the granules again, by an ATP-mediated transport, to be stored in cells (Fig. 5).Catecholamine secretion is regulated by the sympathetic nervous system: the neurotransmitter acetylcholine stimulates the cholinergic receptors expressed by chromaffin cells with consequent cell depolarization, voltage-dependent Ca^{2+} channel activation and calcium influx, thus inducing the exocytosis of secretory vesicles and the release of their content. Through the blood circulation, catecholamines reach their target organs where they are metabolized by monoamine oxidases (MAO) and catechol-O-methyltransferases (COMT) (Molina, 2004).

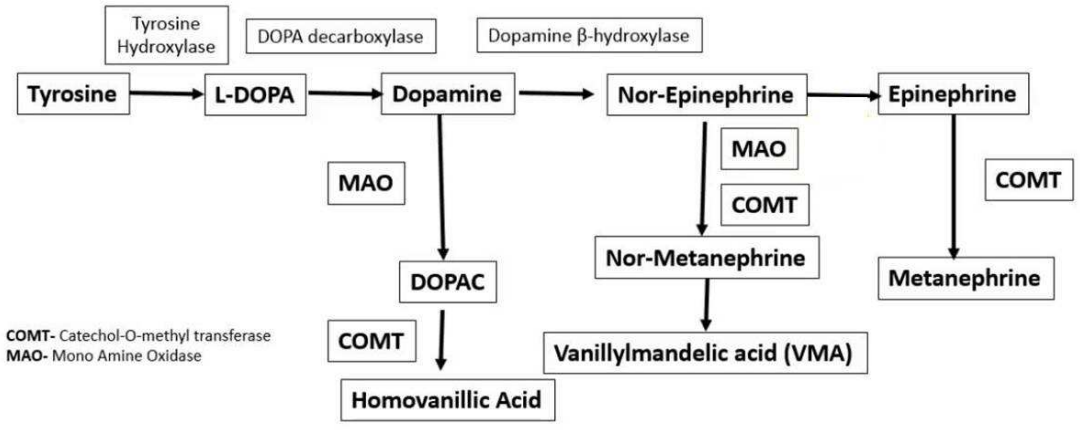


Figure5. Schematic representation of the biosynthesis and metabolism of catecholamines.

2. Adrenocortical carcinoma

Primary adrenocortical carcinoma (ACC) is a rare endocrine malignancy affecting the adrenal cortex (De Lellis *et al*, 2004) presenting high heterogeneity and aggressiveness, often with an unfavorable outcome. Despite immunohistochemical and molecular studies have identified novel biomarkers of diagnostic and prognostic relevance, the discrimination between malignant and benign forms remains challenging, the molecular mechanism underlying the pathology has still to be fully elucidated and the available therapeutic options show limited specificity and efficacy. The integration between different methodologies of study may lead to a better understanding of the tumor biology and behavior, thus offering the potential for classifying the neoplasm and for identifying new therapeutic targets to develop a more personalized approach to treat patients.

2.1. Epidemiology

Among adrenal tumors, that are very common in the general population (4-7% incidence) and prevalently diagnosed as nonfunctional adrenal adenomas (ACAs) (Young, 2007), adrenocortical carcinoma is quite rare. The estimated incidence is 0.5-2 per million cases per year in adults, (Kebebew *et al*, 2006; Golden *et al*, 2009, Fassnacht *et al*, 2013; Kerkhofs *et al*, 2013), with a median age of diagnosis in the fifth to sixth decades and a female-to-male ratio of 1.5-2.5:1 (Luton *et al*, 1990; Michalkiewicz *et al*, 2004; Fassnacht & Allolio, 2009). However, a bimodal age distribution can be described, as a first peak of occurrence is observed in the first decade of life (Wooten & King, 1993; Wasserman *et al*, 2012). Pediatric ACC shows an incidence of 0.2% of all childhood cancers (Miller *et al*, 1995), with a prevalence in children younger than 5 years and older than 10 years (53% and 37%, respectively) (Wieneke *et al*, 2003). A specific population of children in Southern Brazil represents an exception, showing an incidence of ACC 10-15 time greater (Michalkiewicz *et al*, 2004; Pianovski *et al*, 2006a) that has been related to the presence of a specific single germline mutation in the gene encoding p53 (*TP53*, p.R337H) (Latronico *et al*, 2001; Ribeiro *et al*, 2001; Seidinger *et al*, 2011).

The majority of adrenocortical carcinomas occurs sporadically, but multiple well-defined genetic syndromes have been associated with an increased susceptibility to

ACC. The most common is Li-Fraumeni syndrome (LFS), a familial disease caused by germline mutations of *TP53*, which associates with an increased susceptibility to neoplasms like breast cancer, leukemia, brain tumors and sarcomas (Li &Fraumeni, 1969). Among these, approximately 3-10% of LFS-associated cancers in children are ACC (Wagner *et al*, 1994; Varley *et al*, 1999;Rodriguez-Galindo *et al*, 2005), whilst in adults the prevalence of *TP53* mutation ranges from 3 to 7% (Herrmann *et al*, 2012; Raymond *et al*, 2013a). Beckwith-Wiedemann syndrome (BWS) also increases the risk for ACC, which can occur, mostly during childhood, in 5-15% of cases (Wiedemann, 1983; Lapunzina, 2005). The genetics of this disease bases on the alteration of DNA methylation of the 11p15 locus, which harbors the *IGF2* and *CDNKC* genes and the nontranslated RNA *H19* (Weksberg*et al*, 2005), generally leading to the loss of the maternal locus and to the amplification of the paternal one (Barlaskar & Hammer, 2007). Multiple endocrine neoplasia type 1 (MEN1) is due to inactivating mutations of *MEN1* gene and classically associates with hyperparathyroidism, neuroendocrine tumors, pituitary adenomas and adrenal lesions in 20-55% of cases (Waldmann *et al*, 2007). Among these, roughly 10% are distinct adrenal tumors, with a 1.4% overall rate of ACC (Gatta-Cherifi *et al*, 2012). Alterations in mismatch repair genes and microsatellites instability are hallmarks of Lynch syndrome, which increases the risk of malignancies in patient, with an occurrence of ACC in 3% of cases (Raymond *et al*, 2013b). To a less extent, ACC associates also with Familial Adenomatous Polyposis (FAP), neurofibromatosis type 1 and Werner syndrome (Else, 2010). More recently, ACC has been also reported in patients with Carney Complex (Anselmo *et al*, 2012; Morin *et al*, 2012) and, even if very rarely, described in conjunction with congenital adrenal hyperplasia (CAH) (Bauman & Bauman, 1982; Barzon *et al*, 2007).

2.2. Clinical features and presentation

There are three typical clinical scenarios in which ACC can be discovered in adults: approximately 40-60% of cases present the signs and symptoms of hormone excess (hypercortisolism, virilization in women, feminization in men) due to the presence of functional tumors, which generally associate with a rapidly progressive Cushing's syndrome (Luton *et al*, 1990;Abiven *et al*, 2006; Fassnacht & Allolio, 2009; Ishikura *et al*, 2010). The second common clinical presentation is represented by

nonspecific symptoms related to the mass growth, such as early satiety, abdominal fullness and flank or abdominal pain, when a nonfunctional tumor is present (Luton *et al*, 1990; Fassnacht & Allolio, 2009). In these cases, systemic symptoms can be also present (fever, weight loss, anemia, anorexia) and distant metastases can occur, principally in lung, liver and bones (Abiven *et al*, 2006; Libel *et al*, 2007). Finally, roughly 20-30% of ACCs are incidentally discovered during imaging for other unrelated medical issues (Fassnacht & Allolio, 2010; Else *et al*, 2014a).

ACCs are generally unilateral large tumors, measuring more than 5-6 cm and with a weight higher than 100 grams; however, in some cases smaller tumors can show malignant features, like loss of homogeneity, presence of foci and irregular edges. Grossly, they may appear encapsulated or adherent to or infiltrating the surrounding structures, with the cut surface ranging from brown to yellow depending on the cell lipid content. Fibrous tissue bundles often separate the tumor mass in lobules, hemorrhage and necrosis are commonly present and vascular invasion can also be observed. The histological evaluation shows a less ordered architecture compared to benign adenomas: compact cells predominate, arranging in trabecular and diffuse patterns, and mitotic activity, often with atypical forms, can be usually seen. Blood vessel and capsule invasion are commonly observed and, together with distant metastases, they define malignancy (McNicol, 2008; Else *et al*, 2014) (Fig. 6).

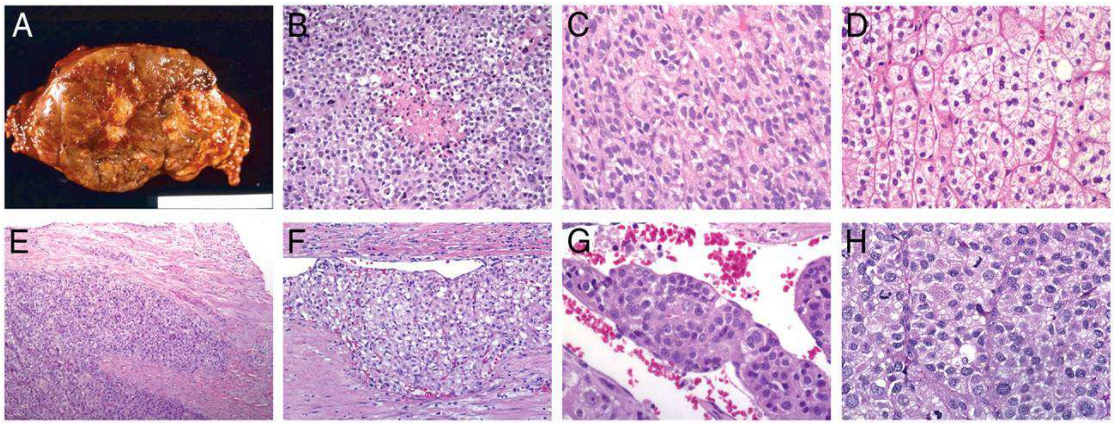


Figure 6. Gross presentation (**A**) and histological sections of ACC with the typical observed features: an hypercellular population showing tumor necrosis, solid growth pattern, abundant eosinophilic cytoplasm and mitotic figures is present (**B**, **C**, **H**) compared to ACA (**D**). Malignancy is defined by direct invasion of the tumor capsule (**E**) and blood vessels (**F**, **G**). Adapted from Else *et al*, 2014a.

Adrenocortical carcinomas typically exhibit a large degree of intratumor heterogeneity, as well as different mitotic/proliferative capacity: in fact, numerous areas and nodules with different histological phenotypes can be commonly observed. Three distinct variants of ACC has been described: the oncocytic variant, characterized by large and pleomorphic cells with abundant granular and eosinophilic cytoplasm (Erlandson & Reute, 1991; Macchi *et al*, 1998; Hoang *et al*, 2002; Song *et al*, 2004; Ohtake *et al*, 2010; Wong *et al*, 2011); the myxoid variant, presenting extracellular deposits of myxoid material (Brown *et al*, 2000; Suresh *et al*, 2005; Karim *et al*, 2006; Raparia *et al*, 2008; Papotti *et al*, 2010; Hsieh *et al*, 2011; Zhang *et al*, 2011; Sheng *et al*, 2012); and the sarcomatoid variant, in which spindle cell areas or specialized sarcomatous component are present (Coli *et al*, 2010).

2.3. Diagnosis and classification

The diagnosis of adrenocortical carcinoma needs investigation of clinical, biological and imaging features before surgical intervention and pathological examination after tumor removal.

Since signs and symptoms can vary among patients and part of ACCs are incidentally discovered, a preoperative biochemical evaluation, consisting in blood and urine measurement of steroid hormones potentially produced by the tumor, is suggested for suspected ACC. This allows to establish or exclude the presence of hormone excess (glucocorticoids, mineralcorticoids, androgens), to confirm the adrenocortical origin of the tumor and to define the malignancy of the lesion (particularly in the presence of androgen or estrogen production). Moreover, the steroid hormone assessment provides molecular markers for further patient follow-up and surveillance and can indicate the necessity for post-surgical replacement therapy (Else *et al*, 2014a). Remarkably, urine steroid analysis may represent a new potential approach to discriminate ACCs from ACAs since a differential steroid precursor and metabolite profile can be observed in malignant versus benign tumors (Arlt *et al*, 2011). In addition, metanephrine urinary and blood level assessment can support differential diagnosis from pheochromocyoma.

Traditional imaging techniques, such as contrast-enhanced computed tomography (CT) or Magnetic Resonance Imaging (MRI), are the instrumental

choice for initial diagnosis and staging of an adrenal mass basing on tumor size, as well as for follow-up, since they allow to detect recurrence and metastasis (Bharwani *et al*, 2011). Functional imaging, performed by Positron Emission Tomography(PET) with FDG ([^{18}F]fluorodeoxyglucose) or MTO ([^{11}C]metomidate), may also be used to confirm the diagnosis of malignancy or the cortical origin of the lesions (Minn *et al*, 2004; Zettinig *et al*, 2004).

The best validating score used as standard for the diagnosis of malignancy in adrenal tumors still remains the Weiss system with its modifications (Weiss, 1984; Aubert *et al*, 2002; Lau & Weiss, 2009). It is based on the microscopically evaluation of the following nine morphologic items concerning tumor architecture, nucleus and invasion:

- nuclear grade
- mitotic rate (>5 per 50 high-powered field)
- atypical mitotic figures
- eosinophilic tumor cell cytoplasm (>75% of tumor cells)
- diffuse architecture (>33% of tumor)
- necrosis
- venous invasion
- sinusoidal invasion
- capsular invasion

Each item scores 1 when present and the sum of all positives items defines the final score. A score ≥ 3 represents the threshold to classify adrenal tumors as ACCs, since they often behave in a malignant fashion, with recurrence or metastases in roughly 80% of cases. Instead, tumors with a score lower than 2 can be classify as ACAs; however the diagnosis of tumors with a score of 2/3remains challenging: in fact, sometimes tumors not classified as ACC can develop a malignant behavior (Pohlink *et al*, 2004) and, conversely, some tumors diagnosed as ACC do not behave as predicted (Giquel *et al*, 2001; Lucon *et al*, 2002). Also, in case of oncocytic or myxoid ACC variants the Weiss system results inadequate in defining malignancy (Papotti *et al*, 2010; Duregon *et al*, 2011; Wong *et al*, 2011).

Considering the limitations and criticisms of the Weiss scoring, attemps have been made to refine its criteria and to improve diagnosis accuracy (Volante *et al*, 2009). Among these, the disruption of reticular network, assessed by reticulin histochemical staining, seems to specifically discriminate ACCs from ACAs

(Duregon *et al*, 2013a). In addition, many immunohistochemical methods have been developed to improve the differential diagnosis of ACC, mainly focusing on tumor cell proliferation and mitotic count. The most widely used is the Ki67 proliferation index (Cattoretti *et al*, 1992;Morimoto *et al*, 2008). Tumors with a Ki67 ≥ 5% are generally considered ACC. Since some ACCs can show a loss of adrenocortical differentiation, a battery of immunostains can aid in confirming the adrenocortical origin of the tumor, such as α-inhibin (Arola *et al*, 2000), calretinin (Zhang *et al*, 2008), synaptophysin, melanA (MART-1) (Ghorab *et al*, 2003), SF-1 (Sbiera *et al*, 2010; Duregon *et al*, 2013b).Furthermore, other molecular markers, like the combined expression of the microRNA miR483-3p and Smad4, have been investigated and confirmed as potential diagnostic complements to the Weiss score, particularly in case of borderline tumors (Wang *et al*, 2014).

Tumor size, lymph nodes involvement and the presence of distant metastases are the parameters commonly used for adrenocortical carcinoma staging, based on the traditional TNM (Tumor, Node, Metastasis) classification system (MacFarlane, 1958; Sullivan *et al*, 1978), modified in 2004 by the World Health Organization (WHO) and Union for International Cancer Control (UICC) (De Lellis *et al*, 2004) and further revised by the European Network for the Study of Adrenal Tumors (ENSAT) (Fassnacht *et al*, 2009). This system defines 4 stages for ACC (Tab. 1): tumors strictly localized to the adrenal gland are considered as stage I (≤ 5 cm diameter) and II (> 5 cm diameter); infiltration in the surrounding tissue or involvement of locoregional lymph nodes characterize the stage III, whereas stage IV is defined by the presence of distant metastases.

STAGE	UICC/WHO (2004)	ENSAT (2009)
I	T1, N0, M0	T1, N0, M0
II	T2, N0, M0	T2, N0, M0
III	T3, N0, M0 T1-2, N1, M0	T1-2, N1, M0 T3-4, N0, M0
IV	T1-4, N0-1, M1 T3-4, N1, M0 T4, N0, M0	T1-4, N0-1, M1

Table 1. TNM classification systems (UICC/WHO versus ENSAT modifications) for ACC staging. T1, tumor ≤ 5 cm; T2, tumor > 5 cm; T3, tumor infiltration into surrounding tissue; T4, tumor invasion into adjacent organs or venous tumor thrombus (ENSAT classification). N0, negative lymph nodes; N1, positive lymph node(s). M0, no distant metastases; M1, presence of distant metastases.

A recent study from ENSAT suggested to move stage III tumors to stage IV when lymphonodes are compromised (Libé *et al*, 2015); moreover, stage IV has been proposed to be further stratified in a, b, c subgroups. In fact, the proposed staging combined with grading, R status, age and symptom parameters (GRAS classification) have been shown to refine the prognostication of the tumor classification (Libé *et al*, 2015).

2.4. Prognosis and predictive markers

Despite ACC prognosis is generally unfavorable, with a median survival less than 12 months in advanced stage (Sidhu *et al*, 2004; Kebebew *et al*, 2006), a marked difference among patient exists for disease progression, recurrence and overall survival, thus mainly being due to the pathology heterogeneity. In fact, even in case of advanced disease, survival can range from few months to several years and an exceptional prolonged survival (> 10 years) has been observed in a restricted subset of patients with recurrent and metastatic ACC (Hermsen *et al*, 2008). Age at diagnosis, increased cortisol production, as well as the tumor growth rate, correlate with a decreased overall survival (Abiven *et al*, 2006; Assié *et al*, 2007; Berruti *et al*, 2014; Else *et al*, 2014b). Also, it has been shown that an increased intra-abdominal fat associates with worsening survival (Miller *et al*, 2012).

The three major criteria to define the disease free survival for localized ACCs and the overall survival for metastatic carcinomas are represented by the complete surgical resection (R0), the ACC grading (related to the proliferation index) and the staging. Tumors completely resected associate with a 5-year survival ranging from 16 to 55%, whereas this percentage decreases to 5% and the median survival to 12 months in case of incomplete tumor removal (Schulick& Brennan, 1999; Paton *et a*l, 2006; Bilimoria *et al*, 2008; McCauley & Nguyen, 2008; Erdogan *et al*, 2013). Tumor staging is mandatory to assess prognosis, since ACCs with stage I-II have a better outcome than stage III and IV, according to different series (Icard *et al*, 2001; Fassnacht *et al*, 2009; Lughezzani *et al*, 2010; Kerkhofs *et al*, 2013, Libé *et al*, 2015).Finally, the proliferation index, such as the percentage of Ki67 and the mitotic count, can aid in defining ACC prognosis: in fact, Ki67 has been found to represent a single factor predicting recurrence in localized ACCs after R0 resection

(Beuschlein *et al*, 2015) and to be an important prognostic parameter of overall survival in stage IV ACCs (Libé *et al*, 2015); high tumor grade, defined as more than 20 mitoses per HPF, represents another unfavorable prognostic factor (Miller *et al*, 2010; Else *et al*, 2014b). As reported above, a combination of the new proposed staging, grading, R status, age and symptom parameters resulted in a more robust prognostication (Libé *et al* 2015).

Global gene expression analysis have identified several potential biomarkers that could aid in improving diagnosis and prognosis in addition to the classical histological parameters. Transcriptome studies (de Reynies *et al*, 2009; Giordano *et al*, 2009; Laurell *et al*, 2009; Assié *et al*, 2010) have described different expression profiles discriminating ACCs from ACAs, and identified molecular characteristics able to stratify ACC patients into two subgroups, namely clusters C1A and C1B, associated with different outcomes: the C1B group presents a marked better 5-year survival rate and a transcription signature characterized by the expression of genes related to cell metabolism, intracellular transport, apoptosis and differentiation. Instead, the poor outcome group C1A generally presents an increased expression of transcriptional control and cell cycle-associated genes and high histologic grade, as well as *TP53* and *CTNNB1* mutations (Ragazzon *et al*, 2010; Ip *et al*, 2015). Moreover, a combination of three genes (*BUB1B*, *PINK1*, *DLG7*) seems to be predictive of clinical outcome, identifying subgroups of ACC with different free-disease and overall survival regardless to disease stage and grade (de Reyniès *et al*, 2009; Fragoso *et al*, 2012). DNA methylation and microRNAs expression can also discriminate two ACC subgroups with different outcomes, and hypermethylation was associated with poor survival (Barreau *et al*, 2013; Assié *et al*, 2014). Other factors have been reported as associated with poor prognosis in ACC patients, including overexpression of pituitary tumor transforming gene 1 (PTTG1) (Demeure *et al*, 2013), low expression of transforming growth factor β signaling mediator SMAD and GATA6 (Parviainen *et al*, 2013) and cyclin E overproduction (Tissier *et al*, 2004).

2.5. Molecular pathology

The rarity and heterogeneity of adrenocortical carcinoma are the main challenging aspects to be faced in order to elucidate the molecular mechanisms underlying the disease evolution, from the onset to the development and progression. Once the molecular mechanisms have been elucidated it might be possible to develop new specific diagnostic and prognostic tools, as well as to improve patient follow-up and treatment. Over recent decades a number of studies have identified different biomarkers with diagnostic and prognostic impact by employing various approaches. Molecular studies based on the assessment of candidate genes led to the elucidation of the genetics of rare syndromes associated with adrenocortical tumors, as well as to the identification of the major molecular pathways altered in ACC. The recent advent of high-throughput methodologies, including genome-wide sequencing, exome sequencing, transcriptome, miRNome and methylome, allowed to identify subgroups of tumor characterized by distinct and specific genetic markers, molecular pathway activation patterns and clinical behavior. Moreover, the dysregulation of signaling pathways involved in organogenesis and homeostatic maintenance of the adrenal cortex seems to also play a pivotal role in adrenocortical disease.

2.5.1. Genetics

Adrenocortical tumors show a complex landscape of genetic alterations which are cumulative towards malignant transformation. ACCs are particularly characterized by a high degree of chromosomal instability, one of the main hallmarks of cancer (Hanahan & Weinberg, 2011) which leads to a rapid accumulation of somatic mutations (the mutator phenotype phenomenon) (Loeb, 2011), thus contributing to the disease progression (acceleration of the mutagenesis process and acquiring of selective advantage) and the development of resistance to therapy. The genetic dissection of ACC, including the identification of genomic aberrations, specific gene expression profiles, mutations and epigenetic alterations, allowed to define the molecular signature of malignant transformation of adrenocortical cells and a better classification of adrenocortical carcinomas.

- **Chromosomal aberrations**

The first characterization of the gross morphological observation of ACC was performed thanks to karyotyping studies, demonstrating the presence of a large number of chromosomal aberrations, such as segmental duplications, rearrangements and aberrant chromosomes. Further cytogenetic and flow cytometry studies corroborated these findings, demonstrating that most ACCs exhibit aneuploidy/polyploidy compared to ACAs, which are almost always diploid (Klein *et al*, 1985; Bowlby *et al*, 1986; Limon *et al*, 1987; Marks *et al*, 1992). Clonal studies have shown that ACCs are mostly of monoclonal origin, whereas ACAs can be both monoclonal and polyclonal (Beuschlein *et al*, 1994; Gicquel *et al*, 1994; Blanes & Diaz-Cano, 2006), thus suggesting the hypothesis of a multistep process for adrenocortical tumorigenesis, in which a growth advantage of some clones is established: the starting event may be the proliferation of a polyclonal cell population triggered by paracrine/endocrine stimuli, with a successive accumulation of mutations that are thought to accelerate the neoplastic progression of normal cells toward benign lesions and, eventually, to carcinoma (Bernard *et al*, 2003; de Fraipont *et al*, 2005).

With the advent of the Comparative Genomic Hybridization (CGH) techniques these studies were extended to the sub-chromosomal levels, revealing a complex pattern of chromosomal aberrations in ACCs compared to ACAs, with multiple regions of gains and losses (Kjellman *et al*, 1996; Zhao *et al*, 1999; Dohna *et al*, 2000; Sidhu *et al*, 2002; Gruschwitz *et al*, 2010; Barreau *et al*, 2012), that were shown to often harbor oncogenes and oncosuppressor genes, respectively. Chromosomal gains were frequently observed at chromosomes 4, 5, 9, 12 and 19, while chromosomal losses were most commonly seen at chromosomes 1p, 17p, 22, 2q, 11q. The presence of genetic aberrations in ACC were correlated with tumor size (Kjellman *et al*, 1996; Zhao *et al*, 1999; Sidhu *et al*, 2002), thus corroborating the hypothesis that they may cumulate during the neoplastic progression. Microsatellite studies have also allowed to demonstrate that ACCs frequently present allelic losses (Loss Of Heterozigosity, LOH) or disequilibrium in the *TP53*17p13 region (85%), the *MEN1* 11q13 locus (92%) and the Carney Complex 2p16 region (90%) (Kjellman *et al*, 1996; Gicquel *et al*, 2001). Some other alterations (amplifications at chromosomes 6q, 7q and 12q, and losses in chromosomes 3, 8, 10p, 16q and 19q) were associated with a decreased overall survival in ACC patients (Stephan *et al*, 2008). In a more

recent study, a higher-resolution CGH array was used to assess the diagnostic and prognostic value of chromosomal abnormalities in adrenocortical tumors (Barreau *et al*, 2012): alterations were observed more frequently in ACCs (44%) compared with ACAs (10%) and chromosomal gains at 5, 7, 12, 16, 19 and 20 and losses at 13 and 22 were confirmed. Within these regions a group of genes, including fibroblast growth factor 4 (FGF4), cyclin-dependent kinase 4 (CDK4) and cyclin E1 (CCNE1), was identified to be potentially involved in adrenocortical tumorigenesis. Frequent gains at 9q34 region, which includes the Steroidogenic Factor 1 (SF-1), were also found in adenomas: the same region was previously shown to be gained in pediatric ACCs (Figueiredo *et al*, 1999; James *et al*, 1999; Pianovski *et al*, 2006b). According to mRNA overexpression and strong immunostaining, SF-1 was suggested to be involved in adrenal tumorigenesis (Figueiredo *et al*, 2000; Almeida *et al*, 2010). Moreover, Barreau *et al* (2012) developed a diagnostic tool to differentiate ACCs from ACAs (100% sensitivity, 83% specificity) by using a combination of DNA copy number estimates at six loci (5q, 7p, 11p, 13q, 16q, and 22q). Chromosome 1, 5, 7 and 22 were further confirmed as discriminating between adenomas and carcinomas (Ronchi *et al*, 2013), while frequent recurrent copy number variations (CNVs) at 5p15 and deletions at 22q12.1 have been recently identified (Juhlin *et al*, 2015). Notably, these regions contain *TERT* and *ZNRF3* genes respectively, the latter being the most commonly altered gene in ACC (Assié *et al*, 2014; Juhlin *et al*, 2015; Zheng *et al*, 2016).

- **Gene mutations**

Although adrenocortical carcinoma commonly occurs sporadically, it can manifest in the setting of some rare heritable genetic syndromes which have been well defined at the level of their genetic basis, contributing to the identification of driver gene mutations relevant to the molecular pathogenesis of ACC.

***TP53* and the 17p13 locus.** The chromosomal region 17p13 harbors the gene encoding the oncosuppressor p53, which mainly acts by halting the cell cycle and/or inducing apoptosis and senescence in response to DNA damage or other stress stimuli. It represents one of the most frequently mutated gene in human cancer (Hollstein *et al*, 1991; Vogelstein *et al*, 2000; Suzuki & Matsubara, 2011), with both tumor-specific mutations of *TP53* gene and alterations of the negative

regulators of the p53 protein, thus leading to the final effect of inactivation of the oncosuppressor signaling pathway. Germline mutations of *TP53* are found in about 70% of patients with Li-Fraumeni syndrome, which associates with an increased susceptibility to ACC. For this reason, the presence of *TP53* alterations have been investigated in sporadic ACCs. A specific germline mutation in the exon 10 of *TP53* gene (p.R337H) was found in the Southern Brazilian population and it is present in up to 90% of ACC patients, with the higher prevalence in children (Latronico *et al*, 2001; Ribeiro *et al*, 2001). While germline *TP53* mutations are rare in adult ACC patients, somatic mutations have been described in 25-70% of cases (Reincke *et al*, 1994; Barzon *et al*, 2001a; Libè *et al*, 2007; Waldmann *et al*, 2012) and LOH at the 17p13 locus can be described in more than 50% of cases (Gicquel *et al*, 2001; Soon *et al*, 2008a). According to these findings, *TP53* inactivation has been proposed to follow the classic Knudson's two-hit hypothesis for a tumorsuppressor gene, in which both alleles are inactivated: in the presence of a germline or somatic *TP53* mutation, a second genetic event, such as a promoter region methylation or LOH, determines the inactivation of the second allele. However, since *TP53* point mutations and 17p13 LOH do not completely overlap, an alternative inactivating mechanism has to be involved. More recently the presence of somatic *TP53* inactivation has been demonstrated to represent a molecular signature of ACC and to correlate with poor outcome in patients (Ragazzon *et al*, 2010). Furthermore, also the presence of some less common variants of *TP53* polymorphisms have been associated with a poor outcome in adult ACC patients (Ignaszak-Szczepaniak *et al*, 2006; Heinze *et al*, 2014). The relatively high frequency of *TP53* mutations in ACC (~15%) has been confirmed in other recent studies investigating the genomic landscape of ACC (De Martino *et al*, 2013; Assié *et al*, 2014; Ragazzon *et a*l, 2014; Juhlin *et al*, 2015; Zheng *et al*, 2016), which identified other cell cycle-related genes also altered in ACC, including the oncosuppressors *CDKN2A* and *RB1* and the oncogenes *MDM2* and *CDK4*. These findings indicate that a global impairment of the cell-cycle regulation constitutes a key element in sustaining cancer cell proliferation in ACC.

***IGF2* and the 11p15 locus.** Genetic and epigenetic alterations at the chromosomal locus 11p15 underlie the Beckwith-Wiedemann Syndrome (BWS), a somatic overgrowth syndrome associated with an increased risk of developing neoplasms, including ACC. This chromosomal region contains the genes *CDKN1C* (the Cyclin-Dependent Kinase inhibitor 1C - *p57kip2* -, a negative cell cycle regulator), *IGF2* (the Insulin-like Growth Factor 2, mainly expressed during fetal life and responsible for fetal growth) and *H19* (a nontranslated RNA that acts as a transcriptional repressor of IGF2), which are structurally organized in a cluster and expressed monoallelically in normal individuals: *IGF2* is maternally imprinted, thus only the paternal allele is expressed; contrarily, the paternal alleles of *CDKN1C* and *H19* are silenced by imprinting and only the maternal ones are expressed (Fig. 7). In BWS multiple epigenetic and structural changes at 11p15 (i.e. duplications, translocations/inversions, microdeletions, DNA methylation changes, uniparental isodisomy and mutations of *CDKN1C*) lead to a biallelic expression of *IGF2*and to inactivation of *CDKN1C*and *H19* (Weksberg *et al*, 2010). In ACC, *IGF2* is the most widely known overexpressed gene compared to both benign adenomas and normal adrenals (Giordano *et al*, 2003; de Fraipont *et al*, 2005; Libé *et al*, 2007; Almeida *et al*, 2008; Giordano *et al*, 2009). In sporadic case of ACC, *IGF2* overexpression and downregulation of *CDKN1C* and *H19* are observed in roughly 90% of cases (Gicquel *et al,* 1997; Giordano *et al*, 2003, 2009) and have been demonstrated to be caused by somatic structural alterations of the 11p15 locus, such as paternal isodisomy (loss of the maternal allele and duplication of the paternal allele) (Fig. 7), LOH of the maternal allele (Wilkin *et al*, 2000) and, more rarely, loss of imprinting due to demethylation of the maternal allele (Ogawa *et al*, 1993; Rainier *et al*, 1993). LOH or paternal isodisomy at 11p15 locus associate with a higher risk of tumor recurrence and correlate with the Weiss score (Gicquel *et al*, 2001; de Reyniès *et al*, 2009).

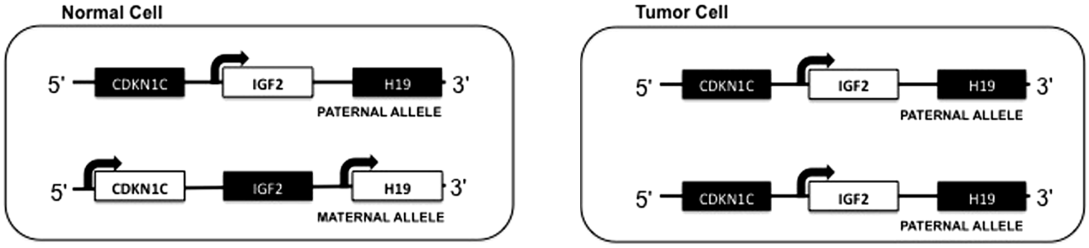


Figure 7. Molecular mechanism underlying IGF2 overexpression in ACC: in normal cells, *IGF2* is imprinted on the maternal allele, while CDKN1C and H19 are imprinted on the paternal allele. In ACC, the paternal isodisomy mechanism results in biallelic IGF2 expression and in transcriptional silencing of *CDKN1C* and *H19*. Adapted from Lerario *et al*, 2014.

Wnt/β-catenin-related genes. Familial Adenomatous Polyposis (FAP) or Gardner's Syndrome, is characterized by the development of multiple colonic polyps and is caused by germline inactivating mutations of the APC gene (Nishisho *et al*, 1991; Half *et al*, 2009). It has been demonstrated that, in many cases, it leads to the development of bilateral adrenocortical nodular hyperplasia, general non-functional and benign, although ACCs have also been described (Marshall *et al*, 1967; Marchesa *et al*, 1997; Kartheuser *et al*, 1999; Smith *et al*, 2000; Bläker *et al*, 2004; Gaujoux *et al*, 2010). APC is a downstream regulator of the Wnt pathway, a network of proteins involved in regulating fundamental cell processes during embryogenesis and tissue morphogenesis and homeostasis, such as migration, proliferation, differentiation, survival, stem cell self-renewal and fate (Moon *et al*, 2004; Kim *et al*, 2013). Wnt signalization is mediated by the interaction between a Wnt ligand and a Frizzled family receptor expressed on cell membrane, thus inducing a reaction chain which results in the inactivation of the β-catenin destruction complex. This latter is a multiprotein complex, constituted by APC protein, axin and GSK3-β enzyme which constitutively phosphorilates β-catenin, addressing it toward a proteasome-mediated degradation. The β-catenin still present in the cytosol localizes at the cell membrane, being part of adherent junctions in epithelial tissues. Once the pathway is activated, β-catenin is stabilized and accumulates in the cytoplasm, from which it is further translocated to the nucleus, where it acts as a transcriptional factor, regulating the expression of target genes (Fig. 8). In Gardner's syndrome, APC loss causes an abnormal constitutional activation of the Wnt pathway, leading to tumor development in many organs, including adrenals. A constitutively activated Wnt signalization has been frequently observed in many types of cancer (Karim *et al*, 2004), associating with cell proliferation, cell motility, epithelial-to-mesenchymal transition and resistance to apoptosis (Kim *et al*, 2013).

Although in sporadic ACTs somatic APC mutations are rare events (Gaujoux *et al*, 2010), *CTNNB1* (β-catenin) activating mutations have been found in both ACAs and ACCs (Tissier *et al*, 2005;Gaujoux *et al*, 2008;Masi *et al*, 2009) and the up-regulation of β-catenin was confirmed by immunohistochemical nuclear staining (Tissier *et al*, 2005). *CTNNB1*isone of the main driver genes in ACC (10-16%), but other factors that are part of the Wnt/β-catenin pathway seem to be involved in adrenal carcinoigenesis (De Martino *et al*, 2013; Assié *et al*, 2014; Juhlin *et al*, 2015; Zheng *et al*, 2016):in particular, the *ZNRF3* gene, encoding a negative regulator of the Wnt/β-

catenin pathway (a E3 ubiquitine ligase), has been demonstrated to be the most frequently mutated gene in ACC (> 20%), with inactivation mostly due to homozygous deletion or missense and nonsense mutations. Notably, *CTNNB1*and *ZNRF3* mutations are mutually exclusive, as well as *CTNNB1* and *TP53* mutations (Assié *et al*, 2014; Juhlin *et al*, 2015; Zheng *et al*, 2016).

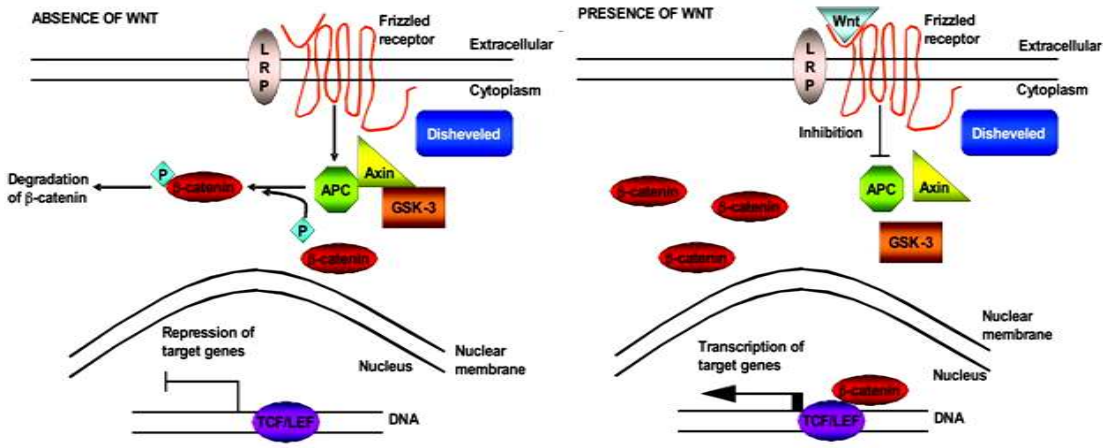


Figure 8. Representation of the canonical Wnt signaling pathway: in the absence of signal, the destruction complex (GSK3β, APC, Axin) phorylates β-catenin, which becomes a target for ubiquitination and degradation by the proteosome. Binding of Wnt ligand to a Frizzled/LRP receptor complex leads to stabilization of hypophosphorylated β-catenin, which translocates into the nucleus, where it interacts with TCF/LEF proteins to activate transcription.APC, adenomatous polyposis coli; GSK-3, glycogen synthase kinase 3; LRP, lipoprotein receptor–related protein; P, phosphate; TCF/LEF, T cell–specific transcription factor/lymphoid enhancer-binding factor. Adapted from Soon *et al*, 2008b.

***MEN1* and the 11q13 locus.** Germline inactivating mutations of the *MEN1* tumor suppressor gene at chromosome 11q13 cause the Multiple Endocrine Neoplasia type 1 (Chandrasekharappa *et al*, 1997), an autosomal dominant disease characterized by the onset of tumors in endocrine tissues, including adrenals: in fact, approximately 40% of patients develops bilateral adrenocortical nodules, generally nonfunctional and benign (Skogseid *et a*l, 1992, 1995; Barzon *et al*, 2001b; Waldmann *et al*, 2007), but also secreting ACAs have been described, whereas ACCs are considered as a rare manifestation of this syndrome (Skogseid *et al*, 1992, 1995; Griniatsos *et al*, 2011). In sporadic ACCs, MEN1 somatic mutations are relatively unusual (~ 7%) (Assié *et al*, 2014; Zheng *et al*, 2016), whereas LOH of 11q13 locus has been frequently observed (Schulte *et al*, 2000; Kjellman *et al*, 1999), suggesting that this region may harbor other unrecognized suppressor genes involved in adrenal tumorigenesis. *MEN1* encodes menin, a protein involved in the maintenance of cell homeostasis of various endocrine organs, as well as in regulating several crucial signaling pathways by

controlling gene transcription, also through epigenetic mechanisms (Feng *et al*, 2017 **in press**). Interestingly, other genes involved in histone modification (*MLL*, *MLL2*, and *MLL4*) and chromatin remodeling (*ATRX*, *DAXX*)has been found altered in ACC (Zheng *et al*, 2016), suggesting a role for epigenetic deregulation in adrenocortical carcinogenesis.

***PRKAR1A* gene.** Protein Kinase cAMP-dependent Type 1 Regulatory Subunit α (PRKAR1A) is the main mediator of cAMP signaling (Bossis & Stratakis, 2004) and the presence of mutations of its gene is one of the main cause of the Carney Complex Syndrome (Kirschner *et al*, 2000). *PRKAR1A* has been also demonstrated to be involved in endocrine tumorigenesis (Bertherat, 2001) and, particularly, in the adrenal tumor development: LOH of 17q22–24, the locus harboring the *PRKAR1A* gene, was found in 23% of ACAs and 53% of ACCs and direct sequencing of *PRKAR1A* gene revealed inactivating mutations in 10% of ACAs, with parallel decrease of mRNA and proteins levels (Bertherat *et al*, 2003). In a more recent study, *PRKAR1A* inactivating mutations were also found in ACCs (11%) (Zheng *et al*, 2016), thus expanding the role of PKA signaling in adrenocortical malignancy development.

▪ Epigenetics

In the last two decades has been widely demonstrated that the presence of cancer-specific mutations is not sufficient to exhaustively explain the mechanisms underlying tumor development and progression. Epigenetic changes, including DNA methylation and microRNA expression dysregulation, are thought to play a central role in almost every step of tumorigenesis and tumor evolution, and a complex relationship between genetics and epigenetics seems to exists to final determine the acquisition of hallmark properties of cancer (Timp & Feinberg, 2013; Chatterjee *et al*, 2017 **in press**). Particularly for what concerns tumor aggressiveness, the dynamism of epigenetics marks appears to better reflect and to potentially sustain the phenotypical transitions taking place during cancer cell invasion and metastasis. Epigenetic studies of ACC revealed the presence of specific methylation and microRNA profiles which allows to better classify the malignancy, with both diagnostic and prognostic significance.

DNA methylation. The best characterized epigenetic modification of DNA in mammals is the addition of a methyl group to cytosines, typically occurring at CpG dinucleotides. In normal cells, DNA methylation occurs predominantly in repetitive genomic regions, while CpG island are generally unmethylated, particularly those associated with promoters upstream tumor suppressor genes. In tumor cells, instead, repeat-rich heterochromatin becomes hypomethylated, thus contributing to genomic instability, loss of parental imprinting and reactivation of transposable elements; on the other hand, *de novo* methylation of CpG islands frequently occurs, resulting in the transcriptional silencing of growth-regulatory genes (Robertson, 2005) (Fig. 9). The role of an aberrant methylation pattern in promoting tumorigenesis has been investigated also in ACC, focusing on both candidate gene approaches and genome-wide methylation level analysis. In sporadic ACCs, DNA methylation of the *H19* promoter has been correlated to abnormal expression of both *H19* and *IGF2* genes (Gao *et al*, 2002), whereas *TP53* promoter methylation does not seem to be an important event in ACC (Sidhu *et al*, 2005), by contrast to what happens in other types of cancer. At genome-wide level, the first evidence of methylation as regulator of gene expression in ACC was the observation of a global hypomethylation in primary and metastatic ACCs compared to adenomas and normal adrenals, with a group of 57 genes down-regulated and hypermethylated (Rechache *et al*, 2012). These findings were confirmed by another study in which,

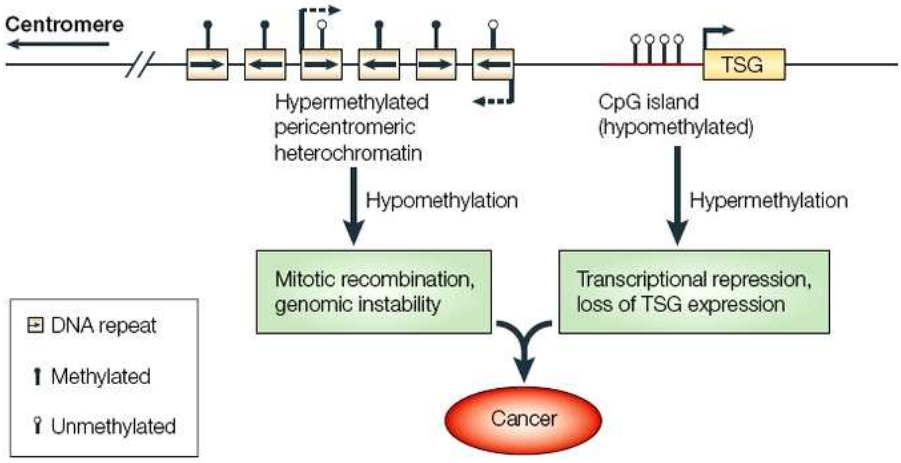


Figure 9. Schematic representation of a genomic DNA region in normal cells. Repeat-rich pericentromeric heterochromatin is hypermethylated, while hypomethylated CpG islands associate with active transcription of tumor suppressor genes (TSG). In tumor cells, global hypomethylation contributes to genomic instabilityby increasing mitotic recombination events. *De novo* methylation of CpG islands also occurs and can result in the transcriptional silencing of growth-regulatory genes. Adapted from Robertson, 2005.

among 27˙578 CpG sites analyzed, 212 CpG islands in promoter regions of genes involved in regulating cell cycle, apoptosis and transcription, were found significantly hypermethylated in ACCs compared to ACAs and normal tissues (Fonseca *et al*, 2012). From a third study comes a further confirmation of ACC-specific hypermethylation in promoter regions, also correlating with prognostic features in patients (Barreau *et al*, 2013). Furthermore, two subgroups of ACC were identified: a "CIMP (*CpG Island Methylator Phenotype*)-high" group, showing high methylation levels associated with *TP53* inactivation; and a "CIMP-low" group, characterized by lower methylation and Wnt signaling activation. These findings indicate that different mechanisms are responsible for the transcriptional dysregulation and that ACCs with similar phenotype can be heterogeneous concerning the molecular mechanisms related to tumorigenesis. More recently, the prognostic value of CpG island methylation of some candidate genes has been confirmed to be an independent predictor for recurrence and death in ACC, together with the classic clinical parameters (Jouinot *et al*, 2017).

MicroRNAs. MicroRNAs (miRNAs) are a class of evolutionary conserved, small (18-25 nucleotides) non-coding RNAs that post-transcriptionally regulate gene expression by directly targeting mRNAs: they specifically bind the mRNA 3′-UTR regions, affecting their stability and translation, or, as recently demonstrated, they may target protein coding and 5'-UTR regions (Vasudevan, 2012). After being synthesized into the nucleus, miRNAsare exported to the cytosol and act within multiproteic effector complexes, the so-called RNA-induced silencing complexes (RISCs), which are guided through complementary base pair to their targets (Fabian *et al*, 2010; Czech & Hannon, 2011). Several miRNAs have been identified as regulators of genes involved in crucial biological processes, including organogenesis, hematopoiesis, cell development, proliferation and invasion, and miRNA dysregulation, such as overexpression or deletion, has been associated with initiation and development of many types of tumor (Lujambio & Lowe, 2012; Markopoulos *et al*, 2017; Fang *et al*, 2017): in fact, genomic instability andaberrations of mechanisms related to miRNA processing and epigenetic regulation have been described as involved in miRNA expression deregulation (Fig. 10) (Calin *et al*, 2004; Lin & Gregory, 2015). Furthermore, miRNA profiling has been shown to be potentially used as tumor biomarker easily available, since miRNAs can be directly

assayed in biological fluids (Ferracin *et al*, 2010; Xiao *et al*, 2013), also in blood as circulating cell-free miRNAs (Schwarzenbach *et al*, 2014).

The relevance of miRNAs has been investigated also in adrenocortical tumors. Different studies have demonstrated that ACCs present distinct miRNA expression profiles compared to ACAs and normal adrenals (Soon *et al*, 2009; Tömböl *et al*, 2009; Doghman *et al*, 2010; Özata *et al*, 2011; Patterson *et al*, 2011; Schmitz *et al*, 2011; Assié *et al*, 2014; Duregon *et al*, 2014; Feinmesser *et al*, 2015). Among all miRNAs assessed, miR-184, miR-210, miR-503 and miR-483 resulted significantly up-regulated in ACCs, whereas a specific down-regulation was observed for miR-214, miR-375, miR-511, miR-195 and miR-335 (Soon *et al*, 2009; Tömböl *et al*, 2009; Özata *et al*, 2011; Schmitz *et al*, 2011). miR-483 is the most consistently overexpressed miRNA found in ACC: its gene is located at 11p15.5, within the second intron of *IGF2* gene, and the high expression of miR-483-5p associates with overexpression of *IGF2* (Patterson *et al*, 2011), as well as poorer prognosis (Soon *et al*, 2009). Up-regulation of miR-483-3p was specifically observed in pediatric ACC (Doghman *et al*, 2010), as well as in adults, where has been also shown to improve the diagnostic accuracy of Weiss score when combined with Smad4 expression (Wang *et al*, 2014). The oncogenic mechanism of miR-483-3p has been linked to its ability to modulate

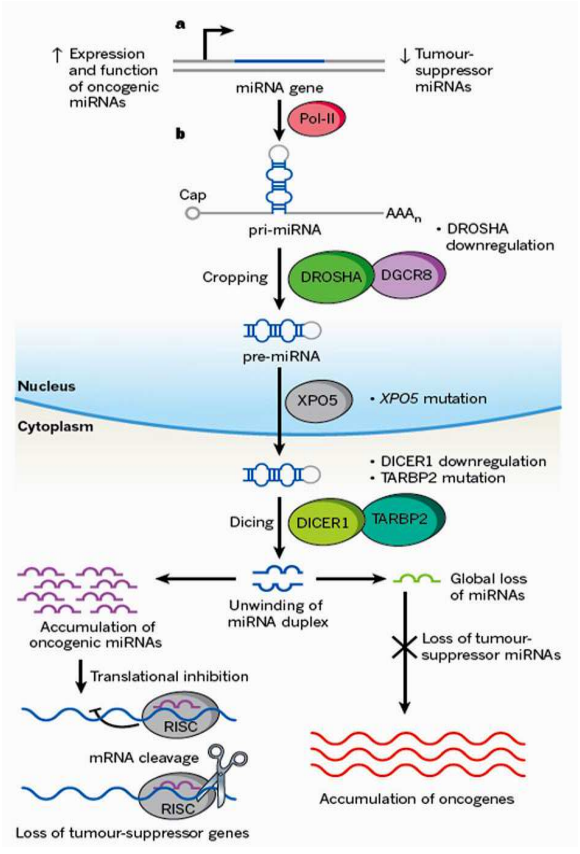


Figure 10. Representation of possible miRNA dysregulation mechanisms in cancer cells. Globally, miRNA downregulation, loss of tumor suppressor miRNAsand accumulation of oncogenic miRNAs take place, thus resulting in the promotion of cancer development. Genomic alterations, mutations and epigenetic changes can affect miRNA expression. The global levels of miRNAs can be also impaired at the biogenesis and maturation level, through alterations of the processing machinery. This results in the final accumulation of oncogenic miRNAs, which act by inhibiting tumor suppressor gene expression, and loss of tumor suppressor miRNAs, with the consequential accumulation of oncogenes. Adapted from Lujambio & Lowe, 2012.

the pro-apoptotic proteins BCC3/PUMA, thus protecting cells from apoptosis (Veronese *et al*, 2010). Similar observations were further made also in the human adrenocortical carcinoma cell line NCI-H295R (Ozata *et al*, 2011), confirming the role of miR-483-3p as apoptotic regulator. Among the overexpressed miRNAs, high miR-210 was also associates with ACC aggressiveness and poor prognosis (Duregon *et al*, 2014) and it results of particular interest since it plays a crucial role in cell response to hypoxia (Chan *et al*, 2012), one of the major cancer hallmark. miR-195 is another main deregulated miRNA in ACC, particularly showing down-regulation which significantly associates with poor overall survival (Soon *et al*, 2009). Evidence suggests that it promotes apoptosis while inhibits proliferation, as demonstrated also in NCI-H295R cells (Ozata *et al*, 2011). miR-195 has been found down-regulated also in childhood ACC together with miR-99a and miR-100, the latter ones having as targets several components of the IGF1 signaling pathway (Doghman *et al*, 2010). A specific group of miRNAs belonging to the imprinted DLK1/MEG3 cluster (consisting of multiple maternally expressed noncoding RNA genes and paternally expressed protein-coding genes) were found down regulated in non-aggressive ACC compared to the aggressive ones (Chabre *et al*, 2013; Assié *et al*, 2014). Deregulated expression of a set of specific miRNAs, including some of the previous ones, and their ability to define malignancy have been recently validated (Koperski *et al*, 2017). Other studies have investigated the role of miRNA-processing enzyme in ACC tumorigenesis and a significant overexpression of *TARBP2*, *DICER1* and *DROSHA* transcripts has been described in ACC (Caramuta *et al*, 2013). Another regulator of miRNA biogenesis, namely LIN28, has been found underexpressed in aggressive ACC compared to their benign counterparts (Faria *et al*, 2015): this is a RNA-binding protein which blocks miRNA processing by Drosha in the nucleus and Dicer in the cytosol (Tsialikas & Romer-Seibert, 2015). Finally, miRNAs have been evaluated as ACC prognosis markers in different studies assessing the circulating miRNA levels in ACC patient blood, either plasma or serum (Chabre *et al*, 2013; Patel *et al*, 2013; Szabo *et al*, 2014; Salvianti *et al*, 2017), and several miRNAs differentially expressed in ACC and ACA have been identified: has-miR-483-p5 levels increase in ACC patients and correlate with tumor stage and prognosis, also differentiating non-aggressive from aggressive carcinomas; on the other hand, low levels of hsa-miR-195 can discriminate between adenomas and carcinomas, being predictive of recurrence risk in ACC patients and confirming the previous data on

tumor samples. In addition, some other circulating miRNAs have been described, such as the down-regulated hsa-miR-335 (Chabre *et al*, 2013) and the overexpressed hsa-miR-100, hsa-miR-181b, hsa-miR-184, hsamiR-210 (Szabo *et al*, 2014) and hsa-miR-34a (Patel *et al*, 2013). Although the global impact of miRNA deregulation on ACC pathogenesis and evolution has to be fully elucidated (Igaz *et al*, 2015;Cherradi, 2016) and further validation studies are needed, miRNAs might represent promising non-invasive biomarkers for adrenocortical malignancy.

- **Integrated genomic characterization of ACC**

Studying the genomics of adrenocortical tumors at different levels has provided several markers able to define a molecular malignancy signature, allowing to better discriminate ACC from ACA and to be predictive for patient outcome. Globally, they revealed a modulation of gene expression leading to the alteration of pivotal signaling pathways regulating cell cycle, chromosomal maintenance, cell survival, inflammation, immunity. So far, integrating genomics of ACC allowed to discriminate different subgroups that correlate with specific phenotype and prognosis (Faillot & Assié, 2016). Moreover, by combining different genomic approaches (whole genome sequencing, exome sequencing, SNP array, DNA methylation analysis, mRNA expression array, miRNA sequencing), a comprehensive "omics"-based ACC classification has been generated in two independent international cohorts (Assié *et al*, 2014; Zheng *et al*, 2016), which concordantly identified two main molecular subgroups of ACC, one counting the most aggressive tumors (C1A cluster), and the other defining the most indolent forms (C1B cluster), confirming what shown in the previous studies. A third molecular subgroup, associating with an intermediate prognosis, could be defined between the two. Globally, the two studies confirmed that chromosomal instability is the main signature of ACC, with LOH of the *IGF2* locus (11p15) as the most frequent. The assessment of copy-number alterations and LOH revealed recurrent high-level amplifications of *TERT*, *TERF2*, *CCNE1* and *CDK4* and deletions of *ZNRF3*, *CDKN2A*, *RB1*. An additional deletion peak was detected at the chromosomal locus related to the long noncoding RNA *LINC009*. Exome and RNA sequencing defined the driver events in ACC: both studies identified *TP53*, *CTNNB1* and *MEN1* as the most frequently mutated genes, together with DAXX (Assié *et al*, 2014) and *PRKAR1A* and *RPL22* (Zheng *et al*, 2016). Collectively, gene alterations

(mutations, high-amplifications, deletions) result in affecting three major pathways in ACC: β-catenin pathway (cell growth and proliferation), p53/Rb signaling (cell cycle regulation) and chromatin remodeling (epigenetic regulation). DNA methylation profiles were able to well defined the ACC subgroups, particularly associating with patient prognosis: the molecular group with the better outcome resulted as non-CIMP, while the higher aggressive ACCs presented hypermethilation of CpG island in gene promoters (CIMP), with a further discrimination between CIMP-high and CIMP-low (Assié *et al*, 2014; Zheng *et al*, 2016). Finally, also miRNA expression profiles were able to discriminate different groups of ACC associated with different patient outcome. These results indicate that a specific ACC classification might be used to integrate the molecular signature with the classical clinical and pathological parameters, thus improving not only the diagnosis and prognosis accuracy, but also the global management of ACC patients, with the possibility of developing new personalized therapies (Fig. 11).

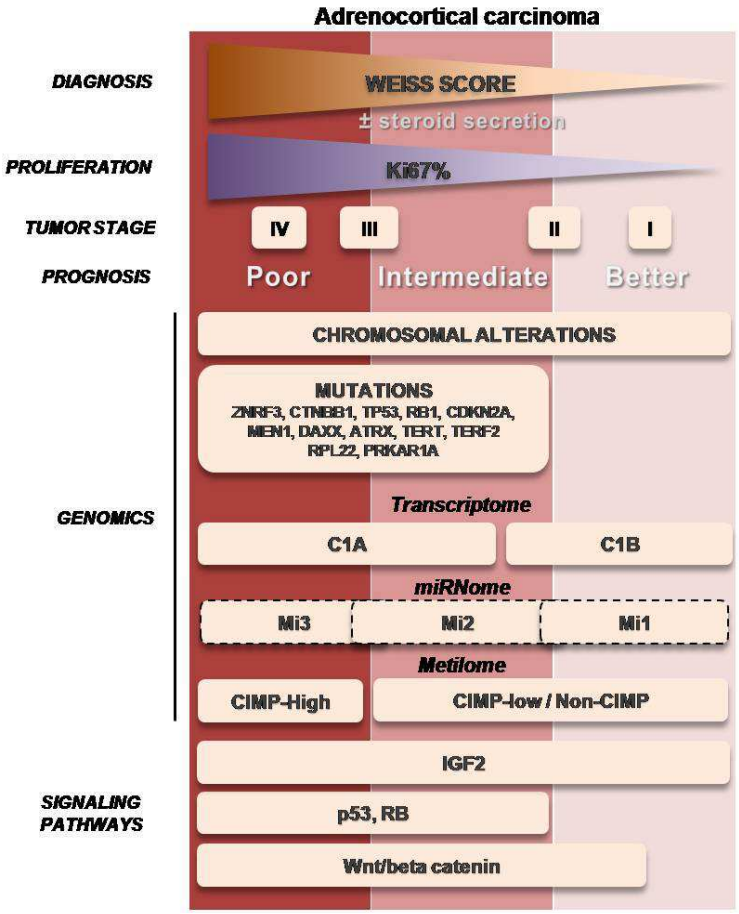


Figure 11. Potential ACC re-classification resulting from the integration of the specific molecular features with the classical clinical and pathological parameters. Adapted from Armignacco *et al*, 2017.

2.5.2. Cell signaling pathways: the cross-link between the normal homeostatic adrenal maintenance and neoplasia

Tissue development and homeostatic maintenance are strictly regulated by specific factors and molecular mechanisms. This is even more true for the adrenal cortex, in which a morphological and functional differentiation has to be maintained between the three zones throughout life. The normal adrenal homeostasis is mediated by a stem/progenitor cell population which has been demonstrated to be located in the peripheral capsular and subcapsular compartment and that is responsible for the whole adrenal cortex replenishment, with a centripetally displacement of cells toward the cortico-medullary boundary (Vinson, 2003; Kim *et al*, 2009; King *et al*, 2009; Huang *et al*, 2010; Wood & Hammer, 2011; Freedman *et al*, 2013; Vidal *et al*, 2016). Notably, the dysregulation of the signaling pathways underlying adrenal organogenesis and homeostasis are the same mainly involved in adrenocortical disease, including IGF2, Wnt and Protein Kinase A (PKA) pathways, as well as telomere protection and maintenance mechanisms, that have been found to be implicated in both adrenal function and dysfunction (Penny *et al*, 2017).

IGF2/IGF-1R. As previously described, *IGF2* is one of the most common up-regulated genes in adrenocortical carcinoma, but it is also highly expressed in the fetal adrenal (Voutilainen & Miller, 1988; Rainey *et al*, 2001). IGF2 is part of the IGF family of ligands (counting also IGF1) and, together with the IGF receptors (IGF1-R, IGF2-R and insulin receptor isoform A, IR-A), the IGF binding proteins (IGFBP 1-6) and the IGF binding proteins proteases, constitutes the IGF signaling pathway. The interaction between the secreted ligand IGF2 and the IGF-1R activates the downstream AKT/PI3K and MAPK signaling to regulate cell metabolism, differentiation, proliferation and apoptosis (Kha & Lackner, 2010) (Fig. 12). In human adrenal, the IGF pathway mediates ACTH-induced prenatal growth, as well as fetal and adult steroidogenesis and homeostatic maintenance (Han *et al*, 1992; l'Allemand *et al*, 1996; Mesiano & Jaffe, 1997). The main role of IGF2 lies in fetal development, and the evidence that IGF2 expression rapidly drops after birth (Bolgorosky *et al*, 2009) suggests a possible mechanisms of fetal/embryonic program reactivation in ACC, with the establishment of a paracrine/autocrine mitogenic effect of the IGF2/IGF1-R axis, as also observed in ACC cell lines (Logié *et al*, 1999). In association with IGF2, also IGFBP2 results increased in ACC and correlates with

tumor stage (Boulle *et al*, 1998, 2001). Despite the marked increase of IGF2 expression in ACCs compared to ACAs, it has been demonstrated in mice that *Igf2* overexpression alone is not sufficient for the neoplastic transformation (Weber *et al*, 1999; Drelon *et al*, 2012; Heaton *et al*, 2012), thus suggesting that additional genetic and epigenetic alterations are needed for adrenocortical carcinogenesis. The potential involvement of other growth promoting pathways beyond IGF2 has been recently confirmed in a study assessing the phenotypical and molecular features of both ACCs and adrenocortical cells overexpressing or not IGF2 (Guillaud-Bataille *et al*, 2014).

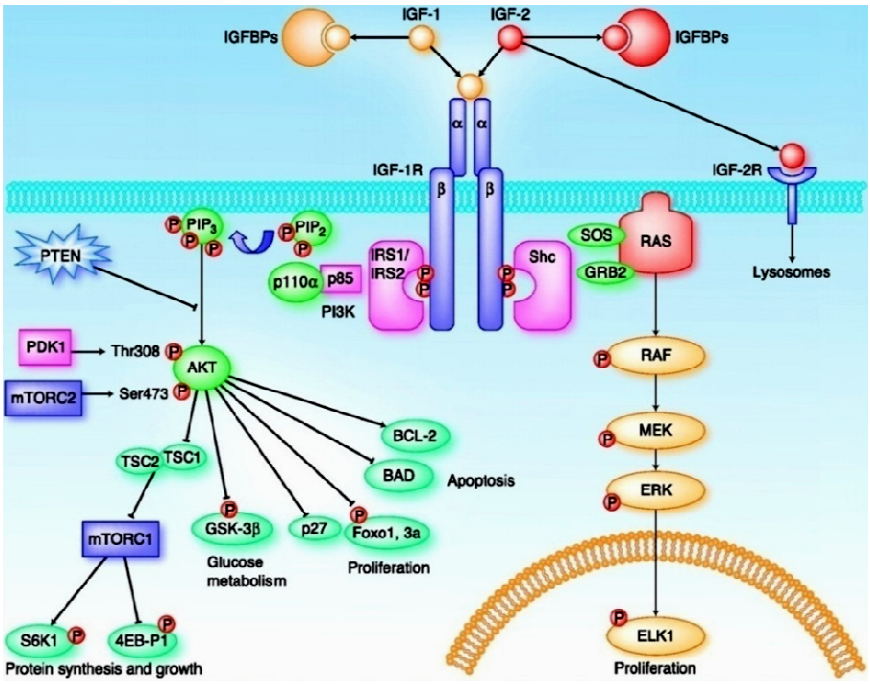


Figure 12. Representation of the IGF1-R signaling pathway. Both IGF1 and IGF2 are capable to bind the IGF1 receptor and their bioavailability is modulated by IGFBPs or, for IGF2, by its binding to IGF2-R, that leads to receptor-mediated internalization and degradation of IGF-2 in lysosomes. Once bound to its ligand, IGF1-R undergoes receptor cross-linking and autophosphorylation, thus creating multiple sites for cytosolic adaptor binding and inducing the intracellular signalization involved in the regulation of cell metabolism, proliferation and survival. Adapted from Kha & Lackner, 2010.

Wnt/β-catenin. The canonical Wnt signaling is widely implicated in adrenocortical homeostasis and disease: evidence in mouse models suggest that it is essential for embryonic adrenal development and that it regulates adrenal stem/progenitor maintenance, proliferation, adrenal zonation and steroidogenesis (Kim *et al*, 2008; Walczak *et al*, 2014). Once translocated into the nucleus, β-catenin interacts with the Steroidognic Factor 1 (SF-1) and constitutes a transcriptional

complex inducing the expression of two specific target genes, DAX1 (Dosage-sensitive sex reversal, Adrenal hypoplasia congenita critical region on the X chromosome, gene 1) and inhibin-α, which are involved in regulating adrenal development and adrenocortical cell stem niche maintenance (Parker & Schimmer, 1997; Parker *et al*, 2002; Gummow *et al*, 2003; El-Khairi *et al*, 2011; Scheys *et al*, 2011). Moreover, SF-1 also interacts with the GATA transcription factors: GATA-4, which regulates, in the fetal adrenal, the expression of gene related to steroidogenesis (*inhibin-a*, *CYP17, StAR*) (Tremblay & Viger, 2003) and that, under LH control, drives gonadal differentiation (Looyenga & Hammer, 2006); and GATA-6, which induces adrenal differentiation and regulates the expression of steroidogenesis enzymes (*StAR*, *CYP11A1*, *CYP17*) in the adult gland (Jimenez *et al*, 2003; Tremblay & Viger, 2003). Deregulation of these factors may play a role in adrenal tumorigenesis: in fact, increased GATA-4 mRNA and protein expression have been described in both ACAs and ACCs, while GATA-6 seems to be down-regulated, mainly in carcinomas (Barbosa *et al*, 2004; Kiiveri *et al*, 2004; Kiiveri *et al*, 2005). Indeed, SF-1 seems to play an important role in adrenocortical proliferation (Figueiredo *et al*, 2000; Doghman *et al*, 2007; Almeida *et al*, 2010): it has been shown an overexpression in pediatric ACCs, also related to frequent gains of its chromosomal region 9q34 (Figueiredo *et al*, 1999; James *et al*, 1999; Pianovski *et al*, 2006b), and SF-1 immunostaining allows to identify cell adrenocortical origin with high diagnostic accuracy, as well as high prognostic value (Sbiera *et al*, 2010; Duregon *et al*, 2013b).

As previously illustrated, Wnt/β-catenin is one of the main cell signaling pathways altered in ACC, mostly resulting in the up-regulation of the β-catenin trascriptional activity. The Wnt pathway presents a complex degree of regulation, which includes ligand availability, secretion of different types of frizzled receptor inhibitors and autocrine regulation of Wnt ligands. More recently, a key regulation system involving the R-spondin-ZNRF3/RNF43 signaling module has been described (Fig. 13): ZNRF3 and RNF43 are membrane E3 ligases which negatively regulate Wnt by promoting ubiquitination and degradation of Wnt receptors; R-spondin proteins (RSPO1-4), instead, serve as antagonists of ZNRF3/RNF43, suppressing their inhibitory action. In cancer cells, the maintenance of a sustained Wnt/β-catenin signaling requires to overcome ZNRF3/RNF43-mediated feedback

inhibition and mutations of ZNRF3/RNF43 or translocations of RSPO2/RSPO3 have been identified in various cancers (Hao *et al*, 2016).

Notably, as already described, *CTNNB1* and *ZNRF3* are commonly and mutually exclusive altered in ACC, thus leading to an aberrant activation of Wnt. The effects of constitutively active β-catenin have been investigated also in adrenocortical cell lines and in mouse models: NCI-H295R cells harbor an activating β-catenin mutation and it has been shown that knocking down or targeting β-catenin induces cell growth arrest *in vitro* and complete tumor regression *in vivo* (Dogman *et al*, 2008; Gaujoux *et al*, 2013; Salomon *et al*, 2015). Moreover, mice with adrenal over-activated Wnt signaling develop microscopic and macroscopic adenomas, which evolve to large tumors when these mice are crossed with adrenal specific *Igf2* overexpressing mice (Heaton *et al*, 2012). These results, further validated in a similar model of adrenocortical-specific β-catenin stability and *Igf2* overexpression (Drelon *et al*, 2012), indicate a causative role for β-catenin in adrenocortical tumorigenesis, potentially synergic with the IGF2 signaling.

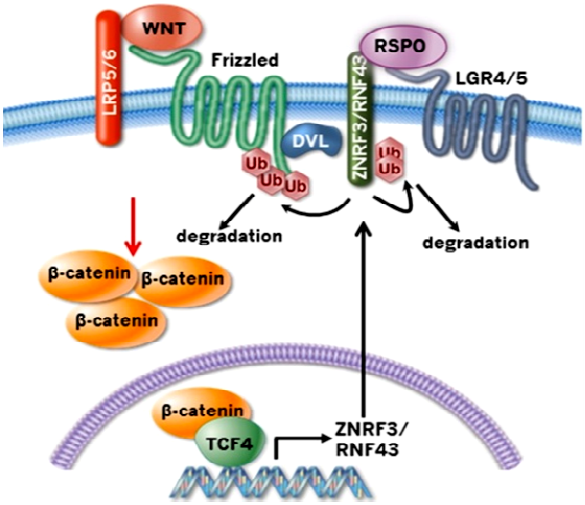


Figure 13. Representation of the feedback negative control of Wnt by the R-spondin-ZNRF3/RNF43 signaling module. In the nucleus, β-catenin acts as regulator of transcription of target genes, including ZNRF3/RNF43, which translocates to the plasma membrane and promotes Frizzled receptors degradation, switching off Wnt/β-catenin signaling. R-spondin counteracts this mechanism by binding LGR4/5 and ZNRF3/RNF43 and inducing ubiquitination and degradation of ZNRF3/RNF43 itself. Adapted from Hao *et al*, 2016.

cAMP/PKA. The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling is crucially involved in adrenocortical growth and maintenance, as well as in glucocorticoid and androgen production. The pathway is activated later on the binding of adrenocorticotropic hormone (ACTH) to its receptor, namely the Melanocortin 2 receptor (MC2R), a 7-transmembrane G-protein coupled receptor which activates a Gsα protein (G protein subunit α) to promote the conversion of ATP to cAMP through the adenylate cyclase. Once cAMP binds the two regulatory subunits of PKA, the other two catalitic subunits are free to activate the target

substrates responsible of inducing the transcription of cAMP-responsive genes, including those involved in proliferation and steroid production in adrenal *zona fasciculata* and *reticularis* (Fig. 14).

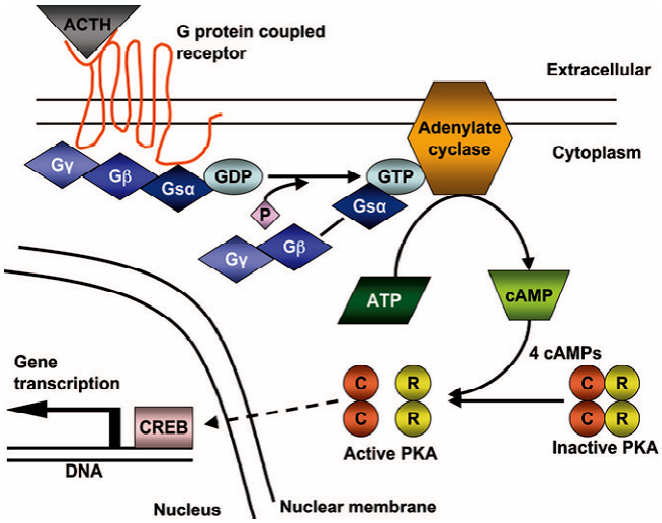


Figure 14. Representation of the ACTH-cAMP–PKA pathway. In the presence of ACTH, cAMP production is promoted, thus activating PKA (C, catalitic subunits; R, regulatory subunits) which phosphorilates CREB (cAMP-response element), inducing the transcription of specific target genes. Adapted from Soon *et al*, 2008b.

Alterations of this pathway are etiologically involved in several endocrine diseases and an increased PKA activation is supposed to stimulate adrenocortical growth and glucocorticoid production: for example, activating mutations of the *GNAS1* (Guanine Nucleotide binding protein Alpha Stimulating 1) gene, encoding for Gsα, cause the McCune-Albright Syndrome which, among other manifestations, can present cortisol-producing bilateral adrenocortical hyperplasia (Weinstein *et al*, 1991). Somatic activating *GNAS1* mutations have been also described in ACTH-independent macronodular adrenal hyperplasia (Fragoso *et al*, 2003) and cortisol producing adenomas (Almeida *et al*, 2012; Sidhu *et al*, 2013; Goh *et al*, 2014;). It has been hypothesized that alterations of MC2R may contribute to adrenocortical tumorigenesis, but actually activating *MC2R* mutations have never been described in ACTs (Latronico *et al*, 1995; Light *et al*, 1995) and what has been observed is that ACCs frequently exhibit allelic losses of region 18p11.2 and downregulation of *MC2R* (Reincke *et al*, 1997a). Contrarily, cortisol-secreting ACAs overexpress *MC2R* (Reincke *et al*, 1997b), thus suggesting a role for the receptor in cell differentiation. More commonly, secreting adrenocortical diseases show alterations of the PKA regulatory subunits, particularly the PRKAR1A (Kirschner *et al*, 2000; Yates *et al*, 2013). The recent identification of frequent *PRKAR1A* inactivating mutations in ACC includes this gene in the set of the main drivers in adrenal malignancy (Zheng *et al*, 2016).Consistently, recent evidence indicates that PKA is able to counteract the

WNT-induced carcinogenesis, whereas a decrease in PKA activity associates with higher levels of WNT-related gene expression, thus favoring the acquisition of malignant features (Drelon *et al*, 2016).

Other growth factors. The ***Fibroblast Growth Factors*** (***FGFs***) constitute another family of ligands commonly involved in development, homeostasis and repair processes, as well as in cancer (Carter *et al*, 2015). They can activate several different pathways depending on cell context, including Ras/MAPK (cell proliferation and differentiation), PI3K/AKT (anti-apoptotic signaling) and PKC (cell migration) (Turner & Grose, 2010). Studies in mice revealed that FGF signaling is essential for both fetal adrenal development and adult gland growth (Guasti *et al*, 2013) and it has been proposed that their signalization may be implicated in ACC biology, since some expression profile analysis have identified an enrichment of FGFR1 and FGFR4 receptors in ACCs compared to ACAs (de Fraipont *et al*, 2005; Laurell *et al*, 2009; Brito *et al*, 2012). Indeed, b-FGF2 seems to be the best candidate to be evaluated in adrenocortical tumors, as it is highly expressed in adrenal tissue and it has been showed to have a powerful mitogenic action in fetal and adult adrenal cell cultures (Mesiano *et al*, 1991; Feige *et al*, 1998), as well as in the adrenocortical cell line NCI-H295R (Boulle *et al*, 2000).

The ***Transforming Growth Factor-β1***(***TGF- β1***) is another modulator of cell growth involved in the regulation of fetal and adult adrenal. A decreased expression of its mRNA has been observed in ACC, whereas it is not true for its receptor (Bocuzzi *et al*, 1999; Arnaldi *et al*, 2000).

The ***Epidermal Growth Factor Receptor*** (***EGFR***) is a tyrosine kinase-coupled receptor which mediates a signalization involved in the regulation of cell fate, proliferation, survival, cell cycle control, differentiation and motility through the Ras/Raf/Mek/Erk pathway (Shields *et al*, 2000). Immunohistochemistry studies have shown that EGFR overexpression is almost ubiquitous in ACCs (Kamio *et al*, 1990; Edgren *et al*, 1997; Adam *et al*, 2010), even if frequency of somatic activating mutations is low. Recently, it has been shown that co-inhibiting EGFR and IGF-1R signaling significantly decrease cell proliferation and viability (Xu *et al*, 2016).

Telomeres, *TERT* and *TERF*2 expression. The adrenal gland acquires its final structure and function thanks to a remodeling process of the fetal zone soon after

birth and throughout childhood, in which a balance between proliferation, apoptosis and differentiation is established in order to reach the steroidogenic cell number sufficient to sustain the global function of the gland. Normal cells can replicate within a limited number of division, after that they enter a quiescent phase, the so-called "replicative senescence". This phenomenon is due to the telomere shortening at the chromosome ends: their function of stabilizing chromosomes and protecting their ends from nuclease activity and DNA damage response (de Lange, 2004; Verdun & Karlseder, 2007) is progressively lost, driving cells toward quiescence. However, cells with a high proliferative rate, such as stem/progenitor cells, germ cells or neoplastic cells, can avert telomeres shortening by activating the enzyme telomerase. This is a ribonucleoprotein complex consisting of a protein component TERT (Telomerase Reverse Transcriptase), with reverse transcriptase activity, and a ribonucleotide subunit TERC (Telomerase RNA Component), which serves as a template for telomere elongation, catalyzing the extension in the 5'→3' direction (Bekaert *et al*, 2004; Cohen *et al*, 2007; Shay, 2016). Telomerase and telomere maintenance play a pivotal role in adrenal homeostasis, with increased telomerase expression and telomere length in populations supposed to be part of the adrenal stem niche (Else, 2009). Instead, normal adult adrenal cells lose telomerase activity and telomeres shorten every division, thus inducing a decline of the cell replicative potential (Yang *et al*, 2001). Nevertheless, it has been demonstrated that restoring telemerase activity by transfecting cells with the hTERT subunit prevents telomeres from shortening and induces a rapid cell proliferation; contrarily, when neoplastic cells are deprived from telomerase, they progressively lose their malignant properties (Sun *et al*, 2004). Notably, the investigation of the telomere length maintenance mechanism (TMM) in adrenal tumors demonstrated that malignant tissues present at least one TMM compared to benign tumors and normal adrenal, as also confirmed in adrenocortical cell lines (Else *et al*, 2008). Moreover, it has been shown that ACCs have a significantly higher telomerase activity compared to ACAs and it correlates with tumor dimension (Mannelli *et al*, 2000). The role of telomeres in the etiology of adrenocortical neoplasia is even more supported by the recent ACC genomic characterization (Assié *et al*, 2014; Pinto *et al*, 2015; Zheng *et al*, 2016): *TERT* has been identified as one of the candidate driver genes showing frequent high-level amplifications, together with current focal amplifications of *TERF2*

(Telomeric Repeat Binding Factor 2), thus indicating that this key stem cell pathway is strongly involved in promoting cancer cell survival.

2.6. Therapeutic approaches

Despite the recent advances in the understanding of ACC molecular pathophysiology, as well as in multimodality treatment, the curative options for this malignancy still remain limited to the complete surgical resection, aided by adjuvant therapies aimed to decrease the chance of recurrence. In case of unresectable or metastatic ACC, when tumor aggressive behavior frequently associates with an extremely poor prognosis, all therapy is considered as a palliative to improve patient quality of life and minimize side effects related to antineoplastic therapies. However, the assessment of ACC genomic landscape could provide new insights into the genetic and biologic heterogeneity of the tumor, thus allowing to identify a set of new potential therapeutic targets exploitable for the development of more specific and effective drugs.

2.6.1. Surgery

Complete surgical resection (R0) is the treatment of choice for localized ACCs, with particular attention to preserve the tumor capsule intact in order to avoid cancer cells dissemination. Preoperative evaluation, including biochemical evaluation and imaging, and operative planning are pivotal to assure the best outcome. The optimal surgical approach needs considerations about tumor dimension and secretion, lymph nodes involvement and invasion of adjacent organs, depending on the single case (Else, 2014). Open adrenalectomy (OA) with lymph node dissection is regarded as the standard treatment for ACC (Bellantone *et al*, 2015), especially in case of infiltrating tumor or suspected lymph nodes. Laparoscopic adrenalectomy (LA) may also be performed in case of stage I-II ACCs with a diameter < 8-10 cm, even if the choice between OA and LA remains controversial considering the data obtained from retrospective series investigating surgery efficacy and safety (Gonzalez *et al*, 2005; Brix *et al*, 2010; Leboulleux *et al*, 2010; Miller *et al*, 2010; Porpiglia *et al*, 2010). However, it has to be considered that, despite also in patients with metastatic disease surgery seems to improve survival

(Livhits *et al*, 2014), even after complete resection the recurrence rate remains between 19 and 34% basing on tumor stage, often with metastases occurring (Bellantone *et al*, 1997; Schulick & Brennan, 1999; Icard *et al*, 2001).

2.6.2. *Mitotane adjuvant treatment*

Due to the high rate of local recurrence, surgery is routinely followed by adjuvant treatment with the adrenolytic drug mitotane [1-(o-chlorophenyl)-1-(p-chlorophenyl)-2,2-dichloroethane, o,p'-DDD]: this is a synthetic derivative of the insecticide DDT (dichlorodiphenyltrichloroethane) and it is the only drug approved by the U.S. Food and Drug Administration and European Medicine Executive Agency for the treatment of adrenocortical carcinoma (Schteingart *et al*, 2005). Mitotane shows a relative specificity for the *zona fasciculata* and *reticularis* of the adrenal cortex, where it exerts its cytotoxic function following the metabolization into the metabolites 1,1-(o,pdichlorodiphenyl)-2,2dichloroethene (o,p'DDE) and 1,1-(o,pdichlorodiphenyl)acetic acid (o,p'DDA) (Martz & Straw,1980; Cai *et al*, 1995a, 1995b). Active mitotane metabolites inhibit the adrenocortical steroidogenic pathways, mainly targeting enzymes involved in cortisol metabolism, such as StAR and the cholesterol side-chain cleavage enzymes CYP11A1 and CYP11B1 (Cai *et al*, 1997; Lindhe *et al*, 2002; Lin *et al*, 2012). In cells, mitotane is able to decrease cell proliferation and cortisol secretion (Lindhe & Skoseid, 2010) and it is thought to act primarily by affecting the mitochondrial function, altering mitochondrial respiratory chain activity and thereby inducing an apoptotic process (Sinsheimer & Freeman, 1987; Hescot *et al*, 2013; Poli *et al*, 2013). The endoplasmic reticulum has been recently identified as another key target of mitotane (Sbiera *et al*, 2015). However, the exact pharmacological mechanism of its antitumor action remains to be fully elucidated, despite it has been also investigated by microarray and proteomic techniques (Stigliano *et al*, 2008; Zsippai *et al*, 2012; Lehmann *et al*, 2013).Some studies have identified a functional relation between the expression of the *RRM1* (Ribonucleotide Reductase Large Subunit 1) gene and mitotane sensitivity in adrenocortical carcinoma cell lines (Volante *et al*, 2012; Germano *et al*, 2015), hypothesizing that RRM1 may interfere with mitotane metabolism. However, this mechanism still remains to be clarified.

Mitotane adjuvant treatment administered soon after surgery has been shown to significantly improve the median tumor-free survival in patients with completely resected ACC (Terzolo *et al*, 2007; Terzolo *et al*, 2012; Fassnacht *et al*, 2013). However some evidence indicates that only a subgroup of patients may benefit, probably the one with cortisol-producing tumors (Bertherat *et al*, 2007). Contrarily, the efficacy of mitotane in case of not completely resected, metastasized or recurrent ACC is well established and mitotane response, measured as stable disease or partial remission after the treatment, is observed in only 25-30% of patients (Else, 2014).The major factor influencing the therapy response is represented by the target plasma concentration of mitotane, ranging from 14 to 20 mg/l: several studies showed that patients with advanced ACC who reached mitotane plasma levels higher than 14 mg/l have less recurrence and a prolonged recurrence-free and overall survival (Terzolo & Berruti, 2008; Hermsen *et al*, 2011; Terzolo *et al*, 2013). Thus, monitoring mitotane plasma concentration is of great importance for the therapy management. However, mitotane doses usually used to reach the therapeutic window often associates with a range of significant toxicity, which commonly leads to gastrointestinal, neurological, metabolic and endocrine adverse effects (Else, 2014), thus inducing drug withdrawal in some cases. For advanced ACC, multiple combination regimen have been investigated, particularly the EDP (Etoposide, Doxorubicine, Cisplatin) plus mitotane (Berruti *et al*, 1998) and the Streptozotocin plus mitotane (Khan *et al*, 2000), the first one currently being the standard treatment option (FIRM-ACT phase III trial, Fassnacht *et al*, 2012). However, the improvement of disease control with polychemotherapy remains quite modest and chemoresistance often develops. Notably, mitotane has been shown to strongly induce P450-34A (CYP34A) (Kroiss *et al*, 2011; Chortis *et al*, 2013), a drug-metabolizing microsomal enzyme expressed in liver and gut: this may lead to significant drug interaction, decreasing the efficacy of combination strategies and partially explaining the development of chemoresistance.

2.6.3. Radiotherapy

Historically, ACC was considered resistant to radiotherapy and the use of this treatment remains controversial considering efficacy versus side effects related to the normal tissue toxicity in proximal radiosensitive structures, such as kidneys,

stomach, intestine and spinal cord (Milgrom & Goodman, 2012). However, advances in radiotherapy techniques, particularly the possibility to modulate the radiation intensity, have made the treatment safer and promising results have been obtained in ACC patients in both adjuvant and palliative settings (Fassnacht *et al*, 2006; Hermsen *et al*, 2010; Sabolch *et al*, 2011, 2015; Habra *et al*, 2013). Studies *in vitro* suggest a possible synergistic inhibition of cell growth by the combination of irradiation and mitotane administration (Cerquetti *et al*, 2008). Nevertheless, the radiotherapy efficacy still needs to be firmly established.

2.6.4. *Targeted therapy*

The limited effectiveness of the traditional available therapies for ACC has led to explore new modes of treatment, starting from the molecular alterations described for this malignancy to be exploited as pharmacological targets (Costa *et al*, 2016). Tyrosine-Kinase (TK) receptors and their downstream effectors mediating the main signaling pathways altered in ACC were the most promising molecular targets (Fig. 15), and many studies have been performed to investigate the efficacy of different inhibitors. The most data concern the IGF-1R antagonists: among all, linsitinib (OSI-906), a small molecule inhibitor of both IGF-1 and insulin receptor, generated great expectation basing on early clinical results (Barlaskar *et al*, 2009). Despite it reached a phase III trial, it has been prematurely stopped since it did not show any improvement of overall survival compared to placebo (Fassnacht *et al*, 2015). A possible explanation may be the previous exposure of most of the enrolled patients to mitotane, thus activating CYP3A4 and affecting linsitinib efficacy. Also, IGF-1R may not be a major driver in adrenocortical carcinoma and other compensatory growth-promoting pathways can activate. All the other previous trials with IGF-1R inhibitors, including the antibodies Figitumumab and Cixutumumab, were precociously stopped due to the lack of response (Haluska *et al*, 2010; Naing *et al*, 2011). However, the IGF-2 pathway remains of great interest since one of its downstream components, the mammalian target of rapamycin (mTOR), has been shown to be a potential therapeutic target in ACC (Doghman *et al*, 2010; De Martino *et al*, 2014). Particularly, targeting mTOR together with other effectors of proliferation signaling seems to be the best way to avoid the activation of compensatory growth pathways (De Martino *et al*, 2012; Doghman & Lalli, 2012;

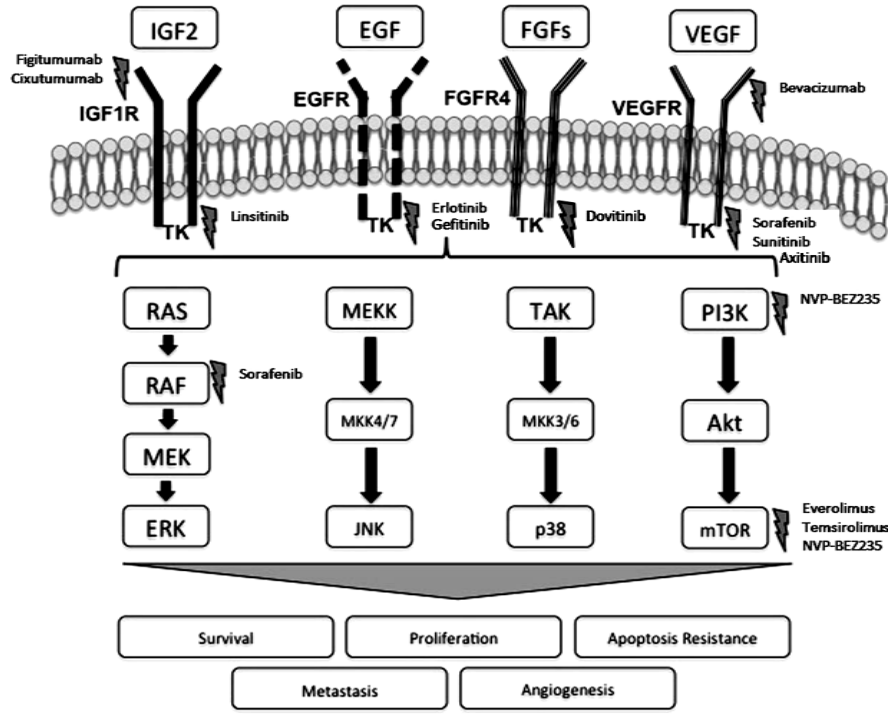


Figure 15. TK receptors abnormally activated in ACC and their downstream effectors addressed for targeted therapy with specific compounds in preclinical studies and clinical trials. Adapted from Lerario *et al*, 2014.

Naing *et al*, 2013). Other TK inhibitors have been tested in ACC in order to target the growth signal trasduction: erlotinib plus gemcitabine was used to block EGFR signaling (Quinkler *et al*, 2008),as well as gefitinib (Samnotra *et al*, 2007), both with disappointing results. Likely, this is due to the low number of *EGFR* activating mutations in ACC. However, in a recent study, it has been shown the potential role of EGFR inhibition in reducing cell growth and survival (Gagliano *et al*, 2015). Imatinib, a platelet-derived growth factor receptor (PDGFR) inhibitor, also failed in inducing response in ACC patients (Gross *et al*, 2006), even if better results have been achieved in metastatic patients by using a combined therapy (Halperin *et al*, 2014). As already described, FGFR4 overexpression and amplification associate with worse outcome in ACC patients. A phase II study has shown a partial response of patients with metastatic or non resectable ACC to the FGFR TK inhibitor dovitinib (Jesùs Garcia-Donas *et al*, 2014). One of the main feature of tumorigenesis is sustained angiogenesis and targeting Vascular Endothelial Growth Factor (VEGF) and its receptor generated encouraging results in several types of cancer. Since both VEGF and VEGFR was shown to be increased in ACC (Bernini *et al*, 2002; Zacharieva *et al*, 2004; Xu *et al*, 2011), specific TK inhibitors, such as axitinib,

sorafenib, sunitinib and bevacizumab, have been tested in ACC but, again, with no meaningful clinical benefits (Wilhelm *et al*, 2004; Wortmann *et al*, 2010; Berruti *et al*, 2012; Kroiss *et al*, 2012; O'Sullivan *et al*, 2014). More recently, the TK receptor c-Met, activated by the Hepatocyte Growth Factor (HGF), has been shown to associate with ACC growth and chemoresistance development; moreover, pharmacologically targeting this pathway, such as with the use of cabozantinib, an inhibitor of both VEGFR and c-Met, allows to significantly suppress cell proliferation and tumor growth (Phan *et al*, 2015).

Steroidogenesis represents another target of interest in the development of new therapies for ACC. The nuclear transcription factor SF-1, which plays a critical role in adrenal development and growth, has been shown to induce both proliferation in adrenocortical carcinoma cells and tumor growth *in vivo* (Doghman *et al*, 2007); on the other hand, SF-1 inverse agonists are able to inhibit proliferation in SF-1 expressing cells (Doghman *et al*, 2009). Such results, together with evidence of SF-1 overexpression in ACC tumor samples (Sbiera *et al*, 2010) and the identification of new SF-1-related genes (Mizutani *et al*, 2015), indicate the potential use of this factor as a novel therapeutic target. Another target involved in steroid biosynthesis is represented by the Acetyl-CoA acetyltransferase 1 (ACAT1), the enzyme catalyzing cholesterol ester formation: a phase I trial (NCT01898715) is currently ongoing to investigate a ACAT1 inhibitor (ATR-101-001) in patients with advanced ACC (Aung *et al*, 2015). Intracellular cholesterol availability is crucial for steroidogenesis, so disrupting cholesterol uptake could have therapeutic potential in ACC. A promising approach seems to be the use of synthetic high-density lipoprotein (HDL) nanoparticles inhibiting cholesterol transporters (Subramanian *et al*, 2016).

Basing on recent advances in unraveling ACC molecular pathophysiology, new potential pharmaceutical target are coming out: Wnt/β-catenin pathway is one of the best candidates, and pre-clinical data support the use of anti-Wnt monoclonal antibody in many types of cancer (He *et al*, 2004). So far, no clinical trials with Wnt inhibitors have been performed yet, but there is evidence that the small-molecule inhibitor PKF115-584 decreases proliferation in ACC cells *in vitro* (Doghman *et al*, 2008) and that silencing β-catenin results in cell proliferation decrease, cell cycle alteration and apoptosis induction (Gaujoux *et al*, 2013). Another strategy could be targeting the immune system: interleukin-13 receptor alpha2 (IL-13Ra2) has been

found overexpressed in ACC compared to ACAs and normal adrenals (Jain *et al*, 2012) and a phase I study has been recently conducted with systemic interleukin-13-Pseudomonas exotoxin in metastatic ACC patients, with 1 patient out of 5 reaching a stable disease for more than 5 months before progression. (Liu-Chittenden *et al*, 2015). Other three clinical trials are currently enrolling patients to investigate the immunotherapy inhibition of lymphocyte programmed cell death protein 1 (PD-1) in adrenocortical carcinoma (NCT02673333, NCT02720484 and NCT02720484).

A reliable approach to identify new therapeutic compounds to treat ACC may be the use of drugs already approved for other diseases, mainly those demonstrated to also exert an anti-neoplastic effect. Among all, some pharmacological classes of antidiabetics, such as thiazolidinediones (TZDs) and metformin, have been widely investigated for their potential effect on solid tumors, for both cancer prevention and therapy (Tuccori *et al*, 2017), and different studies have described some effectiveness also in ACC.

Thiazolidinediones (**TZDs)** are peroxisome proliferator-activated receptor-gamma (PPARγ) agonists approved for the treatment of metabolic syndrome and type 2 diabetes, since they are able to improve insulin sensitivity, as well as to regulate adipocyte differentiation and fat redistribution (Myazaki *et al*, 2002). In addition, TZDs have been shown to exert anti-cancer effects and accumulating evidence suggests a parallel PPARγ-independent action, with a selective inhibition of IGF-1R signaling (Mughal *et al*, 2017). TZDs, particularly Rosiglitazone and Pioglitazone, seem to be effective also in ACC, since they are able to decrease adrenocortical cancer proliferation and invasiveness, as well as to induce cell differentiation and apoptosis (Betz *et al*, 2005; Ferruzzi *et al*, 2005; Cantini *et al*, 2008; Luconi *et al*, 2010; Cerquetti *et al*, 2011). Moreover, they seem to affect steroidogenesis by inhibiting CYP11B2 enzyme expression and aldosterone production (Uruno *et al*, 2011). However, the exact molecular mechanism still needs to be fully elucidated.

Metformin is the first-line therapy in the treatment of type 2 diabetes mellitus, acting as insulin-sensitizer and glucose-lowering. In addition, it has been demonstrated to reduce tumor incidence in diabetic patients (Evans *et al*, 2005; Decensi *et al*, 2010; Monami *et al*, 2011; Johnson & Bowker, 2011) and ongoing clinical trials are currently testing metformin efficacy in cancer prevention and therapy (Pollak, 2013; Quinn *et al*, 2013; https://clinicaltrials.gov). Poli *et al.*

investigated the role of metformin in ACC treatment, showing a significant effect in reducing cell proliferation both *in vitro* and *in vivo*, mediated by metformin inhibition of the IGF2/IGF-1R signaling and a concomitant activation of the intrinsic apoptosis pathway (Poli *et al*, 2016). The global effect likely impairs mitochondria and cancer cell metabolism, as also confirmed in a more recent study analyzing the transcriptome and metabolome of ACC H295R cells treated with metformin (Udhane *et al*, 2017). Furthermore, the anti-cancer effect of metformin has been proven in a clinical case of metastatic and chemoresistent ACC (Brown *et al*, 2017), thus providing new evidence for using this drug as a potential therapeutic adjuvant in adrenocortical carcinoma treatment.

The identification of novel biomarkers would help in developing more effective therapeutic strategies for adrenocortical carcinoma. Few studies have analyzed the proteomic profile of ACC (Yang *et al*, 2013; Kjellin *et al*, 2014; Poli *et al*, 2015), identifying protein patterns differentially expressed in ACC compared to ACA and normal adrenal tissues. Calreticulin, a Ca^{2+} binding protein involved in processes such as cell adhesion, autoimmunity and heat shock, has been identified as overexpressed in ACC compared to ACA and shows significant correlation with tumor stage (Yang *et al*, 2013). Other proteins identified as differentially expressed in ACC mostly associate with mitochondrial function and glucose metabolism (Kjellin *et al*, 2014; Poli *et al*, 2015), suggesting that a shift toward aerobic glycolysis occurs also in adrenocortical carcinoma and tahat mithocondria play a crucial role in adrenal tumorigenesis. Moreover, also proteins involved in cytoskeletal organization and cell migration, such as adenylyl cyclase-associated protein 1 (CAP-1) and Fascin-1 have been found overexpressed in ACC (Poli *et al*, 2015). Taken together, these findings could drive the development of novel specific ACC therapies.

2.7. Experimental models of ACC

Developing new specific and effective treatment for adrenocortical carcinoma still remains challenging, not only for the lack of well-defined molecular targets, but also because just few cell lines are currently available and very often novel therapeutic strategies showing promising results *in vitro* fail when translated into

the clinics. This is due to the patient heterogeneity and the specific therapeutic response of individual tumors on one hand, and to the acquisition of genomic and phenotypic alterations during long-term cell culture expansion on the other, thus resulting in a significant variability between different laboratories, particularly when these same cells are used to generate xenograft models (Hantel *et al*, 2016). However, getting proper preclinical models is crucial for the screening of new drugs.

One of the first established cell model of ACC is represented by the SW-13 adrenocortical cell, that were derived from a small cell carcinoma in the adrenal cortex (Leibovitz *et al*, 1973). Nevertheless, they do not show any steroid production and the adrenocortical origin of the primary tumor has been repeatedly questioned.

The most widely used human ACC cell line is the NCI-H295R: it has been established from an invasive primary adrenocortical carcinoma and further characterized for adrenal steroid production and tumorigenicity in nude mice (Gazdar *et al*, 1990; Logié *et al*, 2000), showing the ability to reproduce the morphological features of the original patient tumor. NCI-H295R xenografts provided a good model for the dysregulated IGF2/IGF1R pathway typically observed in ACC (Barlaskar *et al*, 2009; Hantel *et al*, 2012). They have been also used after β-catenin inactivation using a doxycyclin (Dox) inducible shRNA plasmid to study *in vivo* the role of Wnt/ β-catenin signaling in adrenocortical tumorigenesis and progression (Gaujoux *et al*, 2013), as well as for preclinical evaluation of therapy regimens (Doghman & Lalli, 2013; Hantel *et al*, 2014) and for the development of new therapeutic strategies (Gaujoux *et al*, 2013; Hantel *et al*, 2012; Szabó *et al*, 2014). More recently, a mouse xenograft model of metastatic adrenocortical carcinoma has been established: SF-1-inducible H295R cells tagged with both firefly luciferase and GFP were generated to perform intrasplenic injection in mice followed by splenectomy, thus inducing metastases traceable by chemoluminescence (Morin *et al*, 2017).

The selection taking place during the multiple *in vitro* passages grossly changes the cancer cell biological properties. Thereby, the research in this field is now focusing on the development of patient-derived tumor xenograft (PDTXs), that could allow to reproduce the patient's specific tumor features, actually reflecting ACC heterogeneity (Hantel *et al*, 2016). Accordingly, a model of adult ACC (both

xenograft and cell line) has been recently obtained from a PDTX referred as MUC-1 (Hantel *et al*, 2016). Also, a pediatric tumor model of ACC (SJ-ACC3) has been established by implanting the tumor tissue of a 11-years old patient into immuno-incompetent mice, eventually obtaining a reproducible xenograft model showing the patho-morphological features of the original patient tumor (Pinto *et al*, 2013).

Interestingly, a transgenic mouse model of metastatic adrenocortical cancer has been recently generated through the specific adrenocortical expression of SV40 large T-antigen, which selectively inactivates p53 in the adrenocortex, thus resulting in a spontaneous development of metastatic adrenal tumor after 8 months (Batisse-Leigner *et al*, 2017).

3. Beyond the tumor mass: the role of tumor microenvironment in sustaining tumor progression

A growing tumor mass can be considered as a dynamic "pseudoorgan" consisting of heterogeneous cancer and stromal cell populations, among which an active molecular and metabolic interaction is continuously established, thus contributing to favor the adaptation of cancer cells to different challenging conditions linked to tumor growth, such as physical pressure, oxidative stress, changes in nutrient and oxygen availability, immune surveillance. The features and behavior of the tumor surrounding environment significantly contribute to disease progression, allowing cancer cells for reprogramming their metabolism, dynamically adapting to a variable and somewhat hostile microenvironment and eventually acquiring the ability to successfully metastasize to distant sites. This is a complex process that primarily depends on tumor heterogeneity, which is due on both unique combinations of genetic and epigenetics alterations within the tumor cell populations, and on the ability of cancer cells to engage different metabolic strategies to overcome different tricky environmental conditions. These alterations allow cancer cells to acquire self-sufficiency in growth signals and a limitless replicative potential on one hand, and to become resistant to antigrowth signals and to programmed cell death on the other one, meanwhile developing the ability to induce angiogenesis and eventually invade the surrounding tissues to further metastasize. The so called "oncometabolism" has now entered among the defining hallmarks of cancer (Hanahan & Weinberg, 2011; Fouad & Aanei, 2017): tumor cell metabolic flexibility underlies the balance between energy-producing and energy-consuming processes to manage the need for essential nutrients (Pavlova & Thompson, 2016). Glucose represents a key fuel source and its uptake is usually increased in cancer cells: it is preferentially metabolized through glycolysis, that can be both anaerobic, when the molecular oxygen is limiting (for example, in the core of hypoxic areas of the tumor mass distant from the peripheral blood vessels) and aerobic, during the establishment of the "Warburg effect" (Warburg *et al*, 1927), which allows to rapidly produce ATP and to divert the glycolytic intermediates into various biosynthetic pathways, thus sustaining cell proliferation. Glutamine metabolism also supplies essential substrates for cancer growth, providing both the carbon intermediates for the assembly of macromolecules and the reduced nitrogen

for the *de novo* biosynthesis of nitrogen-containing compounds, including nucleotides, glucosamine-6-phosphate and essential amino acids. Mitochondria-dependent oxidative phosphorylation (OXPHOS) is also used to produce ATP starting from glucose, fatty acids or glutamine and serves alongside glycolysis to fulfill the high anabolic demand of tumor cells (Obre & Rossignol, 2015). Moreover, tumor cells can utilize alternative opportunistic modes to acquire nutrients from the environment, such as macropinocytosis of extracellular proteins, engulfment and digestion of living cells ("entosis") and phagocytosis of apoptotic products (Pavlova & Thompson, 2016). Such distinct metabolic strategies can vary depending on specific surrounding conditions but also on tumor type, referring to both tissue origin and cancer subtypes (Elia *et al*, 2016). In addition, the specific supportive tumor microenvironment is emerging as a crucial driver of cancer progression: stromal cells are recruited through paracrine stimuli produced by cancer cells and are induced to alter their metabolism to in turn fuel cancer growth. Thus, a system of dynamic reciprocal interactions between tumor and its TME is constantly established.

3.1. The tumor microenvironment: a metabolic cooperation and competition in a complex cell and molecular network

In solid cancers, the tumor microenvironment (TME) constitutes a complex network of cells and soluble factors and comprises two main components, that are present in variable proportion depending on tumor location and stage: a cell component, including fibroblasts, myofibroblasts, mesenchymal stem cells (MSCs), adipocytes, pericytes, endothelial cells and cells of haematopoietic origin, both lymphoid (T cells, B cells, natural killer cells) and myeloid cells (macrophages, neutrophils and myeloid-derived suppressor cells); and the extracellular matrix (ECM), composed of collagens, glycoproteins and proteoglycans, scaffolding and maintaining the tissue architecture. The neovasculature developing within the tumor bulk is also part of the TME structure (Fig. 16). However, it is larger in size e less organized compared to its normal counterpart and fails to deeply penetrate the tumor, thus resulting in a progressive deprivation of oxygen and energy precursors from the periphery toward the core of the mass. The resultant hypoxia and

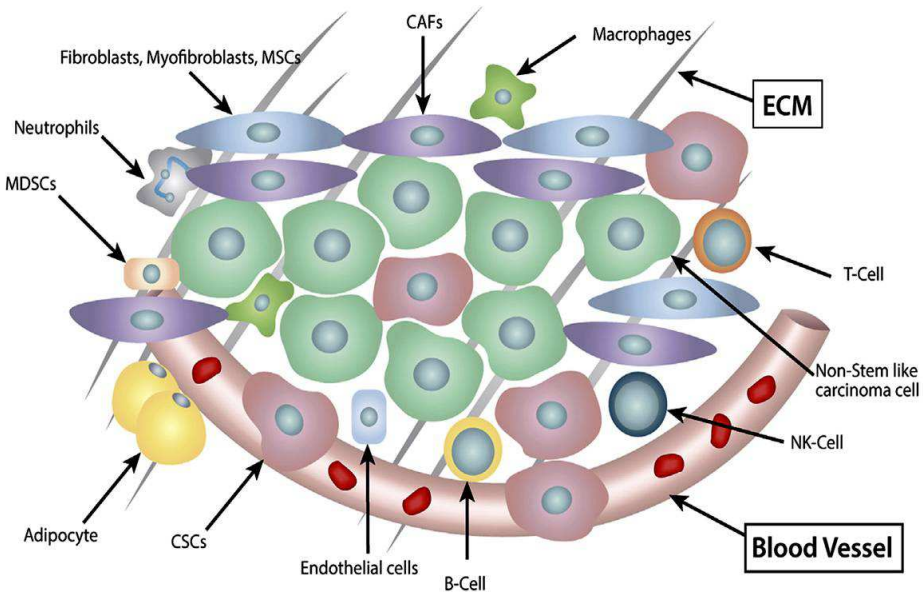


Figure 16. Schematic representation of the tumor microenvironment components. MSCs: mesenchymal stem cells; CAFs: cancer-associated fibroblasts; MDSCs: myeloid-derived suppressor cells; NK: natural killer cells; CSCs: cancer stem cells; ECM: extracellular matrix. Adapted from McCuaig *et al*, 2017.

nutritional stress in turn drive a metabolic remodeling in both cancer and neighbor host cells, thus creating a pro-tumorigenic TME, mainly characterized by the lowering of the extracellular pH due to H+ and lactate released in the extracellular compartment (Chiche *et al*, 2010; Peppicelli *et al*, 2017). In fact, hypoxic cancer cells metabolizing glucose through anaerobic glycolysis produce and secrete lactate, which is thereby available for well-oxygenated cancer cells, fueling their mitochondrial metabolism. This system is regulated by the expression of appropriate intake/release transporters, particularly the lactate exporter monocarboxylate transporter 4 (MCT4), whose expression is increased in hypoxic tumor regions, and the importer MCT1, up-regulated in normoxic cells (Sonveaux *et al*, 2008). This kind of symbiotic metabolism is also established between cancer and surrounding cells (Gupta *et al*, 2017), making lactate one of the main soluble factors acting within this crosstalk (Romero-Garcia *et al*, 2016): according to the Warburg effect, cancer cells produce and secrete large amounts of lactate in the extracellular microenvironment, where it can be picked up by cancer-associated fibroblasts (CAFs) and MSCs and used as energy source (Koukourakis *et al*, 2006). The opposite process has also been described (Pavlides *et al*, 2009): tumor cells induce CAFs to divert their metabolism toward aerobic glycolysis (namely the "reverse Warburg effect"), thereby secreting pyruvate and lactate, which in turn can be consumed by

cancer cells through Krebs cycle and OXPHOS (Chiarugi & Cirri, 2016). The presence of high amounts of lactate in the microenvironment results in lowering the extracellular pH to 6.0–6.5, thus contributing to acidosis and stimulating malignant progression of cancer cells: acidosis, in fact, induces the activation of matrix metalloproteinases (MMPs), a family of endopeptidases capable of degrading and remodeling the ECM, aiding cell migration and invasion (Kato *et al*, 2007). In addition, lactic acidosis stimulates angiogenesis through the activation of the VEGF/VEGFR2 signaling pathway and of endothelial cells, thus promoting tube formation (Xu *et al*, 2002; Hunt *et al*, 2007; Vegran *et al*, 2011; Porporato *et al*, 2012). The increased lactic acid production, together with the limited availability of nutrients and hypoxia, contributes to the metabolic competition in the TME, which particularly affects the tumor-infiltrating immune cell antitumor function, resulting in immune suppression: T cell proliferation, differentiation and activation is inhibited (Dang *et al*, 2011; Wang *et al*, 2014; Chang *et al*, 2015; Ho *et al*, 2015; Brand *et al*, 2016) and tumor-associated macrophages (TAMs) are polarized toward the pro-tumorigenic M2 phenotype (Colegio *et al*, 2014; Laoui *et al*, 2014).

In addition to lactate transferring, cancer cells are also able to get other essential nutrients from the microenvironment (Lyssiotis & Kimmelman, 2017 **in press**), including glutamine, one of the most utilized amino acid: a phenomenon termed "glutamine addiction" has been described for cancer cells, which decrease glutamine synthesis whereas increase the up-take and catabolism of glutamine produced by stromal cells, mainly CAFs and adipocytes (Wise & Thompson, 2010; Ko *et al*, 2011; Lyssiotis *et al*, 2013; Son *et al*, 2013; Meyer *et al*, 2016; Yang *et al*, 2016). Globally, the amino acid metabolism is altered in the TME, as observed for alanin, tryptophan, arginin and cysteine (Platten *et al*, 2012; Zhang *et al*, 2012; Salimian Rizi *et al*, 2015; Sousa *et al*, 2016).

Highly aggressive cancer cells have been shown to have a characteristic pattern of lipid deposition, which associates with the presence of several alterations in lipid- and cholesterol-associated pathways (Beloribi-Djefaflia *et al*, 2016): they can either increase the up-take of exogenous lipids and lipoproteins or up-regulate their endogenous biosynthesis. Moreover, an increased exogenous fatty acid uptake and consumption through the fatty acid β-oxidation is also observed, strongly contributing to survival and metastatic spreading of cancer cells. Lipid molecules, in fact, not only represent a carbon sink, but are also energy-rich molecules that can

support cancer growth in a nutrition deprived environment. Within the TME setting lipid can be provided as free fatty acids by adipocytes as a result of a metabolic reprogramming: in fact, cancer-associated adipocytes are prompted to up-regulate lipolysis and release fatty acids, that can be used by cancer cells for both energy production and lipid biosynthesis (Romero *et al*, 2015). Concomitantly, this process assures to maintain the terminal electron chain and ATP production in the hypoxic TME, as well as to sustain the glycolytic flux thanks to $NADP^+$ generation (Liu *et al*, 2010; Santos & Schulze, 2012).

In addition to the basic molecular building block, non-cancer cells are able to release, mainly through the activation of autophagy mechanisms, larger molecules such as dipeptides (Chaudhri *et al*, 2013) and exosomes, small exocytotic vesicles containing nutrient cargo, including amino acids, fatty acids and tricarboxylic acid cycle metabolites (Zhao *et al*, 2016; Achreja *et al*, 2017), that can be used by cancer cells to address their biosynthetic and bioenergetic needs. A similar symbiotic process has also been described for entire organelles, particularly the transfer of mitochondria and/or mitochondrial DNA from the stromal to the cancer cells, (Spees *et al*, 2006; Tan *et al*, 2015; Moschoi *et al*, 2016).

3.2. Focusing on the adipose tumor microenvironment: cancer-associated adipocytes and adipose stem cells

In recent years, the significant role of adipocytes and adipose progenitors as active players in the tumor microenvironment has been demonstrated for many types of solid and hematological malignancies: far from being just passive bystanders, adipose cells secrete various factors that can mediate both local and systemic effects, with particular implication for tumor initiation and growth, as well as for local invasion and metastasis. This is due to the establishment of a dynamic adipocyte-cancer cell crosstalk that leads to phenotypical and functional changes of both cell types, further enhancing tumor progression (Duong *et al*, 2017).

Adipose tissue represents one of the main component of the human body and, depending on its biological functions, it can be classified into two main types: the white adipose tissue (WAT), localized subcutaneously and surrounding visceral organs, which is specialized in storing energy and constitutes an important

endocrine organ, regulating insulin sensitivity and lipid metabolism; and the brown adipose tissue (BAT), which can be found in paracervical and supraclavicular regions and mainly regulates thermogenesis in response to food intake and cold (Cypess *et al*, 2009; Virtanen *et al*, 2009). This two types of adipose tissue also differ for their morphological features (Fig. 17): WAT has a highly complex cellular composition, consisting of mature adipocytes and stroma-vascular cells, including pre-adipocytes, fibroblasts, pericytes, endothelial cells and immune cells (Eto *et al*, 2009). Due to their size, adipocytes are considered as the major component of WAT and are characterized by the presence of a unique large intracellular lipid droplet that constitutes the primary site of energy storage. On the other hand, BAT is composed of cells with multiple lipid droplets, rich in mitochondria and located in highly innervated and vascularized depots. BAT adipocytes are specialized in heat generation by thermogenesis and lipid oxidation and specifically express the mitochondrial Uncoupling Protein 1 (UCP1) (Cristancho & Lazar, 2011; Cinti, 2012; Sanchez-Gurmaches & Guertin, 2014). A third type of adipocytes have been recently described, namely brite or beige, which predominantly reside in the subcutaneous WAT in the mouse, but share some common features with brown adipocytes, such as the expression of UCP-1, the multilocular morphology, increasing in number after exposure to cold or exercise or (Harms & Seale, 2013; Lee *et al*, 2014). Finally, several « atypical » adipose tissue depots exist, including the one which can be found in the bone marrow: its adipocytes possess unique profiles ascribable to beige or brown-like adipocytes (Naveiras *et al*, 2009; Scheller *et al*, 2015).

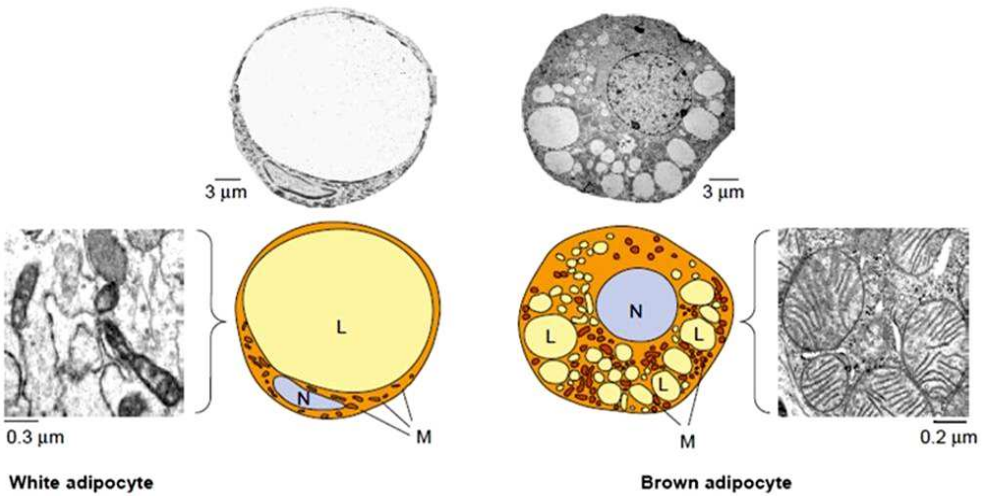


Figure 17. Schematic representation of the differential morphology of white and brown adipocytes. L: lipids; N: nucleus; M: mitochondria. Adapted from Villarroya *et al*, 2005.

Reciprocal transformation between the different types of adipose tissue in response to a variety of metabolic and environmental challenges have been described (Giordano *et al*, 2014). Therefore, adipose tissue constitutes a dynamic organ with a great capacity to adapt its extent depending on the energy status of the organism and in response to different pathophysiological conditions (Pellegrinelli *et al*, 2016). Besides its basal function of energy storing, WAT works as an endocrine organ, secreting hormones, growth factors, chemokines, pro-inflammatory molecules and adipokines, that play a critical role in modulating tumor microenvironment: in fact, they mediate many biological processes through both local and distant effect that can contribute to tumor growth, differentiation and progression. Due to the adipose tissue distribution in different organs, adipocytes can often be found in close contact with cancer cells in many tumor types, such as breast, colon, renal, prostate and ovarian cancer among others, and such a neighborhood commonly associates with a more aggressive tumor behavior (Finley *et al*, 2009; Nieman *et al*, 2011; Wang *et al*, 2012; Zhang *et al*, 2016). Increasing evidence, in fact, confirms the establishment of an active crosstalk between adipose tissue and cancer cells: the adipocytes closer to malignant cells undergo profound phenotypic and functional alterations, such as a decrease in cell number and size compared to those distant from the tumor mass. Moreover, at the tumor center, an increased ratio of fibroblast-like cells can be observed, thus suggesting a process of trans- or de-differentiation of adipocytes induced by cancer cells (Dirat *et al*, 2011; Tan *et al*, 2011). Consistently, adipocytes co-cultured *in vitro* with cancer cells show delipidation and loss of adipose specific markers such as adiponectin, FABP4 and hormone-sensitive lipase (HSL), but they have also been shown to acquire some CAF-associated markers (Bochet *et al*, 2013). In addition, alterations of adipocyte secretome have been described, notably the up-regulation of osteopontin, matrix metalloproteinase 11 and inflammatory cytokines, such as TNF-alpha, IL-6 and IL-1β (Ribeiro *et al*, 2012). Conversely, the secreting adipokines, as well as the released fatty acids, are transferred to cancer cells, where they are able to stimulate adhesion, migration, and invasion and to sustain energy production, respectively (Nieman *et al*, 2013). Finally, this reciprocal crosstalk results in the local production of specific chemokines that can influence immune and inflammatory responses. In particular, immune-tolerant macrophages displaying anti-inflammatory responses are

recruited, thus contributing to a more favorable microenvironment for tumor growth (Sturtz *et al*, 2014).

The characteristic plasticity of the adipose tissue is due to the presence of adipose-derived stem cells (ASCs) within the stromal vascular fraction, which constitute the multipotent progenitor pool of adipose tissue, retaining the stem potential to differentiate toward different cell lineages, such as adipogenic, osteogenic, condrogenic, myogenic, cardiomyogenic and neurogenic-like cell types (Baglioni *et al*, 2009; Gwak *et al*, 2009; Zayan *et al*, 2010). Besides their central role in maintaining tissue homeostasis and providing the reservoir of regenerative cells, ASCs also influence the tumor microenvironment through the expression and secretion of growth factors, cytokines, chemokines and inflammatory factors, thus contributing to preserve cancer hallmarks and promoting cancer progression (Ilmer *et al*, 2014; Freese *et al*, 2015).

3.2.1. *The role of mature adipocytes in cancer progression*

Several *in vitro* and *in vivo* studies indicate that, in the TME setting, adipocytes are modified by cancer cells and acquire metabolic and functional characteristics distinct from those of their naïve counterpart. The so called cancer-associated adipocytes (CAAs) are then able to support and promote tumor growth, as shown in the case of different tumor types, including breast (Elliott *et al*, 1992; Manabe *et al*, 2003), ovarian (Nieman *et al*, 2011), prostate (Tokuda *et al*, 2003; Moreira *et al*, 2015), colon (Amemori *et al*, 2007) cancer and melanoma (Kwan *et al*, 2014). This interaction is mainly mediated by the production and release of different factors by CAAs (Fig. 18). They mainly consist of fatty acids and adipokines, the latter ones constituting a class of pleiotropic molecules, such as leptin, adiponectin, insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF), implicated in many physiological or pathological processes, including cell survival and proliferation, as well as cancer invasion and metastasis.

- **CAAs support tumor growth**

Several models of crosstalk between adipocyte and cancer cells have largely reported a tumor growth-promoting effect, which seems to mainly be mediated by

the interaction of adipokines with their receptors expressed by tumor cells. **Leptin** is a peptide hormone that, besides its neuroendocrine function in controlling food intake, can impact a wide range of biological processes, including angiogenesis, bone formation and modulation of immune response. Evidence supports the potential role for this adipocyte-secreted factor in tumorigenesis: in fact, its receptor (Ob-R) is found highly expressed in many cancers, and several *in vitro* studies showed that leptin can induce cancer cell proliferation via activation of ERK1/2 and JNK pathways (Garofalo & Surmacz, 2006). On the other hand, it has been observed that the production of **adiponectin,** another major adipokine involved in the regulation of glucose levels and fatty acid oxidation, is reduced in CAAs, thus also contributing to cancer cell proliferation. In fact, adiponectin acts by mediating antiangiogenic and tumor growth-limiting effects that are opposite to those of leptin, such as activation of AMPK, inhibition of PI3K/Akt and ERK1/2 pathways, down-regulation of leptin-induced STAT3 phosphorylation, inhibition of NF-kB and Wnt/β-catenin signaling and decrease of ROS production (Dalamaga *et al*, 2012). Thus, the leptin:adiponectin ratio seems to be the major factor influencing tumor growth. Adipocytes also produce growth factors, particularly **IGF-1** and **HGF,** which are able to promote cell growth and survival through the binding to their specific receptors expressed by tumor cells. The IGF1/IGF-1R interaction, which activates the growth-inducing PI3K/Akt and ERK pathways, has been shown to regulate breast cancer cell growth (D'Esposito *et al*, 2012), whereas HGF seems to mediate a paracrine interaction between adipocytes and tumor cells expressing the c-Met receptor, thus promoting tumor progression, as observed in breast and ovarian cancer (Rahimi *et al*, 1994; Takayama *et al*, 1997; Edakuni *et al*, 2001; Wong *et al*, 2001).

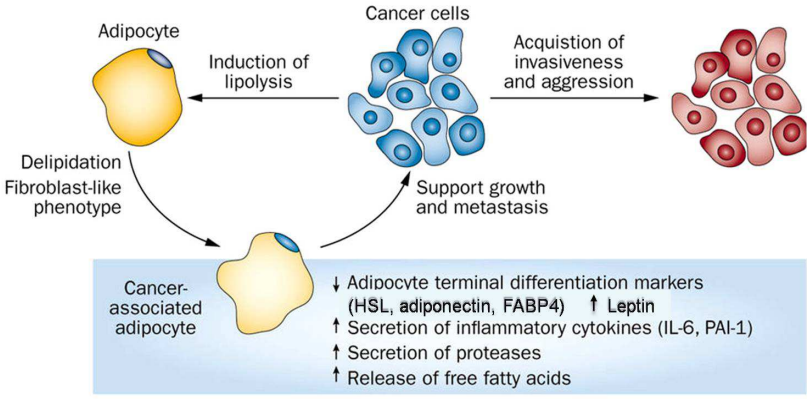


Figure 18. Schematic representation of the crosstalk and the reciprocal functional influence between adipocytes and cancer cells within the TME. Adapted from Park *et al*, 2014.

As already mentioned, cancer cells are capable to induce a metabolic shift from oxidative phosphorylation to glycolysis in cancer-associated fibroblast as well as in the other cells of the tumor stroma, resulting in the production of lactate and pyruvate, factors that sustain cancer metabolism and proliferation. This process may represent one of the mechanisms by which adipocytes are able to fuel cancer growth, since they have been shown to release lactate through the monocarboxylate transporters, particularly under hypoxic conditions (Pérez de Heredia *et al*, 2010). However, lipids seem to be the main source of energy provided by adipocytes to cancer cells: as described in different types of solid cancers, CAAs appear smaller in size and with a decreased lipid content, thus suggesting that a lipolytic process is induced by cancer cells, finally resulting in adipocyte delipidation and release of free-fatty acids (FFAs), which can be in turn metabolized by tumor cells through β-oxydation (Dirat *et al*, 2011; Nieman *et al*, 2011, 2013; Park *et al*, 2014; Balaban *et al*, 2017; Wang *et al*, 2017; Wen *et al*, 2017). The existence of such an active exchange of FFAs between tumor cells and CAAs is supported by the observation of an increased level of free acid-binding proteins (FABPs) in several cancers, including breast, prostate, ovarian and colorectal carcinomas (Guaita-Esteruelas *et al*, 2017 **in press**).

The dynamism of modifications and interactions taking place within the tumor microenvironment requires a steady supply of oxygen and nutrients to assure tumor growth and expansion. Angiogenesis is a crucial process for supplying oxygen and nutrient delivery to the tumor mass. This process is tightly regulated by both tumor and stromal cells, including adipocytes: they can actively participate in angiogenic modulation through the direct production of the classical angiogenic factors (i.e. VEGFA) (Mick *et al*, 2002) or the release of leptin, which has been shown to directly promote proliferation and differentiation of endothelial cells and to up-regulate VEGF in cancer cells (Gonzalez-Perez *et al*, 2010, 2013).

- **CAAs promote cancer invasion and metastasis**

Besides their role in controlling tumor cell proliferation and survival, growth factors and cytokines released within the TME strongly contributes to tumor cell migration and invasion, orchestrating the complex cascade of events underlying dissemination and metastasization (Odenthal *et al*, 2016). In this setting, adipocytes seem to play a crucial role, contributing to the remodeling of extracellular matrix and promotion of

tumor homing. Several *in vitro* models showed that the presence of CAAs is able to increase the invasive ability of cancer cells in different types of solid tumors, and this is mostly mediated by the secretion of soluble factors (Dirat *et al*, 2011; Tan *et al*, 2011; Ribeiro *et al*, 2012; Bochet *et al*, 2013; Moreira *et al*, 2015). The most abundantly adipocyte-secreted cytokines are represented by interleukin 6 (IL-6), IL-8 and monocyte chemotactic protein-1 (MCP-1): they are produced at high levels by CAAs and determine the establishment of a chronic inflammatory state in the tumor-surrounding adipose tissue, which promotes tumor dissemination, as well as cancer cell homing and seeding at distant organs (Finley *et al*, 2009; Dirat *et al*, 2011; Nieman *et al*, 2011; Pramanik *et al*, 2013; Laurent *et al*, 2016). In addition, leptin secretion also seems to contribute to cancer cell migration and invasion via IL-18 expression and secretion (Li *et al*, 2016) and to affects both innate and adaptive immunity, thus facilitating the immunomodulatory mechanisms allowing tumor escape (Delort *et al*, 2015). Furthermore, fatty acid transfer between CAAs and cancer cells via exosome secretion has been shown to further sustain migration and invasion (Lazar *et al*, 2016).

Besides adipokines and growth factors, adipocytes also produce some of the components of the ECM, which undergoes significant structural and molecular remodeling during tumor progression, driving cell migration. In particular, CAAs have been shown to up-regulate their secretion of collagen VI under cancer cell induction (Iyengar *et al*, 2005) and to produce a number of MMPs, predominantly MMP-11 (Chavey *et al*, 2003), which is actively involved in cancer migration and invasion (Zheng *et al*, 2016). MMp-11 also seems to directly affect adipocyte functions by negatively regulating adipogenesis and enhancing de-differentiation, thus leading to the accumulation of fibroblast-like cells in the tumor microenvironment (Andarawewa *et al*, 2005).

3.2.2. The role of ASCs in cancer development, growth and progression

As discussed above, adipose tissue is a rich source of ASCs, which are part of the ubiquitously distributed mesenchymal stem cells (MSCs), constituting the pool of stem and progenitor cells responsible for tissue homeostasis, repair and regeneration. To exert such a function, MSCs are recruited by the local

microenvironment, being able to migrate along chemoattractant gradients (Ilmer *et al*, 2014). As already discussed, in the tumor microenvironment an exchange of soluble factors is dynamically established, thus facilitating the recruitment of immunomodulatory components and stromal cells of various origin, contributing to some of the hallmarks of cancer, such as angiogenesis, invasion and metastasis, and apoptosis resistance. There is increasing evidence that ASCs might be crucial in supporting cancer development and progression, influencing and being influenced by the tumor microenvironment (Freese *et al*, 2015). Many studies, in fact, showed that ASCs recruited to the TME can differentiate toward different cell types, such as endothelial cells, vascular complexes and tumor stroma, facilitating cancer cell growth (Muehlberg *et al*, 2009; Kucerova *et al*, 2011; Chandler *et al*, 2011, 2012; Nowicka *et al*, 2013). CXCL12 (also known as stromal-derived factor-1 - SDF-1) and its receptor CXC Receptor-4 (CXCR4) axis might be involved in driving ASC migration and incorporation into the TME. As recently shown, high levels of CXCL12 in the TME can attract CXCR4-positive immune and stromal cells to the tumor site to assist tumor development (De Palma *et al*, 2005; Orimo *et al*, 2005; Jin *et al*, 2006; Du *et al*, 2008; Beider *et al*, 2014). The other way round, CXCL12 secreted by ASCs in the TME strongly promotes tumor proliferation, migration and invasion through multiple signaling pathways triggered by the interaction with CXCR4 expressed by cancer cells (Zhao *et al*, 2010; Ji *et al*, 2013). CXCL12 is a homeostatic chemokine normally controlling hematopoietic cell trafficking, adhesion, immune surveillance and development through the interaction with its cognate receptor CXCR4, a member of the seven-transmembrane domain G-protein–coupled receptor (GPCR) superfamily. Both are overexpressed in various cancer types and their interaction leads to the activation of intracellular pathways regulating cell survival, proliferation and chemotaxis, such as MAPK, PI3K/AKT, NF-kB (Guo *et al*, 2016) (Fig. 19). In addition, such interaction has been correlated with angiogenesis and EMT induction, promoting cancer metastasis (Guo *et al*, 2016). Moreover, CXCL12 is physiologically expressed by mesenchymal stroma niches in different organs, including liver, lung and bone marrow, where CXCR4-positive cancer cells can be recruited to initiate metastasis (Guo *et al*, 2016). Recently it has been shown that CXCL12 can also bind CXCR7 with high affinity (Ghanem *et al*, 2014), thus mediating different potential effects than CXCR4: CXCR7 overexpression has been reported in different cancer types (Hattermann *et al*, 2010; Deutsh *et al*, 2013; Hu *et*

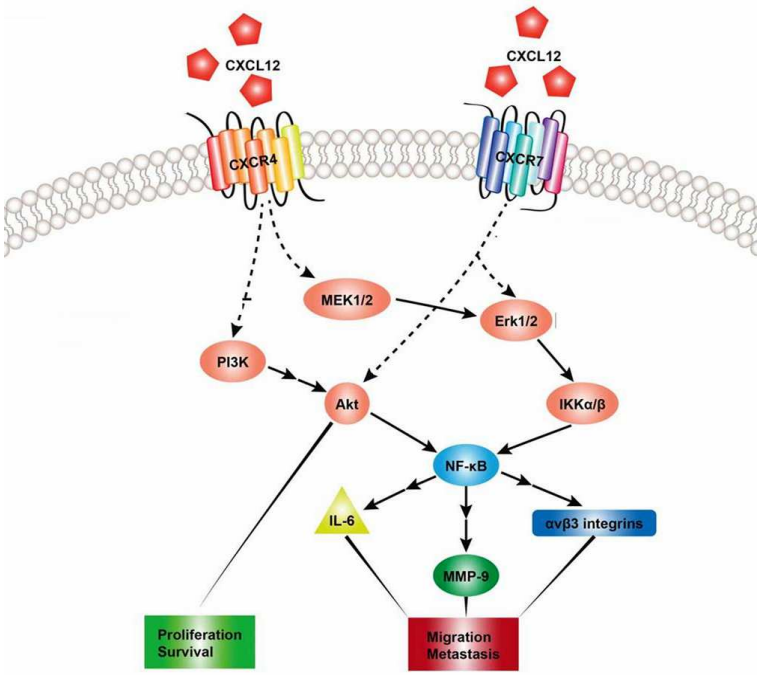


Figure 19. Schematic representation of the downstream pathways regulated by CXCL12/CXCR4/CXCR7 axis. Adapted from Liao *et al*, 2013.

al, 2014; Lin *et al*, 2014; Wu *et al*, 2015; Shi *et al*, 2017), promoting cancer development and progression through the activation of AKT, MAPK and JAK2/STAT3 signaling. Furthermore, CXCR7 has been associated with the activation of genes related to lipid metabolic pathways and chemoresistance (Hekmann *et al*, 2014).

Once recruited to the tumor, ASCs are able to integrate into the stroma undergoing a transition into CAF- or myofibroblast-like cells, which associates with the expression of specific markers like tenascin-C and a-SMA, as demonstrated in different cancer types (Jeon *et al*, 2010; Jotzu *et al*, 2010; Cho *et al*, 2011, 2012; Do *et al*, 2012; Park *et al*, 2013). Therefore, activated ASCs may support the altered tumor metabolism in the contest of the establishment of the reverse Warburg effect. In addition, the role of ASCs in the TME has been linked to increased angiogenesis and cancer invasion: particularly in hypoxic conditions, ASCs have been shown to promote angiogenesis by secreting VEGF, thus sustaining tumor growth (Rasmussen *et al*, 2011). Moreover, ASC secretion of different factors, such as platelet-derived growth factor (PDGF), CCL5, transforming growth factor (TGF)-β IL-6, IL-8, has been associated with increased ECM remodeling, EMT induction in cancer cells and increased cancer migration, invasion and anchorage-independent

growth (Pinilla *et al*, 2009; Walter *et al*, 2009; Devarajan *et al*, 2012; Welte *et al*, 2012; Scherzed *et al*, 2013; Reggiani *et al*, 2017).

Finally, ASCs seem to be crucial in regulating the immunomodulatory mechanisms allowing tumor cells for thwart immune surveillance: they inhibit natural killer (NK) cell activation and suppress B-cell functions (Bochev *et al*, 2008) and secrete various cytokines, including IL-4, IL-10, IL-8, TGF-β, which contribute to regulate T cell activity and to create a tumor microenvironment characterized by specific modulation of inflammation, thus promoting tumorigenic activity (Razmkhah *et al*, 2010).

4. Tumor progression and metastatic colonization: circulating tumor cells as biomarkers of tumor evolution

The main cause of cancer-related death in patients is represented by metastasis: cells from primary tumors acquire the ability to disseminate through the blood circulation and to colonize distant organs, giving rise to a systemic disease and making treatments ineffective in most of the cases. Differently from what originally hypothesized, the acquisition of metastatic traits is an early event in the evolutionary story of a tumor, since invasive and motile cells can enter the circulation and seed distant organs long before a tumor is diagnosed, and the presence of metastatic disease at diagnosis almost always associates with poor prognosis, as well as development of chemoresistance. Current evidence suggests that metastasis-initiating cells are pre-selected within the primary tumor through genetic and, predominantly, epigenetic changes, undergoing further selection under the stress pressure related to tissue invasion, immune surveillance or hypoxia. This is consistent with the observation that metastasis share some genomic traits compared to their primary tumor and support the hypothesis that a clonal expansion in the primary tumor itself gives rise to metastasis-competent cells, that continue to evolve in parallel during their dissemination to distant organs (Klein, 2009; Greaves & Maley, 2012; Vanharanta & Massagué, 2013; Gundem *et al*, 2015; Naxerova & Jain, 2015).

Experimental and clinical observations indicate that metastatic colonization is a highly inefficient process, in which only a minority of the cells shed by the tumors can survive and form macrometastases. This is due to multiple obstacles that circulating tumor cells (CTCs) have to face before reach and seed a distant site, including escaping their primary microenvironment and invading the surrounding tissue, successfully entering the circulation, adapting to a new microenvironment and surviving in a latent dormancy state, to eventually break out as an overt metastasis (Fig. 20) (Massagué & Obenauf, 2016). Therefore, the metastatic process follows multiple steps and shows a number of bottlenecks driving the fate of the most aggressive cells: in the early phases, cytoskeletal rearrangements, combined with ECM remodeling and interactions with the supportive tumor niche, allow cancer cells to invade and migrate through the stroma. In response to various signaling factors released by the surrounding stromal cells, cancer cells can undergo

epithelial-to-mesenchymal transition (EMT): this is a reversible phenotypic change characterized by the loss of intracellular adhesion and epithelial polarization, associated with gained cell motility and invasiveness. Such a process can promote cancer cell intravasation and acquisition of stem-like traits, as well as extravasation and organ colonization in its reverse process (mesenchymal-to-epithelial transition - MET) (Thiery *et al*, 2009; Tam & Weimberg, 2013). Once in the blood circulation, CTCs are exposed to a severe stress linked to the loss of adherence to the ECM, the mechanical and hemodynamic shear forces, as well as the innate immune system defense attack and the oxidative stress, all processes resulting in a dramatically reduction in the number of viable cells able to successfully disseminate. However, metastatic cells can carry out different strategies to protect themselves and survive into the circulation (Strilic & Offermanns, 2017): they can form microaggregates with platelets, reducing the mechanical stress and hiding from the immune surveillance; they can undergo reversible metabolic changes to withstand oxidative stress; they can acquire activating mutations in anti-apoptotic and pro-survival pathways or lose tumor suppressors. The eventual seeding to distant organs and the metastatic overgrowth need further modification and adaptation: cells successfully extravasated have to settle in a new microenvironment, where specialized supportive niches allow cancer cells (now called disseminated tumor cells - DTCs) to survive in a latent dormancy state and to retain their tumor-initiating capacity (Massagué & Obenauf, 2016).

Despite the development of several organ-specific targeted anti-metastatic therapies, their efficacy is often disappointing: tumor shrinkage is only partially

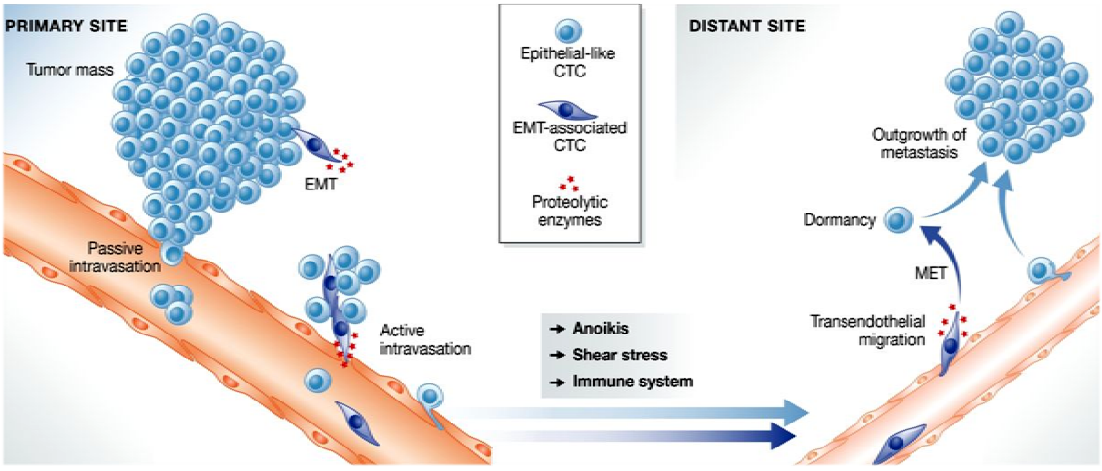


Figure 20. Schematic representation of the metastatic cascade. Adapted from Joosse *et al*, 2015.

achieved and a residual disease is left behind. This is mainly due to the capability of tumor cells to adapt their intracellular pathways under the stress of therapy, expressing a large number of secreted factors (known as therapy-induced secretome) able to protect the drug-sensitive cells and to selectively stimulate the drug-resistant minority (Holohan *et al*, 2013). Therefore, preventing metastasis rather than treat them would be more effective in high-risk patients. In this scenario, liquid biopsy represents the new frontier for cancer early diagnosis, prognosis and treatment. This is a minimally invasive procedure carried out during a standard blood draw, accessing different types of tumor material, including CTCs, cell-free DNA of tumor origin (ct-DNA), RNA and exosomes, feasible for molecular characterization. Analyzing this material may allow to profile the single patient tumor evolution, consistently with the idea of personalized medicine.

4.1. Biology, detection and clinical implications of circulating tumor cells

Circulating tumor cells can be considered as a surrogate of tumor sample that and reflect tumor evolution during progression and metastatic potential, as well as the effectiveness and progression of systemic therapies, allowing for monitoring patients during the follow up or after surgery of during treatment. Indeed, CTCs have been proposed as real-time "liquid biopsy", since they could allow to profile the disease complexity at any stage of tumor progression (Alix-Panabières & Pantel, 2013). Several new high-throughput technologies have been developed to analyze in very deep detail the functional and phenotypic traits of heterogeneous cell populations at the single-cell level. This has largely simplified the study of CTC biology, improving the knowledge about the mechanisms underlying their ability to disseminate and providing new information to make cancer treatment more relevant and patient-centric.

4.1.1. CTC Biology

As already mentioned, CTCs have to overcome a number of physiological hurdles to escape their primary site and enter the blood flow. According to the most widely recognized theory, cells undergo EMT to actively dissociate from the

primary tumor and enter the blood vessels (Pantel & Speicher, 2016), under paracrine signals coming from the surrounding microenvironment. Cytoskeletal changes, together with the loss of tight and adherens junctions, underlie the loss of cell polarity and enable epithelial cells to become more invasive and motile (Joosse *et al*, 2015). This process typically associates with the down-regulation of epithelial markers, such as EpCAM and E-cadherin, the alteration of keratin expression and the up-regulation of mesenchymal markers, such as vimentin. The concomitant up-regulation of matrix metalloproteinases (MMPs) contributes to facilitate migration through the local ECM and intravasation (Lamouille *et al*, 2014). This is consistent with the observation that CTCs with mesenchymal traits predict poor outcome and resistance to standard chemotherapy (Aktas *et al*, 2009; Yu *et al*, 2013; Krawczyk *et al*, 2014; Li *et al*, 2017). However, the role of EMT in CTCs is still unknown: on one hand, tumor cells undergoing EMT seem to be more resistant against share stress (Mitchell & King, 2013), whereas on the other one, several studies have correlated the overexpression of EpCAM to an increased risk of metastasis and reduced survival (Cimino *et al*, 2010; Spizzo *et al*, 2011) and have demonstrated the prognostic relevance of EpCAM-positive CTCs (Rack *et al*, 2014; Janni *et al*, 2016). It might be possible that these cells have some degree of co-expression of epithelial and mesenchymal markers, consistent with their plasticity (Alix-Panabières *et al*, 2017). Indeed, increasing evidence indicates that CTCs represent heterogeneous cell populations (Lapin *et al*, 2017; Lindsay *et al*, 2017). This is consistent with the fact that cancer EMT occurs through a spectrum of intermediate states, from the full transition taking place at the tumor invasive front to the complete absence in the main tumor bulk (Nieto *et al*, 2016). Thus, CTCs may derived from cells that underwent EMT in the primary tumor or may acquire intermediate EMT phenotypes in circulation, allowing for cell survival.

In addition to single CTCs, CTC clusters, namely circulating tumor microemboli (CTM), can also be found in patients' blood: they are groups of 2 to more than 50 cells able to extravasate and generally correlate with very poor clinical outcome (Hou *et al*, 2012; Aceto *et al*, 2014, 2015; Au *et al*, 2016). They are thought to have greater metastatic potential and to provide a survival advantage to single cells, since their cell junctions may help to avoid anoikis (Paoli *et al*, 2013). In addition, single CTCs or clusters can aggregate with platelets, thus facilitating extravasation and

protecting cell from mechanical stress and immune surveillance (Strilic & Offermanns, 2017). Moreover, CTCs are able to suppress T-cell proliferation and cytokine production by expressing PD-L1 (Mazel *et al*, 2015; Nicolazzo *et al*, 2016; Anantharaman *et al*, 2016).

Despite all these survival strategies, CTCs remain a rare event: they persist in the circulation for an estimated time of 1.0-2.4 hours (Meng *et al*, 2004) and can be detected in a range of 1-10 cells per millions (10^6–10^7) of surrounding normal peripheral mononuclear blood cells in the circulation of patients with advanced disease (Alix-Panabieres *et al*, 2012). Thus, isolation and characterization remain challenging, particularly in order to determine the traits underlying cell heterogeneity and to identify the specific markers of metastatic properties.

4.1.2. CTC isolation and characterization

Over the past two decades, many techniques have been developed for CTC detection and novel strategies are continuously proposed to improve isolation yield. CTC enrichment relies on two main approaches: the use of label-dependent techniques, exploiting the detection of surface marker through immunoselection; and the application of label-independent techniques based on unique mechanical and physical differences of CTCs when compared to the blood cells, including size, deformability and electrical properties (Fig. 21) (Joosse *et al*, 2015; Salvianti *et al*, 2016).

The most widely used technology is represented by the CELLSEARCH® system (Veridex, USA), which is based on the immunomagnetic capture of EpCAM-expressing cells, identifying as CTCs cells positive for nuclear DAPI (4′,6-diamidino-2-phenylindole) and cytokeratins, and negative for CD45 (lymphocyte marker). This system allows CTCs automatic count and it has been approved by the USA FDA for the use of CTC enumaration as prognostic marker in metastatic breast, colon and prostate cancers (Cristofanilli *et al*, 2005; Cohen *et al*, 2008; de Bono *et al*, 2008). Although this is the only clinically validated platform for CTC analysis, analogous EpCAM-based systems are also available (Nagrath *et al*, 2007; Talasaz *et al*, 2009; Saucedo-Zeni *et al*, 2012). However, all these methods show the intrinsic bias of the

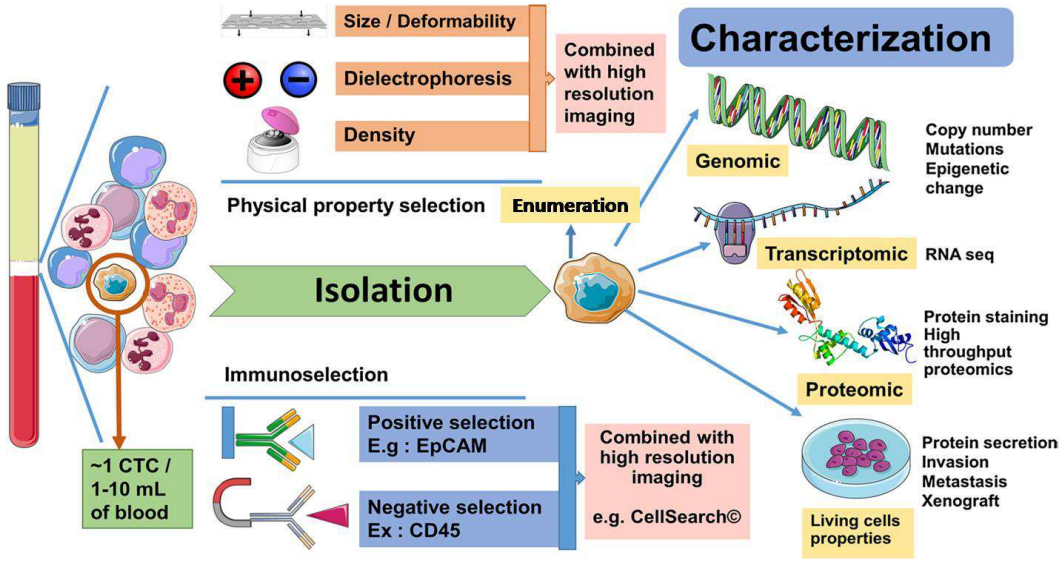


Figure 21. Schematic representation of CTC detection approaches and downstream applications for molecular characterization. Adapted from Cabel *et al*, 2017.

lack of univocal CTC biomarkers: EpCAM itself is not expressed by all tumor types and, as previously described, a subset of epithelial cancer cells undergo EMT, likely losing the expression of this epithelial marker. Alternatively, several approaches relying on physical properties of cells have been developed, such as the use of density gradient centrifugation, which allows to separate cells on the basis of the-sedimentation coefficient (Low & Wan Abas, 2015). Size-based methods, instead, exploit the dimensions of CTCs, which are generally bigger than the other blood cells, with a diameter ranging from 17 to 52 µm. Among all filtration devices, those employing microfilters with a selective porous membrane (8 µm pore diameter), represent a relatively simple way to obtain an enriched pool of cells within a single processing step, preserving cell integrity for CTC identification and further characterization (Vona *et al*, 2000; Desitter *et al*, 2011). In addition, CTC clusters can also be separated this way. However, this approach shows some drawbacks, including the loss of smaller tumor cells, the risk of filter clogging due to high concentration of blood cells and the requirement of CTC detachment from the membrane to perform downstream analysis. Other methods combining cell size and microfluidics have been developed (Hou *et al*, 2013; Sollier *et al*, 2014), as well as size- and deformability-based technologies (Tan *et al*, 2009, 2010; Che *et al*, 2017). Moreover, a chip for the specific isolation of CTC cluster is available, based on the separation by different flow speed compared to single cells (Sarioglu *et al*, 2015).

CTC identification and enumeration are commonly performed by morphologic investigation together with fluorescence immunocytochemistry (ICC). However, conventional microscopic examination is cost- and time-consuming, whereas automated cell image capturing and analysis systems present filter set limitations (Joosse *et al*, 2015). The use of multiplex PCR could help in targeting the numerous tumor-associated mRNA transcripts, such as EMT-associated and stem cell markers for CTC identification (Kasimir-Bauer *et al*, 2012). Another strategy is represented by the enumeration and analysis of proteins specifically secreted by viable tumor cells (EPISPOT, EPithelial Immuno SPOT) through a system of fluorochrome-labeled antibodies targeting the proteins of interest (Alix-Panabieres *et al*, 2007; Alix-Panabieres, 2012).

CTC enrichment and identification represents the first step to eventually obtain single cells suitable for molecular and functional characterization, since simple enumeration does not to reflect the complexity of genetic and epigenetic changes occurring during tumor progression. Many strategies have been applied for this purpose (Shapiro *et al*, 2013; Navin, 2014), including manual low-throughput micromanipulation and laser-capture microdissection, flow cytometry and the most recently developed semiautomated DEParray (Di-Electro-Phoretic Array; Silicon Biosystems, Italy) system (Peeters *et al*, 2013). Once isolated, CTCs can be analyzed for chromosomal rearrangements or changes in gene copy number by using techniques such as interphase fluorescence in situ hybridization (FISH) and array-CGH (comparative genome hybridization). Additionally, next generation DNA sequencing can be performed to study the CTC genome-wide mutation spectrum (Heitzer *et al*, 2013; Carter *et al*, 2017). Other traditional techniques have been applied to CTC characterization, such as RNA *in situ* hybridization, multiplexed quantitative PCR and gene expression microarray (Smirnov *et al*, 2005; Xi *et al*, 2007; Payne *et al*, 2012; Yu *et al*, 2013); moreover, the increasing feasibility of the advanced single-cell expression profiling approaches are now allowing to shed new light on CTC clonal evolution and heterogeneity (Sandberg, 2013; Lohr *et al*, 2014; Wang *et al*, 2014; Miyamoto *et al*, 2015). A question remain about how and when such techniques will become predictive, specific and inexpensive enough to be adopted for the routinely clinical practice.

Due to CTC rarity, their functional characterization still remains technically challenging. Cell line and organoid culture have been established with low efficiency and are available only for a handful of cancers (e.g. Cayrefourcq *et al*, 2015). CTC functionality has been tested both in culture and *in vivo* in mouse xenograft models, measuring the invasive and metastatic properties of isolated cells (Baccelli *et al*, 2013; Friedlander *et al*, 2014), as well as the response to chemotherapeutics (Hodgkinson *et al*, 2014; Yu *et al*, 2014; Khoo *et al*, 2016). However, further studies are needed to standardize the current culture techniques and *in vivo* strategies.

4.1.3. Clinical validity and utility of CTCs

The diagnostic, prognostic and predictive value of CTC has been investigated in various cancer types, demonstrating a clinical validity for CTC detection and characterization, at least in some solid tumors (Cabel *et al*, 2017).

CTC count, mainly based on the use of the CELLSEARCH® technology, has been investigated as diagnostic in early non-metastatic cancers: most of the studies reported very low CTC detection rate, depending on the limited specificity and sensitivity on the technique (Loh *et al*, 2014; van Dalum *et al*, 2015; Bidard *et al*, 2016). The ISET filtration system, instead, showed better performances in lung cancer early detection and differential diagnosis (Ilie *et al*, 2014; Fiorelli *et al*, 2015). On the contrary, high level of evidence showed a strong prognostic value for CTC count determined by CELLSEARCH®: in both metastatic and non-metastatic cancers, particularly breast, colon, prostate and lung cancer, elevated baseline CTC count represents an independent prognostic factor for both overall survival and free-progression survival (Cabel *et al*, 2017). Moreover, several reports suggest a predictive value for CTC detection in monitoring the efficacy of therapies: particularly in metastatic breast, colon and prostate cancers, a decreased CTC count after treatment associates with better prognosis (Cristofanilli *et al*, 2004; Cohen *et al*, 2008; de Bono *et al*, 2008; Hou *et al*, 2012; Bidard *et al*, 2014; Goldkorn *et al*, 2014). However, CTC analysis remains still limited when applied to non-metastatic cancers, due to the low rate of CTC detection and to the significance to be attributable to their presence in non-invasive stages of tumors.

Although its clinical validity, CTC detection has not been approved for the routinely clinical practice, since a clinical utility, meaning an improvement in patient's outcome, has not yet been validated. Several ongoing clinical trials are investigating the utility of CTC in the therapeutic decision, basing on CTC count and variation, as well as on the presence of molecular processes and biomarkers specifically related to CTCs or to the metastatic spread (Cabel *et al*, 2017). The improvement in the sensitivity of CTC detection techniques and the development of *in vitro* (CTC cultures) and *in vivo* xenograft models for drug screening will be crucial to finally launch CTCs in the clinical practice.

4.2. CTCs in adrenocortical carcinoma

To date, only one preliminary study has been performed to assess the presence of circulating tumor cells in the peripheral blood of patient with adrenocortical carcinoma compared with benign adenomas (Pinzani *et al*, 2013). In a cohort of 24 patients (14 ACC and 10 ACA), CTCs were isolated by using the ScreenCell® filtration device and detected in all ACC samples, unlike in ACA samples, thus indicating the potential use of CTC detection for the differential diagnosis of adrenocortical tumors. Adrenocortical origin of isolated cells was confirmed by immunocytochemistry performed against typical neuroendocrine markers, such as synaptophysin and MART-1/MelanA, but also against the more adrenocortex-specific Steroidogenic Factor-1 (SF-1) (Fig. 22). CTC count in pre-surgical and post-surgical samples of ACC patients revealed that surgery significantly affects the number of CTCs (Fig. 23A), even if no significant correlation with the length of follow-up was evident. When patient clinical features were considered, a

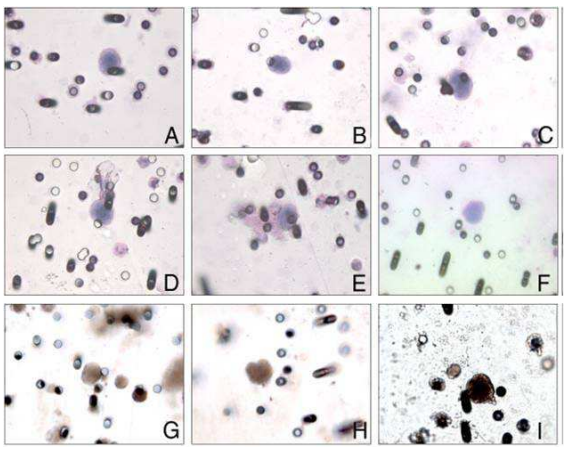


Figure 22. CTC detection in blood samples from ACC patients. A–F: Hematoxylin/eosin staining was performed on filters to identify and count CTCs on the basis of established morphological criteria (cell size > 16 µm; nucleocytoplasmic ratio > 50%; irregular nuclear shape; hyperchromatic nucleus; basophilic cytoplasm. Positivity related to MART-1 (G), synaptophysin (H), and SF-1 (I) immunocytochemistry confirmed CTC adrenocortical origin**.** Adapted from Pinzani *et al*, 2013.

statistically significant linear correlation was found only with the tumor diameter (R2=0.362; R=0.602; *P*=.023; n=14), while no correlation was present with the other parameters analyzed, such as Ki67, age, stage and Weiss score. Moreover, CTC number were significantly higher in patients with metastatic disease (Fig. 23B).

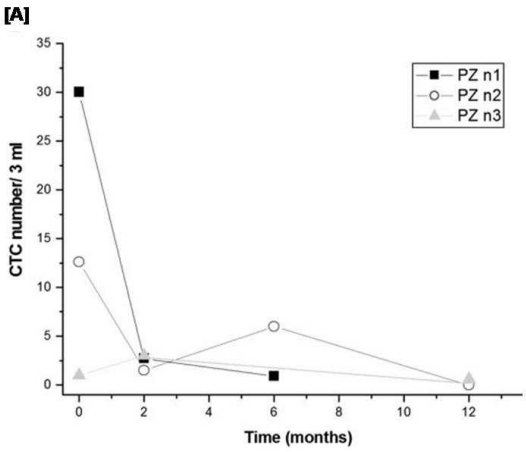


Figure 23. A): Absolute values of CTC count evaluated in ACC patients show a significant decrease of CTC number after surgery in samples collected during follow-up compared to pre-surgery samples. B): Stage and diameter were used to stratify ACC patient (n=14) in two classes, using stage 4 or diameter median value as cut-off. Adapted from Pinzani *et al*, 2013.

[B]

Tumor	Stage < 4	Stage 4	*P*	Diameter < 8.8 cm	Diameter ≥ 8.8 cm	*P*
Postsurgical CTCs, n/3 mL						
Mean (SD)	2.1 (2.1)	11.7 (14.5)	.031	1.8 (2.0)	8.3 (11.2)	.006
Median [interquartile range]	1.1 [0.7–3.0]	5.8 [2.4–27.0]		1.0 [0.5–2.3]	3.0 [2.2–9.0]	
Patients, n (%)	10 (71)	4 (29)		7 (50)	7 (50)	

Although limited by the small cohort of patients analyzed, this preliminary study suggests that CTCs may be a useful marker in aggressive/metastatic ACC for both prognosis and monitoring of progression and response to treatments. Therefore, in the setting of non-invasive liquid biopsy, CTCs could provide meaningful information to elucidate the metastatic process and the different molecular and cell factors involved. The possibility to investigate gene and protein expression, as well as epigenetic changes, starting from single-cell samples, would help in identifying specific biomarkers associated with CTC heterogeneity and metastasizing potential (Salvianti & Pinzani, 2017; Siravegna *et al*, 2017). This knowledge may aid the development of more effective treatments specifically targeting the metastatic disease. Moreover, information provided by CTC analysis could be complemented by additional genomic information coming from cell-free circulating tumor DNA, which has been recently demonstrated to be detectable in blood sample also in ACC (Creemers *et al*, 2017). Thus, the "liquid biopsy" analysis would provide a more comprehensive understanding of ACC heterogeneity and patient-related disease progression.

OBJECTIVES

Molecular features underlying adrenocortical carcinoma heterogeneity and evolution are just being unraveling, showing that driver gene mutations, epigenetic alterations and dysregulation of signaling pathways related to cell survival, growth and proliferation, play a crucial role in determine malignancy. However, even if molecular alterations are essential to provide a survival and growth advantage to cancer cells within the tumor mass, they cannot exhaustively enucleate the mechanisms by which malignant cells acquire the ability to migrate and metastasize to distant sites, thus allowing cancer progression. The adaptation of cell metabolism to dynamically changing environmental conditions related to tumor evolution represents a key hallmark of cancer. Tumor microenvironment constitutes the ideal soil to establish a constant crosstalk between tumor and stromal cells, thus inducing reciprocal metabolic and functional alterations, eventually supporting tumor growth and progression. Studying the dynamics of such interactions would help in elucidating cancer biology and developing more effective targeted therapies addressing cancer-microenvironment crosstalk. Since adrenal glands present a substantial component of adipose tissue, the interactions with the surrounding adipose microenvironment may play a pivotal role in adrenocortical tumorigenesis and ACC progression, as widely described for other tumor types, where cancer cells are in close contact with adipocytes.

The first objective of this thesis (Results - Part I) was to reproduce *in vitro* a system of reciprocal interactions between the adrenocortical tumor cell line NCI-H295R and the human adipose-derived stem cells (ASCs) or *in vitro* differentiated adipocytes in order to evaluate:

i. the paracrine effects of the adipose component on H295R cell behavior, including variations of cell proliferation and motility/migration ability;
ii. the influence of H295R cells on ASC proliferation, gene expression and ability to effectively differentiate toward a white mature and functional adipocyte phenotype
iii. the potential cell signaling pathways and factors involved in this reciprocal interaction.

The second part of the thesis (Results - Part II), instead, investigates the possibility to isolate and characterize circulating tumor cells (CTCs) in blood samples from patients with adrenocortical carcinoma, in order detect specific molecular features to be compared with those of the primary tumor, allowing to track tumor evolution and progression. Particularly, the objectives included:

i. the collection of blood samples from ACC patients, possibly at different time points during the follow-up, for CTC isolation, identification and enumeration;
ii. the cytological analysis of CTCs to confirm their adrenocortical origin;
iii. the design of an experimental workflow to obtain CTC DNA to perform downstream analysis with techniques such as targeted next generation sequence (NGS) and digital-droplet PCR (ddPCR) and to confirm the presence of the original mutations previously found in the primary lesions.

RESULTS

PARTI I - Investigating the crosstalk between adrenocortical carcinoma cells and adipose microenvironment

Materials and methods

- **Cell cultures.** The human adrenocortical carcinoma cell line H295R was obtained from the American Type Culture Collection (ATCC) and cultured in DMEM/F-12 medium with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin-100 µg/ml streptomycin and a mixture of insulin/transferrin/selenium (ITS) (Sigma-Aldrich). Human primary adipocyte-derived stem cells (ASCs) were isolated from the stromal vascular fraction (SVF) derived from subcutaneous adipose tissue biopsies as described elsewhere (Baglioni *et al*, 2009): briefly, adipose tissue biopsies underwent mechanic and enzymatic processing until obtaining the SFV, which was plated and, after having removing the non-adherent contaminating cells, cultured in DMEM with 20% FBS, 2 mM L-glutamine, 100 U/ml penicillin-100 µg/ml streptomycin and 1 µg/ml amphotericin-B (Sigma-Aldrich). All cells were incubated at 37°C in a humidified 5% CO_2 atmosphere.

- **Co-culture system.** H295R and ASCs where co-cultured in the setting of different experiments by using a specific tissue culture inserts for 6-well plates having a porous membrane with 0,4 µm diameter pores (ThinCert™, Greiner Bio-One), thus allowing to maintain the two cell types separated, while the medium soluble components can be exchanged (Fig. R1). H295R cells and ASCs were seeded, except where differently indicated, in the cell culture inserts (10^5cells/insert) and in the well (8×10^4) respectively, each in its own complete medium. At the starting time of the co-culture, the inserts containing H295R were transferred into the wells containing ASCs and all cells were grown in DMEM plus 10% FBS. H295R and ASCs cultured alone in the same conditions of the co-cultured ones were used as relative controls for each type of assay.

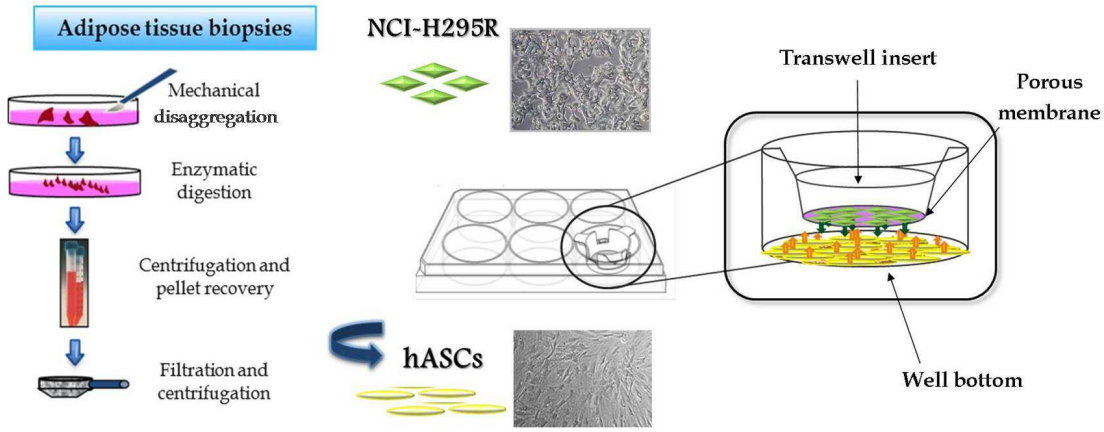


Figure R1. Schematic representation of the described co-culture system

- **Viable cell count.** H295R and ASCs seeded in the co-culture system were 24 hour starved and put together to start the co-culture. At different time points (0, 1, 2, 3, 6, 9 days) cells were trypsinized and counted by an haemocytometer, after dead cell exclusion with trypan blue staining. The mean number of viable cells was obtained by counting four replicates in at least three different experiments.

- **Glucose up-take measurement.** H295R and ASCs, co-cultured or grown alone for 7 days, were washed twice with PBS and incubated overnight in serum free and low glucose (0,55 mM) medium. After PBS wash, cells were incubated with Hepes buffer (140 mM NaCl, 20 mM Hepes-Na pH 7.4, 2,5 mM MgSO4, 1 mM CaCl2, 5 mM KCl) containing 2-deoxy-[^{3}H]D-glucose [1µCi/µl] (Perkin-Elmer) for 10 minutes at 37°C. Cells were then washed with cold PBS and were lysed in 100 mM NaOH for 1 hour at 37°C. After resuspension of samples in Insta-Gel Plus cocktail (Perkin-Elmer), radioactivity was measured on a scintillation beta counter.

- **Evaluation of conditioned media.** Conditioned media related to 72 hour mono- or co-culture of both H295R and ASC were assessed to quantify glucose, lactic acid and cortisol content by using a routinely detection method at the central analysis laboratory (Azienda Ospedaliero Universitaria Careggi, Florence).

- **Transmission electron microscopy.** After 7 day mono- or co-culture, H295R and ASCs were trypsinized and centrifuged at 1200 rpm for 5 minutes. Pellets were fixed in cold 2.5% glutaraldehyde and 2% formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature, and post-fixed in cold 1% osmium

tetroxide in 0.1 M phosphate buffer (pH 7.4) for 1 h at room temperature. Pellets were dehydrated in graded acetone, passed through propylene oxide, and embedded in epoxy resin. Ultrathin sections were stained with gadolinium acetate and alkaline bismuth subnitrate and examined under a JEM 1010 electron microscope (Jeol) at 80 kV.

- **In vitro adipocyte differentiation and lipid content quantification.** ASCs were seeded onto glass coverslips in 6-well plates (5×10^4 cells/well) and cultured, alone or together with H295R (10^5 cells/insert), in the presence of adipogenic medium (DMEM plus 10% FBS, 0,5 mM 3-isobutyl-1-methylxanthine, 1 mM dexamethasone, 10 mM insulin and 1µM rosiglitazone - DIM cocktail). ASCs cultured in DMEM with 10% FBS were used as negative control. After 10 day differentiation, the intracellular lipid content was measured by AdipoRed™ assay according to the manufacturer's instructions. Fluorescence emission was measured by 485/572 nm excitation/emission. The specific absorbance related to the differentiated adipocytes was calculated as fold increase on the unspecific absorbance related to the undifferentiated ASCs. Coverslips were finally mounted on microscope slides in the presence of ProLong® Gold antifade reagent with DAPI (Thermo Fisher Scientific Inc.) and the fluorescence related to the lipid droplets was acquired with a Leica DM4000 epifluorescence microscope (Leica Microsystems GmbH). This same protocol was slightly modified to obtain *in vitro* differentiated mature adipocytes suitable for co-culture with H295R: ASCs were cultured for 7 days in the presence of DIM cocktail and then cultured alone or in the presence of H295R for additional 9 days in complete DMEM/F12. The same experimental procedure already described was used for measuring the intracellular lipid content and to acquire fluorescence images.

- **SDS-PAGE and Western Blot Analysis.** After 7 day mono- or co-culture, cells were lysed in RIPA buffer (20 mM Tris pH 7.4, 150 mM NaCl, 0,2 mM EDTA, 1 mM OVA, 0,5% Triton-100 in ddH2O) supplemented with phosphatase and protease inhibitors. After protein measurement by Coomassie method, equal amounts of proteins for each sample (30 µg) were separated by SDS–PAGE and transferred onto PVDF membranes (Immobilon, Merck Millipore). Each membrane was incubated overnight at 4°C with primary antibodies, at the appropriate

dilutions, against the following proteins: IGF-1Rβ, Fascin-1, RhoA, GAPDH, Actin, GLUT-1, GLUT-4, SDF-1/CXCL12 (Santa Cruz Biotechnology, Inc.), phospho-p44/42 MAPK (Thr202/Tyr204) and p44/42 ERK1/2 (Cell Signalling Technology, Inc.), FAK (GeneTex, Inc.), MEK-1 (EDM-Millipore), Actin α-Smooth Muscle (clone 1A4; A2547; Sigma-Aldrich). The further peroxidase-secondary IgG incubation (1:2000 dilution) (Sigma-Aldrich) was performed at room temperature for 1,5 hours. Image acquisition and densitometric analysis were performed with Quantity One software on a ChemiDoc XRS instrument (BIO-RAD). All Western blots were repeated in at least 3 independent experiments. GAPDH or Actin were used as internal loading control to normalize protein expression.

- **RNA extraction, reverse transcription and quantitative real-time PCR.** RNA extraction from H295R, ASCs and mature adipocytes, co-cultured or grown alone for 7 days, was performed using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. Briefly, pellets of cells, collected by trypsinization and centrifugation, were resuspended in RTL-1% β-mercaptoethanol buffer, added with 70% ethanol, column purified through sequential centrifugation steps and finally eluted in 50 µl RNase-free H_2O. RNA concentration was assessed on a Nanodrop spectrophotometer (Thermo Fisher Scientific). For each RNA sample, cDNA was obtained by performing reverse transcription PCR starting from 250 ng RNA in 50 µl final volume reaction (Taqman RT-PCR kit; Applied Biosystems) using the following cycling conditions: 10 minutes at 25°C, 30 minutes at 48°C, 3 minutes at 95°C, hold 4°C. Further quantitative real-time PCR was carried out using primers and probes for the following genes: BMI-1, Nanog, OCT-4, Leptin, AdipoQ, FABP4, HSL, IL-8, IGF2, IGF1R, LepR, DPP4, CXCR7, CXCL12, IL-6, MCP-1, HSD11β1, GAPDH (Taqman Gene Expression Assay; Applied Biosystems). RT-PCR reactions, performed in triplicate for each gene, were carried out in 12,5 µl final volume on a ABI Prism 7900 Sequence Detector (Applied Biosystems) with the following cycling conditions: 15 seconds at 95°C plus 1 minute at 60°C for 40 cycles. The amount of target genes, normalized to the endogenous reference gene (GAPDH) and related to a calibrator (Stratagene), was calculated by $2^{-\Delta\Delta Ct}$.

- **Wound healing assay.** H295R cultured alone or in co-culture with ASCs for 7 days were trypsinized, seeded in a 12-well plate ($1.5x10^6$ cells/well) and grown up

to confluence. A sterile plastic 10 µl pipette tip was used to scratch the confluent cells monolayer in each well to generate a cell-free zone and, after a wash in PBS to eliminate the detached cells, fresh medium was added and cells were incubated for 48h. In each well, migration was assessed under an inverted light microscope and quantified using a specific tool (MRI Wound Healing Tool) of the ImageJ software at designated time points, i.e. 0, 24 and 48 hours post-scratch. The percentage of migration rate was expressed as (1 − residual area)/(initial area) × 100.

- **Invasion assay.** The cell invasion assay was performed with a basement membrane-coated CytoSelect™ 24-well cell invasion assay kit (Cell Biolabs), according to the manufacturer's instructions. Briefly, H295R cells, previously cultured alone or co-cultured with ASCs for 7 days, were trypsinized and plated in the upper chamber of the invasion plate ($3x10^5$ cells/well) in serum free culture medium. Complete culture medium (DMEM/F12 plus 10% FBS) was added to the lower chamber. Cells were incubated for 48h at 37°C in 5% CO_2 atmosphere. The non-invasive cells were then removed from the inside of the inserts, that were then transferred to a clean well containing a Cell Stain Solution and incubated for 10 min at room temperature. The inserts were washed in water, allowed to air dry and transferred to an empty well containing an Extraction Solution. After 10 min incubation on an orbital shaker, samples were transferred to a 96-well microtiter plate and the OD 560 nm was measured in a microplate reader (VICTOR multilabel plate reader; Perkin-Elmer). The OD 560 nm value related to the Extraction Solution alone was used as the background subtraction factor. The assay was repeated in three different co-culture experiments.

- **Immunofluorescence.** H295R cells were seeded on glass coverslips in 6-well plates (10^5 cells/well) and cultured alone or together with ASCs grown in cell culture inserts ($8×10^3$cells/insert). After 7 days, H295R were fixed in 4% PFA, permeabilized in 0,2% PBS-Tryton and incubated with the ActinGreen™ 488 ReadyProbes® Reagent (Thermo Fisher Scientific, Inc.) for 40 minutes to stain F-actin cytoskeleton. Coverslips were then mounted on microscope slides in the presence of ProLong® Gold antifade reagent with DAPI (Life Technologies). Fluorescence was acquired with a Leica DM4000 epifluorescence microscope (Leica

Microsystems GmbH). Coverslips incubated with only 5% PBS-horse serum were used as negative controls.

- **ELISA assay.** The conditioned medium related to ASCs cultured alone or together with H295R for 9 days was analyzed with an ELISA kit for the detection of human SDF-1a (CXCL12A) (Thermo Scientific™ Pierce™), according to the manufacturer's instructions. Briefly, samples were incubated in the assay plate containing the anti-SDF-1a antibody for 2,5 hours at room temperature. After washings, samples were incubated with the biotinylated antibody for 1 hour at room temperature. Subsequent streptavidin-reagent and TMB substrate incubation were performed before measuring the OD 450/550 nm on a microplate reader (VICTOR multilabel plate reader; Perkin-Elmer). Each point was performed at least four times in at least three different experiments. Sample absorbance values were interpolated on a standard curve to obtain the relative concentrations. The assay sensitivity was 80 pg/ml. Each value was normalized on the relative cell number.

- **Statistical analysis.** Statistical analysis was performed using SPSS 22.0 software (SPSS, Inc.). The Kolmogorov–Smirnov test was used to verify the normal distribution of data, expressed as mean ± SE. Student's t test was applied for comparison of two classes of data. A $P < 0,05$ value was considered as statistically significant.

Results

1) Cell proliferation and morphology of both H295R and ASCs are altered during co-culture

An *in vitro* co-culture system was set up to assess the reciprocal influence of H295R adrenocortical cancer cells and human adipose-derived stem cell (ASCs) on cell behavior and characteristics. The use of specific 6-well inserts with a porous membrane allowed for culturing the two cell types in the same medium, avoiding direct cell contact but maintaining a constant exchange of soluble factors between the two compartments.

First, H295R and ASCs were co-cultured up to 9 days and monitored at designated time points (0, 2, 3, 7, 9) to assess whether a variation in the proliferation rate was induced compared to the single cultures. Further gene and protein expression analysis was performed to investigated the possible intracellular pathways involved. Microscopy observation allowed to evaluate morphological variations.

H295R co-cultured with ASCs showed a significant increased in cell proliferation at day 9 co-culture, compared to H295R cultured alone, evaluated at the same time point (8.60 vs. 6.21-fold) (Fig. R2, A), indicating that a pro-proliferative signaling is triggered in the presence of ASCs. Western blot analysis was performed on protein samples extracted from H295R after 7 day mono- or co-culture in order to investigate whether the IGF-1R/MAPK signaling, which sustains the proliferative autocrine loop in adrenal cancer, was involved in mediating such proliferation gain. Although the expression of the IGF1 receptor was not altered, a significant increase of MEK and p-ERK expression was observed, indicating an up-regulation of this intracellular signaling when ASCs are present. Moreover, at the same time point, cells in co-culture showed a significant increase in glucose uptake (1.26-fold), measured by incorporation of 2-deoxy-[^{3}H]D-glucose, thus indicating a higher metabolic activity of H295R stimulated by ASCs (Fig. R2, B, C).

Under conventional light microscope observation, H295R co-cultured with ASCs appeared differently distributed on the well surface compared with H295R cultured alone: unlike the wide, foci-presenting clusters typically observed in our H295R cultures, cells formed smaller spaced clusters and acquired a well defined sharp shape (Fig. R3), thus suggesting a variation in cell-cell contact dynamics (Fig. R3).

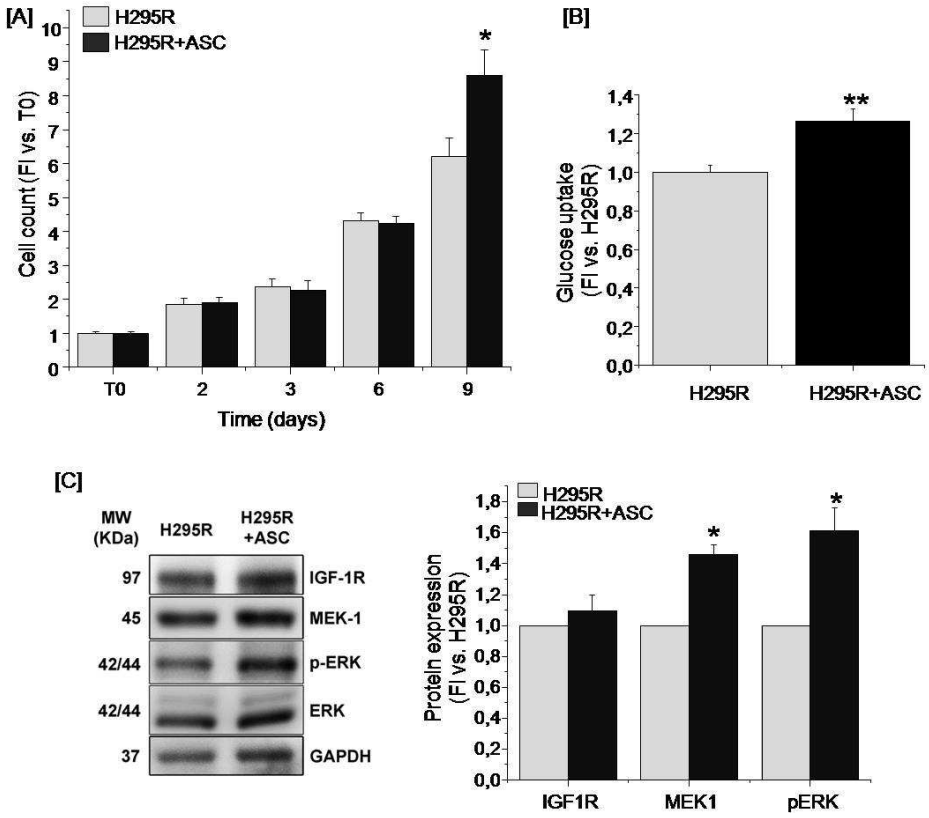


Figure R2. The pro-proliferative effect of ASC co-culture on H295R. A) H295R alone (H295R) or co-cultured with ASCs (H295R+ASC) were assessed for cell proliferation at the indicated time points (2, 3, 6 and 9 days) by direct cell count. The proliferative rate was calculated as fold increased versus T0. Glucose uptake measurement (B) and Western Blot analysis of IGF-1R, MEK1, total ERK and pERK expression (C) were compared in cell samples derived from H295R after 7 day mono- or co-culture. For Western Blot analysis, GAPDH was used as internal loading control. Both protein expression and glucose up-take were calculated as fold increase versus H295R alone. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$, H295R+ASCs vs. H295R.

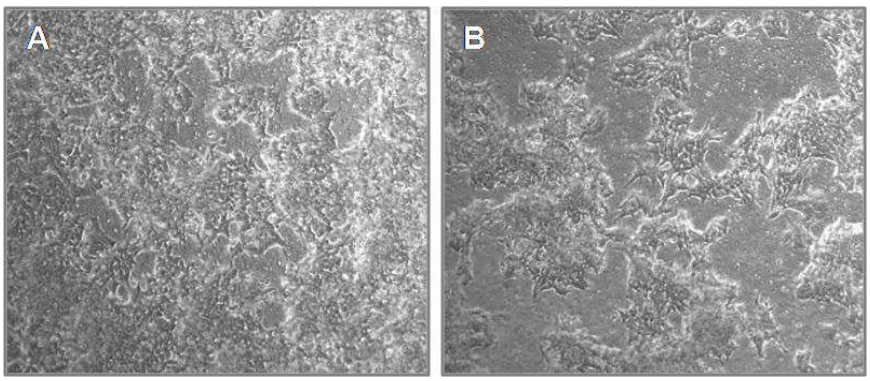


Figure R3. H295R cultured with ASCs show a different distribution compared to the control. Representative images of H295R cultured alone (A) or in the presence of ASCs (B). Original magnification: X10.

Transmission electron microscopy was also performed on ultrathin cell sections to evaluate H295R ultrastructure following the two culture conditions. The main difference was represented by a dramatic decrease of lipid droplets in H295R cultured in the presence of ASCs compared to H295R mono-colture (Fig. R4), thus indicating a possible increase in lipid metabolism.

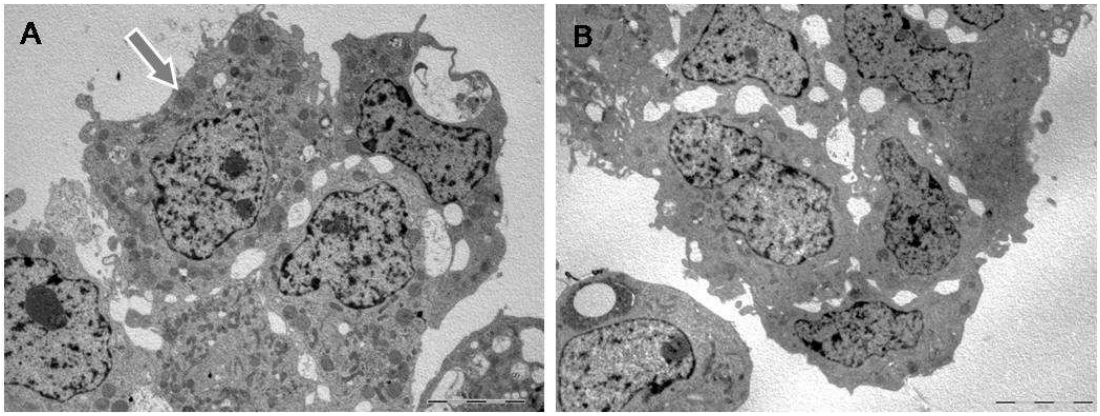


Figure R4. H295R ultrastructure modifications after co-culture. Representative images of control H295R (A) and H295R co-cultured for 7 days with ASCs (B) showing a considerable loss of lipid droplets (arrow). Scale bars = 5 µm.

Proliferative and morphological aspects were concomitantly evaluated in **ASCs** after mono- or co-culture in the setting of the same experiments described above for H295R. ASC proliferation was assessed at the same designated time points and a statistically significant increase in the proliferative rate was observed starting from day 6 co-culture (3.49 vs. 2.69-fold), with a significant difference at day 9 (10.12 vs. 3.85-fold) (Fig. R5, A). Also for ASCs, glucose up-take was significantly increased in co-culture compared to control ASCs (2.06-fold) (Fig. R5, B), indicating that the presence of H295R induces ASCs to enhance glucose metabolism. Consistently, the insulin-independent glucose transporter GLUT-1 appeared up-regulated in ASCs co-cultured with H295R compared to ASCs alone, as assessed by Western Blot analysis (Fig. R5, B, inset). Due to their mesenchymal stem origin, ASCs express a set of specific markers, including Bmi-1, Nanog and Oct-4, which associate with the stem potential maintenance. Following co-culture, the expression of all the three stem genes was significantly decreased (2.8, 3.1 and 1.3-fold, respectively) compared to control ASCs, as assessed by Taqman assay (Fig. R5, C). This suggests that soluble factors secreted by tumor cells and exchanged within the co-culture system are able

to recruit and induce ASC increased proliferation as well as commitment toward adipose lineage, likely acquiring a phenotype metabolically more prone to sustain cancer cell growth. Consistently with this hypothesis, a considerable over-expression of αlpha-Smooth Muscle Actin (α-SMA), a protein specifically indentifying myofibroblast-like cells, was detected by Western Blot analysis in protein samples extracted from ASCs cultured for 7 days with H295R compared to the control (Fig. R5, D).

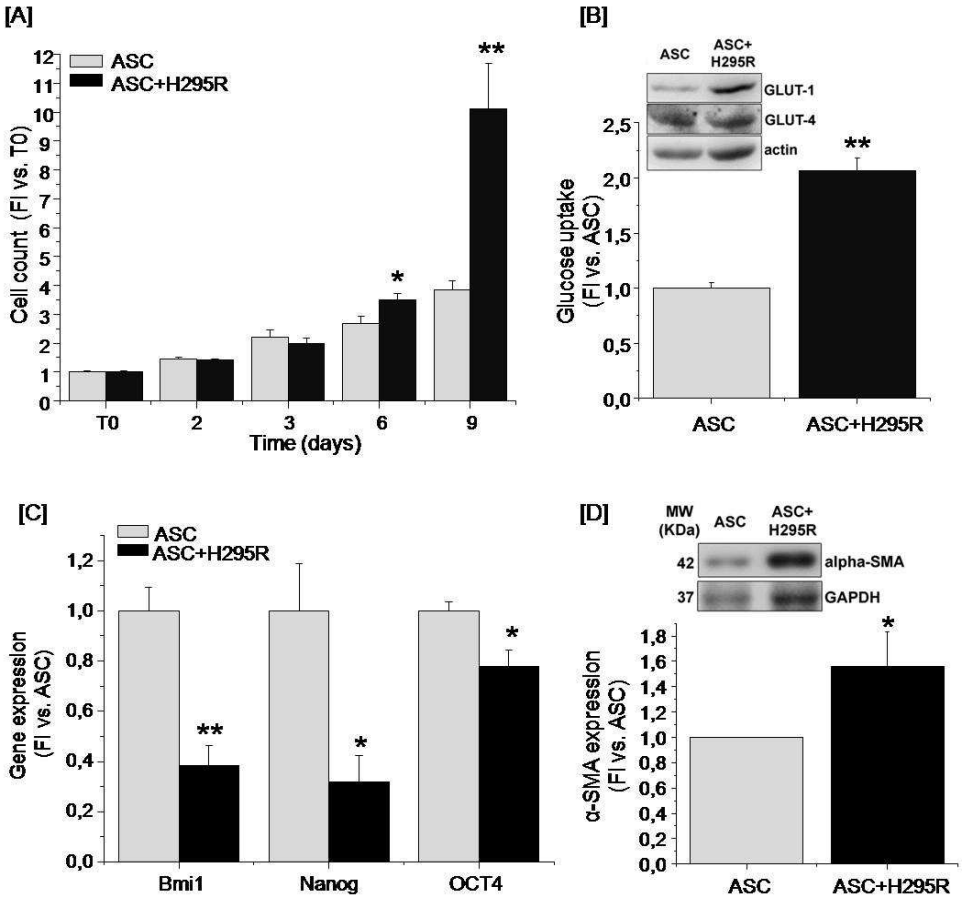


Figure R5. The presence of H295R prompts ASC proliferation and drives ASC differentiation toward a myofibroblast-like phenotype. A) ASCs alone (ASC) or co-cultured with H295R (ASC+ H295R) were assessed for cell proliferation at the indicated time points (2, 3, 6 and 9 days) by direct cell count. The proliferative rate was calculated as fold increased versus T0. Glucose uptake measurement and Western Blot analysis of GLUT-1, GLUT-4 and α-SMA expression (B, D) were compared in cell samples derived from ASCs after 7 day mono- or co-culture. For Western Blot analysis, GAPDH or Actin were used as internal loading control. Both protein and gene expression (C) were calculated as fold increase versus ASCs alone. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$, ASCs+H295R vs. ASCs.

A macroscopic change in cell morphology was observed under light microscopy in ASCs co-cultured with H295R compared to the controls: they appeared more densely distributed into the well and showed a more elongated shape, starting to form cell bundles (Fig. R6).

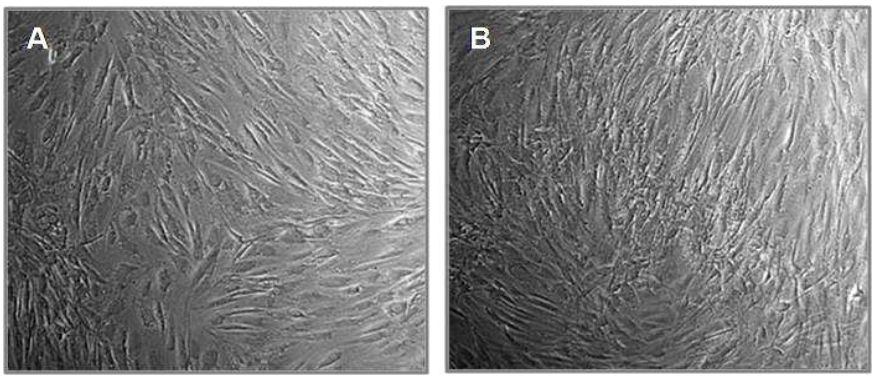


Figure R6. ASCs cultured in the presence of H295R show a different gross morphology compared to the control. Representative images of ASCs cultured alone (A) or in the presence of H295R (B). Original magnification: X10.

Transmission electron microscopy, performed on ultrathin sections of ASC samples, allowed to appreciate several ultrastructure modifications between cells cultured by the two conditions. ASCs cultured alone showed wide portions of the plasma membrane organized in microvilli and cytoplasm rich in dense lysosome-like bodies. Mitochondria appeared elongated (length > 1 µm) and often branched, with typical transverse cristae. Rough endoplasmic reticulum was not of particular extent, as well as Golgi's apparatus. Small cytoplasmic vesicles was also observed, presumably endosomes or lysosome precursors (Fig. R7, A, B). ASCs co-cultured with H295R, instead, showed cytoplasmic structures typically associated with a metabolic active cell: rough endoplasmic reticulum was diffusely represented, with huge amount of ribosomes on its surface. Golgi's cisternae became more and larger and lysosome-like bodies were significantly less numerous. Meanwhile, the presence of lipid droplets was observed. Interestingly, mitochondria were different in size and shape, becoming smaller and roundish (Fig. R7, C, D, E). Thus, the presence of cancer cells induces substantial changes in ASCs, altering gene expression and morphological features toward a phenotype more differentiated and metabolically active.

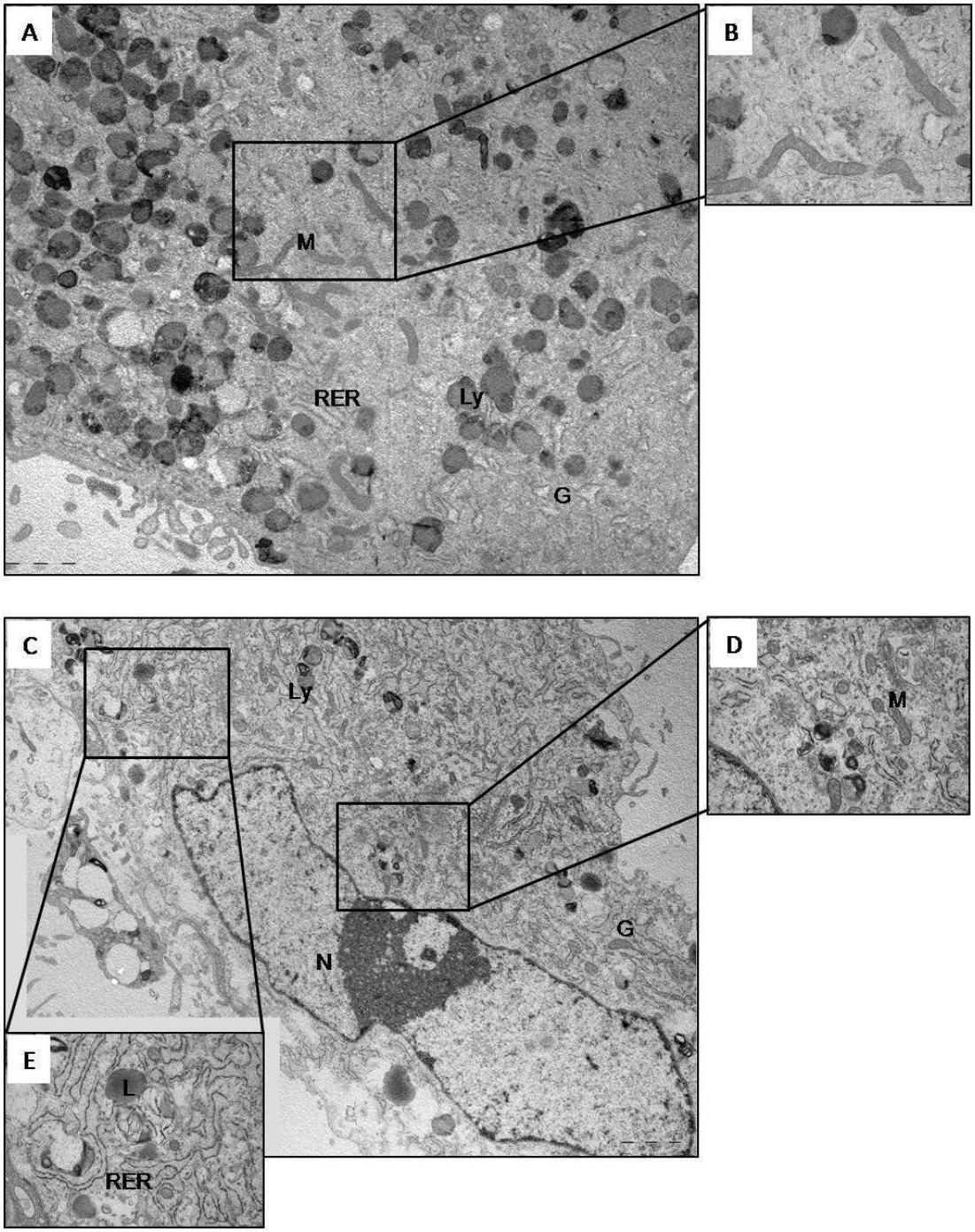


Figure R7. ASC ultrastructure shows substantial changes after co-culture. Representative images of ASCs cultured alone (A; zoom in: B) or in the presence of H295R (C; zoom in: D, E) for 7 days. Different fields with the same original magnification were digitally merged to reconstruct a wider cell portion. N=nucleus; M=mitochondria; RER=rough endoplasmic reticulum; Ly=lysosome; G=Golgi cisternae; L= lipid droplet. Scale bars = 2 µm (A, C) and 1 µm (B, D, E).

2) H295R cell migration and invasion ability is gained in the presence of ASCs

Once ascertained that a reciprocal interaction is established between the two cell types in co-culture, we focused on cancer cell features in order to investigate whether the presence of ASCs could affect cancer cell motility and migration. H295R ability to heal an induced wound was assessed by wound healing assay after 7 day co-culture with ASCs. The percentage of migrating cells, evaluated at 24 and 48 hour post-scratch, was significantly higher in H295R previously co-cultured with ASCs compared to the control (Fig. R8, A, B). Moreover, the same H295R stimulated by ASCs showed also a statistically significant higher capacity to migrate through a basement membrane compared to H295R cultured alone, as assessed by a specific invasion assay (Fig. R8, C).

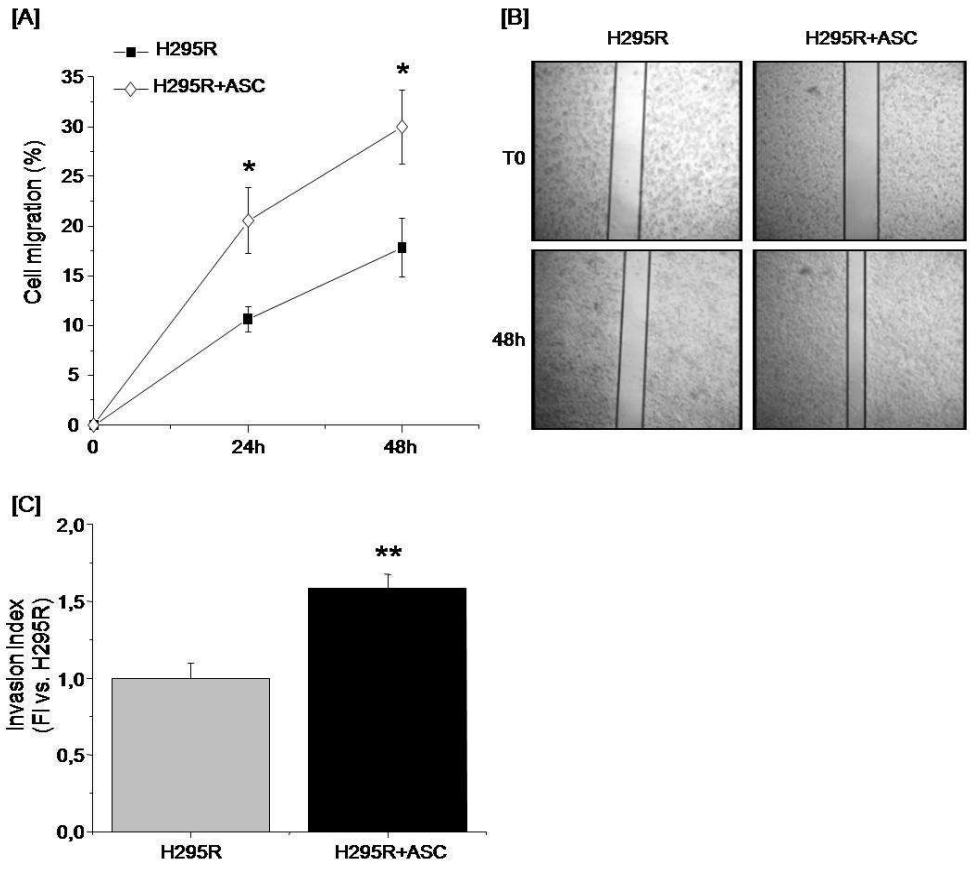


Figure R8. H295R migration and invasion ability is gained after ASC co-culture. A) Quantification of the scratched areas was carried out by using the ImageJ MRI-Wound Healing Tool and the percentage of migrating cell was calculated as following: (1 − residual area)/(initial area) × 100. B) Representative images of wound healing assay at 0 and 48 hour post-scratch in H295R previously cultured alone or in the presence of ASCs for 7 days. Original magnification: X5. C) Invasion was assessed by specific a colorimetric assay and the invasion index was calculated as fold increase vs. H295R. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$, H295R+ASC vs. H295R.

We further performed Western blot assay to assess the expression of proteins associated with cell migration, particularly Focal Adhesion Kinase (FAK), RhoA and Fascin-1, which are involved in the formation of lamellipodia, filopodia and focal adhesion structures. H295R co-cultured with ASCs showed a statistically significant increased expression in all the three proteins compared to the control (Fig. R9, A). Also, we evaluated the actin cytoskeleton organization by performing immunofluorescent staining of F-actin: H295R cultured in the presence of ASCs showed an altered morphology compared to H295R cultured alone, acquiring a more elongated and polarized shape. Moreover, their cytoplasmic stress fibers appeared less defined and F-actin seemed to form focal structures rather than homogeneously distribute along the plasma membrane (Fig. R9, B). This suggests that a cytoskeletal re-organization supporting cancer cell migration is induced during co-culture.

3) *The presence of H295R impairs ASC ability to efficiently differentiate toward mature white adipocytes*

Focusing on the other cell compartment of our co-culture system, we evaluated the functional properties of ASCs when cultured in the presence of H295R compared to ASC mono-colture. As already mentioned, ASCs constitute the stem reservoir for the adipose tissue. Under appropriate stimulation, they are able to differentiate toward white adipocytes, with a substantial change in cell morphology, gene expression and adipokine secretion. To assess this property in our system, we cultured ASCs, alone or together with H295R, in the presence of a specific medium inducing adipogenic differentiation. After 10 days, we analyzed the expression of specific markers related to adipose functionality, such as Adiponectin (AdipoQ), FABP4 and Hormone Sensitive Lipase (HSL) genes: H295R co-culture drastically reduced the expression of all the three genes in the obtained differentiated adipocytes, as showed by Taqman assay (Fig. R10, A). As adipocyte differentiation correlates with the amount of intracellular lipids stored in typical triglyceride droplets, we evaluated the lipid content in adipocytes induced to differentiate in the presence or not of H295R. Quantification of cell triglyceride content by AdipoRed assay showed that adipocytes obtained during H295R co-culture significantly

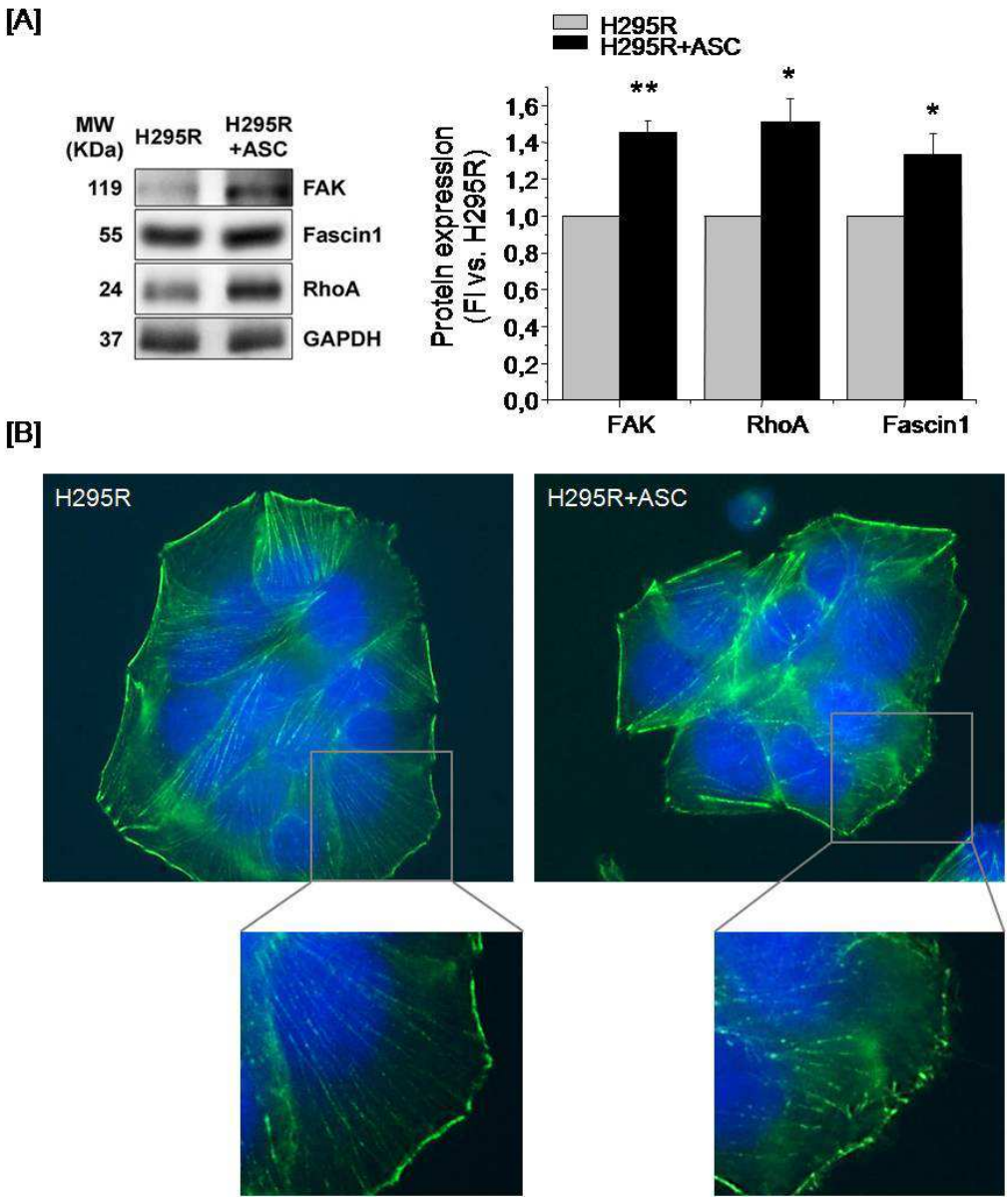


Figure R9. Expression of migration-related proteins and F-actin immunofluorescent staining in H295R after mono- or co-culture. A) Western blot analysis of FAK, RhoA and Fascin-1 were performed on cell lysates of H295R cultured alone (H295R) or together with ASCs (H295R+ASC) for 7 days. Densitometric quantification of protein bands was carried out using GAPDH as internal loading control. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$. B) Representative images of immunofluorescence performed on H295R after mono- or co-colture stained for the F-actin cytoskeleton (green). DAPI staining (blue) was used to visualize cell nuclei. Original magnification: X100; zoom in: X2.

decreased their lipid content compared to the control (Fig. R10, B), thus indicating that the presence of cancer cells affects the ability of ASCs to efficiently differentiate toward mature adipocytes, which instead achieve an intermediate state between undifferentiated ASCs and mature adipocyte. Consistently, images of the same

adipocytes acquired by epifluorescence microscopy revealed a considerable difference in lipid droplet size: while ASCs cultured alone produced adipocytes with larger lipid droplets stuffing the entire cytoplasm, adipocytes differentiated from ASCs co-cultured with H295R showed significantly smaller lipid droplets. (Fig. R10, C).

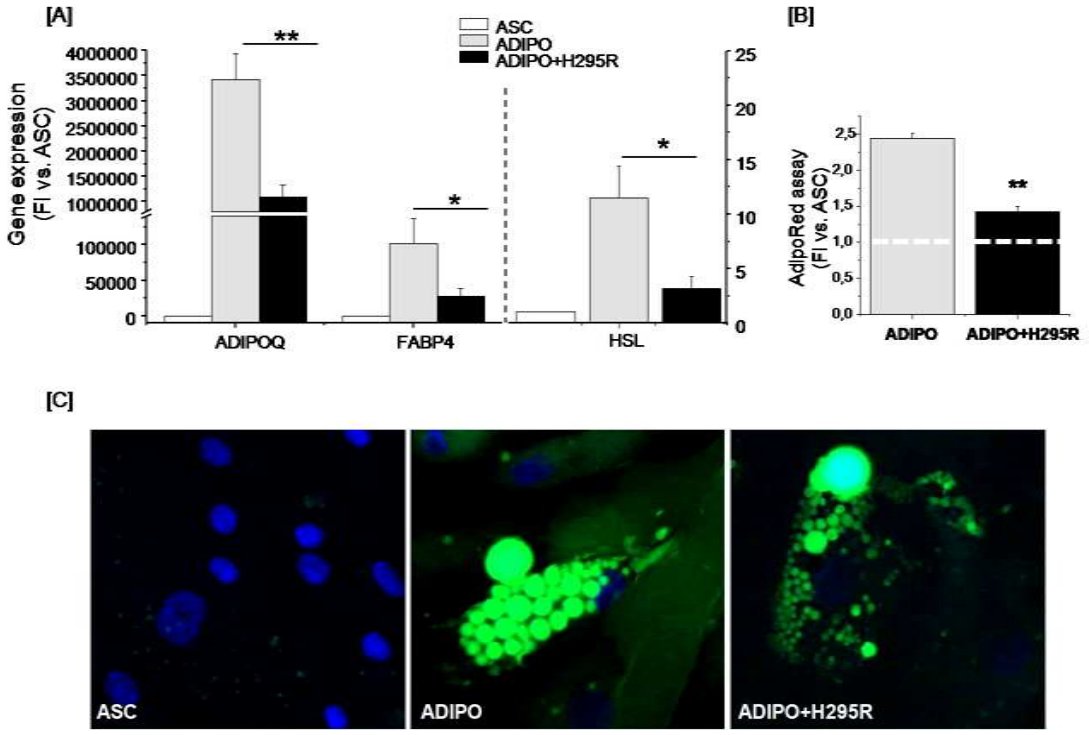


Figure R10. The presence of H295R affects ASC ability to efficiently differentiate toward mature white adipocytes. A) Taqman assay was performed on cell samples from ASCs, used as negative control, and adipocytes differentiated *in vitro* from ASCs alone (ADIPO) or co-cultured with H295R (ADIPO+H295R) for 10 days. Gene expression related to AdipoQ, FABP4 and HSL was calculated as fold increase vs. ASCs. AdipoQ- and FABP4-fold increase refer to the left Y axis, whereas HSL fold increase to the right one. B) Adipocyte lipid content, assessed by AdipoRed assay, was calculated as fold increase vs. ASC, whose value = 1 is indicated by the white dashed line. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$, ADIPO+H295R vs. ADIPO. C) Representative images of AdipoRed-related fluorescence staining intracellular lipid droplets (green) in samples from ASC (negative control), ADIPO and ADIPO+H295R. Cell nuclei were counterstained with DAPI (blue). Original magnification: X20.

However, the morphology of adipocytes differentiated during co-culture may also be consistent with that of brown adipocytes (see *Introduction*, *chapter 3*). As there is evidence in the literature that the induction of white adipose tissue (WAT) browning may play an important role in cancer (Kir *et al*, 2014; Petruzzelli *et al*, 2014;

Singh *et al*, 2016), one could speculate that a similar process could take place in our co-culture system. In support of this hypothesis, we found that adipocytes differentiated in vitro in the presence of H295R express the Uncoupling Protein 1 (Ucp-1) gene compared to ASCs used as negative control (19.92 ± 5.04-fold; $P < 0.05$). However, further analysis is needed to confirm such a differentiation shift from WAT to BAT characteristics.

4) *The potential molecular interaction underlying H295R/ASCs crosstalk*

As already described, our co-culture system allows the two cell types to interact thanks to the exchange of soluble molecules through the porous membrane separating the two compartments. Therefore, one of the main purposes was to investigate the factors produced and secreted by both ASCs and H295R potentially involved in their crosstalk.

Metabolic adaptation is one of the key process occurring within the tumor microenvironment, with lactate playing a central role in mediating tumor/stroma cross-interaction and in sustaining tumor progression (see *Introduction*, *chapter* 3). Basing on the results obtained on cell proliferation during co-culture, we assessed the concentration of glucose and lactic acid in the conditioned media of cells co-cultured or cultured alone for 72 hours, in order to evaluate whether a metabolic change toward aerobic glycolysis was occurring. In our *in vitro* reproduced microenvironment, we measured decreased glucose levels, accordingly with the previously observed increase of glucose up-take in both cell types; instead, lactic acid concentration significantly increased, particularly in the media related to co-cultured ASCs (Tab. R1). This suggests on one hand that the H295R-induced higher proliferation in co-cultured ASCs may rely on a metabolic shift toward glycolysis; on the other one, that the augmented proliferation of H295R in co-culture could be fueled by lactate produced by ASCs and released into the medium. However, further investigation is needed to confirm the role of lactate in H295R/ASCs co-culture system.

Conditioned medium	ASC	ASC+H295R	H295R	H295R+ASC
GLUCOSE	1.0	0.57 ± 0.1 *	1.0	0.75 ± 0.22
LACTIC ACID	1.0	1.78 ± 0.03 *	1.0	1.49 ± 0.21 *

Table R1. Glucose and lactic acid content in conditioned media from mono- and co-cultures. Immuno-enzymatic assay was performed to meseaure glucose and lactic acid concentration in conditioned media from both ASCs and H295R cultured alone or in co-culture. The absolute concentration was normalized on the relative cell number and expressed as fold increase vs. the mono-colture. * $P < 0.05$, co-culture vs. mono-culture condition.

Other soluble factors specifically produced by ASCs or H295R, and involved in potentially prompting cell proliferation, were further investigated. Among all the adipokines produced by ASCs, leptin and interleukin-8 (IL-8) have been associated with increased cancer proliferation, migration and invasion (Delort *et al*, 2015; Wang *et al*, 2015; Cao *et al*, 2016). Accordingly, gene expression analysis of cell samples from ASCs co-cultured with H295R for 7 days revealed a statistically significant increment of both leptin and IL-8, a cytokine involved in upregulation of leptin production, compared to the control (Fig. R11, A). A concomitant increase of leptin receptor (Ob-R) gene expression was observed in H295R in the same co-culture setting compared to H295R cultured alone (Fig. R11, B). Moreover, the same H295R in co-culture showed a decreased expression of both IGF2 and its receptor, IGF-1R (Fig. R11, B). Thus, the Leptin/Ob-R signaling may underlie the increased proliferation of cancer cells in our system, indeed activating a pro-proliferative pathway alternative to the IGF2 paracrine-autocrine loop typically up-regulated in adrenocortical carcinoma (Cantini *et al*, 2008).

To elucidate the molecular mechanisms underlying the higher motility/migratory ability of H295R when co-cultured in the presence of ASCs, we investigated the SDF-1 (CXCL12)/CXCR4/CXCR7 axis in our system, since it is one of the main activated pathways within the TME, sustaining tumor proliferation, migration and invasion (see *Introduction*, *chapter* 3). We assessed the level of gene and protein expression of the chemokine SDF-1, as well as its secretion, in ASCs co-cultured with H295R for 7 days compared to the control: differently from what expected, SDF-1 gene expression was decreased after co-culture (Fig. R12, A), as well as protein expression (Fig. R12, B).

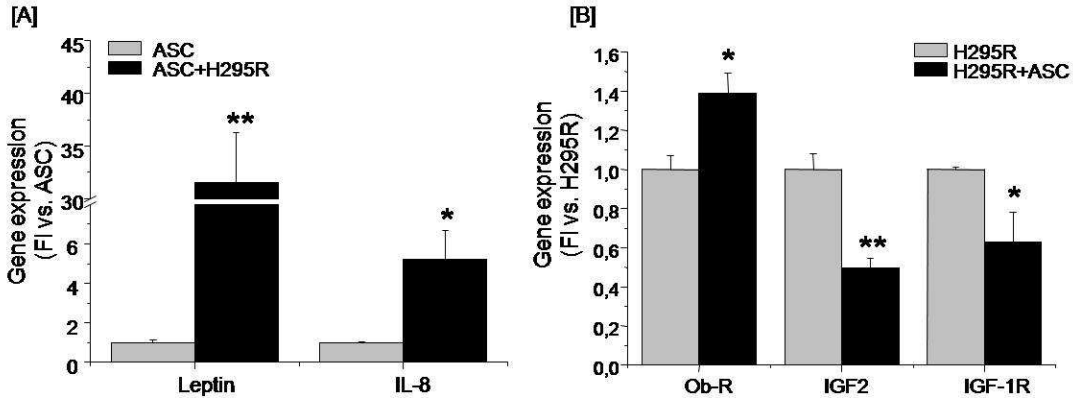


Figure R11. Potential molecular crosstalk underlying cancer cell proliferation. A) Leptin and IL-8 gene expression was assessed by Taqman assay in cell samples derived from 7 day ASC mono- (ASC) or co-culture (ASC+H295R). The relative expression level was calculated as fold increase vs. ASC. B) Gene expression of Ob-R, IGF2 and IGF-1R in H295R cultured alone (H295R) or together with ASCs (H295r+ASC) for 7 days, was calculated as fold increase vs. H295R. Data are expressed as the mean ± SE in least three independent experiments. * $P < 0.05$; ** $P < 0.001$, co-culture vs. mono-culture.

However, when assessed in the conditioned media, the levels of the secreted SDF-1 were significantly higher in ASCs co-cultured compared to ASCs cultured alone (Fig. R12, C). Thus, we hypothesized that, in the setting of the co-culture, high amount of secreted SDF-1 induce a negative loop inhibiting the further production of SDF-1 by ASCs. In addition, we observed a concomitant decreased gene expression of the Dipeptidyl Peptidase 4 (DPP4), a peptidase able to degrade multiple chemokines, including SDF-1. Therefore, lower levels of DPP4 could assure a higher availability of SDF-1, thus concurring to the negative regulation of this chemokine production. On the other hand, we assessed the expression of the SDF-1 receptors, namely CXCR4 and CXCR7, in H295R co-cultured or not with ASCs: after 7 day co-culture, H295R decreased CXCR4 expression, while increased CXCR7 expression. Thus, in our system a shift between CXCR4 and CXCR7 expression occurs, being the axis activation likely mediated CXCR7-downstream pathways.

Finally, we investigated the presence of factors potentially involved in the immunomodulatory mechanisms that have widely been described to occur following the crosstalk between tumor cells and their stromal microenvironment, contributing to tumor progression. Interleukin- 6 (IL-6) and Monocyte Chemoattractant Protein-1 (MCP-1) are two of the main inflammatory cytokines produces by ASCs. We assessed the expression of both IL-6 and MCP-1 genes in ASCs co-cultured with H295R for 7 days, observing a significant decrease compared

to control ASCs (Fig. R13, A). This suggests that adrenocortical cancer cells may be able to achieve immune-tolerance by affecting adipose inflammatory cytokine production. Another mechanism could be linked to increased levels of cortisol production: in fact, this glucocorticoid is specifically produced by adrenocortical cells and exerts anti-inflammatory effects. Accordingly, we detected higher level of cortisol in the conditioned media of to H295R co-cultured with ASCs compared to the control. Concomitantly, an increased expression of the Hydroxysteroid 11-beta Dehydrogenase type 2 (HSD11B2) gene, encoding for the enzyme that converts cortisol to cortisone, was detected in co-cultured H295R compared to the mono-culture (Fig. R13, B). Interestingly, HSD11B2 expression was even higher when H295R were co-cultured with ASCs induced to differentiate toward mature adipocytes, i.e. a mixed culture consisting of both undifferentiated ASCs and pre-adipocytes.

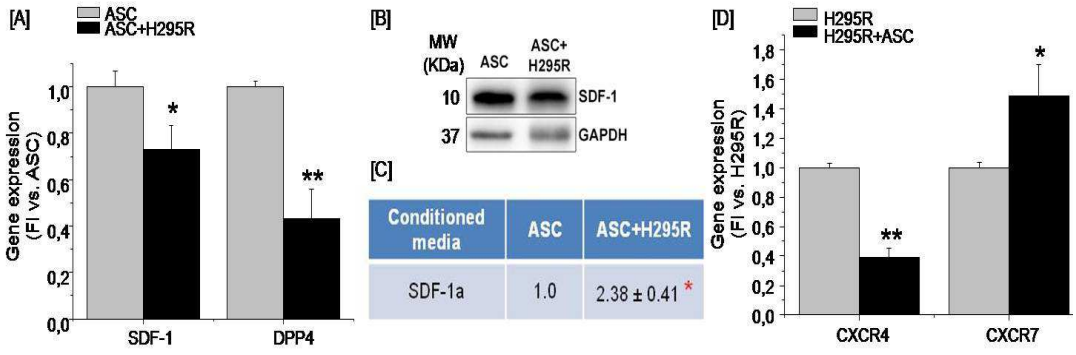


Figure R12. SDF-1/CXCR4/CXCR7 axis in H295R/ASCs co-culture. Gene expression related to SDF-1, and DPP4 (A) and CXCR4 and CXCR7 (D) genes was assessed by Taqman assay in ASCs and H295R respectively, after 7 day co-culture compared to the single culture. Expression levels were calculated as fold increase vs. the relative controls. B) Western blot analysis of SDF-1 in ASCs co-cultured compared to the control. GADPH was used as internal loading control. C) Extracellular levels of SDF-1 measured by ELISA assay in the conditioned media of ASCs after 7 day mono- or co-culture. The absolute concentrations were normalized on the relative cell number and expressed as fold increase vs. ASC. Data are expressed as the mean ± SE in least three independent experiments. * $P < 0.05$; ** $P < 0.001$, co-culture vs. mono-culture.

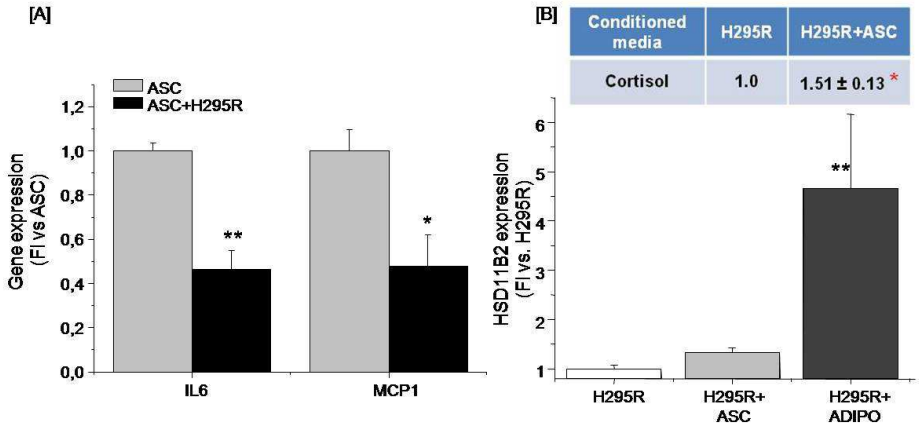


Figure R13. Factors potentially involved in immunomodulatory mechanisms in co-culture. Gene expression related to IL-6, and MCP-1 (A) and HSD11B2 (B, lower panel) genes was assessed by Taqman assay in ASCs and H295R respectively, after 7 day co-culture compared to the single culture. Expression levels were calculated as fold increase vs. the relative controls. C) Extracellular levels of cortisol measured in the conditioned media of H295R after 7 day mono- or co-culture. The absolute concentrations were normalized on the relative cell number and expressed as fold increase vs. H295R. Data are expressed as the mean ± SE in least three independent experiments. * $P < 0.05$; ** $P < 0.001$, co-culture vs. mono-culture.

5) A reciprocal interaction is established also between H295R and "in vitro" differentiated adipocytes

Taking together, all findings described above illustrate that, during co-culture, H295R are able to induce significant morphological and functional alterations in ASCs, which in turn concur to increase cancer cell proliferation and invasiveness. Since a physiological adipose microenvironment would consist of mature adipocytes with their stem pool of ASCs, we decided to investigate whether co-culturing H295R with *in vitro* differentiated adipocytes induced some effects on the two cell compartments. At this purpose, we induced ASCs to differentiate for 7 days in the presence of the appropriate adipogenic medium, keeping culturing the obtained pre-adipocytes in the presence of H295R for additional 9 days. First, we evaluated the proliferative rate of H295R co-cultured with adipocytes compared to the control: even if we observed only a slight non statistically significant increase in cell proliferation in co-cultured H295R compared to the control (5.01 ± 0.28 vs. 4.68 ± 0.86-fold), Western Blot analysis revealed a higher expression of MEK and p-ERK (Fig. R14, left panel), indicating that MAPK-mediated pathway is enhanced by the

presence of adipocytes. Moreover, the expression of the migration-related proteins FAK, RhoA and Fascin-1 was also increased (Fig. R14, right panel), suggesting that also mature adipocytes contribute to cancer cell migration/invasiveness.

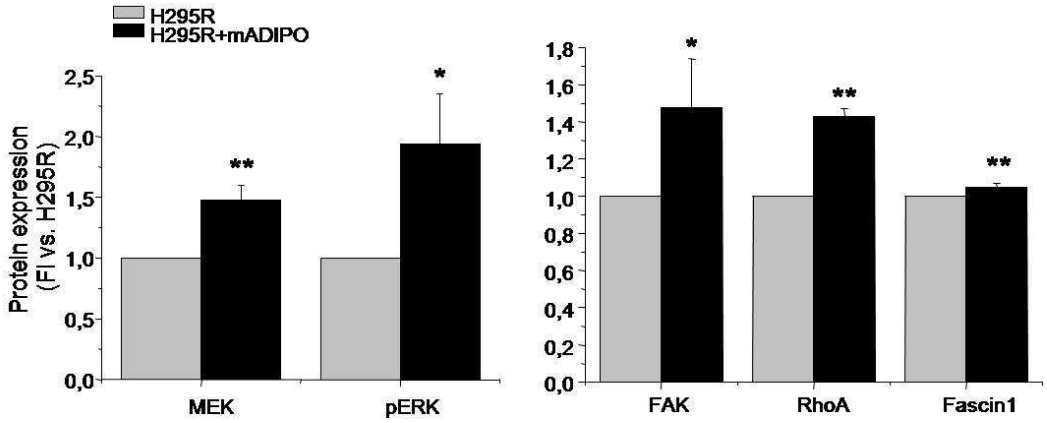


Figure R14. Expression of proliferation- and migration-related proteins in H295R co-cultured with mature adipocytes. Protein expression related to MEK, p-ERK, FAK, RhoA and Fascin-1 was assessed by Western blot analysis in cell samples from H295R co-cultured for 9 days with previously *in vitro* differentiated adipocytes (H295R+mADIPO) compared to the control (H295R). Bar charts represent protein expression quantified by densitometric analysis of protein bands normalized on GAPDH, used as internal loading control, and calculated as fold increase vs. H295R. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$, co-culture vs. mono-culture.

We further evaluated the effect of H295R on adipocyte functionality: by assessing Adiponectin (AdipoQ) and FABP4 gene expression, we observed a statistically significant decrease in the expression of both genes in adipocytes co-cultured with H295R compared to adipocytes cultured alone (Fig. R15, A). This was accompanied by a significant decrease in lipid content, quantified by AdipoRed assay and confirmed by the observational analysis of adipocyte lipid droplets by epifluorescence microscopy (Fig. R15, B, C). Such a decrease in the lipid content in adipocytes interacting with cancer cells is consistent with the widely described delipidation process occurring within the tumor microenvironment. Also, accordingly to what observed in the literature, adipocytes co-cultured with H295R expressed significantly higher levels of leptin compared to the control (4.50 ± 0.3-fold; $P < 0.001$).

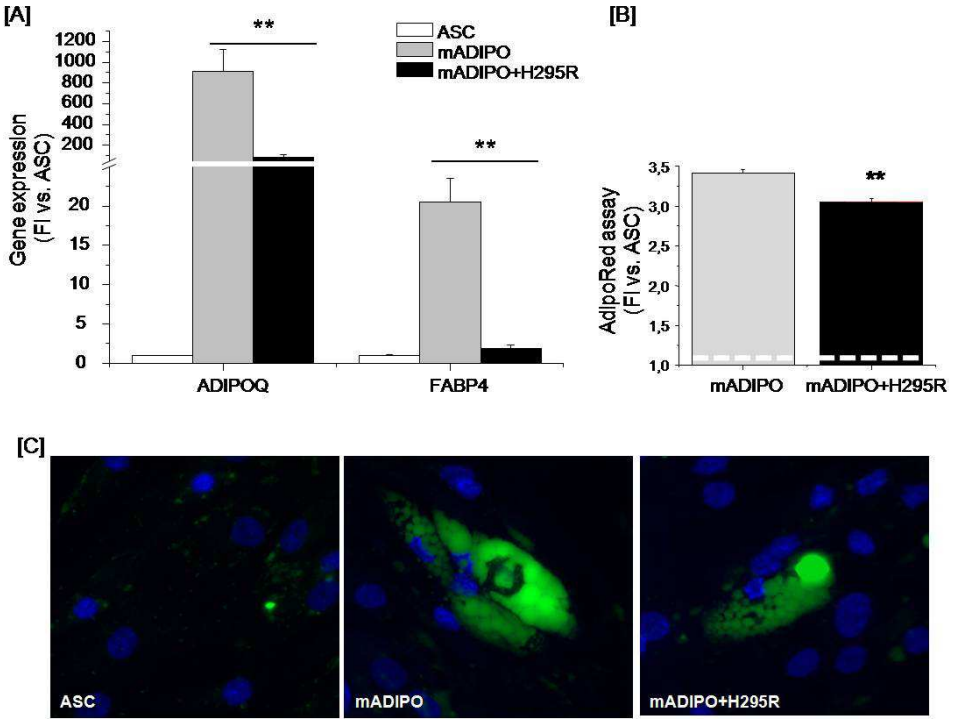


Figure R15. The presence of H295R affects the functionality of mature adipocytes. A) Taqman assay was performed on cell samples from ASCs, used as negative control, and *in vitro* differentiated adipocytes cultured alone (mADIPO) or co-cultured with H295R (mADIPO+H295R) for 9 days. Gene expression related to AdipoQ and FABP4 is expressed as fold increase vs. ASCs. B) Adipocyte lipid content, assessed by AdipoRed assay, was calculated as fold increase vs. ASC, whose value = 1 is indicated by the white dashed line. Data are expressed as the mean ± SE in at least three independent experiments. * $P < 0,05$; ** $P < 0,001$, co-colture vs. mono-colture. C) Representative images of AdipoRed-related fluorescence, staining intracellular lipid droplets (green) in samples from ASC (negative control), mADIPO and mADIPO+H295R. Cell nuclei were counterstained with DAPI (blue). Original magnification: X40.

Discussion

In the complex setting of a cancer mass, several metabolic and molecular interactions are continuously established within the tumor microenvironment (TME) between cancer cell and the surrounding stroma, consisting of both different cell types and extracellular matrix components. Such a dynamic crosstalk leads to substantial modification in both cancer and stromal cell behavior, eventually supporting tumor growth and progression. The role of adipocytes and adipose progenitors as active players within this dynamic crosstalk has been demonstrated for many types of malignancy, with particular implication for tumor initiation and growth, as well as for local invasion and metastasis (Duong *et al*, 2017). Studying such interactions would help in elucidating canceralso the biology of adrenocortical carcinoma: in fact, adrenal glands present a substantial component of adipose tissue, thus the cross-talk with the surrounding adipose microenvironment may play a pivotal role in adrenocortical tumorigenesis and ACC progression. Unraveling the metabolic and molecular dynamics occurring within the ACC TME would allow to develop new therapeutic strategies addressing the specific cancer cells-microenvironment crosstalk.

In this study we reproduced a simplified *in vitro* tumor microenvironment by co-culturing the adrenocortical tumor cells H295R with cells of adipose lineage, both adipose-derived stem cells (ASCs) and *in vitro* differentiated adipocytes, in order to evaluate the paracrine effects of the adipose component on H295R cell behavior, as well as the influence of H295R cells on the adipose compartment. The co-culture system we employed, consisting of specific 6-well inserts with a porous membrane, was particular helpful to address our aim, since it allowed to maintain physically separated the two cell types, concomitantly permitting the exchange of soluble factors between the two compartments. Thus, in the setting of the same experiments, we assessed different features in the two cell compartments at the same time.

First investigating the co-culture effect on cancer cells, we evaluated the H295R proliferation in the presence an adipose microenvironment, meaning ASCs or mature adipocytes, compared to H295R mono-culture, demonstrating that cancer cells enhance their proliferation rate when co-cultured with adipose cells. This increase can be likely mediated by the up-regulation of the MAPK intracellular

signaling, as supported by the observed protein over-expression of MEK and p-ERK. Beyond this effect on cell proliferation, we demonstrated that the presence of adipose cells trigger H295R migration and invasiveness, as shown by both wound healing and invasion assays, as well as by the increased expression of migration-related proteins, particularly Focal adhesion kinase (FAK), RhoA and Fascin1. These three proteins are involved in the cytoskeletal re-organization underlying cell motility, inducing the formation of specialized structures, such as focal adhesion, lamellipodia and filopodia. A variation in cell-cell interactions and the stimulation of cytoskeleton re-organization was indeed evident in H295R stimulated by the presence of ASCs. Notably, the three proteins have been found over-expressed in a variety of human tumors, associating with cancer aggressiveness. Particularly, FAK seems to play a crucial role in the TME, promoting tumor progression and metastasis through effects on both cancer and stromal cells: this is a cytoplasmic protein tyrosine kinase that exerts multiple functions controlling cell movement, invasion, survival, gene expression related to the epithelial-to-mesenchymal transition (EMT), and cancer stem cell self-renewal (Sulzmaier *et al*, 2014). RhoA is part of the Rho GTPases family, an ubiquitously expressed division of GTP-binding proteins involved in the regulation of cytoskeletal dynamics and intracellular signaling, whose abnormal expression and activation have major consequences for cancer progression and metastasis (Jansen *et al*, 2017). Finally, Fascin-1, a filamentous actin-binding protein, is a crucial in organization and functionality of cell protrusions for cell migration, and has been proposed as a prognostic biomarkes in several cancer types (Adams, 2015; Min *et al*, 2015; El-Balat *et al*, 2016). Notalby, it has been demonstrated to be a potential biomarker in adrenocortical carcinoma (Poli *et al*, 2015). Therefore, in our co-culture system Fascin -1 up-regulation becomes particularly meaningful.

If on one hand we were interested in exploring how the adipose microenvironment may sustain cancer cell aggressiveness, on the other one we also wanted to evaluate the alterations induced by cancer cells on both ASCs and mature adipocytes as part of their crosstalk. The main finding observed was that H295R are able to actively "recruit" ASCs, affecting their morphology and functionality and driving their differentiation toward a phenotype more prone to sustain tumor growth and progression. Accordingly to what demonstrated in other cancer types (Jeon *et al*, 2010; Jotzu *et al*, 2010; Cho *et al*, 2011, 2012; Do *et al*, 2012; Park *et al*, 2013),

H295R-stimulated ASCs enhanced their proliferation and acquired a myofibroblast-like phenotype, concomitantly decreasing their stem markers. Accordingly, morphologic alterations were clearly observed in ASCs after co-culture with H295R. Moreover, when induced to differentiate in the presence of cancer cells, ASCs ability to give rise to mature adipocytes was impaired. Instead, they achieved an intermediate state between undifferentiated ASCs and mature adipocytes, which probably serves more efficiently to cancer cell sustaining. As alternative hypothesis, it may be possible that ASC differentiation is shifted by cancer cells toward browning, since we observed the expression of the brown adipocyte-specific Uncupling Protein-1 (UCP-1) gene in adipocytes differentiated in the presence of H295R. This may be consistent with evidence indicating the potential occurrence of white adipose tissue browning in cancer, particularly related to cancer-associated cachexia: brown adipocytes, in fact, are responsible for thermogenesis, that has been hypotesized to underlie energy wasting and fat and muscle atrophy (Kir *et al*, 2014; Petruzzelli *et al*, 2014; Singh *et al*, 2016).

The ability of cancer cells of altering the adipose microenvironment was further confirmed by co-culturing H295R and *in vitro* differentiated adipocytes, which showed a decreased functionality, measured in terms of decreased expression of specific adipogenic markers (i.e. Adiponectin and Fatty Acid Binding Protein 4 - FABP4 - genes), as well as a loss of intracellular lipid content. This could indicate a lipolytic process induced by cancer cells and the release of free-fatty acids (FFAs), as widely described in other cancer types (Dirat *et al*, 2011; Nieman *et al*, 2011, 2013; Park *et al*, 2014; Balaban *et al*, 2017; Wang *et al*, 2017; Wen *et al*, 2017). FFAs, in fact, represents the main energy source provided by adipocytes within the TME to fuel cancer cells. Alternatively, the decreased expression of Adiponectin and FABP4, could be interpreted as an induced de-differentiation of adipocytes toward a more plastic and metabolically active cells, such as the fibroblast-like adipocyte-derived cells described in other types of cancer (Chirumbolo & Bjørklund, 2016; Zoico *et al*, 2016).

Metabolic adaptation represents one of the key process allowing tumor cell survival and proliferation. Lactate, produced at high levels in the TME following the reverse Warburg effect (Pavlides *et al*, 2009; Chiarugi & Cirri, 2016), has been described as one of the major players mediating tumor/stroma cross-interaction and

sustaining tumor progression. Alterations in glucose and lactic acid concentration were observed in the extracellular compartment of our co-culture system: consistently with a possible metabolic shift toward aerobic glycolysis, glucose level decreased in the conditioned media of both H295R and ASCs, with a concomitant increase of glucose up-take by cells, whereas lactic acid concentration was higher, particularly when measured in the media related to ASCs. Thus, lactate may represent a key factor in mediating cell proliferation in a complex intercellular interaction, in which ASCs recruited by H295R increase glycolysis, producing lactate which, in turn, can fuel cancer cell metabolism.

Since our co-culture system actually allows a "soluble" interaction between the two cell compartments, the secreted factors released into the extracellular media are the obvious mediators of the observed effects. Adipose cells produce a variety of hormones, growth factors, chemokines, and adipokines, that play a critical role in modulating tumor microenvironment. Among them, leptin appears to be strongly involved in carcinogenesis and cancer metastasis, promoting cell proliferation and invasion, as well as angiogenesis (Duong *et al*, 2017). Leptin over-expression was observed in both ASCs and mature adipocytes co-cultured with H295R, which in turn up-regulated the expression of leptin receptor. This could explain the IGF-1R-independent up-regulation of the MAPK signaling observed in H295R co-cultured with ASCs, together with the decreased level of IGF2 gene expression: leptin may trigger a pro-proliferative pathway alternative to the IGF2 paracrine-autocrine loop typically up-regulated in adrenocortical carcinoma (Cantini *et al*, 2008).

Another pathway that seems to be involved in the crosstalk between adrenocortical cancer cells and the adipose microenvironment is represented by the CXCL12 (SDF-1)/CXCR4/CXCR7 axis, which has been shown to affect tumor progression by an autocrine/paracrine control of cancer cell survival, proliferation and migration (Guo *et al*, 2016). In our system, along with an increased concentration of SDF-1 measured in the conditioned media of co-cultured ASCs, we observed a de-regulation of CXCR4 and up-regulation of CXCR7 in co-cultured H295R. This is accordant with several evidence showing CXCR7 over-expression in a variety of cancers (Hattermann *et al*, 2010; Deutsh *et al*, 2013; Hu *et al*, 2014; Lin *et al*, 2014; Wu *et al*, 2015; Shi *et al*, 2017) and a role for CXCR7 in negatively regulating CXCR4 (Uto-Konomi *et al*, 2013). Interstingly, in a mouse model of metastatic

neuroblastoma, CXCR7 has been shown to specifically mediate cells cancer homing to the adrenal glands (Mühlethaler-Mottet *et al*, 2015). Also, CXCR7 seems to be specifically required for transendothelial migration (Mazzinghi *et al*, 2008), mediating cancer cell interaction with the endothelia.

To further investigate tumor adaptation within the TME, we also considered the immunomodulatory mechanisms possibly activated by the cross-interaction between H295R and adipose cells. The expression of genes encoding two of the main adipose inflammatory cytokines, Interleukin- 6 (IL-6) and Monocyte Chemoattractant Protein-1 (MCP-1), was decreased in ASCs after co-culture with H295R, thus suggesting that adrenocortical cancer cells may be able to achieve immune-tolerance through the modulation of cytokine production by adipose cells. In fact, lowering IL-6 levels could allow to mild the mobilization of anti-tumor T-cells (Fisher *et al*, 2014), while low levels of MCP-1 have been associated to modest monocyte infiltration, resulting in tumor formation (Nesbit *et al*, 2001). The increased cortisol production and the up-regulation of its metabolizing enzyme (Hydroxysteroid 11-beta Dehydrogenase type 2 - HSD11B2) that we observed in co-cultured H295R, may also concur to establish a low-level inflammation in the TME, supporting tumor progression. Some evidence, in fact, indicates that high levels of cortisol and HSD11B2 promote tumorigenesis and mediate immunomodulation in other types of cancer (Sidler *et al*, 2011; Voisin *et al*, 2017;Wu *et al*, 2017).

In conclusion, our findings demonstrate that an active metabolic and functional crosstalk is established between adrenocortical cancer cell and cells from the adipose lineage. However, as an *in vitro* experimental model, our co-culture system present some limitations, fist of all represented by the pore size (0.4 µm diameter) of the insert membrane, which allows soluble factors and exosomes to pass from a compartment to the other, but likely prevents the exchange of larger secreted vesicles. Some type of experiments required H295R to be cultured inside the inserts thus, considering their small dimensions, larger pores should be avoid not to risk cell migration through the membrane. An alternative method to study the effect of secreted factors on cell behavior would be the use of conditioned media of one cell type directly in the mono-culture of the other. However, in this case the possibility to follow a dynamic intercellular crosstalk would be loss. Another point is represented by the cell models of adrenocortical carcinoma and adipose

microenvironment employed: H295R are not effectively reproducing the specific characteristic of ACC, whereas ASCs are derived from different individuals and would show variations in their functionality and differentiation ability. However, even being a simplified model of tumor microenvironment, our models allows to detect substantial modification in the two different compartments, pointing out the potential role of the surrounding adipose microenvironment in sustaining adrenocortical tumorigenesis and cancer progression.

PART II - Detection and characterization of circulating tumor cells in adrenocortical carcinoma

Materials and methods

- **Patients and samples.** Fourteen patients evaluated at our University Hospital for adrenocortical carcinomas who underwent surgery and are currently under follow-up were enrolled for this study, which was approved by the Local Ethical Committee. All patients agreed by written informed consent. Blood samples were collected in K2-EDTA tubes during standard blood draws, kept at 4°C and processed within 3 hours. For most of the patients, samples related to different time points were collected.

- **Blood filtration and CTC isolation.** Blood samples were processed by using two different ScreenCell® filtration devices for CTC isolation, namely ScreenCell Cyto and MB kits, allowing for cytomorphological analysis and DNA extraction respectively. The filtration was performed according to the manufacturer's instructions. Briefly, with the Cyto kit, 3 ml of blood were diluted in a specific dilution buffer for red blood cell lysis and cell fixing. A total volume of 7 ml of diluted blood was transferred into the filtration unit and filtered through the microporous filter, adding 1,6 ml PBS in order to remove red blood cell debris. At the end of the filtration process, each track-etched microfilter was released from the filtration module, placed on absorbing paper and washed with PBS, allowed to air dry and further stored at 4°C. For each patient, the blood sample was filtered in duplicate, while a third parallel filtration was performed by using the ScreenCell® MB kit: 6 ml blood, diluted with 1 ml of specific dilution buffer, were filtered as described above. The filter was finally released in the provided Eppendorf tube and stored at -20°C (Fig. R16).

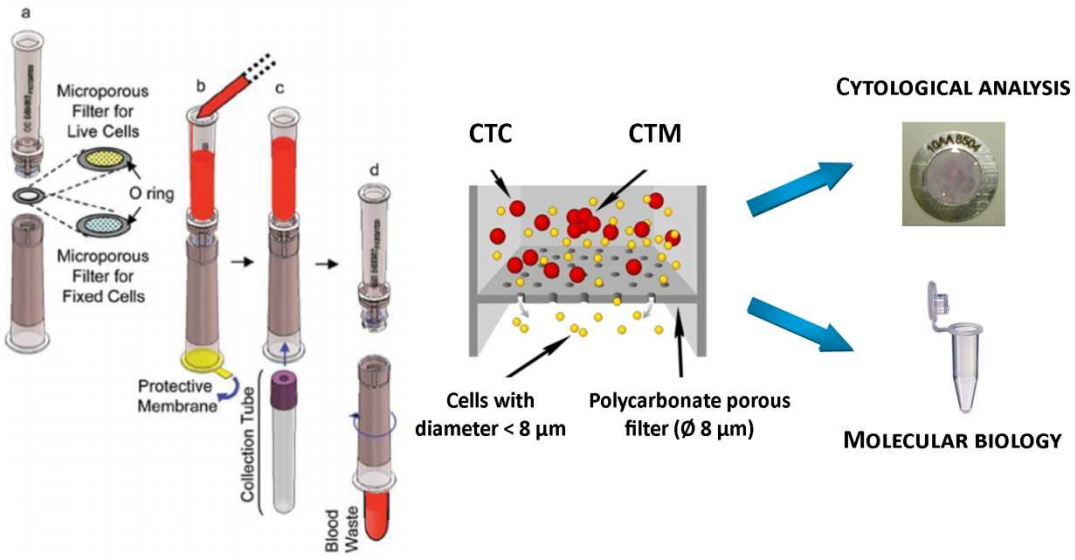


Figure R16. Schematic representation of the described CTC isolation workflow.

- **CTC identification, enumeration and characterization.** Track-etched filters was used to perform cytological studies, including hematoxylin/eosin staining and immunocytochemistry. Each filter was incubated with Hematoxylin S (Merck KGaA) for 2 minutes at room temperature, washed in distilled water and incubated with Shandon Eosin Y (Thermo Fisher Scientific) for 1 minute. Excess of eosin was removed with distilled water and the filter was allowed to air dry, placed on a standard microscopy glass slide and mounted with Faramount mounting medium (DAKO) and 7 mm circular cover slip. CTCs were identified and enumerated under light microscope according to the following morphological criteria: cell size ≥ 16 µm, nucleo-cytoplasmic ratio ≥ 50%, irregular nuclear shape, hyperchromatic nucleus, basophilic cytoplasm. To confirm cell adrenocortex origin, immunocytochemistry was performed as following: filters were hydrated with Tris-buffered saline (TBS; pH 7.4) and incubated with permeabilizing buffer (TBS-0,2% Triton 100X) for 5 minutes at room temperature. Heat-induced epitope retrieval was performed by incubating filters in a bath with Target Retrieval Solution (pH 9.0) (Dako) at 99°C for 20 minutes. Each filter was incubated overnight with 70 µl monoclonal mouse anti-human SF-1 (Upstate; dilution 1:50 in TBS-1% BSA), washed with TBS and immersed in distilled water. Staining was achieved by treating each spot with 70 µl EnVision Detection System Peroxidase/DAB, Rabbit/Mouse (K5007; Dako) for 40 minutes at room temperature, followed by chromogen 3.3' diaminobenzidine

(Dako) for 10 minutes at room temperature. Filters were rinsed with water and nuclei were counterstained with Hematoxylin S.

- **Genomic DNA purification from CTCs and whole genome amplification.** CTCs isolated with ScreenCell MB kit were processed according to the manufacturer's protocol for DNA extraction, purification and amplification: filters were incubated with an appropriate lysis buffer at 56°C for 10 minutes and centrifuged 1 minute at 12000 g. The DNA within the flow-through was column purified through sequential centrifugation steps and finally eluted in 23 µl AE buffer (QIAamp DNA Micro Kit; Qiagen). Whole genome amplification was performed by using the GenomePlex® Single Cell Whole Genome Amplification kit (Sigma-Aldrich): DNA was fragmented by 1 hour incubation at 50 °C with an appropriate working solution, followed by 4 minutes at 99°C, and used for library preparation. Further amplification was performed in a final volume of 75µl mix in a thermal cycler as following: initial denaturation at 95°C for 3 minutes, 30 seconds at 94°C plus 5 minutes at 65°C for 25 cycle, hold at 4°C. Negative and positive controls were amplified along with samples.

- **Targeted NGS sequencing.** Tumor DNA from patients previously assessed for mutations was sequenced by NGS on a Ion PGM system (Thermo Fisher Scientific) with a targeted panel designed with the Ion AmpliSeq Designer tool (Thermo Fisher Scientific) and including 9 genes described as mutated in ACC (ZNRF3, TP53, CTNNB1, CDKN2A, MEN1, RB1, CDK4, GNAS, PRKACA) and a selection of 173 heterozygous SNPs distributed along chromosome arms associated with LOH in ACC. Libraries were prepared according to the Ion AmpliSeq protocol: the selected genes and SNPs were amplified by two multiplex PCR using 2 pools of primer pairs (355 total) in order to obtain amplicons with an average size of 150 bp. 100 ng genomic DNA were used for each PCR reaction. Libraries were further amplified, purified and sequenced by semiconductor sequencing technology (Ion Torrent, Thermo Fisher Scientific). Vcf files were annotated with Annovar. Data were analyzed by using the RStudio software.

- **Digital-droplet PCR (ddPCR).** The QX200 droplet digital PCRsystem (Bio-Rad) was used for mutant detection in selected tumor and CTC DNA samples according to the manufacturer's instructions. Two primer and probe pairs were

designed to amplify and target specific wild type and mutated sequences within the genes ZNRF3 (forward primer: 5'-CCGGTTTCACAGGAAGTG; reverse primer: 5' GACTCTTCAGCAATGGCTAA; wt probe: 5'-TGCAGCACCACAC, HEX-conjugated; mut probe: 5'- TGCAGCAACACACCT, FAM-conjugated) and TP53 (forward primer: 5'-GAGTCTTCCAGTGTGATG; reverse primer: 5'-CACCATCCACTACAACTAC; wt probe: 5'-TCATGCCGCCC HEX-conjugated; mut probe: 5-TCATG+C+T+G+CCCA FAM-conjugated) (Eurogentec). ddPCR reactions were performed in 20 µl mix using 18 ng DNA with the following cycling conditions: 10 minutes at 95°C, 30 seconds at 94°C plus 1 minute at 57°C for 40 cycles; 10 minutes at 98°C, hold at 4°C. Mutant and wild type allele concentration (copies/µl; CMUT and CWT, respectively) were determined basing of the number of positive and negative droplet for each fluorophore in each sample and mutant allele frequency (MAF) was calculated as following: MAF =CMUT/(CMUT +CWT).

Results

1) CTCs can be detected in the peripheral blood of ACC patients with different clinical features

We collected blood samples from 14 patients diagnosed for adrenocortical carcinoma, who underwent surgery and are currently under follow-up. Our cohort includes patients with different features related to disease presentation (tumor size, Weiss score, Ki67, stage), outcome (tumor recurrence or metastasis) and post-surgical treatment, as summarized in Tab. R2. For some of the patients, the mutational profile of the primary tumor was also available, since they were analyzed in the setting of an European cohort for the study of the genomic landscape of ACC (Assié *et al*, 2014). Where present, gene mutations and homozygous deletions are indicated.

For most of the patients, we performed blood draw at different time points, in order to have multiple samples over time. Blood was filtered to isolate circulating tumor cells (CTCs) by using the ScreenCell filtration devices, as described in the section above (see *Materials and methods*), and the circular microfilters were analyzed for CTC detection and characterization. Following hematoxylin/eosin staining, filters were assessed for CTC identification using the described cytomorphometric parameters. In our samples, we detected both single CTCs and CTC clusters, namely circulating tumor microemboli (CTM), considering morphometric parameters such as size (> 16 µm), irregular and hypercromatic nuclei and high nucleocytoplasmic ratio (Fig. R17, A, B, C). In some samples we also detected the so-called cancer-associated macrophages-like cells (CAML), characterized by large size, multilobular or multiple nuclei and voluminous cytoplasm (Fig. R17, D). The relative number of CTC, CTM and CAML detected for each sample of each patient is indicated in Tab. R2. Further immunocytochemistry analysis was performed to assess the adrenocortical origin of the detected CTCs: a nuclear positivity for the Steroidogenic Factor-1 (SF-1) was observed, indicating the adrenocortical nature of cells, as also confirmed by comparing the SF-1 immunohistochemistry performed on tissue samples from the primary tumor of the same patients (Fig. R18). Thus, CTCs can be detected in the peripheral blood of ACC patients, even years after surgical resection of the primary tumor.

Patient	Tumor size (cm)	Weiss	Ki67 (%)	Stage	Recurrent/ metastatic	Chemotherapy	Point mutations	Homozygous deletions	Blood sample	CTC	CTM	CAML
1	15	6	10	II	-	Mitotane	na	na	I	2	-	1
2	7,5	7	30	II	recurrent	Mitotane	ZNRF3	na	I	-	-	-
									II	2/3 (nuclei)	-	-
									III	-	-	-
									IV	-	-	-
3	na	na	na	na	recurrent	Mitotane	na	na	I	3/4 (nuclei)	-	-
					lung metastasis	Radiotherapy			I	-	9	-
4	14,5	7	15	II	-	Mitotane	-	ZNRF3	I	3/4	3	-
									II	3/4	-	-
									III	-	-	-
									IV	-	-	-
5	4	na	60	I	-	Mitotane	na	na	I	-	-	-
6	14	8	70	II	-	-	na	na	I	1	-	-
									II	9	-	1
									III	-	-	7
7	3	7	20	I	-	-	na	na	I	-	-	-
									II	-	-	-
8	3	3	7	I	-	-	na	na	I	2	13	3
9	9,5	7	30	III	-	Mitotane	-	ZNRF3	I	-	-	-
									II	-	-	-
10	4	3	5	I	-	-	GNAS	na	I	2 (nuclei)	-	-
11	13	6	10	III	breast metastasis	Mitotane	na	na	I	4/5	-	-
12	18	8	15	III	-	Mitotane	PRKAR1A	na	I	2	3	-
									II	2	1	-
									III	-	-	-
13	3	5	20	III	-	Mitotane	TP53; CTNNB1; ATRX	na	I	-	-	-
14	11	na	30	na	recurrent disseminating		na	na	I	-	1	-
									II	3	-	-

Table R2. Characteristics of ACC patients analyzed for CTC detection and characterization. na = not available; — = none.

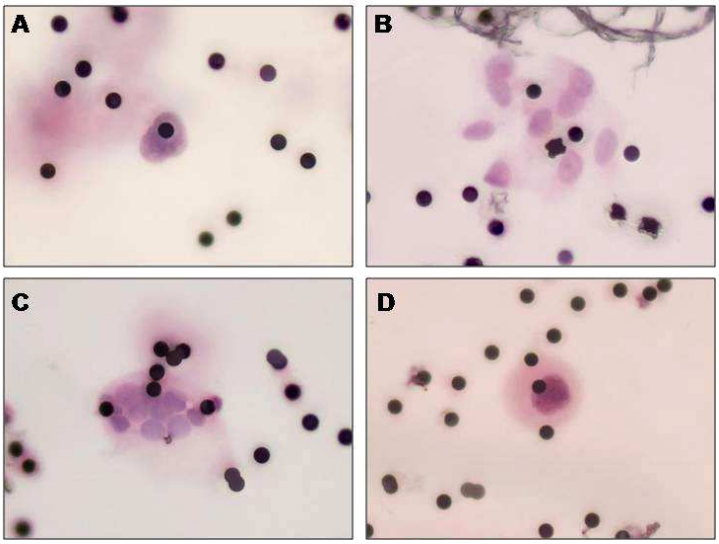


Figure R17. Representative images of hematoxylin/eosin staining of CTCs (A), CTM (B, C) and CAML (D), detected in the blood samples of ACC patients. Original magnification. X40

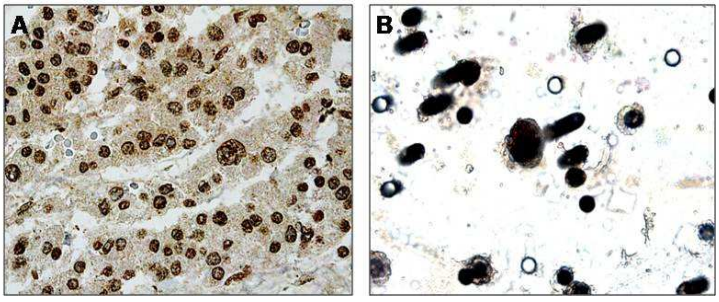


Figure R18. Representative images of nuclear SF-1 staining performed on primary tumor samples (A) and on isolated CTCs (B) in the same patient.

2) *Experimental design for the molecular characterization of isolated CTCs: preliminary results*

The principal aim of this study was to set up an experimental workflow to characterize at the molecular level the isolated CTCs, in order to compare their genetic profile with the primary tumor, thus eventually allowing to follow ACC evolution and progression in the single patient. This workflow includes a first step of CTC isolation and DNA extraction and amplification, aimed to obtain a sufficient amount of DNA feasible for further downstream applications, in particular targeted NGS and digital-droplet PCR (ddPCR) (Fig. R19). The purpose was to assess the CTC DNA for the presence of single point mutations already detected in the primary tumor of the same patients, and to eventually obtain a single cell genetic profile to be compared to that of the primary tumor.

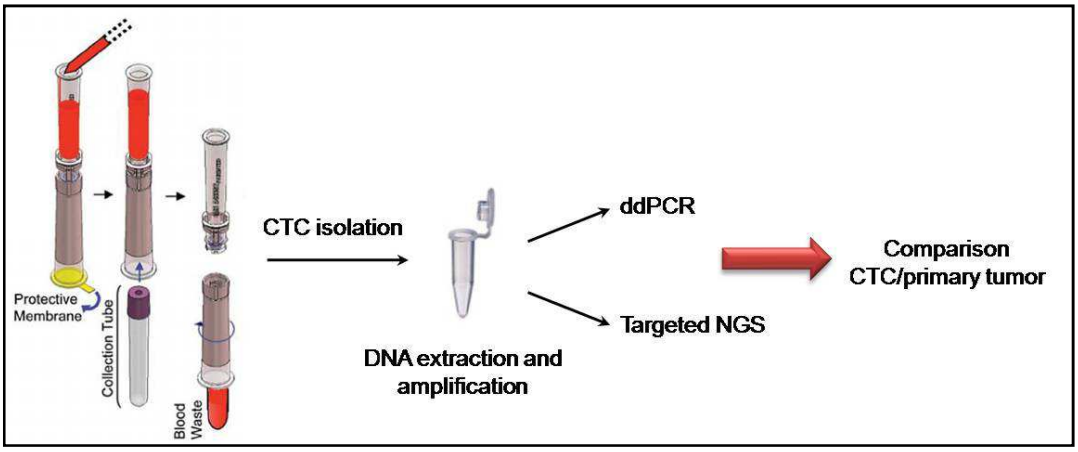
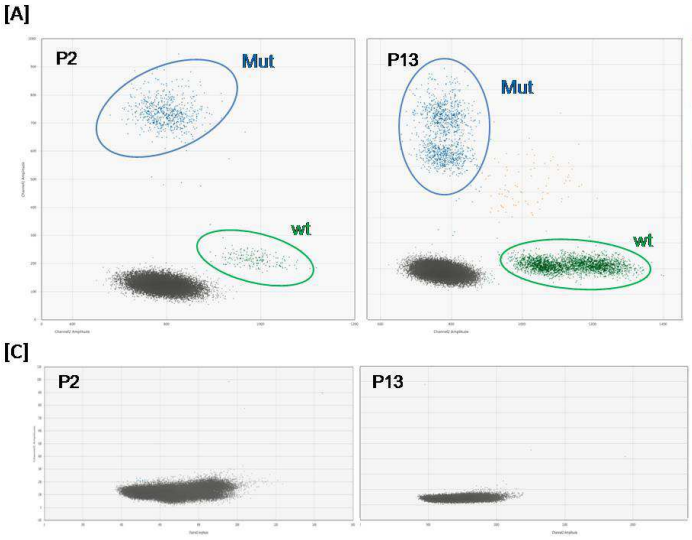


Figure R19. Schematic representation of the experimental workflow for the molecular characterization of circulating CTC isolated from blood sample of ACC patients.

We started from patients whose tumor samples were already analyzed by NGS assay and SNP array (as part of European cohort for the study of the genomic landscape of ACC; Assié *et al*, 2014), in order to detect the presence of mutations and chromosome alterations (i.e. loss of heterozygosity, LOH). In particular, we selected two patients showing mutations in *ZNRF3* and *TP53* genes, respectively (see Table R2, patient 2 and patient 13). Notably, these two genes have been described among the most frequently altered genes in ACC (Assié *et al*, 2014; Juhlin *et al*, 2015; Zheng *et al*, 2016). We used a dedicated ScreenCell filtration device to isolate CTCs and extract DNA. Further whole genome amplification was performed to increase the amount of DNA. We designed a specific ddPCR assay for each gene in order to target the specific mutation in each patient, and we tested the assays on the relative DNA tumor samples. We were able to detect the mutated allele in both patients (Fig. R20, A), thus we could confirm the specificity of our assays. Notably, the allelic frequency of the mutations detected by ddPCR was comparable to that obtained with the previous NGS assay (Fig. R20, B). On the contrary, the results of ddPCR performed on CTC DNA from the same patients were disappointing: no signal was detected in any of the samples, even related to the wild type alleles (Fig. R20, C). Since our CTC isolation system does not allow to obtain a pure sample of CTC, but other blood cells, including lymphocytes, still remain after filtration, at least we should detect germline DNA in our samples. Thus, some technical issue related to CTC DNA purification and amplification affects our system and needs to be investigated.



[B]

Sample	Mutation	Allelic frequency NGS	ddPCR
P2	ZNRF3	0,78	0,81
P13	TP53	0,24	0,29

Figure R20. ddPCR analysis assessing the presence of selected mutations in tumor and CTC-derived DNA. A specific ddPCR assay was designed for each of the selected mutations in ZNRF3 and TP53 related to patient 2 (P2) and patient 13 (P13) of our cohort. The mutated and wild type alleles are detected thanks the complementary hybridization of a specific probe conjugated with a different fluorophore, FAM for the mutated (blue signal) and HEX for the wild allele (green signal). The grey signal represent the double-negative droplets.

To further assess the chromosomal alterations and the mutational state of ACC-related genes in both tumor and CTC samples, we designed a target NGS assay, which included genes and Single Nucleotide Polymorphisms (SNPs) selected on the basis of the previous results on the larger European cohort of ACC patients. In particular, we selected 9 genes with a high percentage of mutation and prognostic value in ACC (*ZNRF3*, *TP53*, *CTNNB1*, *CDKN2A*, *MEN1*, *RB1*, *CDK4* *GNAS*, *PRKACA*) and a selection of 173 SNPs related to 11 chromosome arms (1p, 1q, 2p, 2q, 9q, 11p, 11q, 17q, 18p, 18q, 22q) previously identified as discriminating of chromosomal and noisy LOH profiles, or harboring the genes frequently found deleted in ACC. The SNP selection criteria included heterozygosity (average > 0.4999) and at least 100 kb distance one to the other in order to avoid the linkage disaequilibrium effect and to achieve as much coverage as possible for each chromosome arm. As for ddPCR, we first tested the new NGS panel on ACC tumor samples to compare the results to the previous analysis. We obtained encouraging preliminary results about SNPs, confirming that the new NGS assay can replicate the previous SNP profiles in all the cases of chromosomal, quite or noisy LOH (Fig.

R21). Further data analysis needs to be done to validate the NGS assay, including the evaluation of gene mutations.

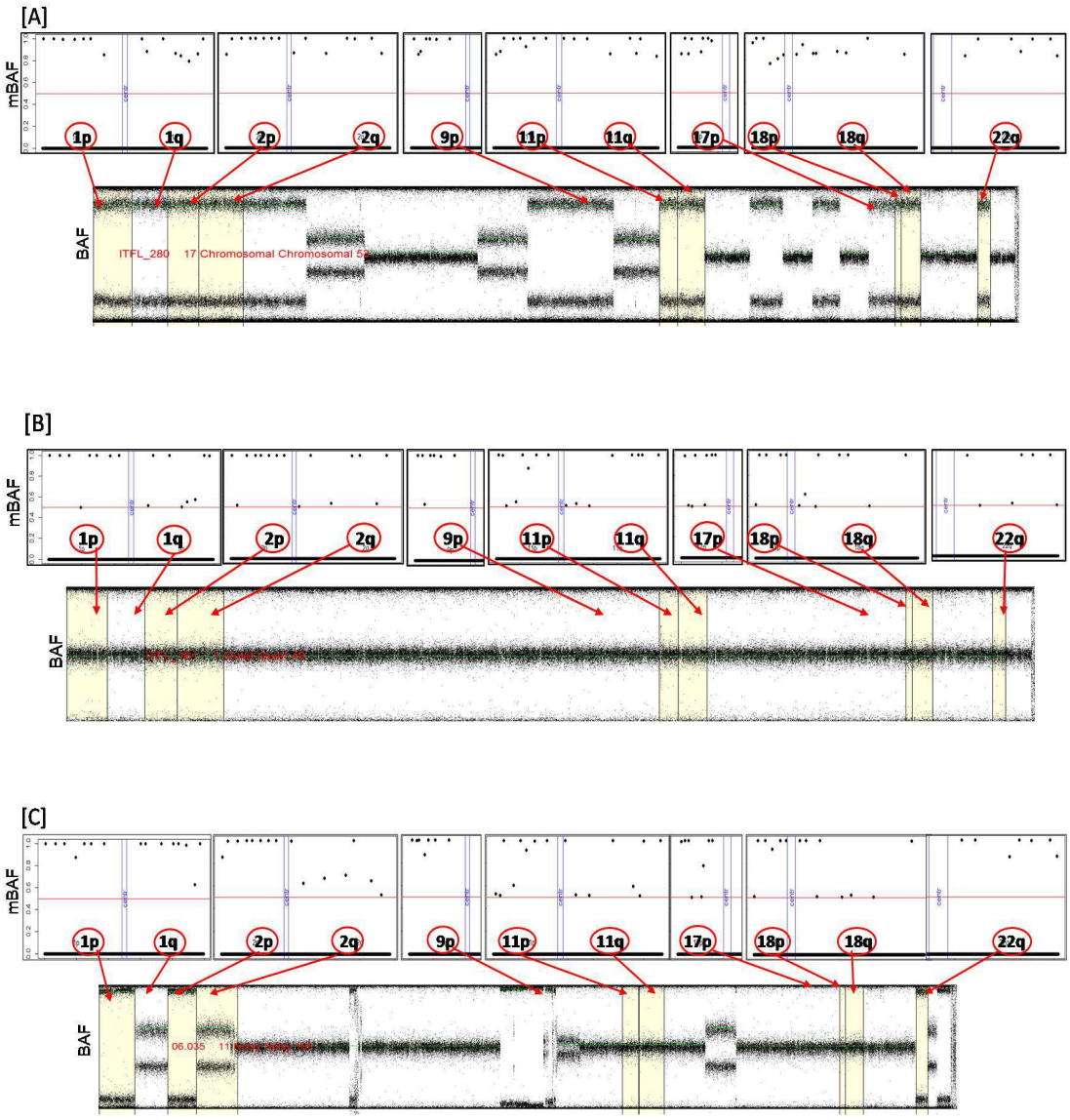


Figure R21. Representative images of chromosomal (A), quiet (B) and noisy (C) LOH profiles related to tumor samples of three different ACC patients. The BAF (B Allele Frequency) plots refer to the previous NGS analysis; the mBAF (mirrored B Allele Frequency) plots are related to the analysis with the new targeted NGS panel. The chromosome arms harboring the analyzed SNPs are indicated on the mBAF plots and linked to the correspondent ones on the BAF plots by the red arrow.

Discussion

The molecular and metabolic evolution within the primary tumor mass, together with a variety of stimuli produced in the surrounding microenvironment, underlies cancer cell ability to acquire metastatic traits and, eventually, disseminating through the blood circulation and colonizing distant organs. Since metastasis represents the main cause of death in cancer patients, preventing cancer cell spreading rather than treat secondary neoplasms would be more effective in high-risk patients. In this setting, the detection and characterization circulating tumor cells (CTCs) could serve as real-time "liquid biopsy" (Alix-Panabières & Pantel, 2013), allowing to profile the disease complexity at any stage of tumor progression. This would particularly helpful in the case of advanced adrenocortical carcinoma, aiding in developing more effective therapeutic strategies targeting specific traits of cells that retain the metastatic potential.

CTC detection and count has been already shown to be a potential marker for differential diagnosis in ACC (Pinzani *et al*, 2013). Here, we aimed to develop an experimental design to characterize CTCs at the molecular level, in order to compare the genetic profile with that of the primary tumor and identify potential markers allowing for early detection of metastatic disease.

CTC isolation by size, associated with the positivity for the adrenal specific marker SF-1, remains the most useful tool in the case of ACC, since adrenocortical carcinoma have been shown to be EpCAM-negative (Went *et al*, 2004), thus making poorly efficient the EpCAM immunoselection-based systems for CTC detection. Using a dedicated filtration device, we were able to assess the presence of CTCs in blood samples from ACC patients under follow-up and with different characteristics of disease presentation. We were able not only to detecte single CTCs, but also CTC clusters (CTM), which have been associated with a more aggressive tumor behavior and poor clinical outcome (Hou *et al*, 2012; Aceto *et al*, 2014, 2015; Au *et al*, 2016). Moreover, we also identified cancer-associated macrophages-like cells (CAML), which seems to be predictive of disease outcome in other cancer types (Adams *et al*,2014, 2016; Mu *et al*, 2017). The specific nuclear positivity for SF-1 in isolated cells confirmed the adrenal origin of the detected CTCs. However, some limitations are still present: first of all, our filtration system do not allow single CTC isolation, since a percentage of blood cells is still retained

on the filters. Employing more sensitive techniques for CTC isolation by size, followed by a specific selection for SF-1-positive cells, will help in overcoming this particular issue.

Concerning CTC molecular characterization, we are still far from have enough amount of high quality DNA from CTCs suitable for downstream applications, such as digital-droplet PCR and next generation sequencing. However, our preliminary results with the designed assay addressing specific alterations in the primary tumors of ACC patients, are encouraging: in fact, we could confirm the presence of both point mutations and the specific LOH profiles in tumor samples of selected patients. Improving technical aspects related to DNA extraction, purification and amplification of CTC DNA will allow to pursue in this promising field, looking forward the application of "liquid biopsy" and CTCs for personalized therapy also in ACC.

CONCLUSIONS AND FUTURE PERSPECTIVES

Despite immunohistochemical and molecular studies have identified novel biomarkers of diagnostic and prognostic relevance for adrenocortical carcinoma, the discrimination between malignant and benign forms remains challenging, the molecular mechanism underlying the pathology has still to be fully elucidated and the available therapeutic options show limited specificity and efficacy. A better understanding of the tumor biology and behavior would help in identifying new therapeutic targets to develop a more personalized approach to treat patients. Beyond molecular alterations occurring within the tumor mass, including driver gene mutations, epigenetic alterations and dysregulation of signaling pathways related to cell survival, growth and proliferation, the role of the surrounding stroma has been widely recognized as crucial in sustaining tumor metabolic adaptation and evolution. Thus, unraveling the dynamics occurring in the setting of the ACC tumor microenvironment would help in elucidating the biology of adrenal tumorigenesis, shedding new light on the mechanisms driving cancer progression. Particularly, this would give a chance to address the metabolic and functional interactions potentially established between adrenocortical carcinoma cancer cells and their adipose microenvironment, allowing to uncouple such a crosstalk. Moreover, identifying more specific features of cells developing and retaining more aggressive traits could allow to monitor tumor progression, preventing tumor dissemination and metastasis. In this scenario, the "liquid biopsy" may represent the possibility to follow tumor evolution, with promising implications for the clinical practice.

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REVIEW

Adrenocortical carcinoma: the dawn of a new era of genomic and molecular biology analysis

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Abstract Over the last decade, the development of novel and high penetrance genomic approaches to analyze biological samples has provided very new insights in the comprehension of the molecular biology and genetics of tumors. The use of these techniques, consisting of exome sequencing, transcriptome, miRNome, chromosome alteration, genome, and epigenome analysis, has also been successfully applied to adrenocortical carcinoma (ACC). In fact, the analysis of large cohorts of patients allowed the stratification of ACC with different patterns of molecular alterations, associated with different outcomes, thus providing a novel molecular classification of the malignancy to be associated with the classical pathological analysis. Improving our knowledge about ACC molecular features will result not only in a better diagnostic and prognostic accuracy, but also in the identification of more specific therapeutic targets for the development of more effective pharmacological anti-cancer approaches. In particular, the specific molecular alteration profiles identified in ACC may represent targetable events by the use of already developed or newly designed drugs enabling a better and more efficacious management of the ACC patient in the context of new frontiers of personalized precision medicine.

Keywords Adrenal cancer · Genomics · Molecular biology

Introduction

In spite of its rarity (1–2 cases per million prevalence), adrenocortical carcinoma (ACC) is an aggressive malignancy of the steroidogenic component of the adrenal gland with poor prognosis, in particular when already metastatic at diagnosis. The current poor and heterogeneous prognosis is due to the lack of both reliable clinically diagnostic criteria and specific/effective therapeutic treatments [1, 2]. To date, in fact, the radical surgery (R0) represents the only effective therapeutic option [3]. In advanced carcinoma, therapy with mitotane, an adrenolytic derivative of the insecticide DDT, is recommended, since it associates with a more prolonged disease-free interval after surgery [4].

ACC diagnosis on the tumor mass [5] is based on histopathological criteria within the Weiss Score system (Table 1), which can also provide a prognostic index [6–8] together with the tumor resection status (R0/R1) [3] and the tumor staging at the diagnosis [9]. This new classification system (Table 2) has been proposed by the European Network for the Study of the Adrenal Tumors (ENSAT) [10]: it revises the original TNM classification to identify four tumor stages associated with different disease outcomes. The proliferation index, evaluated by the cell positivity to Ki67 staining, is also considered as an independent prognostic factor [11]. Although the staging system offers a good prognostic tool, ACC outcome is often highly variable and heterogeneous, even within the same stage: some cases are less aggressive and can be successfully treated by surgery, while others can progress despite an apparently complete mass removal. These aspects, along with the few available

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New insights in the clinical and translational relevance of miR483-5p in adrenocortical cancer

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ABSTRACT

Adrenocortical cancer (ACC) is a rare aggressive malignancy. Recent ACC integrated genomics analysis contributed to redefine the risk groups on molecular basis, including tumor microRNAs (miRs), detectable also in the bloodstream. We developed a quantitative real-time (RT) assay for the measurement of miR483 and miR483-5p absolute levels in plasma samples. miR483/miR483-5p levels were evaluated in plasma samples of 27 patients with ACC before surgery and at follow-up.

Statistically significant differences in miR483-5p and miR483 levels were found between stage 1/2 and stage 3/4 ACCs in pre-surgery and post-surgery samples. ROC curve analysis of miR483-5p levels gave a prediction of the clinical stage (accuracy 0.917±0.084), with the best cut-off value of 0.221 ng/ml, prognosticating overall and recurrence-free survival. In a multivariate Cox analysis (HR 16.2, 95%CI[1.39-188.6, P<0.026]), miR483-5p was the only variable that significantly predicted recurrence, but not overall survival. In addition, miR483 and miR483-5p levels correlated with the number of circulating tumor cells (CTCs) detected in the same blood samples, independently of the timing of sampling. In conclusion, we demonstrated that miR483-5p absolute plasma levels in ACC patients are powerful molecular markers that may help in the follow-up of patients after surgery and chemotherapy, and contribute to more accurately classify and predict tumor progression.

INTRODUCTION

Adrenocortical carcinoma (ACC) is a rare but often aggressive tumor, with dismal prognosis and limited therapeutic options. A complete resection of the mass is the mainstay treatment associated with adjuvant mitotane therapy at advanced stages. Although mitotane mechanisms of action are still poorly understood, its use has been proven to significantly increase disease-free survival [1].

Over the last years, the diagnostic and prognostic relevance of the pan-genomics profile clustering of tumors has been demonstrated for adrenocortical cancer [2, 3]. In addition to the profiles derived from the analysis of the characteristics of the primary tumor mass, information provided by the liquid biopsy components, i.e. miRNA (miR), free circulating DNA, and circulating tumor cells (CTCs), can better characterize tumors at a molecular level [4]. The liquid biopsy is a non-invasive test on blood samples, and provides biological material to monitor easily

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RASSEGNA

Caratterizzazione genomica del carcinoma surrenalico

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Sommario L'ultimo decennio ha visto lo sviluppo esponenziale della genomica, ossia dell'insieme di tecniche di analisi molecolare dei campioni biologici. Tale approccio molecolare ha rivoluzionato la biologia e la genetica anche del carcinoma della corteccia surrenalica (ACC). L'applicazione di tali tecniche all'analisi di ampie casistiche di ACC ha portato all'identificazione di alterazioni nell'espressione genica, nello stato di metilazione, nella struttura cromosomica, dotate di valore prognostico e predittivo, permettendo di formulare una classificazione molecolare della malattia. Tale classificazione potrebbe rivestire un ruolo rilevante non solo per migliorare l'accuratezza della prognosi, ma soprattutto per identificare nuovi bersagli terapeutici utilizzabili per la cura di questo tumore, che ha ad oggi limitati e poco efficaci approcci terapeutici farmacologici.

Parole chiave Carcinoma cortico-surrenalico · Genomica · Marcatori molecolari

Introduzione

Il carcinoma della corteccia surrenalica (*adrenal cortex carcinoma*, ACC) è un tumore maligno che interessa la componente steroidogenica della ghiandola surrenalica. Ha una incidenza rara (1 caso su 2.000.000) ma elevata aggressività, soprattutto se si presenta in forma metastatica alla diagnosi. Ad oggi, l'unica terapia efficace è rappresentata dalla rimozione chirurgica radicale della massa (R0); nelle forme avanzate vi è l'indicazione al trattamento con mitotane, un derivato del DDT (diclorofeniltricloroetano) ad azione adrenolitica. Il mitotane, quando utilizzato in terapia adiuvante, si è dimostrato in grado di determinare un aumento dell'intervallo libero da malattia dopo resezione chirurgica.

La diagnosi è effettuata sulla massa tumorale mediante esame istologico che si basa su criteri istopatologici, in particolare lo score di Weiss, che ha anche un significato prognostico (Tabella 1a). La prognosi è strettamente legata anche alla rimozione completa del tumore (R0) e allo stadio di presentazione alla diagnosi. Tale sistema di classificazione (Tabella 1b), proposto dallo *European Network for the Study of Adrenal Tumors* (ENSAT) [1], si basa sui criteri TNM che identificano 4 stadi a cui corrispondono differenti curve di sopravvivenza. Un ulteriore importante criterio prognostico sembra essere l'indice proliferativo valutato in base alla percentuale di positività cellulare del marcatore Ki67.

Nonostante la classificazione in stadi abbia un buon potere prognostico, il comportamento degli ACC, anche all'interno dello stesso stadio, è spesso molto variabile. Infatti, alcuni carcinomi sono scarsamente aggressivi e curabili con la sola asportazione chirurgica, mentre altri progrediscono nonostante un'apparente rimozione completa.

L'ACC è un tumore di difficile gestione per l'endocrinologo clinico. Oltre ad essere raro, presenta un'estrema variabilità sia in termini di secrezione steroidea e pertanto di esordio clinico, sia in termini di andamento clinico, tanto che ancora oggi risulta difficilmente prevedibile. La sua rarità contrasta con l'alta frequenza delle forme adenomatose (*adrenocortical adenoma*, ACA) benigne, spesso diagnosticate incidentalmente e dalle quali non sembra derivare. Ra-

Proposto da Rossella Elisei.

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Metformin as a new anti-cancer drug in adrenocortical carcinoma

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare heterogeneous malignancy with poor prognosis. Since radical surgery is the only available treatment, more specific and effective drugs are urgently required. The anti-diabetic drug metformin has been associated with a decreased cancer prevalence and mortality in several solid tumors, prompting its possible use for ACC treatment.

This paper evaluates the *in vitro* and *in vivo* anti-cancer effects of metformin using the ACC cell model H295R.

Metformin treatment significantly reduces cell viability and proliferation in a dose- and time-dependent manner and associates with a significant inhibition of ERK1/2 and mTOR phosphorylation/activation, as well as with stimulation of AMPK activity. Metformin also triggers the apoptotic pathway, shown by the decreased expression of Bcl-2 and HSP27, HSP60 and HSP70, and enhanced membrane exposure of annexin V, resulting in activation of caspase-3 apoptotic effector. Metformin interferes with the proliferative autocrine loop of IGF2/IGF-1R, which supports adrenal cancer growth. Finally, in the ACC xenograft mouse model, obtained by subcutaneous injection of H295R cells, metformin intraperitoneal administration inhibits tumor growth, confirmed by the significant reduction of Ki67%.

Our data suggest that metformin inhibits H295R cell growth both *in vitro* and *in vivo*. Further preclinical studies are necessary to validate the potential anti-cancer effect of metformin in patients affected by ACC.

INTRODUCTION

Despite its rarity (1:2 million prevalence), adrenocortical cancer (ACC) deserves more consideration on account of its aggressive behavior and poor prognosis when metastatic at diagnosis. To date, the best curative option is radical surgery. In case of advanced ACC, mitotane is the only available medical treatment [1, 2]. Nevertheless, mitotane has proved to be of limited efficacy and poor tolerability, thus often reducing the patient's compliance. The drug's therapeutic range is reached in less than 50% of treated patients [1]; moreover, specific polymorphisms of the CYP2B6 gene have been associated with reduced circulating levels of mitotane, further decreasing its efficacy [3]. The mechanism by which mitotane acts on cancer cells, as well as on the normal adrenal, is still far from being fully elucidated. In such a scenario, it is mandatory to combine mitotane with other drugs, in order to improve the therapeutic efficacy and reduce treatment toxicity. Of the possible drugs that could be associated with mitotane, those safely and widely administered for treating other diseases, and eventually found to also exert anti-neoplastic effects, should offer the best therapeutic option. Metformin (1,1-dimethylbiguanide) is a biguanide cationic compound commonly used as an insulin-sensitizer and glucose-lowering drug in the treatment of type 2 diabetes (T2D). Epidemiological studies and meta-analyses on large cohorts of diabetic patients have demonstrated a significant association between metformin and a reduced incidence

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2D-DIGE Proteomic analysis identifies new potential therapeutic targets for adrenocortical carcinoma

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ABSTRACT

Adrenocortical carcinoma (ACC) is a rare aggressive tumor with poor prognosis when metastatic at diagnosis. The tumor biology is still mostly unclear, justifying the limited specificity and efficacy of the anti-cancer drugs currently available. This study reports the first proteomic analysis of ACC by using two-dimensional-differential-in-gel-electrophoresis (2D-DIGE) to evaluate a differential protein expression profile between adrenocortical carcinoma and normal adrenal. Mass spectrometry, associated with 2D-DIGE analysis of carcinomas and normal adrenals, identified 22 proteins in 27 differentially expressed 2D spots, mostly overexpressed in ACC. Gene ontology analysis revealed that most of the proteins concurs towards a metabolic shift, called the Warburg effect, in adrenocortical cancer. The differential expression was validated by Western blot for Aldehyde-dehydrogenase-6-A1,Transferrin, Fascin-1,Lamin A/C,Adenylate-cyclase-associated-protein-1 and Ferredoxin-reductase. Moreover, immunohistochemistry performed on paraffin-embedded ACC and normal adrenal specimens confirmed marked positive staining for all 6 proteins diffusely expressed by neoplastic cells, compared with normal adrenal cortex.

In conclusion, our preliminary findings reveal a different proteomic profile in adrenocortical carcinoma compared with normal adrenal cortex characterized by overexpression of mainly metabolic enzymes, thus suggesting the Warburg effect also occurs in ACC. These proteins may represent promising novel ACC biomarkers and potential therapeutic targets if validated in larger cohorts of patients.

INTRODUCTION

Adrenocortical tumors are rather common, with a prevalence of about 9% in the older age, and mostly benign adenomas (ACA). On the other hand, adrenocortical carcinomas (ACC) are rare and aggressive tumors with an incidence of 1-2 cases/million people/year and poor prognosis (5-year survival rate around 30%) [1-4]. Survival rate drops below 10% when metastasis is found at diagnosis.

In spite of recent studies on the genetics and molecular biology of this neoplasia [5-9], its pathogenesis is still not completely understood: new diagnostic and prognostic biomarkers as potential therapeutic targets are

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