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Angiogenesis in Adrenocortical Tumors

Sofia Borges Oliveira

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Angiogenesis in Adrenocortical Tumors

Sofia Borges Oliveira

Master's thesis in Biochemistry presented to
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MSc

Angiogenesis in Adrenocortical Tumors

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Resumo

A maioria dos tumores adrenocorticais (ACT) têm um comportamento benigno e não funcionante, e, portanto, são maioritariamente encontrados acidentalmente. Ao contrário dos adenomas (ACA), os carcinomas adrenocorticais (ACC) são raros e geralmente agressivos. O diagnóstico diferencial entre estas duas entidades é baseado em características histológicas pouco especificas, e até ao momento não foi reconhecido nenhum marcador molecular que permitisse o seu diagnóstico diferencial. Do mesmo modo, o prognóstico é baseado em parâmetros incapazes de contornar a heterogeneidade molecular dos ACC, conduzindo a estratégias clínicas inadequadas. A angiogénese é um processo biológico frequentemente estudado no contexto tumoral. Sendo um mecanismo complexo, são várias as vias de sinalização envolvidas na iniciação, crescimento e manutenção dos vasos sanguíneos, entre as quais, a via do VEGF e do Ang-Tie.

O objetivo deste estudo foi avaliar o papel das vias do VEGF e Ang-Tie na angiogénese dos ACT, de forma a identificar marcadores moleculares, ou um conjunto destes, que possa contribuir para o diagnóstico e/ou prognóstico dos ACC.

A expressão das proteínas envolvidas na angiogénese, nomeadamente o VEGF, VEGF-R2, Ang1, Ang2, Tie1, Tie2 e o CD34, foram avaliadas através da técnica de imunohistoquímica em ACC (n=22), ACA com síndrome de Cushing (n=8) e ACA não funcionantes (n=13). A marcação obtida foi quantificada através de uma ferramenta de análise morfométrica, exceto para a avaliação do VEGF. Os resultados obtidos foram correlacionados com as características clínicas dos tumores.

Entre os marcadores moleculares estudados, apenas o CD34, o Ang1 e o Ang2 demonstraram apresentar diferenças significativas entre os ACA e os ACC. A expressão do CD34 foi menor nos ACC quando comparada com os ACA, enquanto que a expressão do Ang1 e o Ang2 foi mais elevada nos tumores malignos. No entanto, não foi demonstrada a utilidade destes marcadores no diagnóstico de ACC. Embora, a expressão de Tie1 tenha sido mais elevada em ACC de doentes com invasão venosa e tempo de sobrevida mais curto.

Em conclusão, este estudo descreve pela primeira vez o papel da via do Ang-Tie na angiogénese dos ACC. A expressão mais elevada de Ang2 em tumores malignos pode estar relacionada com uma maior permeabilidade vascular e facilidade de disseminação tumoral. Além disso, a expressão mais elevada de Tie1 em ACC de doentes com pior prognóstico aponta para um possível alvo terapêutico para o

tratamento dos ACC. No futuro, serão necessários estudos funcionais e mecanísticos para validação dos resultados obtidos.

Palavras-chave: Tumores Adrenocorticais, Diagnóstico, Prognóstico, Angiogénese, Via do VEGF, Via do Ang-Tie.

Abstract

The majority of adrenocortical tumors (ACT) are benign and non-functioning, and therefore, found incidentally. In contrast to adenomas (ACA), adrenocortical carcinomas (ACC) are rare and usually aggressive. The differential diagnosis of these two entities is based on unspecific histological features, and so far, no molecular marker has been identified to improve ACC diagnosis. Similarly, prognosis is predicted on factors with insufficient capacity to overcome the molecular heterogeneity of ACC, leading to an inaccurate clinical strategy. Angiogenesis is a biological process frequently studied in a tumor context. Being a complex mechanism, there are several signaling pathways involved in the initiation, growth, and maintenance of blood vessels, including the VEGF and Ang-Tie pathway.

The aim of this study was to evaluate the role of the VEGF and Ang-Tie pathways in ACT, in order to identify molecular markers that may contribute to the diagnosis and/or prognosis of adrenocortical carcinomas.

The expression of proteins involved in angiogenesis, namely VEGF, VEGF-R2, Ang1. Ang2, Tie1, Tie2 and CD34 was assessed by immunohistochemistry in ACC (n=22), ACA with Cushing's syndrome (n=8) and non-functioning ACA (n=13). Immunochemistry staining was quantified using a morphometric analysis tool, except for VEGF. The results were correlated with clinical features associated with the tumors.

Among the molecular markers studied, only CD34, Ang1 and Ang2 were found to be significantly different between ACC and ACA. CD34 expression was lower in ACC when compared to ACA, while Ang1 and Ang2 were higher in ACC. However, these molecular markers did not prove to be sufficiently accurate for ACC diagnosis. Higher Tie1 expression was observed in ACC of patients with venous invasion and shorter overall survival.

As conclusion, we described for the first time the role of Ang-Tie pathway in ACC angiogenesis. The higher expression of Ang2 in ACC could be related with higher vascular permeability, thus facilitating tumor spreading. In addition, the higher Tie1 expression in ACC patients with poor prognosis may represent a possible therapeutic target for ACC treatment. In the future, functional and mechanistic studies are needed to validate these data.

Key words: Adrenocortical Tumors, Diagnostic, Prognostic, Angiogenesis, VEGF pathway, Ang-Tie pathway.

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Abbreviations

ACC	Adrenocortical carcinoma
ACA	Adrenocortical adenoma
ACAc	Adrenocortical adenoma with Cushing syndrome
ACAn	Non-functioning adrenocortical adenoma
ACT	Adrenocortical tumors
AUC	Area under the curve
Ang1	Angiopoietin 1
Ang2	Angiopoietin 2
BSA	Bovine serum albumin
CT	Computed tomography
CYB5A	Cytochrome B5A
CYP11A1	Cholesterol side chain cleavage enzyme
CYP11B1	11β-hydroxylase
CYP11B2	Aldosterone synthase
DAB	3,3'-diaminobenzidine
DHE	Dehydroepiandrosterone
DHEA-S	Dehydroepiandrosterone sulfate
ENSAT	European Network for the study for the Adrenal Tumors
IHC	Immunohistochemistry
MAPK	Mitogen-activated protein kinase
PBS	Phosphate buffer saline
PI3K-AK	Phosphatidylinotisol-3 kinase
RGB	Red-Green-Blue
ROC	Receiver operating characteristic
StAR	Steroidogenic acute regulatory protein
Tie1	Tyrosine kinase with immunoglobulin-like and EGF-like domain 1
Tie2	Tyrosine kinase with immunoglobulin-like and EGF-like domain 2
TNM	Tumor-node-metastasis
UICC	Union for International Cancer Control
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor A
VEGF-B	Vascular endothelial growth factor B
VEGF-C	Vascular endothelial growth factor C
VEGF-EG	Endocrine gland-derived vascular endothelial growth factor

VEGF-R1	Vascular endothelial growth factor receptor 1
VEGF-R2	Vascular endothelial growth factor receptor 2
VEGF-R3	Vascular endothelial growth factor receptor 3

Chapter 1

Introduction

1.1. Adrenal gland

Adrenal glands are endocrine organs located in the upper pole of each kidney. Histologically, this organ is divided into two distinct regions with different embryological origins: the cortex, with mesodermal origin, and the medulla, derived from neural crest. The cortex corresponds to the outer region and constitutes approximately 90% of the total volume of the gland. In the adult, the cortex is divided into three morphologically and functionally distinct zones: glomerulosa, fasciculata and reticular. Each zone has a characteristic enzymatic profile which allows the production of different steroid hormones from cholesterol, predominantly aldosterone, cortisol and androgens (Figure 1). The medulla constitutes the center of the adrenal gland and it is the region responsible for secreting catecholamines, namely norepinephrine and epinephrine (Dutt et al., 2021).

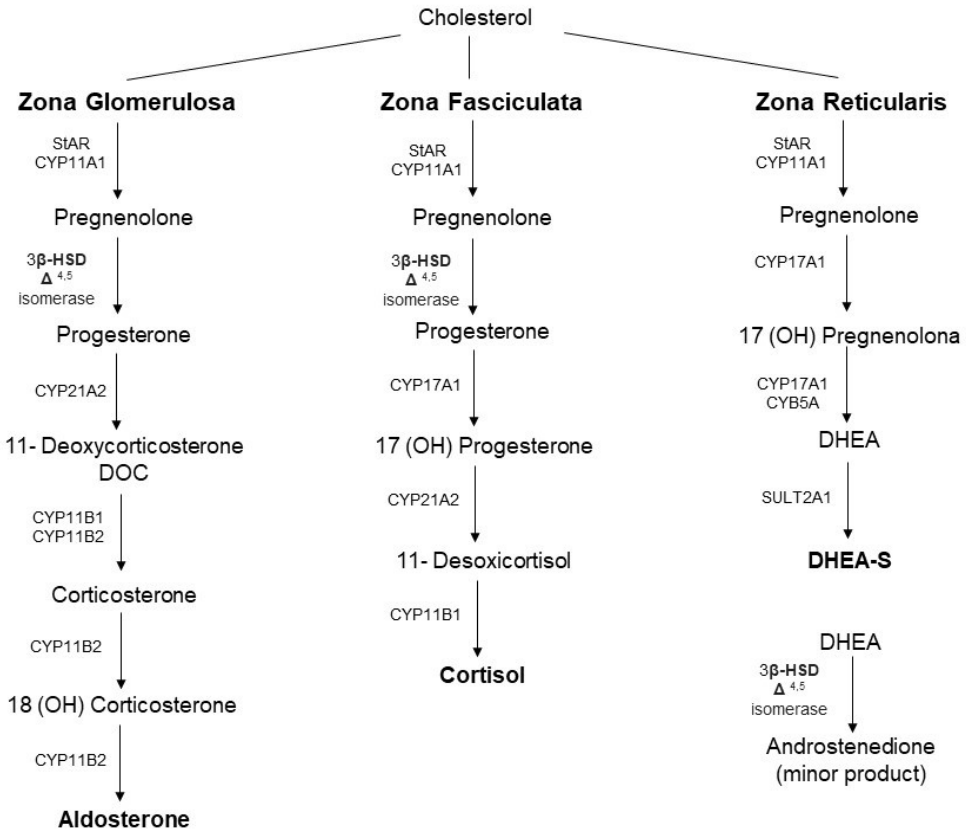

Figure 1 – Steroidogenesis in the different zones of the adrenal cortex. 3β-HSD: 3 β-hydroxysteroid dehydrogenase, CYB5: cytochrome B5A, CYP11A1: cholesterol side chain cleavage enzyme, CYP11B1: 11β-hydroxylase, CY11B2: aldosterone synthase, CYP17A1: 17α-hydroxylase, CYP21A2: 21α-hydroxylase, DHEA: dehydroepiandrosterone, DHEA-S: dehydroepiandrosterone sulfate, StAR: steroidogenic acute regulatory protein and SULT2A1: sulfotransferase 2A1.

1.2. Adrenocortical Tumors

Adrenocortical tumors (ACT) are tumors originating in the adrenal cortex, affecting 3 to 10% of the human population (Lehmann & Wrzesinski, 2012). ACT can be classified according to its biological behavior in benign and malignant, and according to its functionality in functioning and in non-functioning tumors. Functioning tumors secrete steroid hormones autonomously, whereas, non-functioning tumors do not secrete steroids or produce it at insufficient levels to give rise to clinical manifestations (Pignatelli, 2011). Depending on the steroid hormone secreted, functional tumors can lead to the development of different clinical syndromes. Aldosterone-secreting tumors are responsible for the development of Conn's syndrome, which the main symptom is hypertension (Seccia et al., 2005). On the other hand, tumors with hypersecretion of cortisol lead to Cushing syndrome, and consequently to clinical manifestations such as obesity, hyperglycemia and muscle weakness (Gladding et al., 2005). The androgens secreting tumors, mainly dehydroepiandrosterone sulfate (DHEA-S) and androstenedione, result in the development of virilizing syndromes (Pingle et al., 2020).

The majority of these tumors have a benign behavior and are non-functioning tumors, and therefore, are often diagnosed incidentally in the course of imaging examinations performed for non-related conditions. Adrenal tumors discovered in these circumstances are referred as incidentalomas. Radiological studies show that the frequency of incidentalomas is approximately 4% in individuals aged between 40-60 years and that it tends to increase with age (Fassnacht et al., 2016; Terzolo et al., 2011).

In contrast to adenomas, adrenocortical carcinomas (ACC) are rare, with an annual incidence of 0.7-2 cases per million population. Although they can occur at any age, there are two peaks of incidence: in early childhood (under 5 years) and between 40-50 years, with a slight predominance in females (F:M ratio 1.5-2.5:1) (Fassnacht et al., 2013; Libé, 2015). Most ACC are functional (50-60%), frequently exhibiting the capacity of producing cortisol, androgens or the combination of both hormones (Else et al., 2014). About 40-60% of the individuals manifest symptoms resulting from the hormone hypersecretion and so, the functionality of the tumor may contribute to an early diagnosis. However, in many cases the production of hormones is not always clinically evident. This may be due to inefficient steroidogenesis of ACC, manifesting with increased secretion and release of steroid precursors (Arlt et al., 2011; Else et al., 2014; Pereira et al., 2020).

The classification of adrenocortical tumors into benign and malignant represents a difficulty for the clinicians (Pignatelli, 2011). The differentiation between the two entities

by imaging modalities is mainly based on the size of the lesion. In addition to the size, a value of less than 10 Hounsfield units on unenhanced computed tomography (CT) supports the diagnosis of ACC. After surgical resection, which is recommended for all adrenal tumors larger than 4 cm on radiological examination, the differential diagnosis is based on macro- and microscopic criteria (Mazzaglia et al., 2020). Macroscopic features include the size and weight of the tumor, the integrity of the capsule and the presence of hemorrhage or necrosis. For microscopic evaluation, the Weiss system is the most commonly tool used for ACC diagnosis. This is a multi-parametric system that includes 9 histopathological parameters such as mitotic rate, nuclear grade, disarrangement of cellular architecture, proportion of clear cells, nuclear pleomorphism, abnormal mitoses, venous, sinusoidal and capsule invasion (Table1). In 2002, the modified Weiss system was proposed which includes only 5 of the 9 histological criteria, removing the most subjective parameters and difficult to interpret. Thus, the modified system is based on the following criteria: necrosis, capsule invasion, mitotic rate, abnormal mitoses and proportion of clear cells (Table 1) (Lau & Weiss, 2009; Mizdrak et al., 2021). These histological features are poorly specific and overlap between the two entities, contributing to the inaccuracy of the diagnosis.

Table 1 – Weiss system and modified Weiss system for diagnosing adrenocortical carcinoma [adapted from (Lau & Weiss, 2009).

Histological Criteria	Weiss Score	Modified Weiss Score
Mitotic rate >5 por 50 high-power fields	×	×
Cytoplasm (Clear cells) Clear cells comprising 25% or less of the tumor	×	×
Abnormal mitoses Presence of abnormal distribution of chromosomes or excessive number of mitotic spindles	×	×
Fuhrman nuclear grade grade 3 or 4	×	
Diffuse architecture > 1/3 of the tumor demonstrates a patternless sheets of cells	×	
Capsular Invasion Nests or cords of tumor extending into or through tumor capsule	×	×
Venous Invasion	×	

Sinusoidal Invasion	×	
Necrosis	×	×

×= indicates the histopathological feature used in Weiss system and modified Weiss system for diagnosing ACC.
Weiss Sore: a score of 3 or more suggests malignancy.
Modified Weiss Score calculation: 2 × mitotic rate + 2 × clear cells + 2 × abnormal mitoses + necrosis + capsular invasion. A score of 3 or more correlates with a malign behavior.

ACC are usually aggressive, and are associated with a poor prognosis, with a 5-year mortality rate of 75-90% (Nakamura et al., 2015). This clinical outcome is mainly due to the difficulty of diagnosing malignant tumors at earlier clinical stages and, the lack of effective treatments.

Individual variability in tumor progression and survival was previously described. This heterogeneity is related to the different biological and molecular profiles of carcinomas, reinforcing the need to identify molecular markers with prognostic value that would allow adjusting and personalizing clinical strategies (Assié et al., 2014; Jouinot & Bertherat, 2018). In the absence of these molecular markers, European Network for the Study of Adrenal Tumors (ENSAT) tumor stage, age and tumor proliferation index, assessed by ki-67 immunohistochemistry, are the main prognostic factors of adrenocortical carcinomas (Else et al., 2014; Jouinot & Bertherat, 2018). The tumor-node-metastasis (TNM) proposed by the ENSAT is the currently used staging system for ACC (Table 2) and, seems to have an improved prognostic accuracy when compared with the previously system used for disease staging proposed by the Union for International Cancer Control (UICC) (Lughezzani et al., 2010).

Table 2 – Classification of adrenocortical carcinoma (ACC) from European Network for the Study of Adrenal Tumors (ENSAT).

ENSAT stage	T	N	M
I	1	0	0
II	2	0	0
III	1,2	1	0
	3,4	0,1	0
IV	1-4	0,1	1

T1: tumor <5 cm; T2: tumor >5 cm; T3: tumor infiltration into surrounding tissue; T4: tumor invasion in adjacent organs
N0: no positive lymph nodes; M0: no distance metastasis; N1: positive lymph nodes; M1: presence of distance metastasis.

1.3. Angiogenesis

Angiogenesis is the biological mechanism by which new blood vessels are formed from pre-existing vasculature, and it is crucial in embryonic and fetal development. In the adult, angiogenesis is a transient mechanism, necessary in biological processes such as wound healing and in the female reproductive cycle. Beyond physiological processes, angiogenesis plays a central role in pathological conditions, namely in tumor growth and metastasis, being a well-recognized hallmark of cancer (Chung & Ferrara, 2011; Hanahan & Weinberg, 2011).

Angiogenesis, either in normal or tumor tissues, usually occurs via one or more of the following mechanisms:

1. Sprouting angiogenesis, one the most well characterized mechanism leading to angiogenesis, relies on endothelial cells function specification into either tip or stalk cells. Tip cells are derived from the parent vessel, degrade the basement membrane, extend large filopodia which can sense angiogenic factor gradients, such as vascular endothelial growth factor (VEGF), and migrate along the chemotactic paths. In contrast, stalk cells proliferate behind tip cells to form the sprout body, start the process of lumen formation, and connect with neighboring vessels (Duran et al., 2017; Lugano et al., 2020; Mentzer & Konerding, 2014).
2. Intussusceptive is a process that consists in the splitting of pre-existing vessels into two new vessels. It starts with the formation of transluminal tissue pillars through the invagination of opposing capillary endothelial cells into the vascular lumen, creating a zone of contact. Commonly, intussusceptive and sprouting angiogenesis are complementary mechanisms (Makanya et al., 2009; Mentzer & Konerding, 2014).
3. Recruitment of endothelial progenitor cells and vasculogenesis, a process through which endothelial progenitor cells are required in response to several growth factors, cytokines and/or hypoxia-inducible factors. Endothelial progenitor cells differentiate into mature endothelial cells and are incorporated into the angiogenic sprout, thus contributing to new blood vessel formation (Kolte et al., 2016; Patan, 2004).
4. Vasculogenic mimicry: malignant tumor cells form de novo vessel-like structures without endothelial cells. The newly formed channels mimic the embryonic vascular network pattern, being able to provide enough blood supply to the tumor tissue (Fernández-Cortés et al., 2019; Ge & Luo, 2018).

Angiogenesis is a complex mechanism regulated by multiple signaling pathways. Among these, VEGF and Ang-Tie pathways are particularly important and have been the focus of multiple studies, especially in the context of cancer (Saharinen et al., 2011). During malignant transformation, when tumor cells exceed the limiting distance of the adjacent vasculature, the balance between pro-angiogenic and anti-angiogenic molecules, which is responsible for the quiescent phenotype of the vasculature, is disrupted. This deregulation is called the angiogenic switch and translates into the capacity of tumor progression and possible metastasis of tumor cells (Baeriswyl & Christofori, 2009). VEGF receptor and Tie ligands are widely distributed and were shown to play a coordinated role in endothelial cell proliferation and vessel wall assembly in normal and pathological conditions.

1.3.1. VEGF Pathway

In mammals, the VEGF system includes five secreted ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor) and three primary tyrosine kinase receptors receptor tyrosine kinases (VEGFR-1, VEGFR-2 e VEGFR-3) (Takahashi & Shibuya, 2005). The VEGF system also includes the cell-surface proteins, heparan sulfate proteoglycans and neuropilin-1 and -2, which operate as VEGF coreceptors (Chiodelli et al., 2011; Guo & Vander Kooi, 2015).

VEGF-R1 and VEGF-R2 are expressed in vascular endothelial cells, while VEGF-R3 seems to be prominently expressed in lymphatic endothelial cells (Cross et al., 2003). VEGF ligands have different affinities for one of the three VEGF-R. As tyrosine kinases receptors, upon dimerization by a VEGF ligand, the VEGF-Rs auto-phosphorylate, a phenomenon which in turns activates downstream signaling pathways including mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinotisol-3 kinase (PI3K-AK) pathway, and the phospholipase-C-γ pathway. Those pathways drive various intracellular effects in endothelial cells, such as migration, proliferation and cell survival. The activation of phospholipase-C-γ pathway via VEGF-A-VEGF-R2 binding was reported to be a key signal for endothelial proliferation (Shibuya, 2011).

In tumoral context, hypoxia is one of the main stimuli responsible for VEGF expression, which is produced and secreted by tumor cells and by the adjacent stroma. An increased expression of this protein correlates with progression, invasion, metastasis and tumor recurrence in several types of cancer, including breast cancer and colorectal cancer (Apte et al., 2019; Goel & Mercurio, 2013).

In 2001, a new VEGF was identified, the endocrine-gland-derived VEGF (EG-VEGF). This ligand does not show any structural homology to the VEGF family, but displays several biological similarities to VEGF ligands, including hypoxic regulation and ability to induce fenestration in target cells. Moreover, EG-VEGF expression is restricted to steroidogenic tissues (adrenal, ovary, testis and placenta) and its effects seem to be restricted to endothelial cells derived from these organs (LeCouter et al., 2001).

1.3.2. Ang-Tie pathway

Ang-Tie signaling pathway regulates vascular permeability and remodeling during tumor angiogenesis and metastasis. Ang/Tie signaling seems to complement VEGF signaling pathway by controlling later stages of angiogenesis and by being involved in vascular maturation (Figure 2) (Augustin et al., 2009).

The angiopoietin family includes two type 1 transmembrane protein receptors: Tie1 and Tie2 and four ligands: Ang1, Ang2, Ang3 and Ang4. Ang1 and Ang2 have been identified as the main ligands for Tie receptors, while the Ang3 and Ang4 biological function is still poorly characterize (Eklund & Saharinen, 2013; Fagiani & Christofori, 2013; H. J. Lee et al., 2004).

Ang1 binds and activates Tie2 resulting in Tie internalization and ligand release. Then it leads to Tie2 tyrosine residues phosphorylation that in turn recruits adaptor proteins and ignites PI3K/Akt and MAPK signaling pathways, promoting pro-survival, anti-permeability, and anti-inflammatory effects on endothelial cells (Davis et al., 1996). Tie2 is not required for the endothelial cells' differentiation but is rather reported as necessary for cell maintenance (Dumont et al., 1994).

Ang2 that shares approx. 60% amino acid homology with Ang1, binds to Tie2 with a similar affinity as Ang1. Ang2 seems to block the Ang1-induced Tie2 phosphorylation. Ang2 is upregulated during tumor angiogenesis and so was considered as a potential antiangiogenic target. However, recent studies found Ang2 to have a dual function, acting as Tie2 antagonist in the presence of Ang1 or acting as Tie2 agonist in the absence of Ang1 (Yuan et al., 2009). Different studies reported that it is unlikely that Tie2 can act differently when binding to Ang1 and Ang2, since both angiopoietins interact with Tie2 in a structurally similar manner and pointed out that other still unidentified mechanisms were likely to be involved (Seegar et al., 2010). One of the proposed mechanisms involve the Tie1 receptor (Seegar et al., 2010).

Contrary to Tie2, the Tie1 has been less well characterized. Tie1 is considered an orphan receptor and is mainly expressed at vascular bifurcations and branching points,

with no yet identified in vivo ligand (Porat et al., 2004). It is well known, however, that Tie1 has an important role in vascular development, since it is inactivation causes late embryonic lethality and vasculature maturation failure (La Porta et al., 2018; Puri et al., 1995). Cells expressing both receptors are responsive to chemotactic signals and able to promote vessel branching and sprouting that is required for angiogenesis. On the other hand, Tie1 is absent in stable and quiescent mature vessels (Seegar et al., 2010).

A mechanistic study indicated Tie1 as being responsible for angiopoietin's differential function. In mature vessels, as Tie1 is absent, Tie2 can be activated by either Ang1 or Ang2, to promote vessel stability. On active angiogenesis sites, Tie1 and Tie2 form a complex and Ang2 fails to activate Tie2, allowing vessel branching to be promoted. On the other hand, Ang1 is able to dissociate Tie2 from the Tie-Tie2 complex, activating Tie2 and thus enhancing vascular stability (Seegar et al., 2010; Zhang et al., 2019).

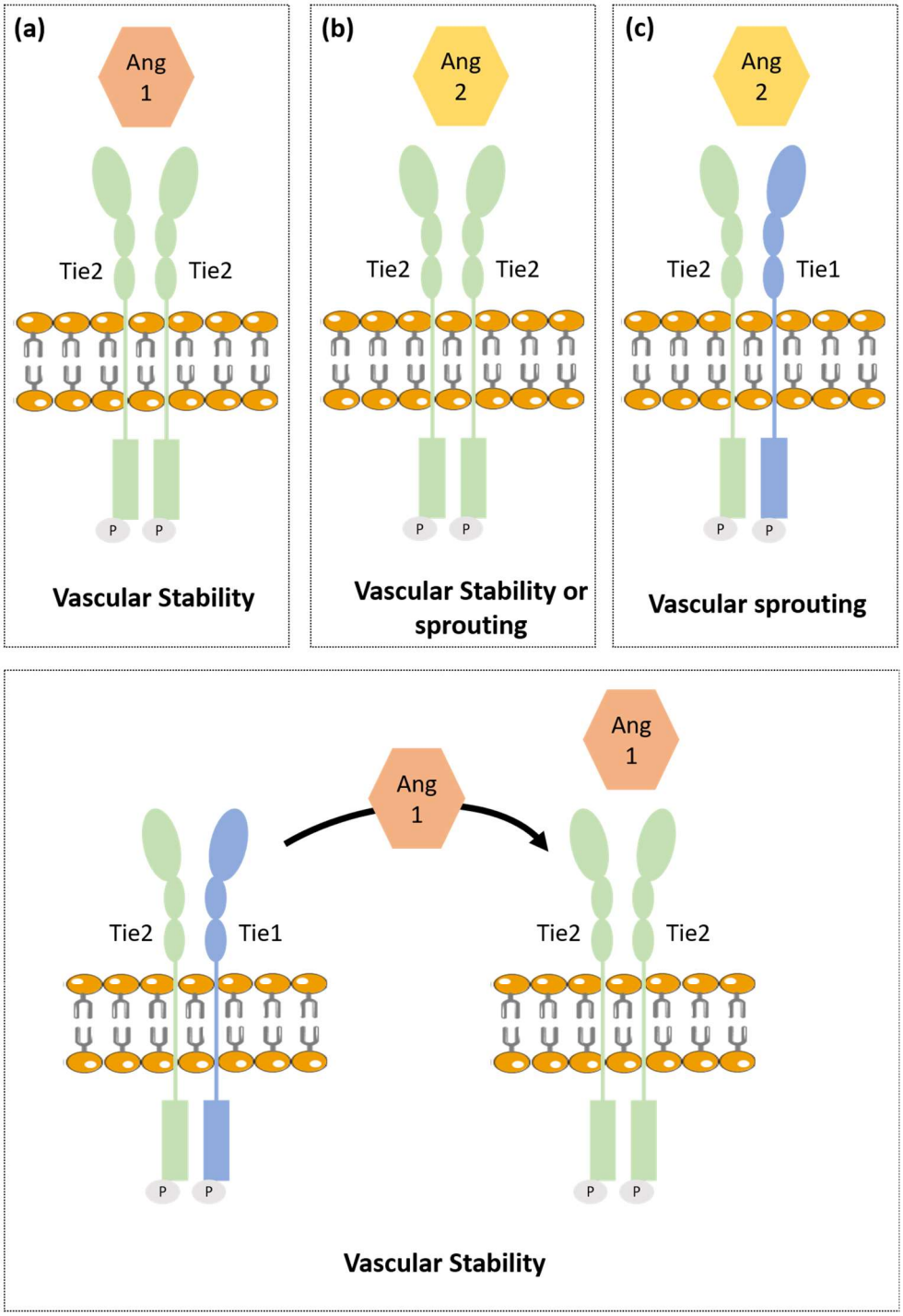

Figure 2 - Schematic illustration of the vessel stability regulation by Ang-Tie signaling. (a) Ang1 binds to Tie2 promoting vasculature stability. (b) Ang2 has a dual function acting as agonist or antagonist of Tie2 to promote vascular stability or sprouting, respectively. (c) Tie1 forms a complex with Tie2. Upon Ang2 stimulation, Tie1 and Tie2 remain associated and Ang2 induces vascular sprouting. (d) Ang1 stimulation promotes Tie2 clustering leading to vascular stability. This schematic illustration includes the most consensual theories; however, this pathway is not yet fully understood. Besides that, this figure is a schematic representation that is not intended to translate the real chemical conformation of the proteins (Pereira et al., 2021).

1.4. Angiogenesis in Adrenocortical Tumors

In adrenocortical tumors, angiogenesis has been previously characterized through the VEGF system (Table 3).

Table 3 – VEGF system findings in adrenocortical tumors [adapted from (Pereira et al., 2021).

	Group Comparisons	Results	Ref.
VEGF	Patients with ACT vs. Healthy individuals	↑ VEGF serum levels in patients with ACT	(Kolomecki et al., 2001; Zacharieva et al., 2004)
	Aldosterone secreting ACA vs. Non-functioning ACA	↑ VEGF tumor expression in aldosterone producing ACA	(Bernini et al., 2002)
	Cortisol secreting ACA vs. Aldosterone secreting ACA	↑ VEGF serum levels in patients with cortisol secreting ACA	(Zacharieva et al., 2004)
	ACC vs, Normal adrenal glands	↑ VEGF expression in ACC	(Bernini et al., 2002; Kroiss et al., 2011)
	ACC vs. ACA	↑ VEGF serum levels in ACC ↑ VEGF tumor expression in ACC	(Bernini et al., 2002; de Fraipont et al., 2000; Kolomecki et al., 2001; Xu et al., 2011)
	Patients with recurrent ACC vs Patients with non -recurrent ACC	↑ VEGF serum levels in recurrent ACC ↑ VEGF tumor expression in recurrent ACC	(Bernini et al., 2002; Zacharieva et al., 2004)
	Localized ACC vs. Invasive ACC	No difference in VEGF tumor expression	(de Fraipont et al., 2000)
VEGF-R2	ACC vs Normal adrenal glands	↑ VEGF-R2 tumor expression in ACC	(Kroiss et al., 2011)
	ACC vs ACA	↑ VEGF-R2 tumor expression in ACC	(Xu et al., 2011)

ACA - Adrenocortical Adenomas; ACC - Adrenocortical Carcinoma; ACT - Adrenocortical Tumors; VEGF - Vascular endothelial growth factor; VEGF-R2 - Vascular endothelial growth factor receptor 2; ↑ - increased levels or expression.

Patients with ACT were found to present higher VEGF serum levels as compared to healthy controls (Holmes & Zachary, 2005; Zacharieva et al., 2004) In addition, Kolomecki *et al.* demonstrated that VEGF serum levels were significantly higher in patients with non-functioning malignant tumors than in patients with non-functioning ACA. Noteworthy, VEGF serum levels in patients with ACC were shown to decrease

after tumor surgical resection and increase in patients who experienced tumor recurrence (Kolomecki et al., 2001). de Fraipont *et al.* found that cytosolic VEGF-A concentrations were higher in ACC when compared to ACA, although not being significantly different when localized and more invasive ACC were compared (de Fraipont et al., 2000). Nevertheless, cytosolic VEGF-A concentrations were higher in recurrent as compared to non-recurrent ACC after primary tumor resection (de Fraipont et al., 2000).

Tumor VEGF expression was also found to be higher in ACC as compared to normal adrenal glands and ACA (Bernini et al., 2002; Kroiss et al., 2011; Xu et al., 2011). VEGF receptor 2 tumor expression was also found to be higher in ACC when compared with ACA and normal adrenal glands (Kroiss et al., 2011; Xu et al., 2011).

Bernini *et al.* assessed the vascular density of adrenocortical tumors through the expression of CD34, a transmembrane glycoprotein used to evaluate the vascular system, and found that tumor VEGF expression was not directly correlated with vascular density, which was lower in ACC as compared to ACA and normal adrenal tissue. The fact that a higher VEGF expression was not shown to be associated with increased vascular density in ACC, was somehow unsurprising since a high vascular density already characterizes normal adrenal cortex tissue. What surprised researchers was that despite ACC lower vascular density, patients still had a very short survival time (Bernini et al., 2002).

Other studies reported that although no differences in vascular density were noticed when ACC, ACA and normal adrenal glands were compared, blood vessels perimeter and area were higher in ACC when compared to (Diaz-Cano et al., 2001; Sasano et al., 1998). In addition, endothelial cell proliferation was higher in ACC (Sasano et al., 1998).

On an opposed direction, another group reported vascular density to be higher in malignant ACT as compared to benign ACT (Zhu et al., 2014) Another study observed that its series VEGF expression was positively correlated with vessel density (Xu et al., 2011). Pereira *et al.* also reported ACC to present a higher vascular density, but only when compared to cortisol secreting ACA (Pereira et al., 2018). This could, however, be derived from cortisol anti-angiogenic effects (Logie et al., 2010). There is additional evidence supporting that adrenocortical angiogenic status could be tightly related to the tumor's hormonal functionality. Bernini *et al.* found that VEGF tumor expression was higher in aldosterone secreting ACA as compared to non-functioning ACA and normal adrenal glands (Bernini et al., 2002). In addition, in another study patients with cortisol-

secreting ACA were found to have higher circulating VEGF levels than in patients with aldosterone secreting adenomas (Zacharieva et al., 2004).

The discovery of EG-VEGF, a steroidogenic organ specific VEGF, brought some enthusiasm to the scientific community as a potential explanation to the contradictory angiogenic patterns in ACTs as well as a potential target for ACC treatment. Heck *et al.* characterized the expression of EG-VEGF and its receptors [prokineticin receptor 1 (PKR1) and 2 (PKR2)] in a large number of ACC, ACA, and normal adrenal glands. In this study, EG-VEGF and both receptors PKR1 and PKR2 were found to be present in the majority of ACT. Moreover, the nuclear protein expression of either EG-VEGF or PKR1 or both in ACC was reported to be associated with higher mortality, suggesting that these could be used as prognostic markers for overall patient survival (Heck et al., 2015).

The importance of the Ang-Tie pathway in fetal human adrenal (HFA) development and maintenance of adrenal vasculature in adults, has been previously demonstrated, however, the role of this pathway in adrenocortical tumors is still unknown (Pereira et al., 2021).

Chapter 2

Aim

To evaluate the role of VEGF and Ang-Tie pathways in ACT angiogenesis and the potential usefulness of its molecular intervenient for diagnosis and/or prognosis of ACC.

Chapter 3

Material and Methods

1.1. Case Selection

Adrenal tissue sections were obtained during adrenalectomy from patients with ACT (n=43), including ACC (n=22) and ACA (n=21). Benign tumors included cortisol producing adenomas (ACAc) (n=8) and non- functioning adenomas (ACAn) (n=13). A summary of the patients and tumors characteristic is given in table 4. The study was approved by the ethics committee of Centro Hospitalar Universitário do São João (Ethical approval code: CE 62-14), Instituto Português de Oncologia (Ethical approval code: CE 14/2020) and Centro Hospitalar Universitário do Porto (Ethical approval code: 173-DEFI/180-CE).

Table 4 – Patients and tumors characteristics.

	ACC	ACA	
		N/F	Cushing
N	22	13	8
Age at surgery	54 ± 11	34 ± 8	59 ± 12
Sex F:M	15:7	9:3	7:1
Tumor size (cm) (range)	10 ± 5.6 (2.7-20)	4.1 ± 2.2 (1.8-9.5)	3.5 ± 0.98 (2.4-5)
Weiss score (range)	3-8	0-1	0
ENSAT score			
1	15%	NA	NA
2	31%		
3	31%		
4	23%		
Functionality			
N/F	9%	100%	0
Cortisol	9%	0	100%
Aldosterone	5%	0	0
Androgens	9%	0	0
Cortisol + Androgens	5%	0	0
Unknown	63%	0	0

ACA: Adrenocortical Adenomas; ACC: Adrenocortical Carcinomas; ENSAT: European Network for the Study of Adrenal Tumors; NA: Not Applicable; NF: Non-Functioning.

1.2. Immunohistochemistry

The immunohistochemistry (IHC) technique was performed for the detection of the proteins involved in angiogenesis, CD34, VEGF, VEGF-R2, Ang1, Ang2, Tie1 and Tie2 as described below.

For that, 3 μm formalin-fixed paraffin-embedded tissue sections were mounted on adhesive microscope slides (Superfrost® Plus, Menzel-Gläzer). Tissue sections were deparaffinized in xylene, hydrated in graded alcohols and underwent antigen retravel, as described in table 5.

Table 5 – Antigen retrieval and washing solutions for each primary antibody.

Antibody	Antigen Retrieval	Washing Solutions
CD34	Microwave treatment in 0.01M citrate buffer at pH 6.0 with 0.05% Tween 20 during 15 minutes	PBS 0.05% Tween 20
VEGF	Pressure cooking boiling for 3 minutes in 0.01M citrate buffer at pH 6.0	PBS
VEGF-R2	Pressure cooking boiling for 3 minutes in 0.01M citrate buffer at pH 6.0	PBS
Ang1	Microwave treatment in 0.01M citrate buffer at pH 6.0 during 15 minutes	PBS
Ang2	Microwave treatment in 0.01M citrate buffer at pH 6.0 during 15 minutes	PBS 0.05% Tween 20
Tie1	Pressure cooking boiling for 3 minutes in 0.01M citrate buffer at pH 6.0 with 0.05% Tween 20	PBS 0.05% Tween 20
Tie2	Pressure cooking boiling for 3 minutes in 0.01M citrate buffer at pH 6.0 with 0.25% Triton-X	PBS 0.05% Triton-X

Ang1: angiopoietin 1; Ang2: angiopoietin 2; PBS: Phosphate buffer saline; Tie1: tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF: Vascular endothelial growth factor; VEGF-R2: vascular endothelial growth factor receptor 2.

After that, the sections were rinsed in the respective washing solution (Table 5), followed by a treatment with 3% hydrogen peroxide (H_2O_2) (MERK, Germany) in methanol to inhibit endogenous peroxidase for 15 minutes. For Ang2, tissue sections were also incubated with 10% bovine serum albumin (BSA) at room temperature for 30 minutes to block non-specific reactions.

Then the tissues sections were incubated overnight at 4 °C with the primary antibody, as described in Table 6.

Table 6 – Primary antibodies used in immunohistochemistry in this study.

Antibodies	Source	Reference and vendor	Dilution
CD34	Rabbit	Ref. ab81289; Abcam, Cambridge, United Kingdom	1:2000
VEGF	Rabbit	Ref. ab52917; Abcam, Cambridge, United Kingdom	1:100
VEGF-R2	Rabbit	Ref. ab2349; Abcam, Cambridge, United Kingdom	1:100
Ang1	Rabbit	Ref. ab8451; Abcam, Cambridge, United Kingdom	1:400
Ang2	Rabbit	Ref. ab153934; Abcam, Cambridge, United Kingdom	1:400
Tie1	Mouse	Ref. ab201986; Abcam, Cambridge, United Kingdom	1:100
Tie2	Mouse	Ref. ab24859; Abcam, Cambridge, United Kingdom	1:100

Ang1: Angiopoietin 1; Ang2: Angiopoietin 2; Tie1: Tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: Tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF: Vascular endothelial growth Factor; VEGF-R2: Vascular endothelial growth factor receptor 2.

The detection of the immune reaction was performed by incubation, for 60 minutes, with the commercial Dako Real™ EnVision™ Detection System (K5007, Dako, Denmark), which includes a dextran backbone with peroxidase molecules and goat secondary antibody against rabbit and mouse immunoglobulins. 3,3'-diaminobenzidine (DAB) was used as chromogen and hematoxylin as nuclear counterstaining (Table 7). The tissue sections were dehydrated in graded alcohols and xylene before being mounted.

Table 7 – Time of DAB reaction and hematoxylin dilution used.

Antibody	Time of DAB reaction		Hematoxylin dilution in H_20
	DAB	DAB	

	(k5007, Dako, Denmark)	(k346811-2, Dako, North America)	(HX90507449, MERCK, Germay)
CD34	2 minutes		1:2
VEGF	2 minutes		1:2
VEGF-R2	2 minutes		1:2
Ang1		1 minute	ND
Ang2		1 minute	ND
Tie1	2 minutes		1:2
Tie2		1 minute	1:2

Ang1: Angiopoietin 1; Ang2: Angiopoietin 2; DAB: 3,3'-diaminobenzidine; ND: Not diluted hematoxylin; Tie1: Tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: Tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF: Vascular endothelial growth factor; VEGF-R2: Vascular endothelial growth factor receptor 2.

As a positive control, kidney tissue was used for Tie1, VEGF and CD34, lung tissue for Tie2 and Ang1; placenta for Ang2 and breast tissue for VEGF-R2. The omission of the primary antibody incubation was used as negative control.

1.3. Computerized Image analysis

To evaluate the stained area for the CD34, VEGF-R2, Ang1, Ang2 and Tie1 computerized image analyses were performed. First, slides were scanned for each marker using the image acquisition Olympus® VS110™ virtual slide scanning system and captured using the image acquisition software VS-ASW (version 2.3 for Windows).

IHC images were analyzed using the software ImageJ (National Institutes of Health, USA), that allows the separation of the stained area from each histological image based in Red-Green-Blue (RGB) system, through a color deconvolution plug-in (H-DAB), as shown in figure 3. The software quantifies the stained area, which corresponds to the area stained by respective antibody and also the total tissue area. The percentage of the stained area for each protein was calculated through the ratio between the stained area and the total tissue.

VEGF localization within the cells varied across the different tumor. Therefore, a computerized analysis as performed for the remaining proteins would not be reliable in translating these cellular phenomena. So instead, we evaluated the protein expression localization within the cell for each tumor: cytoplasm and nuclear expression or cytoplasm expression only.

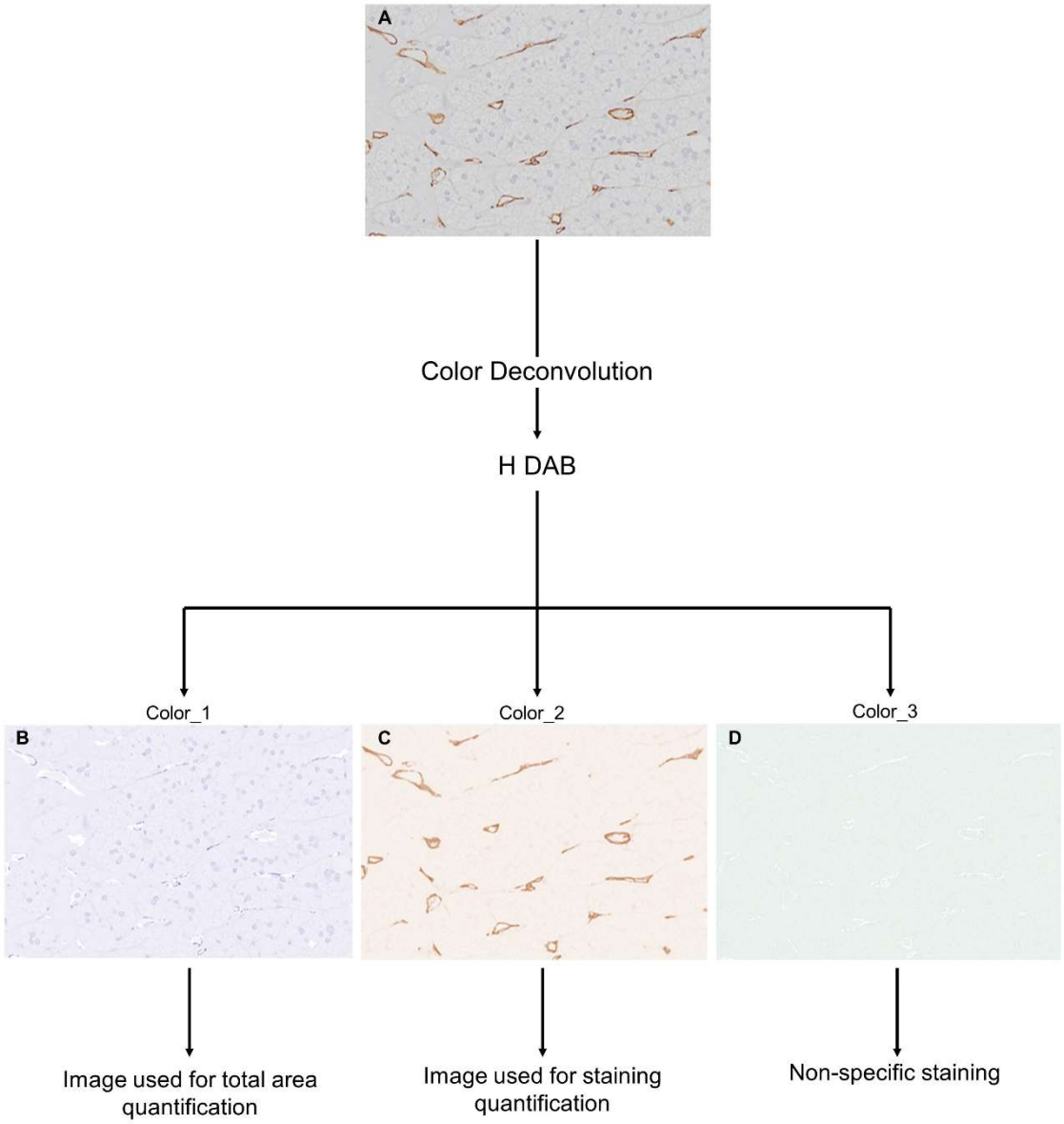

Figure 3 - Representation of color deconvolution plug-in (H-DAB) on Image J. From the scanned image (A) H DAB plug-in allows isolating hematoxylin staining (B), DAB staining (C) and non-specific staining (D). Thus, it is possible to quantify the percentage of the area stained by DAB and the total area.

1.4. Statistical analysis

All ordinal data are represented as mean ± standard error of the mean (SEM). To evaluate variables normality the D' Agostinho & Pearson test was used. For variables that pass this test, one way ANOVA test with the Post-hoc Tukey was used to compare the means of 3 groups and, the t-test to compare 2 groups. For variables that did not pass the normality test, the Kruskal Wallis with a Post-hoc Dunn's was used to compare 3 groups and the Mann-Whitney test to compare 2 groups. The two different VEGF expression patterns were compared among the different groups through χ^2. Correlation between continuous variables were evaluated using the Pearson Test or the Spearman Test depending on the variables. Kaplan-Meier method was used to compare overall

survival of patients depending on the VEGF expression localization. For the remaining markers, Cox regression model, adjusted for age, was used for overall survival analyses.

The area under the receiver operating characteristic (ROC) curve (AUC) was used to determine the diagnosis accuracy of the angiogenic markers. AUC values ranging between 0.90 and 1.00 were considered excellent, good when 0.80 and 0.90, fair when 0.70 and 0.80, poor when 0.60 and 0.70 and failed if below 0.60. All statistical analysis were performed using the GraphPad Prism (version 8.01) except for the Cox regression analysis that was performed using the SPSS software (version 26.00 for Windows). A $p<0.05$ was considered statistically significant.

Chapter 4

Results

4.1. Immunostaining results

4.1.1. CD34 expression

ACT tumor blood vessel density was evaluated by immunohistochemistry staining for CD34 (Figure 4 A-C). The percentage of CD34 stained area was significantly lower in ACC when compared with total ACA (ACAc + ACAn) (4.023 ± 0.408% vs 8.735 ± 0.918%, $p<0.001$) (Figure 4E). Similarly, the vascular area was significantly lower in ACC when compared to both ACAc (9.947 ± 1.431%, $p<0.01$) and ACAn (7.988 ± 1.188%, $p<0.001$) (Figure 4F). No differences were observed between the two groups of adenomas.

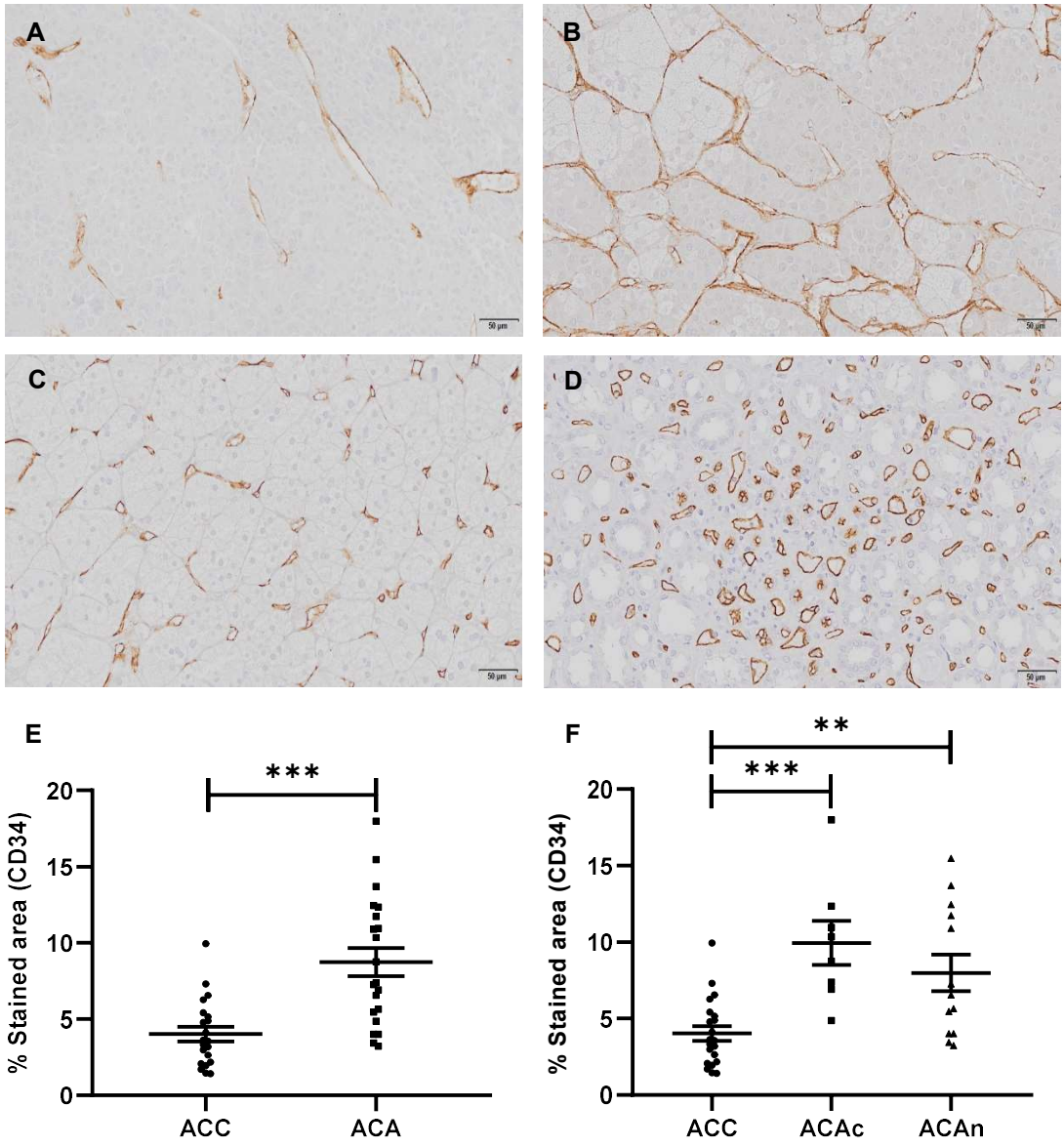

Figure 4 - Immunohistochemistry staining for CD34 (200×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (kidney); E: Graphic representation of the stained area between ACC and ACA (ACAc + ACAn) (T-test: *** $p<0.001$); F: Graphic representation of the stained area in ACT groups (ANOVA: *** $p<0.001$, ** $p<0.01$).

4.1.2. VEGF expression

VEGF expression was present in all ACT, however its location within the cells varied across the tumors (Figure 5 A-C). VEGF expression was found in the nucleus and in the cytoplasm in 38% of ACC and ACAn (Table 8). The remaining 62% of the cases only showed VEGF expression in the cytoplasm. In contrast, VEGF expression in ACAc was exclusively found in the cytoplasm (Table 8). However, the different VEGF localization pattern was not significantly different between ACT groups (p=0.09) (Table 8). Due to the different method adopted for IHC VEGF expression analysis, the VEGF results will be presented separately from the other angiogenic markers.

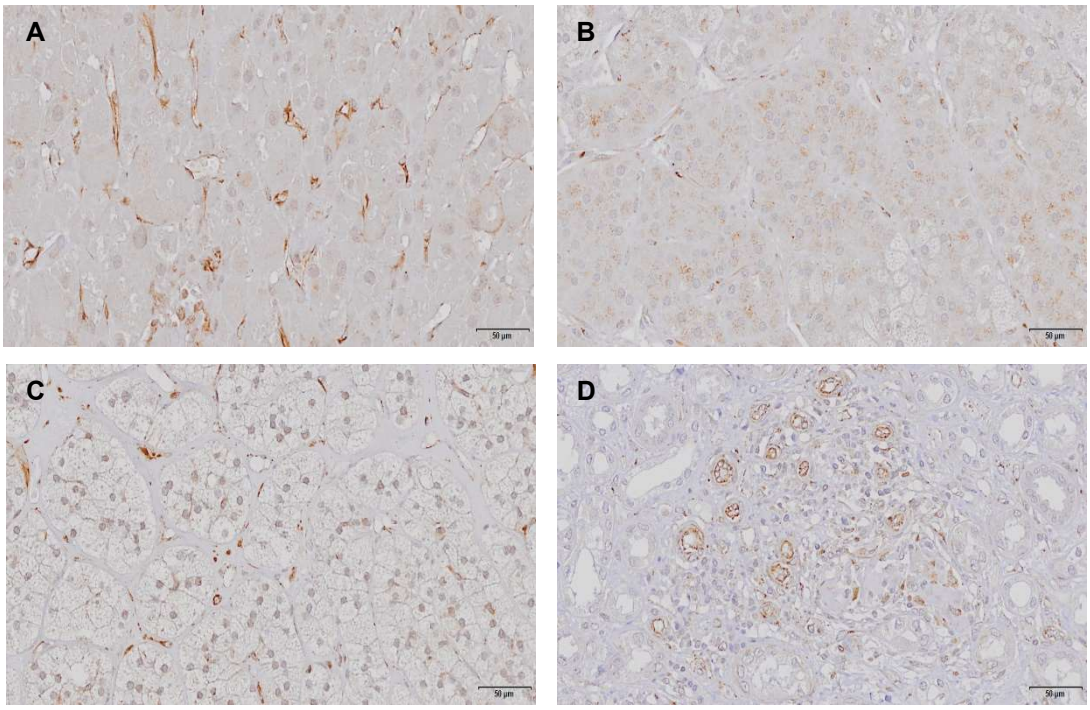

Figure 5 - Immunohistochemistry staining for VEGF (264×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (kidney).

Table 8 – VEGF immunostaining localization in the different groups of ACT.

Groups	n	Expression Pattern		p
		Nucleus + Cytoplasm	Cytoplasm	
ACC	21	8 (38)	13 (62)	0.09
ACAc	8	0 (0)	8 (100)	
ACAn	13	5 (38)	8 (62)	

ACAc: Adrenocortical Adenoma with Cushing syndrome; ACAn: Non-functioning Adrenocortical Adenoma; ACC: Adrenocortical Carcinoma.

4.1.3. VEGF-R2 expression

The VEGF-R2 expression was positive in all ACT. In addition to cytoplasm expression, VEGF-R2 nuclear expression was also observed in all ACC, ACAc and ACAn (Figure 6 A-C). The percentage of VEGF-R2 stained area was significantly lower in ACAn (10.639 ± 1.900%) than in ACAc (27.298 ± 4.454%, $p<0.05$) (Figure 6F). The VEGF-R2 stained area was not significantly different between ACC (19.194 ± 2.683%) and any adenoma's group (Figure 6 E-F).

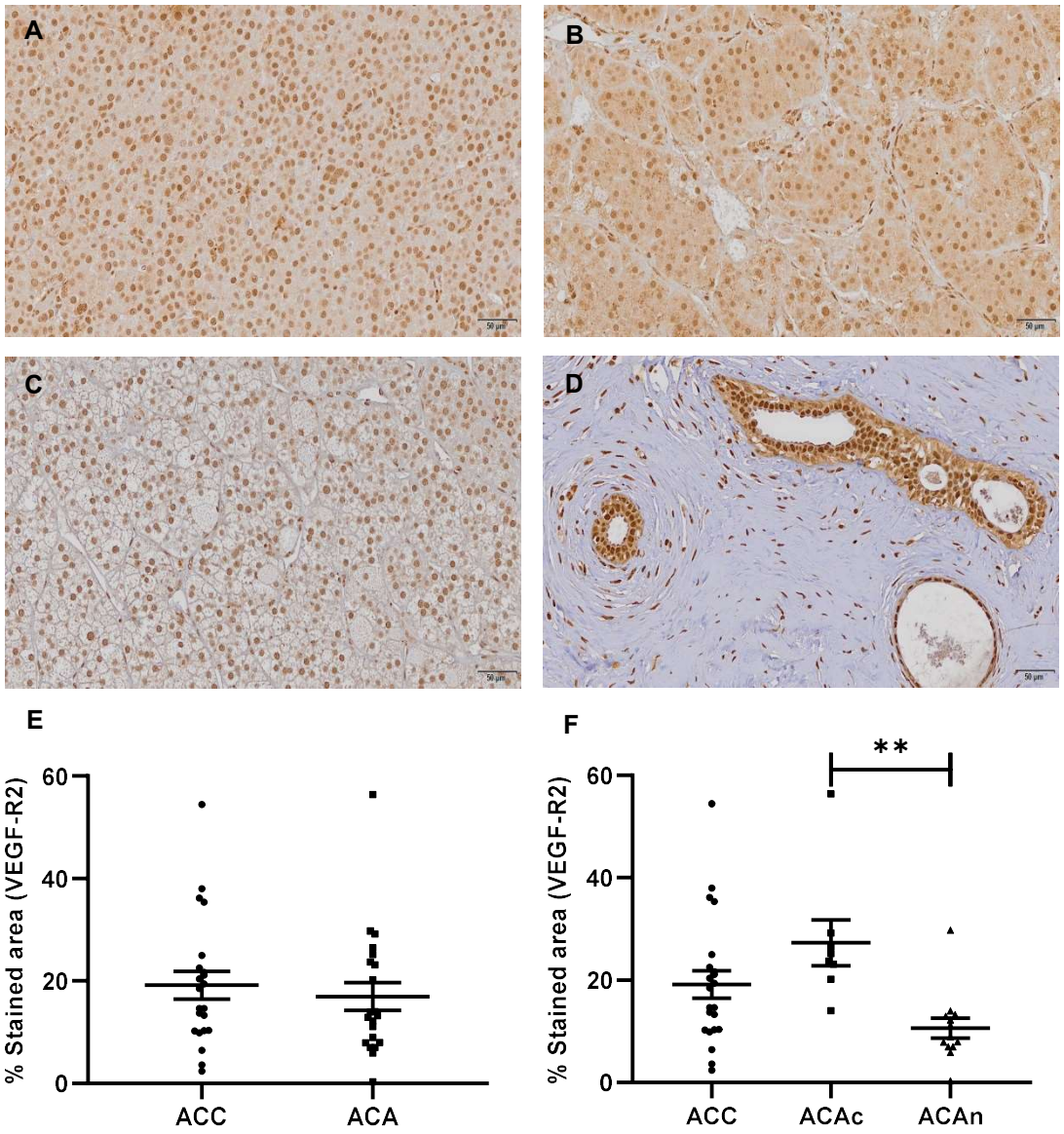

Figure 6 - Immunohistochemistry staining for VEGF-R2 (200×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (breast); E: Distribution of the stained area between ACC and total ACA (ACAc + ACAn) (Mann-Whitney: non-significant); F: Distribution of the stained area in ACT groups (Kruskal-Wallis: ** $p<0.01$).

4.1.4. Ang1 expression

Ang1 was expressed in all ACT. Ang1 expression was significantly higher in ACC (14.479 ± 1.279%) when compared to ACA (10.555 ± 1.371%, $p<0.05$) (Figure 7E). The Ang1 stained area was lower in ACAc (10.087 ± 1.377%) than in ACC, but similar with ACAn (10.843 ± 2.088%), however no significant differences were observed between these groups (Figure 7F).

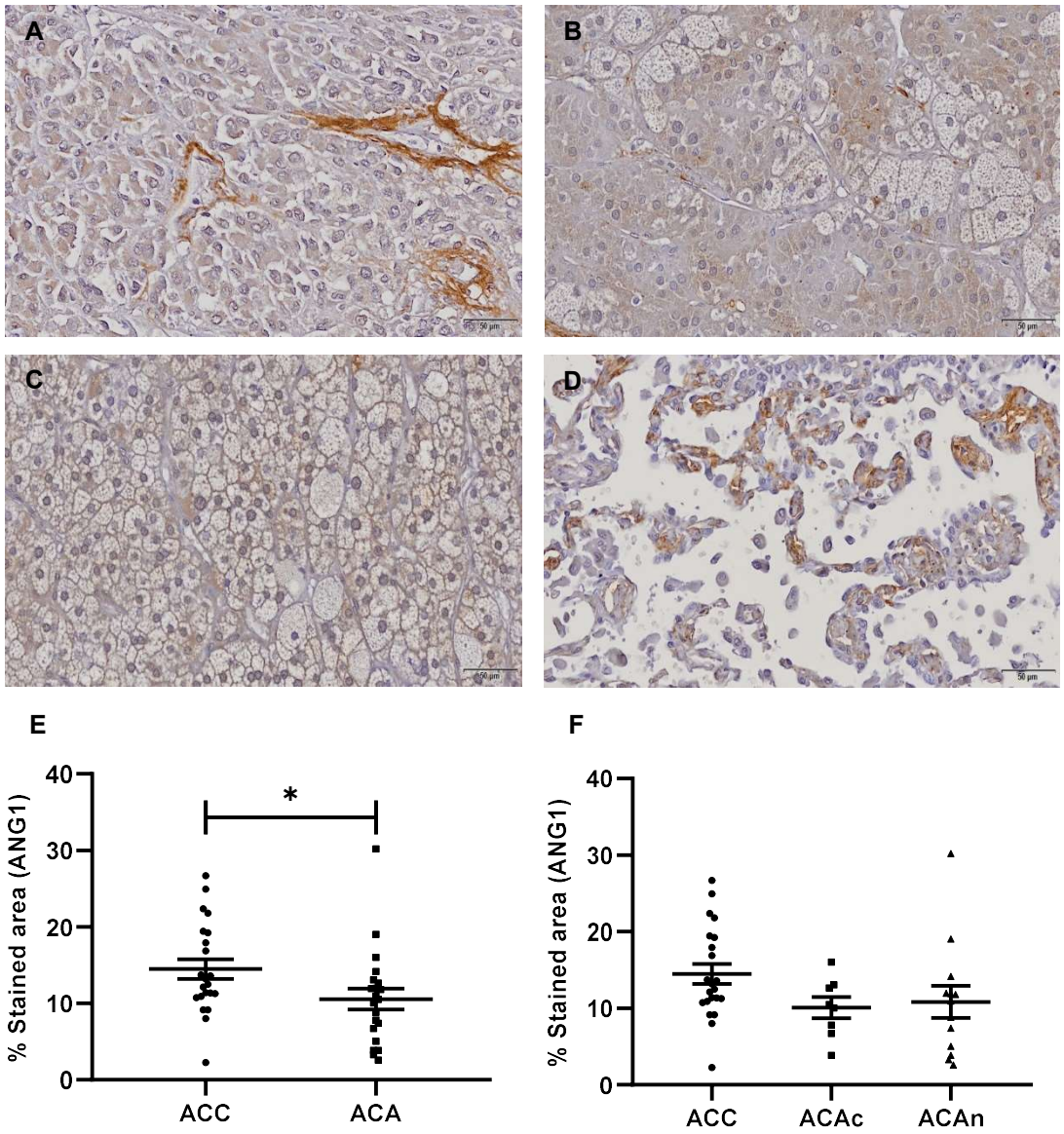

Figure 7 - Immunohistochemistry staining for Ang1 (264×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (lung); E: Distribution of the stained area between ACC and total ACA (ACAc + ACAn) (Mann-Whitney: * $p<0.05$). F: Distribution of the stained area in ACT groups (Kruskal-Wallis: non-significant).

4.1.5. Ang2 expression

Positive staining for Ang2 was observed in all ACT (Figure 8 A-C). The Ang2 stained area was significantly higher in ACC when compared to total ACA (41.731 ± 2.832% vs 31.246 ± 1.869%, $p<0.01$) and to ACAn (30.097 ± 2.428%, $p<0.05$) (Figure 8). No differences were observed between the two groups of adenomas (ACAc: 33.113 ± 2.993%) (Figure 8).

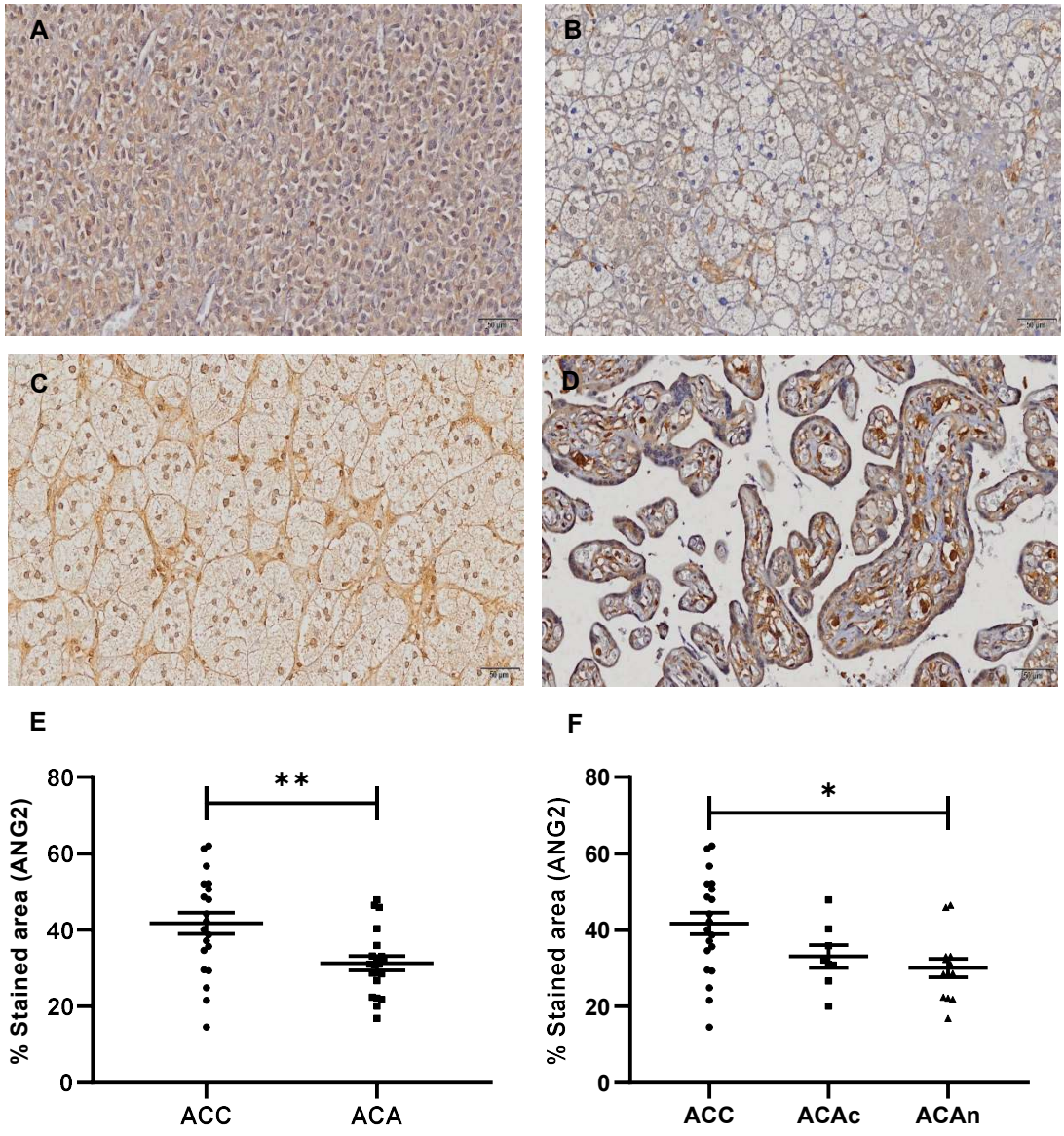

Figure 8 - Immunohistochemistry staining for Ang2 (200×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (placenta); E: Distribution of the stained area between ACC and total ACA (ACAc + ACAn) (T-test: * p<0.05). F: Distribution of the stained area in ACT groups (ANOVA: * p<0.05).

4.1.6. Tie1 expression

Tie1 expression was present in all ACT, except for one ACAn (Figure 9 A-C). No significant differences were observed between ACC and ACA (ACC: 0.373 ± 0.107%, ACAc: 0.933 ± 0.310%, ACAc: 0.933 ± 0.310% and ACAn: 0.489 ± 0.111%) (Figure E-F).

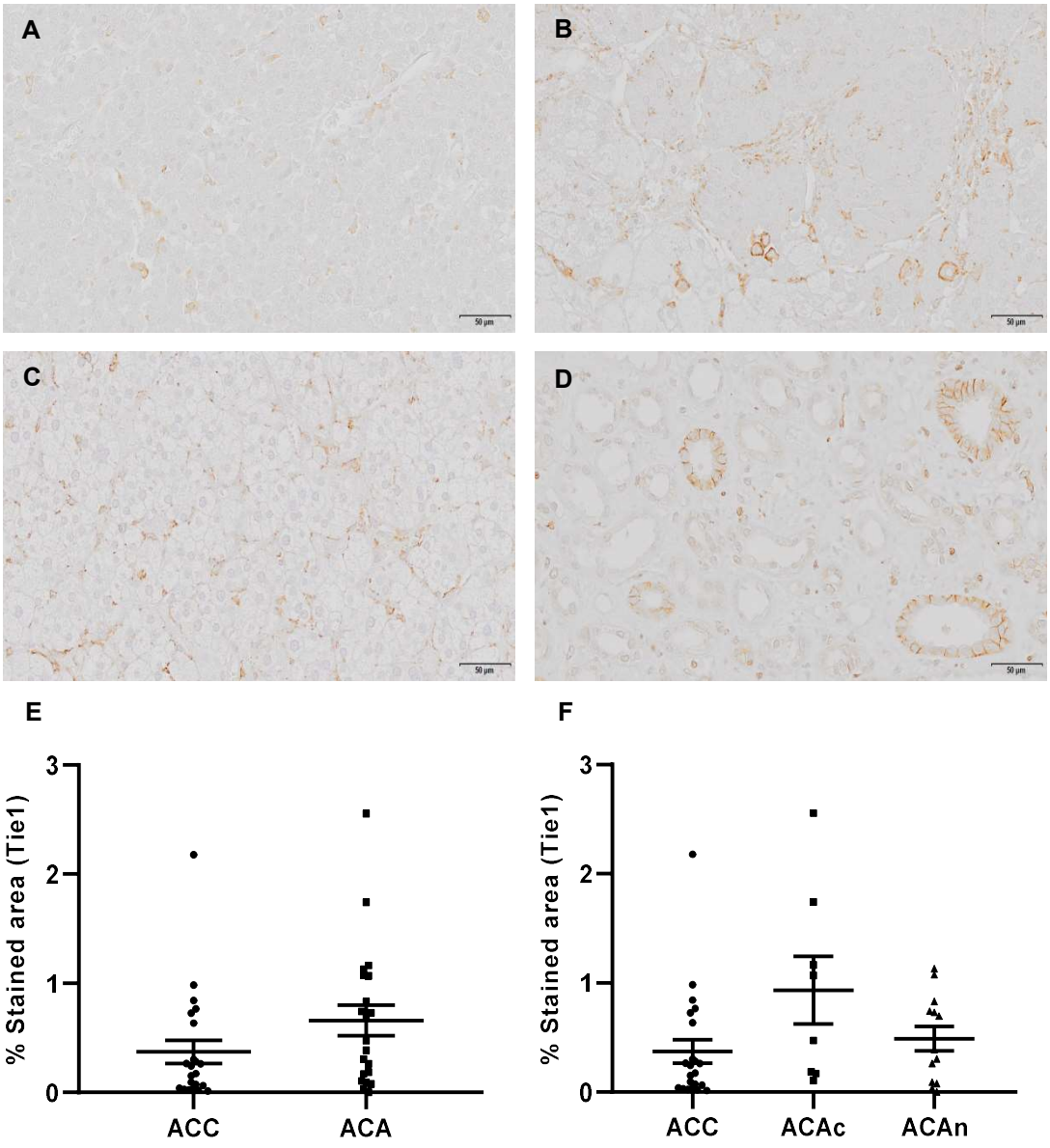

Figure 9 - Immunohistochemistry staining for Tie1 (264×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (kidney); E: Distribution of the stained area in ACC and total ACA (ACAc + ACAn) (Mann-Whitney: non-significant); F: Distribution of the stained area in ACT groups (Kruskal-Wallis: non-significant).

4.1.7. Tie2 expression

Tie2 expression was present in all ACAc and in 57.1% of ACC. In contrast, Tie2 staining was negative in ACAn group and in 42.9% of ACC (Figure 10 A-C). The percentage of Tie2 stained area was significantly higher in ACAc when compared to ACC (3.695 ± 1.682% vs 0.881 ± 0.539%, $p<0.05$) (Figure 10 F). No significant differences were observed between ACC and ACA (1.556 ± 0.805%) (Figure 10 E).

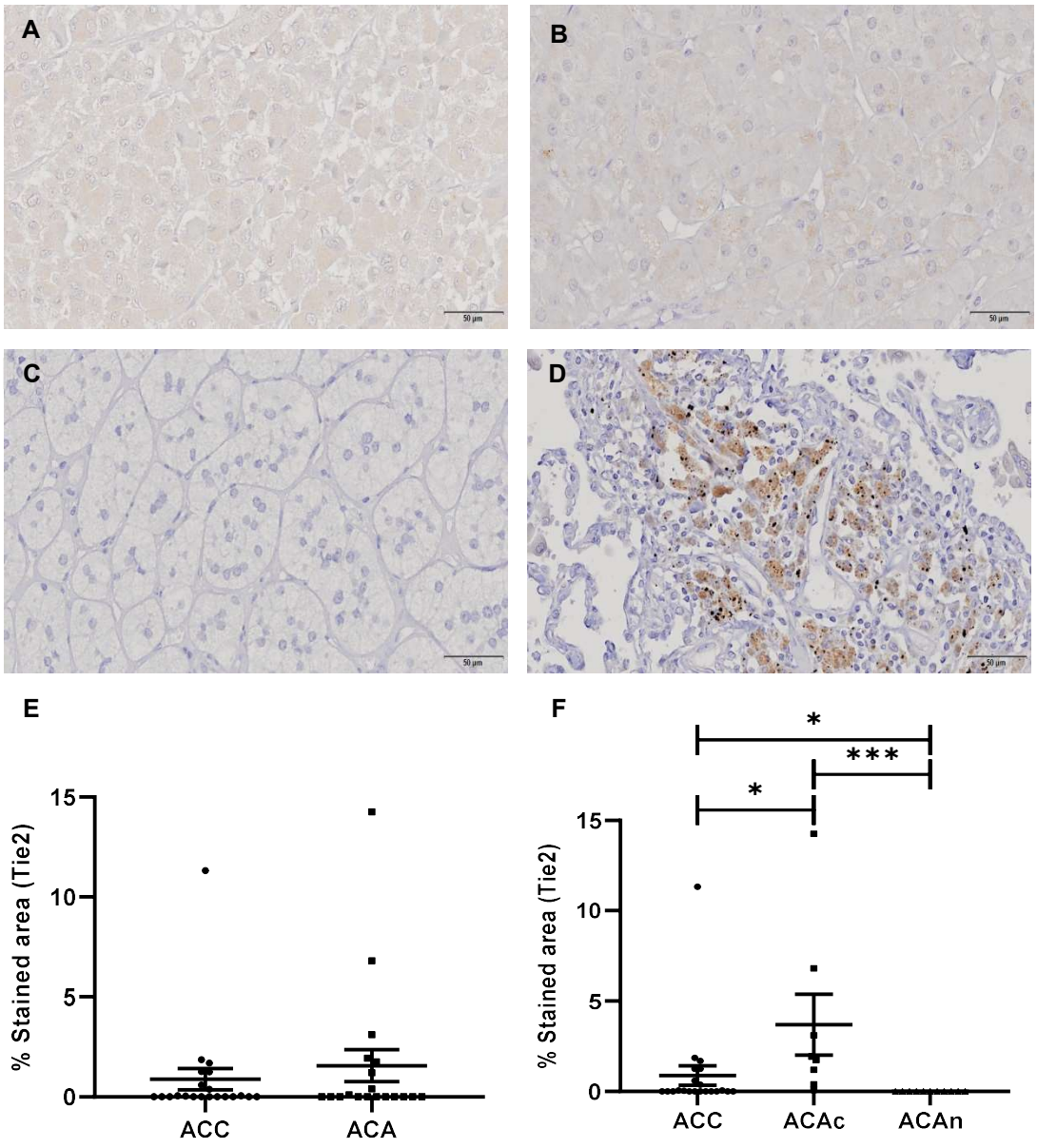

Figure 10 - Immunohistochemistry staining for Tie2 (300×). A: Adrenocortical carcinoma (ACC); B: Adrenocortical adenoma with Cushing Syndrome (ACAc); C: Non-functioning adrenocortical adenoma (ACAn); D: Positive control (lung); E: Distribution of the stained area between ACC and total ACA (ACAc + ACAn) (Mann-Whitney: non-significant); F: Distribution of the stained area in ACT groups (Kruskal-Wallis: * $p<0.05$, *** $p<0.001$).

4.2. Accuracy of the angiogenic markers for ACC diagnosis

The accuracy of each angiogenic marker for the differential diagnosis between ACC vs ACA, ACC vs ACAc and ACC vs ACAn, was assessed using the ROC curves (Figure 11 and Figure 12).

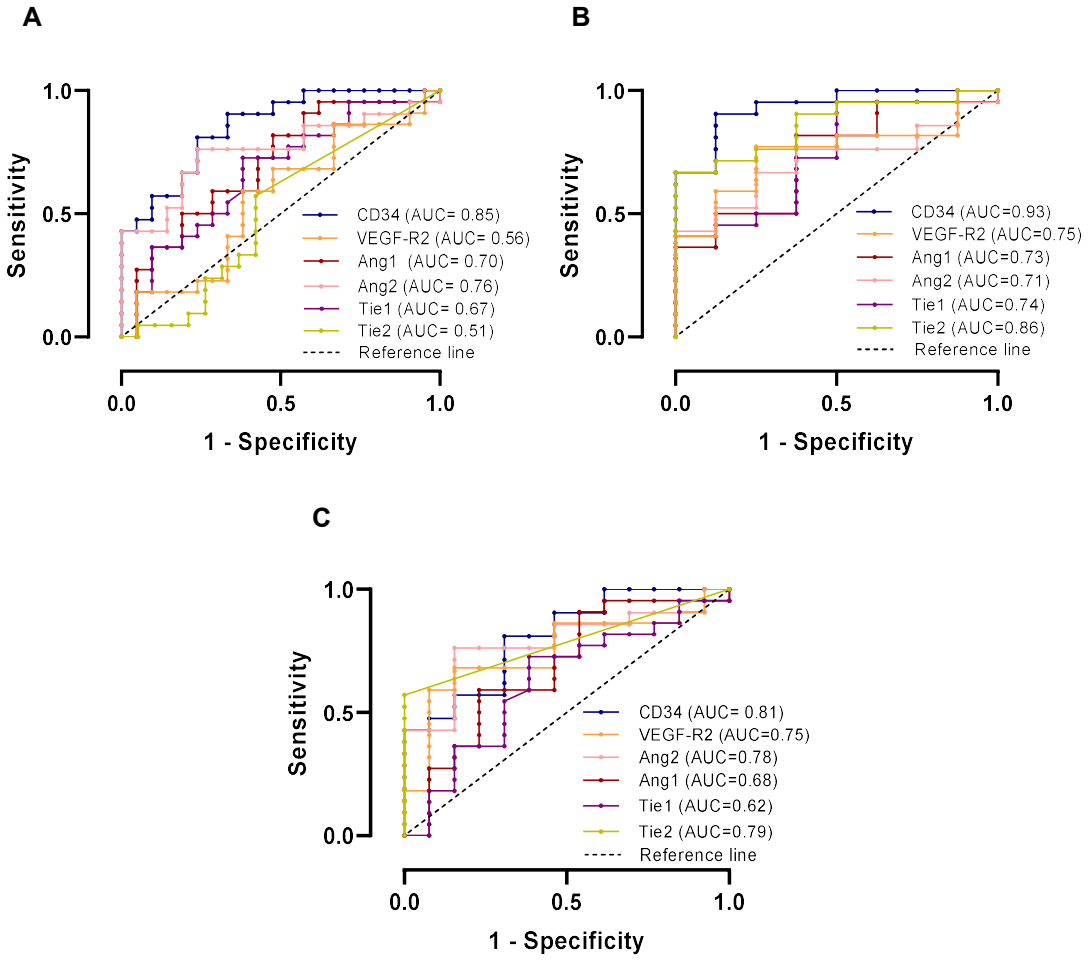

Figure 11 - Graphic representation of ROC curve to distinguish between A: ACC and total ACA (ACAc + ACAn), B: ACC and ACAc, C: ACC and ACAc with the respective area under the curve (AUC).

Analyzing the ROC curves, CD34 was the only molecular marker demonstrated to have a good accuracy for differential diagnosis between ACC and ACA. This molecular marker is particularly accurate for the differential diagnosis between ACC and functioning ACA (ACAc). In addition, Tie2 receptor also shown to be a good marker to distinguish ACC from ACAc.

Although several differences were observed when comparing total ACA and ACC, the remaining molecular markers analyzed showed low accuracy to distinguish ACC from ACA (Figure 11 and Figure 12).

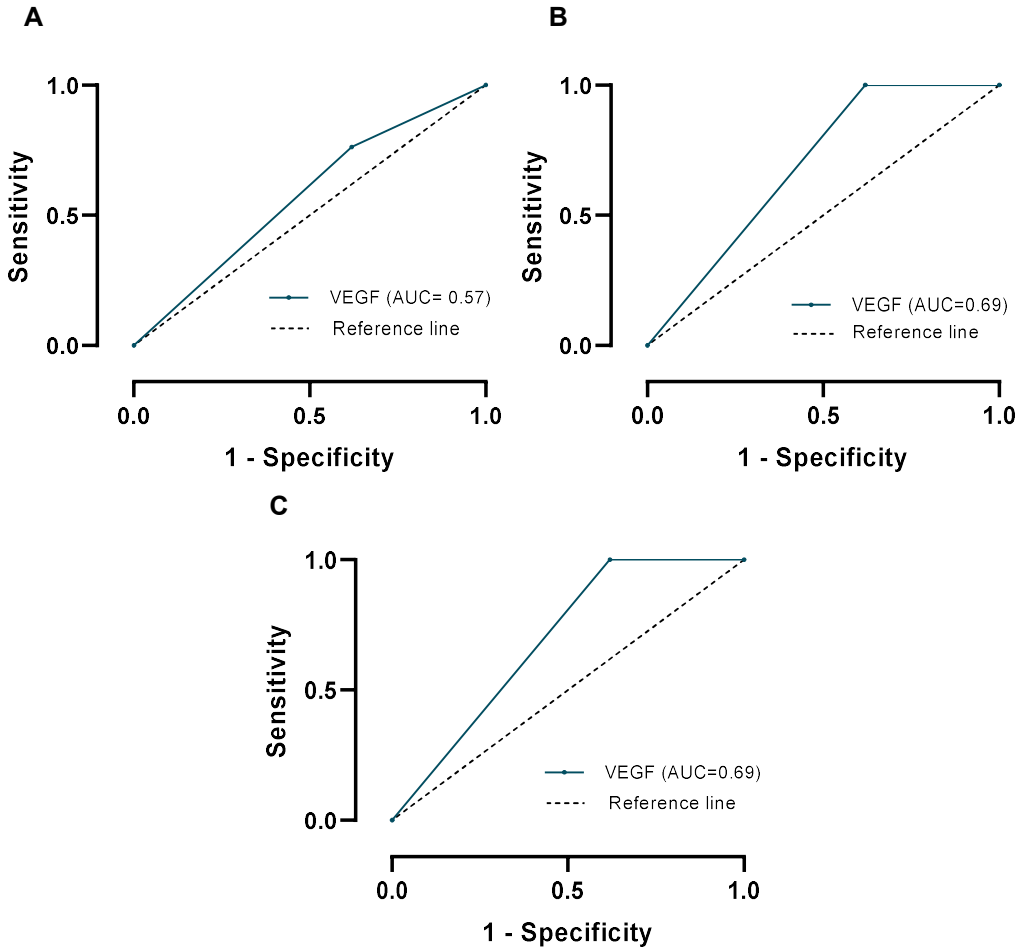

Figure 12 - ROC curves to assess the accuracy of VEGF to distinguish between A: ACC and total ACA (ACAc + ACAn), B: ACC and ACAc, C: ACC and ACAn with the respective area under the curve (AUC).

4.3. Correlation between the angiogenic markers and patients/ tumor characteristics

No significant correlations were identified between the different angiogenic markers and the tumor and patient's characteristics in ACC and ACA (Appendix table 1, 2 and 3).

For ACC group, the correlations between the percentage of stained area of each protein and several tumor parameters, including ENSAT score; presence and absent metastasis, capsule, venous and sinusoidal structures invasion, were evaluated (Table 9).

ACC with venous invasion presented higher Tie1 expression when compared to ACC without venous invasion (0.431 ± 0.133% vs 0.099 ± 0.031%, *p*=0.021*)* (Table 9). No differences were observed between the others angiogenic markers and the remaining analyzed parameters.

Furthermore, no differences were observed between the VEGF cell localization and the ACC parameters analyzed (Appendix table 4).

Table 9 – Percentage of stained area of each angiogenic marker in various parameters including gender, ENSAT score, metastasis, capsular, venous and sinusoidal invasion.

		CD34	VEGF-R2	Ang1	Ang2	Tie1	Tie2
ENSAT score	1-2	2.589 ± 0.538	9.676 ± 3.342	15.477 ± 2.278	44.699 ± 5.810	0.106 ± 0.046	0.284 ± 0.282
	3-4	4.728 ± 0.944	15.771 ± 1.767	14.824 ± 1.473	45.449 ± 5.727	0.206 ± 0.111	0.239 ± 0.178
	p	0.051	0.138	0.945	>0.999	0.731	>0.999
Metastasis	Yes	3.473 ± 0.533	17.458 ± 1.670	14.880 ± 2.207	53.200 ± 2.804	0.116 ± 0.058	0.094 ± 0.094
	No	3.859 ± 0.891	10.958 ± 2.460	15.235 ± 1.889	41.105 ± 5.294	0.179 ± 0.089	0.333 ± 0.219
	p	0.940	0.106	0.940	0.283	0.825	0.471
Capsular Invasion	Yes	3.957 ± 0.710	13.950 ± 1.664	14.030 ± 1.204	42.795 ± 3.700	0.233 ± 0.095	0.193 ± 0.111
	No	2.478 ± 0.548	9.840 ± 4.857	14.585 ± 2.55	45.314 ± 16.004	0.155 ±0.068	0.565 ± 0.564
	p	0.291	0.365	0.945	>0.999	0.734	0.739
Venous Invasion	Yes	3.914 ± 0.628	19.527 ± 2.987	14.099 ± 1.390	41.141 ± 5.186	**0.431 ± 0.133**	0.245 ± 0.162
	No	3.474 ± 0.889	12.871 ± 2.633	14.709 ± 1.704	45.182 ± 3.686	**0.099 ± 0.031**	0.235 ± 0.187
	p	0.351	0.166	0.793	0.536	**0.021**	0.872
Sinusoidal Invasion	Yes	4.158 ± 0.761	16.006 ± 2.871	14.245 ± 1.585	43.456 ± 3.733	0.215 ± 0.084	0.335 ± 0.165
	No	2.808 ± 0.289	12.350 ±2.742	14.590 ± 1.639	43.456 ± 8.110	0.193 ± 0.039	0.014 ± 0.011
	p	0.411	0.446	0.446	>0.999	0.379	0.850

Ang1: Angiopoietin 1; Ang2: Angiopoietin 2; ENSAT: European Network for the Study of Adrenal Tumors; Tie1: Tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: Tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF-R2: Vascular endothelial growth factor receptor 2.

4.4. Accuracy of the angiogenic markers for ACC prognosis

To evaluate the prognostic value of the angiogenic markers CD34, VEGF-R2, Ang1, Ang2, Tie1 and Tie2 we analyzed overall survival through Cox regression, adjusted for age, in patients with ACC (Table 10). Whereas for VEGF, we used the Kaplan-Meier method to compare the overall survival of patients with ACC with or without nuclear expression.

Table 10 – Overall survival in patients with ACC.

Angiogenic marker	HR	95% CI	*p*
CD34	0.69	0.36-1.33	0.27
VEGF-R2	1.12	0.97-1.30	0.13
Ang1	1.07	0.89-1.28	0.48
Ang2	1.03	0.95-1.12	0.49
Tie1	25.93	0.62-1087.29	0.09
Tie2	1.89	0.42-8.45	0.40

Ang1: Angiopoietin 1; Ang2: Angiopoietin 2; HR: Hazard Ratio; Tie1: Tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: Tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF-R2: Vascular endothelial growth factor receptor 2; 95% CI: 95% confidence.

The expression of the analyzed angiogenic markers does not seems to influence the overall survival of the patients with ACC (Table 10). Nevertheless, although no significant differences were observed, most patients with a higher Tie1 expression had a lower survival.

The different VEGF localization pattern observed in ACC does not seems to influence the patients' overall survival (Figure 13).

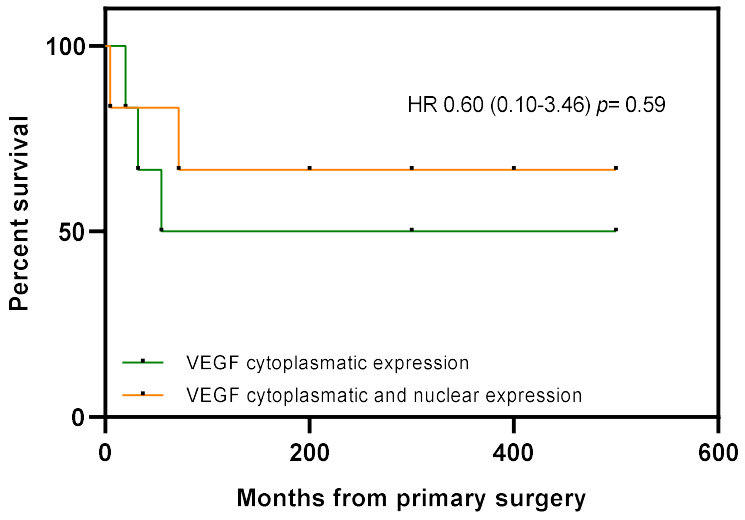

Figure 13 - Kaplan-Meier survival probability by VEGF expression pattern (p>0.05).

Chapter 5

Discussion

The majority of ACT are benign and non-functioning, in contrast to ACC, which are rare and usually very aggressive tumors. After surgery, the current diagnosis is mainly based on the Weiss system that includes subjective histological criteria, resulting in a misclassification in 9-13% of the cases (Viëtor et al., 2021). The difficulty in identifying malignant tumors in early stages, along with the lack of effective treatments, contributes to the poor prognosis in ACC (Jouinot & Bertherat, 2018). Tumor stage, age and ki-67 index are used as prognostic tools, however, there is a variability in patient survival within these prognostic factors, leading to an inaccurate clinical strategy (Else et al., 2014). Therefore, it is imperative to identify accurate molecular markers to improve the differential diagnosis between ACC and ACA and, on the other hand, to allow the prognostic stratification of ACC, and so personalization of adjuvant therapies and follow-up duration.

Angiogenesis is a key step for tumor development and progression, as well as for the spreading of neoplastic cells. The VEGF and Ang-Tie system seems to play a coordinate role in the initiation, development and maintenance of *de* novo vasculature (Lugano et al., 2020; Saharinen et al., 2011).

So, our aim was to evaluate the role of VEGF and Ang-Tie pathways in ACT angiogenesis and the potential usefulness of its molecular intervenient for the diagnosis and/or prognosis of ACC.

VEGF signaling has been associated with tumor progression due to its crucial role in inducing angiogenesis and, consequently, increasing vascularization (Chekhonin et al., 2013). We found different VEGF expression location within the cells. Nuclear expression was found in 38% of ACC and ACAn cases, whereas in the remaining cases, the immunostaining was exclusively present in the cytoplasm. Nevertheless, no significant differences were observed between all the groups. To our knowledge, VEGF nuclear expression has not been previously described in ACT. Contrarily, previous studies observed VEGF nuclear expression in other types of tumors, such as breast cancer and colorectal cancer (Bhattacharya et al., 2017; T. H. Lee et al., 2007). However, VEGF nuclear function and its internalization mechanism are still unclear (Wiszniak & Schwarz, 2021). In this study, we could not associate the different VEGF localization with ACT malignancy, since the nuclear expression was not exclusively observed in ACC. In addition, patients with ACC cases with or without VEGF nuclear expression did not seem to have a different prognosis.

Our study found that VEGF-R2 expression was similar between ACC and ACA. However, the percentage of VEGF-R2 stained area was significantly higher in ACAc

when compared to ACAn. Adrenal cortex is characterized by having high levels of VEGF expression that is needed for the maintenance of the dense and fenestrated vasculature required for efficient steroid secretion. Therefore, higher VEGF-R2 levels in ACAc might be related to tumor functionality (Feige, 2009). In addition to the expected location of the VEGF-R2 in the cytoplasm, we also observed nuclear localization in all ACT. Indeed, VEGF-R2 nuclear expression has been previously described in physiological and pathological conditions and seems to be associated with proliferative capacity of tumor cells, suggesting that nuclear expression may be involved in molecular mechanisms that contribute for tumor progression (Domingues et al., 2011). So, VEGF-R2 nuclear localization in ACT may contribute to amplification of the angiogenic response, however this is not exclusive of malignant tumors. In line with that, ROC curve analyzes suggested that VEGF-R2 has a poor accuracy for the differential diagnosis between ACC and both functioning and non-functioning ACA. Similarly, VEGF-R2 has failed to have a prognostic value in ACC patients.

The Ang-Tie pathway acts as a master regulator of vascular homeostasis, since it is responsible for controlling vascular permeability and remodeling in angiogenesis (Eklund & Saharinen, 2013). A clear association between higher tumor levels of Ang1 and malignancy has never been shown (Bach et al., 2007). In contrast, Ang2 appears to have differential roles in tumor angiogenesis. Ang2 and VEGF seems to act in synergism to promote angiogenesis, while in the absence of VEGF, Ang2 induces blood vessel regression (Fukumura et al., 2018).

Since all ACT cases presented VEGF expression, Ang2 function in ACT may be related with angiogenesis promotion, in particular to vascular permeability. We found that Ang2 expression was higher in ACC than in ACA and so ACC vascular vessels may be more permeable when compared to ACA vessels.

The balance between Ang1 and Ang2 has previously been reported to play an important role to define the tumorigenic outcome. In other types of tumors, including hepatocellular carcinoma and colorectal cancer, higher levels of Ang2 than Ang1 were correlated with malignancy and with a poor prognosis (Fagiani & Christofori, 2013; Tait & Jones, 2004)Whitin ACC group, we observed an increased Ang2 expression relative to Ang1 expression levels, suggesting that the ratio of Ang2:Ang1 was in favor of Ang2, which may be correlated with malignancy. However, we did not find any significant correlation between Ang2 expression and the malignant features studied.

Regarding to Tie1, no significant differences were found between the different groups of ACT. However, within the ACC group, we found that tumors with venous

invasion presented higher levels of Tie1 expression when compared to ACC without venous invasion. Tie1 is expressed in vascular bifurcations and branching points (i.e., in activated endothelium), therefore a possible explanation might be the disruption of the endothelial wall of the venous structure resulting in loss of the endothelium's quiescent state, which triggers higher levels of Tie1 expression. In addition, Tie1 expression was associated with lower survival rates, which might be linked to the Tie1 higher levels in ACC with venous invasion.

Our study found that Tie2 expression was absent in ACAn and in 42.9% of ACC cases, while in ACAc Tie2 expression was present. In addition, Tie2 expression was higher in ACAc than in ACC. Taken together, these results suggest that Tie2 expression might be related with tumor functionality. Tie2 receptor is the main mediator of the biological functions of Ang1 and Ang2, however in higher expression levels, Ang2 bind and activate Tie2 (Eklund & Saharinen, 2013). Since Ang2:Ang1 was in favor of Ang2 in ACT, the activation of Tie2 might be mediated through Ang2 binding.

Using ROC curve analyses, we found that Ang1, Ang2, Tie1 and Tie2 are not accurate markers for the differential diagnosis between ACC and both functioning and non-functioning ACA.

Vascular density evaluation has been considered as a golden standard to assess tumor angiogenesis (Weidner, 1995). There are several molecular markers, such as Factor VIII related antigen, CD31 and CD34, which allow the identification by immunohistochemistry and quantification of the angiogenic status (Pusztaszeri et al., 2006). In our study we used the CD34 marker and we found that all cases have high vascular density, however, it was significantly lower in ACC when compared with ACA. Nevertheless, we found higher levels of Ang2 in ACC when compared to ACA and so, ACC vessel density may be lower but vessels could be more permeable, contributing to tumor cell dissemination. In addition, since the adrenal gland is one of the most densely vascularized organs in adult mammals, the ACC tumor cells may take advantage of that to invade.

Previous studies comparing ACC and ACA vascularity density found inconsistent results (Bernini et al., 2002; Diaz-Cano et al., 2001; Pereira et al., 2018; Sasano et al., 1998; Xu et al., 2011; Zhu et al., 2014). This could be due to the use of different methods for quantification of vessels density and because tumors functionality may also influence the angiogenesis process. So, although we found that CD34 is an excellent marker to distinguish ACC from ACAc, this could be only be related to its steroid production

capacity. In line with that, the Cox regression analyses demonstrated that the CD34 expression in ACC is not related with the patients' overall survival.

Chapter 6

Conclusion

As far as we know, this is the first study that shows that the Ang-Tie pathway may have a role in ACC angiogenesis. Although ACC presented a lower vascular density when compared to ACA, the malignant tumors have a higher expression of Ang2 which is related to higher vascular permeability facilitating the cell spreading. The Tie1 expression was higher in ACC with venous invasion and in patients with lower overall survival, supporting the role of Tie1 in ACC angiogenesis. Therefore, Tie1 may represent a possible target for ACC treatment. In the future, functional and mechanistic studies are needed to validate these results.

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Appendix 1

Appendix Table 1 - Correlation between the angiogenic markers and patient/tumor characteristics in ACC.

Correlation		Coefficient correlation (r)	*p*
Angiogenic marker	**Patient/ Tumor characteristics**		
CD34	Age	0.080	ns
	Tumor size	-0.138	ns
	Weiss score	0.006	ns
	ENSAT score	0.285	ns
VEGF-R2	Age	-0.188	ns
	Tumor size	0.136	ns
	Weiss score	0.279	ns
	ENSAT score	0.441	ns
Ang1	Age	0.376	ns
	Tumor size	0.055	ns
	Weiss score	0.128	ns
	ENSAT score	0.043	ns
Ang2	Age	-0.004	ns
	Tumor size	0.317	ns
	Weiss score	0.152	ns
	ENSAT score	0.336	ns
Tie1	Age	0.096	ns
	Tumor size	0.092	ns
	Weiss score	0.060	ns
	ENSAT score	0.006	ns
Tie2	Age	-0.126	ns
	Tumor size	0.202	ns
	Weiss score	-0.175	ns
	ENSAT score	-0.158	ns

Ang1: angiopoietin 1; Ang2: angiopoietin 2; ENSAT: European Network for the Study of Adrenal Tumors; ns: non-significant. Tie1: tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF-R2: vascular endothelial growth factor receptor 2.

Appendix Table 2 - Correlation between the angiogenic markers and patient/tumor characteristics in ACAc.

Correlation		Coefficient correlation (r)	*p*
Angiogenic marker	**Patient/ Tumor characteristics**		
CD34	Age	-0.538	ns
	Tumor size	0.655	ns
	Weiss score	0.131	ns
VEGF-R2	Age	0.357	ns
	Tumor size	-0.509	ns

	Weiss score	0.393	ns
Ang1	Age	0.429	ns
	Tumor size	0.436	ns
	Weiss score	0.655	ns
Ang2	Age	0.500	ns
	Tumor size	0.127	ns
	Weiss score	0.393	ns
Tie1	Age	-0.250	ns
	Tumor size	0.164	ns
	Weiss score	-0.655	ns
Tie2	Age	0.143	ns
	Tumor size	0.291	ns
	Weiss score	0.393	ns

Ang1: angiopoietin 1; Ang2: angiopoietin 2; Tie1: tyrosine kinase with immunoglobulin-like and EGF-like domain 1; Tie2: tyrosine kinase with immunoglobulin-like and EGF-like domain 2; VEGF-R2: vascular endothelial growth factor receptor 2.

Appendix Table 3 - Correlation between the angiogenic markers and patient/tumor characteristics in ACAn.

Correlation		**Coefficient correlation (r)**	***p***
Angiogenic marker	**Patient/ Tumor characteristics**		
CD34	Age	0.073	ns
	Tumor size	-0471	ns
	Weiss score	-	
VEGF-R2	Age	0.093	ns
	Tumor size	0.311	ns
	Weiss score	-	
Ang1	Age	0.091	ns
	Tumor size	0.471	ns
	Weiss score	-	ns
Ang2	Age	0.091	ns
	Tumor size	-0.471	ns
	Weiss score	-	
Tie1	Age	0.358	ns
	Tumor size	0.101	ns
	Weiss score	-	

Ang1: angiopoietin 1; Ang2: angiopoietin 2; Tie1: tyrosine kinase with immunoglobulin-like and EGF-like domain 1; VEGF-R2: vascular endothelial growth factor receptor 2; Correlation between expression of the angiogenic markers and the Weiss score as not performed, since the score for all ACAn was 0.

Appendix Table 4 - Percentage of ACC samples with different VEGF expression localization pattern according to various parameters including ENSAT score, metastasis, capsular, venous and sinusoidal invasion.

		Cytoplasm + Nucleus expression	Cytoplasm expression
ENSAT Score	1-2	50.00 %	50.00 %
	3-4	50.00 %	50.00 %
	p	>0,99	
Metastasis	Yes	33.33 %	16.67 %
	No	66.67 %	83.33 %
	p	> 0.99	
Capsular Invasion	Yes	85.71 %	71.43 %
	No	14.29 %	28.57 %
	p	> 0.99	
Venous Invasion	Yes	28.57 %	55.56 %
	No	71.43 %	44.44 %
	p	0.36	
Sinusoidal Invasion	Yes	57.14 %	87.50 %
	No	42.86 %	12.50 %
	p	0.28	

ENSAT: European Network for the Study of Adrenal Tumors.

Appendix 2

Publication

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Review

Angiogenesis in the Normal Adrenal Fetal Cortex and Adrenocortical Tumors

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Simple Summary: Pharmacological angiogenesis modulation was robustly demonstrated to be a powerful clinical resource in oncotherapy. Adrenocortical carcinomas (ACC) often have a poor prognosis for which therapeutic options are limited. Understanding the mechanisms that regulate adrenocortical angiogenesis both under physiological conditions and in ACC could provide important clues on how these processes could be modulated for clinical purposes. This report summarizes the current knowledge on adrenal cortex angiogenesis regulation in physiological conditions and ACC. Embryonic adrenal angiogenesis is regulated by VEGF and Ang-Tie signaling pathways. VEGF angiogenic pathway was initially considered a promising therapeutic target for improving ACC prognosis. However, every single VEGF pathway-targeting clinical trial in ACC so far conducted yielded disappointing results. In contrast, the potential of Ang-Tie pathway-targeting in ACC is yet to be explored. Therefore, further investigation on the role and efficacy of modulating both Ang-Tie and VEGF pathways in ACC is still an unmet need.

Abstract: Angiogenesis plays an important role in several physiological and pathological processes. Pharmacological angiogenesis modulation has been robustly demonstrated to achieve clinical benefits in several cancers. Adrenocortical carcinomas (ACC) are rare tumors that often have a poor prognosis. In addition, therapeutic options for ACC are limited. Understanding the mechanisms that regulate adrenocortical angiogenesis along the embryonic development and in ACC could provide important clues on how these processes could be pharmacologically modulated for ACC treatment. In this report, we performed an integrative review on adrenal cortex angiogenesis regulation in physiological conditions and ACC. During embryonic development, adrenal angiogenesis is regulated by both VEGF and Ang-Tie signaling pathways. In ACC, early research efforts were focused on VEGF signaling and this pathway was identified as a good prognostic factor and thus a promising therapeutic target. However, every clinical trial so far conducted in ACC using VEGF pathway- targeting drugs, alone or in combination, yielded disappointing results. In contrast, although the Ang-Tie pathway has been pointed out as an important regulator of fetal adrenocortical angiogenesis, its role is yet to be explored in ACC. In the future, further research on the role and efficacy of modulating both Ang-Tie and VEGF pathways in ACC is needed.

Keywords: angiogenesis; adrenal fetal cortex; adrenocortical tumors; adrenocortical carcinoma; anti-angiogenic drugs

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1. Introduction

Angiogenesis is a dynamic process during which new blood vessels are formed derived from pre-existing vasculature. Angiogenesis is an extensively studied process in tumors and a well-recognized hallmark of cancer [1]. Angiogenesis was previously studied in adrenocortical carcinomas (ACC), although the relative rarity of these tumors represents a limitation to conduct extensive clinical and molecular characterization studies. This review aims to bring together all the available data on angiogenesis regulation during the adrenocortical development and in ACC, which could be potentially useful to identify future research avenues to achieve advances in ACC clinical management and disease prognosis. Data source and study selection approach is described in the Supplementary File S1.

2. Angiogenesis Regulation

Angiogenesis plays a central role in several physiological (e.g., fetal development and wound healing) and pathological processes (e.g., vascular overgrowth for tumor expansion and metastasis) [2–4]. Angiogenesis, either in normal or tumor tissues, usually occurs via one or more of the following mechanisms:

(1) Sprouting angiogenesis, one the most well characterized mechanism leading to angiogenesis, relies on endothelial cells function specification into either tip or stalk cells. Tip cells are derived from the parent vessel, degrade the basement membrane, extend large filopodia which can sense angiogenic factor gradients, such as vascular endothelial growth factor (VEGF), and migrate along the chemotactic paths. In contrast, stalk cells proliferate behind tip cells to form the sprout body, start the process of lumen formation, and connect with neighboring vessels [5–7].

(2) Intussusceptive angiogenesis is a process that consists in the splitting of pre-existing vessels into two new vessels. It starts with the formation of transluminal tissue pillars through the invagination of opposing capillary endothelial cells into the vascular lumen, creating a zone of contact. Commonly, intussusceptive and sprouting angiogenesis are complementary mechanisms [5,8].

(3) Recruitment of endothelial progenitor cells and vasculogenesis, a process through which endothelial progenitor cells are recruited in response to several growth factors, cytokines and/or hypoxia-inducible factors. Endothelial progenitor cells differentiate into mature endothelial cells and are incorporated into the angiogenic sprout, thus contributing to new blood vessel formation [4,9].

(4) Vasculogenic mimicry: malignant tumor cells form de novo vessel-like structures without endothelial cells. The newly formed channels mimic the embryonic vascular network pattern, being able to provide enough blood supply to the tumor tissue [10,11].

Multiple signaling pathways regulate blood vessel growth and maintenance. Among these, VEGF and Ang-Tie pathways are particularly important and have been the focus of multiple studies, especially in the context of cancer [12]. VEGF receptor and Tie ligands are widely distributed and were shown to play a coordinated role in endothelial cell proliferation and vessel wall assembly in normal and pathological conditions.

2.1. VEGF Pathway in Angiogenesis Regulation

In mammals, the VEGF system mainly includes five secreted ligands (VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor) and three primary tyrosine kinase receptors (VEGF-R1, VEGF-R2, VEGF-R3) [13]. The VEGF system also includes the cell-surface proteins, heparan sulfate proteoglycans and neuropilin-1 and -2, which operate as VEGF coreceptors [14,15].

VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells, while VEGF-R3 seems to be prominently expressed in lymphatic endothelial cells [16]. VEGF ligands have different affinities for one of the three VEGF-R. As tyrosine kinases receptors, upon dimerization by a VEFG ligand, the VEGF-Rs auto-phosphorylate, a phenomenon which

in turn activates downstream signaling pathways including mitogen-activated protein kinase (MAPK) pathway, the phosphatidylinositol-3 kinase (PI3K-AKT) pathway, and the phospholipase-C-γ pathway. Those pathways drive various intracellular effects in endothelial cells, such as migration, proliferation, and cell survival. The activation of phospholipase-C-γ pathway via VEGF-A-VEGFR-2 binding was reported to be a key signal for endothelial proliferation [17].

In 2001, a new VEGF was identified, the endocrine-gland-derived VEGF (EG-VEGF). This ligand does not show any structural homology to the VEGF family, but displays several biological similarities to VEGF ligands, including hypoxic regulation and ability to induce fenestration in target cells. Moreover, EG-VEGF expression is restricted to steroidogenic tissues (adrenal, ovary, testis and placenta) and its effects seem to be restricted to endothelial cells derived from these organs [18].

2.2. *Ang-Tie Pathway in Angiogenesis Regulation*

Ang-Tie signaling pathway regulates vascular permeability and remodeling during tumor angiogenesis and metastasis. Ang/Tie signaling seems to complement the VEGF signaling pathway by controlling later stages of angiogenesis and by being involved in vascular maturation (Figure 1) [19].

The angiopoietin family includes two type 1 transmembrane protein receptors: Tie1 and Tie2 and four ligands: Ang1, Ang2, Ang3 and Ang4. Ang1 and Ang2 have been identified as the main ligands for Tie receptors, while the Ang3 and Ang4 biological function is still poorly characterized [20–22].

Ang1 binds and activates Tie2 resulting in Tie2 internalization and ligand release. Then it leads to Tie2 tyrosine residues phosphorylation that in turn recruits adaptor proteins and ignites PI3K/Akt and MAPK signaling pathways, promoting pro-survival, anti-permeability, and anti-inflammatory effects on endothelial cells [23]. Tie2 is not required for the endothelial cells' differentiation but is rather reported as necessary for cell maintenance [24].

Ang2 that shares approx. 60% amino acid homology with Ang1, binds to Tie2 with a similar affinity as Ang1. Ang2 seems to block the Ang1-induced Tie2 phosphorylation. Ang2 is upregulated during tumor angiogenesis and so was considered as a potential antiangiogenic target. However, recent studies found Ang2 to have a dual function, acting as a Tie2 antagonist in the presence of Ang1 or acting as a Tie2 agonist in the absence of Ang1 [25]. Different studies reported that it is unlikely that Tie2 can act differently when binding to Ang1 and Ang2, since both angiopoietins interact with Tie2 in a structurally similar manner and pointed out that other still unidentified mechanisms were likely to be involved [26]. One of the proposed mechanisms involve the Tie1 receptor [27].

Contrary to Tie2, the Tie1 has been less well characterized. Tie1 is considered an orphan receptor and is mainly expressed at vascular bifurcations and branching points, with no yet identified in vivo ligand [28]. It is well known, however, that Tie1 has an important role in vascular development, since its inactivation causes late embryonic lethality and vasculature maturation failure [29,30]. Recent studies proposed that Tie1 forms a complex with Tie2 on the endothelial cell surface and acts as a Tie2 inhibitor [27]. Cells expressing both receptors are responsive to chemotactic signals and able to promote vessel branching and sprouting that is required for angiogenesis. On the other hand, Tie1 is absent in stable and quiescent mature vessels [27].

A mechanistic study indicated Tie1 as being responsible for angiopoientin's differential function. In mature vessels, as Tie1 is absent, Tie2 can be activated by either Ang1 or Ang2, to promote vessel stability. On active angiogenesis sites, Tie1 and Tie2 form a complex and Ang2 fails to activate Tie2, allowing vessel branching to be promoted. On the other hand, Ang1 is able to dissociate Tie2 from the Tie1-Tie2 complex, activating Tie2 and thus enhancing vascular stability [27,31].

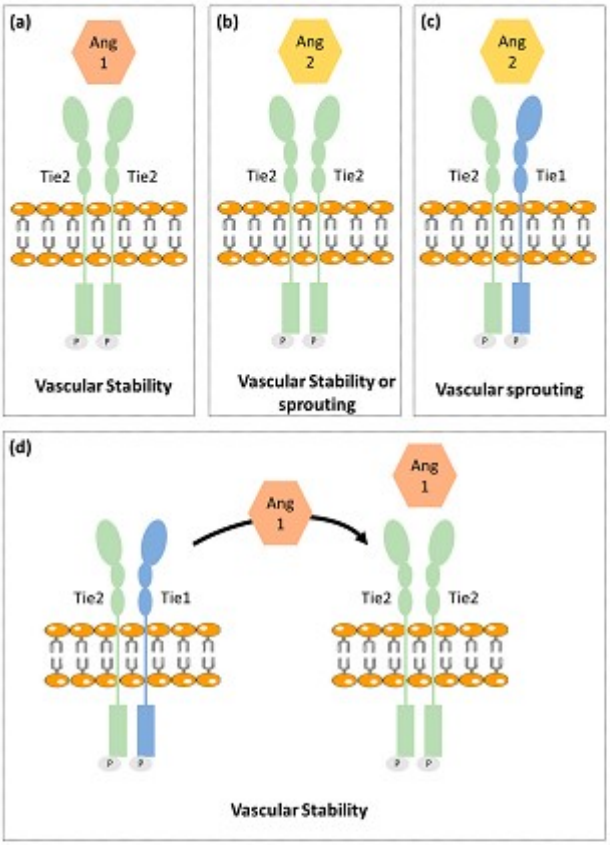

Figure 1. Schematic illustration of the vessel stability regulation by Ang/Tie signaling. (a) Ang1 binds to Tie2 promoting vascular stability. (b) Ang2 has a dual function acting as a Tie2 agonist or antagonist to promote vascular stability or sprouting, respectively. (c) Tie1 forms a complex with Tie2. Upon Ang2 stimulation, Tie1 and Tie2 remain associated and Ang2 induces vascular sprouting. (d) Ang1 stimulation promotes Tie2 clustering leading to vascular stability. This schematic representation includes the most consensual theories; however, this pathway is not yet fully understood. Besides that, this figure is a schematic representation that is not intended to translate the real chemical conformation of the proteins.

3. Angiogenesis in Normal Adrenal Cortex

3.1. Fetal Adrenal Cortex

Human fetal adrenal (HFA) plays a critical role in fetal maturation and perinatal survival. HFA steroid hormones regulate intrauterine homeostasis and appropriate fetal organ systems maturation [32,33].

Contrary to the adult adrenal cortex that includes three distinct zones: glomerulosa, fasciculata and reticularis; the HFA is primarily composed of two single distinct zones: outer zone or definitive zone and inner zone or fetal zone [33,34]. The definitive zone comprises a narrow band of small cells that exhibit typical characteristics of cells in proliferative state. Definitive zone does not produce steroids until the third trimester. However, as gestation advances, definitive zone cells start to accumulate lipids and resemble steroidogenic

active cells. The fetal zone is the largest adrenal cortex zone and consists of large cells that exhibit features characteristic of steroid-secreting cells [33–37]. In ultrastructural studies, a third zone in between definitive zone and fetal zone, named transitional zone, has been described. The transitional zone is composed by cells with intermediate characteristics, but capable to synthetize cortisol and so cells can be considered analogous to fasciculata layer cells of mature adrenal cortex [33,38–41].

Due to the HFA critical role in fetal maturation, the early and extensive vasculature development that occurs in this gland, is not only necessary but also particularly important. Angiogenesis is not only required for HFA growth and maturation, but it is also necessary for the influx of steroid precursors and trophic factors into the gland to enable mature steroids synthesis and secretion into circulation. Indeed, the fetal adrenal gland is one of the most highly vascularized organs in the human fetus [41].

Previous studies have reported that VEGF-A, FGF-2, Ang1, Ang2, and Tie2 are expressed in HFA since midgestation and to have a putative role in adrenal gland angiogenesis [34,42,43].

Ang2 expression in HFA is markedly higher when compared to the mature adrenal gland, whereas Ang1 and Tie2 expression seem to be similar in both fetal and adult adrenals. Thus, supporting higher angiogenesis activity and vascular instability in developing adrenal glands [42].

Ang2, FGF-2 and VEGF-A expression are mainly expressed in the gland periphery suggesting that the HFA periphery is the primary site of angiogenesis, in parallel to cell proliferation [42,43]. Further supporting this hypothesis, a dense network of irregular capillaries was also observed at the HFA periphery [44].

On the contrary, Ang1 is mainly expressed in the fetal zone, suggesting that the inner adrenal zone presents a greater vessel maturity. Tie2, was exclusively identified to be present in endothelial cells throughout the gland [42,43].

Adrenocorticotropic hormone (ACTH), the main regulator of HFA growth and function, also seems to be implicated in angiogenesis control. In vitro studies found that ACTH upregulates VEGF-A, FGF-2 and Ang2 in the HFA, therefore controlling angiogenesis while simultaneously exerting growth and secretion stimulatory actions [42,43,45,46].

The steroidogenic factor 1 has a critical role in adrenal development, steroidogenesis, and also in gonadal differentiation [47]. In addition, steroidogenic factor 1 also seems to be implicated in HFA angiogenesis regulation by direct interaction and activation of the Ang2 gene promoter. Furthermore, the authors demonstrated that steroidogenic factor 1 and Ang2 are strongly co-expressed in HFA periphery in early stages of development [48].

Overall, these findings support that the adrenal gland growth, steroidogenesis and blood vessel formation, are synchronized phenomena [42,43,45,46].

3.2. *Adult Adrenal Cortex*

The adrenal gland is one of the most vascularized organs in adult mammalian organisms. Its developed intrinsic vasculature is required for an efficient secretion of steroid hormones into the systemic blood flow. The adrenal gland is supplied by three different arterial branches derived the abdominal aorta: inferior phrenic artery, middle adrenal artery and renal artery. The arterial blood enters in the adrenal gland and flows centripetally through the adrenal cortex into the adrenal medulla [49,50].

Previous studies have found that adrenocortical cells highly express VEGF-A and EG-VEGF—a VEGF specific of steroidogenic organs, both having been pointed out as important molecules for maintenance of the dense and fenestrated vasculature of the adrenal cortex. This expression also seems to be regulated by ACTH [51–55].

In addition, the vasculature of the adrenal cortex seems to be coordinated with the mass of the adrenal cortex, since it suffers fluctuations decreasing or increasing along regression or expansion of the adrenal cortex, respectively [52].

4. Angiogenesis in Adrenocortical Tumors

Adrenocortical tumors (ACT) are common adrenal tumors affecting 3% to 10% of the human population [56]. The majority of ACT are benign non-functioning adrenocortical adenomas (ACA), while malignant ACC are rare with an incidence of 0.7 to 2 per million per year [56]. ACC most often have a poor prognosis and are frequently already metastasized when first diagnosed. ACC pathogenesis is still largely unclear, which results in a lack of biomarkers available for diagnosis and in limited treatment options [57,58].

The status of the VEGF pathway in adrenocortical tumors has been already addressed in multiple studies (Table 1).

Table 1. VEGF pathway findings in adrenocortical tumors.

	Patient Group Comparisons	Results
VEGF	Patients with ACT vs. Healthy individuals	↑ VEGF serum levels in patients with ACT [59,60]
	Aldosterone secreting ACA vs. Non-functioning ACA	↑ VEGF tumor expression in aldosterone producing ACA [61]
	Cortisol secreting ACA vs. Aldosterone secreting ACA	↑ VEGF serum levels patients with cortisol secreting ACA [60]
	ACC vs. Normal adrenal glands	↑ VEGF expression in ACC [61,62]
	ACC vs. ACA	↑ VEGF serum levels in ACC ↑ VEGF tumor expression in ACC [59,61,63,64]
	Patients with recurrent ACC vs. Patients with non-recurrent ACC	↑ VEGF serum levels in recurrent ACC ↑ VEGF tumor expression in recurrent ACC [60,63]
	Localized ACC vs. Invasive ACC	No difference in VEGF tumor expression [63]
VEGF-R2	ACC vs. Normal adrenal glands	↑ VEGF-R2 tumor expression in ACC [62]
	ACC vs. ACA	↑ VEGF-R2 tumor expression in ACC [64]

ACA—Adrenocortical Adenomas; ACC—Adrenocortical carcinomas; ACT—Adrenocortical tumors; VEGF—Vascular endothelial growth factor; VEGFR—Vascular endothelial growth factor receptor; ↑—Increased protein levels or expression.

Patients with ACT were found to present higher VEGF serum levels as compared to healthy controls [59,60]. In addition, Kolomecki et al. demonstrated that VEGF serum levels were significantly higher in patients with non-functioning malignant tumors than in patients with non-functioning ACA. Noteworthy, VEGF serum levels in patients with ACC were shown to decrease after tumor surgical resection and increase in patients who experienced tumor recurrence [59]. de Fraipont et al. found that cytosolic VEGF-A concentrations were higher in ACC when compared to ACA, although not being significantly different when localized and more invasive ACC were compared [63]. Nevertheless, cytosolic VEGF-A concentrations were higher in recurrent as compared to non-recurrent ACC after primary tumor resection [63].

Tumor VEGF expression was also found to be higher in ACC as compared to normal adrenal glands and ACA [61,62,64]. VEGF receptor 2 tumor expression was also found to be higher in ACC when compared with ACA and normal adrenal glands [62,64].

Bernini et al., however, found that tumor VEGF expression was not directly related with vascular density, which was lower in ACC as compared to ACA and normal adrenal tissue. The fact that a higher VEGF expression was not shown to be associated with increased vascular density in ACC, was somehow unsurprising since a high vascular

density already characterizes normal adrenal cortex tissue. What surprised researchers was that despite ACC lower vascular density, patients still had a very short survival time [61].

Other studies reported that although no differences in vascular density were noticed when ACC, ACA and normal adrenal glands were compared, blood vessels perimeter and area were higher in ACC when compared to ACA [65,66]. In addition, endothelial cell proliferation was higher in ACC [66].

On an opposed direction, another group reported vascular density to be higher in malignant ACT as compared to benign ACT [67]. Another study observed that in their series VEGF expression was positively correlated with vessel density [64]. Pereira et al. also reported ACC to present a higher vascular density, but only when compared to cortisol secreting ACA [68]. This could, however, be derived from cortisol anti-angiogenic effects [69]. There is additional evidence supporting that adrenocortical angiogenic status could be tightly related to the tumor's hormonal functionality. Bernini et al. found that VEGF tumor expression was higher in aldosterone secreting ACA as compared to non-functioning ACA and normal adrenal glands [61]. In addition, in another study patients with cortisol-secreting ACA were found to have higher circulating VEGF levels than patients with aldosterone secreting adenomas [60].

The discovery of EG-VEGF, a steroidogenic organ specific VEGF, brought some enthusiasm to the scientific community as a potential explanation to the contradictory angiogenic patterns in ACTs as well as a potential target for ACC treatment. Heck et al. characterized the expression of EG-VEGF and its receptors [prokineticin receptor 1 (PKR1) and 2 (PKR2)] in a large number of ACC, ACA and normal adrenal glands. In this study, EG-VEGF and both receptors PKR1 and PKR2 were found to be present in the majority of ACT. Moreover, the nuclear protein expression of either EG-VEGF or PKR1 or both in ACC was reported to be associated with higher mortality, suggesting that these could be used as prognostic markers for overall patient survival [53].

New prognostic and diagnostic markers are needed to improve ACC clinical practice. As described in this section, the usefulness of angiogenic factors for ACC diagnosis and/or prognosis was already investigated. From those, VEGF was the one with more consistent and replicable results, being increased in ACC when compared with ACA [59,61,63,64], in particular in the recurrent malignant tumors [60,63]. However, due to the rarity of ACC, the number of patients included in each study is small. So, in the future, to validate this result, multi-center studies are needed to increase the samples/participants' number and to uniformize the methodological approach to analyze the VEGF tumors expression in ACT. Stratified analysis according to tumors functionality are needed since in previous studies, it showed to influence VEGF levels.

5. Anti-Angiogenic Agents' Efficacy in Adrenocortical Carcinomas Treatment

The demonstration that patients with ACC had high VEGF circulating levels and tumor expression, along with the recent evidence on the efficacy of anti-VEGF drugs for other types of neoplasia treatment, such as, advanced colorectal cancer [70], opened the promising perspective of using this drug class agents for ACC treatment as well.

The first report using an anti-angiogenic agent for the treatment of patients with ACC was released in 2010 (Table 2). Ten patients with advanced ACC, refractory to several cytotoxic chemotherapies, were treated with the monoclonal anti-VEGF antibody bevacizumab in combination with capecitabine, an adrenolytic agent. The results were disappointing since the disease progressed in all patients [71].

A phase II clinical trial using sorafenib in combination with paclitaxel was conducted in ten patients with advanced ACC after treatment with mitotane plus one or two chemotherapy lines. Sorafenib is a tyrosine kinase inhibitor (TKI) drug that inhibits several receptors, such as VEGFR2, VEGFR3, platelet-derived growth factor receptor (PDFGR) and RAF-1, a key enzyme in the MAPK-ERK signaling pathways. The sorafenib plus paclitaxel drug combination was demonstrated to be ineffective in patients with ACC, as progressive

disease was observed in nine consecutive patients leading to clinical trial interruption 2 months after initiation [72].

In another trial 35 patients with ACC refractory to mitotane and cytotoxic chemotherapies were treated with the TKI sunitinib, a drug that inhibits multiple receptors, such as VEGFR1 and VEGFR2, c-KIT, Fms-like tyrosine kinase 3, and PDGFR. Six of the thirty-five patients in that trial died of progressive disease. Of the remaining twenty-nine patients, five patients had stable disease, and 23 patients had progressive disease on first evaluation (12 weeks). Three of the five patients with stable disease on first evaluation, had disease progression later. In addition, authors reported that concomitant mitotane had a negative impact on treatment outcome, by lowering sunitinib blood levels. Therefore, sunitinib only demonstrated to have a modest efficacy in the treatment of patients with advanced ACC, while the efficacy in patients without mitotane exposure needs to be further assessed [73].

In a phase II clinical trial, axitinib, a potent VEGFR-1, VEGFR-2, and VEGFR-3 selective inhibitor, was administrated to thirteen patients with metastatic ACC previously treated with at least one chemotherapy regimen, with or without mitotane. No patient in trial achieved a partial or complete response, and only eight patients experienced stable disease for more than 3 months [74].

Thalidomide is an immunomodulatory agent with anti-angiogenic properties by targeting TNF-α, ILs, VEGF, bFGF. The effectiveness of thalidomide was investigated in a trial that included twenty-seven patients with advanced ACC refractory to mitotane and other systemic drug treatments. Twenty-five of the twenty-seven patients experienced clinical or radiological disease progression at the time of first evaluation. So, thalidomide also, only showed to be marginally effective in patients with refractory advanced ACC [75].

Lenvatinib is another TKI drug that inhibits multiple receptors including VEGFR-1, VEGFR-2 and VEGFR-3, FGFRs, PDGFR-α, KIT and RET. The efficacy of lenvatinib in combination with pembrolizumab, an immune checkpoint inhibitor, was also investigated in eight patients with ACC and progressive and/or metastatic disease after receiving previous treatment interventions. None of the eight patients had to discontinue the treatment in result of toxicity. One patient had stable disease, lasting for 8 months, two patients had a partial response while receiving therapy and five patients developed progressive disease, so lenvatinib plus pembrolizumab combined therapy was demonstrated to achieve positive responses in a subset of patients without significant toxicity [76]. Phase II clinical trials with larger patient cohorts are still needed to confirm these conclusions.

The clinical efficacy and safety of the TKI cabozantinib was investigated in a retrospective cohort study in sixteen patients with advanced ACC after other treatments having failed. Cabozantinib is a multi-inhibitor of c-MET, VEGFR-2, AXL, and RET. At first evaluation, two patients had partial response and six had stable disease. At four months evaluation, half of patients were alive and progression free [77]. Although these results were not brilliant, they were superior to the ones previously reported for other anti-angiogenic agents.

Although previous studies using anti-angiogenic therapies did not show encouraging results in patients with ACC, there are several registered clinical trials using VEGF-R inhibitors ongoing or due to be initiated. Two phase II clinical trials designed to test the efficacy of cabozantinib in patients with advanced ACC (NCT03370718 and NCT03612232) are currently recruiting, and a phase II clinical trial to test the efficacy of the VEGFR-2 inhibitor apatinib plus camrelizumab an immune checkpoint inhibitor, is registered (NCT04318730).

Table 2. Clinical studies using anti-angiogenic drugs for the treatment of patients with adrenocortical carcinomas.

Anti-Angiogenic Drug	Mechanism of Action	Study Type	Patient Population	Results	Ref.
Bevacizumab (+capecitabine)	Monoclonal anti-VEGF antibody	Observational retrospective cohort study	Patients with refractory ACC (n = 10)	PFS: 59 days OS: 124 days	[71]
Thalidomide	Immunomodulatory agent that targets TNF-α, ILs, VEGF, bFGF	Observational retrospective cohort study	Patients with refractory ACC (n = 27)	PFS: 11.2 weeks (4.4–22.8 weeks) OS: 36.4 weeks (5.1–111.1 weeks)	[75]
Lenvatinib (+pembrolizumab)	Multi-TKI that inhibits VEGFR-1, VEGFR-2 and VEGFR-3, FGFRs, PDGFR-α, KIT, RET	Observational retrospective cohort study	Patients with recurrent and/or metastatic ACC (n = 8)	PFS: 5.5 months OS: NA	[76]
Cabozatinib	TKI that targets VEGFR-2 and c-Met	Observational retrospective cohort study	Patients with refractory metastatic ACC (n = 16)	PFS: 16.2 weeks (2.8–61 weeks) OS: 56 weeks (5.6–83.1 weeks)	[77]
Sorafenib (+paclitaxel)	Multi-TKI inhibitor that VEGFR-2 VEGFR-3, PDGFR and RAF-1	Phase II, single-arm, open label clinical trial	Patients with refractory metastatic ACC (n = 10)	Trial interrupted due disease progression in all enrolled patients	[72]
Sunitinib	Multi-TKI that inhibits VEGFR-1 and VEGFR-2, c-KIT, FLT3 and PDGFR	Phase II, single-arm, open label clinical trial	Patients with advanced ACC after mitotane or others cytotoxic drugs (n = 35)	PFS: 2.8 months (5.6–11.2 months) OS: 5.4 months (14.0–35.5 months)	[73]
Axitinib	Selective inhibitor of VEGFR-1, VEGFR-2 and VEGFR-3	Phase II, single-arm, open label clinical trial	Patients with metastatic ACC previously treated with at least one chemotherapy regimen (n = 13)	PFS: 5.48 months (1.8–10.92 months) OS: 13.7 months	[74]

FPS and OS median and ranges were included in the table, when available in the original manuscript. ACC—Adrenocortical carcinoma; bFGF—basic fibroblast growth factor; FGFRs—fibroblast growth factor receptors; FLT3—FMS-like tyrosine kinase 3; ILs—interleukins; NA—not available; OS—Overall Survival; PFS—Progression-free survival; TKI—Tyrosine kinase inhibitor; TNF-α—Tumor necrosis factor alpha; VEGF—Vascular endothelial growth factor; VEGFR—Vascular endothelial growth factor receptor; PDGFR—platelet-derived growth factor receptor.

It is important to highlight that although the VEGF pathway is one of the most important angiogenic pathways, this is not the only pathway involved in angiogenesis regulation. As far as we are aware, no clinical trial has tested the effect of drugs targeting the Ang-Tie pathway in patients with ACC.

In 2014, one single patient with ACC was enrolled in a phase 1b clinical trial designed to test the efficacy of trebananib, a dual Ang1 and Ang2 inhibitor plus VEGFR inhibitors (bevacizumab or motesanib) in various solid tumors. Stable disease was achieved in the patient with ACC. However, since this clinical trial included patients with different solid tumors no further data specifically related to this patient is available [78].

Further investigation of molecules involved in Ang-Tie pathway in ACC tissues is needed in order to understand whether this pathway has a role in the pathophysiology of this type of tumor. This knowledge is needed to provide a rationale for conducting clinical trials targeting both Ang-Tie and VEGF pathways in patients with ACC.

There are no doubts that angiogenesis is an important process in ACC progression and the rationale for the use of anti-angiogenic drugs in ACC treatment is unquestionable.

However, so far clinical trials to test the efficacy of these drugs were conducted in patients with advanced/metastatic ACC that precluded the possibilities of success, since even if the angiogenic capacity of the tumor is decreased, the disease is already in an uncontrolled stage. In contrast, the efficacy of anti-angiogenic drugs in non-advanced ACC is unknown yet. Therefore, ex-vivo studies could be useful to assess these drugs efficacy as compared to mitotane, which is the only drug licensed for ACC treatment.

In addition, whenever possible, conducting studies to evaluate whether there is a correlation between drug efficacy and the tumor molecular profile, would provide additional insights on the mechanisms responsible for successful or unsuccessful treatment and support future clinical trials.

6. Conclusions

Angiogenesis is well-known to be required for cancer cell expansion and considered an important hallmark of cancer [1]. Adrenal glands have a very dense vascular network that is necessary to support their hormonal secretion functions. Therefore, it may not be surprising that this normal adrenal gland high vascular density is unlikely to be further increased in the context of adrenocortical neoplasia.

Although several molecules within the VEGF pathway were identified as prognostic markers and promising targets for ACC treatment, the cohort studies and clinical trials so far concluded yielded disappointing results. The most promising results were observed for cabozantinib, a multi-TKI inhibitor, which induced stable or partial response in half of the patients with advanced ACC [77]. Given this effect and its overall safety with a tolerable side effect profile, two clinical trials to assess the efficacy of this drug are now recruiting (NCT03370718 and NCT03612232). Both trials require the discontinuation of mitotane and exclude patients with mitotane levels higher than 2 mg/L. Future clinical trials should take in consideration prior mitotane exposure on the study design, since previous or concomitant mitotane use may influence the investigational drug treatment outcomes due to its impact on drug metabolism [73,79].

Another registered clinical trial will assess the efficacy of the anti-angiogenic drug Apatinib combined with an immunomodulatory agent (PD-1 inhibitor: camrelizumab) in ACC (NCT04318730), a drug that was previously tested alone and was demonstrated to induce a stable disease or an objective response in 52% of the patients [80]. Since this drug combination elicited impressive clinical results in many solid tumors [81–83], there is a great expectation for this clinical trial outcomes.

Furthermore, the Ang-Tie pathway which is known to have an important role on fetal adrenal gland angiogenesis should also receive attention [42,43]. The role of Ang-Tie pathway in adrenocortical tumors has not yet been investigated. Future studies and clinical trials investigating the role of Ang-Tie pathway in adrenocortical tumors and the efficacy of targeting Ang-Tie or both Ang-Tie and VEGF pathways in ACC treatment are needed.

Supplementary Materials: The following are available online at https://www.mdpi.com/2072-6694/13/5/1030/s1. Supplementary File S1: Data Source and Study Selection.

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