Steroid Hormones and Cancer Immunity – learning from Adrenocortical Carcinoma

Steroidhormone und Tumorimmunität im Nebennierenrindenkarzinom

Doctoral Thesis

for a Doctoral Degree
at the Graduate School of Life Sciences,
Julius-Maximilians-Universität Würzburg,
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Würzburg, August 2021

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Parts of this thesis have been published in the following article:

Landwehr LS, Altieri B, Schreiner J, Sbiera I, Weigand I, Kroiss M, Fassnacht M, Sbiera S. Interplay between glucocorticoids and tumor-infiltrating lymphocytes on the prognosis of adrenocortical carcinoma. *Journal for Immunotherapy of Cancer* vol. 8,1 (2020): e000469. doi:10.1136/jitc-2019-000469

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List of Abbreviations

3β-HSD	3β-hydroxysteroid dehydrogenase
ACC	Adrenocortical carcinoma
ACTH	Adrenocorticotropic hormone
APC	Antigen presenting cell
BSA	Bovine serum albumin
BWS	Beckwith-Wiedemann syndrome
CBG	Corticosteroid binding globulin
CDKN2A	Cyclin dependent kinase inhibitor 2A
CI	Confidence interval
CO_2	Carbon dioxide
CRH	Corticotropin-releasing hormones
CS	Cushing´s syndrome
CT	Computer tomography
CTCAE 4.0	Common terminology criteria for adverse events 4.0
CTLA-4	Cytotoxic T lymphocytes antigen 4
CTNNB1	β–catenin
CYP11B1	11β-hydroxylase
CYP17A1	17α-hydroxylase
CYP21A2	21-hydroxylase
CYP3A4	Cytochrome P450 3A4
DAB	3,3′-diaminobenzidine
DAPI	4′,6-diamidin-2-phenylindol
DC	Dendritic cell
DCR	Disease control rate
DDT	Dichlorodiphenyltrichloroethane
DHEA	Dehydroepiandrostendione
DHEAS	Dehydroepiandrosterone sulfate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco’s phosphate buffered saline
DST	Dexamethasone suppression test
EDP	Etoposide, doxorubicin and cisplatin
EDTA	Ethylenediaminetetraacetic acid

ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
ESE	European Society of Endocrinology
EtOH	Ethanol
FAP	Familial adenomatous polyposis
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
FIRM-ACT	First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment
FoxP3	Forkhead-box protein P3
g	Gravitation
GC	Glucocorticoid
GR	Glucocorticoid receptors
H	Hour
H_2O	Water
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HPA	Hypothalamic-pituitary-adrenal
HR	Hazard ratio
HRP	Horseradish peroxidase
HSC	Hematopoietic stem cell
HSD11B1	Hydroxysteroid 11β-dehydrogenase type 1
HSD11B2	Hydroxysteroid 11β-dehydrogenase type 2
Hz	Hertz
IFN-γ	Interferon γ
IL	Interleukin
Ig	Immunoglobulin
irRECIST	Immune-related response evaluation criteria in solid tumors
IV	Intravenously
$KHCO_3$	Potassium bicarbonate
L	Liter
LDL	Low density lipoprotein
LFS	Li-Fraumeni syndrome
LS	Lynch syndrome
MAGE-A3	Melanoma antigen A3
MC2R	Melanocortin type 2 receptor

MEN	Multiple endocrine neoplasia
MEN1	Menin 1
MeOH	Methanol
MHC	Major histocompatibility complex
Min	Minute
mL	Milliliter
mM	Millimole
MMR-D	Mismatch repair deficiency
MRI	Magnetic resonance imaging
MSI	Microsatellite instability
NaN_3	Sodium azide
NH_4Cl	Ammonium chloride
NK	Natural killer
NPR	Non-progression rate
NY-ESO-1	New York esophageal squamous cell carcinoma-1
o,p´DDD	1 – (*o*-chlorodiphenyl) – 1 – (*p*-chlorophenyl) – 2,2 – dichloroethane
OD	Optic density
ORR	Overall response rate
OS	Overall survival
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
PFA	Paraformaldehyde
PFS	Progression-free survival
pg	Picogram
PR	Partial response
PRKAR1A	Protein kinase cAMP-dependent type I regulatory subunit α
PRR	Pattern recognition receptor
PVN	Paraventricular nucleus
RB1	Retinoblastoma 1
RECIST	Response evaluation criteria in solid tumors
RFS	Recurrence-free survival
Rh	Recombinant human
RNA	Ribonucleic acid

RPL22	Ribosomal protein L22
RT	Room temperature
SD	Stable disease
Sec	Second
SF-1	Steroidogenic factor 1
St-M	Streptozotocin plus mitotane
StAR	Steroidogenic acute regulatory
TCR	T cell receptor
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor β
TIL	Tumor-infiltrating lymphocytes
TNF-α	Tumor necrosis factor α
TP53	Tumor suppressor p53
TRAE	Treatment-related adverse events
TMB	Tumor mutational burden
VEGFR2	Vascular endothelial growth factor receptor 2
Vs	Versus
ZF	Zona fasciculata
ZG	Zona glomerulosa
ZNRF3	Zinc and ring finger 3
ZR	Zona reticularis
µl	Microliter
µM	Micromole
°C	Degree Celsius
α	Alpha
β	Beta
γ	Gamma
δ	Delta
ε	Epsilon
ξ	Zeta
κ	Kappa
ø	Phi
X^2	Chi-square

Summary

Adrenocortical carcinoma (ACC) is a rare, but highly aggressive endocrine malignancy. Tumor-related hypercortisolism is present in 60 % of patients and associated with worse outcome. While cancer immunotherapies have revolutionized the treatment of many cancer entities, the results of initial studies of different immune checkpoint inhibitors in ACC were heterogeneous. Up to now, five small clinical trials with a total of 121 patients have been published and demonstrated an objective response in only 17 patients. However, one of the studies, by Raj et al., reported a clinically meaningful disease control rate of 52 % and a median overall survival of almost 25 months suggesting that a subgroup of ACC patients may benefit from immunotherapeutic approaches. Following the hypothesis that some ACCs are characterized by a glucocorticoid-induced T lymphocytes depletion, several studies were performed as part of the presented thesis. First, the immune cell infiltration in a large cohort of 146 ACC specimens was investigated. It was demonstrated for the first time, and against the common assumption, that ACCs were infiltrated not only by FoxP3$^+$ regulatory T cells (49.3 %), but also that a vast majority of tumor samples was infiltrated by CD4$^+$ T_H cells (74 %) and CD8$^+$ cytotoxic T cells (84.3 %), albeit the immune cell number varied heterogeneously and was rather low (median: 7.7 CD3$^+$ T cells / high power field, range: 0.1-376). Moreover, the presence of CD3$^+$-, CD4$^+$- and CD8$^+$ ACC-infiltrating lymphocytes was associated with an improved recurrence-free (HR: 0.31 95 % CI 0.11-0.82) and overall survival (HR: 0.47 96 % CI 0.25-0.87). Particularly, patients with tumor-infiltrating CD4$^+$ T_H cells without glucocorticoid excess had a significantly longer overall survival compared to patients with T cell-depleted ACC and hypercortisolism (121 vs. 27 months, $p = 0.004$). Hence, the impact of glucocorticoids might to some extent be responsible for the modest immunogenicity in ACC as hypercortisolism was reversely correlated with the number of CD4$^+$ T_H cells. Accordingly, CD3$^+$ T cells co-cultured with steroidogenic NCI-H295R ACC cells demonstrated *in vitro* an enhanced anti-tumoral cytotoxicity by secreting 747.96 ±225.53 pg/ml IFN-γ in a therapeutically hormone-depleted microenvironment (by incubation with metyrapone), versus only 276.02 ±117.46 pg/ml IFN-γ in a standard environment with glucocorticoid excess.

Other potential biomarkers to predict response to immunotherapies are the immunomodulatory checkpoint molecules, programmed cell death 1 (PD-1) and its ligand PD-L1, since both are targets of antibodies used therapeutically in different cancer entities. In a subcohort of 129 ACCs, expressions of both molecules were heterogeneous (PD-1 17.4 %, range 1-15; PD-L1 24.4 %, range 1 - 90) and rather low. Interestingly, PD-1 expression significantly influenced ACC patients´ overall (HR: 0.21 95 % CI 0.53-0.84) and progression-free survival (HR: 0.30 95 % CI 0.13-0.72) independently of established factors, like ENSAT tumor stage, resection status, Ki67 proliferation index and glucocorticoid excess, while PD-L1 had no impact.

In conclusion, this study provides several potential explanations for the heterogeneous results of the immune checkpoint therapy in advanced ACC. In addition, the establishment of PD-1 as prognostic marker can be easily applied in routine clinical care, because it is nowadays anyway part of a detailed histo-pathological work-up. Furthermore, these results provide the rationale and will pave the way towards a combination therapy using immune checkpoint inhibitors as well as glucocorticoid blockers. This will increase the likelihood of re-activating the immunological anti-tumor potential in ACC. However, this will have to be demonstrated by additional preclinical *in vivo* experiments and finally in clinical trials with patients.

Zusammenfassung

Das Nebennierenrindenkarzinom (ACC) ist ein seltenes, aber äußerst aggressives endokrines Malignom. Ein tumorbedingter Hyperkortisolismus liegt bei 60 % der Patienten vor und ist mit einer schlechteren Prognose assoziiert. Während Krebsimmuntherapien die Behandlung vieler Krebsentitäten revolutioniert haben, waren die Ergebnisse der ersten Studien zu verschiedenen Immun-Checkpoint-Inhibitoren beim ACC heterogen. Die fünf klinischen Studien mit insgesamt 121 Patienten zeigten ein objektives Ansprechen bei nur 17 Patienten. Eine Studie von Raj et al. berichtete über eine klinisch bedeutsame Krankheitskontrollrate von 52 % und ein medianes Gesamtüberleben von fast 25 Monaten. Diese beträchtliche Anti-Tumor-Aktivität legt nahe, dass eine Subgruppe von ACC-Patienten von immuntherapeutischen Ansätzen profitieren könnte. Der Hypothese folgend, dass einige ACCs durch eine Glukokortikoid-induzierte T Lymphozyten-Depletion gekennzeichnet sind, wurden im Rahmen der vorliegenden Arbeit mehrere Studien durchgeführt. Zunächst wurde die Immunzellinfiltration in einer großen Kohorte von 146 ACC-Proben untersucht. Entgegen der verbreiteten Annahme konnte erstmals gezeigt werden, dass ACCs nicht nur von FoxP3$^+$ regulatorischen T Zellen (49,3 %), sondern die Mehrheit der ACCs von CD4$^+$ T_H (74 %) und CD8$^+$ zytotoxischen T Zellen (84,3 %) infiltriert wurde, wenngleich die Immunzellanzahl heterogen und eher gering war (7,7 CD3$^+$ T Zellen/HPF). Darüber hinaus war die Präsenz von CD3$^+$-, CD4$^+$- und CD8$^+$ ACC-infiltrierenden Lymphozyten mit einem rezidivfreien (HR: 0,31; 95 % CI 0,11-0,82) und verbesserten Gesamtüberleben (HR: 0,47; 95 % CI 0,25-0,87) assoziiert. Insbesondere Patienten mit tumorinfiltrierenden CD4$^+$ T_H Zellen ohne Glukokortikoid-Überschuss hatten im Vergleich zu Patienten mit T Zell-depletiertem ACC und Hyperkortisolismus ein signifikant längeres Gesamtüberleben (121 vs. 27 Monate; p = 0.004). Daher könnte die Wirkung von Glukokortikoiden für die moderate Immunogenität verantwortlich sein, da Hyperkortisolismus umgekehrt mit der Zahl der CD4$^+$ T_H Zellen korreliert war. Dementsprechend zeigten CD3$^+$ T Zellen, die mit steroid-produzierenden NCI-H295R ACC-Zellen co-kultiviert wurden, *in vitro* eine erhöhte anti-tumorale Zytotoxizität in einem durch Metyrapon-induzierten Mikromilieu ohne Glukokortikoide im Vergleich zu einem Glukokortikoid-Überschuss (IFN-γ Sekretion: 747,96 pg/ml vs. 276,02 pg/ml). Andere potenzielle Biomarker zur Vorhersage des Ansprechens auf Immuntherapien sind die immunmodulatorischen Checkpoint-Moleküle, Programmed cell death 1 (PD-1) und sein Ligand PD-L1, da beide Ziele von Antikörpern sind, die therapeutisch bei verschiedenen Krebsentitäten eingesetzt werden. In einer Subkohorte von 129 ACCs waren die Expressionen beider Moleküle heterogen (PD-1 17,4 %, PD-L1 24,4 %). Interessanterweise beeinflusste die PD-1-Expression signifikant das Gesamtüberleben (HR: 0,21; 95 % CI 0,53-0,84) und das progressionsfreie Überleben (HR: 0,30; 95 % CI 0,13-0.72) unabhängig von etablierten Faktoren, wie dem ENSAT Tumorstadium, Resektionsstatus, Ki67 Proliferationsindex und Glukokortikoid-Überschuss, während PD-L1 keinen Einfluss hatte. Zusammenfassend liefert diese Studie mehrere mögliche Erklärungen für die heterogenen Ergebnisse der Immun-Checkpoint-Therapie bei fortgeschrittenem ACC. Darüber hinaus ist die Etablierung von PD-1 als prognostischer Marker anwendbar als Teil einer detaillierten histo-pathologischen Untersuchung. Zudem liefern diese Ergebnisse die Rationale und ebnen den Weg für eine Kombinationstherapie von Immun-Checkpoint-Inhibitoren sowie Anti-Glukokortikoiden, um die Wahrscheinlichkeit einer Reaktivierung des immunologischen Anti-Tumor-Potenzials beim ACC zu erhöhen. Dies muss jedoch durch zusätzliche präklinische *in vivo* Experimente und schließlich in klinischen Studien mit Patienten nachgewiesen werden.

1 Introduction

1.1 Hypothalamus pituitary adrenal axis

The human endocrine system regulates internal homeostasis by maintaining a dynamic equilibrium through release of hormones. These signaling molecules are responsible for vital functions including metabolism, growth and development, reproduction, the immune system and response to stress and environmental influences. These physiological processes are orchestrated by the hypothalamic-pituitary-adrenal (HPA) axis through the release of glucocorticoids (GCs) and negative feedback mechanisms (Tsigos and Chrousos 2002, McEwen 2007).

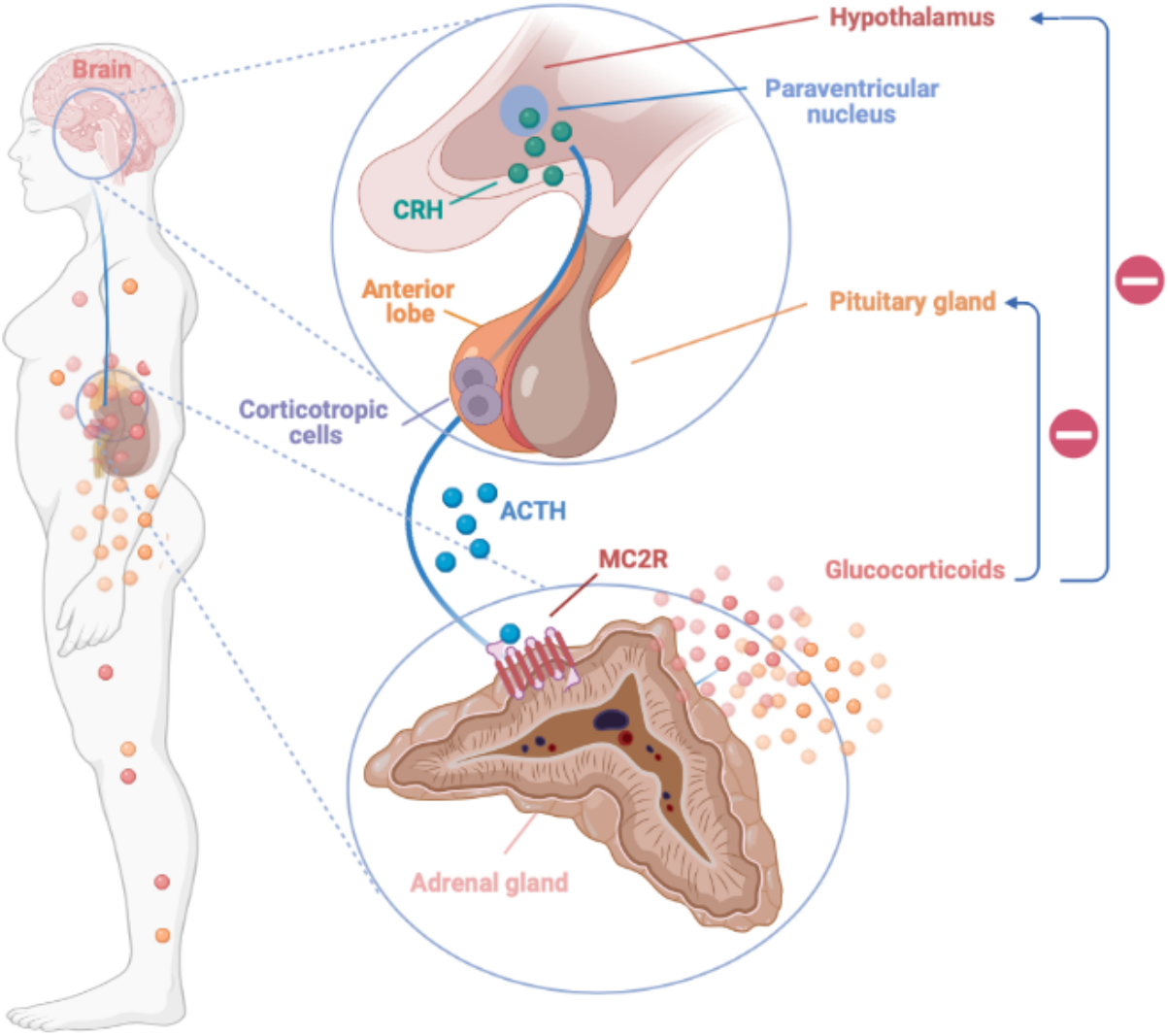


Figure 1.1: HPA axis
Schematic illustration of the HPA axis. CRH, synthesized by the paraventricular nucleus (PVN) of the hypothalamus, stimulates corticotroph cells of the anterior pituitary lobe to produce ACTH that stimulates via MC2R adrenocortical cells to generate glucocorticoids. These effector hormones inhibit by binding to MC2R and GR the secretion of ACTH in the pituitary and CRH in the hypothalamus via a negative feedback mechanism. (Created with BioRender.com)

Hypophysiotropic neurons of the hypothalamic paraventricular nucleus (PVN) synthesize and secret the corticotropin-releasing hormone (CRH) that stimulates the secretion of adrenocorticotropic hormone (ACTH) by the corticotroph cells in the anterior pituitary lobe (Sapolsky, Romero and Munck 2000). Circulating ACTH in turns binds to the melanocortin type 2 receptor (MC2R) in the adrenal cortex stimulating biosynthesis of glucocorticoids, such as cortisol (Spiga et al. 2017). The regulation of glucocorticoid pulsatility is ensured by inhibition of HPA-mediated stress response through negative feedback mechanisms (Russell and Lightman 2019). Binding of glucocorticoids to mineralocorticoid- and glucocorticoid receptors (MR and GR) in the brain and the peripheral tissue results in inhibition of secretion of hypothalamic CRH and pituitary ACTH (Gjerstad, Lightman and Spiga 2018).

1.2 Adrenal glands

The adrenal glands are paired endocrine glands that are located suprarenal and surrounded by a fatty capsule within the renal fascia in the retroperitoneum (Silverman and Lee 1989). They consist of two distinct tissues with different embryonic origins – the outer cortex and the inner medulla. While the cortex is derived from the intermediate mesoderm, the medulla develops from the ectoderm derived from the neural crest. The central medulla that is driven by sympathetic nervous system is surrounded by the larger cortex, which is entirely encapsulated by connective tissue (Mesiano and Jaffe 1997, Xing et al. 2015b).

Both adrenal medulla and cortex synthesize a variety of physiologically vital hormones. While chromaffin cells of the medulla produce catecholamines, including epinephrine and norepinephrine, the cortex is responsible for the secretion of different steroid hormones originating from three different cortical layers – *zona glomerulosa*, *zona fasciculata* and *zona reticularis*. Cells from the *zona glomerulosa* (ZG) aligned in oval groups, immediately under the fibrous capsule, produce mineralocorticoids, like aldosterone, and regulate the blood pressure. The middle layer, *zona fasciculata* (ZF), constitutes 80 % of the cortical volume and consists of cells containing abundant lipid droplets. These droplets serve as cholesterol storage and are arranged in radially oriented columns. These cells synthesize glucocorticoids, mainly cortisol, to regulate stress and immune response. The *zona reticularis* (ZR) directly adjacent to the medulla comprises irregularly clustered small cells that produces androgens, such as dehydroepiandrostendione (DHEA), which control reproductive organs (Mesiano and Jaffe 1997, Auchus and Rainey 2004).

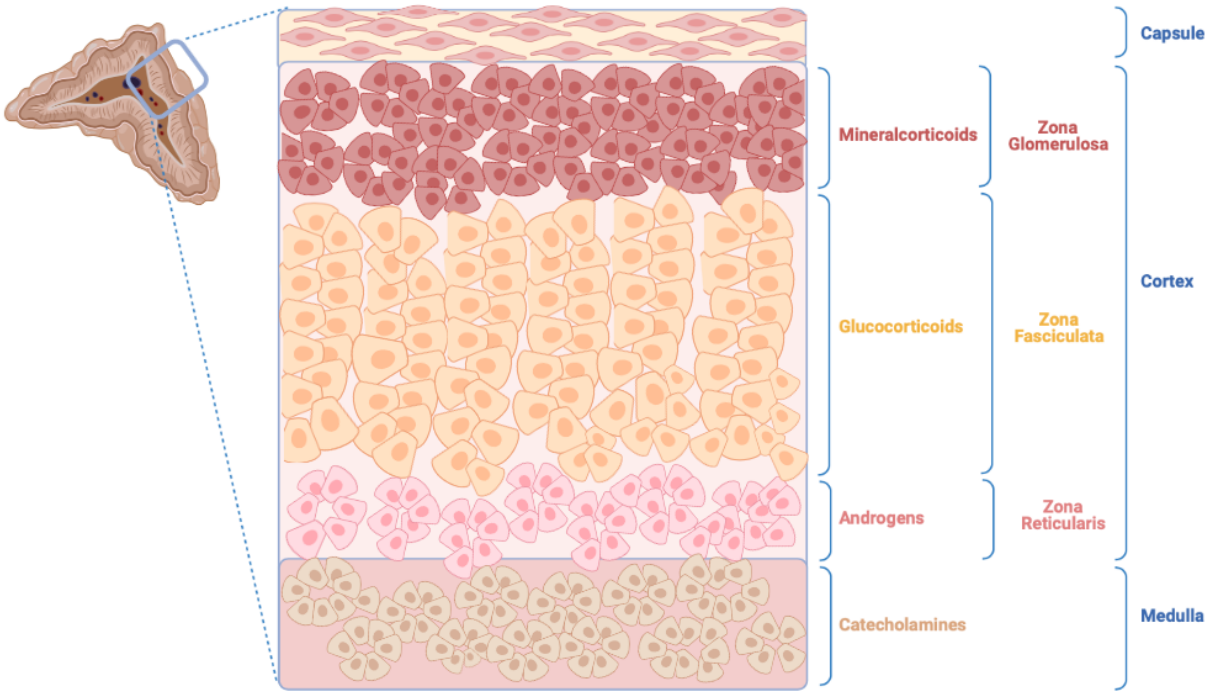


Figure 1.2: Histology of the human adrenal gland
The adrenal gland is surrounded by a fibrous capsule and consist of an outer cortex, which is subdivided into three zones - *zona glomerulosa, zona fasciculata and zona reticularis* -, and the inner medulla. (Created with BioRender.com)

1.3 Adrenal steroidogenesis

Biosynthesis of steroid hormones is a dynamic process originating from cholesterol precursor via consecutive involvement of mitochondrial and microsomal enzymes. The initial cholesterol substrate is synthesized *de novo* within the cell from acetate, from cholesterol esters provided by intracellular lipid droplets or, mainly, supplied by low density lipoproteins (LDLs), and to a lesser extent by high density lipoproteins (HDLs). Lipophilic cholesterol is solubilized by binding to steroidogenic acute regulatory (StAR) protein that facilitates the transport to the inner mitochondrial membrane. As a first, rate-limiting regulatory step, cholesterol is converted by cytochrome P450 side chain cleavage enzyme (P450scc) CYP11A1 into pregnenolone. Pregnenolone is further converted into progesterone by 3β-hydroxysteroid dehydrogenase (3β-HSD). Both pregnenolone and progesterone are catalyzed by microsomal 17α-hydroxylase (CYP17A1) into 17α-hydroxypregnenolone and 17α-hydroxyprogesterone, respectively. 17α-hydroxypregnenolone can also be converted into 17α-hydroxyprogesterone by 3β-HSD. Subsequently, 17α-hydroxyprogesterone is converted into 11-deoxycortisol by 21-hydroxylase (CYP21A2) located in the smooth endoplasmic reticulum. The last step in adrenal steroidogenesis is the conversion of 11-deoxycortisol into active cortisol by mitochondrial enzyme 11β-hydroxylase (CYP11B1).

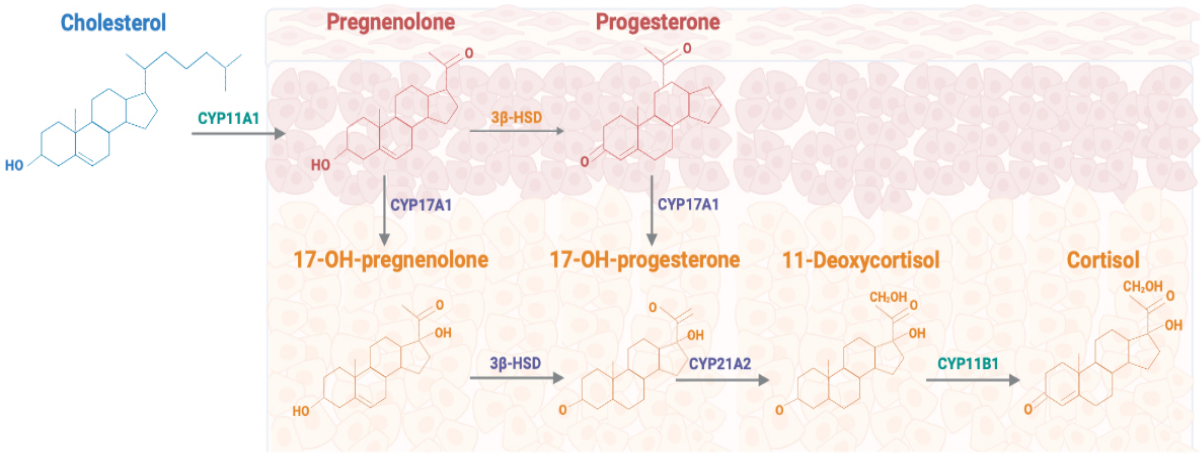


Figure 1.3: Adrenal glucocorticoid synthesis
Glucocorticoids are synthesized from cholesterol via different enzymes localized to mitochondria and the endoplasmic reticulum. Cholesterol is converted to pregnenolone by cytochrome P450 side chain cleavage enzyme (P450scc; CYP11A1) in the *zona glomerulosa*. Pregnenolone is further enzymatically processed via 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17α- hydroxylase (CYP17A1) into 17α-hydroxypregnenolone. This metabolite is converted into 11-deoxycortisol by 21-hydroxylase (CYP21A2) and lastly into active cortisol by 11β-hydroxylase (CYP11B1) in the *zona fasciculata*. (Created with BioRender.com)

Most endogenous glucocorticoids present in the extracellular space are inactive and bound to corticosteroid binding globulin (CBG). Unbound glucocorticoids are lipid soluble enabling diffusion through hydrophobic cell membrane. In the cytoplasm, cortisol can be inactivated into cortisone by 11β-hydroxysteroid dehydrogenase type II (HSD11B2), whereas type I (HSD11B1) reverts these metabolites into the active form. Cytoplasmic cortisol binds the glucocorticoid receptor (GR) and translocate to the nucleus intending genomic and non-genomic effects.

1.4 Adrenal tumors

Tumors of the adrenal gland are very common in the human population with a prevalence of about 3 to 10 % (Kloos et al. 1995). These neoplasms are classified according to their malignant properties, endocrine functionality and histological origin.

While benign adenomas are the most prevalent adrenocortical tumor (Fassnacht et al. 2016, Sherlock et al. 2020, Kebebew 2021), only 1 % of them are malignant lesions of the adrenal cortex (Bornstein, Stratakis and Chrousos 1999). Adrenocortical tumors, both benign and malign, differ according to their endocrine activity. They appear as functionally inactive tumors with non-hypersecretion of hormones or are hormonally active leading to clinical symptoms. Depending on pathological hormone excess of aldosterone, corticosteroids or androgen/estrogens, disease pattern of primary hyperaldosteronism, Cushing´s syndrome (CS) or virilization/feminization, respectively, are consequently developed (Luton et al. 1990, Fassnacht, Kroiss and Allolio 2013, Mansmann et al. 2004). Pheochromocytomas that comprise tumors of the adrenal medulla are characterized by an excessive release of catecholamine, but only 10 to 17 % show a malignant phenotype (Lenders et al. 2014).

1.5 Adrenocortical carcinoma

1.5.1 Incidence and Epidemiology

Adrenocortical carcinoma (ACC) belongs to the most aggressive/severe endocrine malignancies. It is a very rare disease with an incidence of 0.6 to 2 cases per million habitants per year (Kebebew et al. 2006, Kerkhofs et al. 2013) and affects 0.02 to 0.2 % of all cancer-related deaths (Mansmann et al. 2004). The bimodal distribution considering age at diagnosis indicates an increased incidence during the first and fifth decade of life (Kebebew et al. 2006). While tumorigenesis appears at any age with ultimate incidence between the age of 40 and 60 years, the median age at diagnosis is 46 years with a facile propensity towards females (55 to 60 %) (Else et al. 2014).

1.5.2 Molecular pathogenesis

Several international multicenter studies investigated precisely the genetic aberrations and pathogenesis of ACC; thereby, realizing that pathogenic driver mutations appear in ACCs, however, in a minor variety (Assié et al. 2014, Juhlin et al. 2015, Zheng et al. 2016). The most frequently altered genes in ACCs are shown in Table 1.1 (Assié et al. 2014, Juhlin et al. 2015, Zheng et al. 2016).

Table 1.1: Frequently altered genes in adrenocortical carcinoma

Gene		Function	% of Tumor
TP53	Tumor Suppressor p53	P53/Rb signaling	16-21
CTNNB1	β-Catenin	Wnt/β-catenin pathway	10-20
ZNRF3	Zinc and Ring Finger 3	Wnt/β-catenin pathway	10-21
CDKN2A	Cyclin Dependent Kinase Inhibitor 2A	p53/Rb signaling	11-15
TERT	Telomerase Reverse Transcriptase	Telomere regulation pathway	14-15
PRKAR1A	Protein Kinase cAMP-Dependent Type I Regulatory Subunit α	cAMP-dependent signaling	8-11
MEN1	Menin I	Wnt/β-catenin pathway	7
RPL22	Ribosomal Protein L22	RNA processing	7
RB1	Retinoblastoma I	p53/Rb signaling	7

However, in adults, most of the ACCs are sporadic, albeit referable to heredity genetic mutations predisposing individuals for Li-Fraumeni syndrome (LFS; (Raymond et al. 2013a), Lynch syndrome (LS) (Raymond et al. 2013b), Beckwith-Wiedemann syndrome (BWS), multiple endocrine neoplasia 1 (MEN 1) (Langer et al. 2002) and familial adenomatous polyposis (FAP) (Else et al. 2014). Patients

diagnosed with LFS have an early-onset and predisposition to cancer. LFS is associated with a germline mutation of the *TP53* tumor suppressor gene encoding transcription factor p53 that regulates the cell cycle, apoptosis and genomic stability (Raymond et al. 2013a). Alterations in genes involved in DNA mismatch repair genes – *MLH1*, *MSH2*, *MSH6*, *PMS2* and *EPCAM* – are associated to LS. LS patients have higher risk of several cancers including colorectal cancer (Lynch and de la Chapelle 2003), endometrial cancer (Møller et al. 2017) and ACC (Raymond et al. 2013b). Mutations resulting in mismatch repair deficiency (MMR-D) lead to a hypermutated phenotype and high levels of microsatellite instability (MSI). Patients diagnosed with FAB have an increased susceptibility for the development of cancer, particularly colorectal cancer and in few cases ACC, caused by mutations in the *APC* gene or *MUTYH* gene (Nielsen et al. 2007, Gaujoux et al. 2010, Pilati et al. 2017).

1.5.3 Clinical management

Clinical characteristics of patients with ACC are quite variable. Hence, 10 to 30 % of ACCs are diagnosed incidentally by imaging for other purpose and 20 to 30 % of patients appear with rather non-specific, para-neoplastic symptoms due to clinical impairments, such as abdominal or back pain, hypoglycemia or leukocytosis. However, the majority, 40 to 60 % of patients, appear with clinical signs of hormone excess (Luton et al. 1990, Else et al. 2014, Fassnacht et al. 2018).
Hypercortisolism is the most common presentation affecting 50 to 80 % of hormone-secreting ACC. Those functional ACCs become clinically apparent due to plethora, diabetes mellitus, muscle atrophy and osteoporosis. Hypokalemia and hypertension appear also frequently caused by activation of the renal hydroxysteroid 11β-dehydrogenase 2 (HSD11B2) system due to glucocorticoid-mediated mineralocorticoid receptor activation. These are symptoms of progressive Cushing´s syndrome, generally indicative of a malign tumor progression. The second most common pathophysiologically secreted hormones in ACC patients are androgens (40 to 60 % of functional tumors). Female patients present with hirsutisms, virilization and menstrual irregularities, while 1 to 3 % of male ACC patients rarely present with gynecomastia and testicular atrophy. Interestingly, concurrent hypersecretion of cortisol and androgens is apparent in approximately 50 % of all functional ACC tumors. Very few tumors (2.5 %) that autonomously hypersecrete aldosterone are known; whereas mineralocorticoid effects resulting from increased cortisol levels were commonly observed (Else et al. 2014).

At the time of diagnosis, ACC tumors measure commonly 10 to 13 cm in diameter (Sturgeon et al. 2006). In order to clinically assess the extent of this malignant adrenal tumor, a globally recognized standard classification is introduced by the European Network for the Study of Adrenal Tumors (ENSAT). This defined ENSAT four-stage system refers to the extent by tumor size and localization.

Table 1.2: Tumor classification of the European Network for the Study of Adrenal Tumors

I	T1, N0, M0
II	T2, N0, M0
III	T3 – T4, N1
IV	T1 – T4, N0 – N1, M1

Tumor: T1: tumor ≤ 5 cm; T2: > 5 cm; T3: tumor invasion in surrounding tissue; T4: Tumor invasion in adjacent organs or venous tumor thrombus in vena cava or renal vein; *Lymph nodes*: N0: negative lymph nodes; N1: positive lymph nodes; *Metastasis*: M0: no distant metastasis; M1: present distant metastasis

Stage I, tumors ≤ 5 cm, and stage II, everything beyond (> 5 cm), are related to local tumors within the adrenal gland. More advanced stage III describes tumor invasion in para-adrenal tissue and adjacent organs or an involvement of loco-regional lymph nodes. Patients with distant metastasis are classified as most advanced stage IV (Fassnacht et al. 2009). An overview of the ENSAT classification is shown in Table 1.2.
Metastatic lesions originated from primary ACC commonly occur in liver (40 to 90 %), lung (40 to 80 %) or bone (5 to 20 %), while brain and skin are rarely affected (≤ 5 %) (Allolio et al. 2004).

The initial work-up generally includes physical examination and anamnesis with particular focus on potential hereditary contributions and symptoms for hormone excess. In order to clarify the hormone status, biochemical examination of steroid hormones is performed among basic blood parameters. Staging necessarily requires diagnostic imaging using contrast-enhanced computer tomography (CT) or magnetic resonance imaging (MRI) (Bharwani et al. 2011, Fassnacht et al. 2018). Upon the occurrence of abnormalities, such as an adrenal mass and/or a hormonal excess, differential laboratory workup needs to be addressed. Therefore, according to the Endocrine Society Clinical Practice Guideline for diagnosing subclinical CS-related hypercortisolism, either a 24-hour urine cortisol, a midnight salivary cortisol measurement or, preferably, an 1 mg overnight dexamethasone suppression test (DST) is recommended. After DST, serum cortisol value above 1.8 µg/dl (50 nmol/liter) implies hypercortisolism due to autonomous cortisol secreting ACC tumor (Nieman et al. 2008). To determine androgen and/or estrogen excess, blood serum is assessed whether testosterone, dehydroepiandrosterone sulfate (DHEAS) and/or estradiol levels, respectively, are increased (Fassnacht et al. 2018). At last, hypersecretion of mineralocorticoids is initially assessed by screening for elevated aldosterone levels, while renin is suppressed (Williams and Reincke 2018).

In addition to imaging and biochemical diagnostic, pathological assessment is an essential advance for the classification of malignant potential of adrenocortical tumors using the Weiss scoring system. Adrenal tumors with abundance of ≥ 3 alterations in growths pattern, necrosis, mitotic rates, atypical

mitotic figures and invasion are categorized as malignant with potential metastatic tendency (Aubert et al. 2002). The presence of specific proteins, like steroidogenic factor-1 (SF-1) (Sbiera et al. 2010), α-inhibin (Arola et al. 2000), calretinin (Jorda, De and Nadji 2002), synaptophysin (Komminoth et al. 1995) and Melan-A (Ghorab et al. 2003), verifies the adrenocortical origin and differentiation. Its aggressiveness can additionally be assessed via the proliferation index Ki67 (Beuschlein et al. 2015).

After comprehensive diagnostic assessment, therapy is conducted depending on tumor characteristics and malignancy. A complete surgical resection (R0), preferably via open adrenalectomy, is the best curative approach for long-term local control of ACC (Miller et al. 2012, Gaujoux, Mihai and ENSAT 2017).

Radiological follow-up imaging is performed at regular intervals of three months for two years and, subsequently, every three to six months for another three years (Fassnacht et al. 2018).

Even after complete tumor excision, 19 to 70 % of ACC patients remain at high risk for local recurrences and progression (Lombardi et al. 2012, Glenn et al. 2019). Therefore, the European Society of Endocrinology (ESE) and ENSAT defined the Clinical Practice Guidelines on consistent management of ACC based on clinical trials (Fassnacht et al. 2018). While ACC patients with low/intermediate risk, ENSAT stage I+II and Ki67 ≤ 10 %, only be advised considering mitotane, patients with ENSAT stage III+IV or Ki67 > 10 % that have a high risk for recurrence are treated with the adjuvant mitotane. Mitotane is a derivate of the organochlorine insecticide dichlorodiphenyltrichloroethane (DDT) and an isomer of 1 – (*o*-chlorodiphenyl) – 1 – (*p*-chlorophenyl) – 2,2 – dichloroethane (*o,p´*DDD) that harbors adrenolytic activity (Bergenstal 1960). Although its pharmacological mechanism and adrenolytic effect requires further investigation, its ability to inhibit several enzymes involved in adrenocortical steroidogenesis pathway is substantiated (Corso et al. 2020). It is the only approved drug by the U.S. Food and Drug Administration (FDA) and European Medicine Agency (EMA) for treatment of ACC. By progressively increasing mitotane plasma concentration, the therapeutic window set between 14 to 20 mg/l ensured best efficacy. Levels above are associated with neurological toxicity and severe adverse events (Haak et al. 1994, van Slooten et al. 1984, Hermsen et al. 2011).

By microscopically residual tumor (resection status 1 (R1)), ACC patients are treated with adjuvant mitotane considering radiation therapy (Fassnacht et al. 2018).

Patients with advanced ACC not amenable for complete surgical resection (R0) of tumor burden, are either treated with mitotane monotherapy plus local therapies (radiotherapy, radiofrequency ablation, cryo- or microwave ablation and (chemo-)embolization) or mitotane plus a combination of chemotherapeutics etoposide, doxorubicin and cisplatin (EDP-M) (Fassnacht et al. 2018). However, the efficacy of mitotane monotherapy is unsatisfactory in patients with advanced disease by an objective response rate of 20.5 % (Megerle et al. 2018). Therefore, the preferred guideline-based standard therapy is derived from the First International Randomized Trial in Locally Advanced and Metastatic Adrenocortical Carcinoma Treatment (FIRM-ACT) investigating favorable regimens. This phase III

trial compared EDP-M to streptozotocin plus mitotane (St-M) in 204 patients with advanced ACC. Patients treated with EDP-M demonstrated a higher response rate (23.2 vs. 9.2 %) and longer progression-free survival than patients treated with St-M (5.0 vs. 2.1 months; hazard ratio (HR) 0.55; 95 % confidence interval (CI) 0.43 to 0.69) (Fassnacht et al. 2012). Streptozotocin or gemcitabine-based chemotherapy were only considered as second-line therapies in advanced disease (Sperone et al. 2010, Henning et al. 2017, Khan et al. 2000, Fassnacht et al. 2012). For patients with sufficient response, subsequent surgery is worth considering.

In addition to the previously described therapies aimed directly at tumor disease, severe comorbidities caused by hormone excess need to be considered. Although mitotane also controls steroid biosynthesis by inhibiting multiple steroidogenic enzymes, additional inhibitors of steroidogenesis are commonly used to regulate pathological hormone levels.
Ketoconazole positively effects hypercortisolism, while improving mineralocorticoid-induced hypertension and hyperandrogenemia by inhibiting cytochrome p450 (CYP) enzymes 20,22-desmolase (CYP11A1), 17α-hydroxylase (CYP17A1), 11β-hydroxylase (CYP11B1) and 18-hydroxylase (CYP11B2). Another potent inhibitor, metyrapone, addresses high cortisol and aldosterone levels by targeting CYP11B1 and CYP11B2 (Daniel and Newell-Price 2015). Aldosterone-producing ACCs causing hypertension and hypokalemia are treated with spironolactone, a mineralocorticoid receptor antagonist. Spironolactone inhibits the synthesis of 11-deoxycorticosterone and aldosterone ensuring normalizing mineralocorticoid levels, while moderately blocking androgen synthesis as well. Estrogen excess is commonly treated with aromatase inhibitors or tamoxifen, a selective estrogen receptor modulator (Else et al. 2014).

The poor prognosis and still limiting treatment options requires further investigations. Studies considering targeted therapies directing receptors and intracellular enzymes are unsatisfactory as well. For instance, although the insulin-growth factor 2 (*IGF2*), highly expressed gene in ACC, served as promising target of insulin-growth factor receptor 1 (IGF-1R) antagonist (Boulle et al. 1998, Arnaldez and Helman 2012, Guillaud-Bataille et al. 2014), several studies investigating figitumumab (Haluska et al. 2010), cixutumumab (Naing et al. 2013) or linsitinib (Fassnacht et al. 2015) in advanced ACC were disappointing. Another approach focusing on multikinase inhibitor, sunitinib, only achieved stable disease in 5 of 35 refractory ACC patients (Kroiss et al. 2012, Else et al. 2014). The combination of multityrosine kinase inhibitors and mitotane that induces cytochrome P450 3A4 (CYP3A4) has negative effects on treatment response because these inhibitors are in turn metabolized by CYP3A4 (Kroiss et al. 2011). In line with this reciprocal interaction, a retrospective study on cabozantinib investigated its efficacy in advanced ACC by discontinuation of mitotane (< 2 mg/l; (Kroiss et al. 2020). Cabozantinib is a multityrosine kinase inhibitor of c-MET, vascular endothelial growth factor receptor 2 (VEGFR2), AXL and RET that motivates a therapeutic rational in ACC (Phan et al. 2015). Hence, two patients

partially responded, five were stable and eight progressive, while the median progression-free survival was four months and overall survival more than one year (14.5 months; (Kroiss et al. 2020). Consequently, a prospective phase II study of cabozantinib monotherapy is ongoing (*ClinicalTrials.gov identifier (NCT number):* NCT03612232).

Recently, the activation of an anti-tumoral immune response with immunotherapies has revolutionized the therapy of many cancer entities resistant to standard cancer therapies. Results of first clinical trials with immune checkpoint inhibitor monotherapies in ACC are heterogeneous with median progression-free survival times of 1.8 until 6.75 months (Carneiro et al. 2019, Raj et al. 2019, Le Tourneau et al. 2018, Habra et al. 2019). However, Raj et al. demonstrated a median overall survival of 25 months in 39 ACC patients suggesting that at least a subgroup of patients benefits from this immunotherapeutic approach (Raj et al. 2019).

1.6 Human immune system

The human immune system comprises various biological processes that protect the organism from disease. It is organized by a complex liaison of multiple organs, different cell types and signaling factors. This tightly regulated network enables recognition of pathogens including bacteria, viruses or fungi, toxins and degenerated cells; thereby, being able to distinguish them from own tissue and ensure self-tolerance. Their elimination is regulated by a harmonizing defense mechanism comprising the innate and adaptive immunity. While the innate immunity immediately mediates the initial protection against common microbes, the adaptive immunity that develops slowly is more specialized (Abbas, Lichtman and Pillai 2014).

Once the physical and chemical defense barriers are overcome, phagocytic cells resident in almost all tissue act as sensor for invading microbes. Phagocytic cells are the major components of the innate immunity including neutrophils, monocytes differentiating into macrophages and dendritic cells (DCs), natural killer (NK) cells and mast cells (Abbas et al. 2014). They detect microbes by common pathogens associated molecular patterns (PAMPs) binding to pattern recognition receptors (PRRs; (Akira, Uematsu and Takeuchi 2006). After initiation of the inflammatory cascade through the release of cytokines and opsonization of pathogens by the complement system, further immune cell recruitment and clearance of microbes are promoted (Medzhitov and Janeway 2000, Dunkelberger and Song 2010). Phagocytic cells enable antigen presentation of cellular or exogenously phagocytosed peptides (Neefjes et al. 2011). These antigen presenting cells (APCs) initiate the adaptive immune response divided into cell-mediated and humoral immunity.

Cell-mediated immunity is assisted by thymus-derived T lymphocytes. Once T cell receptors (TCRs) interact with antigens presented by major histocompatibility complex (MHC) molecules on APCs, the immunologic effector pathway that specifically eliminates microbes through secretion of cytokines or direct cell-cell contact starts.

The humoral immunity is mediated by bone marrow-derived B lymphocytes. After direct recognition of antigens, B lymphocytes stimulate the generation of an immunologic memory. They proliferate and differentiate into plasma cells that produce immunoglobulins (Ig) against specific antigens or into memory B cells that respond upon re-exposure (Abbas et al. 2014).

1.7 Cancer immunity - immunoediting and tumor evasion

Since Paul Ehrlich in 1909 hypothesized that degenerated cells are continuously emerging within the human body and the immune system controls the development of carcinoma ensuring beneficial homeostasis of health, there have been many multifaceted discussions about cancer immunology and immune surveillance (Ehrlich 1909). About fifty years later, Frank Macfarlane Burnet and Lewis Thomas reconsidered the natural immunologic sentinel of neoplasia as tumor cell-specific neoantigens

initiate an immunologic reaction that eliminate nascently transformed cells (Burnet 1957, Thomas 1959, Burnet 1964, Burnet 1971).

However, the immune system comprises a dual role in cancer. While immunosurveillance only ensures suppression of tumor growth (Smyth, Godfrey and Trapani 2001), the innate and adaptive immune system may also endorse the emergence of tumors escaping immune recognition and elimination (Shankaran et al. 2001). This host-protective and tumor-promoting character of the immune system throughout tumorigenesis is defined as cancer immunoediting. It describes a dynamic process that includes three sequential phases – *elimination, equilibrium* and *escape* (Dunn et al. 2002, Dunn, Old and Schreiber 2004b).

The *elimination phase* portrays the classical model of cancer immunosurveillance, in which the innate and adaptive immune system dominate over nascent tumor cells.

However, apart from continuous elimination of tumor cells, many tumor cells acquire different genetic and epigenetic alterations and become resistant to immune reactions. These tumor cell variants evolve a non-immunogenic phenotype that are capable for surviving in an immunologically intact microenvironment. This immune-mediated dormancy describes the *equilibrium phase*.

If tumor cell variants acquire the ability to circumvent immune recognition, resist to immune effector mechanisms and/or induce an immunosuppressive state within the tumor microenvironment, they enter the *escape phase*. Subsequently, these poorly immunogenic and immunoevasive transformed tumor cells become a clinically apparent tumor (Dunn, Old and Schreiber 2004a, Schreiber, Old and Smyth 2011, Mittal et al. 2014).

The reciprocal interactions of the immune system and tumor illustrate that *de novo* malignancies arise predominantly in an immunosuppressed microenvironment impeding the elimination or equilibrium phase. This association could also be demonstrated by some clinical incidents. Patients diagnosed with the acquired immunodeficiency syndrome (AIDS) or recipients of solid organ transplant, who both experienced an immunosuppressive regime, show a higher risk for cancer. Even if cancer in these patients is prevalently associated with viral etiology, like cervical cancer caused by human papilloma virus, (non)-Hodgkin´s lymphomas by Epstein-Barr virus or Kaposi´s sarcoma by herpesvirus, they have an increased propensity to develop non-viral associated cancers, like Merkel cell carcinoma, lung cancer or melanoma (Grulich et al. 2007, Engels et al. 2011, Yarchoan and Uldrick 2018).

In addition, the impact of the immune system on tumor disease is also elucidated as intratumoral immune response predicts cancer patient's prognosis (Pagès et al. 2010). Many studies have shown a correlation of tumor-infiltrating lymphocytes (TILs) with patients' survival, like in colorectal cancer (Galon et al. 2006), melanoma (Thomas et al. 2013) and ovarian cancer (Zhang et al. 2003). Hence, cancer immunoediting illustrates the interaction of the immune system and cancer, while it enables several possibilities to sculpture cancer immunogenicity.

1.8 Lymphocytes and their role as tumor-infiltrating lymphocytes

Major regulators specifically targeting tumor cells are T lymphocytes. They are originated from pluripotent hematopoietic stem cells (HSCs) in the bone marrow that give rise to lymphoid and myeloid progenitor cells. The common lymphoid progenitor cells migrate to the thymus to mature into naïve T lymphocytes (Klein et al. 2009). This process requires sequential stages.

T cell receptor gene rearrangement and the T cell precursor selection are crucial for infinite antigen recognition. Therefore, while migrating through the thymus, recombination of genomic TCR DNA sequences resulted in highly specific repertoire of T lymphocytes. Moreover, T cell precursors encountering peptide MHC complexes on thymic APCs, including cortical and medullary thymic epithelial cells and DCs, undergo an essential selection process (Zhang, Sun and Zhao 2007). This ensures T cells with TCRs of intermediate affinity towards MHC complex (positive selection), while TCRs with negligible or high affinity experience apoptotic cell death (negative selection). Positive and negative thymic selection resulted in a tolerant immune system that efficiently respond to microbes or nascent tumor cells, but avoid autoimmune reaction ensuring non-immunogenic self-tolerance. Positively selected naïve T cells translocate to peripheral secondary lymphoid organs, including lymph nodes, mucosa-associated lymphoid tissues, spleen and tonsils (Klein et al. 2009).

Upon recognition of developing tumor and microenvironment remodeling, an anti-tumor immune response is initiated. This results in generation of proinflammatory signals that recruit innate immune cells to tumor site. Tumor cell debris is ingested by DCs that migrate to tumor-draining lymph nodes. The recruitment of more immune cells is mediated by cytokines, interleukin 12 (IL-12) and interferon γ (IFN-γ), which gradually promotes tumor eradication (Garris et al. 2018).

In the lymph node, the adaptive immune response is initiated by interaction of resident naïve T lymphocytes and transient APCs presenting tumor-specific antigens. This antigen recognition requires a number of auxiliary molecules.

On naïve T lymphocytes, the TCR composed of α and β chain (a smaller fraction harbors γ and δ chains) is associated with a non-polymorphic membrane protein complex, collectively known as CD3 (γ-, δ-, ε-, ξ-subunits), that non-covalently initiates an intracellular signal upon TCR ligation (Rudolph, Stanfield and Wilson 2006). Depending on T cell, CD4 or CD8 co-receptors that simultaneously bind residues of MHC complex class II or I, respectively, stabilize the T cell and APC interaction (Abbas et al. 2014). Antigen recognition induces the synthesis of cytokines, mainly IL-2, by APCs that in turn stimulate the expression of co-stimulators. The potent co-stimulatory receptor CD28 expressed on all naïve T cells interacts with its ligand CD80 (B7-1) and CD86 (B7-2) on APCs (Greenwald, Freeman and Sharpe 2005). Subsequently, antigen-specific T lymphocytes and APCs increasingly synthesize IL-2 cytokines enhancing proliferation. Proliferation starts within 1 to 2 days resulting in clonal expansion and inducing downstream signaling cascades for differentiation of naïve T cells into tumor antigen-specific effector

T cells with distinct functions that act at primary tumor site and directly eradicate antigen-bearing tumor cells (Abbas et al. 2014).

Four different effector T cells represent the cell-mediated immune response. $CD4^+$ T helper (T_H) cells and $CD8^+$ cytotoxic T cells constitute the two major subtypes, while γδ T cells and NK T cells are less prominent (Abbas et al. 2014).

1.8.1 $CD3^+$ $CD4^+$ T_{helper} cells

Upon antigen recognition, $CD4^+$ T_H cells regulate the immune response and differentiate into several distinct subtypes - T_H1, T_H2, T_H9, T_H17, T_{fH}, T_{reg} - under the influence of specific cytokines (Zhou, Chong and Littman 2009).

The generation of IFN-γ and IL-12 in response to an intracellular antigen promotes the differentiation of naïve T cells into T_H1 helper cells (Trinchieri 2003). This subtype is responsible for phagocyte-mediated ingestion and elimination of microbes mediated by generation of IFN-γ as it stimulates mononuclear phagocytes and enhances their antigen-presenting and phagocytic potencies. IFN-γ and IL-2 also result in opsonizing IgG antibodies enhancing phagocytosis and stimulate the maturation of $CD8^+$ cytotoxic T cell precursors (Abbas et al. 2014). The recruitment of mononuclear phagocytes and the priming and expansion of $CD8^+$ cytotoxic T cells that enable tumor eradication characterize T_H1 cells as potent anti-tumor mediator.

In presence of IL-4, naïve T cells differentiate into T_H2 helper cells that impact the humoral immune response. T_H2 cells induce IgE isotype switching and secretion of B cells by IL-4 that upregulates high-affinity IgE receptors on mast cells and induces degranulation. Its production of IL-5 results in activation of eosinophils, which bind to IgE and release their granule enzymes for elimination of extracellular helminthic parasites. Moreover, IL-13 promote the expulsion of parasites from mucosal organs and mediates the alternative macrophage activation. The enhanced mobilization of innate cells, mainly eosinophils and macrophages, to tumor site demonstrates their anti-tumor ability. As shown by Mattes et al., an induction of T_H2 immunity via adoptive transfer resulted in an eosinophil-dependent. In contrast, tumor-promoting effect of T_H2 cells was observed in pancreatic cancer (Ochi et al. 2012).

T_H17 cell lineage differentiation occurs by inflammatory cytokines, IL-1, IL-6, IL-23, and transforming growth factor β (TGF-β). In response to extracellular fungi and some bacteria, T_H17 cells produce IL-17 that mediate an inflammatory immune response by recruiting neutrophils and to some extent monocytes to site of antigen recognition, while IL-22 ensured epithelial barrier function (Zhou et al. 2009, Abbas et al. 2014). Induction of T_H17 cell-associated pro-inflammatory processes results in a tumor-sculpting, but also tumor-suppressive microenvironment. While chronic pro-inflammatory stimuli and pro-angiogenic IL-17 expression promote tumorigenesis *in vivo* (Numasaki et al. 2005), T_H17 cells also endorse the recruitment of different leucocytes to tumor site and priming of $CD8^+$ cytotoxic T cells (Muranski et al. 2008, Martin-Orozco et al. 2009).

As shown in tumor mouse models and human tumors as well, tumor-specific $CD4^+$ T_H cells migrated into the tumor stroma upon antigen recognition and activation (Pardoll and Topalian 1998, Corthay et al. 2005). Interestingly, tumor-infiltrating $CD4^+$ T_H cells are potent to eliminate tumor cells *in vivo* in absence of $CD8^+$ cytotoxic T cells (Mumberg et al. 1999, Perez-Diez et al. 2007). However, under physiological circumstances, $CD4^+$ T_H and $CD8^+$ cytotoxic T cells are both involved in an effective tumor defense (Chen and Mellman 2013, Shankaran et al. 2001).

1.8.2 $CD3^+$ $CD8^+$ cytotoxic T cells

The other major effector T cell lineage of the cell-mediated immunity is comprised by $CD8^+$ cytotoxic T lymphocytes. These cells recognize class I MHC-associated antigens via their TCR and CD8 co-receptor. Most of the tumors express class I MHC molecules, whereas the occurrence of class II molecules declined, including ACCs (Marx et al. 1996, Wolkersdörfer et al. 2005). Upon antigen recognition, $CD8^+$ cytotoxic T cells are activated to exocytose granules consisting of perforin and granzyme B targeting the altered cells. Perforin disrupts the integrity of the targeting cell membrane that facilitates access of granzyme B to cytosol, which induces the apoptotic cascades (Abbas et al. 2014). However, most of the tumor-derived antigens are self-tolerant autoantigens, which implicates that the immune system classifies these altered tumor cells as body´s own, and, consequently, will not be lysed. Rosenberg et al. demonstrated in studies, in which $CD8^+$ cytotoxic T cells are stimulated by tumor antigens *in vitro* und subsequently readministered to patients, a tumor antigen-specific immune response resulting in tumor regression (Rosenberg et al. 2008). This verified the direct anti-tumoral effect of $CD8^+$ cytotoxic T cells.

Additionally, many clinical studies, such as on breast cancer (Mahmoud et al. 2011) or colorectal cancer (Galon et al. 2006), verified an improved overall survival of patients, whose tumor is infiltrated by $CD8^+$ cytotoxic T cells (Galon et al. 2006, Fridman et al. 2012, Pagès et al. 2010).

1.8.3 $CD3^+$ $CD4^+$ $FoxP3^+$ regulatory T cells

For maintenance of immunologic self-tolerance and avoidance of hyperreactivity, $CD4^+$ $CD25^+$ $FoxP3^+$ regulatory T (T_{reg}) cells are crucial for balancing immune response. Forkhead-box protein P3 (FoxP3) transcription factor expressed in $CD4^+CD25^+$ T_{reg} cells functions as major lineage-specific transcription factor that induces downstream to TGF-β signaling upon antigen priming in peripheral lymphoid organs. Additionally, high levels of IL-2 produced by antigen-activated T cells act on IL-2 receptor on T_{reg} cells inducing its immunosuppressive abilities via a negative feedback mechanism (Chinen et al. 2016). The potent inhibitory cytokines, IL-10 and TGF-β, counteract the pro-inflammatory response as they suppress the activation of lymphocytes, DCs and macrophages (Fontenot, Gavin and Rudensky 2017, Zhou et al. 2009). Moreover, T_{reg} cells express the cytotoxic T lymphocyte-associated protein 4 (CTLA-4), an immune checkpoint molecule, that bind to CD80 (B7-1) and CD86 (B7-2) molecules on APCs and prevents co-stimulation via CD28 resulting in activation of effector T cells (Pentcheva-Hoang et al.

2004). These immunosuppressive mechanisms are utilized by the tumor in order to evade tumor-directed immune responses. Interestingly, the number of $CD4^+CD25^+FoxP3^+$ T_{reg} cells infiltrated within the tumor and its microenvironment or the systemic circulation is increased (Wolf et al. 2003, Togashi, Shitara and Nishikawa 2019). In several tumor entities, tumor-infiltration by these immunosuppressive T_{reg} cells is correlated with patients´ outcome. In ovarian cancer (Curiel et al. 2004) or breast cancer (Bates et al. 2006), $CD4^+CD25^+FoxP3^+$ T_{reg} are associated with a worse overall survival. By contrast, several studies of other cancer entities could not demonstrate an impact of $CD4^+CD25^+FoxP3^+$ T_{reg} cell infiltration on survival or their presence is even correlated with better survival in head and neck cancer (Badoual et al. 2006). Studies on tumor mouse models demonstrated that an inhibition of $CD4^+CD25^+FoxP3^+$ T_{reg} cells resulted in an increased anti-tumor immune response, while improving therapeutic response of cancer immunotherapy (Ghiringhelli et al. 2004, Hermans et al. 2003).

1.9 Impact of steroid hormones on the immune system

Glucocorticoids are distributed throughout the body, which indicates the wide variety of its physiological influences. Apart from different effects on systemic homeostasis, their immunosuppressive and immunomodulatory forces arose scientific interest (Cain and Cidlowski 2017).

Upon antigen recognition, pro-inflammatory mediators are secreted for the initiation of an immune response. However, in presence of GCs, these signaling pathways that induce the immunological cascade are impaired. This includes basically a minor expression of adhesion molecules and chemoattractants that results in sparsely recruiting and trafficking of leukocytes towards infection site (Cronstein et al. 1992, Ince, Weber and Scheiermann 2018). Particularly, neutrophil granulocytes that usually strive to the site of infection are influenced by GCs resulting in a severely diminished ability to adhere to vascular endothelium and to transmigrate permeable blood vessels. Additionally, the enhanced mobilization of neutrophils from the bone marrow and their little impairment of apoptosis results in neutrophilia (Cavalcanti et al. 2007, Cavalcanti et al. 2006). With regard to less frequent basophil and eosinophil granulocytes, GCs even induces their cell death (Schleimer and Bochner 1994, Yoshimura et al. 2001). Monocytes and macrophages, key components of the innate immune system, show impaired pro-inflammatory potentials in presence of GCs. Since GCs inhibit transcription of several pro-inflammatory cytokine, like IL-1, IL-6, IL-8, IL-12, tumor necrosis factor (TNF) α, granulocyte-macrophage colony-stimulating factor (GM-CSF), whilst promoting anti-inflammatory mechanisms via IL-10 and TGF-β,. Accordingly, GC-mediated gene programming shifts pro-inflammatory classical M1-like macrophages towards an anti-inflammatory M2-like alternatively activated subtype (Ehrchen et al. 2007, Martinez et al. 2008). *In vitro* studies demonstrated that GM-CSF-treated monocytes (M1 phenotype) undergo apoptosis in presence of GCs, while M-CSF-treated monocytes (M2 phenotype) remain vital (Achuthan et al. 2018). Although the number of circulating monocytes and resident macrophages is reduced, their motility and phagocytic abilities are increased by GCs (Ehrchen et al.

2007). The GC-induced impairments are also observed in DCs. While their maturation is hampered, their immature precursors and resident types undergo GC-induced apoptosis. Due to the reduced production of pro-inflammatory cytokines caused by GCs, antigen presentation by MHC class II molecules is hampered. This in turn negatively influences the recognition of novel microbe- or tumor-derived antigens and also the activation of the adaptive immunity by interfering with TCR signaling (Cao et al. 2013).

The interaction between GR and TCR signaling influences a common reduction of circulating T cells. Even already during thymopoiesis, GCs impacts the threshold for positive and negative selection (Mittelstadt, Monteiro and Ashwell 2012) and thymocytes are sensitive to GC-induced apoptosis (Wang et al. 2006). In the circulation, GCs induce a reduction of T cells by inhibition of IL-2 signaling and promoting lymphocytes apoptosis. Besides this, GCs differently influence their differentiation. While GCs preferentially suppress T_H1 and T_H17 cells, they promote a shift towards T_H2 cells. The GC-mediated T_H2 cell polarization is stimulated by a reduced production of T_H1 cell-promoting cytokine IL-12 by APCs and attenuating of IL-12 receptor expression in T cells, while T_H2 cell-promoting cytokines, IL-4, IL-10 and IL-13, are increased by T_H2 cells (Elenkov 2004, Calcagni and Elenkov 2006, Cain and Cidlowski 2017).

Other immunoregulatory properties encouraged by GCs are observed on $CD4^+$ $CD25^+$ $FoxP3^+$ T_{reg} cells. Hence, GCs promote their differentiation and immunosuppressive activity by upregulation of the major transcription factor FoxP3 (Karagiannidis et al. 2004).

Studies on the effects of GCs on humoral immunity demonstrate that immature B cells are more susceptible to GC-induced apoptosis than their mature form (Garvy et al. 1993, Cain and Cidlowski 2017). Consistently, patients after adrenalectomy or under treatment with GR antagonist mifepristone are characterized by an expansion of immature B cells (Igarashi et al. 2005). Although there is a substantial variability considering levels of immunoglobulins, GCs may promote the class-switch recombination IgE, while other Ig isotypes are rather decreased or remain unaffected (Settipane, Pudupakkam and McGowan 1978, Zieg et al. 1994).

1.10 Immunotherapies in cancer treatment

In order to control tumor disease via immunological means, cancer immunotherapies are supposed to increase the efficiency of immune effector cells, to target tumor-specific antigens and/or to overcome tumor-mediated immunosuppression. Therefore, multiple approaches of immunotherapies are actively investigated.

These includes prophylactic vaccines that prevent carcinogenic infections, like the vaccine against human papillomavirus against cervical cancer, or therapeutic vaccines that stimulate a specific immune response targeting tumor-associated antigens, like melanoma antigen (MAGE) A3 or New York esophageal squamous cell carcinoma (NY-ESO)-1 (Decoster, Wauters and Vansteenkiste 2012, Thomas et al. 2018). Additionally, clinical trials on personalized recombinant cancer vaccines that promote

immune response to tumor-specific neoantigens identified via next-generation sequencing of genomic tumor DNA show promising results (Ott et al. 2017, Sahin et al. 2017). Another approach focuses on native or engineered oncolytic viruses that selectively replicate in tumor cells, while they induces tumor debulking and T cell activation leading to tumor lysis (Kaufman, Kohlhapp and Zloza 2015).
Further advances in adoptive T cell transfer of *in vitro* expanded and/or genetically engineered autologous or allogenic tumor-specific lymphocytes are very sustainable directing anti-tumor activity (Rosenberg et al. 2008). Another therapeutic approach with monoclonal antibodies targeting CD20 on leukemia and lymphoma cells (Rituximab) or human epidermal growth factor receptor 2 (HER2) on breast cancer cells (Trastuzumab) are routinely appointed to specifically target tumor cells (Hilchey et al. 2009, Slamon et al. 2011). At last, studies on molecular or cellular mediators of tumor-induced immunosuppression, such as CTLA-4, programmed cell death 1 (PD-1), programmed cell death ligand 1 (PD-L1) or T_{regs}, particularly contribute to the scientific progress (Dougan and Dranoff 2009, Sharma and Allison 2015, Wei, Duffy and Allison 2018).
These mediators regulate the immune system by ensuring self-tolerance, while simultaneously providing comprehensive protection against microbes and/or neoplasia. Currently, the most potent T cell immune checkpoint molecules are the CTLA-4 and PD-1. They provide the fundamental precondition for several pioneering cancer research advances for that James Patrick Allison and Tasuku Honjo ultimately were honored by the Nobel Prize in Physiology and Medicine in 2018 (Waldman, Fritz and Lenardo 2020).

The first described negative immune regulator, CTLA-4, is present within intracellular vesicles in naïve T cells, while thoroughly expressed on the surface of immunosuppressive $CD4^+FoxP3^+$ T_{reg} cells. Upon antigen recognition in the lymphoid tissue, T cells display CTLA-4 on their surface. Due to the fact that CTLA-4 is structurally and biochemically similar to the co-stimulatory surface protein CD28 on T cells, they both favor ligand CD80 (B7-1) and CD86 (B7-2) expressed by APCs; even though, CTLA-4 presents with higher affinity. Consequently, CTLA-4 inhibits T cell activation by directly antagonizing CD28 and competing with co-stimulatory ligands, which in turn reduces IL-2 secretion resulting in hampered T cell proliferation. Additionally, CTLA-4 prevents the conjugation of T lymphocytes and APCs (Schneider et al. 2006), while recruiting inhibitory effectors (Fraser et al. 1999, Greenwald et al. 2001). Among its impact on the conventional $CD4^+$ T_H lymphocytes, CTLA-4 expressed on $CD4^+FoxP3^+$ T_{reg} cells is crucial for direct and indirect immunosuppression (Tai et al. 2012, Wing et al. 2008).
Due to these distinct immunoregulatory abilities, CTLA-4 came to the fore of research considering anti-tumoral immunity. James Patrick Allison and colleagues illustrated that neutralizing the inhibitory effects of CTLA-4 using a CTLA-4-targeting antibody results in effective anti-tumoral immune response *in vivo* (Leach, Krummel and Allison 1996). Although several preclinical studies present heterogeneous, tissue-specific results, clinical trials on monoclonal CTLA-4 antibodies demonstrated its therapeutic effectivity in metastatic melanoma (Grosso and Jure-Kunkel 2013). Ultimately, ipilimumab,

a human IgG1κ anti-CTLA-4 monoclonal antibody, received FDA approval for non-resectable stage III/IV melanoma in 2011 (Hodi et al. 2003, Hodi et al. 2010, Grosso and Jure-Kunkel 2013). Remarkably, the median overall survival of patients with advanced melanoma that were treated with the immune checkpoint inhibitor ipilimumab was 11.4 months and 22 % of all patients experienced at least a three-year survival rate (Schadendorf et al. 2015). However, results of clinical trials in other tumor entities, such as non-small cell lung cancer (Lynch et al. 2012) or prostate cancer (Kwon et al. 2014), were not as impressive as in melanoma.

Whereas CTLA-4 regulates immune homeostasis predominantly within lymphoid tissue, the additional T cell immune checkpoint molecule, PD-1, influences T cell activation subsequently within peripheral tissue. The human immunoinhibitory receptor, PD-1, is expressed on activated T cells, B cells, NK cells and myeloid cells and binds its B7 homologues ligand PD-L1 (B7-H1/CD274) or PD-L2 (B7-DC/CD273) presented on APCs. Upon engagement, PD-1 and its ligand negatively regulate the TCR signaling causing inhibition of T lymphocyte proliferation and IL-2 and IFN-γ cytokine secretion (Freeman et al. 2000, Okazaki et al. 2013). Additionally, the PD-1/PD-L1 axis regulates the differentiation of $CD4^+$ T_H cells into $CD4^+FoxP3^+$ T_{reg} cells and promotes its immunosuppressive functions (Francisco et al. 2009). These immunoinhibitory phenomena are adopted by tumor cells whilst upregulating PD-L1 (Keir et al. 2008). This results in T cell exhaustion and creates a non-immunogenic tumor microenvironment favoring tumor growth and metastatic spread (Wherry and Kurachi 2015).
Once PD-1 and PD-L1 were involved in negatively regulating T cells and in generating an immunosuppressive tumor microenvironment, several preclinical studies appeared. Studies on different cancer cell lines overexpressing PD-L1 demonstrated its involvement in tumor escape due to their insusceptibility to cytotoxic $CD8^+$ T cell response and enhancement in tumorigenesis. Neutralizing the PD-1/PD-L1 pathway using anti-PD-L1 antibody reversed these effects and enhanced T cell cytotoxicity towards tumor cells (Iwai et al. 2002).
These research advances pave the way for clinical trials on PD-1 and PD-L1 inhibition enhancing anti-tumoral immunity (Hargadon, Johnson and Williams 2018, Gong et al. 2018).
Results of a phase I clinical trial on anti-PD-1 (MDX-1106) demonstrated the safety and tolerability, while promoting anti-tumor response in several advanced tumor diseases (Brahmer et al. 2010). In 2014, pembrolizumab and nivolumab, both IgG4 humane anti-PD-1 monoclonal antibodies, received their FDA approval for refractory and unresectable melanoma (Weber et al. 2015, Hargadon et al. 2018, Robert et al. 2015a). Their therapeutic applications have since been extended to many other tumor entities, such as non-small-cell lung cancer (Garon et al. 2015), head and neck squamous cell carcinoma (Ferris et al. 2016, Cohen et al. 2019), Hodgkin-lymphoma (Moskowitz et al. 2016, Ansell et al. 2015). In comparison to CTLA-4 inhibitor ipilimumab, the PD-1 inhibitor, pembrolizumab, convinced by a prolonged six months progression-free survival and less toxicity in patients with advanced melanoma (Robert et al. 2019, Robert et al. 2015b). Hence, anti-PD-1 therapy imposes with long-term immune-

mediated patients´ outcome and a broader clinical benefit than through CTLA-4 blockade (Robert et al. 2019, Topalian et al. 2019). The chance that tumor cells acquire the ability to express PD-L1 that enables them to suppress anti-tumoral immunity (Han, Liu and Li 2020).

The first monoclonal IgG4 antibody targeting PD-L1, atezolizumab, was approved in 2016 for treatment of urothelial carcinoma (Rosenberg et al. 2008). The next year, avelumab and durvalumab drew attention for the treatment of different cancer entities, such as Merkel cell carcinoma (Kaufman et al. 2016) or urothelial carcinoma (Powles et al. 2017), respectively (Hargadon et al. 2018).

The interaction partners as well as the cell-specific expression of the described immune checkpoint molecules that were targeted by different immunotherapies are illustrated in Figure 1.4.

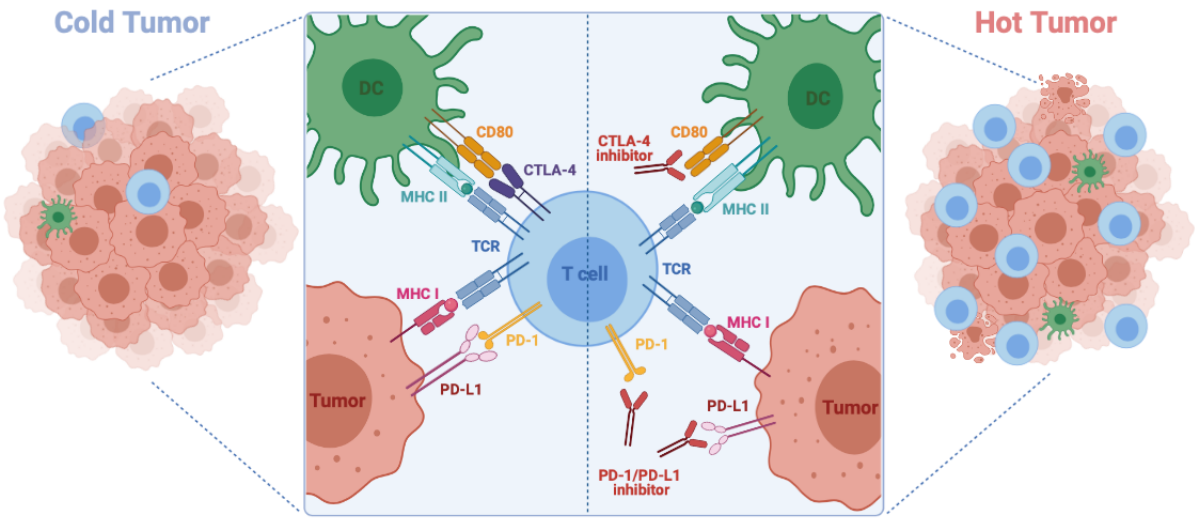


Figure 1.4: Immune checkpoint regulation in immunogenic "cold" and "hot" tumors
T cell receptor interacts with peptide-MHC molecules of antigen-presenting cells/dendritic cells or tumor cells, followed by co-stimulation through binding of CD28 and CD80. In immunogenic "cold" tumors, co-stimulation is inhibited by binding of CTLA-4 to CD80 and PD-1 to PD-L1. This immunosuppressive status can be reversed by PD-1-, PD-L1- and CTLA-4 inhibitors resulting in an immunogenic "hot" tumor. (Created with BioRender.com)

These scientific advances in anti-tumor therapies targeting immune checkpoint molecules resulted in five clinical trials in ACC patients with heterogeneous treatment response as described in detail in 4.2 (Le Tourneau et al. 2018, Carneiro et al. 2019, Habra et al. 2019, Raj et al. 2019, Klein et al. 2021).

1.11 Hypothesis/Objectives

By re-analyzing the RNA sequencing of 78 ACC patients generated by The Cancer Genome Atlas (TCGA) consortium, two distinct subgroups of ACC patients were identified. The comprehensive cluster analysis provided insight into the overexpression of genes related to steroidogenesis or the immune system. Both, the "steroid phenotype" or "immune phenotype", do not only differ in terms of gene expression but also regarding prognosis.

In order to advance the understanding of the pathogenesis and clinical prognostic of ACC, this thesis focuses on the interplay of steroid hormones and the immune system and the study of glucocorticoid-induced T cell depletion. A more profound analysis of differences between the "steroid" and "immune" ACC phenotype will contributes to improved therapeutic immunogenic strategies (Landwehr et al. 2020).

In addition, biomarkers as PD-1 and its ligand PD-L1, currently considered predictive of response to immunotherapy in other tumor entities, are also investigated. Predictive markers might lead to increased prognostic and therapeutic benefits and enable the elucidation of resistance mechanisms, especially with regard to immunotherapy of ACC patients.

To further understand the impact of steroid hormones on immune response and to overcome their immunosuppressive resistance mechanism, the anti-tumoral immune system might be reactivated by a combination of inhibitors of immune checkpoints and steroidogenesis.

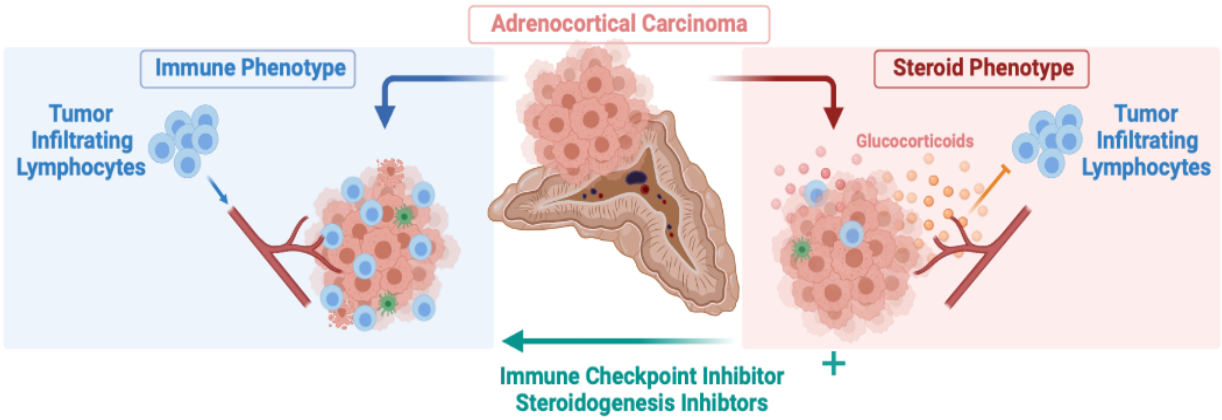


Figure 1.5: Hypothesis of "Immune Phenotype" vs. "Steroid Phenotype"
(Created with BioRender.com)

The main questions this thesis aims to answer are the following:

I) Can we identify the above mentioned "immune phenotype" by an upregulation of tumor-infiltrating lymphocytes and is this indeed, associated with an improved survival of these patients? And vice versa, is there a "steroid phenotype", characterized by an increase of glucocorticoids that lead to T cell depletion within the tumor microenvironment and causes worse outcome?

II) Is the expression of immune checkpoint molecules PD-1 and PD-L1 a relevant prognostic biomarker in ACC patients?

III) Is it possible to see *in vitro* a positive effect on antitumoral cytotoxicity in a co-culture system of human ACC and T cells, when these cells are treated with an inhibitor of steroidogenesis?

2 Materials & Methods

2.1 Materials

2.1.1 Human cell lines

For *in vitro* co-culture experiments, different human cell lines were used and listed in Table 2.1.

Table 2.1: Human cell lines

Name	Origin	Company
NCI-H295R	Adrenocortical carcinoma	ATTC
CU-ACC1	Adrenocortical carcinoma	provided by K. Kiseljak-Vassiliades
CU-ACC2	Adrenocortical carcinoma	provided by K. Kiseljak-Vassiliades
JIL-2266	Adrenocortical carcinoma	in house

2.1.2 Human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated from lithium-heparin full blood provided by healthy donor for *in vitro* co-culture experiments as shown in Table 2.2.

Table 2.2: Human peripheral blood mononuclear cells

Cells	Origin	Blood collection	Company
PBMCs	ACC patients	Lithium-heparin	Sarstedt
PBMCs	Healthy Donor	Lithium-heparin	Sarstedt

2.1.3 Human tissue

For chromogenic and fluorescent immunohistochemical staining, formalin-fixed and paraffin-embedded (FFPE) tumor tissue from ACC patients and five human tonsils from healthy donors were used and listed in Table 2.3.

Table 2.3: Human tissue

Tissue	Origin
Tonsil	Healthy donor
ACC	ACC patients

2.1.4 Cultivation media, solutions and buffer

2.1.4.1 Cultivation media

For *in vitro* experiments, cultivation media different tumor cell lines, viable primary cells and PBMCs were listed in Table 2.4. CU-ACC1-, CU-ACC2-, JIL-2266 cells, viable tumor cells and PBMCs were freshly supplemented with 1 % Penicillin/Streptomycin (10 mg/ml, Sigma) and 0.5 % Amphotericin-B (250 µg/ml, Sigma). PBMCs were additionally cultured in 20 U/ml rh IL-2 (Gibco).

Table 2.4: Cultivation media

Cells	Cultivation medium	Company
NCI-H295R	DMEM / F12 (1:1) [+] L-Glutamine, [+] 15mM HEPES	Gibco
	Supplemented with: Nu-Serum (2.5 %) 1x Insulin-Transferrin-Selenium	 Coning Gibco
CU-ACC1 / CU-ACC2	F12 nutrient ham: DMEM high-glucose (3:1)	Gibco Gibco
	Supplemented with: 10 % FBS 0.5 µg/ml insulin 0.4 µg/ml hydrocortisone 8.4 ng/ml cholera toxin 24 µg/ml adenine 10 ng/ml EGF 1 % Penicillin/Streptomycin 0.5 % Amphotericin-B	 Sigma Sigma Sigma Sigma Sigma Invitrogen Sigma Sigma

JIL-2266	F12 nutrient ham:	Gibco
	DMEM high-glucose (3:1)	Gibco
	Supplemented with:	
	10 % FBS	Sigma
	0.5 µg/ml insulin	Sigma
	0.4 µg/ml hydrocortisone	Sigma
	8.4 ng/ml cholera toxin	Sigma
	24 µg/ml adenine	Sigma
	10 ng/ml EGF	Invitrogen
	1 % Penicillin/Streptomycin	Sigma
	0.5 % Amphotericin-B	Sigma
Viable cells	F12 nutrient ham:	Gibco
	DMEM high-glucose (3:1)	Gibco
	Supplemented with:	
	10 % FBS	Sigma
	0.5 µg/ml insulin	Sigma
	0.4 µg/ml hydrocortisone	Sigma
	8.4 ng/ml cholera toxin	Sigma
	24 µg/ml adenine	Sigma
	10 ng/ml EGF	Invitrogen
	1 % Penicillin/Streptomycin	Sigma
	0.5 % Amphotericin-B	Sigma
PBMCs	RPMI-1640	Sigma
	[+] L-Glutamine, [+] Sodium	
	Supplemented with:	
	10 % human AB serum	Sigma
	1 % Penicillin/Streptomycin	Sigma
	0.5 % Amphotericin-B	Sigma
	20 U/ml rh IL-2	Gibco
Freezing medium	Cultivation medium	
	Supplemented with:	
	10 % DMSO	Sigma

2.1.4.2 Solutions and buffers

Table 2.5: Solutions and buffers

Solution / Buffer	Composition	Company
Buffers for immunohistochemical staining:		
Blocking solution	DPBS (1x) [-] $MgCl_2$, [-] $CaCl_2$	Gibco
	10 % normal goat serum	Sigma
	1 % BSA	Sigma
Citric acid monohydrate buffer	DPBS (1x) [-] $MgCl_2$, [-] $CaCl_2$	Gibco
	10 mM citric acid monohydrate pH 6.5	Sigma
PBS	DPBS (1x) [-] $MgCl_2$, [-] $CaCl_2$	Gibco
Buffers for T lymphocyte response:		
Erythrocyte lysis buffer	H_2O distilled	
	155 mM ammonium chloride	Riedel de Häen
	10 mM potassium bicarbonate	Fluka
	10 µM EDTA	Sigma
FACS buffer	DPBS (1x) [-] $MgCl_2$, [-] $CaCl_2$	Gibco
	4 % BSA	Sigma
	0.02 % Na-azide	Fluka
MACS buffer	DPBS (1x) [-] $MgCl_2$, [-] $CaCl_2$	Gibco
	5 % BSA	Sigma
	2 mM EDTA	Sigma
	0.02 % Na-azide	Fulka

T cell A/E buffer	DPBS (1x) [-] $MgCl_2$, [-] $CaCl_2$	Gibco
	0.5 % human serum albumin	Sigma
	2 mM EDTA	Sigma

2.1.5 Chemical reagents, drugs, consumables

Table 2.6: Chemical reagents, drugs, consumables

Name	**Application**	**Company**
Human AB serum	IHC / IF and cell culture	Sigma
CWFS gelatin	IF	Aurion
Dimethyl sulfoxide (DMSO)	Cell culture	Sigma
IL-2	Cell culture	Gibco
IFN-γ	Cell culture	Sigma
Lymphoprep	PBMC isolation	Stemcell
Metyrapone	Cell culture	Sigma
Paraformaldehyde (4 %)	IHC / IF	Merck
ProLong Diamond Antifade Mountant	IHC / IF	Thermo Fisher
ROCK inhibitor	Cell culture	Stemcell
Trypan blue 0.4 %	Cell count	Invitrogen
Trypsin-EDTA solution	Cell culture	Sigma
Türck´s solution	Cell count	Sigma

2.1.6 Antibodies

Primary and secondary antibodies for chromogenic and fluorescent immunohistochemical staining with specified hosts, concentration and clone were listed in Tables 2.7 and 2.9 and their respective combination in Table 2.8.

Table 2.7: Primary and secondary antibodies for fluorescent immunohistochemical staining

Primary ab				
	Host	**Concentration**	**Clone**	**Company**
CD3	mouse	1:50	PS1	Abcam; ab699
CD4	rabbit	1:1000	EPR6855	Abcam; ab133616
FoxP3	mouse	1:40	236A/E7	Abcam; ab20034
CD8	rabbit	1:1000	-	Abcam; ab4055
Secondary ab				
anti-mouse	goat	1:300	Alexa Flour 488	Abcam; ab150117
anti-rabbit	goat	1:200	Alexa Flour 555	Thermo Fisher; A21428

Table 2.8: Combination of fluorescence double staining of tumor-infiltrating lymphocytes

Set	**Combination**
1	$CD3^+CD8^+$
2	$CD4^+FoxP3^+$

Table 2.9: Primary antibodies for chromogenic immunohistochemistry staining

Primary ab	**Host**	**Concentration**	**Clone**	**Company**
PD-1	mouse	1:300	EH33	Cell Signaling; 43248S
PD-L1	rabbit	1:400	E1L3N	Cell Signaling; 13684

2.1.7 Kits

Table 2.10: Kits

Name	Application	Company
HiDef Detection HRP Polymer System	IHC	Cell Marque
Tumor Dissociation	Co-Culture	Miltenyi Biotec
Human CD3 MicroBeads	MACS	Miltenyi Biotec
Human CD4 MicroBeads	MACS	Miltenyi Biotec
Human FoxP3 MicroBeads	MACS	Miltenyi Biotec
Human CD8 MicroBeads	MACS	Miltenyi Biotec
Human CD56 MicroBeads	MACS	Miltenyi Biotec
Human anti-fibroblast MicroBeads	MACS	Miltenyi Biotec
Human IFN-γ ELISA	ELISA	Abcam; ab46025
MassChrom Steroids in Serum/Plasma	LC-MS/MS	Chromsystems Instruments & Chemicals

2.1.8 Equipment

Table 2.11: Equipment

Name	Type & Company
Cameras	Axiocam 503 Color, Carl Zeiss AG, Jena, Germany
	AxioCam MRm, Carl Zeiss AG, Jena, Germany
	Andor Zyla scmos, Oxford Instruments, Abingdon, Great Britain
	SPOT Insight 14.2 color mosaic digital, SPOT Imaging, Sterling Heights, MI, USA
Cell Counter	Countess II, life technologies, Waltham, MA, USA
Centrifuges	Rotixa 120R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany
	Megafuge 1.0R, Heraeus Holding GmbH, Hanau, Germany
	Mikro 200R, Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany

CO_2 Incubators	C150 Binder GmbH, Tuttlingen, Germany
	Forma Scientific $C0_2$ Water Jacketed Incubator, Thermo Fisher Scientific, Waltham, MA, USA
Tumor dissociator	GentleMACs dissociator, Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany
Tumor dissociator tubes	GentleMACs tubes, Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany
MACS column	MACS MS column, Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany
Magnetic stirrer	MMS-3000, bioSan, Riga, Latvia
Mass spectrometer	Orbitrap Fusion, Thermo Fisher Scientific, Waltham, MA, USA
	Qtrap 6500+, AB Sciex Germany GmbH, Darmstadt, Germany
Microscopes	Aperio Versa 8, Leica microsystems, Wetzlar, Germany
	Axio Scope A1, Carl Zeiss AG, Jena, Germany
	Primovert, Carl Zeiss AG, Jena, Germany
	DMIL, Leitz GmbH & Co KG, Stuttgart, Germany
Microtome	Slee Cut 5062 medical, Mainz, Germany
Mini rotator	A. Hartenstein, Würzburg, Germany
MACS separator	OctoMACS separator, Miltenyi Biotec B.V. & Co. KG, Bergisch Gladbach, Germany
pH meter	FiveEasy, Mettler Toledo, Columbus, OH, USA
Pipet controller	Accujet pro, Brand GmbH & Co. KG, Wertheim, Germany
Pipet stepper	HandyStep, Brand GmbH & Co. KG, Wertheim, Germany
Precision balance	Kern ABJ, Kern & Sohn, Balingen, Germany
Steril bank	MSC advantage, Thermo Fisher Scientific, Waltham, MA, USA
	SK-1500, BDK GmbH, Sonnenbühl, Germany
Tissue paraffin embedding system	Shandon Histocentre 3, Thermo Fisher Scientific, Waltham, MA, USA
Thermo shaker	TS-100, A. Hartenstein, Würzburg, Germany
Vortex	Vortex V-1 plus, A. Hartenstein, Würzburg, Germany

2.1.9 Software and databases

Table 2.12: Software and databases

Name	Company
Axiovision	Carl Zeiss AG, Jena, Germany
Excel 2016	Microsoft Corporation, Redmond, WA, USA
GraphPad Prism 8.0 / 9.0	GraphPad Software Inc., La Jolla, CA, USA
ImageJ software	Wayne Rasband, National Institutes of Health, NIH, USA
Leica Versa Aperio software	Leica, Buffalo Grove, IL, USA
PowerPoint 2016	Microsoft Corporation, Redmond, WA, USA
SPSS Statistics 26	IBM Corporation, Armonk, New York, USA
Word 2016	Microsoft Corporation, Redmond, WA, USA
Zeiss Axiovert 135 software	Zeiss, Carl Zeiss AG, Jena, Germany
Zeiss Zen2 software	Zeiss, Carl Zeiss AG, Jena, Germany

2.2 Methods

2.2.1 Patients cohort

The study was conducted as part of the European Network for the Study of Adrenal Tumors (ENSAT) ACC Registry (Fassnacht et al. 2009). Thereby, the principles of the Declaration of Helsinki and the Good Clinical Practice Guidelines were conformed and approved by the ethics committee of the University of Würzburg (#88/11). All patients provided a written informed consent for collecting clinical data, including follow-up and survival data, and tissue material. Control tissues (five non-pathological tonsils) were provided by the tissue biobank of the Institute of Pathology of the University of Würzburg and analyzed on an anonymous basis.

For investigation of $CD3^+$-, $CD4^+$-, $FoxP3^+$- and $CD8^+$ tumor-infiltrating lymphocytes, 146 tumor samples of 109 ACC patients (107 primary tumors, 16 local recurrences and 23 metastases) were included. This composition is defined as cohort I (Landwehr et al. 2020).

For the analyses of PD-1 and PD-L1 immune checkpoint molecules, 129 tumor samples of 119 ACC patients descending from the immune infiltration cohort I were integrated. These samples defined as cohort II comprise 94 primary tumors, 16 local recurrences and 19 metastases.

In 43 % of patients from both cohorts, autonomous glucocorticoid excess was diagnosed by means of pathological 1 mg dexamethasone test (cortisol > 5 μg/dl) in presence of suppressed ACTH (see Table 3.1 and 3.6, (Fassnacht et al. 2016).

Completeness of surgical resection of the primary tumor was based on negative surgical, pathological and imaging reports for any residual malignant tissue. At the time of diagnosis and during follow up examinations at an interval of 3 - 6 months, the presence of local recurrence or metastasis was evaluated by computerized tomography of chest and abdomen.

Metastases were not restricted to any secondary site, but rather evenly distributed. Within the immune infiltration cohort I, metastases from abdomen (n = 7), bone (n = 2), liver (n = 4), lung (n = 6) and lymph nodes (n = 4), were analyzed, of which 19 were allocated to abdomen (n = 6), bone (n = 2), liver (n = 3), lung (n = 5) and lymph nodes (n = 3), within the immune checkpoint cohort II.

2.2.2 Immunohistochemical staining methods

Chromogenic and fluorescent immunohistochemical staining was performed on human full formalin-fixed, paraffin-embedded (FFPE) tumor sections.
Human tumor tissue was pathologically preserved according to standard protocol of the Institute of Pathology at the University of Würzburg, Germany, or other accredited pathological departments. Laboratory fixation and preservation of cells were processed in the Research Division of the Department of Endocrinology at the University Hospital of Würzburg, Germany.

2.2.2.1 Formalin-fixed paraffin-embedded cell pellets

To analyze cell line expressing proteins by immunohistochemical methods, 1 x 10^7 cells were processed to generate FFPE cell pellets. Cells were detached as described in 2.2.4 and 2.2.5 and pelleted by centrifugation at 1000 x g for 5 min at room temperature (RT). Subsequently, cells were washed in Dulbecco´s phosphate buffered saline (DPBS), followed by centrifugation twice at 1000 x g and once at 1300 x g for 5 min each. After the last wash step, cell pellets were resuspended in 2 ml 4 % paraformaldehyde (PFA) and kept in gentle movement at 4 °C for 24 h. In the following, cell suspension was pelleted at maximum resolution (15 000 x g) for 5 min and PFA discarded. Cells were dehydrated by 2 ml of increasing ethanol (EtOH) concentration - 70 %, 85 % and 96 % - and isopropanol each for 20 min with centrifugation at 1000 x g for 5 min in between. 1 ml paraffin was added on cell pellet and incubated in paraffin tank for 1 h at 60 °C. At last, embedded cell pellet was transferred into special cassette and left to set.

2.2.2.2 Microtome sectioning

To generate precisely and continually sliced section from FFPE cell pellet and tumor tissue blocks, a microtome was used. Blocks were cooled at - 20 °C to provide a smooth flat cutting surface and trimmed prior first valuable section. Paraffin sections were cut at a thickness of 4 µm, placed on 50 °C water bath and once expanded transferred onto histology glass slides. Slides were dried and stored at RT until use for immunohistological staining methods.

2.2.2.3 Immunocytochemistry

To localize the presence of a specific protein in single cells *in situ*, 5 x 10^5 cells per well were seeded in four-well chamber slide. After achieving sufficient cell adherence and density, cells were 3 x washed with 1 ml DPBS and fixed by using 400 µl of 4 % PFA for 8 min at RT. Subsequent to three wash steps with DPBS, chamber system was removed. Henceforth, visualization of sub-cellular compartments expressing antigens of interest was succeeded by immunofluorescence staining as described in 2.2.2.4.

2.2.2.4 Immunofluorescence staining

FFPE tissue sections mounted on slides were deparaffinized 3 x in xylene for 5 min each and sequentially rehydrated by descending EtOH concentration – 100 %, 90 %, 80 % and 70 % - each for 3 min, rinsed in distilled water (H_2O) and washed twice in DPBS for 2 min. Subsequently, antigen retrieval was performed in 10 mM citric acid monohydrate buffer (pH 6.5) under pressure for 13 min. Henceforth, all incubation steps were performed in humid conditions and without exposure to light. Tissue was permeabilized in 0.5 % Triton-X for 10 min at RT and non-specific binding sites were blocked by incubating tissue slides in DPBS containing 10 % normal goat serum and 1 % bovine serum albumin (BSA) for 1 h at RT. Subsequently, slides were incubated with primary antibody in an appropriated concentration (Table 2.7) using 1 % BSA in DPBS overnight at 4 °C. Negative controls were performed on human tonsils using 1 % BSA in DPBS without primary antibody. After 3 x 5 min wash with DPBS, tissue was incubated with goat anti-mouse and / or goat anti-rabbit secondary antibody (Table 2.7) in DPBS for 1 h at RT and afterwards, washed 3 x in DPBS. Nuclei were counterstained with 4´,6-diamidino-2-phenylindole (DAPI) in DPBS for 2 min at RT and washed in DPBS without additives. At last, slides were mounted with ProLong Gold Antifade and preserved at 4 °C.

2.2.2.5 Chromogenic immunohistochemical staining

To examine the expression of proteins by chromogenic immunohistochemistry, FFPE tissue sections were deparaffinized and antigen retrieval was performed as described in 2.2.2.4. Henceforth, all incubation necessities were performed under humidified conditions. To block endogenous peroxidase activity, sections were incubated in methanol (MeOH) containing 10 % hydrogen peroxide (H_20_2) at RT for 10 min and 5 x washed with H_2O. Unspecific binding site were blocked by using 20 % human AB serum at RT for 1 h, followed by incubation of primary antibodies in their respective concentration (Table 2.9) overnight at 4 °C. For negative controls, universal isotype anti-mouse and anti-rabbit controls on human tonsil were performed. Slides were washed 3 x for 5 min each in DPBS and signal amplification was achieved by HiDef Detection HRP Polymer System for 20 min. After 3 x 5 min wash with DPBS, tissue slides were incubated with HiDef Detection HRP Polymer Detector for 20 min at RT. Subsequently, slides were rinsed 3 x for 5 min in DPBS and antigen targeting was finalized by 3,3´diaminobenzidine (DAB) substrate-chromogen (DAB Liquid Kit; Dako) for 10 min at RT - horseradish peroxidase (HRP) enzymatically visualizing colored precipitate. Nuclei were counterstained using Mayer´s hematoxylin for 2 min prior to blue coloring in running H_2O for 5 min. Stained tissue slides were dehydrated in 100 % EtOH and dried at 56 °C. After all staining, tissue slides were preserved by ProLong Gold Antifade and coverslips and stored at RT until microscopic evaluation.

2.2.3 Microscopy analysis

Quantitative analyses of fluorescent and chromogenic immunohistochemical staining were described in detail in 2.2.3.1 and 2.2.3.2.

Results of evaluation were collectively determined by investigators and a final score was generated by consensus. The Spearman correlation for interobserver reliability in rating given by different evaluators was specified by a threshold of > 0.85.

2.2.3.1 Quantitative analysis of fluorescence staining

For analysis of immune cell infiltrates, 10 x high power fields (HPFs) of each whole tumor section were chosen avoiding relevant necrosis and microphotographically captured with 40 x magnification objective of an Axiovert 135 microscope (Carl Zeiss). Three independent investigators (Jochen Schreiner, Iuliu Sbiera and Laura-Sophie Landwehr) manually quantified specific antibody-mediated membrane or nucleus staining by using ImageJ software (Wayne Rasband, National Institutes of Health, NIH, USA). By evaluating individual single photographed color channel, antibody-specific positivity of different subtypes of T lymphocytes was ensured. Immune-depleted ACC were defined as negative, any immune cell infiltration as positive.

2.2.3.2 Quantitative analysis of chromogenic immunohistochemical staining

For the assessment of immune checkpoint molecules, full section of each tumor was microscopically scanned by Leica Aperio Versa system with 20 x magnification objective. Two independent investigators (Iuliu Sbiera and Laura-Sophie Landwehr) quantified the membranous expression of both PD-1 and its ligand PD-L1 by using Aperio eSlide Manager (Leica Biosystems).
The expression of PD-1 was analyzed as ratio of PD-1^+ T cells to total number of cells with a threshold of > 1 %. For the quantification of PD-L1, the combined positive score, which defined the ratio of PD-$L1^+$ tumor cells and immune cells to total number of tumor cells, was used.

2.2.4 Cell culture

Cells were cultivated at 37 °C, 95 % relative humidity and 5 % CO_2 atmosphere ensuring *in vivo* state. Cell culture laboratory work was performed under aseptic conditions at laminar flow hood class II (Thermo Fisher Scientific and The Baker Company).

2.2.5 Passaging of tumor cells

Cells were cultured in T25-, T75- or T125 culture flasks and kept at optimal density by maintaining log phase for continued proliferation. In order to prevent contact inhibition of growth, cells were passaged prior of reaching confluence.

After removal of medium, cells were washed with 10 ml DPBS and incubated with 2 ml Trypsin-ethylenediaminetetraacetic acid (EDTA) solution for 1 min ensuring initiation of detachment of cells. Trypsin-EDTA was removed and after additional 3 min, cells were harvested by resuspending in 10 ml medium. Cells were split depending on their requirements – 1:2 (CU-ACC1, CU-ACC2) and 1:3 (NCI-H295R, JIL-2266).

2.2.6 Freezing of tumor and immune cells

For long-time storage, cells were detached by trypsinization as described in 2.2.4 and 2.2.5. Cell suspension from a sub-confluent culture flask was pelleted by centrifugation for 5 min at 1000 x g and resupended in cell culture medium containing 10 % dimethyl sulfoxide (DMSO). PBMC were respuspended in 1 ml MACS buffer with 10 % DMSO. For both tumor and immune cells, 5 x 10^6 cells in a volume of 1 ml were transferred to each cryo-vial and frozen by gradually decreasing temperature to -80 °C using Mr. Frosty™Freezing Container. Within 72 h, cell vials were stored in vapor phase nitrogen freezers until reutilization.

2.2.7 Thawing of tumor and immune cells

For thawing, cryopreserved cells were warmed up in a water bath at 37 °C and directly transferred into 10 ml fresh medium to minimize DMSO toxicity. Tumor cells were pelleted by centrifugation for 5 min at 1000 x g and cultivated in T75 culture flask containing 12 ml medium. For PBMCs, cells were pelleted for 5 min at 400 x g and cultured in media in 6-well plate.

2.2.8 Tumor-specific T lymphocyte response

In order to study the cytotoxic effects of T lymphocytes on tumor cells considering different treatment stimuli, co-culture experiments were performed.

2.2.8.1 Peripheral blood mononuclear cell isolation

To define different phenotypes and to analyze the cytotoxic characteristics of immune cells in ACC patients, primary T cells were isolated from whole blood by density gradient centrifugation using Lymphoprep™ gradient medium.

Therefore, 20 ml venous blood were collected in 3 x 7.5 ml lithium-heparin S-monovette tubes from ACC patients and healthy donors. Collection tubes were centrifuged at 800 x g for 10 min at RT and liquid plasma layer was removed. The remaining blood cells were resuspended each in 3 ml DPBS supplemented with 4 % BSA and 0.02 % sodium-azide (NaN_3) and cell suspension was carefully layered on 12 ml Lymphoprep™ density gradient medium in 2:1 ratio. As the separability of different blood cells is determined by differences in size and density, layered components were centrifuged at 800 x g for 20 min at RT. Upper layer was aspirated and the turbid band of peripheral blood mononuclear cells (PBMCs) – lymphocytes, monocytes and dendritic cells – separated along the density gradient were carefully transferred into new conical tube. Erythrocytes and granulocytes left in the sediment due to higher density were preserved for potential subsequent leucocytes DNA extraction. Isolated PBMCs were washed in 10 ml DPBS supplemented with 0.5 % BSA, 2 mM EDTA and 0.02 % NaN_3. In case of contamination by erythrocytes and granulocytes sediments, PBMCs were treated with erythrocytes lysis buffer containing of 155 mM ammonium chloride (NH_4Cl), 10 mM potassium bicarbonate ($KHCO_3$) and 10 µM EDTA for 10 min on ice. After centrifugation at 400 x g for 10 min at 4 °C and another wash step, PBMCs were cultured at a density of 5 x 10^6 cells / ml in cell medium supplemented with 20 U/ml human recombinant IL-2 in 6-well plate or preserved for long-time storage according to 2.2.6.

2.2.8.2 PBMC priming, activation and expansion

For the generation of tumor-reactive T lymphocytes, PBMCs were co-cultivated with adherent NCI-H295R cells. Therefore, NCI-H295R cells were seeded with a density of 1 x 10^6 cells / well in a 6-well plate containing 2 ml NCI-H295R medium. After 24 h, when NCI-H295R cells ensured adherence, NCI-H295R medium was removed and renewed by 2 ml of T cell A/E medium per well. Three hours prior to co-cultivation, PBMCs isolated as described in 2.2.8.1 were activated by using the human T Cell Activation / Expansion Kit (MACS, Miltenyi Biotec). In a first step, Anti-Biotin MACSiBead Particles were loaded with biotinylated antibodies against human CD2, CD3 and CD28 to mimic antigen-presenting cells and activate resting T lymphocytes from isolated PBMCs. Best activation was achieved by using a 1:2 ratio of loaded Anti-Biotin MACSiBead Particle to PBMCs in T cell A/E medium. The activated PBMCs were added to adherent NCI-H295R cells in a 7:1 ratio in T cell A/E medium

supplemented with 20 U/ml rh IL-2. Activated PBMCs were primed during culturing at 37 °C and 5 % CO_2 for 48 h.

2.2.8.3 Isolation of T cell subpopulations via magnetic-activated cell sorting

For purification of $CD3^+$ T lymphocytes, the Magnetic Activated Cell Sorting (MACS) technology was performed. In a first step, $CD56^+$ Natural Killer cells were depleted and subsequently, $CD3^+$ T cells were positively selected based on surface epitope recognition. The primed and activated PBMCs described in 2.2.8.2 were harvested and washed with 5 ml DPBS. For magnetic labelling, 20 µl of CD56 MicroBeads were added to 1 x 10^7 PBMCs in 80 µl MACS buffer. After incubation for 15 min at 4 °C, PBMCs were washed by adding 2 ml MACS buffer, followed by centrifugation at 300 x g for 10 min. Supernatant was aspirated and CD56 MicroBeads labelled PBMCs were resuspended in 500 µl MACS buffer. For magnetic separation, MS MACS column was rinsed with 500 µl MACS buffer and magnetically labelled PBMC suspension was applied onto column placed in a magnetic field of an OctoMACS Separator. Column was washed 3 x with 500 µl MACS buffer each to avoid unspecific binding. For $CD56^+$ NK cell depletion, $CD56^-$ PBMC effluent was collected in canonical tube, followed by a wash step with 2 ml MACS buffer and centrifugation at 300 x for 10 min 4 °C.
Subsequently, for positive selection of $CD3^+$ T cells, $CD56^+$ NK cell depleted PBMCs were magnetically labelled with CD3 MicroBeads methodically consistent as for CD56. After separation via an OctoMACS Separator, the MS column was removed from the magnetic field and the magnetically labelled cell fraction were flushed out by firmly applying a plunger and 1 ml MACS buffer. $CD56^-CD3^+$ T cells were collected in a canonical tube and cultivated at a density of 1 x 10^6 cells / ml in T cell A/E medium supplemented with 20 U/ml rh IL-2 in a 24-well plate at 37 °C and 5 % CO_2.

2.2.8.4 Co-culture of T cells and tumor cells

For activation of T lymphocytes, $CD56^-CD3^+$ T cells isolated as described in 2.2.8.3 were harvested from culture plate and washed with 5 ml T cell A/E buffer. After centrifugation at 300 x g for 5 min at RT, cells were diluted providing a density of 5 x 10^6 cells / ml per cm^2 of a flat-bottom 96-well plate. The cells were activated with Anti-Biotin MACSiBeads Particles using the Human T Cell Activation / Expansion Kit (Miltenyi Biotec) following manufacturer instruction and as described in 2.2.10.2. Subsequently, $CD56^-CD3^+$ T cell expansion was performed over 7 days. At day 3 after T cell activation, $CD56^-CD3^+$ T cell suspension was gently resuspended to dissolve agglomerates. Cell suspension was split into two equal parts and fresh T cell A/E medium supplemented with 20 U rh IL-2 / ml added. Cell were cultivated at 37 °C and 5 % CO_2. At day 7, cell agglomerates were dissolved by gently resuspending and single cells were counted. T cells were diluted to 1.2 – 2.5 x 10^6 cells per ml by fresh T cell A/E medium supplemented with 20 U rh IL-2 / ml. After these steps, $CD56^-CD3^+$ T cell priming, activation and expansion was successfully completed.

Two days prior to co-cultivation, 1-2 x 10^4 NCI-H295R cells / well in a 96-well plate (F-bottom) and cultured at 37 °C and 5 % CO_2. After 24h, one half of the NCI-H295R cells were treated with 100 µM metyrapone for inhibition of CYP11B1, 11β-HSD1 and 11β-HSD2, the other half with standard NCI-H295R medium without additives. After another 24 h, cell media was removed, NCI-H295R cells were washed with 200 µl DPBS and treated with 100 µl of fresh NCI-H295R medium with and without 100 µM metyrapone, respectively.

The primed, activated and expanded $CD56^-CD3^+$ T cells were diluted in T cell A/E medium ensuring a ratio of 1:2 (2 x 10^4) and 1:5 (5 x 10^4) of tumor and immune cells in a total volume of 200 µl / well. NCI-H295R tumor cells (HLA-A*02:01, HLA-B*15:10 and HLA-C*03:04) and $CD56^-CD3^+$ T cells (healthy donor: HLA-A(1)*02:01, HLA-A(2)*11:01, HLA-B(1)*14:01, HLA-B(2)*15:01, HLA-C(1)*03:04 and HLA-C(2)*08:02) were co-cultivated for 24h and 48h at 37 °C and 5 % CO_2. Cell medium supernatant was separately collected, centrifuged at 300 x g for 5 min at 4 °C and stored at -20 °C for enzyme-linked immunosorbent assay (ELISA).

2.2.8.5 Determination of hormones from adrenocortical carcinoma cells via LC-MS/MS

For determination of hormone status of NCI-H295R cells and adequate hormone inhibition by metyrapone, liquid-chromatography tandem mass spectrometry was performed at the Core Unit Clinical Mass Spectrometry, University Hospital Würzburg. Preseverd cell supernatants were quantified with the MassChrom steroids kit (Chromsystems) on a Qtrap 6500+ (Sciex) mass spectrometer coupled to a 1290 Infinity HPLC system (Agilent). Data analysis was performed using Analyst Software (1.6.3, Sciex) (Kurlbaum et al. 2020).

2.2.8.6 Interferon γ release of T lymphocytes via ELISA

For quantitative measurement of IFN-γ releases during NCI-H295R and $CD56^-$ $CD3^+$ T cell co-culture experiments, an ELISA with monoclonal antibody specific for IFN-γ was performed following manufacturer instructions (Abcam). The absorbance of each well was evaluated using the Victor3™ Multiple Plate Reader using 450 nm wavelength for 5 – 10 min. Standard curves were created by optical density (OD) of serial diluted reference protein and equivalent known concentration. The IFN-γ concentration present in the samples is quantified by extrapolation of linear proportion of the defined standard dilution curve.

2.2.9 Statistics

Statistical analyses were performed using Prism (Version 8 and 9, GraphPad Software Inc., La Jolla, CA, USA) and SPSS (Version 26, IBM Corporation, Armonk, New York, USA).

All statistically generated data were specified as median and range. A threshold of $p < 0.05$ considering significance was used. The cut-off for tumor immune infiltration was defined as "negative" in case of absent tumoral immune cell infiltration, while any infiltration of lymphocytes termed "positive".

Overall survival described the length of time from surgery to last follow-up or death. The period between surgery and the first occurrence of relapse was defined as recurrence-free survival. Progression-free survival was specified as time interval during or after therapy, in which the course of disease did not process.

To estimate and compare event-free survival the Kaplan-Meier method and log-rank test was performed. Cox proportional hazard regression modelling was applied for the identification of clinical factors that independently impacted patients´ outcome. In a first step, for all known or potentially relevant prognostic factors (age, sex, ENSAT stage, resection status, Ki67 proliferation index and autonomous glucocorticoid secretion), a univariate analysis was performed. Additionally, all four immune cell epitopes ($CD3^+$, $CD4^+$, $FoxP3^+$ and $CD8^+$) were included independently due to their subtype character (marked by line, Table 3.3 and 3.4). Hazard ratio (HR) with 95 % confidence intervals (CI) were analyzed by Cox regression as well. For the multivariate analysis, only parameters with a significant impact on patients' prognosis and survival in the univariate analyses ($p < 0.05$) were included.

For differences in tumor infiltration or in immune checkpoint molecules considering tumor localization, ENSAT stage, resection status, tumor size, Ki67 proliferation index, glucocorticoid excess and the number of tumor-infiltrating lymphocytes (only for cohort II), the *t*-test, an unpaired non-parametric Mann-Whitney test with median values and 95 % CI, was performed. Per sample analyses of primary tumor and patient-matched metastasis were evaluated by a non-parametric Wilcoxon matched-pairs signed rank test. The correlations between different variables were performed using Pearson´s chi-squared (X^2) test with Phi (ø) coefficient as a measure for effect size (Landwehr et al. 2020).

To evaluate differences in treatment of steroidogenic NCI-H295R with steroidogenesis inhibitor, metyrapone, or in tumor and immune cell ratios in co-culture experiments, a two-way ANOVA test was implemented.

3 Results

3.1 Tumor-infiltrating lymphocytes in adrenocortical carcinoma

3.1.1 ACC patients´ and tumor characteristics in primary tumors, local recurrences and metastases

Baseline characteristics of ACC patients and tumor specimens of cohort I are given in Table 3.1. The entire cohort was quite representative for ACC patients as female proportion dominated and median age was 47 years (Else et al. 2014), but the percentage of stage IV patients was slightly lower due to the fact that several of these patients were not operated.

Table 3.1: Adrenocortical carcinoma patients´ and tumor characteristics (Cohort I)

	n	Age (year)	Sex (m/f) (n)	Size of tumor (cm)	Ki67 index (%)	GC +/- other steroid	Sex hormone	Other steroid pattern	No steroid excess	Hormone profile n/a
All ACC samples	146	47 (18-77)	45/101	10.0 (0.5-30.0)	10 (0-80)	63 (36/27)	12	7	31	33
Primary tumor	107	47 (18-77)	32/75	11.8 (3.3-30.0)	10 (0-80)	44 (29/15)	9	4	23	27
ENSAT I/II[a]	47	46 (18-77)	13/34	12.0 (3.3-28.0)	10 (0-50)	17 (10/7)	6	3	14	7
ENSAT III[a]	38	47 (18-75)	13/25	10.3 (5.0-30.0)	20 (1-80)	16 (10/6)	3	1	7	11
ENSAT IV[a]	20	47.5 (24-72)	6/14	13.0 (7.0-25.0)	10 (1-30)	11 (9/2)	0	0	3	6
Local recurrence[b]	16	44 (20-70)	6/10	4.5 (1.5-9.4)	12.5 (5-20)	6 (2/4)	2	2	3	3
Distant metastasis[b]	23	41 (19-72)	7/16	1.7 (0.5-4.0)	17.5 (5-40)	13 (8/5)	1	1	4	4

Data represent median values with ranges or total numbers.

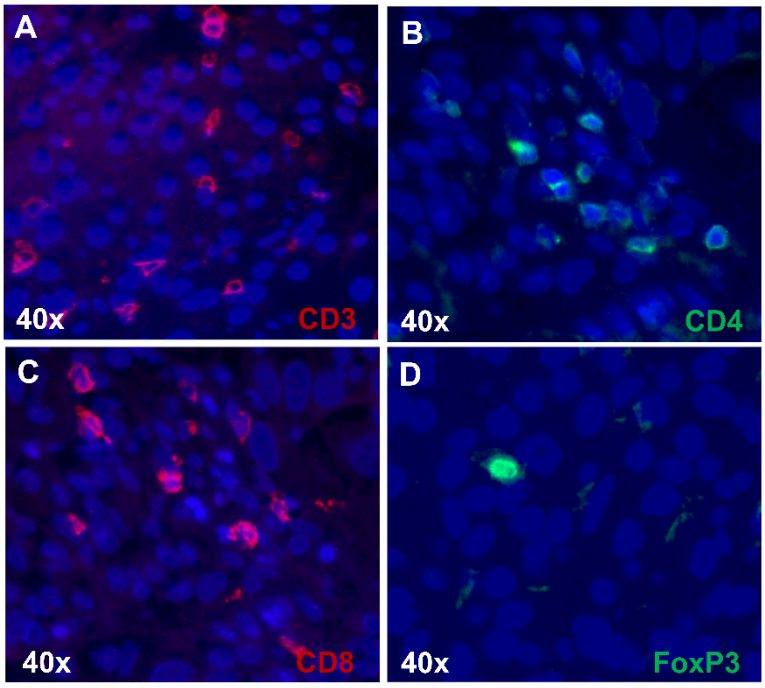
[a] Tumor stage according to the European Network for the Study of Adrenal Tumors (ENSAT) classification at the time of diagnosis (Fassnacht et al. 2009).

[b] In patients who experienced local recurrence or distant metastasis, endocrine activity was classified according to diagnostic analyses at primary diagnosis.

Tumor stage was not applicable in two cases. In 14 cases, no data regarding tumor size available, in 41 cases, proliferation status and in 33 cases hormone data were not determined.

3.1.2 ACC-infiltrating immune cells in primary tumors, local recurrences and metastases

An infiltration of $CD3^+$ T cells was detectable in 86.3 % of the 146 ACC samples using immunofluorescence microscopy, but the median number of these T cells was rather low (median 7.7 cells per HPF; Figure 3.1). $CD4^+$ T_H cells were visualized in 74.0 % of the ACC samples (Figure 3.1), while $CD8^+$ cytotoxic T lymphocytes were the most frequent infiltrating T cell type present in 84.3 % (Figure 3.1). In contrast, only 49.3 % of the ACCs stained positive for $FoxP3^+$ T_{reg} cells (Figure 3.1). Detailed results of the quantification of ACC-infiltrating T lymphocytes are given in Table 3.2.



Immune Cells	% of ACC	n of TILs / HPF
CD3	86.3 %	7.7 (0.1-376.0)
CD4	74.0 %	6.7 (0.2-109.0)
CD8	84.3 %	5.7 (0.1-291.0)
FoxP3	49.3 %	0.8 (0.1-18.0)

Figure 3.1: Tumor-infiltrating lymphocytes in adrenocortical carcinoma
Representative immunofluorescence staining of $CD3^+$- (A), $CD4^+$- (B), $CD8^+$- (C) and $FoxP3^+$- (D) tumor-infiltrating lymphocytes in adrenocortical carcinoma (n = 146). Modified from (Landwehr et al. 2020).

Subsequently, analyses focusing on potential differences in ACC-infiltrating lymphocytes between primary tumors, local recurrences and metastases were performed. Thereby, primary ACC tumors (n = 107) in comparison to total metastases (n = 23) indicated a tendency of higher median number of $CD3^+$- (median 8.5 vs. 5.4 per HPF), $CD4^+$ T_H - (median 7.6 vs. 3.8 per HPF) and $CD8^+$ cytotoxic T cells

(median 6.5 vs. 3.8 per HPF; Table 3.2). Notably, the highest infiltration by $CD3^+$-, $CD4^+$ - and $CD8^+$ T cells were observed in local tumors of ENSAT stage IV as they presented a median of 19.2, 14.4 and 8.2 cells per HPF, respectively. Moreover, local recurrences were frequently infiltrated (87.5 %), although a median of 5.1 $CD3^+$-, 5.0 $CD4^+$- and 5.0 $CD8^+$ T cells per HPF was relatively low (Table 3.2).

Table 3.2: Tumor-infiltrating lymphocyte characteristics (Cohort I)

	CD3		**CD4**		**FoxP3**		**CD8**	
	n (+/-)	**# TILs**	**n (+/-)**	**# TILs**	**n (+/-)**	**# TILs**	**n (+/-)**	**#TILs**
All ACC	26/20	7.7 (0.1-376.0)	108/38	6.7 (0.2-109.0)	72/74	0.8 (0.1-18.0)	123/23	5.7 (0.1-291.0)
Primary Tumors	92/15	8.5 (0.3-375.7)	80/27	7.6 (0.2-85.0)	57/50	1.2 (0.1-18.0)	89/18	6.5 (0.2-291.0)
ENSAT I/II	43/4	8.5 (0.3-280.0)	41/6	7.7 (0.3-54.0)	27/20	0.7 (0.1-18.0)	42/5	7.0 (0.3-226.0)
ENSAT III	31/7	4.0 (0.5-59.3)	24/14	6.3 (0.8-34.3)	19/19	0.3 (0.1-5.5)	31/7	4.0 (0.2-46.2)
ENSAT IV	16/4	19.15 (1.6-376.0)	13/7	14.4 (1.1-85.0)	10/10	1.8 (0.3-17.7)	14/6	8.2 (1.6-291.0)
Local recurrence	14/2	5.1 (2.0-149.9)	12/4	5.0 (1.7-128.8)	9/7	1.1 (0.1-9.7)	14/2	5.0 (1.7-128.8)
Distant metastasis	20/3	5.4 (0.1-35.3)	16/7	3.8 (0.1-28.5)	6/17	0.4 (0.2-1.8)	20/3	3.8 (0.1-28.5)

Data represent total numbers or median values with ranges.
With regard to the quantification of tumor-infiltrating lymphocytes, a definition was made for no infiltration: negative (-) and for any tumor infiltration of T cells: positive (+). Modified from (Landwehr et al. 2020).

In detail, a longitudinal analysis was achieved in 14 ACC patients, in whom both primary tumor and patients-matched metastasis were available. In accordance with the aforementioned results, metastases were significantly lower infiltrated by T cells subtypes in comparison to their matched primary tumors. Hence, a distinct decline of $CD3^+$- and $CD4^+$ T_H cells was observed in metastases of 12 of 14 patients, while $CD8^+$ cytotoxic T cells consistently decreased in all but one single patient (Figure 3.2).
Accordingly, the median number of $CD3^+$-, $CD4^+$-, and $CD8^+$ T cells were higher in primary ACC tumors (4.5, 4.7 and 4.4 T cells per HPF, respectively) than in distant metastases (2.15, 1.15 and 2.0 T cells per HPF, respectively; Figure 3.2) (Landwehr et al. 2020).

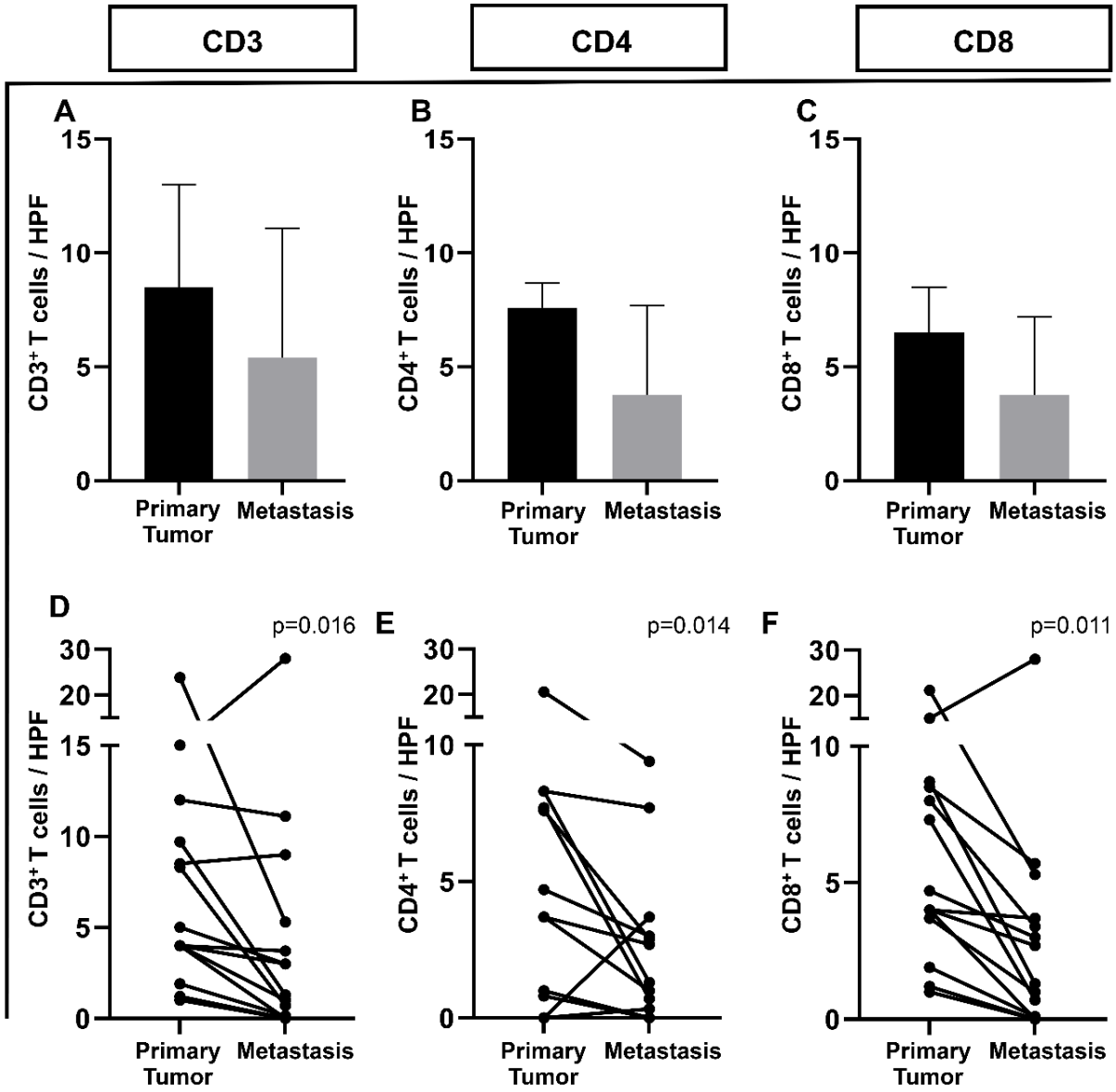


Figure 3.2: Comparison of tumor-infiltrating lymphocytes in primary tumors and metastases of ACC
A-C: $CD3^+$- (A), $CD4^+$- (B) and $CD8^+$- (C) T cells infiltrated in primary localized tumor tissue (n = 107) or distant metastases (n = 23) of entire ACC cohort I. D-F: Per sample analysis, $CD3^+$- (D), $CD4^+$- (E) and $CD8^+$- (F) T cells quantified in primary tumor and patient-matched distant metastasis (n = 14), median 95 % CI. Modified from (Landwehr et al. 2020).

3.1.3 Association of overall and progression-free survival with tumor-infiltrating T lymphocytes

In order to determine the impact of tumor-infiltrating T cells on ACC patients´ clinical outcome, the overall survival in all 107 ACC patients with primary tumor samples was investigated by using univariate analyses. The presence of ACC-infiltrating T cells was significantly associated with an improved overall survival compared to immune-depleted ACC (Figure 3.3, Table 3.3). More in detail, ACC patients with a primary tumor with $CD3^+$ T cell infiltration demonstrated a median survival of 81.8 months, while median survival was only 29.7 months in patients with absent $CD3^+$ TILs (Figure 3.3,

Table 3.3). Similar results were observed for $CD4^+$ T_H- and $CD8^+$ cytotoxic T cell-infiltrated ACCs with a median overall survival of 91.4 vs. 27.0 and 81.8 vs. 21.0 months, respectively (Figure 3.3, Table 3.3). However, in cohort I, no significant association of $FoxP3^+$ T_{reg} cell infiltration and overall survival was ascertainable (Table 3.3).

By using multivariate overall survival analyses, including additional prognostic factors, like ENSAT stage, resection status and Ki67 proliferation index, a significant independent association of $CD3^+$-, $CD4^+$- and $CD8^+$ ACC-infiltrating lymphocytes was noticed in ACC patients with localized, non-metastatic tumors, while this effect was diminished considering all patients with metastatic disease (Figure 3.3, Table 3.3). However, this impact was very potent for both, $CD4^+$- and $CD8^+$ T cells by a three-fold mortality risk reduction (HR 0.3, 95 % CI 0.13-0.66 and HR 0.29, 95 %CI 0.11-0.81, respectively; Figure 3.3, Table 3.3).

Table 3.3: Factors influencing ACC patients' overall survival (Cohort I)

			Univariate analyses (all)			Multivariate analyses (all)			Multivariate analyses (localized, non-metastatic)		
		n	**Hazard Ratio**	**95 % CI**	**p**	**Hazard Ratio**	**95 % CI**	**p**	**Hazard Ratio**	**95 % CI**	**p**
Age		-	0.99	0.97-1.01	0.423						
Sex	Female	75									
	Male	32	1.02	0.58-1.81	0.937						
ENSAT stage	I / II	47									
	III	38	1.77	0.93-3.37	0.081	1.36	0.58-3.14	0.479	1.29	0.54-3.13	0.568
	IV	20	5.28	2.63-10.57	<0.001	4.41	1.41-13.85	0.011			
Resection status	R0 / RX	59									
	RI / II	34	4.94	2.42-7.98	<0.001	2.20	0.94-5.13	0.070	2.67	0.97-7.36	0.057
Ki67 index	0-19 %	52									
	> 20 %	41	2.14	1.17-3.92	0.014	2.53	1.28-5.04	0.008	3.13	1.30-7.52	0.011
GC excess	-	36									
	+	44	1.92	1.01-3.66	0.048	1.37	0.57-3.28	0.477	1.91	0.70-5.23	0.208
CD3	-	15									
	+	92	0.47	0.25-0.87	0.016	0.55	0.23-1.30	0.173	0.19	0.06-0.57	0.003
CD4	-	27									
	+	80	0.39	0.23-0.67	0.001	0.58	0.29-1.18	0.134	0.30	0.13-0.66	0.003
FoxP3	-	50									
	+	57	0.87	0.52-1.48	0.617	0.77	0.41-1.45	0.414	0.64	0.30-1.36	0.246
CD8	-	18									
	+	89	0.47	0.26-0.85	0.013	0.52	0.23-1.14	0.101	0.29	0.11-0.81	0.018

For overall survival analysis, samples from ACC patients with primary tumors and available clinical data were included (n = 107). For multivariate analyses, complete applicable data from 83 (all) and 67 (localized, non-metastatic) ACC tumors were integrated out of 107 and 86 samples, respectively. Male sex, ENSAT stage I/II, low proliferating Ki67 (0-19 %) and lack of glucocorticoid excess: GC (-) were classified as reference category. Modified from (Landwehr et al. 2020).

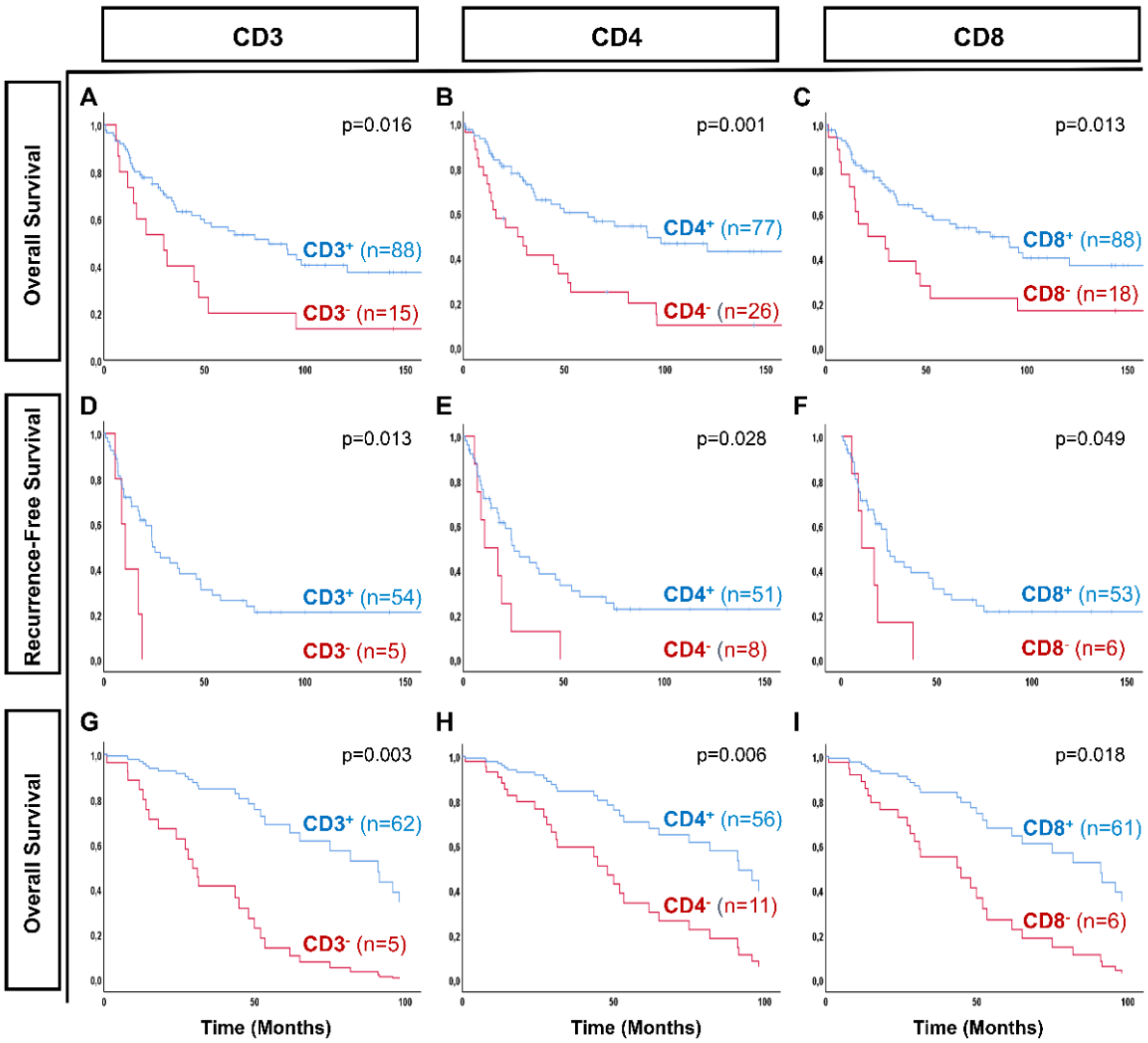


Figure 3.3: Survival analyses in patients with adrenocortical carcinoma according to $CD3^+$-, $CD4^+$- and $CD8^+$ tumor-infiltrating lymphocytes

A-C: Kaplan-Meier overall survival of all ACC patients with primary tumor samples influenced by $CD3^+$- (A), $CD4^+$- (B) and $CD8^+$- (C) TILs was demonstrated. D-F: In a subgroup of patients with localized ACC after complete surgical resection, a Kaplan-Meier recurrence-free survival analysis was performed according to $CD3^+$- (D), $CD4^+$- (E) and $CD8^+$- (F) tumor infiltration (n = 59).

G-I: Using multivariate COX regression, overall survival analyses in ACC patients with localized, non-metastatic primary tumor (n = 67) influenced by $CD3^+$- (G), $CD4^+$- (H) and $CD8^+$- (I) T cell infiltration according to different prognostic factors, including ENSAT stage, resection status and Ki67 proliferation index was performed. Modified from (Landwehr et al. 2020).

Consequently, to investigate the impact of tumor-infiltrating T cells on recurrence-free survival, ACC patients with primary localized, non-metastatic tumors after complete surgical resection (R0/RX status) were investigated (n = 59).

Table 3.4: Factors influencing ACC patients' recurrence-free survival (Cohort I)

			Univariate analyses (all)			**Multivariate analyses (all)**		
		n	**Hazard Ratio**	**95 % CI**	**p**	**Hazard Ratio**	**95 % CI**	**p**
Age		-	0.97	0.95-0.99	0.003			
Sex	Female	38						
	Male	21	1.70	0.92-3.14	0.090			
ENSAT stage	I / II	40						
	III	19	1.56	0.84-2.92	0.162			
Ki67 index	0-19 %	33						
	> 20 %	23	4.14	2.09-8.21	<0.001	3.84	1.90-7.75	<0.001
GC excess	-	26						
	+	22	1.43	0.72-2.84	0.303			
CD3	-	5						
	+	54	0.31	0.11-0.82	0.019	0.42	0.14-1.29	0.130
CD4	-	8						
	+	51	0.43	0.19-0.94	0.033	0.57	0.24-1.34	0.195
FoxP3	-	25						
	+	34	0.75	0.41-1.38	0.357	0.40	0.40-1.44	0.396
CD8	-	6						
	+	53	0.42	0.17-1.03	0.049	0.45	0.17-1.21	0.113

For recurrence-free survival analysis, samples from ACC patients with primary, localized tumors, R0 / RX status and available clinical data were included (n = 59). For multivariate analyses, complete applicable data from 56 ACC tumors (all) were integrated. Low Ki67 proliferation index (0-19 %) was classified as reference category in multivariate analyses.
No data regarding resection status in two patients and in three cases no data about proliferation index accessible. Clinical data considering glucocorticoid activity was not applicable in 11 cases. Modified from (Landwehr et al. 2020).

Similar to the findings on overall survival, $CD3^+$-, $CD4^+$- and $CD8^+$ ACC-infiltrating lymphocytes were associated with a significant longer median recurrence-free survival in comparison to immune-depleted ACC (24.2 vs. 10.7 months, 25.5 vs 10.7 months and 24.2 vs 10.7 months, respectively; Figure 3.4,

Table 3.4). However, in multivariate analysis including Ki67 proliferation index, these significant effects diminished (Table 3.4) (Landwehr et al. 2020).

3.1.4 Influence of glucocorticoids on tumor-infiltrating lymphocytes

To investigate the immunosuppressive effect of glucocorticoids on T lymphocytes, the presence of tumor-infiltrating T cells in primary ACC samples and tumor-induced autonomous glucocorticoid excess were correlated using Pearson correlation coefficient. Of note, among all different analyzed T cell subtypes, only $CD4^+$ TILs displayed a significant negative correlation with glucocorticoids in ACC patients (ø = -0.290, p = 0.009; Table 3.5). Additionally, the amount of $CD4^+$ TILs was significantly higher in hormone-inactive ACC (median 7.7 vs. 1.8 cells per HPF; Figure 3.4, A). Hence, 32 out of 36 primary tumor samples (89 %) from ACC patients without tumor-induced glucocorticoid excess presented a $CD4^+$ T_H cell tumor infiltration, while this occurred in only 64 % with hypercortisolism. Accordingly, 80 % of $CD4^+$ T_H cell-depleted tumors were observed in ACC patients with autonomous glucocorticoid excess.

Focusing on the entire cohort I, these observations were verified in primary tumors, local recurrences and distant metastases (ø = -0.260, p = 0.006; Table 3.5).

Table 3.5: Correlation of $CD4^+$ T_H cells with glucocorticoid excess (Cohort I)

		GC $^-$	**GC $^+$**	**Total**
A	**$CD4^-$**	4	16	20
	$CD4^+$	32	28	60
	Total	36	44	80
B	**$CD4^-$**	0	5	6
	$CD4^+$	25	17	42
	Total	26	22	48
C	**$CD4^-$**	6	22	28
	$CD4^+$	43	41	84
	Total	49	63	112

X^2 (Pearson) correlation of $CD4^+$ T_H cells with glucocorticoid excess in primary tumors (A: ø = -0.290, p = 0.009, n = 107), ACC patients with localized, non-metastatic tumors after complete surgical resection (B: ø = -0,284, p = 0.049, n = 60) and the entire cohort consisting of primary tumors, local recurrences and distant metastases (C: ø = -0.260, p = 0.006, n = 146).

Clinical data considering endocrine activity was not available in 27, 12 and 34 cases, respectively. Modified from (Landwehr et al. 2020).

At last, the overall survival considering not only immune infiltration, but also ACC-induced hypercortisolism was analyzed. These results enabled a more advanced prognostication with four different subgroups (Figure 3.4, B). In detail, tumors with $CD4^+$ T_H cell infiltration and without hypercortisolism presented the most favorable overall survival with a median of 121.0 months. For ACC patients with tumors that were infiltrated by $CD4^+$ T_H cells, but were diagnosed with a glucocorticoid excess showed a median overall survival of only 75.0 months. The prognosis of ACC patients with T cell-depleted tumors was substantially worse and also depended on glucocorticoid activity. The median overall survival of ACC patients with glucocorticoid excess was 44.9 months, while worst clinical outcome with only 27.0 months was observed with hypercortisolism. These results implied a 1.44- and 1.82 times increased mortality risk with tumor-induced glucocorticoid excess in $CD4^+$ T_H cell-infiltrated and -depleted ACCs, compared to $CD4^+$ T_H cell-infiltrated and hormone inactive ACCs, respectively (Figure 3.4, B); (Landwehr et al. 2020).

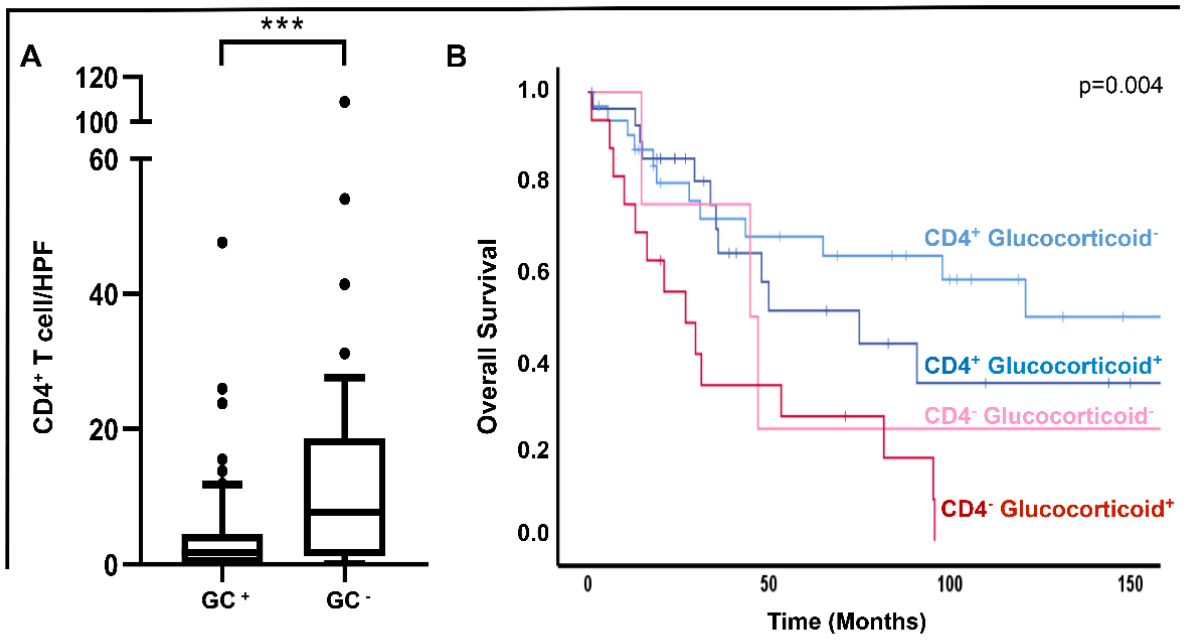


Figure 3.4: Correlation of glucocorticoids and $CD4^+$ T_H cells
A: Using an unpaired non-parametric Mann-Whitney test for the correlation of the number of tumor-infiltrating $CD4^+$ T_H cell with glucocorticoid excess (GC^+) or hormone inactive (GC^-) primary ACCs ($p = 0.0007$; $n = 107$, box/whiskers 10-90 percentile). B: Overall survival in ACC patients with $CD4^+$ T_H cell-infiltrated or -depleted primary tumors considering the presence or absence of glucocorticoid excess ($n = 80$).
Primary tumors with applicable endocrine status were classified as "lymphocytes-infiltrated" in case of definite immune infiltration (blue) and as "lymphocytes-depleted" (red) in the absence of tumor-infiltrating T cells. In addition, phenotypes were further subdivided according to hypercortisolism and hormonal inactivity. Modified from (Landwehr et al. 2020).

3.2 Immune checkpoint molecules in adrenocortical carcinoma

3.2.1 Expression of immune checkpoint molecules in primary tumors, local recurrences and metastases

In the next step, the expression of immune checkpoint molecule PD-1 and its ligand PD-L1 was investigated and will be described first separately in 3.2.2.1 and 3.2.2.2. For these analyses, cohort II, whose baseline characteristics of ACC patients and tumor specimens are given in Table 3.6, was investigated. Cohort II is – as a subgroup of cohort I – obviously quite similar to the main cohort with comparable key characteristics, mean age in fifth decade of life and more females than men (Else et al. 2014). In comparison to a standard cohort of patients with ACC, the proportion of tumors of ENSAT stage IV was again lower due to the fact that only operated patients were included.

Table 3.6: Adrenocortical carcinoma patients´ and tumor characteristics (Cohort II)

	n	Age (year)	Sex (m/f) (n)	Size of tumor (cm)	Ki67 index (%)	GC +/- other steroid	Sex hormone	Other steroid pattern	No steroid excess	Hormone profile n/a
All ACC samples	129	46 (18-77)	41/88	10.0 (0.5-30.0)	16 (0-80)	55 (30/25)	12	7	29	26
Primary tumor	94	47 (18-77)	30/64	12.0 (3.3-30.0)	16 (0-80)	38 (24/14)	9	4	22	21
ENSAT I/II[a]	41	46 (18-77)	12/29	10.0 (3.3-28.0)	16 (0-40)	14 (7/7)	6	3	12	6
ENSAT III[a]	34	46 (18-75)	13/21	10.0 (5.0-30.0)	16 (1-80)	14 (9/5)	3	1	7	9
ENSAT IV[a]	19	46 (24-72)	5/14	10.0 (7.0-25.0)	16 (1-30)	10 (8/2)	0	0	3	6
Local recurrence[b]	16	47 (20-70)	6/10	3.2 (1.5-9.4)	16 (10-15)	5 (2/3)	2	1	2	1
Distant metastasis[b]	19	46 (19-72)	5/14	1.9 (0.5-4.0)	16 (5-40)	11 (4/7)	1	1	4	2

Data represent median values with ranges or total numbers.

[a] Tumor stage at the time of diagnosis according to the European Network for the Study of Adrenal Tumors (ENSAT) classification (Fassnacht et al. 2009).

[b] In patients who experienced local recurrence or distant metastases during mitotane treatment, endocrine activity was classified according the information available at primary diagnosis.

Tumor size was not applicable in nine cases, while proliferation status was not determined in 35 and hormone data in 26 cases.

3.2.1.1 Programmed cell death 1

Using chromogenic immunohistochemical microscopy, the immune checkpoint molecule PD-1 with a threshold of ≥ 1 % was detectable in 17.3 % of all 129 ACC tumors of cohort II (PD-1$^+$ 22 vs. PD-1$^-$ 105; Figure 3.5, Table 3.7). In detail, the expression of PD-1$^+$ tumor-infiltrating lymphocytes was visible in 17.4 % of primary tumors (PD-1$^+$ 16 vs. PD-1$^-$ 76), 18.8 % of local recurrences (PD-1$^+$ 3 vs. PD-1$^-$ 13) and 15.8 % of metastases (PD-1$^+$ 3 vs. PD-1$^-$ 16; Table 3.7). Significant differences between primary tumors, local recurrences or metastases could not be identified (Table 3.7). However, PD-1 expression in TILs was associated with lower ENSAT tumor stage. While local tumors of patients diagnosed with ENSAT I or II showed a higher infiltration of PD-1$^+$ TILs, patients with more advanced stage III and IV appeared with significantly less PD-1 positivity (24.2 % vs. 9.5 and 13.0 %, respectively, p = 0.016; Table 3.7). Three ACC tumors specimens presented with loco-regional, para-adrenal lymph nodes were positive for PD-1, although adjacent tumor tissue was negative or < 1 % (Figure 3.5, G).

Moreover, the expression of PD-1 was independently of tumor size (PD$^+$ 9.5 cm vs. PD$^-$ 9.5 cm; p = 0.668) and resection status (R0 / RX vs. R1 / R2, X^2 = 1.47, p = 0.225) and did not correlate with Ki67 proliferation index (0-19 % vs. >20 %, X^2 = -0.08, p = 0.421) in consideration of all tumor samples (based on Table 3.6 and 3.7). However, glucocorticoids seem to impact the expression of PD-1 in local, non-metastatic tumors, as hormone inactive tumors show a higher expression of PD-1$^+$ TILs (GC$^+$ vs. GC$^-$, X^2 = 4.45, p = 0.035; based on Table 3.6 and 3.7).

Table 3.7: PD-1 immune checkpoint molecule expression (Cohort II)

	% of PD-1$^+$ ACC	PD-1 $^+$	PD-1 $^-$
All ACC samples	17.3	22	105
Primary tumor	17.4	16	76
ENSAT I / II [a]	24.2	15	47
ENSAT III [a]	9.5	4	38
ENSAT IV [a]	13.0	3	20
Local recurrence [b]	18.8	3	13
Distant metastasis [b]	15.8	3	16

Data represent percentage or total numbers.

[a] Tumor stage at the time of diagnosis according to the European Network for the Study of Adrenal Tumors (ENSAT) classification (Fassnacht et al. 2009).

[b] In patients who experienced local recurrence or distant metastases during mitotane treatment, endocrine activity was classified according the information available at primary diagnosis.

Studies concerning its ligand PD-L1, demonstrated a significant correlation with the expression of PD-1 in primary tumors as well as local recurrences and metastases ($X^2 = 26.943$, $p < 0.001$; based on Table 3.7).

3.2.1.2 Programmed cell death ligand 1

By studying the expression of the corresponding immune checkpoint marker PD-L1, ACC tumor specimens of cohort II presented a PD-L1 positivity with a threshold of ≥ 1 % in 24.4 % (PD-L1$^+$ 31 vs. PD-L1$^-$ 96; Figure 3.5, Table 3.8). Focusing on different tumor localization, 25.0 % of the primary tumors (PD-L1$^+$ 23 vs. PD-L1$^-$ 69), 18.75 % of local recurrences (PD-L1$^+$ 3 vs. PD-L1$^-$ 13) and 26.3 % of the metastases (PD-L1$^+$ 5 vs. PD-L1$^-$ 14) expressed PD-L1 (Table 3.8). Three ACC tumors specimens presented with loco-regional, para-adrenal lymph nodes that express PD-L1, while adjacent tumor tissue was negative or < 1 % (Figure 3.5, H).

While PD-L1$^+$ tumor cells were slightly more present in metastases, there was no difference detectable according to ENSAT stage ($X^2 = 1.795$, $p = 0.408$) and resection status ($X^2 = 1.375$, $p = 0.503$; based on Table 3.6 and 3.8). A distinct tendency of more PD-L1 expression was correlated to tumor size ($X^2 = 70.476$, $p = 0.065$; based on Table 3.6 and 3.8). Additionally, high proliferating ACC with a Ki67 index of > 20 % tended to present a higher expression of PD-L1, while less was found in ACC tumors with lower Ki67 proliferation index (0-19 %; $X^2 = 0.612$, $p = 0.082$). In contrast to PD-1$^+$ TILs, ACC tumors positive for PD-L1 expression were not influenced by glucocorticoid excess ($X^2 = 0.561$, $p = 0.454$).

Table 3.8: PD-L1 immune checkpoint molecule expression (Cohort II)

	% of PD-L1$^+$ ACC	PD-L1 $^+$	PD-L1 $^-$
All ACC samples	24.4	31	96
Primary tumor	25.0	23	69
ENSAT I / II [a]	27.4	17	45
ENSAT III [a]	14.6	6	35
ENSAT IV [a]	29.2	7	17
Local recurrence [b]	18.75	3	13
Distant metastasis [b]	26.3	5	14

Data represent percentage or total numbers.

[a] Tumor stage at the time of diagnosis according to the European Network for the Study of Adrenal Tumors (ENSAT) classification (Fassnacht et al. 2009).

[b] In patients who experienced local recurrence or distant metastases during mitotane treatment, endocrine activity was classified according the information available at primary diagnosis.

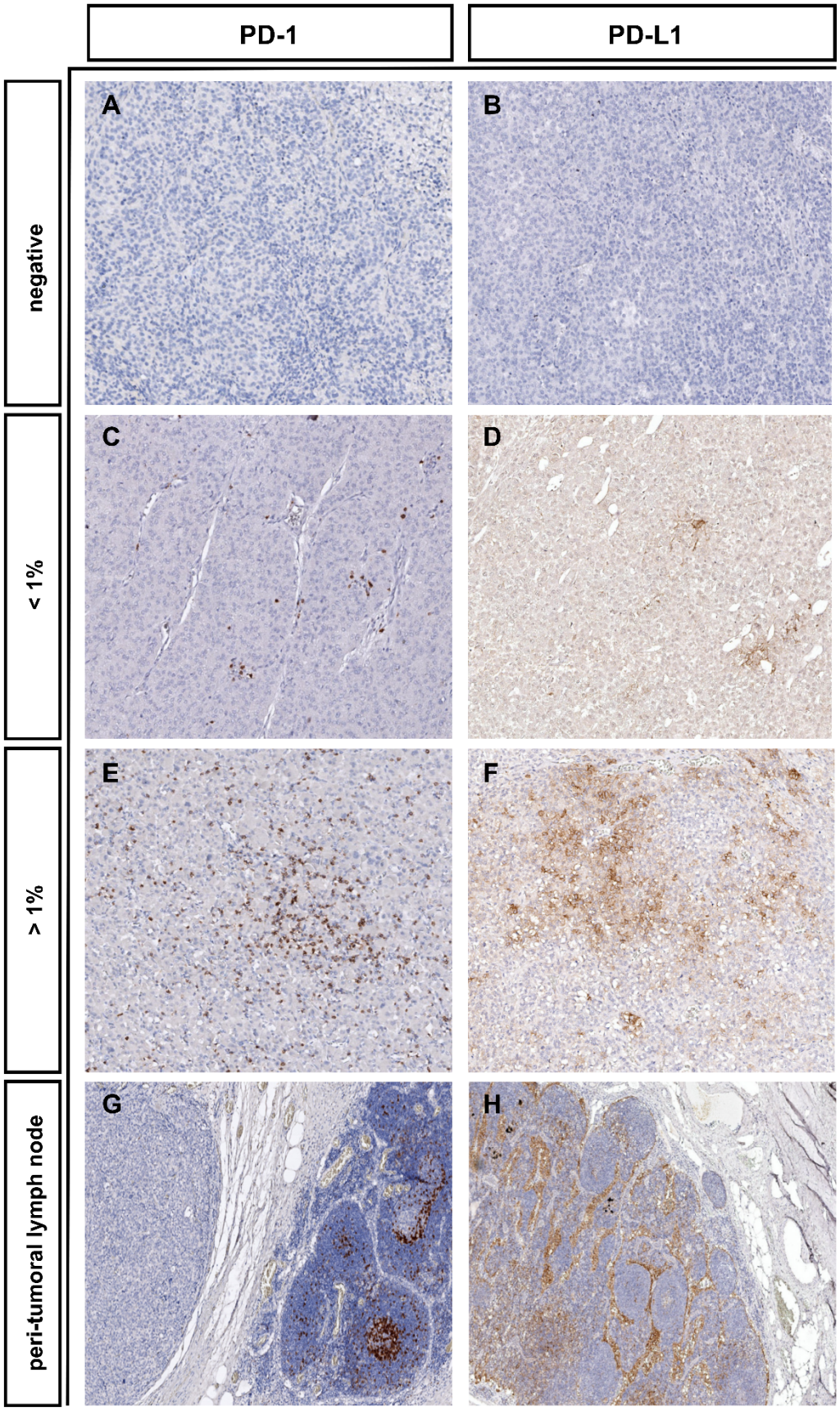


Figure 3.5: Immune checkpoint molecules in adrenocortical carcinoma
Representative chromogenic immunohistochemical staining of immune checkpoint molecules, PD-1 and PD-L1, in adrenocortical carcinoma, n = 129 (A-H).

3.2.2 Association of overall, recurrence and progression-free survival with immune checkpoint molecules

To assess the impact of PD-1 and PD-L1 expression on ACC patients´ outcome, the overall survival in all 94 patients with available primary tumor specimen was studied by using first univariate analyses. The presence of PD-1^{+} ACC-infiltrating lymphocytes was significantly associated with better overall survival compared to PD-1^{-} ACC tumors (HR 0.36, 95 % CI 0.13-1.00, p = 0.050; Figure 3.6, Table 3.9). More in detail, median survival in ACC patients with PD-1^{+} primary tumor was not reached, whereas PD-1^{-} tumors were associated with a median survival of only 50.0 months (Figure 3.6, Table 3.9). With regard to ligand PD-L1, there was not impact on patients´ overall survival (HR 1.00, 95 % CI 0.59-1.69, p = 0.993; Figure 3.6, Table 3.9).

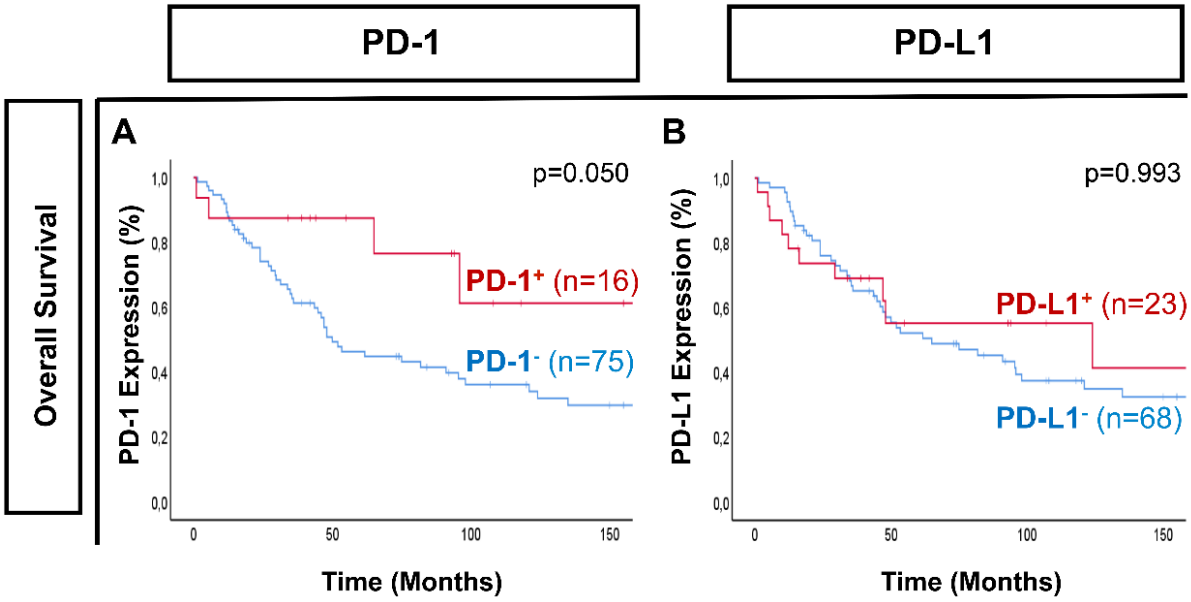


Figure 3.6: Overall survival analyses in patients with adrenocortical carcinoma according to PD-1^{+} and PD-L1^{+} immune checkpoint molecules
Kaplan-Meier overall survival of all ACC patients with primary tumor samples (n = 94) influenced by PD-1^{+}- (A) and PD-L1^{+}- (B) expression (n = 129).

By performing multivariate overall survival analyses that included s prognostic factors, like ENSAT stage, resection status, Ki67 proliferation index and glucocorticoid excess, a significant independent influence of the ENSAT tumor stage (III: HR 2.78, 95 % CI 1.12-6.97 and IV: HR 10.32, 95 % CI 2.55-41.86) and PD-1 expression (HR 0.21, 95 % CI 0.53-0.84) on patients' overall survival was determined (Table 3.9).

Table 3.9: Factors influencing ACC patients' overall survival (Cohort II)

			Univariate analyses (all)			Multivariate analyses (all)		
		n	**Hazard Ratio**	**95 % CI**	**p**	**Hazard Ratio**	**95 % CI**	**p**
Age		-	0.995	0.98-1.01	0.607			
Sex	Female	63						
	Male	30	1.24	0.71-2.16	0.406			
ENSAT stage	I / II	41						
	III	33	2.35	1.23-4.50	0.001	2.78	1.12-6.97	0.030
	IV	19	5.95	2.90-12.19	<0.001	10.32	2.55-41.86	0.001
Resection status	R0 / RX	53						
	RI / II	30	5.23	2.88-9.50	<0.001	1.14	0.41-3.21	0.804
Ki67 index	0-19 %	49						
	> 20 %	33	2.45	1.32-4.54	0.004	2.40	0.99-5.81	0.052
GC excess	-	35						
	+	38	2.04	1.08-3.86	0.029	1.61	0.69-3.75	0.272
PD-1 (> 1 %)	-	75						
	+	16	0.36	0.13-1.00	0.050	0.21	0.53-0.84	0.027
PD-L1 (> 1 %)	-	68						
	+	23	1.00	0.59-1.69	0.993			

For overall survival analysis, only samples from ACC patients with primary tumors and applicable clinical data were included (n = 94). For multivariate analyses, complete available data from 57 ACC tumors were included out of 94 samples. ENSAT stage I / II, resection status R0 / RX, low proliferating Ki67 index (0-19 %), lack of glucocorticoid excess were classified as reference category.

In the following, the influence of PD-1$^+$ expressing TILs on the recurrence-free survival of ACC patients with primary localized, non-metastatic tumors after complete surgical resection was analyzed (n = 62). Thereby, it revealed that PD-1 expression was not significantly associated with longer median recurrence-free survival (HR 0.61, 95 % CI 0.27-1.36, p = 0.222), but rather age and Ki67 proliferation index independently of prognostic markers (Table 3.10).

Considering immune checkpoint molecule PD-L1, there was also no effect of its presence regarding recurrence-free survival of patients with completely resected, non-metastatic primary tumors.

Table 3.10: Factors influencing ACC patients' recurrence-free survival (Cohort II)

			Univariate analyses (all)		
		n	**Hazard Ratio**	**95 % CI**	**p**
Age		-	0.98	0.96-1.00	0.025
Sex	Female	40			
	Male	21	1.71	0.94-3.10	0.081
ENSAT stage	I / II	38			
	III	23	1.53	0.54-2.78	0.158
Ki67 index	0-19 %	38			
	> 20 %	18	3.23	1.67-6.25	0.001
GC excess	-	31			
	+	20	1.16	0.61-2.21	0.654
PD-1 (> 1 %)	-	49			
	+	12	0.61	0.27-1.36	0.22
PD-L1 (> 1 %)	-	44			
	+	16	1.16	0.60-2.25	0.66

For recurrence-free survival analysis, only samples from ACC patients with primary, localized tumors, R0 / RX-state and applicable clinical data were included (n = 62). For multivariate analyses, complete available data from 56 ACC tumors were included. Low proliferating Ki67 (0-19 %) was classified as reference category in multivariate analyses.
In 14 cases, no data regarding resection and in 8 cases, no data about proliferation state available. Clinical data considering glucocorticoid secretion state was not determined in 16 cases.

At last, the impact of PD-1 expression in primary tumors, local recurrences and distant metastases was studied to investigate its impact on the progression-free survival of ACC patients. These patients experienced a significant better progression-free survival of more than two years compared to patients lacking PD-1 expression (34 vs. 9 months, p = 0.020; Figure 3.7, Table 3.11).

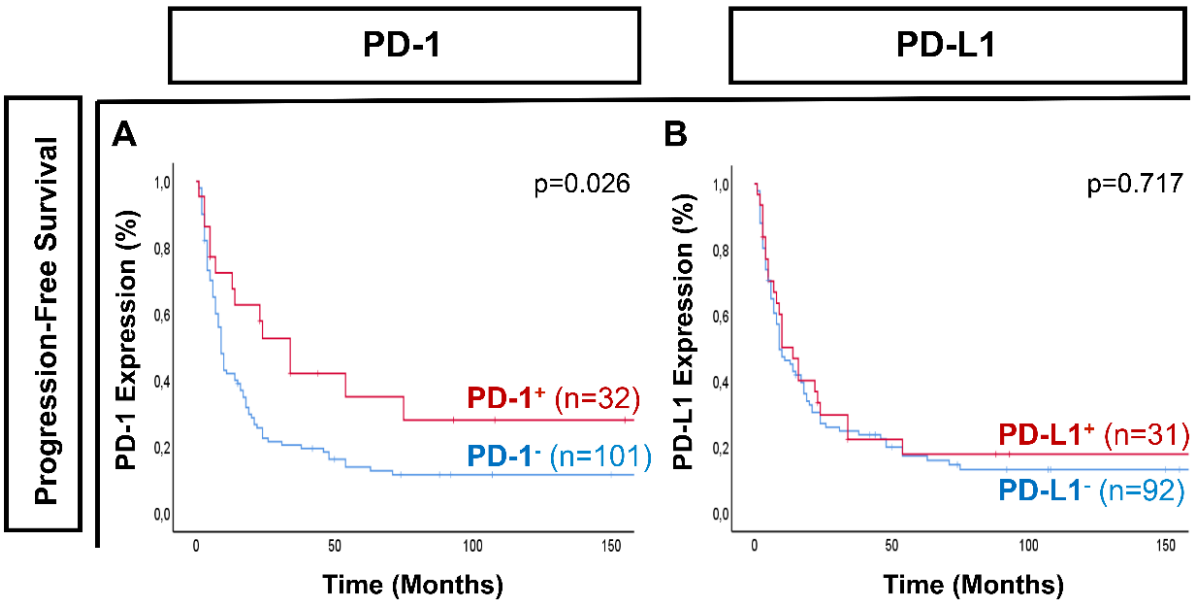


Figure 3.7: Progression-free survival analyses in patients with adrenocortical carcinoma according to PD-1$^+$ and PD-L1$^+$ immune checkpoint molecules

Kaplan-Meier progression-free survival analysis was performed regarding PD-1$^+$- (A) and PD-L1$^+$- (B) expression in primary tumors, local recurrences and metastases (n = 129).

In a multivariate analysis that included additional prognostic factors, such as ENSAT stage, resection status, Ki67 proliferation index and glucocorticoid excess, PD-1 significantly influenced the progression-free survival (Table 3.11).
In contrast, there was no impact of the ligand PD-L1 on patients´ progression-free survival (Table 3.11).

Table 3.11: Factors influencing ACC patients' progression-free survival (Cohort II)

			Univariate analyses (all)			Multivariate analyses (all)		
		n	**Hazard Ratio**	**95 % CI**	**p**	**Hazard Ratio**	**95 % CI**	**p**
Age		-	0.99	0.98-1.00	0.120			
Sex	Female	85						
	Male	40	1.18	0.79-1.77	0.418			
ENSAT stage	I / II	60						
	III	41	1.47	0.95-2.28	0.086	1.82	0.95-3.48	0.069
	IV	24	3.00	1.78-5.03	< 0.001	3.04	1.05-8.81	0.041
Resection status	R0 / RX	75						
	RI / II	34	2.58	1.64-4.04	<0.001	1.19	0.52-2.72	0.673
Ki67 index	0-19 %	56						
	> 20 %	38	2.41	1.49-3.90	> 0.001	2.63	1.41-4.90	0.002
GC excess	-	46						
	+	55	1.63	1.05-2.52	0.029	0.95	0.52-1.72	0.856
PD-1 (> 1 %)	-	101						
	+	22	0.53	0.30-0.93	0.026	0.30	0.13-0.72	0.007
PD-L1 (> 1 %)	-	92						
	+	31	0.92	0.59-1.45	0.717			

For progression-free survival analysis, primary tumors, local recurrences and metastases from ACC patients with applicable clinical data were included (n = 129). For multivariate analyses, complete available data from 67 ACC tumors were included. ENSAT stage I / II, R0 / RX resection status, low proliferating Ki67 (0-19 %) and lack of glucocorticoid excess was classified as reference category in multivariate analyses.
In 20 cases, no data about resection status. Clinical data considering proliferation was not determined in 35 and considering glucocorticoid secretion state in 26 cases.

3.2.3 Association of immune cells and immune checkpoint molecules and the impact on survival

Since the immune checkpoint molecule PD-1 was expressed on tumor-infiltrating lymphocytes as shown in 3.2.1 (Figure 3.5), their mutual correlation was consequently investigated thoroughly. The total cohort of 129 ACC samples, including primary tumors, local recurrences and metastases, demonstrated a significant correlation between the PD-1 expression and $CD3^+$ T cell tumor infiltration ($X^2 = 4.112$, Ø

= 0.180, p = 0.043; Table 3.12, A). These findings were also confirmed in T cell subtypes comprising FoxP3$^+$ T_{reg} - and CD8$^+$ cytotoxic T cells (X^2 = 9.498, Ø = 0.273, p = 0.002 and X^2 = 4.681, Ø = 0.192, p = 0.031, respectively; Table 3.12, C+D), while CD4$^+$ T_H cells only tended to correlate (X^2 = 3.114, Ø = 0.157, p = 0.079; Table 3.12, B).

Table 3.12: Correlation of PD-1$^+$ cells (≥ 1 %) with tumor-infiltrating lymphocytes

A		**CD3$^-$**	**CD3$^+$**	**Total**
	PD-1$^-$	17	88	105
	PD-1$^+$	0	22	22
	Total	17	110	127
B		**CD4$^-$**	**CD4$^+$**	**Total**
	PD-1$^-$	28	77	105
	PD-1$^+$	2	20	22
	Total	30	97	127
C		**FoxP3$^-$**	**FoxP3$^+$**	**Total**
	PD-1$^-$	57	48	105
	PD-1$^+$	4	18	22
	Total	61	66	127
D		**CD8$^-$**	**CD8$^+$**	**Total**
	PD-1$^-$	19	86	105
	PD-1$^+$	0	22	22
	Total	19	108	127

X^2 (Pearson) correlation of PD-1$^{+/-}$ immune checkpoint molecule expression with CD3$^{+/-}$- (A), CD4$^{+/-}$- (B), FoxP3$^{+/-}$- (C) and CD8$^{+/-}$- (D) T cells in primary tumors, local recurrences and metastasis (n = 129).

Additionally, PD-1 expression (+/-) was not only associated with the presence of TILs, but also correlated with the amount of infiltrated CD3$^+$- (15.8 vs. 4.0; p = 0.0004), CD4$^+$ T_H - (7.8 vs. 2.9; p = 0.009), FoxP3$^+$ T_{reg} - (0.3 vs. 0.0; p = 0.009) and CD8$^+$ (17.4 vs. 3.7; p < 0.0001) immune cells.
Next, the percentage portion of PD-1 immune checkpoint molecule (%) was correlated to the number (#) of immune cells infiltrated in ACC (Figure 3.8). While PD-1 and CD3$^+$- (A), CD4$^+$- (B), CD8$^+$- (D) T cells did not interfere (Figure 3.8), FoxP3$^+$ T_{reg} cells significantly correlate with PD-1 (> 1 %) expression (p = 0.020, Figure 3.8).

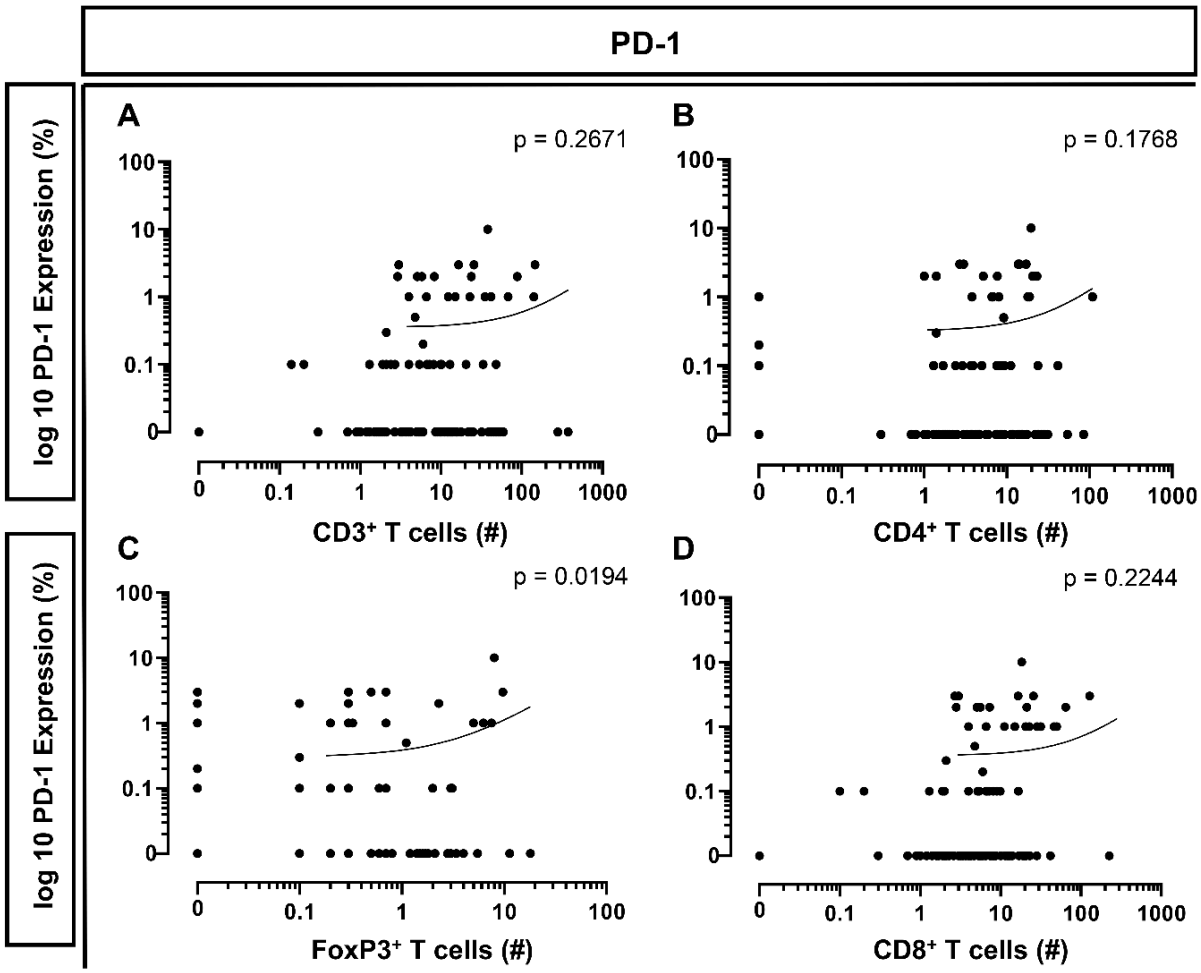


Figure 3.8: Correlation of immune checkpoint molecules PD-1 and number of tumor-infiltrating lymphocytes
Correlation of PD-1 immune checkpoint molecules expression (%) with number of tumor-infiltrating lymphocytes (#), $CD3^+$- (A), $CD4^+$- (B), $FoxP3^+$- (C) and $CD8^+$ T cells (D) per HPF.

In contrast, its ligand PD-L1 demonstrated a superior mutual effect on the number of TILs in ACC specimens. Hence, immune checkpoint molecule PD-L1 expressed in percentage significantly correlated with $CD3^+$- (A), $FoxP3^+$- (C) and $CD8^+$ (D) T cells ($p = 0.0003$, < 0.0001 and < 0.0001, respectively, Figure 3.10), while $CD4^+$ T_H cells were not directly affected (Figure 3.9).

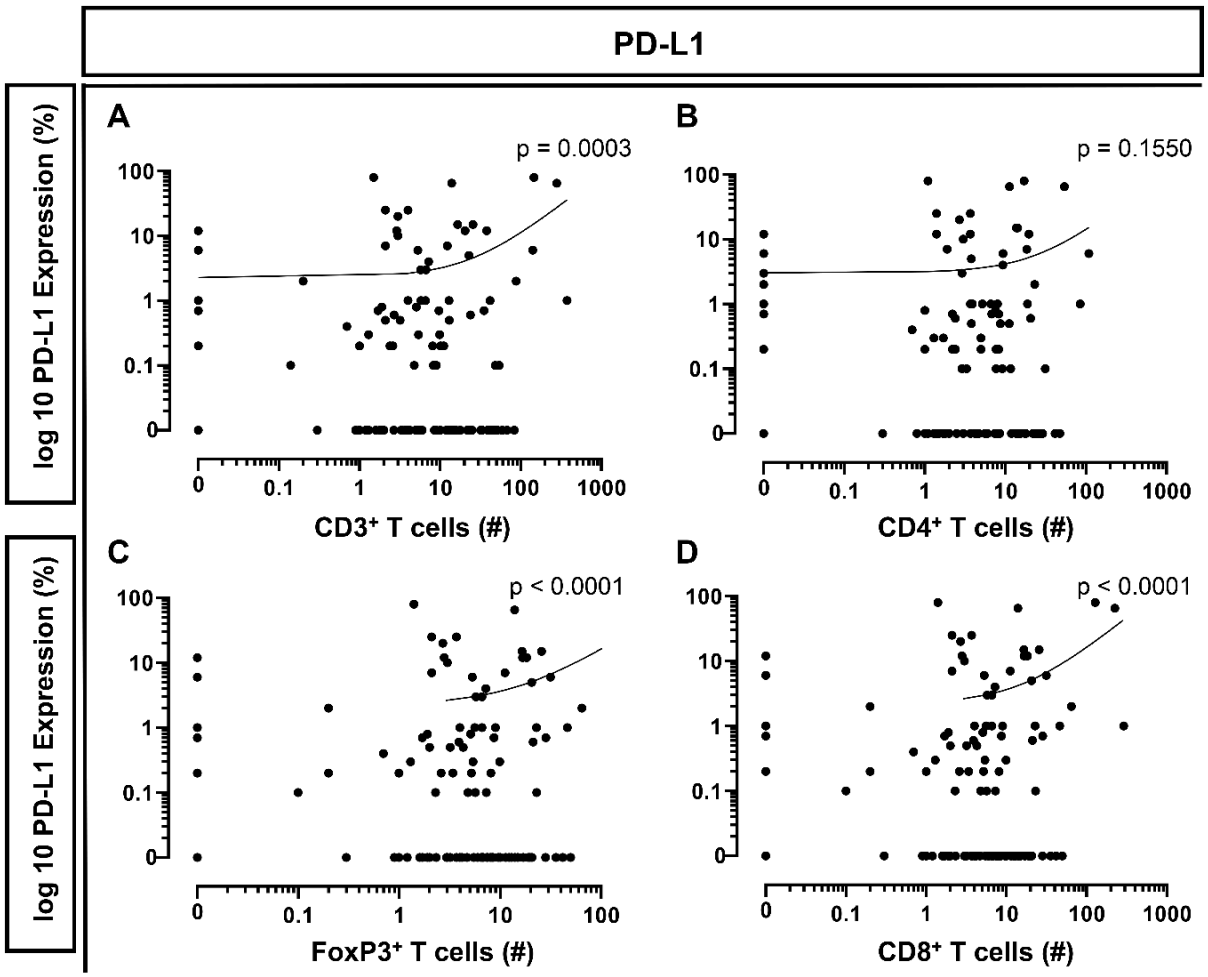


Figure 3.9: Correlation of immune checkpoint molecule PD-L1 and number of tumor-infiltrating lymphocytes
Correlation of PD-L1 immune checkpoint molecules expression (%) with number of tumor-infiltrating lymphocytes (#), $CD3^+$- (A), $CD4^+$- (B), $FoxP3^+$- (C) and $CD8^+$ T cells (D) per HPF.

Next, the association of immune checkpoint PD-1 on patients' overall survival in consideration of $CD3^+$, $CD4^+$-, $FoxP3^+$- and $CD8^+$ T cell infiltration in primary ACC was analyzed (Figure 3.7, Table 3.13). In the univariate analyses, $CD4^+$- and $CD8^+$ T cells and PD-1 immune checkpoint molecule (< 1 %) was significantly associated with overall survival also in cohort II, while $FoxP3^+$ T cells were not (Table 3.13).

In an advanced multivariate analysis, including ENSAT stage, Ki67 index, glucocorticoid excess, PD-1 expression and additionally, individual immune cells, PD-1 (≥ 1 %) expression still positively influenced ACC patients´ overall survival independently of other prognostic factors when adjusted for $CD4^+$- and $FoxP3^+$ T cells (HR 0.20, 95 % CI 0.05-0.81, p = 0.024 and HR 0.18, 95 % CI 0.05-0.71, p = 0.014, respectively, Figure 3.7, Table 3.13).

Table 3.13 Immune cell infiltration and immune checkpoint PD-1 influencing ACC patients' overall survival (Cohort II)

			Univariate analyses (all)			Multivariate analyses[1] (all)		
		n	**Hazard Ratio**	**95 % CI**	**p**	**Hazard Ratio**	**95 % CI**	**p**
CD3	-	13						
	+	80	0.52	0.27-1.01	0.053	0.60	0.15-2.39	0.464
PD-1 (> 1 %)	-	75						
	+	16	0.36	0.13-1.00	0.050	0.26	0.06-1.13	0.073
CD4	-	21						
	+	72	0.47	0.27-0.83	0.009	0.63	0.24-1.66	0.346
PD-1 (> 1 %)	-	75						
	+	16	0.36	0.13-1.00	0.050	0.20	0.05-0.81	0.024
FoxP3	-	41						
	+	52	1.19	0.70-2.05	0.521	1.76	0.80-3.88	0.164
PD-1 (> 1 %)	-	75						
	+	16	0.36	0.13-1.00	0.050	0.18	0.05-0.71	0.014
CD8	-	16						
	+	77	0.53	0.28-0.98	0.044	0.67	0.21-2.16	0.502
PD-1 (> 1 %)	-	75						
	+	16	0.36	0.13-1.00	0.050	0.26	0.06-1.12	0.070

For overall survival analysis, $CD3^+$-, $CD4^+$-, FoxP3-$^+$ and $CD8^+$ T cell infiltrations in primary tumors from ACC patients with applicable clinical data were analyzed (n = 94).

For multivariate analyses, significant prognostic factors from univariate analyses as listened in Table 3.9 were included in addition to PD-1 expression and individual immune cell infiltrates. Complete available data from 57 ACC tumors were included out of 94 samples.

[1] In addition to the described factors, these analyses were adjusted also for ENSAT stage, resection status, Ki67 index and glucocorticoid excess using categorical variables as defined in Table 3.9.

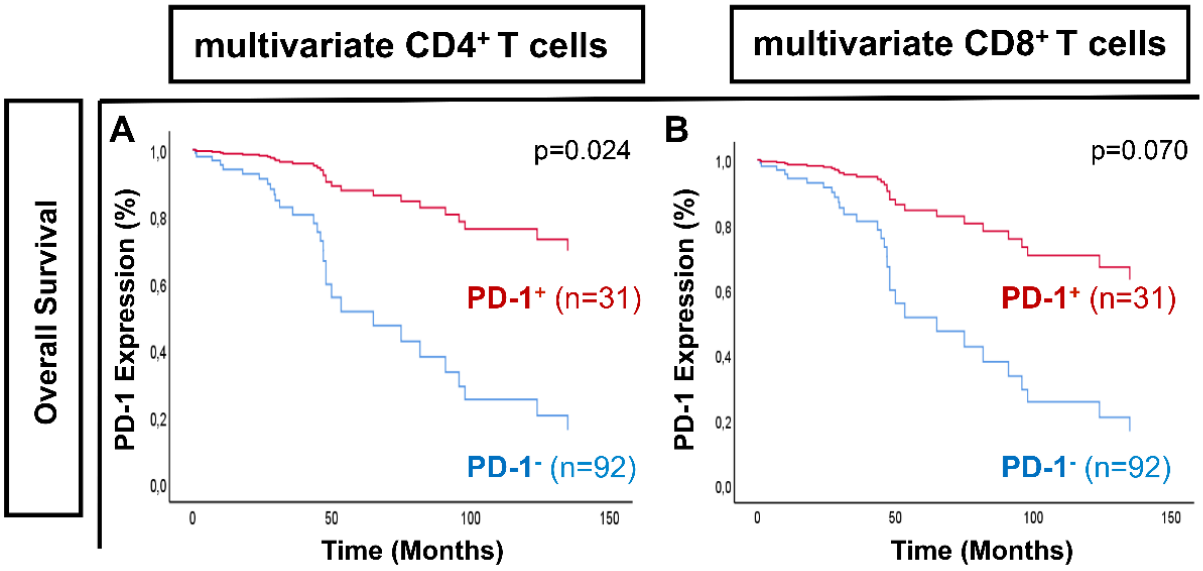


Figure 3.10 Multivariate survival analyses in patients with adrenocortical carcinoma according to PD-1 immune checkpoint molecule

Overall survival in patients with adrenocortical carcinoma according to PD-1^{+} determined by chromogenic immunohistochemistry. Multivariate cox regression for overall survival of ACC patients with primary tumor samples (n = 94) influenced by PD-1^{+} expression including standard prognostic markers, ENSAT stage, resection status, Ki67 index, glucocorticoid excess and individual $CD4^{+}$- (A) and $CD8^{+}$- (B) T cell immune infiltrates.

As previous analyses shown in 3.1.4 demonstrated a significant impact of glucocorticoids on ACC-infiltrating lymphocytes and patients´ survival, its influence on PD-$1^{+/-}$ TILs was examined.

In the immune checkpoint cohort II, glucocorticoids significantly correlate with PD-1 expression in non-metastatic, completely resected primary tumors ($X^2 = 4.454$, ø = -0.296, p = 0.035, based on Table 3.6 and 3.7). Subsequently, the PD-1 expression was analyzed in consideration of the "immune" and "steroid" phenotype as defined in 3.1.4. Thereby, a significant correlation between the PD-1 expression and $CD3^{+/-}$ T cells / $GC^{+/-}$ ($X^2 = 6.568$, Ø = 0.255, p = 0.013), $CD4^{+/-}$ T_H / $GC^{+/-}$ ($X^2 = 5.655$, Ø = 0.247, p = 0.013), $FoxP3^{+/-}$ T_{reg} / $GC^{+/-}$ ($X^2 = 12.050$, Ø = 0.345, p = 0.002) and $CD8^{+/-}$ cytotoxic T cells / $GC^{+/-}$ ($X^2 = 6.885$, Ø = 0.261, p = 0.010) was determined in the entire cohort II (Table 3.14).

In consideration of the ligand PD-L1, its expression is solely significantly correlated with $FoxP3^{+/-}$ T_{reg} / $GC^{+/-}$ ($X^2 = 7.175$, Ø = 0.267, p = 0.048).

Table 3.14: Correlation of PD-1$^+$ cells (≥ 1 %) with "immune"- and "steroid" phenotype

A		**CD3$^+$ GC$^-$**	**CD3$^+$ GC$^+$**	**CD3$^-$ GC$^-$**	**CD3$^-$ GC$^+$**	**Total**
	PD-1$^-$	29	39	7	7	82
	PD-1$^+$	12	7	0	0	19
	Total	41	48	7	7	101
B		**CD4$^+$ GC$^-$**	**CD4$^+$ GC$^+$**	**CD4$^-$ GC$^-$**	**CD4$^-$ GC$^+$**	**Total**
	PD-1$^-$	29	32	7	14	82
	PD-1$^+$	12	5	0	2	19
	Total	41	37	7	16	101
C		**FoxP3$^+$ GC$^-$**	**FoxP3$^+$ GC$^+$**	**FoxP3$^-$ GC$^-$**	**FoxP3$^-$ GC$^+$**	**Total**
	PD-1$^-$	17	17	19	29	82
	PD-1$^+$	11	4	1	3	19
	Total	28	21	20	32	101
D		**CD8$^+$ GC$^-$**	**CD8$^+$ GC$^+$**	**CD8$^-$ GC$^-$**	**CD8$^-$ GC$^+$**	**Total**
	PD-1$^-$	29	37	7	9	82
	PD-1$^+$	12	7	0	0	19
	Total	41	44	7	9	101

X^2 (Pearson) correlation of PD-1$^{+/-}$ immune checkpoint molecule expression with CD3$^{+/-}$ T cells / GC$^{+/-}$ glucocorticoid excess (A), CD4$^{+/-}$ / GC$^{+/-}$ (B), FoxP3$^{+/-}$ / GC$^{+/-}$ (C) and CD8$^{+/-}$ / GC$^{+/-}$ (D) in primary tumors, local recurrences and metastasis (n = 129).
Clinical data considering glucocorticoids secretion was not applicable in 26 cases.

3.3 Impact of steroid hormones on T lymphocyte cytotoxicity in adrenocortical carcinoma *in vitro*

To evaluate the cytotoxic ability of T lymphocytes towards steroidogenic adrenocortical tumor cells, *in vitro* co-culture experiments of $CD56^{-}CD3^{+}$ T cells and NCI-H295R with and without steroidogenesis inhibitor, metyrapone, were performed. Subsequent quantitative measurement of IFN-γ releases mirrored the cytotoxic capacity of T cells with regard to ACC cells in presence or absence of steroid hormones.

Treatment of biotinylated antibodies against human CD2, CD3, and CD28 that mimic antigen-presenting cells and stimulate latent T lymphocytes (described in 2.2.8.2) resulted in activation of human $CD56^{-}CD3^{+}$ T cells visualized by forming immune cell clusters (Figure 3.12, A-C).

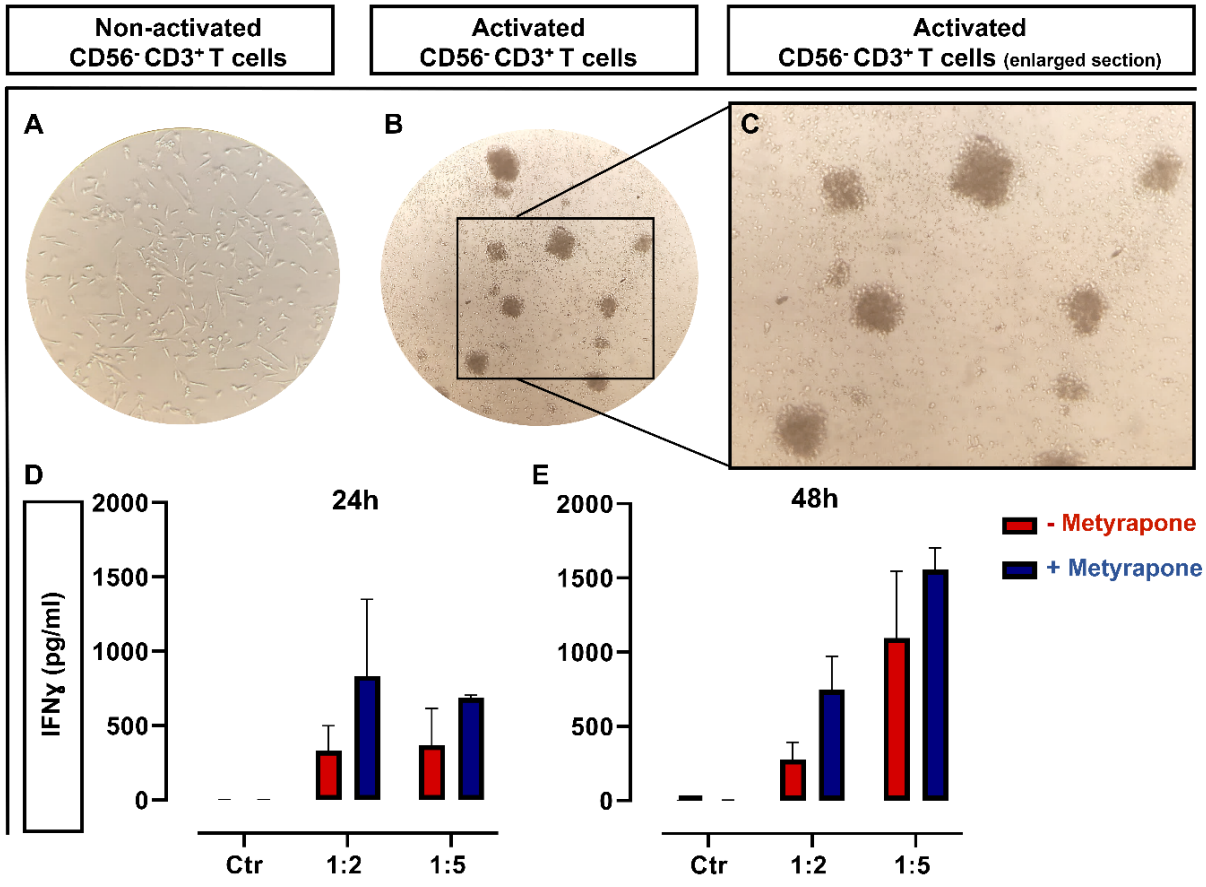


Figure 3.11: Co-culture of NCI-H295R cells with activated $CD56^{-}CD3^{+}$ T lymphocytes
In vitro co-culture of NCI-H295R cells with non-activated (A) and activated (B+C) $CD56^{-}CD3^{+}$ T lymphocytes in a 96-well plate. IFN-γ release of activated $CD56^{-}CD3^{+}$ T lymphocytes co-cultured in an 1:2 and 1:5 ratio with NCI-H29R cells in presence (red) or absence (blue) of steroid hormones/glucocorticoids after 24 h (D) and 48 h (E).

In general, $CD56^-CD3^+$ T cells that were co-cultured with NCI-H295R cells showed a distinct IFN-γ secretion, while its concentration was virtually absent in mono-cultures of NCI-H295R tumor cells that served as negative control ($p > 0.999$; Figure 3.11, D and E).

However, 1 x 10^4 NCI-H295R cells that were co-cultured with 2 x 10^4 or 5 x 10^4 $CD56^-CD3^+$ T cells (1:2 and 1:5 ratio) showed differences in IFN-γ secretion depending on interference of glucocorticoids and period of exposure (24 h or 48 h; Figure 3.11, D and E).

After 24 h, 2 x 10^4 $CD56^-CD3^+$ T cells co-cultured with 1 x 10^4 NCI-H295R cells (2:1 ratio) secreted 834.28 ± 515.05 pg/ml IFN-γ in a hormone inactive microenvironment, while their anti-tumoral efficiency was diminished to 332.37 ± 167.47 pg/ml in presence of glucocorticoids (Figure 3.11, D). The cytotoxic potential was even increased when 1 x 10^4 NCI-H295R cells were co-cultured with 5 x 10^4 $CD56^-CD3^+$ T cells in a 1:5 ratio. Hence, $CD56^-CD3^+$ T cells secreted 687.72 ± 17.70 pg/ml IFN-γ when lacking glucocorticoids, whereas 369.65 ± 246.69 pg/ml IFN-γ was secreted without metyrapone inhibiting endogenous glucocorticoid secretion (Figure 3.11, D).

After 48 h, 2 x 10^4 $CD56^-CD3^+$ T cells exhibited a high IFN-γ concentration of 747.96 ± 225.53 pg/ml in a co-culture system with 1 x 10^4 NCI-H295R (1:2 ratio) with absent glucocorticoids. The IFN-γ release of $CD56^-CD3^+$ T cells was significantly decreased to 276.02 ± 117.46 pg/ml when glucocorticoids were abundant (Figure 3.12, E). When 5 x 10^4 $CD56^-CD3^+$ T cells were co-cultured with NCI-H295R cells treated with metyrapone (1:10 ratio), the IFN-γ concentration was the highest with 1557.13 ± 145.62 pg/ml, while its effect was significantly minor with 1094.52 ± 453.18 pg/ml with glucocorticoid excessive microenvironment (Figure 3.11, E). Overall, there was a significant higher IFN-γ concentration in metyrapone-mediated condition compared to non-treated co-cultured cells elucidating higher anti-tumoral effects of $CD56^-CD3^+$ T cells after 48 h (ANOVA, $p = 0.005$). Additionally, the increasing proportion of $CD56^-CD3^+$ immune cells to NCI-H295R tumor cells, demonstrated a significant stronger IFN-γ release after 24 h and 48 h ($p = 0.006$ and $p < 0.0001$, respectively).

4 Discussion

Profound advances in studying cancer immunity have revolutionized multiple anti-tumor approaches. Both subtypes, $CD4^+$ T_H and $CD8^+$ cytotoxic T cells, are associate partners in preventing and eliminating cancer. Therefore, their anti-tumoral assistance in the tumor microenvironment and within the tumor mass is fundamental (Hanahan and Weinberg 2011, Chen and Mellman 2013). However, the therapeutic efficacy is different among the various tumor entities. The present study provides several potential explanations for the heterogeneous results of the first clinical trials with immune checkpoint inhibitors in advanced ACC.

First, many ACC were characterized by low or absent $CD4^+$ T_H and $CD8^+$ cytotoxic T cell infiltration that could flare up a sufficient anti-tumor response. In addition, the fact that metastases were even less immunogenic than patient-matched primary tumors implied that immune escape mechanisms are even more pronounced in advanced ACC and might increase over time; this in turn means increasing challenges for immunotherapies.

Most of these cancer immunotherapies are targeting immune checkpoint molecules PD-1 and its ligand PD-L1 that are applied as prognostic and/or predictive biomarkers in different cancers. Here, PD-1 expressing TILs were correlated to ACC patients´ clinical outcome, while PD-L1 did not. Therefore, the presence of tumor-reactive $PD\text{-}1^+$ T cells that were able to get reinvigorate by anti-PD-1 therapy might be a therapeutic attempt. Additionally, the intratumoral glucocorticoids might be another key factor for the modest immunogenicity as hypercortisolism was reversely correlated to $CD4^+$ T_H cells, but also $CD8^+$ cytotoxic T cells.

Therefore, a combination therapy of this immune checkpoint blockade with an inhibition of steroidogenesis might efficiently reactivate the immunological anti-tumor potential.

In the following sections, the results of this thesis and the potential consequences for immunotherapeutical concepts will be discussed - also in consideration of immunoregulatory glucocorticoid hormones.

4.1 Tumor-infiltrating lymphocytes in adrenocortical carcinoma

The fraction of tumor-infiltrating lymphocytes varied distinctively among different tumor entities. Tumors, such as cutaneous melanoma (Lee et al. 2016, Leonardi et al. 2020) or non-small-cell lung cancer (Garon et al. 2015, Hellmann et al. 2017, Gataa et al. 2021), that were highly infiltrated by T lymphocytes showed a particularly higher response to immunotherapies (Thorsson et al. 2018, Lee et al. 2016, Leonardi et al. 2020). In contrast, after uveal melanoma, ACCs were characterized by the lowest fraction of leukocytes according to Thorsson et al., who applied a cell type identification by estimating relative subsets to RNA transcript and analyzed the composition of tumor-infiltrating immune cells across different TCGA tumor types (Thorsson et al. 2018).

Consequently, after these *in silico* analyses of the TCGA cohort, this was the first study on immune cell infiltration in a large cohort of ACC, including primary tumors, local recurrences and metastases. $CD3^+$-, $CD4^+$-, $FoxP3^+$- and $CD8^+$ tumor-infiltrating lymphocytes were quantified in a cohort of 146 ACC patients. In contrast to a prevailing view, the majority of different ACC specimens (86.3 %) were infiltrated by $CD3^+$ T lymphocytes (Landwehr et al. 2020). However, the number of infiltrated $CD3^+$ T cells was lower in comparison to many other tumor entities (Eerola, Soini and Pääkkö 2000). This observation was also confirmed by lower quantitative mRNA expression levels of immune-related genes derived from the TCGA dataset (Paré et al. 2018).

However, the presence of TILs was significantly correlated to ACC patients´ overall and recurrence-free survival (Landwehr et al. 2020). Many studies considering other tumor types have demonstrated the potent effects of TILs on patients´ prognosis and survival. This was observed even independently of the treatment of patients. For instance, Balermpas et al. demonstrated an improved clinical outcome in head and neck squamous cell carcinoma depending on the presence of T lymphocytes. Highly infiltrated tumors had a significant better overall survival of 42.7 months, whereas low $CD3^+$ T cell infiltration were related to worse outcome with only 30.3 months (Balermpas et al. 2014). This favorable effect of the presence of TILs was even confirmed in less immunogenic tumor entities. In advanced ovarian cancer, Zhang et al. described a significant association of intratumoral $CD3^+$ T cell and patients´ clinical outcome (Zhang et al. 2003). While immune infiltration was associated with a median duration of 74.5 months until progression, immune-depleted ovarian tumors showed a progression-free survival of only 7.6 months (Zhang et al. 2003).

Similarly, this beneficial impact of tumor immune infiltration on overall and recurrence-free survival was determined in ACC as shown in this present study. Accordingly, ACCs infiltrated by $CD3^+$ TILs showed a median overall survival of 81.8 months, while immune-depleted ACCs were associated with only 29.7 months until death. More in detail, $CD4^+$ T_H and $CD8^+$ T cells presented with comparable median overall survival periods of 91.4 and 81.8 months for immune-infiltrated tumors and only 27.0 and 21.0 months for immune-depleted ACC, respectively. Accordingly, $CD3^+$- and both subtypes $CD4^+$- and $CD8^+$ - TILs were associated with a risk reduction of 53-61 % for death and 57-68 % for recurrence (Landwehr et al. 2020). Consistently, the association of tumor-infiltrating lymphocytes, especially $CD8^+$ T cells, with clinical outcome was described in smaller cohort of 48 pediatric patients with ACC, as well (Parise et al. 2019).

The present study demonstrated the important aspect that was not analyzed by all similar studies, namely the fact that the infiltration of T cells was sincerely an independent prognostic marker and not just a surrogate for staging or histological features like high proliferation. We could clearly show that the effect of TILs on patients´ overall survival determined in localized, non-metastatic ACC was independently of prognostic factors, like ENSAT stage, resection status and Ki67 proliferation index, and resulted in a relative risk reduction for death of 70-81% (Landwehr et al. 2020).

Another interesting observation was the variability of T lymphocytes infiltrating ACC tumors depending on tumor localization. In metastatic lesions, less than half of TILs were quantified in comparison to primary tumors (Landwehr et al. 2020). Similar phenomena were observed in other tumor entities at secondary sides. For instance, a study on breast cancer identified that metastases were overall less infiltrated by T lymphocytes relative to their patient-matched primary tumors (Zhu et al. 2019). These observations might be due to advanced immune escape mechanisms (Beatty and Gladney 2015).

4.2 Immune checkpoint molecule expression – PD-1 and PD-L1 – in adrenocortical carcinoma

Since most of the currently applied cancer immunotherapies are targeting immune checkpoint molecules by neutralizing PD-1 and/or its ligand PD-L1, while reversely enhancing T cell cytotoxicity towards tumor cells, the interest on their expression in many tumor entities aroused. Both PD-1 and PD-L1 play a crucial role in self-tolerance, albeit several tumor types overexpress these immune checkpoint markers in order to evade immune surveillance by T lymphocytes (Han et al. 2020).

It has been shown in many studies, especially in so called immunogenic tumors, such as malignant melanoma (Obeid et al. 2016) or non-small-cell lung cancer (Konishi et al. 2004), that PD-1 was expressed on tumor-infiltrating T lymphocytes and PD-L1 on tumor and mononuclear immune cells. However, PD-1 and PD-L1 expression was also described in less immunogenic tumors, like breast cancer (Buisseret et al. 2017).

In ACC, the expression of both immune checkpoint molecules was heterogeneous and rather low as observed in this study.

Whereas studies on PD-1 expression in ACC were scarcely performed, its ligand PD-L1 came continually more to the fore. For instance, Fay et al. demonstrated in an exploratory biomarker study on 28 ACC patients that 10.7 % of the tumor cells stained positive for PD-L1 when applying a threshold of ≥ 5 % (Fay et al. 2015). Additionally, tumors from ACC patients participating in recent clinical trials on immune checkpoint inhibitor monotherapy in ACC varied in PD-L1 (≥ 1 %, ≥ 5 %) expression from 0 to 75 % (Le Tourneau et al. 2018, Carneiro et al. 2019, Habra et al. 2019, Raj et al. 2019). In addition, based on mRNA expression analyses, the heterogeneous expression of PD-L1 was confirmed in another retrospective study (Billon et al. 2019). However, the largest of these studies included 42 patients with protein expression (Le Tourneau et al. 2018) and 79 with mRNA data (Billon et al. 2019). Nevertheless, these data were consistent to findings from cohort II expressing PD-L1 in 24.0 % of tumors considering a threshold of $\geq$1 %.

Both targets, PD-1 and PD-L1, are applied as prognostic and/or predictive biomarkers in different cancers due to their immunomodulatory character.

Initially, a few studies focused on the immunosuppressive properties of PD-1. Hence, upon ligation of PD-1 to PD-L1, TCR signaling and activation is impaired, while constitutive expression of PD-1 by tumor-infiltrating T cells primarily describes T cell exhaustion and dysfunctional T cell function

resulting in tumor escape mechanism (Simon and Labarriere 2017). A negative correlation of PD-1 and TILs was emphasized by some studies. For instance, in breast cancer, PD-1^+ TILs were associated with a worse overall survival and a 2.7 x higher risk for death (Muenst et al. 2013).

However, different studies highlighted the ambiguous role of PD-1 expression in T cell response. Although its immunoregulatory character was commonly established, PD-1 is first a biomarker for TCR strength and T cell activation (Simon and Labarriere 2017). In this study on ACC, PD-1 expressing TILs were associated with a significant better overall and progression-free survival independently of established prognostic markers compared to PD-1^- ACC. Hence, ACC patients with primary tumor experienced a mortality risk reduction by 79 %, while PD-1 expressing tumors, including primary tumors, local recurrences and metastases, were characterized by a risk reduction for progression by 70 %.

These observations were confirmed in other tumor entities, as well. For instance, in colorectal carcinoma, higher expression of PD-1 significantly correlated with an improved clinical outcome and serves as independent prognostic factor (Li et al. 2016).

In contrast, its ligand PD-L1 was not associated with clinical outcome in this ACC cohort II and was not suitable as prognostic biomarker. These observations were also found by Fay et al. who demonstrated that PD-L1 expressing ACC were not related to 5-year survival by Kaplan-Meier analysis (Fay et al. 2015). However, this study included only 28 patients and, therefore, these data were judged as less reliable.

Several studies considering the prognostic implication of PD-L1 in different tumor types were performed with heterogeneous correlation of PD-L1 expression and patients´ prognosis. In some tumor entities, such as cervical carcinoma (Karim et al. 2009), PD-L1 expression did also not predict patients´ outcome. However, in pancreatic cancer, PD-L1 overexpression was inversely correlated with the number of $CD4^+$ T_H and $CD8^+$ cytotoxic TILs and patients´ impaired outcome, while 1-year postoperative survival in PD-$L1^+$ patients was only 33.5 % (Nomi et al. 2007). Similarly, patients with PD-$L1^+$ hepatocellular carcinoma had a significantly worse overall survival of only 29.6 months compared to 59.4 months for absent or low expressing PD-L1 tumors (Gao et al. 2009). In both entities, PD-L1 expression was established as independent prognostic marker in both tumor entities (Nomi et al. 2007, Gao et al. 2009).

In contrast, PD-L1 expression has both a positive and negative prognostic character as observed in different studies of lung cancer (Azuma et al. 2014, Velcheti et al. 2014) and colorectal cancer (Shi et al. 2013, Droeser et al. 2013).

In non-small-cell lung cancer, Azuma et al. detected a significant shorter overall survival of only 55.9 months in patients with PD-$L1^+$ tumors compared to 72.6 months in PD-$L1^-$ tumors (Azuma et al. 2014), whereas Velcheti et al. observed a significant better patients´ outcome in PD-L1 expressing non-small-cell lung cancer with a mortality risk reduction by 39 % (Velcheti et al. 2014). Similarly, in colorectal

cancer, positive PD-L1 expression resulted in an increased risk for death by 2.8 times independently of other prognostic markers (Shi et al. 2013). On the other hand, Droeser et al. revealed that PD-L1 expression was associated with an improved survival of 32 months compared to only 23 months in patients with mismatch repair-proficient colorectal carcinoma (Droeser et al. 2013).
Therefore, the presented results in ACC might not be the final prove of this association. However, the fact that the smaller series of Fay et al. and the larger cohort described here came to the same conclusion suggest some reliability.

4.3 Influence of glucocorticoids on cancer immunity in adrenocortical carcinoma

Characteristically, ACC patients present with endogenous hypercortisolism in approximately 60 % (Else et al. 2014, Fassnacht et al. 2018). Even in ACC that were diagnosed as hormone inactive tumors according to low serum glucocorticoid levels, intratumoral concentrations due to upregulated steroid synthesis pathways should also be considered (Fiorentini et al. 2019).
Since hypersecretion of ACC-associated glucocorticoids negatively correlated with $CD4^+$ T_H cells in this series, it clearly suggest an immunosuppressive effect of glucocorticoids (Landwehr et al. 2020). This immunoregulating impact of glucocorticoids referred especially to $CD4^+$ TILs that were indispensable for the activation of immunological processes and the regulation of immune responses. The distinctive status of $CD4^+$ T_H cells in the context of endogenous glucocorticoids was also observed in an *in vivo* study. Mice selectively lacking glucocorticoid receptor expression in T lymphocytes rapidly lead to death due to hyperactive T_H1 cells resulting in lethal immunopathologies (Kugler et al. 2013).

As indicated in primary tumors of this study, patients with $CD4^+$ T_H cell-infiltrated tumors without hypercortisolism appealed with a favorable overall survival of 121 months, while the presence of glucocorticoids diminished the median survival to 75 months. Even worse, the outcome of patients with $CD4^+$ T_H cell-depleted ACC with autonomous glucocorticoid excess averages only 27 months (Landwehr et al. 2020). These observations might at least partly explain the considerable effects of glucocorticoids regarding patient's survival indicated in several independent cohorts (Berruti et al. 2014, Puglisi et al. 2018, Vanbrabant et al. 2018). As shown by Berruti et al., overt hypercortisolism is a major prognostic factor as cortisol excess significantly influenced overall (HR: 1.3, 95 % CI 1.04-2.62) and recurrence-free survival (HR: 1.55, 95 % CI 1.15-2.09) (Berruti et al. 2014). In patients with autonomous glucocorticoid, the anti-tumoral immune response may be interfered. This may subsequently result in stronger propensity to acquire recurrences and ACC-related death.
In general, the strong impact of glucocorticoids on several physiological processes including cell differentiation, proliferation, migration and apoptosis is well acquainted and characterized by anti-inflammatory and pro-apoptotic effects (Cain and Cidlowski 2017).
Hence, several studies on glucocorticoids in the context of cancer were performed both *in vitro* and *in vivo*. It has been shown that glucocorticoids enhance tumor cell proliferation in diverse cell lines derived

from different tumor types and germline origin (e.g. mammary- and renal carcinoma, rhabdomyosarcoma and glioblastoma) and in a pre-clinical mouse model of lung carcinoma. Precisely, dexamethasone-induced tumor cell proliferation increased by 40 % compared to naïve tumor cells. In accordance, patient-derived primary leukemia cells were resistant towards apoptotic cell death in presence of dexamethasone or prednisolone (Gündisch et al. 2012). Moreover, glucocorticoids affected peripheral T lymphocyte function as they reduced their cytotoxic potential to eliminate tumor cells. The tumor-associated augmentation of glucocorticoids was recognized in several cancer entities and correlated with malign prognosis. For instance, increased serum cortisol levels were associated with high tumor diameter and grade in renal cell carcinoma. Additionally, there was a tendency towards an impaired prognosis in patients with higher serum cortisol than for those with lower levels (Rasmuson et al. 2001).

In consideration of the therapeutic application of dexamethasone in anti-cancer treatment, a study demonstrated a glucocorticoid-induced enhancement of PD-1 expression in mouse and human. The dexamethasone-induced upregulation of immune checkpoint PD-1 was mediated through the glucocorticoid receptor as its effect could be reversed by GR antagonist mifepristone. Furthermore, they demonstrated an impaired T cell function through inhibition of anti-inflammatory cytokines, IL-2, IFN-γ and TNF-α, by glucocorticoids. This in turn, hampered T cell activation and induced the apoptotic pathways (Xing et al. 2015a).

Thus, autonomous glucocorticoid hypersecretion might be a major contributor to the immunological coldness as in ACC (Bonaventura et al. 2019, Galon and Bruni 2019).

Therefore, this study illustrated the impact of glucocorticoids on the anti-tumor T cell cytotoxicity and, additionally, proposed a potential concept to force immunological “cold” ACC into a “hot” tumor susceptible for immunotherapies. Hence, the cytotoxic effects of T lymphocytes towards NCI-H295R cells was investigated by an *in vitro* co-culture system that mimicked an adrenocortical tumor microenvironment under the influence of immunosuppressive glucocorticoids. Here, $CD3^+$ T cells exposed to high concentration of glucocorticoids showed less anti-tumoral efficiency since its IFN-γ release was only modest. In contrast, in hormone inactive conditions caused by steroid biosynthesis inhibitor metyrapone, the cytotoxicity of T lymphocytes targeting non-steroidogenic ACC cells was almost double. This therapeutic approach is promising to enhance the intratumoral immunity by enabling immune cell infiltration and pave the way for reactivation of these tumor-infiltrated T cells.

Since the activation of glucocorticoid receptor pathway was associated to chemotherapy resistance, a study performed in pancreatic ductal adenocarcinoma and ovarian cancer investigated the effect of a potent glucocorticoid receptor antagonist in combination with paclitaxel chemotherapy. Thereby, in 19 % of patients with pancreatic ductal adenocarcinoma a disease control at 24 weeks was observed. In patients with ovarian cancer, a disease control rate of even 31 % was reached (Munster et al. 2019). Another study focusing on glucocorticoid receptor antagonist, relacorilant, on immune activation demonstrated that immunosuppressive effects of endogenous glucocorticoids can be reverted. In

presence of relacorilant, $CD8^+$ T cells activation and pro-inflammatory cytokine, like IFN-γ and TNF-α, release was observed in PBMCs from patients with solid tumors (Greenstein et al. 2020).
Interestingly, a recently published *in silico* study by Muzzi et al. focused on the understanding of immune pathways by gene expression considering steroid interferences. Therefore, they performed a pan-cancer analysis of the TCGA RNAseq data. Similarly, this study identified a high expression of immune-related mediators on patients with low steroid phenotype. Additionally, these patients were characterized by an increased immune cell infiltration compared to those patients with high steroid phenotype (Muzzi et al. 2021). These findings suggested that steroid profile and activation of these immunological biomarkers is worth considering for immunotherapeutic targeting.

4.4 Clinical implications of present research results

4.4.1 Immune checkpoint therapy in adrenocortical carcinoma

Immune checkpoint inhibitors that stimulate a relevant anti-tumor immune response broaden the therapeutic options in many tumor entities and in some tumors their efficacy even revolutionized the entire treatment concept (Waldman et al. 2020).
They obtained FDA approval for their efficacy against an extensive spectrum of cancers and even ascended to first-line therapy in localized and metastatic melanoma and locally advanced and metastatic non-small-cell lung cancer (Weber et al. 2015, Weber et al. 2017, Hellmann et al. 2017). Immune checkpoint inhibitors targeting PD-1, nivolumab and/or pembrolizumab, encourage with beneficial clinical outcome. For instance, a phase II study on PD-1 inhibitor, pembrolizumab, for relapsed/refractory classic Hodgkin lymphoma demonstrated an overall response rate of 69.0 % and a decline in tumor burden in more than 90% of patients (Chen et al. 2017).
A combination of CTLA-4 and PD-1 targeting inhibition was even more effective as shown in a phase III study on advanced melanoma. Accordingly, the median progression-free survival was 11.5 months in patients with combined treatment of nivolumab and ipilimumab, while monotherapy with nivolumab or ipilimumab resulted in 6.9 or 2.9 months, respectively (Larkin, Hodi and Wolchok 2015).

Consequently, the intense consideration of cancer immunity likewise increased in ACC resulted in fife clinical trials investigating immune checkpoint therapy in advanced disease with overall heterogeneous results (Table 4.1).
Le Tourneau et al. published in October 2018 the first multicenter phase Ib study investigating avelumab, PD-L1 antibody, in 50 patients with metastatic ACC, previously treated with ≥ 1 platinum-based chemotherapy. 50 % of patients continued with concomitant mitotane use. The primary endpoint was defined as overall response rate (ORR) according to Response Evaluation Criteria In Solid Tumors (RECIST; version 1.1) and immune-related response criteria (irRECIST; (Eisenhauer et al. 2009, Wolchok et al. 2009). 74% of patients have been treated with at least two prior lines of systemic therapy.

While patients previously treated with one (13 %) systemic therapy showed an ORR of 15.4 %, patients with three (19 %) prior lines had no response to treatment. Overall, partial response (PR) was assessed in three patients (6 %), while 21 patients (42 %) had best response achieving stable disease (SD) with a disease control rate (DCR) of 48 %. Median progression-free survival (PFS) was 2.6 months and 10.6 months overall survival (OS) with one-year OS rate of 43 % (Le Tourneau et al. 2018).

Carneiro et al. performed a phase II multicenter study of nivolumab, PD-1 antibody, in 10 patients with locally advanced or metastatic ACC that were previously first line treated (n = 8) or treatment naïve (n = 2). Primary endpoint was defined as ORR using RECIST criteria (version 1.1). Four patients showed hormone producing tumors (two cortisol, one aldosterone, one testosterone and androstenedione), while six were nonfunctional. In total, one patient had an unconfirmed PR (10 %) and two were stable (20 %) with a DCR of 30 %. While the median PFS was 1.8 months with 20 % six-month PFS, the OS was 21.2 months with 56 % six-month OS (Carneiro et al. 2019).

The third trial was a single center phase II study of pembrolizumab, PD-L1 antibody, in 16 patients with advanced ACC and at least one prior line therapy. Ten patients (63 %) had hormonally active tumors (one cortisol, one androgen and six cortisol and androgens). The primary endpoint was defined as non-progression rate (NPR) at 27 weeks assessed by irRECIST. Therefore, 36 % of patients were progression-free at six-month evaluation. Considering 14 patients (two patients excluded due to lost to follow up and toxicity), two had PR and seven SD with an ORR of 14 % (Habra et al. 2019).

The most recent phase II single center study was performed by Raj et al. considering pembrolizumab in 39 patients with unresectable or metastatic ACC. In 31 % of patients, previous treatment with ≥1 prior therapy was observed, but throughout trial no concomitant mitotane was accepted. ORR was defined as primary endpoint using RECIST (version 1.1). Nine patients (23 %) experienced PR, of whom two had mixed response, and seven (18 %) a SD with an ORR of 23 %. The median PFS was 2.1 months and the median OS 24.9 months with a two-year OS of 50 % (Raj et al. 2019).

In total, these four clinical monotherapy trials comprise 115 ACC patients with advanced disease. Although none of them experienced a complete response, PRs were observed in fifteen ACC patients with an ORR of 13 % and 37 patients (32 %) showed SD. Thus, 45 % of ACC patients under ICI therapy experienced an overall clinical benefit. The median PFS ranges between 1.8 and 2.6 months and the median OS between 10.6 and 24.9 months.

Although treatment-related adverse events (TRAEs) categorized according to Common Terminology Criteria for Adverse Events v4.0 (CTCAE 4.0) were observed in all studies, only 40 % presented with TRAEs of ≥ grade 3. No treatment-related deaths were described and the clinical efficacy with improved safety profile was noteworthy compared with other standard therapies. Neither the extent of previous treatments, nor concomitant mitotane have any impact on ICI therapy response. However, autonomous hypercortisolism might correlate with an impaired treatment response (Le Tourneau et al. 2018, Carneiro et al. 2019, Habra et al. 2019, Raj et al. 2019).

Table 4.1: Overview of current clinical trials on immune checkpoint therapy in ACC

Immune checkpoint inhibitor	Type of study	Number of patients	PD-L1 expression[a]	Results		Reference
Avelumab (anti-PD-L1)	Phase Ib expansion	50	15/42	PR SD OS PFS	6% 42% 10.6 2.6	Le Tourneau et al., 2018
Nivolumab (anti-PD-1)	Phase II	10	6/8	PR SD OS PFS	10% 20% 21.2 1.8	Carneiro et al., 2019
Pembrolizumab (anti-PD-1)	Phase II	16	0/14	PR SD OS PFS	12.5 12.5 n.r. 6.75	Habra et al., 2019
Pembrolizumab (anti-PD-1)	Phase II	39	7/21	PR SD OS PFS	23% 17.9% 24.9 2.1	Raj et al., 2019
Ipilimumab and Nivolumab (anti-CTLA-4, anti-PD-1)	Phase II	6	N/A	PR SD OS PFS	33% 33% n.r. 5.5	Klein et al., 2021

Data represent percentage or total numbers. Partial response (PR) and stable disease (SD) are indicated in %, overall survival (OS) and progression-free survival (PFS) in months.
[a] Expression of PD-L1$^+$ tumors of total immunohistochemically analyzed tumors is indicated.
n.r. not reported

As systemic glucocorticoids are routinely required for the treatment of toxicities and immune-related adverse events, some studies on the effect of steroids on the outcome of immune checkpoint inhibitors therapies were published. In a meta-analysis including 4045 cancer patients of 16 studies, patients with steroid application show 1.54 times higher risk for death in comparison to those without concomitant use of steroids. In contrast, when steroids were prescribed for immune-related adverse events, the outcome was not negatively affected (Petrelli et al. 2020).

Recently, one prospective multicenter clinical trial investigated a combination immunotherapy using ipilimumab, anit-CTLA-4, and nivolumab, anti-PD-1, in six ACC patients with advanced disease, who had received prior systemic therapy (> 28 days washout) or were treatment naïve. The primary endpoint was defined as clinical benefit, including CR, PR or SD, according to RECIST 1.1 criteria. Two patients with microsatellite instable phenotype have an ongoing partial response of 10 and 25 months,

respectively, while two others were stable resulting in a total disease control of 66 % (Klein et al. 2021). An overview of all published five trials including mono- and combined therapies of immune checkpoint inhibition in ACC patients are shown in Table 4.1.

In the light of these heterogeneous results, it has to be taken into consideration that ACC patients were heavily treated with first-, second-, or even third line therapy prior to immunotherapy. Therefore, a randomized phase III clinical trial on immune checkpoint mono- or combination versus standard EDP chemotherapy (Fassnacht et al. 2012) is probably necessary to judge the true value of this approach in ACC. However, it could be reasonable first to further improve the available immunotherapeutical concepts, which will be discussed in the next section.

4.4.2 Current and prospective concepts to better predict response to immune therapy in adrenocortical carcinoma

Although results from the first small clinical trials of immunotherapy in advanced ACC were – as just described - heterogeneous and only modest, they are pioneering work for prospective research. While a larger proportion of patients do not present clinically meaningful responses in advanced ACC, a few, however, show promising disease control rates and longer survival on immune checkpoint inhibitor therapy. Therefore, selecting the right patients for immunotherapy considering genetic and molecular biological characteristics is a pivotal objective in current ACC research. Hence, tumor-specify biomarkers that predict response to immunotherapy are of major interest.

These immune checkpoint therapies intend the (re)-activation of an anti-tumoral immune response (Waldman et al. 2020). Since the presence of tumor resident T cells is crucial for sufficient immune response (Gide et al. 2019, Badalamenti et al. 2019) and immunotherapies target immune checkpoint molecules, the study of $CD3^+$-, $CD4^+$-, $FoxP3^+$- and $CD8^+$ tumor-infiltrating lymphocytes and PD-1 / PD-L1 immune checkpoint molecules as predictive biomarkers were consequently reasonable. Hence, the predictive impact of both, TILs and PD-1 / PD-L1, was certainly confirmed in other tumor entities, like melanoma (Tumeh et al. 2014, Uryvaev et al. 2018) and non-small-cell lung cancer (Haratani et al. 2017, Thommen et al. 2018).

For instance, in metastatic melanoma, the infiltration of $CD4^+$- and $CD8^+$ T lymphocytes prior to therapy predicted significantly the response to PD-1 immune checkpoint therapy (Tumeh et al. 2014, Uryvaev et al. 2018). Hence, tumors with less $CD8^+$ T cell infiltration showed a response rate of only 30 %, while highly infiltrated melanoma reached 90 %. With regard to $CD4^+$ TILs, higher amounts predicted a 66.7 % response to anti-PD-1 treatment (Uryvaev et al. 2018).

Moreover, Thommen et al. defined via transcriptional analyses in human non-small-cell lung cancer, three distinct $CD8^+$ cytotoxic T cell subtypes when considering PD-1 expression. The $CD8^+PD\text{-}1^+$ T cells recruited $CD4^+$ T_H cells and showed highly proliferative abilities. In addition, they were strong predictors for both clinical outcome and response to PD-1 inhibition when compared to $CD8^+PD\text{-}1^{-/low}$

TILs (Thommen et al. 2018). Interestingly, in the present study, the presence of $CD3^+$-, $CD4^+$-, $FoxP3^+$- and $CD8^+$ ACC-infiltrating T cells expressing PD-1 was approved, just like their prognostic impact on patients´ survival. Therefore, considering TILs, but also PD-1 expression might be prognostically valuable to predict success in immunotherapy response.
Since more than 80 % of FDA approvals for common immune checkpoint therapies are linked to PD-1 and/or PD-L1 expression, their histo-pathological companion diagnostic was established for routine clinical practice prior therapy (Evans et al. 2018, Davis and Patel 2019). In contrast, immunofluorescent $CD4^+$ T cell expression is not yet routinely evaluated and part of standard pathology assessment as prognostic factor in solid tumors (Taube et al. 2020). Therefore, established histo-pathological examination of PD-1 might be a feasible tool easily to apply.

Moreover, TILs expressing PD-1 might also be favorable as they were associated with therapeutically promising neoantigens. Hence, the presence of TILs expressing PD-1 enabled the identification of tumor-reactive mutant-specific $CD8^+$ cytotoxic T cells that were associated with favorable therapeutic chances as shown in melanoma tumors (Inozume et al. 2010, Gros et al. 2014, Gros et al. 2016). Hence, PD-1 expressing $CD8^+$ TILs were commonly associated with targeting patient-specific neoantigen with high affinity TCR. These T cells were able to eliminate autologous tumor cells and might be of therapeutic relevance for personalized therapies by using neoantigen-reactive T lymphocytes derived from peripheral blood as demonstrated in melanoma patients (Gros et al. 2016).

However, to date, clinical data of ACC patients on immune checkpoint therapy from this cohort are not yet available. Therefore, to answer the question if tumor-infiltrating lymphocytes and immune checkpoint molecules are reliable biomarkers predicting clinical benefit of ACC patients to immunotherapies, is, so far, impossible.
Additionally, the described clinical trials of immune checkpoint therapies in ACC patients have addressed the issue to some extent, but the number of patients with annotated clinical data was too small. Consequently, at this time, a final answer can not be given.
However, in this evolving field of cancer immunotherapies it is scientifically reasonable to consider further promising predictive factors that are already investigated in other tumor entities and might be transferable to ACC.
This includes essentially tumor-specify biomarkers, like tumor mutational burden (Yarchoan, Hopkins and Jaffee 2017, Samstein et al. 2019) and DNA mismatch repair deficiency or microsatellite instability (Latham et al. 2019) or other immune checkpoint molecules, like PD-L2, CTLA-4, TIM-3, IDO, Lag-3 (He and Xu 2020), that are commonly established predictors for the likelihood of response to cancer immunotherapy.
For instance, patients with MMR-D/MSI-H colorectal carcinoma that are associated with high tumor mutational burden and immune cell infiltration showed a significant longer progression-free survival

after pembrolizumab therapy compared to standard chemotherapy (André et al. 2020, Le et al. 2020). Therefore, anti-PD-1 pembrolizumab was approved by the FDA in 2018 for patients with MMR deficient / MSI-high in solid tumors.

With regard to ACC, current research based on the TCGA mRNA dataset suggests an intermediate TMB on average (Colli et al. 2016). Within the small clinical trial of combining ipilimumab and nivolumab in 6 ACC patients, Klein et al. demonstrated that both responders were MSI-H (Klein et al. 2021). However, Raj et al., who included a larger cohort of 39 ACC patients, did not detect any significant correlation between TMB/MMR-D/MSI-H and response to treatment as 78 % of patients with objective response were microsatellite stable (Raj et al. 2019). Moreover, several patients with Lynch syndrome are associated with ACC positive for germline MSH mutations (Raymond et al. 2013b). A pan-cancer study on MSI detected a higher prevalence of MSI in ACC (4.3 %) compared to the average of 39 tumor entities (Bonneville et al. 2017). Hence, ACC patients could benefit from genetic counseling, especially in case of occurrence of familiar Lynch syndrome-associated cancers, for predicting benefit from immunotherapy.

It is obviously that current immunotherapies are efficient only in a subset of patients with ACC. Therefore, one crucial question for the future of immunotherapeutical concept will be if at least one reliable predictor of response can be identified that allow patient selection.

4.5 Limitations and strengths of the study

The present study provides valuable insights into the immunological characteristics of ACCs under the influence of immunosuppressive glucocorticoid hormones. However, the study has a several limitations. First, the retrospective design of the study leads to all shortcoming of such study, including but limited to incompleteness of clinical data. For instance, in 33 of 146 patients no hormonal work-up was performed prior surgery and in some more patients these diagnostic procedures were not performed according the highest standards. Second the number of patients and tumor samples are relatively low compared to other tumor entities. Especially, patient material from distant metastases was very scarce due to the fact that ACC patients of advanced ENSAT stage IV were less common considered for surgery (Fassnacht et al. 2018). However, the utmost rarity of ACC prevented the collection of a larger cohort.
Third, while considering the correlation of tumor-infiltrating lymphocytes and glucocorticoid excess, the question for a quantitative correlation or even for an intratumoral steroid excess arises. Due to the fact that intratumoral glucocorticoid concentrations might also be significantly elevated in clinically hormone inactive ACC in comparison to other solid tumor entities, although without systemically measurable plasma cortisol levels. However, such a method is not yet available and our group is currently aim to establish the measurement of intratumoral steroids via LC-MS/MS.
Moreover, different sampling locations may affect the results of the quantification of tumor-infiltrating $CD3^+$-, $CD4^+$-, $FoxP3^+$- and $CD8^+$ T lymphocytes and PD-1 and PD-L1 immune checkpoint molecules. However, we minimized the selection bias by excluding necrotic tumor specimens and by analyzing whole tumor sections.
Another challenge was to define the cut-offs of PD-1 and PD-L1 expression and the specificity and sensitivity of their respective antibodies that varied among different studies. Therefore, cut-offs and antibodies that were used in common peer-reviewed cancer studies and/or in recent clinical trials on immune checkpoint monotherapy in ACC were chosen.
Although the present study investigated the expression of PD-1 and its ligand PD-L1 (and also TILs) for consideration as prognostic markers, their application for predicting treatment response was unfeasible, because only very few patients of this cohort have been treated with checkpoint inhibitors. However, a correlation of data from patients that have been treated recently with anti-PD-1 and/or anti-PD-L1 immune checkpoint therapy is ongoing.
Furthermore, other checkpoint molecules, like CTLA4, could have been analyzed and integrated in this multi-dimensional approach. However, since CTLA-4 regulates the T cell proliferation at an early stage of immune response and primarily in lymph nodes, studying its expression in ACC patients is rather impeded.
At last, with regard to the *in vitro* co-culture experiments, the amount of available PBMCs after isolation from donor li-heparin full blood was limiting. Additionally, due to the fact that co-culturing of different cell models with appropriate HLA-matching T lymphocytes only occurred in a non-physiological

environment, lacking other influencing factors, like other immune cells or cytokines, has to be considered when interpreting results.

Despite these limitations, the study has also several strengths that should be acknowledged, as well. In the light of the rarity of ACC, this series is actually extremely large and most likely no other center will collect within the next years more patients for such a detailed characterization.
Moreover, a considerable advantage of this study is the comprehensive analysis of even four crucial T cell subtypes of the adaptive immune system. Accordingly, the specific establishment of immunohistochemical antibodies is another major prerequisite for all subsequent measures. The evaluation of antibodies was collectively assessed by two / three independent investigators and a final score was generated by consensus.
An outstanding quality of this study is the very well characterized patient material. This includes not only the availability of clinical data, like the pathological examination and detailed hormonal analyses, but also a sufficiently long period of follow-up. This facilitates that enough events in the form of local recurrences and deaths were available enabling comprehensive multivariate analyses.
Another notable strength is the fact that this study could characterize the immunological nature of ACCs that was defined *in silico* on mRNA levels of the TCGA-ACC cohort in an independent cohort with direct evidence using immunohistochemical methods.

4.6 Outlook

Clinical trials have shown that only a small subset of ACC patients benefit from immune checkpoint therapies. Therefore, an improved understanding of the specific resistance mechanisms in ACC is required for optimization of immunotherapies.

In accordance with our hypothesis, we are convinced that intratumoral glucocorticoid excess plays an essential role in T cell depletion and may be therapeutically targetable. Therefore, one key overarching aim must be to identify molecular factors that impair the activation of anti-tumoral abilities of the adaptive immune system and to progress approaches to overcome resistance of ACC against immunotherapy. In this context, there are several ideas that should be addressed on ongoing research projects. Some of them will shortly introduced here:

Based on the prior *in vitro* studies that demonstrated an enhanced anti-tumoral T cell cytotoxicity towards therapeutically inhibited steroidogenic ACC cells, we will investigate the effects of a combination of immune checkpoint inhibition and blocking of the steroidogenesis. Therefore, we will expand our established co-culture system by including another steroidogenic cell line CU-ACC1 (Kiseljak-Vassiliades et al. 2018), the newly established patient-derived non-steroidogenic cell line JIL-2266 (Landwehr et al. 2021) and primary ACC cells with patients´ HLA-matched T lymphocytes. Therapeutically, we will apply a novel selective glucocorticoid receptor antagonist relacorilant (CORCEPT Therapeutics, (Greenstein et al. 2020)), the inhibitor of steroidogenesis metyrapone and the PD-1 antibody nivolumab +/- PD-L1 antibody avelumab to study the impact on cytotoxicity and IFN-γ release of T lymphocytes.

To monitor the activity of glucocorticoid-mediated transcription, a custum-made GR Nanostring NCounter array (Veldman-Jones et al. 2015) in T lymphocytes and tumor cells will be performed to compare gene expression profiles in autonomously glucocorticoid secreting and/or hormonaly non-functioning ACCs.

To more physiologically verify the potential of overcoming glucocorticoid-induced T cell depletion by combining inhibition of immune checkpoint and steroid biosynthesis, *in vivo* experiments are required. Therefore, in collaboration with Prof. Pierre Val (Clermont-Ferrand, France) genetic mouse models that spontaneously develop ACC will be investigated. The AdTAg mice represent a phenotype with glucocorticoid excess (Batisse-Lignier et al. 2017), whereas a newly developed mouse model with double knock-out of ZNRF3 and TP53 has no endocrine phenotype. Both mouse models should be treated in different mono- and combitherapies with anti-PD1 and metyrapone. The primary endpoint of these *in vivo* studies is the immune cell infiltration in primary ACC and lymph nodes, while the adrenal weight, size and number of metastases (liver and lung) and the number/phenotype of circulating immune cells are the secondary endpoint.

However, novel therapeutic strategies to shape the tumor immunogenicity towards a immunological "hot" tumor are required to increase meaningful responses to immunotherapies in ACC. First, a combination of ACC-specific radionucleotid therapy with [^{131}I] iodometomidate/ [$^{123/131}$I] IMAZA

(Heinze et al. 2021) with immunotherapy might induce clinically relvant anti-tumor activity in tumor sections and spheroid models *in vitro* and *in vivo* mouse experiments.

Another approach might be the evaluation of tumor-specific mutant antigens – conventional and cryptic HLA neoantigens – in mouse and human. For conventional neoantigens, following *in silico* prediction (POLYmorphic loci reSOLVER (Shukla et al. 2015) and netMHCpan (Jurtz et al. 2017)) of high-affinity HLA-peptides, candidates will be validated *in vitro* via FACS analyses using fluorescence-labelled HLA dextramer-peptide complexes and patients circulating and tumor-infiltrating T lymphocytes.

For cryptic neoantigens, MHC class I peptides will be isolated from ACC homogenisates and analyzed via nanoHPLC-coupled to ESI tandem mass sepctrometry. Candidates will subsquently correlated to *in silico* MHC binding prediction tools (Erhard et al. 2020).

Both neoantigen-targeting approaches will be a crucial prerequisite for the development of ACC specific vaccines that might also strengthen other immunotherapeutic approaches.

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Appendix

Figure 1.1, 1.2, 1.3, 1.4 and **1.5** were created with BioRender.com.

Table A.1: Clinical data of adrenocortical carcinoma patients included in this thesis
Indicated are the age at diagnosis (years), sex (f = female, m = male), ENSAT stage, resection status (0 = complete surgical resection chirurgically and histo-pathologically examined, X = complete surgical resection chirurgically examined, 1/2/3 = incomplete surgical resection), the Ki67 proliferation index (%) and glucocorticoid excess (0 = GC inactive, 1 = GC active). N/A: not available

Nr.	Age	Sex	ENSAT stage	Resection status	Ki67 proliferation	Glucocorticoid excess
1	28	f	2	0	20	N/A
2	41	m	3	0	30	0
3	41	m	3	0	N/A	0
4	35	f	3	0	30	1
5	29	f	4	2	1	0
6	37	f	2	0	N/A	1
7	24	f	4	2	20	1
8	40	m	2	0	3	1
9	73	f	3	1	40	N/A
10	34	f	2	1	N/A	1
11	47	f	2	0	5	1
12	18	m	3	0	15	1
13	19	m	3	0	N/A	1
14	26	m	2	0	20	N/A
15	49	f	3	0	10	1
16	38	f	2	0	1	N/A
17	35	m	3	1	80	1
18	35	f	2	0	30	0
19	44	m	3	0	3	N/A
20	47	m	3	0	5	0
21	50	m	3	0	10	0
22	60	m	2	1	N/A	0
23	18	f	2	X	40	1
24	55	f	4	2	N/A	1
25	45	f	3	0	3	N/A
26	53	f	3	0	5	1
27	40	f	2	2	N/A	1
28	31	f	4	0	10	1
29	31	f	4	1	N/A	1
30	33	f	2	0	2	N/A
31	61	f	2	X	30	0
32	62	f	4	2	N/A	0
33	32	f	3	0	30	0
34	52	f	3	0	5	1

Nr.	Age	Sex	ENSAT stage	Resection status	Ki67 proliferation	Glucocorticoid excess
35	44	f	2	N/A	5	1
36	70	m	4	N/A	N/A	N/A
37	72	m	4	2	N/A	N/A
38	27	f	4	2	10	1
39	27	f	4	N/A	N/A	1
40	65	f	2	0	30	1
41	49	f	4	2	20	1
42	51	f	3	0	10	1
43	68	m	2	0	5	0
44	47	f	3	X	15	0
45	49	m	4	0	20	1
46	20	f	4	N/A	N/A	1
47	45	m	3	1	1	N/A
48	72	m	2	0	10	1
49	61	f	2	0	15	0
50	49	f	2	0	10	1
51	53	f	2	N/A	N/A	1
52	48	m	2	N/A	10	N/A
53	49	m	2	N/A	10	N/A
54	39	f	2	0	10	0
55	42	f	1	0	10	1
56	33	m	2	0	30	0
57	56	m	3	0	N/A	0
58	51	m	2	0	20	0
59	60	m	2	0	10	0
60	55	f	4	2	3	N/A
61	56	f	3	0	N/A	N/A
62	31	f	3	1	15	1
63	34	f	3	1	N/A	1
64	43	f	4	2	10	1
65	51	m	1	0	10	1
66	55	m	1	0	N/A	1
67	46	f	2	0	20	0
68	40	m	3	0	10	0
69	40	f	4	0	N/A	1
70	41	f	4	0	N/A	1
71	N/A	f	N/A	N/A	N/A	N/A
72	75	f	2	0	8	N/A
73	55	f	3	0	5	1
74	18	f	2	0	15	0
75	20	f	2	1	N/A	0
76	58	f	2	0	2	0
77	34	m	4	2	30	N/A
78	32	f	2	0	0	0

Nr.	Age	Sex	ENSAT stage	Resection status	Ki67 proliferation	Glucocorticoid excess
79	35	f	2	0	N/A	1
80	36	f	2	X	40	1
81	34	f	2	0	40	1
82	23	f	2	0	N/A	N/A
83	24	f	2	0	N/A	N/A
84	59	m	2	0	N/A	0
85	51	m	2	X	30	1
86	65	f	2	0	5	1
87	37	f	2	0	N/A	0
88	36	f	2	0	30	0
89	55	f	3	0	3	0
90	74	f	3	0	20	N/A
91	77	m	2	0	10	0
92	50	m	3	1	30	1
93	19	f	2	0	30	0
94	69	f	2	X	40	1
95	63	f	3	1	20	1
96	46	m	3	0	40	N/A
97	50	m	2	N/A	5	N/A
98	49	f	4	2	8	N/A
99	62	f	2	0	10	1
100	51	f	3	1	50	1
101	53	f	3	N/A	N/A	1
102	47	f	3	1	20	1
103	34	m	3	X	20	0
104	74	f	3	0	N/A	0
105	32	f	2	0	5	0
106	33	f	3	0	1	0
107	57	f	2	0	4	0
108	30	f	3	X	30	1
109	22	f	2	0	N/A	0
110	62	f	4	2	20	1
111	66	f	2	0	10	0
112	72	f	2	0	20	0
113	64	f	4	3	10	N/A
114	39	m	3	0	30	1
115	45	f	2	1	50	1
116	46	f	2	N/A	N/A	1
117	60	f	3	0	N/A	1
118	46	m	4	2	10	1
119	53	f	3	1	30	N/A
120	53	f	3	0	30	N/A
121	75	f	3	X	20	N/A
122	50	m	2	1	5	0

Nr.	Age	Sex	ENSAT stage	Resection status	Ki67 proliferation	Glucocorticoid excess
123	51	m	2	0	15	0
124	47	m	2	0	2	0
125	51	f	3	1	50	1
126	31	m	4	2	10	1
127	34	f	2	0	5	N/A
128	36	f	3	0	10	0
129	32	f	4	N/A	N/A	0
130	32	f	4	2	10	0
131	60	f	3	2	20	N/A
132	60	f	N/A	N/A	N/A	N/A
133	60	f	3	2	20	N/A
134	54	f	2	0	40	0
135	32	f	2	0	5	0
136	33	f	2	N/A	5	0
137	71	f	4	2	25	1
138	71	f	2	0	8	0
139	39	f	2	N/A	N/A	0
140	32	m	4	2	N/A	N/A
141	36	m	2	N/A	N/A	N/A
142	52	f	2	1	20	1
143	54	f	2	0	N/A	1
144	35	m	3	0	20	1
145	36	m	3	0	N/A	1
146	55	m	2	0	5	N/A

Danksagung

An dieser Stelle gebührt mein herzlichster Dank allen Menschen, die mich auf meinem Weg begleitet und unterstützt haben.

Mein ganz besonderer Dank gilt Prof. Dr. Martin Fassnacht, der mir die Möglichkeit gab, diese Doktorarbeit zu verwirklichen und mich herzlich in seiner Arbeitsgruppe aufnahm. Währenddessen unterstützte und förderte er mich durch sein medizinisch-naturwissenschaftliches Fachwissen, seine Leidenschaft zur aktiven Mitgestaltung des transnationalen Forschungsfortschrittes und sein Begrüßen vom wissenschaftlichen, kollaborativen Austausch. Durch seine Leidenschaft zur Wissenschaft und Herzlichkeit hat er sehr zum Erfolg dieser Forschungsarbeit und vieler weiterer Projekte beigetragen.

Großer Dank gilt auch Prof. Dr. Thomas Dandekar, der dieses Projekt und die Forschungsarbeit darüber hinaus durch seine wertvollen Ideen und Inspirationen bereicherte, sowie durch seine Freundlichkeit und Hilfsbereitschaft unterstützte. Zudem danke ich ihm sehr für die Übernahme des Zweitgutachtens.

Außerdem möchte ich mich herzlich bei PD Dr. Niklas Beyersdorf für den wissenschaftlichen Austausch und die geschätzte Unterstützung, besonders im Bereich der Immunologie und Onkoimmunologie, bedanken.

Bei Prof. Dr. Felix Beuschlein möchte ich mich besonders für seine fachliche Unterstützung, insbesondere im Bereich der endokrinen Onkologie, und die motivierenden Diskussionen im Rahmen des DFG Sonderforschungsbereiches Transregio 205 bedanken.

Großer Dank gilt auch PD Dr. Silviu Sbiera, der mir stets mit hilfreichen Ratschlägen bei laborexperimentellen und fachspezifischen Fragen zur Seite stand. Durch seine Offenheit, seine Hilfsbereitschaft und seinen Humor hat er sowohl das Forschungsprojekt, als auch den Arbeitsalltag besonders bereichert.

Neben meinem Promotionskomitee, möchte ich besonders Prof. Dr. Dr. Matthias Kroiss für die äußerst wertgeschätzte Betreuung, die fachliche Unterstützung und seine bedeutend fröhliche Motivation danken. Durch sein unermüdliches Engagement und seine Kreativität hat er sehr zum Erfolg dieser Forschungsarbeit und vieler weiterer Projekte beigetragen.

Ebenfalls bedanken möchte ich mich bei unserer gesamten Arbeitsgruppe der Endokrinologie für die schöne und fröhliche Zeit. Nur durch die gemeinsame Teamarbeit von Forschungslabor, Krankenstation, Ambulanz, Routinelabor und Massenspektrometrie-Unit ist dieses Forschungsprojekt ermöglicht worden. Ein großer Dank gilt auch Isabel und Christina für die schöne Zeit in Würzburg, die unvergesslichen Kongressreisen und unsere wunderbare Freundschaft.

Bei Dr. Silke Appenzeller und Dr. Sabine Herterich bedanke ich mich sehr für ihre stetige Unterstützung hinsichtlich einiger Fragestellungen zu genetischen Sequenzierungen und Dr. Stefan Kircher bezüglich seiner hilfreichen Ratschläge histo-pathologischer Natur.

Meinen Freunden und meinem Freund gilt ein großer Dank für viele wundervolle und bedeutende Momente, die wertvolle Motivation und die liebe Verbundenheit.

Zum Abschluss möchte ich mich von ganzem Herzen bei meiner liebevollen Familie bedanken, die mich auf all meinen Wegen voller Liebe und Halt begleitet hat. Ich bin voller Dankbarkeit für eure bedingungslose Unterstützung, die unvergessenen Momente und eure liebevolle Herzlichkeit!
Herzlichen Dank, schön, dass ihr alle ein Teil davon seid!

Affidavit

I hereby confirm that my thesis entitled "Steroid Hormones and Cancer Immunity – learning from Adrenocortical Carcinoma" is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eid statt, die Dissertation „Steroidhormone und Tumorimmunität im Nebennierenrindenkarzinom" eigenständig, d.h. insbesondere selbstständig und ohne Hilfe eines kommerziellen Promotionsberaters, angefertigt und keine anderen als die von mir angegebenen Quellen und Hilfsmittel verwendet zu haben.

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