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Characterization of circulating and adrenal miRNA expression in adrenal Cushing's syndrome

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München 21.07.2023 Ru Zhang

place, date Signature doctoral candidate

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List of abbreviations

24h urinary free cortisol	UFC
3’ untranslated region	3’UTR
5’ untranslated region	5’UTR
Adrenocortical carcinoma	ACC
Adrenocorticotropic hormone	ACTH
Area Under the Curve	AUC
Corticotropin releasing hormone	CRH
Cortisol-producing adenoma	CPA
Cushing’s disease	CD
Cushing’s syndrome	CS
Glucocorticoids	GCs
Hypothalamic-pituitary-adrenal axis	HPA axis
Late-night salivary cortisol	LNSLC
Low dose dexamethasone suppression test	LDDST
Massive macronodular adrenocortical disease	MMAD
Micronodular bilateral adrenal hyperplasia	MiBAH
microRNA sequencing	miRNA seq
Next-Generation Sequencing	NGS
Primary bilateral macronodular adrenocortical hyperplasia	PBMAH
Primary pigmented nodular adrenocortical disease	PPNAD
Real-time quantitative polymerase chain reaction	q-PCR
Receiver Operating Characteristic analysis	ROC analysis
RNA sequencing	RNA seq
Zona fasciculata	zF
Zona glomerulosa	zG
Zona reticularis	zR

List of publications

Publications summarized in this thesis

Paper I

Vetrivel S, **Zhang R**, Engel M, Altieri B, Braun L, Osswald A, Bidlingmaier M, Fassnacht M, Beuschlein F, Reincke M, Chen A, Sbiera S, Riester A. **Circulating microRNA Expression in Cushing's Syndrome**. Front Endocrinol (Lausanne). 2021 Feb 22;12:620012. doi: 10.3389/fendo.2021.620012. eCollection 2021.

Paper II

Vetrivel S*, **Zhang R***, Engel M, Oßwald A, Watts D, Chen A, Wielockx B, Sbiera S, Reincke M, Riester A**.** ***Characterization of Adrenal miRNA-Based Dysregulations in Cushing's Syndrome***. Int J Mol Sci. 2022 Jul 12;23(14):7676. doi: 10.3390/ijms23147676.

*Authors contributed equally to this publication

Further publications during my doctoral study

Zhang R*, Rubinstein G*, Vetrivel S, Kunz S, Vogel F, Bouys L, Bertherat J, Kroiss M, Deniz S, Osswald A, Knösel T, Bidlingmaier M, Sbiera S, Reincke M, Riester A. ***Steroid profiling using liquid chromatography mass spectrometry during adrenal vein sampling in patients with primary bilateral macronodular adrenocortical hyperplasia***. Front Endocrinol. 2022 Dec 06. doi: 10.3389/fendo.2022.1079508.

*Authors contributed equally to this publication

Contribution to the publications

1.1 Contribution to paper I

For the paper I entitled “Circulating microRNA Expression in Cushing's Syndrome”, I performed most of the experiments. In detail, I validated the findings of the miRNA seq with real-time quantitative polymerase chain reaction (q-PCR) of the discovery group. I isolated miRNA from serum samples of patients of the validation cohort and of patients before and after low dose dexamethasone test (LDDST), performed reverse transcription and q-PCR of these isolated miRNAs. In addition, I calculated q-PCR results, designed figures and calculated parts of the statistic.

Dr. rer. nat Sharmilee Vetrivel participated conception, experiments planning, Next-Generation Sequencing (NGS) data analyses, q-PCR data analysis and manuscript writing of this project. Therefore, I’m the second author of this paper.

1.2 Contribution to paper II

For the paper II entitled “Characterization of Adrenal miRNA-Based Dysregulations in Cushing's Syndrome”, I performed all the experiments except RNA isolation from human adrenal (performed by collaborators at Max Planck Institute of Psychiatry in Munich), luciferase assays and animal handling (performed by our collaborator in Technical University Dresden) and drafting the original manuscript.

In detail, I performed cDNA preparation and q-PCR in isolated human adrenal RNAs samples to validate miRNA seq results, then performed parts of the *in silico* analysis of miRNA targets, analysis of the RNA sequencing (RNA seq) data and validated them using q-PCR. Next, I isolated RNA from adrenocorticotropic hormone (ACTH) stimulated murine adrenals and validated the selected miRNA by q-PCR. To proof the mRNA-miRNA interaction I performed *in vitro* studies. For this experiment, I used luciferase assays in HAC15 cell line to investigate the interactions between miR-1247-5p and Cyb5a. In addition, I calculated the data, did statistical analyses, designed figures, and wrote most of the sections “material and methods” and “results” of the manuscript. At last, I performed supplementary experiments (standard curve of q-PCR) and answered questions regarding the methodology when the manuscript was in revision.

Dr. rer. nat Sharmilee Vetrivel, who shared the first authorship with me, performed the bioinformatic analyses of the NGS data, used *in silico* prediction software

for miRNA target analyses, designed and verified the plasmid constructs needed for the luciferase assays, performed dual luciferase assays in HELA cells, helped in the project conception, made associated figures and drafted the other part of the manuscript. Therefore, we shared the first authorship.

1.3 Contribution to paper III (Apendix A)

For the paper III entitled "Steroid profiling using liquid chromatography mass spectrometry during adrenal vein sampling in patients with primary bilateral macronodular adrenocortical hyperplasia", I calculated the data, did all the analysis and drafted the manuscript. Dr. German Rubinstein as a clinician scientist did the patient selection, collected clinical data, and reviewed the manuscript. We shared the first authorship.

2. Introduction

2.1 Hypothalamic-pituitary-adrenal (HPA) axis

Hypothalamic-pituitary-adrenal (HPA) axis represents a complex set of feedback loops regulating the hormones secreted by the hypothalamus, the anterior pituitary gland and adrenal glands. This axis plays a central role in homeostasis, stress response, energy metabolism and neuropsychiatric function (1).

The HPA axis regulates the peripheral concentrations of steroid hormones under the control of an ultradian, circadian and stress-related fashion (2). Once the hypothalamus receives the signal of stress, corticotropin releasing hormone (CRH) neurons in the hypothalamic paraventricular nucleus releases CRH (3), which is transported to the pituitary gland via hypophyseal portal system. CRH binds to the specific receptor on the pituitary gland and subsequently activates the synthesis and secretion of ACTH. Circulating ACTH acts on the cortex of adrenal gland and stimulates the adrenal cortex to produce cortisol. Circulating cortisol regulates CRH release and ACTH secretion by negative feedback and thereby maintains the relative homeostasis of circulating cortisol (Figure 1).

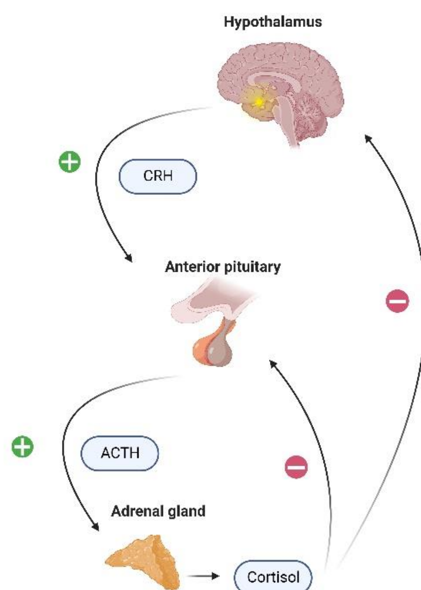

Figure 1 Regulation of Hypothalamic-pituitary-adrenal axis (Adapted from "Hypothalamic-Pituitary-Adrenal Axis", by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates)

2.2 Adrenal gland

2.2.1 Morphology and Histology

Humans have two adrenal glands located at the top of each kidney. The right adrenal is pyramidal and the left adrenal gland is crescentic in shape.

The adrenal gland is composed of two different types of tissue, the cortex and the medulla. Among them, the cortex makes 85% of adrenal tissue while the medulla makes 15% (4). The adrenal cortex is divided into three separate histological and functional zones (Figure 2): zona glomerulosa (zG), zona fasciculata (zF) and zona reticularis (zR) (5). These different zones are associated with the synthesis and secretion of different steroids, with zG producing mineralocorticoids, zF producing glucocorticoids (GCs) and zR producing androgens. The medulla mainly consists of chromaffin cells and ganglion cells, producing catecholamines.

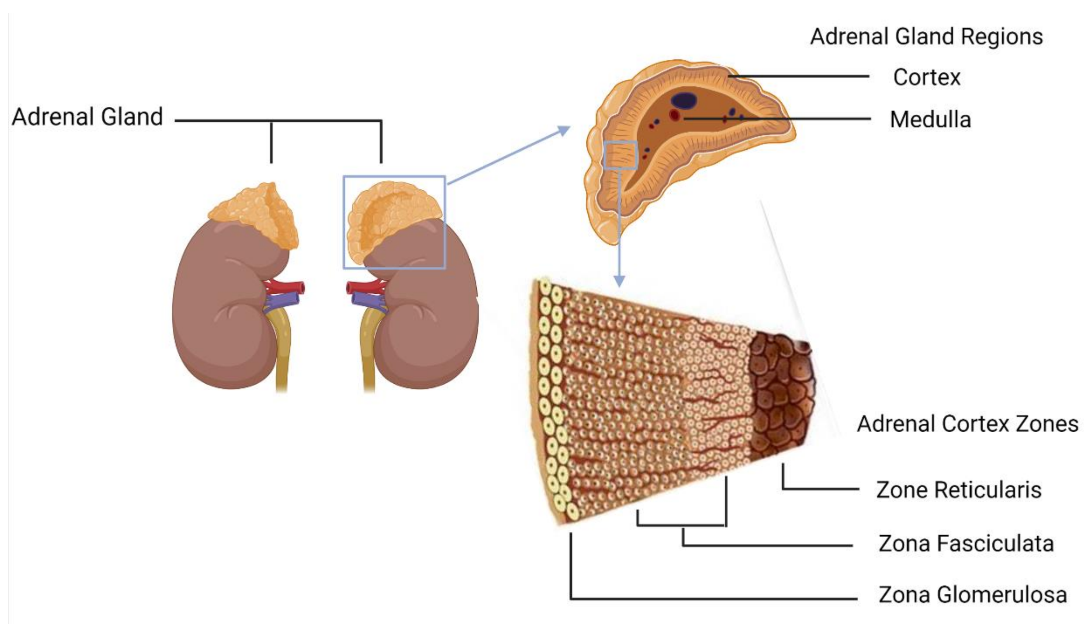

Figure 2 Morphology and histology of adrenal (created with BioRender.com).

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2.2.2 Physiology of Glucocorticoids (GCs)

GCs are essential in many biological processes, such as metabolism (6), bone metabolism and growth (7-9), anti-inflammation and immunomodulation (8, 10), reproduction (11), development (12), mood and cognitive functions (13).

Cortisol, as the most important glucocorticoid in humans, is pulsatively released throughout the day with peak at around 8 am and nadir around midnight. Acute stress results in short spikes in circulating cortisol levels. The regulation of corti-

sol is crucial and can be influenced by many different stressors. These stressors include physical and mental stimuli such as hunger, cold, trauma, infection, depression and anxiety (14). Besides the regulation of cortisol secretion via the previously described HPA axis, there are several other and especially intra-adrenal mechanisms which are important for the fine regulation of the adrenal stress response via cortisol secretion.

2.3 Cushing's syndrome (CS)

CS is an endocrine and metabolic disorder caused by increased levels of circulating glucocorticoids. This disorder includes not only excess of circulating glucocorticoids but also disturbed diurnal rhythm of cortisol secretion (15). Long time exposure to high glucocorticoids result in increased morbidity and mortality (16).

2.3.1 Clinical presentations

CS presents a broad range of symptoms, depending on the exposure times to GCs, level of GCs in circulation and the subtype of CS. The symptoms are listed in table 1 based on organ systems. As the symptoms are not specific, diagnosis is often delayed (see below).

Table 1 Clinical presentations of Cushing's syndrome based on systems.

System	Clinical presentations
General symptoms	Fatigue, abnormal growth in children (lagging height but gaining weight)
Adipose	Weight gain, altered fat distribution (central adiposity and moon faces but slender limbs, buffalo hump, moon face) (17)
Metabolism	Diabetes/impaired glucose tolerance (18, 19), dyslipidemia (20)
Musculoskeletal system	Proximal muscle weakness, osteoporosis/fracture (21, 22)
Infection	Increased infections in affected systems (23)
Skin and hair	Purple striae on abdomen, buttons, upper arm/leg breasts, thin skin, hyperpigmentation, hirsutism (women), balding (women), acne, poor wound healing, easy bruising, flushed face (24)
Cardiovascular system	Hypertension (25, 26), cerebrovascular accident, myocardial infarction, thrombus, edema (27, 28)
Urinary system	Increased incidence of urinary stones (29)
Reproductive system	sexual dysfunction, hypogonadism (21, 22); masculinization

	(women), menstrual disorders (women) (30), infertility (31)
Neuropsychiatric system and cognition	Exacerbation of existing personality/disorder, depression, anxiety and irritability, memory loss, cognitive difficulty, headache, insomnia (32)

2.3.1 Morphology and Histology

CS could be caused by endogenous or exogenous factors. Exogenous factor is the most common cause while endogenous cause is rare (33, 34), with an incidence of 1.8–3.2 per million people per year (35). However endogenous CS is more complex in subtyping. ACTH-dependent CS are responsible for 80% of endogenous CS and are caused by ACTH-secreting pituitary adenoma or non-pituitary tumors with ectopic ACTH production. 20% of endogenous CS are ACTH-independent CS and caused by autonomous cortisol-producing adrenal pathologies (36, 37). Moreover, ACTH-independent CS has four main subtypes which are summarized in Table 1.

Table 2 Subtypes of endogenous Cushing's syndrome (33, 36, 37).

	Proportion
ACTH-dependent CS	80% (33, 36)
Cushing's disease (CD)	70% (37)
CS with ectopically secreting ACTH from non-pituitary tumor	10% (37)
Unknown source of ACTH	5% (37)
ACTH-independent CS	20% (33, 36)
Cortisol-producing adenoma (CPA)	10% (37)
Adrenocortical carcinoma (ACC)	5% (37)
Micronodular bilateral adrenal hyperplasia (MiBAH)	Very few (37)
Primary bilateral macronodular adrenocortical hyperplasia (PBMAH)	Very few (37)

2.3.2 Diagnosis

Due to the high variability of clinical presentations and considerable clinical overlaps with other metabolic diseases, diagnosing and subtyping of CS remains challenging.

Patients with clinical suspicion of CS are subjected to the initial biochemical screening tests by endocrinologists. The first-line screening tests recommended for CS diagnosis by the Endocrine Society's guidelines (38) include 24h urinary free cortisol (UFC, reflecting daily cortisol secretion), low dose dexamethasone

suppression test (LDDST, assessing feedback inhibition of cortisol on the HPA axis) and late-night salivary cortisol (LNSLC, evaluating the diurnal rhythm in cortisol secretion) (39). The combination of assays is more sensitive and specific in diagnosing CS in comparison to serum cortisol (40). In addition, dexamethasone-CRH test and desmopressin test are used as the second-line test for CS screening (39, 41). The workflow of CS diagnosis is summarized in figure 3.

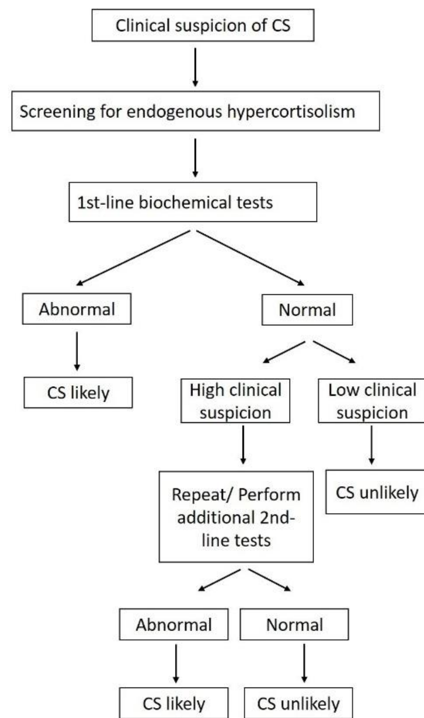

Figure 3 Workflow of CS diagnosis (38).

After the diagnosis of CS is established, the next main step is to find out the subtype of CS. Mostly, the ACTH concentration could distinguish ACTH-dependent from ACTH-independent CS (42). Imaging (MRI or CT) and additional biochemical tests (e.g. high dose dexamethasone test, CRH test) can further help to differentiate between subtypes of CS (42).

2.3.3 Management

Surgical removal of ACTH-secreting pituitary adenoma, ectopic ACTH-secreting tumor or cortisol producing tumors of adrenal pathologies is recommended as the best treatment of CS when surgery is possible (43). The success rate of surgery depends on the size of tumor and the experience of the surgeon, so that not all patients could achieve completely remission. Additionally, some patients have recurrence of CS after surgery. No surgical therapy is possible when the primary source of ACTH production is occult. For these patients who have persistent or recurrent hypercortisolism, additional treatments are recommend-

ed, such as re-operation (the same or alternative types), radiation therapy or medical treatment (13, 43, 44) .

Currently available pharmacological therapies of CS include pituitary-directed drugs, steroidogenesis inhibitors and glucocorticoid receptor antagonists. However, most of these drugs have severe side-effects and therefore, their application is limited. If these therapies failed to control hypercortisolism or if both adrenals are affected by cortisol producing nodules (e.g. in patients with PBMAH), bilateral adrenalectomy could be performed as a last therapeutic option (43, 45). In consequence, the patients have to substitute hydrocortisone life-long.

2.4 MicroRNA (miRNA)

miRNAs are single stranded, small non-coding RNAs that are 21-24 nucleotides in length and were first described in in *Caenorhabditis elegans* in 1990s (46, 47). Since their first discovery, miRNAs have been subject of many studies to investigate their physiological and pathological role in biological processes.

There are two biogenesis pathways of miRNAs in mammals: canonical pathway (mediated by RNA polymerase II, most common pathway of miRNA biogenesis) and non-canonical pathway (mediated by RNA polymerase III). Majority of the identified miRNAs are present in the intracellular environment, only a small quantity of miRNAs is reported to be in the extracellular fluids, commonly called extracellular miRNA or circulating miRNA, if they are in blood circulation. The source of circulating miRNAs remains unknown, but current research supports the hypothesis that circulating miRNAs are actively secreted by cells via extracellular vesicles and protein carriers (48), and that only a small portion are passively released from necrotic or damaged cells (49, 50).

miRNAs are widespread in human body, a total of 1917 mature human miRNAs have been identified and included in miRbase (a database included all published miRNA sequences and associated annotation, https://www.mirbase.org/, last visit on Nov 10th, 2022) (12). According to a study published in 2009, over 60% of human protein-coding genes is estimated to be regulated by miRNAs (51). This indicates that gene regulation mediated by miRNAs is common in various biological processes.

2.4.1 Circulating miRNA as biomarkers

The discovery of miRNAs in biological fluids suggests that miRNAs might also act as signaling molecules outside the cell. In addition, miRNA expression shows unique patterns in different cells and tissues, and even at different stag-

es of biological process, indicating that miRNAs can serve as biomarkers for certain pathologies (52). As potentially valuable biomarkers for specific biochemical process in medicine, circulating miRNAs show various advantages: 1). Endogenous circulating miRNAs are resistant to plasma RNase (53); 2). The collection of circulating mRNAs is minimally invasive; 3). The concentrations of miRNAs can be measured relatively cheap with standard lab techniques already included into clinical practice (54); 4). miRNA profiles are highly sensitive and specific (55).

2.4.2 miRNAs mediate gene regulation in tissue

miRNAs regulate gene expression at post-transcriptional level by binding to the mRNA. The seed sequence (or seed region) is an evolutionarily conserved region which is mostly located at 2-7 nucleotides from the 3’ end of miRNA. This region plays an essential role in miRNA target recognition, determination of miRNA targeting efficacy and specificity in the miRNA-mRNA interaction (56, 57). Canonically, miRNA targets the 3’ untranslated region (3’UTR) of target mRNAs by base-pairing of the seed sequence through contiguous and Watson-Crick base-pairing (58). mRNA degradation or translation repression is the most common result of miRNA-mRNA interaction (61). 5’untranslated region (5’UTR), coding regions and promoter regions are the other uncommon binding sites of targets mRNAs (62). The binding of miRNAs to 5’UTR and coding regions could also result in silencing effects on gene expression, while miRNA interaction within promoter region has also been reported to activate transcription (63). The canonical miRNA-mRNA interaction is schematized in Figure 4.

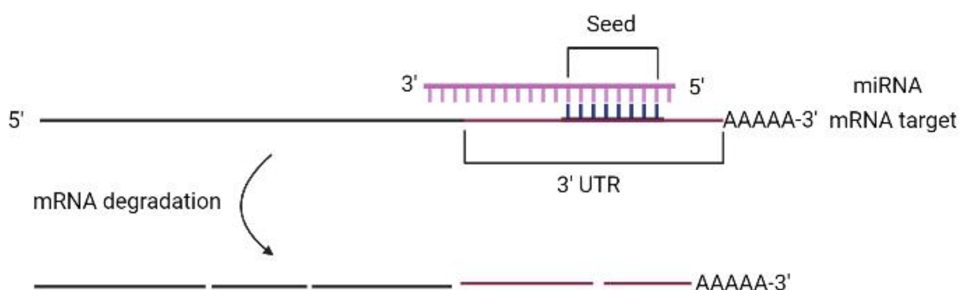

Figure 4 miRNA-mRNA interaction (created with BioRender.com).

As mentioned above 1917 mature human miRNAs target 60% human genes, hinting that miRNA-mRNA targeting isn’t a one-to-one interaction. One miRNA may have multiple mRNA targets containing the cognate miRNA-binding site (64) and one mRNA also could be regulated by multiple miRNAs (65).

2.4.3 miRNA in CS

The above described time-consuming diagnosis of CS denotes the risk of delayed or missed diagnosis of CS, with crucial implications for morbidity and mortality (40). Therefore, specific molecular biomarkers could help in the diagnosis of CS. In 2013, Dhaval Patel et al. were the first to publish their study on the roles of serum miR-34a and miR-483-5p as candidate biomarkers in distinguishing benign from malignant adrenocortical tumors (11). Plasma miRNAs were studied to differentiate CD from ectopic CS in 2020 (66).

Epigenetic alterations are crucial during tumorigenesis. As an eminent part of epigenetic mechanism, miRNAs also play important roles in tumorigenesis. Although studies have identified specific miRNAs and their targets in different forms of adrenal pathologies, such as primary pigmented nodular adrenocortical disease (PPNAD) (67), massive macronodular adrenocortical disease (MMAD) (68) and PBMAH (69), no common miRNA was found among these studies. This indicates that different underlying pathologies in CS have specific miRNAs profiles. Comprehensive understanding of the effect of miRNA in different subtypes of CS may provide an understanding of miRNA-based targeting in CS subtypes.

2.5 Aims

The overall purpose of this dissertation is to have a comprehensive understanding of the role of miRNAs in CS. The specific aims of this research project are:

- To identify specific biomarkers for diagnosing and subtyping of CS (ACTH-dependent and ACTH-independent) by performing a profile of circulating miRNA by miRNA seq (Publication I).
- To explore adrenal miRNA profile in ACTH-dependent and ACTH-independent CS by adrenal miRNA seq (Publication II)
- Study miRNA-mRNA interactions and involved pathways in adrenal glands of patients with CS using *in silico* predictions, pathway analysis, and luciferase assays. (Publication II)

3. Materials, methods and major findings

To achieve the described aims, biological samples (serum and adrenal tissue) were collected from patients who were registered in at least one of the following biobanks: German Cushing's Registry (CUSTODES), European Network for the Study of Adrenal Tumor (ENS@T - http://www.ensat.org/) and/or Network of Excellence for Neuroendocrine Tumors (NeoExNet). The CUSTODES was founded by Prof. Dr. Martin Reincke and the Adrenal Gland Section of the German Society for Endocrinology (DGE). Several centers throughout Germany are actively involved. This joint register is used to evaluate data on occurrence, diagnosis, symptoms, therapy and long-term prognosis of CS and aims to gain a better understanding of this rare disease by improving diagnostic methods, medical care and optimizing long-term therapy. The NeoExNet, which is funded as part of the m4 Cluster of Excellence (http://www.m4.de), is a clinical registry and associated biobank recruiting patients with endocrine tumors, especially patients with CS, with aims to systematically examine the complaints, symptoms, secondary diseases and mortality of various neuroendocrine tumors. ENS@T is a European register and biobank of adrenal-related tumors, devoted to the research on adrenal tumors.

For the first project of this dissertation, two medical centers were involved: LMU Klinikum, Ludwig-Maximilians-Universität in Munich and the Hospital of the University of Würzburg.

We included a cohort of patients with CS (CD and CPA) and controls as discovery cohort (10 controls, 5 patients with CPA and 10 patients with CD), and another cohort of patients with CS and controls as validation cohort (11 controls, 11 patients with CPA and 9 patients with CD). CS was diagnosed following the current guidelines (38). Both pre- and post-operative blood samples from patients with CS and blood samples from controls were collected. Serum miRNAs were isolated from all the blood samples using miRNeasy Serum/Plasma Kit (Qiagen GmbH, Hilden, Germany). miRNAs extracted from the discovery cohort were divided into two aliquots, one was processed for NGS and another was stored at −80°C until further use. All miRNAs (from both discovery cohort and validation cohort) were reverse transcribed with TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). Real-Time Quantitative PCR (q-PCR) was performed with TaqMan Fast Universal PCR Master Mix (2x) (CN:4352042; Thermo Fisher Scientific) on a ThermoFisher QuantStudio™ 5 Real-Time PCR System to confirm miRNA expressions. Further, we evaluated the candidate miRNAs with Receiver Operating Characteristic (ROC) analysis.

At last, the influence of short-term glucocorticoids on the circulating miRNA candidates was investigated.

Results of the first project showed distinct circulating miRNA profiles in patients with CD and CPA. Circulating miR-182-5p was identified as a possible biomarker for CD. We also found that circulating miRNA expression could be affected by dexamethasone, which underlies the hormonal regulation in circulating miRNA.

The second project of this dissertation focused on the role of miRNAs in the adrenal glands including the miRNA-mRNA interactions and involved pathways.

Normal adrenal tissue from 8 controls who underwent adrenalectomy for pheochromocytoma, 7 patients with CPA and 8 patients with CD who underwent adrenalectomy were collected and stored at −80°C until use. Adrenal miRNAs were isolated using RNeasy Tissue Kit (Qiagen, Hilden, Germany) and stored at −80°C. Synacthen (ACTH1-24) (Sigma Aldrich, Munich, Germany) was intraperitoneally injected into 13-week-old C57BL/6 J female mice (Janvier, Le Genest-Saint-Isle, France) with 1 mg/kg. Murine adrenals were collected at 10, 30, and 60 min of injections. Another group of mice without ACTH injection were used as controls (0 min). All murine adrenals were frozen in liquid nitrogen and stored at −80°C for later RNA extraction. We performed NGS of adrenal miRNA and RNA. q-PCR was used to confirm miRNA and RNA expression. *In silico* prediction and RNA seq data were used to predict and validate miRNA targets, luciferase assay was performed to validate miRNA-mRNA interaction.

miRNA seq showed different adrenal miRNA profiles in patients with CD and patients with CPA. q-PCR confirmation yielded two significantly upregulated miRNA candidates when compared to controls, miR-1247-5p in CPA and miR-379-5p in CD. Further q-PCR analysis revealed that miR-1247-5p was upregulated in CPA and PBMAH (both are ACTH-independent CS) compared to controls, CD and ectopic CS (both are ACTH-dependent CS). miR-379-5p was upregulated in CD and PBMAH compared to controls. *In vivo* experiments showed that adrenal miR-1247-5p and miR-379-5p expression were independent from ACTH stimulation. Therefore, it could be hypothesized that in CPA and PBMAH the upregulated miR-1247-5p might contribute to cortisol regulation independent from ACTH. In CD and PBMAH, where adrenal hyperplasia is found, the upregulated miR-379-5p could be involved in the proliferative pathology. *In silico* analysis, RNA seq data and q-PCR identified Cyb5a as a potential target of miR-1247-5p in CS. However, the target interaction could not be proven conclusively by dual luciferase assay. Finally, comparative pathway analysis of miR-

1247-5p and miR-379-5p targets revealed the Wnt signaling pathway as a commonly regulated pathway of both miRNA candidates in CS.

4. Summary

Endogenous CS is a rare disease with high morbidity and mortality. Several diagnostic tests have been established for its diagnosis. However, timely diagnosis and subtyping of CS forms is challenging. Further, treatments based on specific CS subtypes are limited. After surgery as the first line treatment based on the underlying cause of hypercortisolism (adrenal, pituitary or tumour) the patients have to substitute hydrocortisone and are ins risk of adrenal crisis. Existing medical therapy do not effectively control hypercortisolism in long term and have several side-effects.

In this dissertation, we performed miRNA seq in serum and adrenal samples of patients with CS with the following aims: (1.) to identify circulating biomarkers for CS, (2.) to explore adrenal miRNA profile in ACTH-dependent and ACTH-independent CS and (3.) to study miRNA-mRNA interactions and involved pathways.

In the first project of this dissertation, we compared the circulating miRNA expression profile quantified by NGS in serum samples of control patients and in pre- and postoperative serum samples of patients with the CD (the most prevalent subtype of ACTH-dependent CS) and CPA (the most prevalent type of ACTH-independent CS). 14 miRNAs were found to be differentially expressed in the pre- and postoperative groups. The results were validated by q-PCR in the discovery cohort and in an independent validation cohort. miRNA-182-5p was observed to be significantly upregulated in CD and CPA group compared to controls. ROC analysis for the diagnostic power showed promising results for circulating miR-182-5p to distinguish CS patients from controls (AUC 0.84, $p=0.0002$), and even better for CD patients (AUC 0.87, $p=0.0003$). This suggests that circulating miR-182-5p may function as a biomarker specifically for CD. Further, significant differences in miRNA expression were found in pre and post dexamethasone treatment, hinting at a hormonal regulation of the circulating miRNAs. In conclusion, it could be speculated that the presence or absence of ACTH might be at least as relevant for circulating miRNA expression as hypercortisolism itself.

In the second project, we investigated adrenal miRNA profiles, potential miRNA-mRNA targets and their related pathways in CS. For this, miRNA-based NGS was performed in adrenal tissues taken from patients with CPA, and from patients with CD after bilateral adrenalectomy, and from control subjects. The results revealed different miRNA patterns in CS subtypes. Validation analysis by q-PCR showed an upregulation of miR-1247-5p in CPA (log2-fold change>2.5, $p<0.05$) and of miR-379-5p in CD (log2-fold change>1.8, $p<0.05$). Further vali-

dation analyses in adrenals of patients with PBMAH and ectopic Cushing indicated that miR-1247-5p could contribute to cortisol production in CPA and PBMAH, while miR-379-5p could contribute to adrenal hyperplasia in PBMAH and CD. The expression of miRs-1247-5p and miR-379-5p was not changed in the adrenals of mice after short-term ACTH stimulation test.

For miRNA-mRNA target interaction, *in silico* prediction and RNA seq analyses revealed that both miR-1247-5p and miR-379-5p target specific genes in the Wnt signaling pathway. Additionally, Cyb5a, as a target of miR-1247-5p, was significantly downregulated in all CS samples. Luciferase assay suggests that miR-1247-5p and Cyb5 may interact by a non-canonical way in adrenal tissue.

In summary, this dissertation helps to elucidate the role of circulating and adrenal miRNAs in different subtypes of CS. We found miR-182-5p as a promising minimally invasive biomarker for CS. Target prediction, miRNA-mRNA interaction and pathway analysis gave us a more comprehensive understanding of the role of miRNA in CS pathogenesis.

5. Zusammenfassung (deutsch)

Das endogene Cushing-Syndrom ist eine seltene Erkrankung, die jedoch mit einer hohen Morbidität und Mortalität einhergeht. Obwohl mehrere diagnostische Tests zur Diagnosestellung des Cushing-Syndroms entwickelt wurden, bleibt die Diagnosestellung nach wie vor herausfordernd und zeitaufwändig. Zusätzlich zu den Herausforderungen ein Cushing-Syndrom richtig zu diagnostizieren, sind die Therapieoptionen sehr eingeschränkt. Die Operation, die als Erstlinientherapie abhängig von der jeweiligen Ursache des Hypercortisolismus (Nebennieren, Hypophyse oder Tumor) durchgeführt wird, führt zu einer lebenslangen Hydrocortison-Substitution sowie den Gefahren einer Nebennierenkrise. Eine medikamentöse Therapie führt selten zu einer langfristigen Kontrolle des Hypercortisolismus und ist reich an Nebenwirkungen.

In dieser Dissertation führten wir eine RNA-Sequenzierung in Serum- und Nebennieren-Proben von Patienten mit Cushing-Syndrom mit folgenden Zielen durch: (1.) Identifizierung zirkulierender Biomarker für das Cushing-Syndrom, (2.) Exploration des miRNA-Profils der Nebennieren bei ACTH-abhängigem und ACTH-unabhängigem Cushing-Syndrom, (3.) Untersuchung der miRNA-mRNA Interaktionen und der beteiligten Signalwege.

Im ersten Projekt der Dissertation verglichen wir die Expressionsprofile der zirkulierenden miRNA anhand von NGS-Daten der Serumproben der Patienten mit Morbus Cushing (CD, dem häufigsten Subtyp des ACTH- abhängigen Cushing Syndroms) und der Patienten mit Cortisol-produzierenden Nebennierenadenomen (CPA, dem häufigsten Subtyp des ACTH-unabhängigen Cushing Syndroms). Bei 14 miRNAs wurde eine signifikant unterschiedliche Expression in den prä- und postoperativen Proben gefunden. Die Ergebnisse wurden mittels q-PCR in der Entdeckungskohorte als auch in einer unabhängigen Validierungskohorte überprüft. miRNA-182-5p war signifikant hochreguliert in der CD- und in der CPA-Gruppe im Vergleich zur Kontrollgruppe. ROC-Analysen ergaben vielversprechende Ergebnisse für die diagnostische Aussagekraft von zirkulierendem miR-182-5p zur Unterscheidung von Patienten mit CS und Kontrollen (AUC 0,84, p = 0,0002) und war noch deutlicher in der Unterscheidung von Patienten mit CD und Kontrollen (AUC 0,87, p = 0,0003). Dies deutet darauf hin, dass zirkulierendes miR-182-5p als Biomarker speziell für CD fungieren könnte. Weiterhin wurden signifikante Unterschiede in der miRNA-Expression vor und nach der Behandlung mit Dexamethason gefunden, was auf eine hormonelle Regulation der zirkulierenden miRNAs hindeutet. Zusammenfassend lässt sich vermuten, dass das Vorhandensein oder Fehlen von ACTH für die

zirkulierende miRNA-Expression mindestens so relevant sein könnte wie der Hypercortisolismus selbst.

Im zweiten Projekt untersuchten wir die adrenalen miRNA-Profile, potenzielle miRNA-mRNA-Interaktionen und ihre betroffenen Signalwege bei CS. Dazu wurde miRNA-basiertes NGS in Nebennieren von Patienten mit CPA, in Nebennieren von Patienten mit CD nach bilateraler Adrenalektomie sowie in normalem Nebennierengewebe von Patienten mit Phäochromozytom (Kontrollen) durchgeführt. Die Ergebnisse zeigten unterschiedliche miRNA-Muster in den CS-Subtypen. Die Validierungsanalyse durch q-PCR zeigte eine Hochregulierung von miR-1247-5p in CPA (log2-fold change >2.5, $p<0.05$) und von miR-379-5p in CD (log2-fold change >1.8, $p<0.05$). Weitere Untersuchungen in Nebennieren von Patienten mit PBMAH und ektopem CS deuteten darauf hin, dass miR-1247-5p zur Cortisolproduktion bei CPA und PBMAH beitragen könnte, während miR-379-5p bei PBMAH und CD zur Nebennierenhyperplasie beitragen könnte. Die Expression von miRs-1247-5p und miR-379-5p war in den Nebennieren von Mäusen nach einem Kurzzeit-ACTH-Stimulationstest nicht verändert.

Für die miRNA-mRNA-Interaktion ergaben *in silico* Vorhersagen und RNA-Seq-Analysen, dass sowohl miR-1247-5p als auch miR-379-5p spezifische Gene im Wnt-Signalweg regulieren können. Darüber hinaus wurde Cyb5a als Zielgen von miR-1247-5p in allen CS-Proben signifikant herunterreguliert. Der Luciferase-Assay deutete darauf hin, dass miR-1247-5p und Cyb5 im Nebennierengewebe auf nicht-kanonische Weise interagieren können.

Zusammenfassend trägt diese Dissertation dazu bei, die Rolle von zirkulierenden und adrenalen miRNAs in verschiedenen Subtypen von CS aufzuklären. Wir identifizierten miR-182-5p als vielversprechenden minimal-invasiven Biomarker für CS. MiRNA Expressionspforfile, Analysen von miRNA-mRNA-Interaktion und den entsprechenden Signalwegen erweiterten unser Verständnis von der Rolle von miRNA in der Pathogenese des CS.

6. Paper I

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Circulating microRNA Expression in Cushing's Syndrome

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Context: Cushing's syndrome (CS) is a rare disease of endogenous hypercortisolism associated with high morbidity and mortality. Diagnosis and classification of CS is still challenging.

Objective: Circulating microRNAs (miRNAs) are minimally invasive diagnostic markers. Our aim was to characterize the circulating miRNA profiles of CS patients and to identify distinct profiles between the two major CS subtypes.

Methods: We included three groups of patients from the German Cushing's registry: ACTH-independent CS (Cortisol-Producing-Adenoma; CPA), ACTH-dependent pituitary CS (Cushing's Disease; CD), and patients in whom CS had been ruled out (controls). Profiling of miRNAs was performed by next-generation-sequencing (NGS) in serum samples of 15 CS patients (each before and after curative surgery) and 10 controls. Significant miRNAs were first validated by qPCR in the discovery cohort and then in an independent validation cohort of 20 CS patients and 11 controls.

Results: NGS identified 411 circulating miRNAs. Differential expression of 14 miRNAs were found in the pre- and postoperative groups. qPCR in the discovery cohort validated 5 of the significant miRNAs from the preoperative group analyses. Only, miR-182-5p was found to be significantly upregulated in the CD group of the validation cohort. Comparing all CS samples as a group with the controls did not reveal any significant differences in expression.

Outcome: In conclusion, our study identified miR-182-5p as a possible biomarker for CD, which has to be validated in a prospective cohort. Furthermore, our results suggest that presence or absence of ACTH might be at least as relevant for miRNA expression as hypercortisolism itself.

Keywords: cortisol, ACTH, miRNA, biomarker, cortisol-producing adenoma, miR-182-5p, hypercortisolism, miR-183 cluster

INTRODUCTION

Cushing's syndrome (CS) is a severe disease resulting from prolonged exposure to excessively high levels of cortisol (1). In the majority of patients hypercortisolism is due to ACTH secretion by corticotroph adenomas of the pituitary gland resulting in Cushing's disease (CD) (2). In approximately 20% of cases cortisol is secreted autonomously by the adrenal cortex. Adrenal dependent CS is mostly caused by unilateral cortisol-producing adrenal adenomas (CPA), rare causes are cortisol-secreting adrenocortical carcinomas (ACC), primary bilateral macronodular adrenocortical hyperplasia (PBMAH), bilateral CPAs and primary pigmented micronodular adrenal disease (PPMAD) (3, 4). Prolonged exposure to cortisol causes visceral obesity, resistance to insulin, osteoporosis, altered lipid and glucose metabolism, hypercoagulability, neuropsychiatric disorders and hypertension (5). The clinical features of CS vary widely, and no single specific symptom is present in every patient, making the diagnosis and subtyping of CS difficult and subject to false-positive and false-negative test results (6). However, timely and precise diagnosis of CS is crucial to avoid the high mortality and morbidity of affected patients (7). This clinical situation calls for more reliable, specific, and selective biomarkers for diagnosing CS.

MicroRNAs (miRNAs) are short (20–24 nucleotides) non-coding RNA molecules with diverse cellular regulatory functions including differentiation, proliferation and apoptosis (8). While the majority of miRNAs exist intracellularly, a significant number of miRNAs have been observed in extracellular compartments including plasma, serum, urine, saliva, semen, ascites, amniotic pleural effusions, and cerebrospinal fluid (9, 10). There are two major populations of circulating miRNAs, those vesicle-associated (which represents 90% of circulating miRNAs) and those non–vesicle-associated (11). Circulating miRNA profiles have been found to be very dynamic and abnormal levels of distinct miRNAs could be observed in specific biological stages of diseases and particularly during the development, invasion and metastasis of cancer (12). Circulating miRNAs fulfil several properties of non-invasive biomarkers, such as availability in various bodily fluids, sequence conservation between human and various preclinical models and availability of sensitive technologies for their quantification (13).

In recent years circulating miRNAs have been investigated as potential biomarkers for adrenal diseases with a total of eight studies investigating the pattern of extracellular miRNAs in adrenocortical tumors to date (14). The miRNAs investigated were found to aid in different forms of adrenal adenomas (15). Importantly, circulating miRNAs, miR-34a, and miR-483-5p were identified as candidate serum biomarkers distinguishing between benign and malignant adrenocortical tumors (16). In ectopic ACTH-dependent CS, there has been a single study reporting differences in plasma miR-expression (17). However, to the best of our knowledge, studies comparing different types of CS with each other have not yet been performed. The aim of this study was to compare circulating miRNA expression profiles of patients with different forms of CS and controls and evaluate their applicability as biomarkers. Therefore, we compared circulating miRNA profiles in serum samples of patients with CPA and patients with CD with those of patients in whom CS had been ruled out (controls). In addition to preoperative serum samples, we have also analyzed miRNA profiles in paired pre- and post-operative samples. To investigate possible short-term changes in miRNA profiles, we also analyzed serum samples after 1 mg low-dose dexamethasone testing in control patients.

MATERIALS AND METHODS

Patients and Ethics Approval

We conducted a retrospective, bi-centric study, based on a collection of pre- and post-operative serum samples of patients with Cushing's syndrome and controls at the Endocrinology Unit of the University Hospital of Munich and of the University Hospital of Würzburg. Diagnosis of CS followed current guidelines was based on the presence of relevant clinical features and biochemical confirmation through the following screening tests: increased 24-hour urinary free cortisol; loss of diurnal circadian cortisol rhythm with midnight salivary or serum cortisol concentrations; insufficiently suppressed serum cortisol levels after overnight administration of 1 mg of dexamethasone and suppressed plasma ACTH levels (as shown in **Table 1**). All patients were registered as part of ongoing registries and biobanks (ENS@T, European Network for the Study of Adrenal Tumor; NeoExNet, Exzellenz-Netzwerkes für neuroendokrine Tumoren Muenchen). The study was approved by the Ethics Committees of the University of Munich and Würzburg and written informed consent was obtained from all enrolled patients. All experiments were performed according to current guidelines and protocols. The discovery cohort contained 5 patients with overt adrenal dependent CS (CPA), 10 patients with overt pituitary dependent CS scheduled for surgery (CD) and 10 patients who were submitted in our outpatient clinic with suspicion of CS and in whom CS was ruled out (controls). Serum of the patients with CPA and CD was collected at the time of diagnosis preoperatively and after successful adrenalectomy and transsphenoidal pituitary surgery, respectively. Therefore, in total 40 serum samples of the discovery cohort were used for next-generation-sequencing (NGS): 5 preoperative CPA, 5 postoperative CPA, 10 preoperative CD, 10 postoperative CD, and 10 controls. As a confirmatory cohort, an independent series of 11 patients with CPA, 9 patients with CD and 11 controls were analyzed. Of the 11 controls of the confirmatory cohort also serum samples after 1 mg dexamethasone test were included (**Table 1**). Age and sex did not differ significantly between the groups (**Table 1**). All blood samples were collected in the morning (08:00–11:00 AM). All of our patients with CPA and CD, respectively, revealed postoperative adrenal insufficiency and were in need of a cortisol replacement therapy. At the timepoint of the postoperative blood sampling 87% of the patient still received hydrocortisone between 10 to 25 mg.

TABLE 1 | Clinical characteristics of the patient groups.

		Age at diagnosis/ surgery [years]	Sex [% Female]	BMI [kg/m²]	Hypertension [%positive]	Diabetes [% positive]	baseline ACTH [pg/ml]	Cortisol 24h Urine [nmol/24h]	midnight Cortisol [nmol/l]	Cortisol after 1 mg Dexamethasone [nmol/l]
Discovery Cohort	Controls (n=10)	50 [43;56]	50	33 [30:35]	70	30	3.1 [2.7;3.7]	549 [375;610]	24.8 [17.4;42.2]	27.6 [22.1;29.7]
	CPA (n=5)	58 [48;63]	60	27 [24;28]	60	40	0.4 [0.4;0.4]	876 [506;1346]	242.9 [135.2;397.4]	347.8 [135.2;408.5]
	CD (n=10)	51 [40;57]	50	31 [26;33]	80	50	16.7 [11.0;21.4]	2122 [1991;2868]	165.6 [74.5;460.9]	425.0 [339.5;778.3]
Validation Cohort	Controls (n=11)	45 [29;52]	36	36 [31;40]	54	27	2.2 [2.0;2.9]	270 [223;345]	41.4 [35.9;49.7]	30.4 [24.8;41.4]
	CPA (n=11)	50 [43;53]	72	29 [26;36]	81	36	1.9 [1.6;2.0]	639 [318; 946]	276.0 [182.2;350.5]	532.7 [386.4;623.8]
	CD (n=9)	47 [41;58]	88	26 [25;41]	66	44	14.0 [7.2;21.1]	243 [152; 314]	160.1 [91.1;34.0]	201.5 [121.4;634.8]

Data are given as median with 25th and 75th percentile in brackets.
CPA, cortisol producing adenoma; CD, Cushing's disease; BMI, body mass index.

RNA Extraction

Total RNA isolation was carried out from all serum samples (450 µl) by miRNeasy Serum/Plasma Kit (Qiagen GmbH, Hilden, Germany) and stored at −80°C until further use.

Sample Processing and miRNA Expression Profiling from Serum Samples by Next-Generation Sequencing (NGS)

RNA integrity and absence of DNA was confirmed by Bioanalyzer RNA Nano chips (Agilent Technologies, St. Louis, MO) and Qubit DNA High sensitivity kit, respectively. Sequencing libraries were prepared using the Illumina TruSeq Small RNA Library Preparation Kit. Next generation sequencing was performed on 2 lanes of an Illumina HiSeq2500 (Illumina, San Diego, CA) multiplexing all samples (single end read, 50 bp). The quality of sequencing reads was verified using FastQC0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc) before and after trimming. Adapters were trimmed using cutadapt (18). Reads with <15 bp and >40 bp insert sequences were discarded. Alignment of reads was performed using miRBase V21 (19) with sRNAbench (20). For normalization and identification of differentially expressed miRNAs EdgeR and DeSeq in R was used (21–23). miRNAs with an at least 5 raw count per library were included. Disease groups were compared using the unpaired Mann–Whitney test, and, to decrease the false discovery rate, a corrected p-value was calculated using the Benjamini–Hochberg method. Adjusted $p < 0.05$ and log2 fold of change >1.5 were the cut-off for significance. The RNA seq data generated in this study have been submitted to the NCBI GEO with accession number GSE156693.

Validation of Individual miRNAs

Significantly differentially expressed miRNAs found by NGS were validated by RT-qPCR. Reverse transcription of RNA was performed using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific). As reference miRNA, hsa-miR-16-5p was used (12, 24, 25). Quantitative real-time PCR was performed using the TaqMan Fast Universal PCR Master Mix (2x) (CN: 4352042; Thermo Fisher Scientific) on a Quantstudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific) in accordance with the manufacturer's protocol for TaqMan Advanced miRNA assays (CN: A25576; Thermo Fisher Scientific). The miRNAs along with their respective TaqMan assay IDs are given in **Table S1** (26). Contamination controls contained no cDNA templates.

Statistical Analysis and Software

R version 3.6.1 was used for statistical analyses. To identify miRNAs differentially expressed, generalized linear model (GLM, a flexible generalization of ordinary linear regression that allows for variables that have distribution patterns other than a normal distribution) in the software package edgeR (Empirical Analysis of DGE in R) was employed to calculate *p*-values (25, 27). *p*-values were adjusted using the Benjamini–Hochberg false discovery rate (FDR) procedure (27). GraphPad Prism Version 8 was used for statistical analysis of qPCR results. To quantify the miRNAs in qPCR, the dCt method [delta Ct (cycle threshold) value equals target miRNA's Ct minus housekeeping miRNA's Ct was used (Excel 2016, Microsoft, Redmond, WA, USA). The ANOVA test with Bonferroni correction for three groups and Mann-Whitney test for two groups (CS vs Controls) were used for differentially expressed comparisons (28, 29). Receiver operating characteristic (ROC) analysis was performed on miRNAs that could have potential utility as minimally invasive biomarkers. $p < 0.05$ was considered significant.

RESULTS

miRNA Expression Profiles

NGS was performed in the discovery cohort of 5 CPA (before and after curative surgery), 10 CD (before and after curative surgery), and 10 control serum samples (total n= 40) and detected a total of 411 miRNAs. No significant influence of batch preparation, age, and sex on the miRNA expression profile was found [**Figure S1** (26)]. Differential analysis was performed between the groups (CPA, CD, and controls) to identify any characteristic miRNA profile associated with one of the entities before and after surgery, respectively. No significant changes

were observed based on the comparison of all CS samples pooled together versus control samples (**Figure 1A**). Differential expression levels in three miRNAs, namely miR-185-5p, miR-146b-5p, and miR-342-3p were found when comparing CPA samples with controls (**Figure 1B**), while only miR-182-5p was found to be significantly regulated between CD samples and controls (**Figure 1C**). Post-hoc analyses between CPA and CD revealed that the most pronounced differences included 6 miRNAs: miR-96-5p, miR-146b-5p, miR-183-5p, miR-185-5p, miR-616-5p, and miR-629-5p (**Figure 1D**). Sequencing of the postoperative samples showed significantly different expression of four miRNAs (miR-429, miR-141-3p, miR-215-5p, miR-200a-3p) between CPA and controls [**Figure S2B** (26)]. No significant differences were observed while comparing all postoperative CS samples pooled together with control samples [**Figure S2A** (26)], the postoperative CD samples with controls [**Figure S2C** (26)] and the postoperative CPA serum samples vs CD postoperative samples [**Figure S2D** (26)]. Analysis of the preoperative samples revealed no major difference in the distribution of the top 20 abundant genes compared to the controls [**Figure S3** (26)].

Validation of Selected miRNAs by qPCR

NGS identified 8 miRNAs that were significantly dysregulated in the preoperative group and 4 miRNAs that were significantly altered in the postoperative group (see **Table 2**). qPCR analysis of these miRNAs revealed significant differences in 5 out of the 8 preoperative miRNAs (miR-185-5p, miR-183-5p, miR-182-5p, miR-146b-5p, and miR-96-5p), but in none of the postoperative cohort samples [**Figure S4** (26)]. To further validate the results from the discovery cohort, we assessed the remaining five significant miRNAs in an independent set of samples (CPA, n=11; CD, n=9 controls, n=11). Only miR-182-5p was confirmed to be significantly upregulated in the preoperative group of the independent cohort (**Figure 2B**). The diagnostic utility of miR-182-5p was evaluated using a ROC analysis (**Figure 3**). For CD samples from both discovery and validation cohorts, the area under curve (AUC) was 0.87, (95% confidence interval: 0.7 to 1.0, P = 0.0003), while for the CS samples pooled together the area under curve (AUC) was 0.84 (95% confidence interval:0.7 to 0.9, P = 0.0002). The AUC was further lower for CPA samples, 0.8 (95% confidence interval:0.5 to 0.9, P = 0.003). Further, correlation analyses of the gene expression levels with clinical parameters revealed no significant results (**Table S2**). As none of the postoperative miRNAs were found to be significantly different in the discovery cohort, they were not further analyzed in the validation cohort. Finally, to investigate whether the five selected circulating miRNAs are affected by short-term exogenous glucocorticoids, we analyzed the

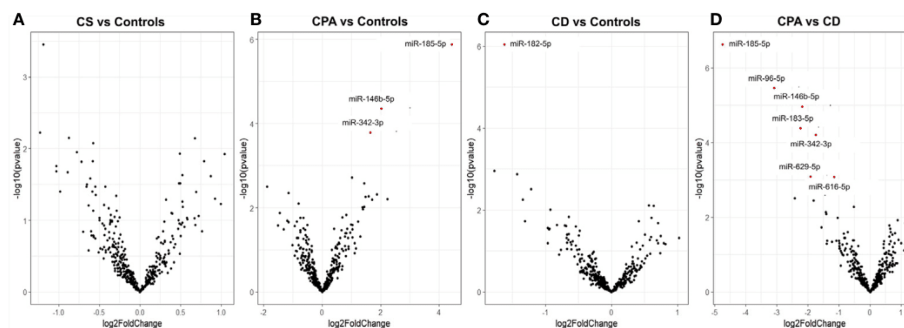

FIGURE 1 | Differentially expressed miRNAs from NGS of preoperative group. Volcano plot showing the relationship between fold change (log2foldchange) and statistical significance (−log10pvalue). The red points in the plot represent the differentially expressed miRNAs with FDR <0.05 considered as statistically significant. CPA, cortisol producing adenoma; CD, Cushing's Disease, CS, Cushing syndrome represents CPA and CD, taken together. Volcano plot showing the relationship between fold change (log2foldchange) and statistical significance (-log10pvalue) for the groups CS vs Controls **(A)**, CPA vs. Controls **(B)**, CD vs. Controls **(C)**, CPA vs CD **(D)**.

TABLE 2 | Comparison of results of RNA Sequencing and qPCR in selected miRNAs.

	RNA sequencing					qPCR		
Gene symbol	Mean Count	Position	CPA vs Controls	CD vs Controls	CPA vs CD	CPA vs Controls	CD vs Controls	CPA vs CD
miR-96-5p	689	122	1.31	−1.77	−3.08***	−0.22	1.77*	−1.99*
miR-146b-5p	842	114	2.03*	−0.16	−2.18**	−0.38	2.57**	−2.95*
miR-182-5p	**5,493**	**46**	**−0.58**	**−1.62*** **	**−1.03**	**0.75**	**1.73***	**−0.98**
miR-183-5p	885	112	0.81	−1.43	−2.23**	0.07	2.20*	−2.14
miR-185-5p	40	313	4.43	−0.31	−4.74***	0.54	1.78*	−1.24
miR-342-3p	1,024	105	1.64	−0.10	−1.75*	0.47	0.31	0.16
miR-616-5p	31	337	0.83	−0.32	−1.15*	0.59	0.99	−0.40
miR-629-5p	37	318	1.59	−0.33	−1.92*	−0.15	−1.02	0.87

**P < 0.05, **P < 0.01, and ***P < 0.001. Gene expression is represented as log2fold change. CPA, cortisol producing adenoma; CD, Cushing's disease. The potential biomarker candidate has been highlighted in bold.*

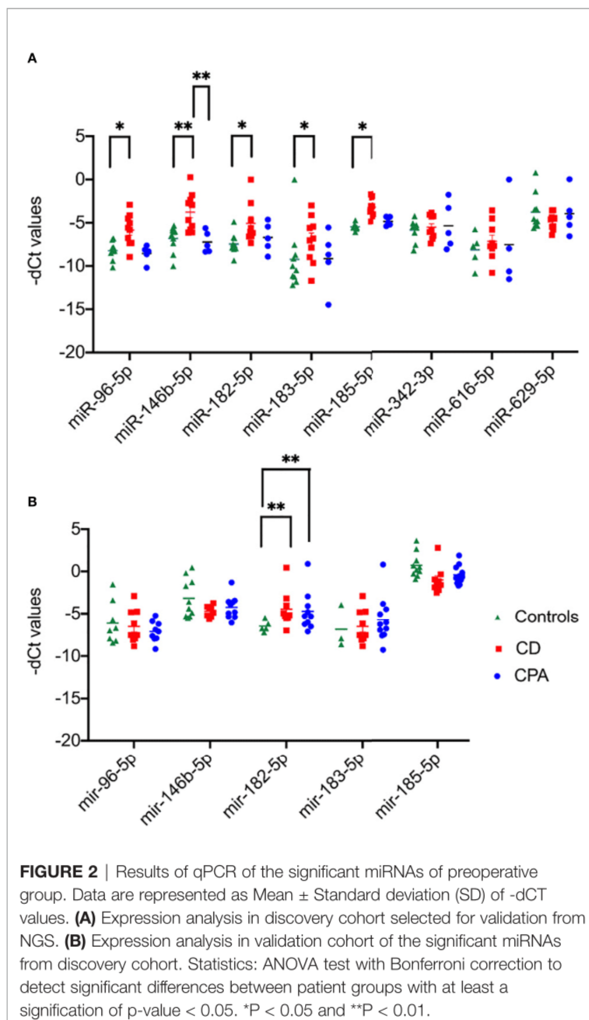

FIGURE 2 | Results of qPCR of the significant miRNAs of preoperative group. Data are represented as Mean ± Standard deviation (SD) of -dCT values. **(A)** Expression analysis in discovery cohort selected for validation from NGS. **(B)** Expression analysis in validation cohort of the significant miRNAs from discovery cohort. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a signification of p-value < 0.05. *P < 0.05 and **P < 0.01.

expression levels of the miRNAs in serum samples before and after 1 mg of dexamethasone was given 9 h before the control patients of the independent cohort. miR-185-5p and miR-96-5p expression was found to be significantly modulated by dexamethasone treatment (**Figure 4**).

DISCUSSION

Diagnosis of CS is still challenging due to the broad symptomatology and the clinical overlap with metabolic syndrome (30). Upon CS diagnosis, treatment is complicated by difficulties in distinguishing between the different subtypes of CS. Of the CS subtypes, CD is the dominant form and a variety of dynamic tests and robust imaging procedures are necessary for diagnosis (5). Unsuspected pituitary and adrenal incidentalomas are increasingly found with the widespread use of abdominal and cranial imaging (31). For pituitary incidentaloma the rate ranges between 3.7% and 37% (32). Therefore, we hypothesized that a potential miRNA based diagnostic should aid in the diagnosis of CS or its subtypes as circulating miRNAs have proven their potential as minimal invasive biomarkers in other diseases (33). Few studies have investigated circulating miRNA profiles in CS, and appropriate controls matched for sex, BMI and comorbidities were missing (17, 34). Therefore, in our study we included clinically relevant control samples to identify a unique circulating miRNA signature associated with CS. The control group consisted of patients submitted with the suspicion of CS in the outpatient clinic but in whom CS was ruled out by several diagnostic tests and follow-up. We decided deliberately for such a control group, as this reflects clinical practice and a new biomarker would have to demonstrate relevance in this clinical scenario. In addition, to ensure technical consistency across the various biological samples, the RNA from different groups, including controls, were isolated in batches and possible batch preparation effect was also analyzed in the NGS data. As can be seen [**Figure S1** (26)], no major changes were found, indicating the changes observed in the NGS data, likely arises from inherent biological differences and not due to batch effect. The sequencing data were also found to not be influenced by both age and sex [**Figure S1** (26)].Only miR-182-5p was confirmed as differentially expressed by qPCR of the discovery and the validation cohorts. ROC analysis for the diagnostic power of miR-182-5p resulted in an AUC of 0.89 in separating CD patients from controls, and an AUC of 0.85 in differentiating all patients with CS from controls. This suggests that circulating miR-182-5p may function as a biomarker specifically for CD. Our observation draws strength from related studies wherein circulating miR-182-5p has been successfully identified as a diagnostic biomarker in colorectal adenocarcinoma (35) and coronary artery disease (36). Furthermore, the overexpression of miR-182-5p in prolactin-secreting pituitary adenomas tissues has also been documented (37). However, the potential use of circulating miR-182-5p as a biomarker in CD should be seen with caution due to the differences observed between NGS and qPCR. While miR-182-5p was found to be significantly downregulated in CD in comparison to controls by NGS (**Table 2**), qPCR in discovery and validation cohorts revealed significant upregulation of CD in comparison to controls (**Table 2** and **Figure 2**). This difference could be possibly explained by the high sensitivity observed with qPCR in comparison to the high throughput genomic profiling technologies including NGS and microarray (38). Concurrently, studies with a wholesome approach to circulating miRNAs, namely through microarray and NGS, are often not able to validate their sequencing and array results (25, 34). miR-182-5p along with miR-96-5p and miR-183-5p, were also found to be deregulated in CPA and CD samples compared to the controls (**Figures 1B, C**). Altogether these miRNas belong to the miR-183 cluster. This miR-183 cluster is known to play a role in a variety of non-sensory diseases, including cancer and neurological, psychiatric and auto-immune disorders (39). Furthermore, the expression profiles of the miR-183 cluster was found to be altered in adrenal tissues of patients with pheochromocytoma (40).

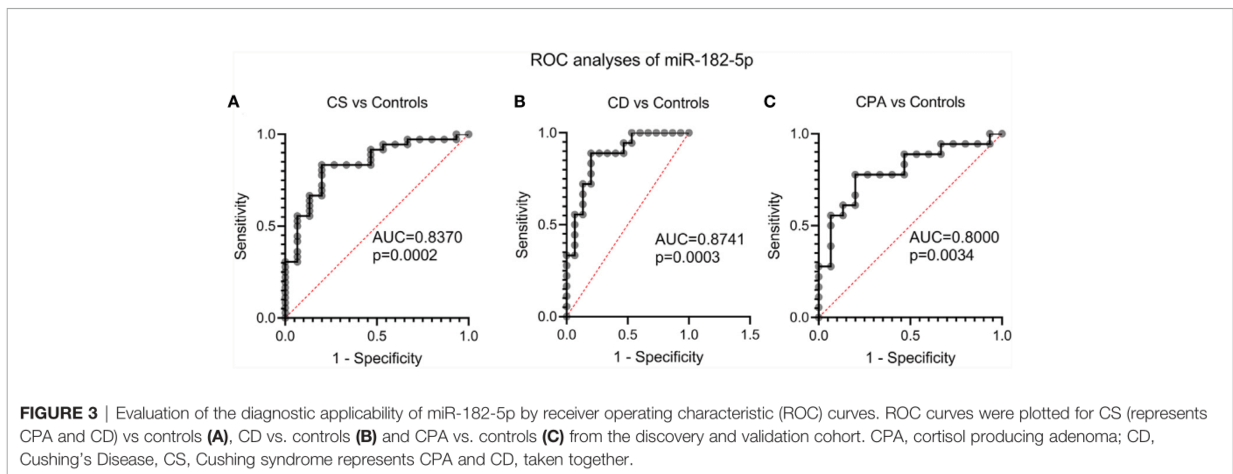

FIGURE 3 | Evaluation of the diagnostic applicability of miR-182-5p by receiver operating characteristic (ROC) curves. ROC curves were plotted for CS (represents CPA and CD) vs controls **(A)**, CD vs. controls **(B)** and CPA vs. controls **(C)** from the discovery and validation cohort. CPA, cortisol producing adenoma; CD, Cushing's Disease, CS, Cushing syndrome represents CPA and CD, taken together.

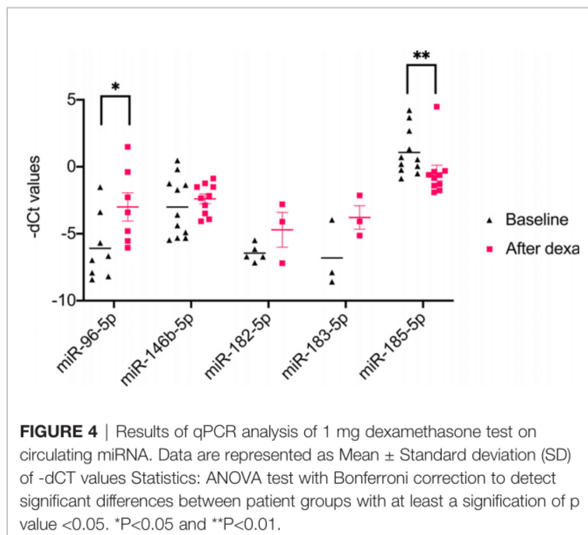

FIGURE 4 | Results of qPCR analysis of 1 mg dexamethasone test on circulating miRNA. Data are represented as Mean ± Standard deviation (SD) of -dCT values Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a signification of p value <0.05. *P<0.05 and **P<0.01.

Surprisingly, NGS did not identify any distinct miRNA expression pattern between CS taken as a single group and the control group (**Figure 1A**). ACTH itself might lead to changes in the circulating miRNA profile that are more relevant as the hypercortisolism which is present in both forms of CS. The modulation of miRNAs by ACTH has been characterized *in mice* (41) but distinct studies of ACTH modulation on circulating miRNAs are missing. We speculate that the similarity in phenotype between CS and this specific control group might have contributed to this observation, too. Additionally, including only patients with benign tumors might have led to the discovery of a low amount of changes in circulating miRNA profiles as cell disruption and release of miRNAs from tumour cells into the blood, typical features of malignant neoplasms, are minimal.

The CPA samples show a far more diverging pattern for their qPCR expression patterns in both the validation and discovery cohort. While only miRNA-146b and miRNA-96-5p were found to be significantly altered between CPA and controls of the discovery cohort, similar differential expression was not observed in the CPA of the validation cohort. The discrepancy might be explained by possible heterogeneity in CPA samples, speculated from previous adenoma studies (34). Decmann et al. recently reported heterogeneity associated with complex underlying pathology to be the possible reason for the observed discordance between NGS and qPCR of adenoma samples.

Finally, we also investigated the influence of short-term regulation of the pituitary-adrenal-axis on circulating miRNAs. For this analysis we focused on the 5 selected miRNAs miR-96-5p, miR-146b-5p, miR-182-5p, miR-183-5p, and miR-185-5p. Of the five selected miRNAs, miR-96-5p was significantly upregulated and miR-185-5p was significantly downregulated in post dexamethasone samples. Hormonal regulation of adrenal related miRNAs has been established in two murine *in vivo* studies for miR-96-5p (41, 42). For miR-185-5p, *in vitro* evidence of the influence of dexamethasone exist through the observed downregulation of the gene miR-185-5p during ameloblast differentiation under the influence of dexamethasone treatment (43). Therefore, it is tempting to speculate that the changes observed in the post dexamethasone samples could be a hint of possible hormonal regulation on the expression of these genes. Thusly, this influence needs to be taken into account in case of possible future application of the miRNAs in diagnostic setting involving dexamethasone.

The origin of circulating miRNAs is still subject to speculation. Since CS is not a malignant disease there is no concrete proof that the miRNAs significantly altered in the circulation are the result of specific tissue dysfunctions. Therefore, the study was limited only to identify and characterize circulating miRNA changes. Concurrently, there have been similar reports of increased miR182-5p expression in prolactin pituitary tumors (37) and ACTH induced up-regulation of miRNA-182-5p in murine adrenal glands. For a comprehensive mechanistic understanding behind 182-5p in CD in depth tissue based molecular analyses is required which is beyond the scope of the current paper (42).

In conclusion, we report distinct miRNA expression profiles associated with CD and CPA through NGS and qPCR confirmed miR-182-5p as the isolated miRNA to be differentially regulated. The identification of only one miRNA as a potential biomarker is speculated to be because of the following reasons: (1) We worked with 3 groups (CPA vs. CD vs. controls), and did an overall analysis and then post-hoc analysis. In this constellation it is more difficult to get significant results. (2) The number of patients per group was relatively low. (3) The controls were not healthy, but showed some similar clinical features as patients with Cushing's syndrome. (4) As CPA and CD is a benign disease, there is no cell rupture and therefore, no release of miRNAs in the circulation. Technical and biological variations observed in the study warrants analysis using larger cohorts and more robust clinically relevant controls before considering miR-182-5p as a biomarker. Furthermore, our results suggest that presence or absence of ACTH might be at least as relevant for hypercortisolism per se.

DATA AVAILABILITY STATEMENT

The data sets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, GSE156693.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committees of the University of Munich and Würzburg. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SV, FB, MR, AC, and SS conceived and planned the experiments. BA, LB, AO, MB, MF, and MR collected the data and provided samples. SV, RZ, ME, and AR performed the experiments. FB, MR, AC, SS, and AR contributed to the interpretation of the results. SV and AR wrote the manuscript. All authors provided critical feedback. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.620012/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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7. Paper II

International Journal of
Molecular Sciences

Article

Characterization of Adrenal miRNA-Based Dysregulations in Cushing's Syndrome

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Abstract: MiRNAs are important epigenetic players with tissue- and disease-specific effects. In this study, our aim was to investigate the putative differential expression of miRNAs in adrenal tissues from different forms of Cushing's syndrome (CS). For this, miRNA-based next-generation sequencing was performed in adrenal tissues taken from patients with ACTH-independent cortisol-producing adrenocortical adenomas (CPA), from patients with ACTH-dependent pituitary Cushing's disease (CD) after bilateral adrenalectomy, and from control subjects. A confirmatory QPCR was also performed in adrenals from patients with other CS subtypes, such as primary bilateral macronodular hyperplasia and ectopic CS. Sequencing revealed significant differences in the miRNA profiles of CD and CPA. QPCR revealed the upregulated expression of miR-1247-5p in CPA and PBMAH (log2 fold change > 2.5, $p < 0.05$). MiR-379-5p was found to be upregulated in PBMAH and CD (log2 fold change > 1.8, $p < 0.05$). Analyses of miR-1247-5p and miR-379-5p expression in the adrenals of mice which had been exposed to short-term ACTH stimulation showed no influence on the adrenal miRNA expression profiles. For miRNA-specific target prediction, RNA-seq data from the adrenals of CPA, PBMAH, and control samples were analyzed with different bioinformatic platforms. The analyses revealed that both miR-1247-5p and miR-379-5p target specific genes in the WNT signaling pathway. In conclusion, this study identified distinct adrenal miRNAs as being associated with CS subtypes.

Keywords: cortisol; ACTH; miRNA; Cushing's; hypercortisolism; pituitary

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1. Introduction

Cushing's syndrome (CS) results from the excessive secretion of cortisol, leading to visceral obesity, resistance to insulin, osteoporosis, and altered lipid and glucose metabolism [1,2]. Excessive production of cortisol by the adrenal glands can be either ACTH-dependent or -independent. In the majority of patients, hypercortisolism is due to ACTH secretion by corticotroph adenomas of the pituitary gland (Cushing's disease, CD) or by ectopic tumors [3]. Approximately 20% of cases are ACTH-independent, where cortisol is secreted autonomously by the adrenal cortex. The pathology of ACTH-independent cases is diverse; they are most often caused by unilateral cortisol-producing adrenocortical adenomas

(CPA). Rare causes are cortisol-secreting adrenocortical carcinomas (ACC), primary bilateral macronodular adrenocortical hyperplasia (PBMAH), bilateral CPAs, and primary pigmented nodular adrenal disease (PPNAD) [4,5]. Irrespective of the subtype, prolonged exposure to cortisol in CS is associated with increased mortality and cardiovascular morbidity in its patients [6]. Treatment is based on the underlying cause of hypercortisolism, with pituitary surgery or adrenalectomy being the preferred choice. Medical therapy options in CS are few and consist of pituitary-directed drugs, steroid synthesis inhibitors, and glucocorticoid receptor antagonists [7]. For the timely diagnosis and targeted management of CS and its subtypes, a comprehensive understanding of cortisol secretion, in terms of canonical signaling pathways as well as upstream epigenetic factors, is needed.

MiRNA molecules have emerged as key epigenetic players in the transcriptional regulation of cortisol production. Briefly, the deletion of *Dicer* in adrenals, a key miRNA processing enzyme, revealed diverse expression changes in miRNAs along with related changes in steroidogenic enzymes such as *Cyp11b1* [8]. Furthermore, key enzymes in the cortisol biosynthesis pathway, namely *Cyp11a1*, *Cyp21a1*, *Cyp17a1*, *Cyp11b1*, and *Cyp11b2*, were also found to be regulated by various miRNAs (miRNA-24, miRNA-125a-5p, miRNA-125b-5p, and miRNA-320a-3p) in in vitro studies [9]. Consequently, various studies have also characterized miRNA expression profiles in CS subtypes. Importantly, miRNA expression in the corticotropinomas of CD patients was found to vary according to USP8 mutation status [10]. Other studies have also identified specific miRNA candidates and associated target genes in the adrenals of patients with PPNAD [11], PBMAH [12,13], and massive macronodular adrenocortical disease [14]. Interestingly, no common miRNA candidates were found among these studies, indicating the specificity of miRNAs to the different underlying pathologies in CS.

There are limited studies directly comparing miRNA expression profiles of ACTH-dependent and ACTH-independent CS patients. Consequently, in our previous study, we found differences in expression profiles when comparing circulating miRNAs in CD and CPA patients [15]. We hypothesized that the presence of ACTH possibly influences the miRNA profile in serum due to the upstream differential expression in the origin tissues. In this study, we aim to further explore this hypothesis by comparing the miRNA expression profile of adrenal tissues in ACTH-dependent and ACTH-independent CS. In brief, miRNA specific sequencing was performed in two prevalent subtypes of CS: in CD, the most prevalent ACTH-dependent form; and in CPA, the most prevalent ACTH-independent form. Specific miRNA candidates related to each subtype were further validated in other forms of CS. To further investigate our hypothesis, the response of miRNA candidates following ACTH stimulation was assessed in mice, and the expression of miRNAs in murine adrenals was subsequently investigated. Finally, an extensive targeted gene analysis was performed based on in silico predictions, RNA-seq data, and luciferase assays.

2. Results

2.1. Differentially Expressed miRNAs

NGS revealed differentially expressed miRNAs between the different groups analyzed (Figure 1). CD and CPA taken together as CS showed a differentially expressed profile (42 significant miRNAs) in comparison to controls. Moreover, individually, CPA and CD were found to show a significantly different expression profile in comparison to controls (n = 38 and n = 17 miRNAs, respectively). Interestingly, there were no significantly upregulated genes in the adrenals of patients with CD in comparison to the control adrenals. A comparative analysis of the top significant miRNAs (log2 fold change (log2 FC) > 1.25 & $p < 0.005$) between the two groups was performed and the representative Venn diagrams are given in Figure 2. Briefly, miR-1247-5p, miR-139-3p, and miR-503-5p were significantly upregulated in CPA, in comparison to both CD and controls. Furthermore, miR-150-5p was specifically upregulated in CPA as compared to CD. Several miRNAs (miR-486-5p, miR-551b-3p, miR-144-5p, miR-144-3p, and miR-363-3p) were found to be significantly downregulated in the groups of CPA and CD in comparison to controls. MiR-

19a-3p and miR-873-5p were found to be commonly downregulated in CPA in comparison to both CD and controls. Principal component analyses based on miRNA sequencing did not identify any major clusters among the samples. Furthermore, the miRNA profile was not different among the CPA samples based on the mutation status of PRKACA (Supplementary Materials Figure S1).

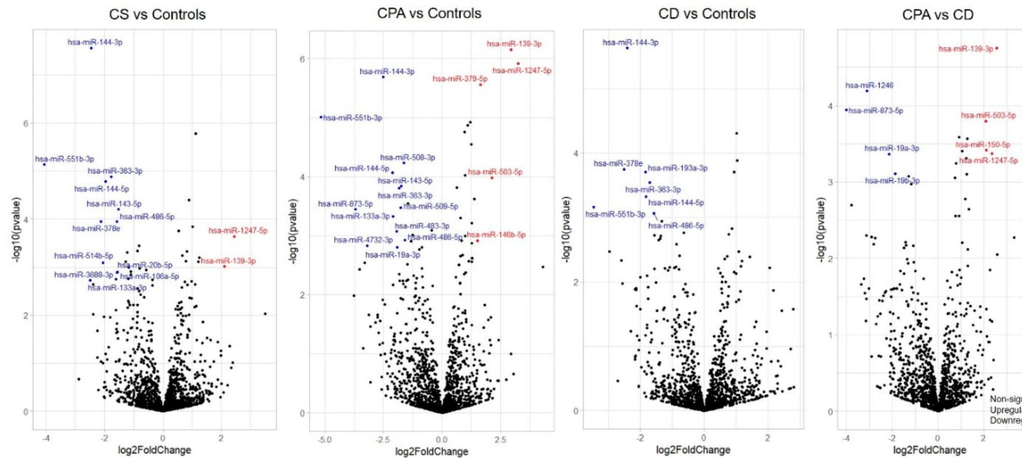

Figure 1. Differentially expressed miRNAs from sequencing. Volcano plot showing the relationship between fold change (log2 fold change) and statistical significance (−log10 *p* value). The red points in the plot represent significantly upregulated miRNAs, while blue points represent significantly downregulated miRNAs. CPA, cortisol producing adenoma; CD, Cushing's disease; Cushing's syndrome represents CPA and CD, taken together.

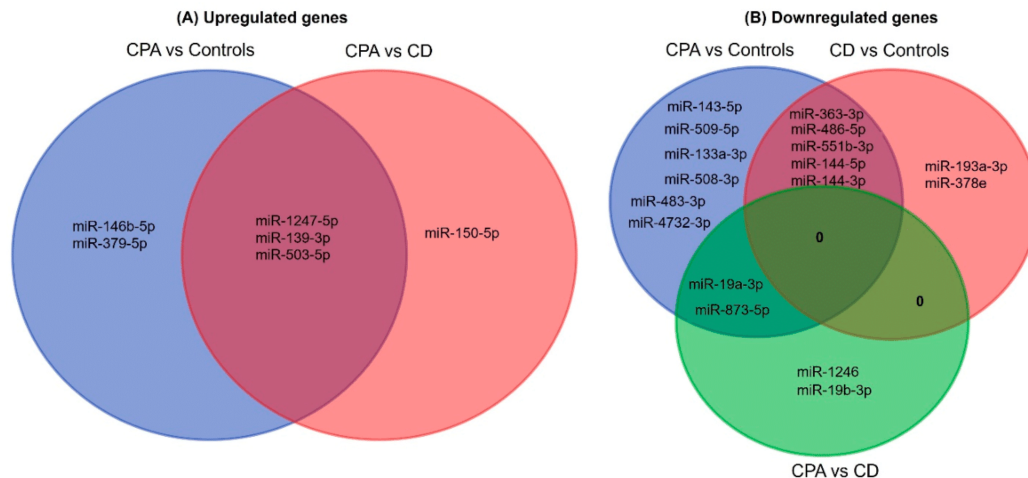

Figure 2. Venn analyses of the common significant miRNAs from each group. The significantly expressed miRNAs from each sequencing analysis were shortlisted and compared between the groups. CPA, cortisol producing adenoma; CD, Cushing's disease.

2.2. Validation and Selection of Candidate miRNAs

For validation by QPCR, the most significant differentially expressed miRNAs (log2 FC > 1.25 & $p < 0.005$) among the groups were chosen (Table S1). According to the current knowledge, upregulated miRNAs are known to contribute more to pathology

than downregulated miRNAs [16]. Since the total number of significantly upregulated miRNAs was six, all these miRNAs were chosen for validation. Contrarily, 25 miRNAs were significantly downregulated among the groups. In particular, miR-486-5p, miR-551b-3p, miR-144-5p, miR-144-3p, and miR-363-3p were found to be commonly downregulated in the CS group in comparison to controls; therefore, these miRNAs were chosen for validation.

Among the upregulated miRNA candidates, miR-1247-5p QPCR expression confirmed the NGS data (Figure 3A, Table S1). Moreover, miR-150-5p and miR-139-3p were upregulated in CPA specifically in comparison to CD, and miR-379-5p was upregulated in CD in comparison to controls by QPCR. In the case of downregulated genes, none of the selected miRNAs could be confirmed by QPCR (Figure 3B). Thus, analysis of the six upregulated and five downregulated miRNAs from NGS yielded two significantly upregulated miRNA candidates, miR-1247-5p in CPA and miR-379-5p in CD, when compared to controls. These miRNA candidates were taken up for further QPCR validation in an independent cohort of other subtypes of CS (Figure 4), namely ACTH-dependent ectopic CS (n = 3) and ACTH-independent PBMAH (n = 10). The QPCR analysis in the other subtypes revealed miR-1247-5p to be consistently upregulated in ACTH-independent CS (PBMAH and CPA) in comparison to ACTH-dependent CS (CD and ectopic CS) and controls. On the other hand, miR-379-5p was upregulated in CD and PBMAH in comparison to controls.

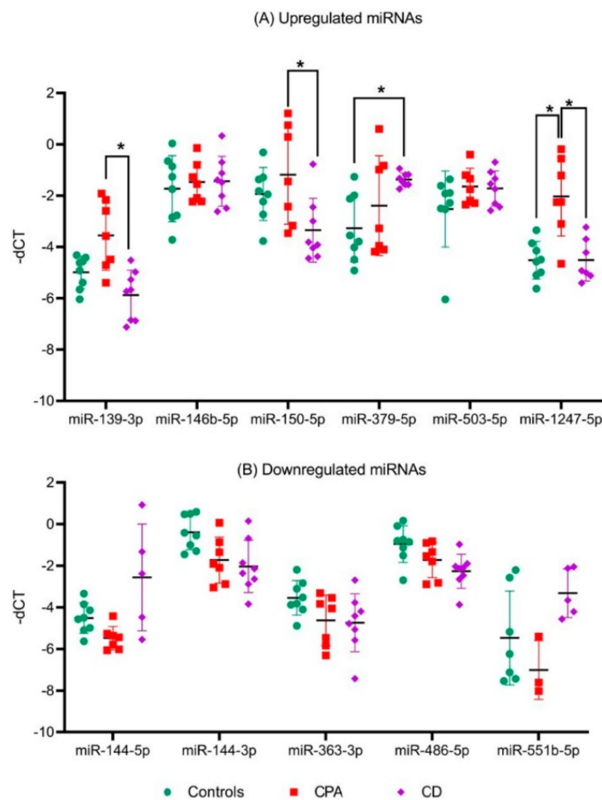

Figure 3. QPCR analyses of significant miRNAs from sequencing analyses. Data are represented as mean ± standard deviation (SD) of −dCT values: (**A**) Expression analysis of significantly upregulated miRNAs; (**B**) Expression analysis of common significantly downregulated miRNAs. Housekeeping gene: miR-16-5p. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a significance of *p*-value < 0.05 (*).

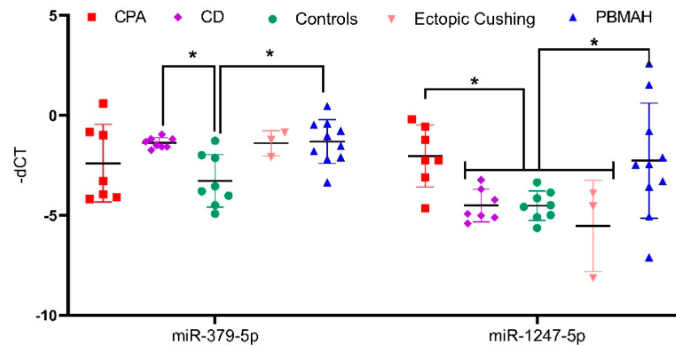

Figure 4. QPCR analyses of significantly upregulated miRNAs from validation QPCR. Data are represented as mean ± standard deviation (SD) of −dCT values. Housekeeping gene: miR-16-5p. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a significance of *p*-value < 0.05 (*).

2.3. In Vivo Assessment of ACTH-Independent miR-1247-5p

To analyze the influence of ACTH on miRNA expression, the expression of miR-1247-5p and miR-379-5p were assessed in the adrenal tissues of ACTH stimulated mice at different time points. For this analysis, miR-96-5p was taken as a positive control, as it has previously been reported to be differentially expressed in ACTH stimulated mice [17]. The analyses revealed that the expression of miR-1247-5p and miR-379-5p did not change at different timepoints of the ACTH stimulation (Figure 5). Meanwhile, the positive control of mir-96-5p showed a dynamic expression pattern with upregulation after 10 min, followed by downregulation at the subsequent 30 and 60 min time points, in concordance with previously reported findings [18].

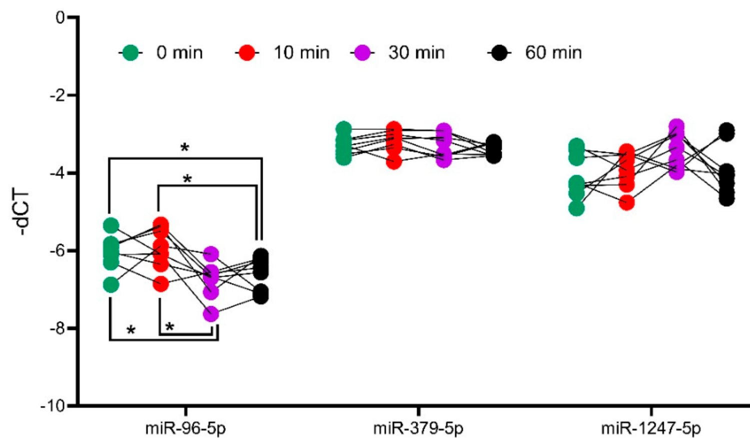

Figure 5. Analysis of miRNA expression in ACTH stimulated mice tissue. QPCR analyses of positive controls, miR-96-5p, and candidates miR-379-5p and miR-1247-5p. Mice were injected with ACTH, and adrenals were collected at different timepoints to assess the impact of ACTH on miRNA expression. Data are represented as mean ± standard deviation (SD) of −dCT values. Housekeeping gene: miR-26a-5p. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a significance of *p*-value < 0.05 (*).

2.4. In Silico Analyses of miRNA Targets

Two diverse approaches were employed for a comprehensive in silico analysis of the miRNA targets. First, the predicted targets of miR-1247-5p and miR-379-5p were taken

from the TargetScan database, which identified miRNA–mRNA target pairs based on sequence analyses [19]. The expression status of these targets was then checked in the RNA sequencing data from CPA vs. controls (miR-1247-5p) and PBMAH vs. controls (miR-379-5p). Targets that showed significant expression changes in the sequencing data were shortlisted (Figure 6A). Among the 1061 predicted miR-1247-5p targets, 28 genes were found to show significant expression changes in CPA (20 upregulated, 8 downregulated). On the other hand, for 124 predicted miR-379-5p targets, 23 genes were found to show significant expression changes in PBMAH (20 upregulated, 3 downregulated). Interestingly, the selected targets were found to be unique for each miRNA, except for FICD (FIC domain protein adenylyltransferase) (Figure 6B).

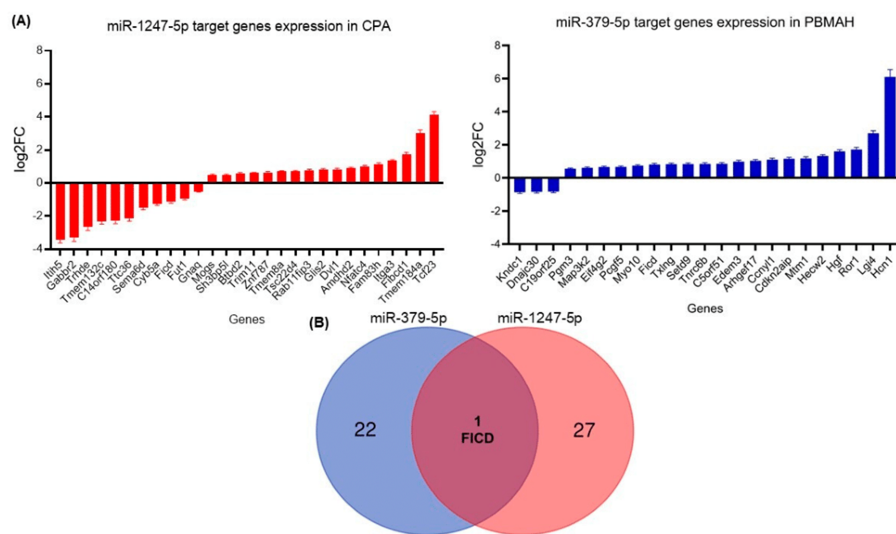

Figure 6. (**A**) Differentially expressed target genes of miRNAs from sequencing. Data are represented as log2 fold change in comparison to the controls. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a significance of p-value < 0.05. (**B**) Venn analyses of common significant miRNA target genes and related pathways. The significantly expressed targets from each sequencing analysis were shortlisted and compared between the groups. Predicted pathways of the targets from the Panther database were shortlisted and compared between the groups.

2.5. In Vitro Analyses of miR-1247-5p Targets

For in vitro analyses, we focused on downregulated targets, as we expect our upregulated miRNA candidates to cause a downregulation of the target mRNAs. For our downregulated mRNAs, only targets of miR-1247-5p were found to have published links to CS, namely Cyb5a, Gabbr2, and Gnaq (Table 1). Therefore, these three targets were then verified by QPCR in the groups of CPA, CD, PBMAH, ectopic CS, and controls (Figure 6). Only Cyb5A was found to be significantly downregulated in ACTH-dependent forms (ectopic CS and CD) as well as in ACTH-independent CS (PBMAH and CPA) in comparison to controls. Consequently, to assess whether Cyb5a is indeed regulated by miR-1247-5p, a dual luciferase assay was performed. To prove our hypothesis, treatment of Cyb5a-WT cells with miR-1247-5p mimic was expected to lead to a reduced luminescence, whereas no effects were expected in cells treated with the miR-1247-5p inhibitor or the Cyb5a-mutant (with a mutation in the miR-1247-5p binding site). As shown in Figure 7, transfection of miR-1247-5p significantly reduced luminescence from Cyb5a-WT in comparison to cells transfected with Cyb5a-WT and miR-1247-5p inhibitors. However, these predicted binding results were not found to be specific, as there were no significant differences when

compared to wells transfected with Cyb5a-WT alone (Figure 8). Consecutively, when the mutated Cyb5a-Mut were transfected along with the mimics and inhibitors, no significant differences in luminescence were observed in the transfected population. Thus, direct interaction between miR-1247-5p and its predicted target gene Cyb5A could not be conclusively proven using this luciferase assay.

Table 1. Analysis of the predicted targets of miR-1247-5p and their expression levels in comparison to controls (log2 fold change). Published literature on target genes in reference to CS is highlighted in bold.

Predicted Targets	Name	RNA-Seq CPA vs. Controls
ITIH5	Inter-alpha-trypsin Inhibitor Heavy Chain Family, Member 5	−3.42
GABBR2 PMID: 31581124	**Gamma-aminobutyric Acid (GABA) B Receptor, 2**	**−3.32**
C14orf180	Chromosome 14 Open Reading Frame 180	−2.31
TTC36	Tetratricopeptide Repeat Domain 36	−2.16
SEMA6D	Semaphorin 6D	−1.52
CYB5A PMID: 31581124	**Cytochrome B5 Type A (Microsomal)**	**−1.26**
FUT1	Fucosyltransferase 1	−0.95
GNAQ PMID: 31581124	**Guanine Nucleotide Binding Protein (G Protein), Q Polypeptide**	**−0.51**

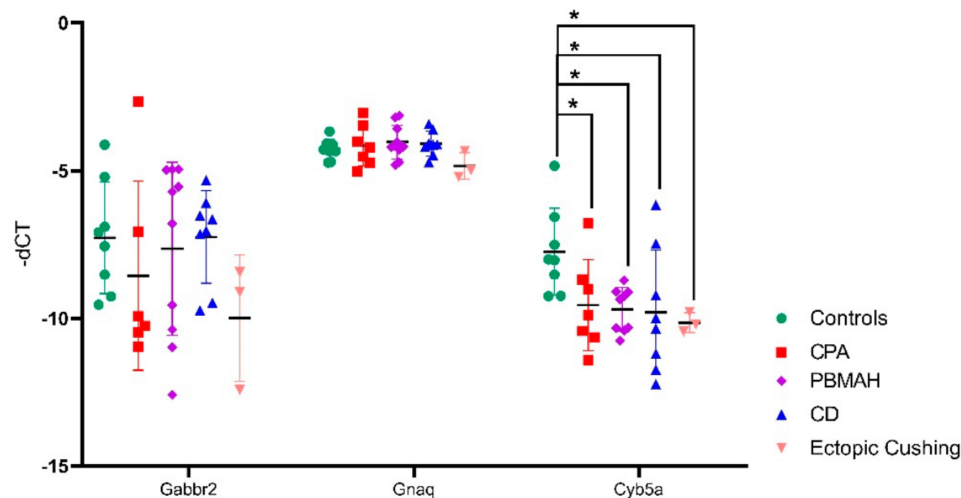

Figure 7. QPCR analyses of the top predicted targets of miR-1247-5p. Data are represented as mean ± standard deviation (SD) of −dCT values. Housekeeping gene: PPIA. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a significance of p-value < 0.05 (*).

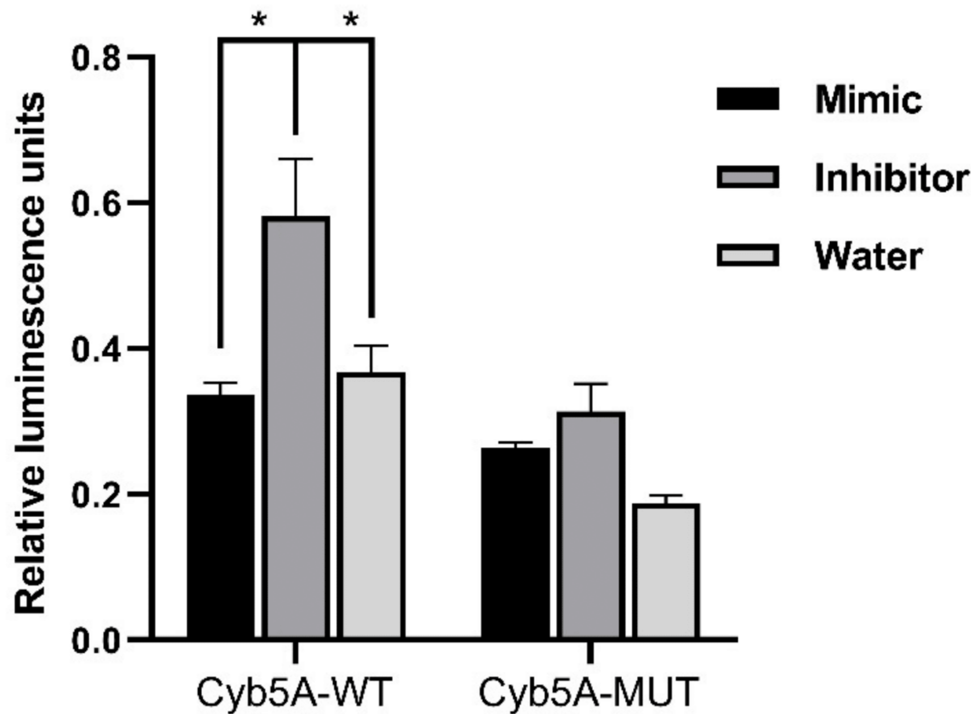

Figure 8. Results of dual luminescence assay on cells transfected with miR-1247-5p mimics and related controls. Cells were transfected with plasmids containing either the WT or Mut miRNA binding sequence in Cyb5a. Either miR-1247-5p mimics or miR-1247-5p inhibitors were transfected together with the respective plasmids. Data are represented as mean ± standard error of mean (SEM) of relative luminescence unit values. Statistics: ANOVA test with Bonferroni correction to detect significant differences between patient groups with at least a significance of p value < 0.05 (*).

2.6. Pathway Analyses of miRNA Targets

For the pathway analysis (Reactome) we used the 28 predicted miRNA-1247-5p targets and the 23 predicted miRNA-379-5p targets from TargetScan, which were significantly differently expressed in the RNA-seq (Figure 6). Concurrently, the pathways commonly enriched by both miRNAs included the WNT signaling pathway and N-acetyl-glucosamine synthesis (Figure 9A). As a complementary approach, in silico analyses were also performed based on the targets from miRTarBase. In this database, targets are shortlisted based on published experimental results. In this analysis, miR-1247-5p (n = 21) and miR-379-5p targets (n = 85) were unique. While the validated targets of miR-379-5p were found to show significant changes in expression in the RNA-seq data from PBMAH (n = 12), none of the validated miR-1247-5p targets were found to show significant expression changes in the RNA-seq data from CPA. Therefore, all the validated targets of the miRNAs were subjected to pathway analyses (Panther). Interestingly, the WNT signaling pathway was also found to be commonly regulated by both miRNAs using this approach (Figure 9B). Finally, the expression status of target genes related to WNT signaling pathways were checked in our RNA-seq data (Figure S2). Given the upregulated status of the miRNAs, a downregulated expression of its target genes was expected. However, a significantly upregulated expression was observed for DVL1 in CPA in comparison to controls and for ROR1 in PBMAH in comparison to controls.

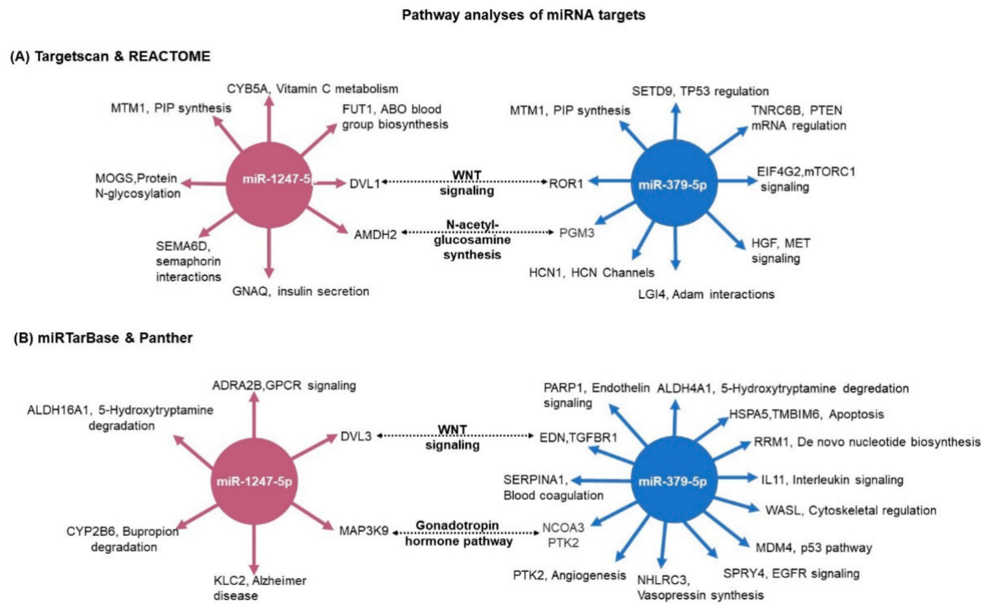

Figure 9. Pathway analyses of miRNA target genes. (**A**) The predicted targets were matched with the RNA-seq expression data. For miR-1247-5p, CPA vs. controls expression data; and for miR-379-5p, PBMAH vs. controls expression data. The significantly expressed target genes were then subjected to pathway analyses by Reactome. The significantly enriched pathway networks ($p < 0.05$) and their related genes are given. (**B**) The experimentally validated target genes from miRTarBase were analyzed for their role in pathways by the Panther database. The target genes and their related pathways are given. The commonly represented pathways are marked in bold.

3. Discussion

MiRNAs are fine regulators of both physiology and pathology and have diverse roles as diagnostic biomarkers as well as therapeutic targets. While circulating miRNAs have been investigated as potential biomarkers for hypercortisolism in CS subtypes (36), comprehensive analyses of their pathological role in CS subtypes have not yet been performed. This study hoped to uncover the pathological role of miRNAs in different CS subtypes as well as identify unique epigenetic targets contributing to hypercortisolism in these subtypes. As such, miRNA sequencing was performed in the ACTH-independent CPA and ACTH-dependent CD, with additional QPCR validation in PBMAH and ectopic CS. As expected, miRNA expression profiles in CD and CPA were very different.

3.1. ACTH-Independent Upregulated miRNAs in CS

Among the analyzed miRNAs, only miR-1247-5p and miR-379-5p showed the most prominent changes in expression levels. Briefly, miR-1247-5p was significantly upregulated in ACTH-independent forms of CS, CPA, and PBMAH (Figures 1 and 3) while miR-379-5p was found to be upregulated in CD and PBMAH, in comparison to controls. Even though CD and PBMAH represent CS subtypes with different ACTH dependence, albeit both with hyperplastic tissue, it is interesting to find a shared miRNA expression status. Concurrently, miRNAs have been identified as dynamic players in regulating the acute effect of ACTH on adrenal steroidogenesis in in vivo murine studies [20,21]. Further diverse miRNAs have been characterized to regulate steroidogenesis in ACTH and dexamethasone treated rats [22] (suppressed ACTH) as well as in in vitro studies [20]. It is possible that miR-379-5p contributes to the adrenal hyperplasia present in both PBMAH and CD by targeting specific

genes related to hyperplasia, and miR-1247-5p by contributing to cortisol production independent of ACTH regulation in CPA and PBMAH.

Interestingly, the miRNA candidates (mir-1247-5p and miR-379-5p) in our study have not been previously characterized in any of these studies. Furthermore, the expression of mir-1247-5p and miR-379-5p were found to be independent of ACTH stimulation, underlying their role in CS independently of the HPA axis control and postulating specific regulatory processes.

3.2. Target Genes of miRNAs in CS

Initially, we focused on the selection of known CS specific target genes that could be directly repressed by miRNAs, thereby contributing to pathology. The predicted target genes of miR-1247-5p and miR-379-5p were assessed for their downregulated expression status in the RNA-seq data. Among the selected target genes, only Cyb5a was found to be significantly downregulated in all forms of CS (Figure 6). Cytochrome b5 (CYB5A) is a marker of the zona reticularis and is an important regulator of androstenedione production [23,24]. Based on its role in adrenal steroidogenesis, it is possible that Cyb5a is downregulated by miR1247-5p. To prove our hypothesis, a dual luciferase assay was performed in HELA cell line to identify a direct interaction between Cyb5a and miR-1247-5p (Figure 7). Unfortunately, a direct interaction could not be proven, indicating that miR-1247-5p perhaps regulates its target genes in different ways.

3.3. Pathway Analyses of miRNA Targets

To identify miRNA specific regulatory processes, comprehensive target and pathway analyses were performed. Independent pathway analyses of the respective targets with two different databases of Reactome and Panther showed the WNT signaling pathway as a common targeted pathway of both mir-1247-5p and miR-379-5p (Figure 8). The WNT signaling pathway represents a crucial regulator in diverse developmental as well as pathological processes with tissue-specific effects [25,26]. Consequently, the WNT pathway has been largely characterized as one of the dysregulated pathophysiological mechanisms in CPA. Mutations in PRKACA, one of the WNT signaling proteins, are present in approximately 40% of CPA [27]. In the case of CD, dysregulated WNT signaling has been characterized as promoting proliferation in ACTH-secreting pituitary adenomas [28]. Moreover, activating mutations in beta catenin, one of the WNT signaling pathways, has been characterized as driving adrenal hyperplasia through both proliferation-dependent and -independent mechanisms [29]. Thus, it could be hypothesized that by targeting specific genes in the pathway, miRNAs drive specific pathophysiological processes in diverse CS subtypes.

3.4. MiRNA Target Genes in WNT Signaling

DVL1 (TargetScan) and DVL3 (miRTar) are the shortlisted target genes of miR-1247-5p in the WNT signaling pathway. These genes are members of canonical WNT pathways and, importantly, activation of the cytoplasmic effector Dishevelled (Dvl) is a critical step in WNT/β-catenin signaling initiation [30,31]. Interestingly, no difference in DVL1 and DVL3 gene expression was found in the analyses of ACTH-secreting pituitary adenomas [32]. Therefore, it could be possible that DVL1 and DVL3 are only targeted by miR-1247-5p specifically in the adrenal of CPA and PBMAH patients, leading to its characterized tumor progression. EDN1, TGFBR1 (TargetScan), and ROR1 (miRTar) were the target genes of miR-379-5p related to the WNT pathway. In epithelial ovarian cancer, Endothelin-1 (EDN-1) was found to regulate the epithelial–mesenchymal transition (EMT) and a chemoresistant phenotype [33]. In the case of receptor tyrosine kinase-like orphan receptor 1 (ROR1), higher expression of the gene was associated with a poor prognosis in ovarian cancer [34]. Concurrently, suppression of TGFBR1-mediated signaling by conditional knockout in mice was found to drive the pathogenesis of endometrial hyperplasia, independent of the influence of ovarian hormones [35]. Therefore, it could be hypothesized that the

dysregulated expression of these factors in adrenals could trigger similar hyperplastic effects mediated by these factors, as in ovarian tissues.

3.5. Bottlenecks and Future Outlook

Interestingly, among these genes, only DVL1 and ROR1 were found to be significantly upregulated in the RNA-seq data (Figure S2). The major regulatory role of miRNAs in gene expression come from their ability to repress gene expression at the level of transcription and translation. There are also reports of miRNA-mediated gene upregulation; however, the physiological evidence of the role is still unresolved [36]. Therefore, it is interesting to see the selected targets of miR-1247-5p and miR-379-5p upregulated. Moreover, it should be noted that most of the experimentally validated miRNA targets were identified by CLIP methods [37]. Crosslinking immunoprecipitation (CLIP) are binding assays that provide genome-wide maps of potential miRNA-target gene interactions based on sequencing. Moreover, these assays do not make functional predictions on the outcome of miRNA binding, and neither do upregulation or downregulation [38,39]. Therefore, in our current experimental setting, we could only identify potential miRNA target genes and speculate on their pathological role based on the published literature and in silico analyses. Furthermore, extensive mechanistic analyses based on these potential targets could help in elaborating the specific epigenetic pathways that fine-tune CS pathology in different subtypes.

4. Materials and Methods

4.1. Sample Collection and Ethics Approval

All patients were registered in the German Cushing's Registry, the ENS@T or/and NeoExNET databases (project number protocol code 379-10 and 152-10). The study was approved by the Ethics Committee of the University of Munich. All experiments were performed according to relevant guidelines and protocols, and written informed consent was obtained from all patients involved. The adrenal samples used in the sequencing (miRNA and RNA) belong to the same patient.

For miRNA-specific next-generation sequencing (NGS), a total of 19 adrenocortical tissue samples were used. The cohort consisted of the following patient groups: ACTH-independent CPA, n = 7; ACTH-dependent hypertrophic adrenals of CD patients after bilateral adrenalectomy, n = 8; normal adjacent adrenal tissue from patients with pheochromocytoma as controls, n = 8. For QPCR validation, the patient groups included adrenal tissue from ACTH-independent PBMAH, n = 10, and ACTH-dependent ectopic CS, n = 3.

In the case of mRNA sequencing, a total of 23 adrenocortical tissue samples were used. This includes the following patient groups: CPA, n = 7; PBMAH, n = 8; normal adjacent adrenal tissue from patients with pheochromocytoma as controls, n = 8.

The clinical characteristics of the patients are given in Table 2. Furthermore, of the eight CPA samples in the study, three of them carried known somatic driver mutations in the PRKACA gene and in the ten PBMAH samples, two carried germline mutations in the ARMC5 gene.

Table 2. Clinical characteristics of the patient groups. Data are given as median with 25th and 75th percentiles in brackets. CPA, cortisol producing adenoma; CD, Cushing's disease.

	n	Age at Surgery (Years)	Sex (%Female)	Baseline ACTH (pmol/L)	Cortisol 24 h Urine (nmol/Day)	Midnight Salivary Cortisol (nmol/L)	Cortisol after 1 mg Dexamethasone (nmol/L)
CPA	7	52 [40; 57]	62	0.4 [0.4; 0.8]	1684 [1449; 2120]	16.0 [12.7; 24.3]	386 [326; 431]
CD	8	42 [37; 52]	75	10.5 [7.8; 12.1]	2211 [1949; 2339]	21.1 [12.0; 27.3]	373 [233; 622]
PBMAH	10	61 [59; 68]	100	0.6 [0.4; 0.7]	994 [619; 1256]	15.6 [9.3; 21.1]	250 [206; 481]
Ectopic	3	69 [62; 71]	66	51.3 [35.0; 68.8]	1168 [939; 22,771]	95.2 [54.8; 538.9]	1169 [884; 1454]
Controls	8	53 [48; 58]	50	NA	NA	NA	NA

The adrenal tissues were stored at −80 °C. Total RNA isolation was carried out from all adrenal cortex samples by an RNeasy Tissue Kit (Qiagen, Hilden, Germany). The isolated RNA was kept frozen at −80 °C until further use.

4.2. MiRNA and RNA Sequencing

RNA integrity and the absence of contaminating DNA were confirmed by Bioanalyzer RNA Nano (Agilent Technologies, Santa Clara, CA, USA) and by Qubit DNA High sensitivity kits, respectively. Sequencing libraries were prepared using the Illumina TruSeq Small RNA Library Preparation Kit. NGS was performed on 2 lanes of an Illumina HiSeq2500 (Illumina, CA, USA) multiplexing all samples (paired end read, 50 bp). The quality of sequencing reads was verified using FastQC0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc, date last accessed: 13 March 2020) before and after trimming. Adapters were trimmed using cutadapt [40]. Reads with <15 bp and >40 bp insert sequences were discarded. An alignment of reads was performed using miRBase V21 [41,42] with sRNAbench [43]. EdgeR and DeSeq in R were used for further analyses [44,45]. MiRNAs with at least 5 raw counts per library were included. RNA-seq was performed by Qiagen, Hilden, Germany. For mRNA, sequencing was performed on Illumina NextSeq (single end read, 75 bp). Adapter and quality trimming were performed by the "Trim Reads" tool from CLC Genomics Workbench. Furthermore, reads were trimmed based on quality scores. The QC reports were generated by the "QC for Sequencing Reads" tool from CLC Genomics Workbench. Read mapping and gene quantification were performed by the "RNA-seq Analysis" tool from CLC Genomics Workbench [46]. The miRNA-seq data generated in this study have been submitted to the NCBI (PRJNA847385).

4.3. Validation of Individual miRNAs

MiRNAs and genes significantly differentially expressed by NGS were validated by QPCR. Reverse transcription of miRNA-specific cDNA was performed by using the TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific, Munich, Germany), and the reverse transcription of RNA genes was done by using the Superscript VILO cDNA synthesis Kit (Thermo Fisher Scientific, Munich, Germany). 50 ng of RNA was used for each of the reverse transcription reactions. Quantitative real-time PCR was performed using the TaqMan Fast Universal PCR Master Mix (2×) (Thermo Fisher Scientific, Munich, Germany) on a Quantstudio 7 Flex Real-Time PCR System (Thermo Fisher Scientific, Munich, Germany) in accordance with the manufacturer's protocol. All QPCR reactions were performed in a final reaction volume of 20 µL and with 1 µL of 1:5 diluted cDNA. Negative control reactions contained no cDNA templates. Gene expression was quantified using the relative quantification method by normalization with reference gene [47]. Statistical analysis using the bestkeeper tool was used to compare and select the best reference gene with stable expression across the human adrenal samples [48]. Reference genes with significantly different Ct values (p-value < 0.01) between the samples were excluded. Furthermore, primer efficiency and the associated correlation coefficient R2 of the selected reference gene were determined by the standard curve method in serially diluted cDNA samples [49]. In the case of miRNA reference genes, miR-16-5p [48,50,51] and RNU6B [52] previously used in similar studies were compared. MiR-16-5p was found to show the most stable expression levels across the samples with a p-value of 0.452 in comparison to RNU6B which had a p-value of 0.001. In the case of RNA reference genes, PPIA [53] and GAPDH [54] were compared. Here, PPIA was found to show the most stable expression levels across the samples with a p-value of 0.019 in comparison to GAPDH which had a p-value of 0.003. Therefore, these genes were used for the normalization of miRNA and RNA expression in human adrenal samples.

4.4. Target Screening

In silico prediction of the possible miRNA targets was performed using the miRNA target database, TargetScan, and miRTarBase [19,37]. The top predicted targets were further

screened based on their expression status in the RNA-seq data from the adrenocortical tissues of CPA, PBMAH, and controls (unpublished data). Pathway analyses of the targets were performed using Reactome [55] and Panther [56] online platforms. The selected downregulated targets were analyzed by QPCR in the adrenocortical samples to confirm their expression status. The successfully validated candidates were then analyzed for regulation by the miRNA using a dual luciferase assay [57].

4.5. Dual Luciferase Assay

The interaction between the predicted 3′-UTR region of Cyb5a and miR-1247-5p was detected using a luciferase activity assay. The 3′UTR sequences of Cyb5a (129 bp) containing the predicted miR-1247-5p binding sites (psiCHECK-2 Cyb5a 3′UTR WT) were cloned into the psiCHECK-2 vector (Promega, Fitchburg, WI, USA). A QuikChange Site-Directed Mutagenesis kit (Agilent Technologies, CA, USA) was used to mutate the miR-1247-5p binding site (psiCHECK-2 Cyb5a 3′UTR mutant) according to the manufacturer's protocol. All the sequences were verified by Sanger sequencing. Then, 200 ng of the plasmid was used for each transfection. Synthetic miR-1247-5p mimics and specific oligonucleotides that inhibit endogenous miR-1247-5p (miR-1247-5p inhibitors) were purchased from Promega and 100 nmol of the molecules were used for each transfection according to the manufacturer's protocol. For the assay, HeLa cells were seeded in 96-well plates and incubated for 24 h. The following day, cells were transfected using the following different conditions: (1) psiCHECK-2 Cyb5a 3′UTR WT + miR-1247-5p mimic; (2) psiCHECK-2 Cyb5a 3′UTR WT + miR-1247-5p inhibitor; (3) psiCHECK-2 Cyb5a 3′UTR WT + water; (4) psiCHECK-2 Cyb5a 3′UTR mutant + miR-1247-5p mimic; (5) psiCHECK-2 Cyb5a 3′UTR mutant + miR-1247-5p inhibitor; (6) psiCHECK-2 Cyb5a 3′UTR mutant + water. Forty-eight hours later, luciferase activity in the cells was measured using the dual luciferase assay system (Promega, Fitchburg, WI, USA) in accordance with the manufacturer's instructions. Renilla luciferase activity was normalized to firefly luciferase activity. Each treatment was performed in triplicate. Any interaction between the cloned gene, Cyb5a (WT and mutant), and miR-1247-5p mimic is accompanied by a decrease in luminescence. This decrease in luminescence would not be observed when the plasmids are transfected with the miR-1247-5p inhibitor, indicating that observed luminescence differences are caused by specific interactions between the plasmid and the miR-1247-5p mimic. Transfection of the plasmid with water corrects any background interactions between the cloned gene and endogenous miRNAs in the culture.

4.6. In Vivo ACTH Stimulation

Experiments were performed on 13-week-old C57BL/6 J female mice (Janvier, Le Genest-Saint-Isle, France). Mice were intraperitoneally injected with 1 mg/kg of ACTH (Sigma Aldrich, Munich, Germany) and adrenals were collected after 10, 30, and 60 min of injections. In addition, control adrenals were collected from mice at baseline conditions (0 min). Mice were killed by cervical dislocation and adrenals were isolated, snap-frozen in liquid nitrogen, and stored at −80 °C for later RNA extraction. MiR-26a was taken as a housekeeping gene in the QPCR [58]. All mice were maintained in accordance with facility guidelines on animal welfare and approved by Landesdirektion Sachsen, Chemnitz, Germany.

4.7. Statistical Analysis and Software

R version 3.6.1 was used for the statistical analyses. To identify RNAs differentially expressed, a generalized linear model (GLM, a flexible generalization of ordinary linear regression that allows for variables that have distribution patterns other than a normal distribution) in the software package edgeR (Empirical Analysis of DGE in R) was employed to calculate *p*-values [45,59]. *p*-values were adjusted using the Benjamin–Hochberg false discovery rate (FDR) procedure [60]. Disease groups were compared using the unpaired Mann–Whitney test, and to decrease the false discovery rate a corrected *p*-value was cal-

culated using the Benjamin–Hochberg method. p adjusted < 0.05 and log2 fold change >1.25 was applied as the cut-off for significance for NGS data. GraphPad Prism Version 8 was used for the statistical analysis of QPCR. To calculate differential gene expression, the dCt method (delta Ct (cycle threshold) value equals target miRNA's Ct minus housekeeping miRNA's Ct) was used (Microsoft Excel 2016, Microsoft, Redmond, WA, USA). For QPCR, an ANOVA test with Bonferroni correction was used [61] to assess significance; p-values < 0.05 were considered significant.

5. Conclusions

In conclusion, while comprehensive information regarding the role of miRNAs in acute and chronic phases of steroidogenesis is available, there is little known about the pathological independent role of miRNAs in the pathology of CS. In our study, we have described ACTH-independent miR-1247-5p and miR-379-5p expression in CS for the first time. Thus, by regulating different genes in the WNT signaling pathway, the miRNAs may individually contribute to the Cushing's pathology in specific subtypes.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ijms23147676/s1.

Author Contributions: Conceptualization, S.V., A.C. and A.R.; methodology, S.V., R.Z. and M.E.; software, S.V. and M.E.; validation, R.Z., A.O., D.W. and B.W.; formal analysis, S.V.; investigation, S.V., R.Z., M.E., A.O. and D.W.; resources, A.C., B.W., M.R. and A.R.; data curation, S.V. and R.Z.; writing—original draft preparation, S.V., R.Z. and A.R.; writing—review and editing, S.S., M.R. and A.R.; visualization, S.V.; supervision, A.R.; project administration, A.R.; funding acquisition, A.C., S.S., M.R. and A.R. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Ludwig Maximilian University, Munich (protocol code 379-10, 152-10 and 20 July2021).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The miRNA-seq data generated in this study have been submitted to the NCBI (PRJNA847385).

Conflicts of Interest: The authors declare no conflict of interest.

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Apendix A: Paper III

Steroid profiling using liquid chromatography mass spectrometry during adrenal vein sampling in patients with primary bilateral macronodular adrenocortical hyperplasia

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